Chapter 143
Isolation and Identification of a Bacterial Strain with Pullulanase Activity and the Cloning of the Pullulanase Gene

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Abstract A novel bacterial strain OPF-0031 with pullulanase activity was isolated from mud sample of the sea. With the analysis of the 16S rDNA sequence and the characterization of biochemical reactions, the strain was identified to be *Bacillus cereus*. A 2641 bp DNA fragment including a pullulanase gene from the genome of OPF-0031, was present. The nucleotide sequence of the gene encoding pullulanase was cloned and analyzed in *Escherichia coli*.

Keywords Gene cloning · Isolation · 16SrDNA · Pullulan hydrolase activity · Phylogenetic dendrogram

143.1 Introduction

Pullulanases are widely distributed among animals, plants, fungi, and bacteria, which hydrolyze the α-1,6-glucosidic linkages in pullulan, amylopectin, starch and related oligosaccharides [1].

Since a variety of microbial pullulanases have been obtained, they have been classified into four groups based on substrate specificities and reaction products [2,

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3]: (i) neopullulanase (EC 3.2.1.135): pullulan hydrolase type I attacks α-1,4 glycosidic linkages in pullulan, forming panose; (ii) isopullulanase (EC 3.2.1.57): pullulan hydrolase type II can hydrolyze α-1,4 glycosidic linkages in pullulan, forming isopanose; (iii) In branched oligosaccharides or pullulan, pullulanase type I (EC 3.2.1.41) specifically attacks α-1,6 glycosidic linkages, linear oligomers or maltotriose were formed, respectively; and (iv) amylopullulanase: pullulanase type II attacks both α-1,6 glycosidic linkages in pullulan and branched substrates and the α-1,4 glycosidic linkages in polysaccharides.

Based on its characteristic, pullulanase has attracted significant attention as a useful tool in starch industry. So far, high value of pullulanase has been applied in the preparing resistant starch, cyclodextrin, maltotriose syrup, beer brewing, and maltotriose syrup.

To date, a large number of different microorganisms with pullulanases activity have been characterized [4–7]. Some of the isolates have been identified to be moderately aerobic thermophilic bacteria *Bacillus acidopullulyticus* [8], Bacillus flavocaldarius KP 1228 [9], *Thermus aquaticus* YT-1 [10], *Thermus caldophilus* GK-24 [11], extreme anaerobic bacterium *Thermotoga maritima* [12], and *Anaerobranca gottschalkii* [13]. Due to high temperatures of 95–105 Centigrade running in the liquefaction process of starch, the absence of thermostable pullulanase has been essential [14]. Most of strains have no potential interest in the industries, for their low expression level or thermolability. Some strategies which include optimization of the culture conditions [15] and the construction of mutant strain et al. were carried out to enhance the enzyme yield from the wild strains by many investigators. To change the culture condition of pullulanase production, some genes of hyperthermophilic strains have been cloned and expressed by the inducer appearance in the host *Escherichia coli* [16] and *B. Subtilis* [17]. Mutant strain, which is catabolite repression resistant, was also screened for enzyme synthesis [18]. However, low biomass yield and complicated culture conditions of recombinant and wild strains lead to higher production costs. Up to now, the only strains used for enzyme industrial production, were *Bacillus acidopullulyticus* and *Bacillus deramificans* from Novo and Genencor respectively. In this study, the work which focuses on the isolation and identification of the microorganism with pullulanase activity was described. Based on the physiological and biochemical characteristics and the analysis of 16S rDNA gene sequence, the strain OPF-0031 was classified to be genus *Bacillus cereus*. Furthermore, the gene encoding pullulanase has been obtained and analyzed, which would be used in the expression of the recombinant enzyme.

143.2 Materials and Methods

143.2.1 Culture Conditions

The enrichment medium contained, per liter: 15 g glutinous rice starch, 10 g peptone, 2 g yeast powder, 0.5 g $MgSO_4 \bullet 7H_2O$, 1 g NaCl, 1 g K_2HPO_4, pH 7.0.

Screening agar plates contained, per liter: 10 g glutinous rice starch, 5 g peptone, 0.1 g Na_2HPO_4, 0.15 g KH_2PO_4, 0.5 g $MgSO_4 \bullet 7H_20$, 1 g NaCl, 18 g agar, pH 7.0.

Fermentation cultures contained, per liter: 15 g soluble starch, 10 g peptone, 5 g yeast powder, 0.5 g $MgSO_4 \bullet 7H_2O$, 1 g NaCl, 1 g K_2HPO_4, pH 7.0.

143.2.2 Pullulan-Utilizing Strain Isolation

5 g soil samples were collected from the sea in China and diluted with 50 mL of sterile physiological water.

1 mL of diluted mud soil were inoculated in 50 mL of sterile enrichment medium in a 250 mL flask and were grown with shaking at 200 rpm in an incubator at 32 °C. After 24 h of incubation, a 1 mL enrichment medium was diluted to 10–7 dilution of the original soil sample with sterile physiological water. The serial dilutions spreaded on solid medium were incubated at 32 °C for 48 h. To ensure the selected isolations containing pullulanase degrading strains, they were inoculated in fermentation cultures and incubated under the same conditions mentioned above.

143.2.3 Enzyme Assays

One unit of enzyme activity was defined as the amount of enzyme that releases 1 mmol of reducing sugars per minute and expressed as U/mL.

