Process Biochemistry 47 (2012) 1807–1812

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

Disruption of the pullulan synthetase gene in siderophore-producing *Aureobasidium pullulans* enhances siderophore production and simplifies siderophore extraction

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ARTICLE INFO

Article history:
Received 27 February 2012
Received in revised form 7 June 2012
Accepted 11 June 2012
Available online 29 June 2012

Keywords:
Siderophore
Pullulan synthetase gene
Pullulan
Gene disruption
Extraction

ABSTRACT

Aureobasidium pullulans HN6.2 can produce a high level of both siderophores and pullulan. However, the generation of pullulan negatively affects siderophore purification. In this study, the pullulan synthetase gene (*PULl*) in the siderophore-producing *A. pullulans* HN6.2 was disrupted and the obtained disruptant DPS1 produced much less polysaccharide but yielded more siderophores than *A. pullulans* HN6.2. Additionally, the transcription level of the *PULl* gene in *A. pullulans* HN6.2 was much higher than that of the *PULl* gene in the disruptant DPS1. However, the transcription level of the gene encoding L-ornithine-N^5-monooxygenase in *A. pullulans* HN6.2 and the disruptant DPS1 was not changed. During the chemical extraction process, more siderophores were recovered in the supernatant of the disruptant DPS1 than in the supernatant of *A. pullulans* HN6.2. Furthermore, ethanol addition for pullulan removal could be omitted during siderophore extraction from the supernatant of the disruptant DPS1, indicating that the siderophore extraction can be simplified.

1. Introduction

Iron is one of the essential elements required by virtually all life forms. In the absence of iron, most microorganisms produce a group of low-molecular-weight compounds called siderophores, which have a specific affinity for ferric ions. They are produced as part of a high-affinity iron transport system, which is responsible for solubilizing, chelating and uptaking nutritional iron into cells. However, too much iron in the cell can be detrimental, because in the presence of oxygen, iron can catalyze the production of cell damaging hydroxyl radicals [1]. Therefore, siderophores have many applications in agriculture, such as the prevention of pathogenic growth in soil, medicine, such as the treatment of iron overload conditions and aluminum overload, the biocontrol of bacterial disease, environmental protection, such as the removal of heavy metals and the biodegradation of petroleum and the carbon and nitrogen cycle in the ocean [2]. In the cell, siderophores also play an important role in iron storage and in detoxification, such as the prevention of iron-mediated oxidative stress, cell growth, germination of conidia, asexual conidiogenesis, sexual development and virulence [1].

Many fungi including *Ustilago maydis*, *Rhodotorula mucilaginosa*, *Aspergillus* spp., *Aureobasidium pullulans*, *Schizosaccharomyces pombe*, even many marine-derived fungi, can produce a wide range of hydroxamate siderophores [3,4]. To date, it has been found that *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans* are the only fungi identified that do not produce siderophores [1]. Yeasts produce only hydroxamate-type siderophores [5] and the biosynthetic pathway for hydroxamate-type siderophores has been identified [6]. In our previous studies [3,7], it was shown that the marine-derived *A. pullulans* HN6.2 was able to produce high levels of siderophores. The crude siderophores produced by the yeast strain HN6.2 were able to inhibit the cell growth of *Vibrio anguillarum* and *V. parahaemolyticus*, isolated from diseased marine animals. In another study [8], the purified siderophores produced by the marine-derived *A. pullulans* HN6.2 were found to be fusigens. The first step of fusigen biosynthesis in *A. pullulans* HN6.2 is catalyzed by ornithine N^5-oxygenase and the gene (*SidA*) encoding the ornithine N^5-oxygenase has been cloned, characterized and disrupted [9].

Pullulan is a linear α-D-glucan in which maltotriose repeating units are interconnected by $\alpha(1\rightarrow 6)$ linkages and it is the water-soluble homopolysaccharide produced extracellularly by *A. pullulans* [10]. Although the biosynthetic pathway for pullulan in *A. pullulans* is still unclear, the pullulan synthetase gene (*PUL1*) of *A. pullulans* has been cloned [11]. In our previous studies [8], it was found that the presence of pullulan in the culture complicated the extraction of siderophores. We speculate that the formation of pullulan, which is also a main product of *A. pullulans*, might lead to a reduced siderophore yield. Thus, if pullulan biosynthesis is arrested, the cells will use additional ATP to synthesize more

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http://dx.doi.org/10.1016/j.procbio.2012.06.024

