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Biosynthesis of 4-vinylguaiacol from crude ferulic acid by *Bacillus licheniformis* DLF-17056

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ABSTRACT

4-vinylguaiacol, a kind of volatile phenolic compound with tobacco flavor, is widely used as a component of edible flavor and intermediate of medicine. Ferulic acid is usually used as substrate for the biosynthesis of 4-vinylguaiacol. However, the price of ferulic acid is high, leading to high production cost. In this study, a feasible low-cost process for the production of 4-vinylguaiacol was developed. The ultrasonic assisted weak alkali was used to extract protein from rice bran, and waste liquid and residue were then mixed to extract crude ferulic acid by alkaline hydrolysis. Subsequently crude ferulic acid without further purification was directly converted into 4-vinylguaiacol via alginate-immo cells of the strain *Bacillus licheniformis* DLF-17056, which was newly isolated and highly active with ferulic acid conversion to 4-vinylguaiacol. 4-Vinylguaiacol could be produced up to 0.76 g/L from 1.0 g/L ferulic acid within 24 h biotransformation. Furthermore, the immobilized biocatalysts retained above 60% initial activity even after 8 times biotransformations. Thereby, it was assumed that our study would contribute to the industrial production of 4-vinylguaiacol from ferulic acid.

1. Introduction

Rice bran, an undervalued by-product of rice milling, contains about 12–15% high-quality protein (Fabian and Ju, 2011). The most common method for the production of rice bran protein is alkali extraction and followed by acid precipitation (Sereewatthanawut et al., 2008). A large amount of waste residue and waste water will be generated in the process of protein extraction. In fact, waste residue of rice bran still contains some valuable components – for example, ferulic acid (FA) (Fabian et al., 2010) and some phytochemicals. However, most of the waste residue from protein extraction is used as an ingredient in animal feeds, or as a fuel in boilers, which is a waste of natural resources.

4-Vinylguaiacol (4-VG) is a volatile phenolic compound, which is widely used in food, beverage, medicine and spice, and plays an increasingly important role in our life. It possesses the spicy clove-like aroma and is an appreciable flavor constituent in German Rauch beers and Belgian wheat (Mathew et al., 2007). Meanwhile, 4-VG has medicinal value because of its possible anti-cancer activity (Jeong and Jeong, 2010) and anti-oxidant activity (Bortolomeazzi et al., 2007). 4-VG can be produced by chemical synthesis, extracted from natural plant, or synthesized by biotransformation. However, the amount of 4-VG available from natural plant is very limited, which can't meet the increase of consumption demand. Currently, most of 4-VG is produced using chemical synthesis in the market, whereas consumers increasingly favor more environmentally friendly and natural products. And the European and USA food legislation have also stated that "natural flavor substances could be prepared by microbial or enzymatic process" (Li et al., 2008). Hence, the biotransformation has become an appropriate alternative to prepare natural 4-VG.

As a precursor, FA was widely used in the biosynthesis of 4-VG due to their similar molecular structures. In recent years, there have been an increasing number of studies to explore the biotransformation of FA to 4-VG (Hunter et al., 2012; Mishra et al., 2014). For example, a strain called *Bacillus aryabhattai* BA03 was isolated from contaminated cryovials, and the strain could convert FA into 200 mg/L 4-VG in 48 h (Paz et al., 2016). However, in all previous studies, refined FA with relatively high price was used, leading to high production cost.

The aim of this research was to establish a feasible approach to achieve a better production of 4-VG. Firstly, the strain DLF-17056 with high-ability of biotransformation of FA to 4-VG was isolated and identified. Subsequently, waste residue from the extraction process of rice bran protein was mixed with waste water to obtain crude FA. Finally, crude FA without further purification was rapidly transformed to 4-VG by using alginate-immobilized cells of *Bacillus licheniformis* DLF-17056. The overall technology we developed has the advantages of simple process, environmental friendliness and good economic benefit.

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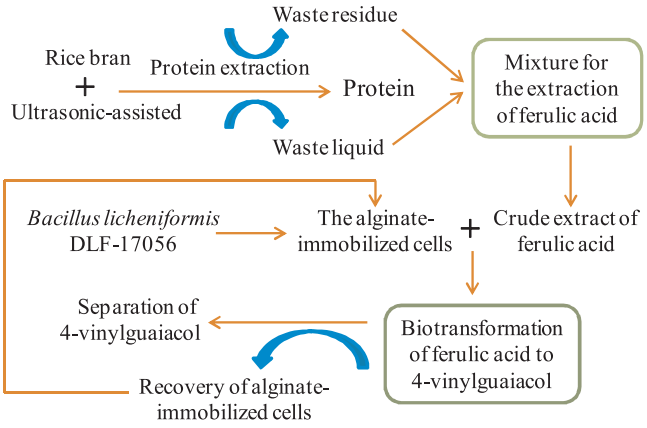

Fig. 1. The overall experiment flow chart.

Meanwhile, it was of great significance in the recycling resource. The simplified experiment process was shown in Fig. 1.

