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Precursors and metabolic pathway for guaiacol production by *Alicyclobacillus acidoterrestris*

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ABSTRACT

Alicyclobacillus acidoterrestris has recently received much attention due to its implication in the spoilage of pasteurized fruit juices, which was manifested by the production of guaiacol. Vanillic acid and vanillin have been accepted as the biochemical precursors of guaiacol in fruit juices. The purpose of this study was to try to find other precursors and elucidate details about the conversion of vanillic acid and vanillin to guaiacol by *A. acidoterrestris*. Four potential substrates including ferulic acid, catechol, phenylalanine and tyrosine were analyzed, but they could not be metabolized to guaiacol by all the thirty *A. acidoterrestris* strains tested. Resting cell studies and enzyme assays demonstrated that vanillin was reduced to vanillyl alcohol by NADPH-dependent vanillin reductase and oxidized to vanillic acid by NAD(P)$^+$-dependent vanillin dehydrogenases in *A. acidoterrestris* DSM 3923. Vanillic acid underwent a nonoxidative decarboxylation to guaiacol. The reversible vanillic acid decarboxylase involved was oxygen insensitive and pyridine nucleotide-independent.

1. Introduction

Alicyclobacillus acidoterrestris are Gram-positive, rod-shaped, nonpathogenic, thermo-acidophilic and spore-forming bacteria, which can cause commercially pasteurized fruit juices/beverages to spoil (Chang and Kang, 2004; Hunniger et al., 2015; Jiao et al., 2015). The spoilage is characterized by the formation of a distinct medicinal or antiseptic off-odor attributed to guaiacol, which at a level of 2 μg/L can be detected by sensory means in fruit juices (Orr et al., 2000; Siegmund and Pollinger-Zierler, 2006). Since the first incidence of apple juice spoilage linked to *A. acidoterrestris* in 1984, this bacteria has been isolated from various fruit juices, such as apple, grapefruit, orange, mango, pear, passion, kiwi and so on (Durak et al., 2010; Oteiza et al., 2011; Wang et al., 2014; Zhang et al., 2013). The origin of *A. acidoterrestris* isolates is considered primarily to be from soil, which can easily come into contact with and adhere to the surface of the leaves and fruit in the orchard (Wisse and Parish, 1998; Yokota et al., 2008). *A. acidoterrestris*-related problems are relatively widespread and have brought about large economic losses to juice manufacturers (Howard, 2006; Torlak, 2014; Walls and Chuyate, 1998). Therefore, there is an urgent need to demonstrate clearly how guaiacol is produced by *A. acidoterrestris*.

It has been accepted that guaiacol is produced from vanillic acid and vanillin in fruit juices by *A. acidoterrestris* (Chang et al., 2015; Chang and Kang, 2004; Smit et al., 2011; Yue et al., 2014). However, details of the conversions are still unavailable. Much of what is known about guaiacol formation by *A. acidoterrestris* is speculated from studies conducted with *Bacillus megaterium*, *Rhodotorula rubra* and *Streptomyces* spp. (Crawford and Olson, 1978; Huang et al., 1993; Lefebvre et al., 1983; Pometto et al., 1981). Additionally, guaiacol may also be formed from ferulic acid, catechol, phenylalanine and tyrosine according to the literature (Huang et al., 1993; Jensen, 2000; Mageroy et al., 2012; Priefert et al., 2001). In this study, the four possible substrates were assayed for their abilities to form guaiacol in the presence of *A. acidoterrestris*. Resting cell studies were conducted to confirm all the products in the metabolism of vanillic acid and vanillin; the enzymes involved in the metabolic pathways were also verified.

2. Materials and methods

2.1. Chemicals

Vanillic acid (≥97.0% purum), vanillin (99%), vanillyl alcohol (≥98%), catechol (≥99%), ferulic acid (≥99%), guaiacol (pharmaceutical secondary standard; traceable to USP and PhEur), *S*-adenosyl-L-methionine (SAM), adenosine triphosphate (ATP) and dithiothreitol (DTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Tyrosine (99%), phenylalanine (99%), the pyridine nucleotides (NAD, NADH, NADP, NADPH) were from Aladdin Industrial Corporation (Shanghai, China). HPLC-grade acetonitrile was bought from Tedia (Fairfield, USA). Other commonly used chemicals and reagents were of analytical grade and from local suppliers at high purity.

