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A UV-induced mutant of *Cryptococcus flavus* GB-1 with increased production of a biodegradable plastic-degrading enzyme

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ABSTRACT

The yeast strain, *Cryptococcus flavus* GB-1, isolated from rice husks as a candidate producer of a biodegradable plastic (BP)-degrading enzyme. The genomic DNA sequence encoding the BP-degrading enzyme of GB-1 was determined. The enzyme was named CfCLE because the deduced amino acid sequence (239 amino acids) was found to be 93% identical to that of CLE of *Cryptococcus* sp. S-2 (accession No. BAK82405.1). The strain GB-1 forms a clear zone (halo) around its colonies on emulsified polybutylene succinate-*co*-adipate (PBSA) agar plates containing soybean oil but not on plates containing glucose. A UV-induced mutant from this strain, designated as GB-1-DMC1, formed halos on emulsified PBSA plates containing glucose and showed 1.53 U/ml of PBSA-degrading activity in liquid culture with glucose, which was more than 2.5-fold higher than that of the parent strain GB-1 (0.58 U/ml). Moreover, using xylose as a carbon source enhanced the PBSA-degrading activity of both strains. Xylose fed-batch cultivation of GB-1 and GB-1-DMC1 using a 5-l jar fermentor for 72 h produced 12.1 and 21.2 U/ml of CfCLE, respectively. Purified CfCLE has a molecular mass of 22 kDa, and its optimal pH and temperature for enzyme activity is 7.8 and 45 °C, respectively. CfCLE degraded various BP cast films.

1. Introduction

Biodegradable plastics (BPs) such as polybutylene succinate (PBS), polybutylene succinate-*co*-adipate (PBSA), poly ε-caprolactone (PCL), and polylactic acid (PLA) can be degraded into water and CO_2 by microorganisms in bioactive environments [1]. However, although these materials are biodegradable, their increased consumption has caused ecological problems due to their incomplete degradation within the desired time [2]. Thus, there is a need to develop methods for the enhanced degradations of BPs. Treatment with BP-degrading enzymes is one such method that is expected to accelerate the degrading reaction. As a candidate producer of BP-degrading enzymes, we previously isolated a yeast strain, *Cryptococcus flavus* GB-1, from a plant surface [3]. On emulsified PBSA agar plates containing soybean oil as a carbon source, *C. flavus* GB-1 degrades emulsified PBSA and forms a clear zone (halo) around its colonies [3].

A cutinase-like enzyme (CLE) of a *C. flavus*-related strain, *Cryptococcus* sp. S-2 (formerly called CS2 lipase), has been reported to degrade various BPs (PBS, PCL, and PLA) [4]. CLE production by *Cryptococcus* sp. S-2 is increased by the addition of 0.5% lactose in CLE production medium containing 1% oil [5]. We previously confirmed that the PBSA-degrading activity of *C. flavus* GB-1 culture broth, using the same CLE production medium, was also relatively high (1.66 U/ml) [6]. Considering these results, we expected the BP-degrading enzyme of this strain to have a structure similar to that of CLE of *Cryptococcus* sp. S-2; consequently, it was provisionally named CfCLE.

CLE of *Cryptococcus* sp. S-2 was first isolated as a lipase [4,5]. There are several reports demonstrating that the lipase productivities of some microorganisms could successfully be enhanced by mutation [7–9]. The enhanced lipase-producing mutants of *Aspergillus niger* were identified as those forming larger halos than the parents around their colonies on the medium containing tributyrin as the substrate and bile salts as a lipase inhibitor [9]. However, in our preliminary trials, the colonies that formed relatively large halos did not always exhibit enhanced CfCLE productivity (unpublished data). This observation demonstrates the

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Table 1
Primers for cloning of *CfCLE1* used in this study.

Oligonucleotide	Sequence	Position[a]
CS2-CLEF1	GCTGGCCTCGGCGTAGCAAAGGCGAT	−1000 to −974
CS2-CLEF2	CTTATTCTACCGCGATGAGGAGCTCCG	−550 to −523
CS2-CLEF3	CAGGTGCCGGCTTATCAGATATAAGAA	−100 to −73
CS2-CLEF4	AGGGTACAGCCAGGGCGCGGCTGCTAC	351 to 377
CS2-CLER1	GCACTAACCGCAGTCTCATCACATGA	−470 to −495
CS2-CLER2	GCGGTATGGCTCGGAGAGGTGAGTGG	−20 to −45
CS2-CLER3	TTGACGGCGTTGAACGCCGCTCCACTC	431 to 404
CS2-CLER4	GCTAGCGAGATATAATGTGCATATGT	881 to 454
Cf-GB-1 CLE F1	TGTACCTGGGCAAATTCATCTACAA	–
Cf-GB-1 CLE R1	ACGCGGTGGGACCTGGTTTATTCGC	–
Cf-GB-1 CLE R2	AAGCAGCGGACAGCGCTGCGAGAGC	–

[a] Positions of the nucleotides in the *CLE1* gene sequence of *Cryptococcus* sp. S-2.

need to develop effective selection methods to detect high BP-degrading enzyme producers.

Recently, we found that *C. flavus* GB-1 colonies did not form halos on emulsified PBSA plates containing glucose as the carbon source (unpublished data). Because CfCLE production by *C. flavus* GB-1 was repressed on glucose-containing plates, we speculated that some of the mutants that form halos on these plates could have enhanced CfCLE productivity. Moreover, our previous studies revealed that the production of a BP-degrading enzyme of a phyllosphere yeast *Pseudozyma antarctica* (PaE) was enhanced by xylose or lactose fed-batch cultivation using a jar fermentor [6,10]. This technique may also be useful for increasing CfCLE production by *C. flavus* GB-1.

