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Effect of inulin on efficient production and regulatory biosynthesis of bacillomycin D in *Bacillus subtilis* fmbJ

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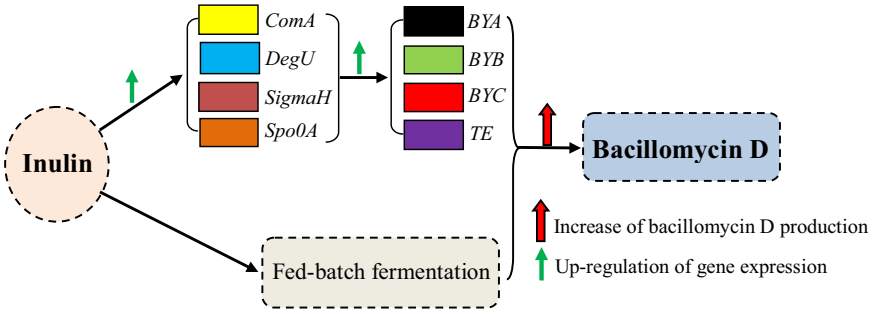
HIGHLIGHTS

- Inulin proved to be efficient in enhancing bacillomycin D biosynthesis.
- Fed-batch fermentation greatly improved the bacillomycin D production.
- Inulin promoted the expression of the bacillomycin D synthetase genes.
- Up-regulation of signal proteins heightened the bacillomycin D production.

GRAPHICAL ABSTRACT



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ABSTRACT

The effect of inulin on the production of bacillomycin D and the levels of mRNA of bacillomycin D synthetase genes: *bmyA* (*BYA*), *bmyB* (*BYB*), *bmyC* (*BYC*), the thioesterase gene (*TE*) and regulating genes: *AbrB*, *ComA*, *DegU*, *PhrC*, *SigmaH* and *Spo0A* in *Bacillus subtilis* fmbJ were investigated. The production of bacillomycin D was enhanced with the increase of biomass concentration. The maximum production and productivity of bacillomycin D were found to be 1227.49 mg/L and 10.23 mg/L h. Inulin significantly improved the expression of bacillomycin D synthetase genes: *bmyA* (*BYA*), *bmyB* (*BYB*), *bmyC* (*BYC*) and the thioesterase gene (*TE*). Also, inulin up-regulated *ComA*, *DegU*, *SigmaH* and *Spo0A* and therefore promoted the high production of bacillomycin D. Our results provided a practical approach for efficient production of bacillomycin D and a meaningful explanation for regulatory mechanism of bacillomycin D biosynthesis.

1. Introduction

Bacillomycin D that contained a β-amino fatty acid with 14–15 carbon atoms and a cyclic peptide with seven amino acids belonged to the iturin family and had strong antifungal, hemolytic and anticancer activities (Gong et al., 2014; Hajare et al., 2013; Nasir and Besson, 2012). Bacillomycin D was cyclic peptide biosynthesized by the non-ribosomal peptide synthetases (NRPSs) *BYA*, *BYB* and *BYC* (Stein, 2005). *BYA*, *BYB* and *BYC* contained amino acid-activating modules that encoded the peptide, forming

Abbreviations: ATP, adenosine triphosphate; BEM, beef extract medium; *BYA*, *bmyA*; *BYB*, *bmyB*; *BYC*, *bmyC*; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonate; HPLC–MS/MS, high performance liquid chromatography–mass spectrometry/mass spectrometry; LM0, a modified Landy medium; NRPSs, non-ribosomal peptide synthetases; PBS, phosphate buffered saline; RP-HPLC, reverse-phase high performance liquid chromatography; RT-qPCR, quantitative real-time reverse-transcriptase polymerase chain reaction; *TE*, thioesterase gene.

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subunits of bacillomycin D synthetase. Particularly, a thioesterase domain encoded by *TE* located at the end of C-terminal of *BYC* is responsible for the cyclization and release of the lipoheptapeptide intermediate (Chen et al., 2009). During the course of bacillomycin D synthesis, signal regulators directly or indirectly affected the activity of bacillomycin D synthetase. Previous research showed that DegU, DegQ, ComA and the minor sigma factors: SigmaB and SigmaH positively influenced the transcriptional activation of the *bmy* promoter (Chen et al., 2009; Koumoutsi et al., 2007).

Many researches focused on the structure, characteristic (Nasir and Besson, 2012) and regulation biosynthesis (Koumoutsi et al., 2007) of bacillomycin D, but no publication has systematically studied the production of bacillomycin D. Therefore, effectively increasing the production of bacillomycin D has great significance on enlarging industrial applications of bacillomycin D. Inulin is a type of carbohydrate, fructan that is composed of fructose. As a kind of renewable raw material, inulin had been reported as a promising substance to produce target products, such bioethanol and fructose syrup (Apolinário et al., 2014; Nakamura et al., 1995).

In this study, effects of different carbohydrates on the production of bacillomycin D were investigated. The fed-batch of inulin aiming at improving bacillomycin D production was performed. To explore the regulatory mechanism of inulin on bacillomycin D biosynthesis, the expression profiling of bacillomycin D synthetase genes (*BYA*, *BYB*, *BYC* and *TE*) and regulating genes (*AbrB*, *ComA*, *DegU*, *PhrC*, *SigmaH* and *Spo0A*) were determined by using quantitative real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) and the relationship between the adenosine triphosphate (ATP) metabolism and inulin consumption was analyzed.