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2.2. Microorganisms and growth medium

A total of thirty strains of *A. acidoterrestris* were used in this study. DSM 3922, DSM 3923, DSM 3924, and DSM 2498 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). AAT10, AAT11, AAT12, AAT13, AAT14, AAT92, AAT93, AAT94, AAT95, and AAT96 were generously provided by Keiichi Goto, Food Research Laboratories, Mitsui Norin Company Limited, Tokyo, Japan (Wang et al., 2010). LC-4, C-ZJB-12-01, C-ZJB-12-04, C-ZJB-12-08, C-ZJB-12-10, C-ZJB-12-11, C-ZJB-12-18, C-ZJB-12-23, C-ZJB-12-28, C-ZJB-12-34, C-ZJB-12-40, C-ZJB-12-42, C-ZJB-12-44, C-ZJB-12-63, C-ZJB-12-71, and C-ZJB-12-73 were obtained from microbiology laboratory of the College of Food Science and Engineering, Northwest A&F University, Yangling, China (Yue et al., 2013; Zhang et al., 2013). Yeast Starch Glucose (YSG) broth containing (w/v) 0.1% glucose, 0.2% yeast extract and 0.2% soluble starch in distilled water was used as the basal medium. The pH was adjusted to 3.7–4.0 with 1 M H_2SO_4.

2.3. Guaiacol formation from selected phenolic compounds and amino acids

Ferulic acid, vanillic acid, vanillin and catechol were separately dissolved in 50% (v/v) ethanol, while phenylalanine and tyrosine were dissolved in 0.1 M sulfuric acid. The concentration of the substrate solution was adjusted to 500 μM. Forty microliters of the filter sterilized (pore size, 0.22 μm) substrate solution was added to 40 mL YSG medium. Then the medium was inoculated with an exponential growth phase preculture (1% inoculum) and incubated on a rotary shaker at 150 rpm at 45 °C. Guaiacol production was determined by HPLC analysis.

2.4. Biotransformation of vanillic acid and vanillin by resting cells

To identify all the intermediate products of vanillic acid and vanillin metabolic pathway in *A. acidoterrestris* DSM 3923, resting cell experiments were carried out. Vanillic acid-induced cells were prepared by inoculating 0.8 L of YSG medium plus 500 μM vanillic acid with an exponentially growing preculture (2% inoculum) and incubating at 45 °C under shaking at 150 rpm for 16 h. Vanillin-induced cells were produced in the same way but replacing vanillic aicd with vanillin. Cultures grown in YSG medium without vanillic acid and vanillin were used as non-induced cells. The resulting cells were harvested by centrifugation at 8000 ×g for 10 min at 4 °C and washed twice with citric acid-disodium hydrogen phosphate buffer (pH 4.5). Then, the cell pellets were resuspended in the same buffer to give a turbidity of McFarland 3 (approximately 9×10^8 cells/mL). Vanillic acid or vanillin was added to a final concentration of 500 μM and the mixture incubated at 45 °C with shaking at 150 rpm. The resting cells without the substrate and the heat killed cells (by autoclaving at 121 °C for 15 min) with the substrate were used as controls. Samples were withdrawn periodically and subjected to HPLC and LC–MS/MS analyses for the substrate and its metabolic intermediates.

2.5. Preparation of cell-free extracts

All subsequent procedures were carried out at 4 °C. The induced or non-induced cell pellets of *A. acidoterrestris* DSM 3923 prepared as described in Section 2.3 were suspended in 20 mM cold sodium phosphate buffer (pH 7.0) containing 2 mM DTT and 5% (v/v) glycerol. The cell suspension was sonicated with an ultrasonic disrupter (JY92-IIN, Scientz, Ningbo, China) for a total exposure time of 5 min with 2 s cooling intervals between two 1 s pulses. To prevent samples from overheating, the vessel containing the cell suspension was surrounded by ice and sea salt. Immediately after sonication, the suspension was centrifuged at 16,000 ×g for 15 min. The supernatant was carefully separated to obtain cell-free enzyme extract. Quantitation of protein content in the extract was performed routinely with the Pierce BCA protein assay kit (Thermo Scientific, USA).