This study aimed to identify the genomic DNA sequence of CfCLE and to enhance the CfCLE productivity of *C. flavus* GB-1 by mutation. Increased CfCLE production of the mutant was demonstrated by fed-batch cultivation using a jar fermentor. Biochemical characterization of CfCLE for the degradation of various BPs was also presented.

2. Materials and methods

2.1. Strains

The strains used in this study include *C. flavus* GB-1 which was isolated from rice husks [3], and its UV-induced mutant, *C. flavus* GB-1-DMC1. These strains were maintained on YM plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, and 2% agar).

2.2. Genomic DNA isolation

C. flavus GB-1 and GB-1-DMC1 were cultivated in 5 ml of YM medium at 30 °C with reciprocal shaking at 160 rpm overnight. The cultures were centrifuged, and genomic DNA was isolated from the cell pellets using Dr. GenTLE for Yeast (Takara, Kyoto, Japan).

2.3. Sequencing of the CfCLE-encoding gene

To determine the nucleotide sequence of the CfCLE-encoding gene, forward primers (CS2-CLEF1, CS2-CLEF2, CS2-CLEF3, and CS2-CLEF4) and reverse primers (CS2-CLER1, CS2-CLER2, CS2-CLER3, and CS2-CLER4) were designed based on the genomic sequence of *Cryptococcus sp.* S-2 *CLE1* (Accession No. AB671329) [11]. The primer sequences used in this study and their positions in the *Cryptococcus sp.* S-2 gene are shown in Table 1. The entire CfCLE-encoding gene of *C. flavus* GB-1 was obtained by PCR amplification with the above primers using genomic DNA of this strain as template. The reaction was performed using KOD-FX (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Amplified DNA fragments were subjected to 1% agarose gel electrophoresis and were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The sequences of the PCR products were determined using BigDye Terminator Cycle Sequence V3.1 Kit with the same primers and Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.4. UV mutagenesis

C. flavus GB-1 was cultivated in 5 ml of YM medium at 30 °C with reciprocal shaking at 160 rpm for 24 h. The culture (1 ml) and 19 ml of sterilized water were mixed in plastic petri dishes (optical density at 660 nm (OD_{660}) was approximately 1.0). The mixture was stirred with a magnetic stirrer and exposed to UV light at a distance of 50 cm for 60 s. After 100 times dilution with sterilized water, the diluted mixtures (50 μl) were spread on selection plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 0.5% emulsified PBSA (Bionolle EM-301, Showa Denko K.K., Japan), and 2% agar). After 48 h cultivation, approximately 500 colonies were growing on the plates. The mutants that constitutively produced BP-degrading enzyme formed a clear zone (halo) around the colonies.

2.5. Flask cultivation

C. flavus GB-1 and GB-1-DMC1 were pre-cultivated in test tubes containing 5 ml of YM medium at 30 °C with reciprocal shaking at 160 rpm for 24 h. Aliquots of the pre-cultures (200 μl) were added into 100 ml-flasks containing YE medium (1% yeast extract) with appropriate carbon sources (6%). Carbon sources and other nutrients were separately autoclaved (121 °C, 20 min). The cultures were incubated at 30 °C with reciprocal shaking at 140 rpm. At various times during the growth, 1-ml aliquots were harvested and centrifuged at 20,000 × *g* for 5 min. The enzymatic activities of the supernatant (crude enzyme solution) were measured as described below. After 96 h cultivation, the cell pellets were dried at 105 °C for 2 h and weighed to determine cell growth.

2.6. Evaluation of BP-degrading activity

BP-degrading activity was determined using a PBSA emulsion as the substrate [3,4,12,13]. The reaction mixture contained 20 mM Tris–HCl buffer (pH 6.8), 0.045% (w/v) emulsified PBSA, and 100 μl of crude enzyme solution in a total volume of 2 ml at 30 °C. The initial OD_{660} was approximately 0.65. The emulsified PBSA solution without enzyme was used as control. Its OD_{660} response was checked during the reaction and was found stable. Its OD_{660} value was subtracted from OD_{660} value of the emulsified PBSA samples with enzyme. One unit (U) of PBSA degrading activity was defined as a 1 OD_{660} decrease per minute in the reaction mixture.

2.7. Scale-up cultivation using a jar fermentor

Pre-cultures (50 ml) of *C. flavus* GB-1 and GB-1-DMC1 in YM medium were grown in 300-ml flasks at 30 °C with reciprocal shaking at 140 rpm for 24 h. The entire pre-culture was then used to inoculate 5-l jar fermentors containing 3 l of CfCLE production medium (0.5% yeast extract, 1.0% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 2% xylose). The culture was incubated for approximately 24 h until the xylose was almost completely consumed. For xylose fed-batch cultivation, CfCLE production was induced by adding feed medium (0.1% yeast extract, 0.2% $(NH_4)_2SO_4$, and 24% xylose) at a feeding rate of 500 ml/d using a peristaltic pump. Xylose and other nutrients were separately autoclaved (121 °C, 20 min).

The cultivation conditions were as follows: dissolved oxygen (DO) was maintained at over 25% of the saturation value; pH was maintained at 5.0 using 14% ammonia solution (which was also