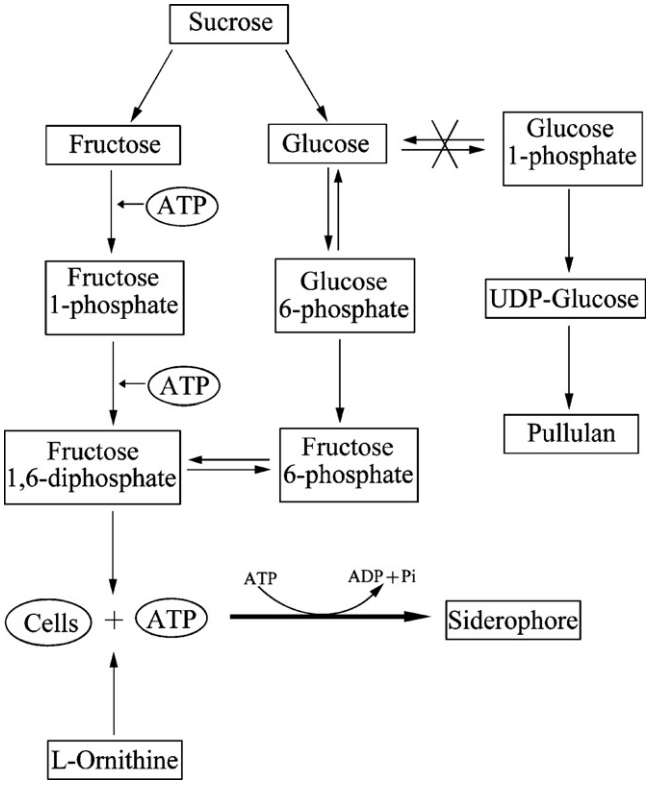

Fig. 1. The relationship between the pullulan biosynthesis pathway and the siderophore biosynthesis pathway in *A. pullulans*. The bold arrow indicates that siderophore biosynthesis is enhanced after pullulan biosynthesis is arrested.

siderophores (Fig. 1). Therefore, in the present study, to increase the yield of siderophores and simplify their purification, we disrupted the *PUL1* gene in *A. pullulans* HN6.2. Then, siderophore production by the disruptants and extraction of siderophores from the cultures were investigated.

2. Materials and methods

2.1. Strains, plasmids and media

A. pullulans HN6.2 isolated from a sea saltern at Yellow Sea was found to produce high levels of siderophores [3]. The marine yeast was maintained and grown in YPD medium [9]. The compositions of the siderophore production medium were described by Chi et al. [9]. Gram-negative and pathogenic bacterium *V. anguillarum* isolated from diseased marine animals was maintained in 2216E medium at 16 °C [12]. The *Escherichia coli* strain used in this study was DH5α [*F*⁻ *endA1 hsdR17*(*rK*⁻/*mK*⁺) *supE44 thi*⁻ *1λ*⁻ *recA1 gyr96*Δ *lacU169*(*φ80lacZ*Δ*M15*)] which was maintained in this laboratory and it was grown in 5.0 ml of Luria broth (LB) in a test tube at 37 °C overnight. The *E. coli* transformants were grown in 5.0 ml of LB medium with 100 μg ampicillin per ml in a test tube at 37 °C overnight. The yeast transformants were grown in HC agar [9]. The plasmid pMD19-T/HPT carrying the promoter of the TEF (translation elongation factor) gene (accession number: U19723), the bacterial hygromycin B phosphotransferase (*HPT*) gene and the terminator (polyA) was constructed in our laboratory [9]. The plasmid pCAMBIA-1381 (accession number: AF234302) carrying the bacterial hygromycin B phosphotransferase (*HPT*) gene and the terminator (polyA) was kindly supplied by Dr. Lining Zhang in Canada. The pMD19-T simple vector, used for the cloning of PCR products, was purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd.

2.2. Isolation of DNA, restriction digestions, and transformation

Yeast genomic DNA for the amplification of the pullulan synthase gene (*PUL1*) in *A. pullulans* HN6.2 was isolated with the TIANamp Yeast Genomic DNA Kits (TIANGEN BIOTECH (Beijing) CO., LTD.). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. [13]. *E. coli* transformants were plated onto LB medium containing 100 μg of ampicillin per ml.