2.6. Conversion of vanillic acid and vanillin by cell-free extracts

The ability of cell-free extracts from vanillic acid-induced cells to convert vanillic acid was examined. The entire reaction mixture (1.2 mL) contained 20 mM phosphate buffer (pH 7.0), 2 mM DTT, 0.2 mM $NAD(P)^+$ or NAD(P)H, 0.5 mM vanillic acid and cell-free extract (5 mg protein). Reaction rates with and without pyridine nucleotides were compared. Incubations were carried out at 45 °C with shaking at 150 rpm. Samples were taken out at 4 h and mixed with 0.6 ml of acetonitrile to allow precipitation of protein. Then the mixtures were centrifuged at 10,000 ×g for 10 min; the supernatant was filtered through a 0.22 μm nylon membrane filter and analyzed for vanillic acid and guaiacol by HPLC. Analyses for vanillin-induced cell extracts transforming vanillin were carried out exactly as above except that vanillic acid was replaced by 0.5 mM vanillin. The conversions of vanillin to vanillic acid and vanillyl alcohol in complete reaction mixture were compared with that obtained by cell-free extracts in which cofactors were omitted. The reverse reaction was also measured by replacing vanillin with vanillyl alcohol.

2.7. Enzyme assays

For measurement of vanillin reductase activity, cell-free extracts (5 mg protein) were incubated with phosphate buffer (20 mM, pH 7.0), 2 mM DTT, 0.2 mM NADPH, and 0.5 mM vanillin in a final reaction volume of 1.2 mL for 30 min at 45 °C. The activity of vanillin dehydrogenase was measured at 45 °C in 1.2 mL of reaction mixtures consisting of 20 mM phosphate buffer (pH 7.0), 2 mM DTT, 0.2 mM NAD^+ or $NADP^+$, 0.5 mM vanillin and cell-free extract (5 mg protein). The reaction was conducted for 30 min. Decarboxylation activity of vanillic acid decarboxylase was determined in the assay buffer (20 mM sodium phosphate, pH 7.0, 2 mM DTT) supplemented with 0.5 mM vanillic acid. Reactions were started by adding cell extract (5 mg protein) and incubating the mixture at 45 °C for 1 h. Oxygen sensitivity of the decarboxylases was tested by performing the enzyme assay both aerobically and anaerobically. Carboxylation assays (Lupa et al., 2005) were conducted in the same buffer containing 10 mM guaiacol and 100 mM sodium bicarbonate and incubated as described above. The heat-inactivated enzyme (121 °C, 15 min) was used as a control. The enzyme products including vanillyl alcohol, vanillic acid and guaiacol were quantified by HPLC. The enzyme specific activity was expressed in terms of nmoles of the corresponding product appeared per min per mg protein.

2.8. Analytical methods

Catechol, vanillin, vanillic acid, vanillyl alcohol, ferulic acid and guaiacol were detected by a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a diode array detector and an Ultimate XB-C18 column (4.6 × 250 mm, 5 μm particle size, Welch Materials, Ellicott, USA) (Chang, 2008). The mobile phases consisted of 0.1% (v/v) formic acid in water (phase A) and acetonitrile (phase B). An isocratic elution of 30% (v/v) phase B was conducted at a flow rate of 1 mL/min and the column temperature was set to 40 °C. The signal was monitored at a wavelength of 275 nm. The analysis of tyrosine and phenylalanine was done by an automatic amino acid analyzer (Hitachi L-8900, Tokyo, Japan) (Shim et al., 2013). LC–MS/MS was performed using an API 4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source that was operated in the positive ion mode for vanillic acid and vanillyl alcohol, and negative for guaiacol.