Microbial Pathogenesis 112 (2017) 63–69

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Screening and identification of lignin-degrading bacteria in termite gut and the construction of LiP-expressing recombinant *Lactococcus lactis*

Haizhu Zhou [a, 1, 2], Wei Guo [b, 1], Bo Xu [a], Zhanwei Teng [a], Dapeng Tao [a], Yujie Lou [a], Yunhang Gao [a, *]

[a] College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China
[b] Jilin Municipal General Station for Animal Husbandry and Veterinary Service, Changchun 130062, China

ARTICLE INFO

Article history:
Received 4 May 2017
Received in revised form
19 September 2017
Accepted 19 September 2017
Available online 21 September 2017

Keywords:
Termite gut
PY12
MX5
LiP
16S rRNA
Recombinant *Lactococcus lactis*

ABSTRACT

Lignin, a common natural polymers, is abundant and complex, and termites can break down and utilize the lignin in their food. In this study an attempt was made to isolate and characterize the lignolytic bacteria from termite (*Reticulitermes chinensis Snyder*) gut. Two strains (PY12 and MX5) with high lignin peroxidase (LiP) activity were screened using the azure B method. By analyzing their 16S rRNA, the strain PY12 was classified as *Enterobacter hormaechei*; MX5, as *Bacillus licheniformis*. We then optimized the different conditions of liquid fermentation medium, and obtained LiP activities of 278 U/L and 256 U/L for PY12 and MX5, respectively. Subsequently, we confirmed the LiP activities of the strains by evaluating their decolorizing effects on various dyes. Finally, we cloned the LiP gene of strain PY12 and successfully transferred it to *Lactococcus lactis*. We believe that our results provide the theoretical and practical basis for the production of genetically engineered bacteria that produce LiP, thus allowing for the utilization of naturally available lignin as an energy resource.

1. Introduction

At present, the global problems of food shortages, environmental pollution and the energy crisis are becoming more serious, making it increasingly important to explore reasonable and effective ways to develop and utilize renewable resources. Lignin-cellulose is the most abundant and cheapest renewable natural resource, and represents a major source of renewable organic matter [1], but it is difficult to be degraded because of its complex structure [2]. First, the lignin is degraded by lignolytic enzymes, releasing cellulose from lignin and hemicellulose, then cellulose is degraded by cellulase. Lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) play a key role in the process of lignin degradation [3]. Many diverse group of microorganisms are capable to degrade lignin, in particular, fungi and bacteria, including actinomycetes [4]. Nevertheless, fungi have the strongest degradation ability.

Termites, social insects that mainly feed on cellulosic material, have their own cellulase system, in addition to cellulase-producing symbiotes in the gut [5–9]. Endo-β-1,4–glucanase, which has strong cellulose degrading activity, was cloned from *Coptotermes formosanus* [5]. The termite gut contains many microorganisms, such as bacteria of the Enterobacteriaceae family and those belonging to the Bacteroides, Staphylococcus, Streptococcus, and Bacillus genera [10,11]. Most bacteria exist in the termite's hindgut, where 65–69% of cellulosic materials are digested [12,13]. Hyodo et al. detected the activity of Lac in several strains of termite symbiotic fungus, which indicates the relationship between Lac and lignin degradation [14]. Furthermore, Zhou isolated 4 strains from termite gut with the ability to degrade lignin and cellulose [15], confirmed the existence of microorganisms in termite gut which can degrade lignin.

Termites act as the most important lignin-cellulose

Abbreviations: LiP, lignin peroxidas; MnP, manganese peroxidase; Lac, laccase; PCR, polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis; VA, veratryl; *E.coli*, *Escherichia coli*; *L. Lactis*, *Lactococcus lactis*.

* Corresponding author.
E-mail addresses: zhouhaizhu@jlau.edu.cn (H. Zhou), gaoyunhang2017@163.com (Y. Gao).

[1] Haizhu Zhou and Wei Guo contributed equally to this work.

[2] Dr. Haizhu Zhou (1982-), mainly engaged in the research on development and utilization of forage resources.

https://doi.org/10.1016/j.micpath.2017.09.047
0882-4010/

decomposers in the natural ecosystem, especially in the tropics. The ability of termite intestinal bacteria to digest the plant cell wall polysaccharide is approximately 30%–40% higher than that of the large herbivores [16,17]. However, few studies exist on the screening and identification of lignin degrading bacteria from termite gut, or on the construction of genetically engineered bacteria. Hence in the present study, we aimed to isolate lignin-degrading bacteria from the termite gut, and use them to genetically engineer an LiP-expressing bacterium and lay the theoretical foundation for industrial production. We hope that our results will provide a solid theoretical basis and technical support for the utilization of lignin-cellulose in the future.