2.3. Construction of the knock-out vector for disruption of the PUL1 gene

Construction of the knock-out vector designed to disrupt the *PUL1* gene was carried out as described by Chi et al. [9,14]. The 5′-arm of the *PUL1* gene was PCR-amplified from the genomic DNA of *A. pullulans* HN6.2 using the primers Pul1F (5′-CTGCAGTCTGCTAGACGTTTCAGAG-3′), in which a *Pst*I site (underlined bases) was added, and Pul1R (5′-CGTCGACGATGTCCTGTTTCTGATG-3′), in which a *Sal*I site (underlined bases) was added after the base C to adjust the annealing temperature. The 3′-arm of the *PUL1* gene was PCR-amplified from the same genomic DNA using the primers Pul2F (5′-GGATCCATGTCTCTTTGACCACCCT-3′), in which a *BamH*I site (underlined bases) was added, and Pul2R (5′-CGCGAATTCCCTTTTCGAGAGTCAAATC-3′), in which an *EcoR*I site (underlined bases) was added after the three bases CGC to adjust the annealing temperature. The primers Pul1F, Pul1R, Pul2F and Pul2R were designed according to the *PUL1* gene (accession number: AF470619). The purified 5′- and 3′-arm fragments were cloned into the pMD19-T simple vector. The recombinant plasmids were transformed into the competent cells of *E. coli* DH5α and the recombinant plasmids extracted from the transformants were digested with *Pst*I, *Sal*I, *BamH*I and *EcoR*1. The 5′-arm fragments obtained were ligated into the plasmid pMD19-T/HPT, digested with *Pst*I and *Sal*I and the resulting plasmid was named pMD19-T/HPT-5′-arm, while the 3′-arm fragments obtained were ligated into the plasmid pMD19-T/HPT-5′-arm digested with *BamH*I and *EcoR*1. The resulting plasmid was named pMD19-T/HPT-PUL and its sequence was verified by DNA sequencing. Finally, the 5′-arm-polyA-HTP-TEF-3′-arm fragments (the knock-out vector) were prepared using PCR with the primers Pul1F and Pul2R as described above.

2.4. Transformation and selection

Transformation of the *A. pullulans* HN6.2 cells was performed using the procedures described by Chi et al. [9]. The putative transformants were verified by cultivation on HC agar (1.5 g of agar per 100 ml of medium in HCS) containing 100 μg of hygromycin B per 100 ml of the medium. After the determination of the siderophore and polysaccharide yield in the supernatant of the putative transformants as described below, it was found that the disruptant DPS1 only synthesized trace amounts of polysaccharide, but it synthesized more fusigen than the wild-type strain. Therefore, the disruptant DPS1 was used in the subsequent investigation.

2.5. Confirmation of the disrupted PUL1 gene

One cell loop of the yeast strain *A. pullulans* HN6.2 and the disruptant DPS1 was transferred into 50.0 ml of YPD medium in a 250-ml flask and aerobically cultivated for 18 h. The cells were collected and washed by centrifugation at 5000 × *g* and 4 °C for 10 min. The genomic DNA was extracted as described above and used as the template for PCR with the primers Y1F (5′-GCCAATCCTCCTTCTCTCTTCACTC-3′)Y1R (5′-ACTCTATCAGAGCTTGGTTGACGGC-3′), Y2F (5′-CCTCCTACATCGAAGCTGAAAGCAC-3′) and Y2R (5′-GTTGTAGATGTAGTCGCCGATACCG-3′). The primers Y1F and Y2R were designed according to the sequences of the 5′-arm and 3′-arm as described above, respectively. The primers Y2F and Y1R were designed according to the sequence of the *HPT* gene as described above. The PCR reactions and conditions were performed as described above and the PCR products obtained were separated on an agarose gel. The sizes of the PCR products were estimated using the Automated Documentation and Analysis System (Gene-Genius, USA). The PCR products were sequenced by Shanghai Sangon Company. The disrupted *PUL1* gene was also analyzed by Southern blotting as described by Watanabe et al. [15].

2.6. Determination of siderophore

One cell loop of the yeast strain *A. pullulans* HN6.2 and the positive disruptant DPS1 was transferred into 50.0 ml of the medium for siderophore production in a 250-ml flask and aerobically cultivated for 120 h. The cultures were centrifuged at 5000 × *g* and 4 °C for 10 min. The supernatants obtained were used as the crude extracellular siderophore solution. The iodine oxidation test [16] was applied to detect the extracellular hydroxymate siderophores. 1 ml of the crude siderophore solutions obtained above was hydrolyzed with 1.0 ml of 6.0 N H_2SO_4 at 130 °C for 30 min to release hydroxylamines from the siderophores, and 1.0 ml of the hydrolysate was buffered by adding 3.0 ml of sodium acetate solution (35 g of sodium acetate per 100 ml). 1 ml of this sample containing a hydroxylamine derivative was added to 4.0 ml of potassium phosphate buffer (100 mM, pH 7.0) and 0.5 ml of acetic acid solution (25.0 g of acetic acid per 100 ml) containing 1.0 g of sulfanilic acid per 100 ml and 0.2 ml of iodine solution in glacial acetic acid (1.3 g of iodine per 100 ml). After standing for 5–7 min at room temperature, the excess iodine was decolorized by the addition of 0.2 ml of 0.1 N sodium thiosulfate solution. The color was developed by the addition of 0.1 ml of α-naphthylamine solution (0.6 g of α-naphthylamine per 100 ml of water) in acetic acid (30 g of acetic acid per 100 ml of water) and measured at 520 nm after standing for 30 min. Calibration curves were prepared using a solution of hydroxylamine hydrochloride which served as the standard. In each case, the absorbance at 520 nm was measured against the appropriate reagent blank.