Cell-free fermentation cultures were obtained by centrifugation (8000×g for 10 min at 4 °C) and incubated with pullulan for determining pullulanase activity by measuring the amount of reducing sugars. To 500 μL of 1 % (wt/vol) pullulan dissolved in 300 μL of 0.2 M phosphate buffer (pH 7.0), 200 μL of culture filtrate was added and the samples were incubated at 32 °C for 30 min. The reaction was terminated and the amount of reducing sugars released was assayed by the DNS. Blanks were prepared to correct for the reducing sugars of fermentation cultures [19].

143.2.4 Identification of Strain OPF-0031

Chromosomal DNA of strain OPF-0031 was obtained with TIANamp Bactria DNA kit (Tiangen Biotech (Beijing) Co., Ltd) and used as template for amplification. Two primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC-GACTT-3′) designed from the conserved zones of 16S rDNA operon in *E. coli*, were used for PCR amplification of the 16S rDNA gene. The genomic DNA of OPF-0031 was used as template with PCR profile carried out at the following temperature: denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 90 s, and the final extension at 72 °C for 90 s. Amplicons were purified using TIANgel Midi Purification (Tiangen Biotech (Beijing) Co., Ltd). After purification, PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. The nucleotide sequences of 16S rRNA gene have been assayed by BLAST search algorithm and public databases of GeneBank.

143.2.5 Molecular Cloning of a Gene Encoding Pullulanase

Two primers BC-1 (5′-GGAATTCCATATGGT-GCAAATTACAAAA-3′) and BC-2 (5′-CGGGATCC-TTATTTAATCGGTTTCTCT-3′) designed from the conserved zones of pullanase gene, were used for PCR amplification and sequencing of the gene encoding pullulanase. The sample with genomic DNA of OPF-0031 as the template was heat-denatured for 7 min at 94 °C and the thermal profile consisted of 32 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, and extension at 72 °C for 3.30 min, and the final extension at 72 °C for 8 min. The PCR product linked with PMD19-T vector for sequencing was cloned into *E. coli* [20]. The recombinant stains were spread on LB agar plates for 16 h at 37 °C of positive clones allowed the identification of recombinant plasmid carrying the pullulanase gene (pulOPF-0031) from OPF-0031. The nucleotide sequences of recombinant plasmid have been assayed by Sangon Biotech (Shanghai) Co., Ltd. After gel electrophoresis, the PCR products was isolated with a TIANgel Midi Purification (Tiangen Biotech (Beijing) Co., Ltd). Recombinant plasmids were purified with a TIANprep Mini Plasmid kit (Tiangen Biotech (Beijing) Co., Ltd).

143.3 Results and Discussion

143.3.1 Isolation and Physiological Characteristics of the Bacterium

A novel Gram+, nonmotile bacteria named OPF-0031 were isolated from mud samples of the sea in China and showed pullulan hydrolysis activity in flask fermentation. The detailed physiological characteristics of OPF-0031 were investigated and compared with *Bacillus cereus* as shown in Table 143.1. Catalase reaction was positive, as well as liquefaction of gelatin, starch hydrolysis, pullulan reduction, voges-proskauer test, urease, methyl red test, and hydrolysis of casein. However, oxidase reaction was negative; so were tests for hydrolysis of chitin. The isolate OPF-0031 could utilize glucose, fructose, and maltose. But arabinose, mannose, sucrose, lactose, galactose, and D-sorbitol were not observed to be resolved. Pellicle was not observed in process of the fermentation, too.

Table 143.1 Comparison of conventional chemical characteristics for strain OPF-0031 and *Bacillus Cereus*

Characteristics	OPF-0031	*Bacillus cereus*
Catalase	+	+
Hydrolysis of gelatin	+	+
Hydrolysis of starch	+	+
Voges-Proskauer test	+	+
Methyl red test	+	+
Urease	+	−
Hydrolysis of casein	+	+
Hydrolysis of chitin	−	+
Glucose	+	+
Fructose	+	+
Maltose	+	+
Arabinose	−	+
Mannose	−	−
Sucrose	−	+
Lactose	−	−
Galactose	−	−
D-sorbitol	−	−
Pellicle	−	−

+ Positive, − negative

143.3.2 Identification of Strain Opf-0031

By means of analyzing nucleotide sequences of known bacteria in NCBI, the isolate OPF-0031 were inferred to be the genus Bacillus and showed a closest match (99 %) with *Bacillus cereus* strain Cr-50 (accession no. JF895490.1). The sequence was deposited in the GenBank database with accession no. JQ824137. Based on the physiological characteristics and its 16SrDNA gene sequence, the strain OPF-0031 was identified as a number of *Bacillus cereus*, and thus named as *Bacillus cereus* OPF-0031.

143.3.3 Molecular Cloning of a Gene Encoding Pullulanase Activity

The 2641 bp PCR fragment was obtained through the method described above. Several recombinants AmpR clones were tested for pullulanase activity. A total of three clones, which carried an insert of 2641 bp pullulanase gene (pulOPF-0031) fragment from *Bacillus cereus* OPF-0031 strain, were detected by colony PCR. The cultivation of three positive clones in LB medium allowed the extraction of the recombinant plasmid. The entire nucleotide sequence of the gene is shown in NCBI.

The sequence of pulOPF-0031 has a very high similarity (99 %) with the pullulanase gene of *Bacillus cereus* F837/76 (accession no. CP003187.1) listed in gene bank. The sequence was deposited in the GenBank database with accession No. JQ707952.

In this study, a bacterial strain, which has been isolated and identified, offers new industrial opportunities in the saccharification of starch. The pullulanase reducing unhydrolysed residues may be more compatible with the amylases in the process of amylopectin hydrolysis at high temperatures. In further research, medium compositions of *Bacillus cereus* OPF-0031 would be optimized and optimal recombinants would also be constructed to improve the yield of pullulanase.

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