2. Materials and methods

2.1. Materials

Adult healthy termites (*Reticulitermes chinensis Snyder*), were donated by Prof. Jianchu Mo, Urban Entomology Research Center, Institute of Agriculture, Zhejiang University; Selective culture medium: Lignin 0.3 g, peptone 1.0 g, NaCl 0.5 g, agar 2.0 g, distilled water 100 mL, autoclaved at 121 °C for 20 min; Liquid culture medium: Beef extract 0.5 g, peptone 0.1 g, straw powder 0.5 g, NaCl 0.5 g, pH7.0, distilled water 100 mL, autoclaved at 121 °C for 20 min; Azure B culture medium: Lignin 10.0 g, $(NH_4)_2SO_4$ 4.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, KH_2PO_4 4.3 g, $CaCl_2$ 0.3 g, Azure B 0.1 g, agar 20.0 g, distilled water 1000 mL, autoclaved at 121 °C for 20 min; pNZ8149 selection culture medium: Peptone 20.0 g, yeast powder 5.0 g, NaCl 4.0 g, sodium acetate 1.5 g, ascorbic acid 0.5 g, agar powder 15.0 g, pH6.8, distilled water 1000 mL, autoclaved at 121 °C for 20 min, bromcresol purple was added to a final concentration 0.004% before the medium was poured into the plate; The primers used in this study are listed in Table 1.

2.2. Screening of lignin-degrading bacteria

The body surfaces of 30 termites were sterilized, their heads and chests were removed under aseptic conditions, and their abdomens were collected to make a homogenate. The homogenate was then diluted using 0.9% normal saline and inoculated into the selective medium with lignin as the sole carbon source, the plates were inverted and cultured at 37 °C for 48 h. Streak plate method was applied to isolate the strains, purified single colonies were inoculated in azure-B medium. The decolorization changes in each medium were observed whenever necessary.

2.3. Measurement of LiP activity

The activated strain was inoculated into 100 mL liquid fermentation medium and cultured at 37 °C for 72 h, this seed liquid was then centrifuged at 4500 rpm for 10 min, and the supernatant was collected as crude enzyme liquid.

LiP activity was determined using the veratryl alcohol (VA) method. The total reaction volume was 4 mL, which contains 50 mmol/L tartaric acid buffer (pH3.0) 2.5 mL, 2 mmol/L VA 1 mL, crude enzyme liquid 0.4 mL, water bath at 30 °C for the entire reaction mixture, then 0.1 mL H_2O_2 solution (0.4 mmol/L) was added to initiate the reaction. The absorbance at 310 nm was measured with spectrophotometer. The crude enzyme liquid was boiled for 15 min as the control. The enzyme activity was defined as the amount required to oxidize all the VA in a minute (1 U).

Table 1
Primers used in this study.

Number	Primers
P1	5′-AGAGTTTGATCCTGGCTCAG-3′
P2	5′-GGTTACCTTGTTACGA CTT-3′
P3	5′-CGGGTACCATGATAGATGGAGGCAACATG-3′ *Kpn I*
P4	5′-CGGAGCTCTCAGAGGCCGAGGGCG-3′ *Sac I*

The "underline" are labelled as restriction enzyme cutting sites in the primers.

2.4. Growth curves

The isolated bacterial strains were inoculated into liquid medium (50 mL), and cultured at 37 °C for 24 h, then 1% of the inoculum was transferred to fresh medium, and cultured under the same conditions. 3 mL bacterial culture was taken every 2 h, to determine the absorbance at 600 nm, the non-inoculated medium was taken as the control.

2.5. Identification of strains PY12 and MX5

The genomic DNA of the strains was extracted according to the instructions of MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Japan), and used as the template for PCR. Bacterial 16S rRNA universal primers (P1 and P2, 10 pmol) were used for PCR reaction in a total volume of 50 μL with dNTP (2.5 mmol/L) 4 μL, 10 × Ex Taq buffer 5 μL, Ex Taq polymerase 5 U. PCR was carried out with an initial heat action step at 94 °C for 3min, and amplifications were achieved through 30 cycles at 94 °C for 45s, 50 °C for 45s and 72 °C for 1 min, with a final extension at 72 °C for 10min. The amplified products were separated on 0.8% agarose gel, then were recovered and sequenced. BLAST software and MegAlign software were used to analyze the homology of the PCR products.

2.6. Optimization of fermentation conditions for producing LiP

The fermentation conditions, namely, carbon source, nitrogen source, pH, fermentation temperature, fermentation time and inoculation amounts, were optimized with a univariate experimental design. LiP activity was determined using the same method as that described in Section 2.3. The details are as follows.

(a) Carbon sources: carboxymethylcellulose (CMC-Na), glucose, straw, lignin + straw, lactose, CMC-Na + straw, lignin, wheat bran;
(b) Nitrogen sources: beef extract, peptone, yeast powder, ammonium tartrate, ammonium sulfate, ammonium sulfate + yeast powder, beef extract + peptone;
(c) Initial pH: pH3.0, pH4.0, pH5.0, pH6.0, pH7.0, pH8.0, pH9.0, pH10.0;
(d) Fermentation temperature: 27 °C, 32 °C, 37 °C, 42 °C, 47 °C, 52 °C;
(e) Fermentation time: 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h, 108 h;
(f) Inoculation amounts: 1%, 3%, 5%, 7%, 9%, 11%.

2.7. Dye decolorization by the two strains

Each strain was inoculated into the liquid culture medium containing 200 mg/L each dye, and cultured at 28 °C for 10 days. The decolorization of dyes were observed by absorption spectrophotometry, and the absorption wavelength of each dye is shown in Table 2. Dye decolorization rate formula: X = (A0-At)/A0 × 100%, where A0 represents the absorbance of the control, and At represents the absorbance of samples.