2. Materials and methods

2.1. Chemicals

Fresh rice bran was obtained from Xing-Wang rice bran oil Ltd (Liaoning, China). 4-VG (98%) was purchased from J&K Chemicals (Beijing, China). FA (> 98%), Vanillin (> 98%) and vanillic acid (> 99%) were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). HPLC-grade methanol was purchased from Merch KGaA (Darmstadt, Germany). Other chemicals were of analytical grade.

2.2. Microorganism

2.2.1. Medium

The growth medium for screening the microorganisms to produce 4-VG followed a previous report described by Ghosh et al (2007) with some minor modifications, and its main components were as follows: glucose 5.0 g/L, yeast extract 2.0 g/L, KH_2PO_4 1.0 g/L, $Na_2HPO_4{\cdot}12H_2O$ 4.0 g/L, $CaCl_2$ 0.05 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, NaCl 0.2 g/L. The pH of the medium was adjusted to 7.0–7.2, and then autoclaved at 115 °C for 20 min.

2.2.2. Isolation and culture conditions

Soil samples used to isolate the strain were collected from different places of Liaoning Province. Firstly, 5.0 g of each soil sample was suspended in 30 mL of sterile water, and then was serially diluted. The dilutions were spread on solid growth medium containing about 1.0 g/L FA, and incubated at 35 °C for 48–72 h. The resulting isolated single colonies were grown aerobically at 35 °C for 24 h in a 250 mL Erlenmeyer flask containing 50 mL of media with a shaking speed of 180 rpm. The target strain was screened out by determination of 4-VG in fermentation broth. Finally, the strain was purified and stored at −20 °C in growth medium containing 30% (v/v) glycerol for further use.

2.2.3. Identification and characterization

Cell morphology of strain DLF-17056 was observed by using a microscope (Leica DM 4000B, Germany). The 16S rDNA gene of strain was amplified and sequenced according to a previously described method (Shi et al., 2006). The primers used for amplification of 16 s rDNA gene were as follows: 27 F (5′-AGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplified PCR products containing the 16S rDNA were sequenced by Sangon Biotechnolgy Co., Ltd (Shanghai, China). The sequences determined in this study were aligned with the 16 s rDNA sequences retrieved from the GeneBank database. The physiological and biochemical properties were investigated by using VITEK 2 Compact (BioMerieux Inc., France). Preliminary physiological and biochemical characterization tests were performed as described in Bergey's Manual of Systematic Bacteriology (8th edn, 1984).

2.3. Fermentation optimization

The affecting factors on biotransformation efficiency were investigated, such as: varieties of carbon source and nitrogen source, concentrations of carbon source and nitrogen source, pH, and temperature. On the basis of above optimization results, FA was added into culture medium to give a concentrations of 1.0, 2.0, 4.0 and 8.0 g/L, respectively to investigate the effect of initial substrate concentration on the biotransformation of FA to 4-VG.

2.4. Preparation of rice bran protein

Rice bran protein was extracted by using ultrasonic assisted weak alkali according to our previous report (Sun et al., 2017).

2.5. Preparation of crude FA from rice bran residue

After extraction of protein, the waste residue of rice bran was mixed with residual wastewater of acid precipitation at a solid/solvent ratio of 1:5-25. Subsequently NaOH was added into the mixture to give a concentration of 0.1–2.0% (w/v). The mixture was heated at 60-80°C for 2–5 h, and was centrifuged at 10,000 × g for 10 min. The FA extract was collected, and was then filtered. Crude FA was collected by solvent extraction (ethyl acetate) and decompressing distillation.

2.6. Immobilization of bacterial cells

The alginate-immobilized cells were prepared according to the method of Zhang et al (2017). Bacteria was cultivated in growth media for 16 h to reach the stationary phase (The growth curve was shown in Fig. S1), and was then centrifuged at 9000 × g for 10 min at 4°C. Subsequently, the cells were fully mixed with a solution of 3% sodium alginate to give the final cell concentration of 5%. Mixture was dropped into 3% calcium chloride solution via an injector at 4°C for 16 h to gelate. The immobilized cells were washed with sterile water and stored at 4°C for further use.

2.7. Biotransformation of crude FA to 4-VG

The alginate-immobilized cell pellets were added into 30 mL of crude FA with a ratio of 1:5 (v/v) in a 250 mL Erlenmeyer flask. And incubation was then performed at 40 °C and pH 7.0 with a shaking speed of 120 rpm. Additionally, alginate-immobilized cell pellets would be recovered and reused after translation.

2.8. Analytical method

The concentrations of FA and 4-VG were determined by using a HPLC (Agilent HPLC-1260 Infinity II, USA) equipped with a C18 column (4.6 mm × 150 mm, 5 μm), and a VWD detector. A previous analysis method described by Yang et al (2009) was used with some modifications. The samples were taken at selected times, centrifuged at 8000 × g for 10 min. The supernatant fluid was diluted 20 times with 30% (v/v) methanol, and then was filtered through 0.22 μm filter membrane. The mobile phase was methanol and 0.5% (v/v) acetic acid solution (30:70, v/v) with a flow rate of 1.0 mL/min, and the detection wavelength was 280 nm. The injection volume of each sample was 10 μL. The bioconversion yield was calculated by the following equation: