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Short Genome Communications

Solid-state fermentative production of aroma esters by *Myroides* sp. ZB35 and its complete genome sequence

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ABSTRACT

Consumers prefer biotechnological food products with high nutritional values and good flavors. Solid-state fermentation is a commonly used technique with a long history. In the present study, *Myroides* sp. ZB35 was used in solid-state fermentative production of aroma volatiles on a rice medium. Using the headspace solid phase microextraction coupled with gas chromatography–mass spectrometry technique and authentic standards, 22 esters with molecular weight ranging from 102 to 172 were identified. At 192 h, the esters reached a total concentration of 1774 μg/kg. Subsequently, the complete genome of ZB35 was sequenced using the PacBio RS II platform. ZB35 has a single circular chromosome of 4,065,010 bp with a GC content of 34.1% and six putative novel esterase genes were found. ZB35 is the first bacterium here discovered being capable of producing so many kinds of aroma esters. The data revealed here would provide helpful information for further developing this strain as a promising source of aroma esters relevant in food and fragrance industries and the source of novel enzymes with potential usages.

Consumers prefer natural products especially in food, medicine, and cosmetics sectors (Xiao et al., 2014a). Solid-state fermentation (SSF) techniques are usually adopted in traditional food industries (Chen et al., 2014; Juodeikiene et al., 2013) and researchers are pursuing good flavor products with selected strains for human consumption or animal feed (Mantzouridou et al., 2015; Vong and Liu, 2017). In this study, SSF was performed using *Myroides* sp. ZB35, the first bacterium previously discovered to be capable of producing aroma esters including isopentyl 2-methylbutanoate, isopentyl 3-methylbutanoate, 2-methylbutyl 2-methylbutanoate, and 2-methylbutyl 3-methylbutanoate (Xiao et al., 2014b).

The seed culture of ZB35 was prepared by growing the bacterium at 30 °C in 50 mL of Luria-Bertani (LB) broth in a 250-mL baffled flask for 12 h with agitation at 120 rpm. SSF experiments were carried out in triplicates using 550-mL glass bottles each containing 15 g of rice (dry weight). The milled *Keng* rice (*Oryza sativa* L. subsp. *japonica*) was obtained from Yanshou Liangzhu Grain and Oil Trade Co., Ltd. (rice-growing area: Yanshou County, Heilongjiang Province, China; crop year: 2016). The grain (length 5.36 ± 0.69 mm, width 2.61 ± 0.12 mm) contains (w/w) 78.5% of carbohydrate, 6.7% of protein, and 1.1% of fat. After sterilization of the bottles containing rice at 121 °C for 20 min, each bottle was supplemented with 10 mL of sterile LB broth and then inoculated with 0.5 mL of seed culture (about 2.3×10^{10} cells), plugged with autoclaved rubber stopper tightly, and then incubated at 30 °C. Control experiments were performed using heat-killed cells as the inoculum. Samples were analyzed once every two days. The bottles were kept closed with the rubber stoppers throughout the experiment to prevent product evaporation. Note that because the members of the genus *Myroides* are aerobic bacteria (Maraki et al., 2012), we kept a very low loading rate of rice in the bottles (Fig. 1 top right).

The volatile compounds were firstly identified by headspace solid phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC–MS). Briefly, HS-SPME was performed using a manual SPME holder with a 75 μm carboxen-polydimethylsiloxane fiber (Supelco) for 30 min at room temperature. Sampling was performed by piercing the rubber stopper with the SPME needle. GC–MS was conducted using Agilent 7890A-5975C equipped with a 30-m DB-5MS column (J&W Scientific). Mass spectra in the electron impact mode were generated at 70 eV and scan mode in the range of 20–450 amu. The compounds were tentatively identified by comparing their mass spectra with those available in the MS data system. For further confirmation, authentic chemical standards were applied to verify the retention time of each compound using Agilent 7890 B GC

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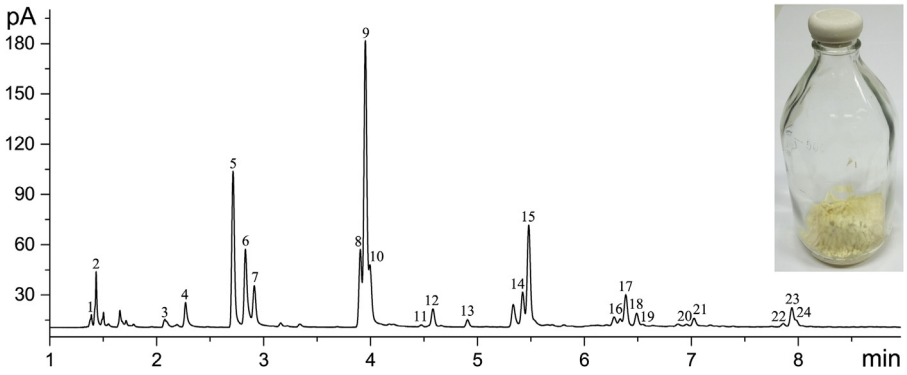

Fig. 1. HS-SPME GC-FID profile of the volatile compounds at 192 h of solid-state fermentation using rice as the main culture medium (top right). The peak numbers are identical to those in Table 1. pA, picoampere.

equipped with a flame ionization detector (GC-FID) and a 30-m HP-5 capillary column (Agilent).

As shown in Fig. 1 and Table 1, 24 volatile compounds were identified and quantified. The concentration of ethyl 3-methylbutanoate (Peak 9) was estimated by the external standard method and the concentrations of other compounds were temporarily estimated by comparing their peak areas with that of ethyl 3-methylbutanoate. Toluene (Peak 6) was detected in all samples including those from control experiments and its concentration remained stable throughout the experiments. We postulated it came from the rubber stopper because toluene is often used in rubber making. The concentrations of other 23 volatile compounds increased gradually during the first 192 h of the SSF experiments and then kept constant. In the previous study, only 4 esters were detected during submerged fermentation using LB broth as the culture medium (Xiao et al., 2014b). However, 18 more kinds of esters were discovered during SSF using rice supplemented with LB medium in this study. The 22 esters reached a total concentration of 1774 μg/kg (of the rice + LB culture medium) at 192 h. Without considering the influences of culture medium, both varieties and concentrations of the esters increased significantly comparing with the previous results of submerged fermentation (Xiao et al., 2014b), indicating that SSF favours the formation of esters. The color of rice changed gradually from white to brown during the experiments. Rice components such as carbohydrate were postulated to be utilized by ZB35 cells to generate some precursors of the esters (Layton and Trinh, 2014).

In order to identify the mechanism of ester production on genomic level, we have sequenced the complete genome of strain ZB35. To date, there are only three complete genomes from the genus *Myroides* available in GenBank database: CP013690 and CP013691 for *Myroides odoratimimus* PR63039, CP010327 for *Myroides* sp. A21, and CP010817 for *Myroides profundi* D25. The information whether these three strains can produce aroma esters is unavailable.

Genomic DNA from *Myroides* sp. ZB35 was extracted using TIANamp Bacteria DNA Kit from TIANGEN Biotech (Beijing) Co., Ltd. Then a 10 kb insert SMRTbell DNA library was constructed and sequenced on the single molecule real-time (SMRT) DNA sequencing platform by the PacBio RS II sequencer (Pacific Biosciences, CA) (Eid et al., 2009). After the filtration of low quality reads, a total of 75,095 qualified reads with mean length of 14,155 bp were *de novo* assembled using the hierarchical genome assembly process (HGAP) (Chin et al., 2013) protocol RS_HGAP_Assembly.3 (Pacific Biosciences, CA) (Jeong et al., 2016), resulting in 261.5 x coverage of one circular chromosome of 4,065,010 bp. The coding sequences (CDSs) were predicted by Prokaryotic Genome Annotation Pipeline (PGAP) version 3.3 software on NCBI (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The features for the complete genome sequence of *Myroides* sp. ZB35 are summarized in Table 2.

Alkanoic acids and alcohols can be synthesized from either fermentative or Ehrlich pathways (Hazelwood et al., 2008; Layton and Trinh, 2014). The biosynthesis pathways of the aroma esters were postulated to be associated with the metabolism of carbohydrates (Layton and Trinh, 2014) and amino acids such as isoleucine, leucine, and valine in *Myroides* sp. ZB35 (Xiao et al., 2014b). Some genes and gene clusters found in the genome of strain ZB35 could contribute to the generation of alkanoic acids and alcohols which can serve as the possible precursors of the aroma esters. As shown in Fig. 2, we postulated the formation pathway of the main ester product ethyl 3-methylbutanoate in *Myroides* sp. ZB35.

Esterification is the rate-limiting step towards aroma ester production, especially when the acid and alcohol substrates exist at very low levels in cells (Xiao et al., 2014b). Alcohol acyltransferases (EC 2.3.1.84) and esterases (EC 3.1.1.1) play roles in ester accumulation in some fungi and plants (Verstrepen et al., 2003). Homologous sequences of alcohol acyltransferase gene cannot be found in the genome of strain ZB35. On the contrary, 6 putative esterases with novel amino acid sequences (with accession numbers APA93062, APA92884, APA91034, APA91179, APA93804, and APA93689 in Fig. 3) can be found and these putative esterases might contribute to the ester biosynthesis in *Myroides* sp. ZB35. Analogues of the other 4 putative esterases (APA91305, APA93104, APA93944, and APA92804 in Fig. 3) can be found in other bacterial species incapable of aroma ester production. Thus these 4 esterases are tentatively excluded from catalyzing the esterification reactions for the aroma esters in strain ZB35. Identification of these novel esterase candidates may be significant, because these enzymes can serve as stereoselective biocatalysts (Romano et al., 2015) and are very useful in the synthesis of optically pure compounds, perfumes, and antioxidants and have important applications in agriculture, food, and pharmaceutical industries (Panda and Gowrishankar, 2005).

In this study, SSF was performed using rice as the main medium component and the abundant esters endowed the rice with a pleasant fruity aroma, revealing the great potential of using this strain as a promising source of aroma esters useful in food and fragrance industries. However, *Myroides* organisms behave as low-grade opportunistic pathogens (Maraki et al., 2012) and toxicological risk assessment should be carried out in future work. The complete genome sequence of *Myroides* sp. ZB35 will help to broaden the current knowledge and speed up its relevant applications especially in the use of aroma esters and novel enzymes.

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