2. Methods

2.1. Microorganism and culture medium

The *Bacillus subtilis* fmbJ strain described in the previous work (Gong et al., 2014) was used in this study. *B. subtilis* fmbJ strain was cultured in beef extract medium (BEM) for seed culture at 37 °C. BEM contained 3 g/L beef extract, 10 g/L peptone and 5 g/L NaCl. Components of the modified Landy medium (LM0) (Landy et al., 1948) were as follows: 1 g/L yeast extract, 5 g/L L-glutamic acid, 1.0 g/L KH_2PO_4, 0.16 mg/L $CuSO_4{\cdot}5H_2O$, 0.5 g/L $MgSO_4{\cdot}7H_2O$. LM0 was used as the fermentation medium, supplemented with different carbohydrates.

2.2. Fermentation methods

The seed culture was carried out in a 250 ml shake-flask containing 100 ml BEM at shaked at 180 rpm, 37 °C for 18 h. 5% (v/v) cultures were then inoculated into LM0 with addition of 20 g/L D-fructose, maltose, sucrose, inulin, alpha-lactose, D-xylose, beta-cyclodextrin, soluble starch and D-glucose, respectively. The fermentations were performed in a 250 ml shake-flask at 33 °C and 180 rpm for 60 h. To further enhance the accumulation of bacillomycin D, the inulin was respectively added at 36, 48, 60 and 72 h during fed-batch fermentation.

2.3. Characterization and determination of bacillomycin D

The supernatant of fermentation broth was obtained after 5000*g* centrifugation, and then was adjusted to pH 2 with 4 M HCl and stayed overnight at 4 °C. After that, 100% methanol was added to the precipitation amassed from the supernatant by re-centrifugating. After adjusting the pH to 7 again using 4 M NaOH, the precipitation was adequately extracted and the crude bacillomycin D samples were prepared after centrifuging at 10,000*g* for 15 min. To calculate the production of bacillomycin D, the bacillomycin D sample was purified using reverse-phase high performance liquid chromatography (RP-HPLC) and the composition of the high-grade bacillomycin D was analyzed by high performance liquid chromatography–mass spectrometry/mass spectrometry (HPLC–MS/MS) as described in previous work (Gong et al., 2014). The typical regression equations of the standard curves were as follows: bacillomycin D: $y = 7.6396x - 2.3576$, $R^2 = 0.9999$; where y represented the peak area (mAU·h) and x represented the concentration of bacillomycin D (mg/L). According to the standard curve, the production of bacillomycin D was determined.

2.4. Thioesterase assay

Thioesterase activity was performed using [5,5′-dithio-bis (2-nitrobenzoic acid)] (DTNB) method, as described previously (Yokoyama et al., 2009), with slight modification. Details were as follows: Cells were harvested at 8000 g for 10 min and resuspended at 4 °C in 50 mL of 0.02 M phosphate buffered saline (PBS) containing 1% Triton X-100, followed by cell lysis using sonication. Cell lysate was centrifuged at 10,000*g* for 10 min. The enzyme concentration was determined by the manufacture's instruction using Branford Assay Kit (Beyotime, Nantong, China). The enzyme activity was measured using lauroyl coenzyme A as substrate. The solution contained 6 ml of 100 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonate (HEPES) (pH 7.4), 20 mM NaCl, 10 mM DTNB and 30 μl of 100 μM substrate was used as the reaction mixture. The reaction was performed by adding 30 μl of thioesterase to reaction mixture at 37 °C in the standard condition and the absorbance of 5-thio-2-nitrobenzoate was measured at 412 nm for 5 min. One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol of lauroyl coenzyme A per minute under the standard condition.

2.5. RNA extraction and RT-qPCR

Total RNA was isolated using Trizol Reagent (Sangon, Shanghai, China) in accordance with the manufacturer's protocol. The quality of RNA was assessed using electrophoresis on a 1.2% agarose gel and examined by NanoDrop2000 (Thermo Scientific, USA). 1 μg of total RNA was used for cDNA synthesis with HiScript™ 1st Strand cDNA Synthesis Kit (Vazyme, USA) according to the manufacturer's instructions. The RT-qPCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using SYBR Premix ExTaqTMII (TaKaRa, Dalian, China) to analyze the expression of target genes (*BYA*, *BYB*, *BYC*, *TE*, *AbrB*, *ComA*, *DegU*, *PhrC*, *SigmaH* and *Spo0A*). The reaction was performed in triplicate with a total volume of 20 μl containing 2× SYBR Premix Ex TaqII10 μl, PCR Primer (10 μM) 1.0 μl and ddH_2O 6.0 μl. The PCR program was 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s. The primers used for amplification of target genes and the reference gene 16sRNA were listed in Table S1.

2.6. Analytical methods

The biomass concentration of cell growth was measured to be as follows. 40 ml fermentation broth was collected and centrifuged at 10,000*g* for 5 min. The resultant cell pellet was then washed with distilled water and re-centrifuged. The wet cells were dried at 85 °C until a constant weight and biomass concentration was determined as the average of the three repeated experiments. The spore number after 60 h of culture was determined as heat-resistant method (Zhu et al., 2012). The cells obtained from the fermentation broth were absolutely lysed in an ice-cold ATP-releasing buffer and centrifuged at 12,000*g* for 10 min. Then, the supernatant was collected for determination of ATP concentration. ATP was