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Biodegradation of C_5-C_8 Fatty Acids and Production of Aroma Volatiles by *Myroides* sp. ZB35 Isolated from Activated Sludge

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In the effluents of a biologically treated wastewater from a heavy oil-refining plant, C_5-C_8 fatty acids including pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, and 2-methylbutanoic acid are often detected. As these residual fatty acids can cause further air and water pollution, a new *Myroides* isolate ZB35 from activated sludge was explored to degrade these C_5-C_8 fatty acids in this study. It was found that the biodegradation process involved a lag phase that became prolonged with increasing acyl chain length when the fatty acids were individually fed to this strain. However, when fed as a mixture, the ones with longer acyl chains were found to become more quickly assimilated. The branched 2-methylbutanoic acid was always the last one to be depleted among the five fatty acids under both conditions. Metabolite analysis revealed one possible origin of short chain fatty acids in the biologically treated wastewater. Aroma volatiles including 2-methylbutyl isovalerate, isoamyl 2-methylbutanoate, isoamyl isovalerate, and 2-methylbutyl 2-methylbutanoate were subsequently identified from ZB35 extracts, linking the source of the fruity odor to these esters excreted by *Myroides* species. To our best knowledge, this is the first finding of these aroma esters in bacteria. From a biotechnological viewpoint, this study has revealed the potential of *Myroides* species as a promising source of aroma esters attractive for food and fragrance industries.

Keywords: *Myroides*, biodegradation, C_5-C_8 fatty acids, aroma esters, pathway

Introduction

Heavy crude oils often contain a high content of molecules bearing various polar groups such as acid and alcohol. These indigenous polar species are mostly amphiphilic and are thus capable of stabilizing emulsions, causing the dispersion of a significant amount of oils and solids into heavy oil refining wastewater. Whilst some of the polar species from crude oils could just be dissolved in the wastewater, most become dispersed through emulsification. There is currently a huge lack of research dedicated to the scientific understanding of breakdown of such emulsions and dispersions and the subsequent separation of the oil from water. This situation together with the fast turnover of a large quantity of the wastewater makes it a hugely challenging task for the fast and efficient processing by the petrochemical industry.

Various technical processes have been developed to deal with the effective treatment of heavy oil refining wastewater. Liaohe Petrochemical Co., the largest heavy oil refining company in China, is currently operating a cyclic activated sludge system to process and purify such wastewater. In the biologically treated effluents, C_5-C_8 fatty acids (FAs) including pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, 2-methylbutanoic acid, and several phenols are often detected by gas chromatography-mass spectrometry (GC-MS) analysis. C_5-C_8 FAs have fetid odors and good solubilities in wastewater and they can cause further downstream pollution. Although they are biodegradable, the removal rates by most existing biotransformation technologies are not entirely satisfactory. There is hence a strong demand for better processing technologies and a crucial part lies in the screening of better microbial strains.

On the basis of the number of carbons, FAs are often classified as short- (less than C_6), medium- (C_6-C_{12}), and long- (more than C_{12}) chain FAs. The biodegradation of short- and medium- chain FAs could be considered as an easy task and there were few publications aiming at it. However in this study, complex degradation phenomena were observed when single or mixed C_5-C_8 FAs were fed to a new *Myroides* isolate. Novel metabolites were also identified from the culture.

The genus *Myroides* was established by reclassification of *Flavobacterium* species in 1996 (Vancanneyt *et al.*, 1996). *Myroides* species are strictly aerobic with good growth on nutrient agar at mesophilic temperatures. Various colony types may occur, but most colonies are yellow pigmented. *Myroides* cells are Gram-negative rods and lack flagella. To date, there are only a handful of studies dedicated to this genus, with most of them dealing with *Myroides*-related infections (e.g. Green *et al.*, 2001; Benedetti *et al.*, 2001) and the discovery of the new species. Several studies have also examined their potential environmental applications (e.g. Maneerat *et al.*, 2006; Li *et al.*, 2012). But overall *Myroides* has not been well-studied so far.

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Materials and Methods

Chemicals

Chemicals used in this work were obtained from commercial sources and used without further purification. Suppliers: butyric acid (99%), Tianjin BASF Chemical Co., Ltd (China); pentanoic acid (99%), Sinopharm Chemical Reagent Co., Ltd (China); hexanoic acid (99%), heptanoic acid (99%), and isoamyl alcohol (98.5%), Tianjin Guangfu technology development Co., Ltd (China); octanoic acid (99%), Tianjin Bodi Chemical Co., Ltd (China); isovaleric acid (99%), 2-methylbutanol (98%), and isoamyl isovalerate (98%), J&K Scientific Ltd (China); 2-methylbutanoic acid (98%), Shanghai Dari Fine Chemicals Co., Ltd (China). Yeast extract and peptone were from Difco (USA).

Following the preparation method of ethyl acetate (Norris, 1924), 2-methylbutyl 2-methylbutanoate, 2-methylbutyl isovalerate, and isoamyl 2-methylbutanoate were synthesized using concentrated sulfuric acid as the catalyst. Briefly, 0.4 ml of alcohol, 0.4 ml of carboxylic acid, and 0.2 ml of sulfuric acid were mixed and controlled at 95°C for 0.5 h in a water bath. Saturated sodium carbonate was then applied to neutralize the sulfuric acid. The products were confirmed by GC-MS and the yields were about 60%.

Microorganism screening and identification

Mineral-salts media (minimal media, MM) supplemented with desired FA were used in the enrichment process. For example, MM including 2-methylbutanoic acid contained (per L deionized water) 200 mg of 2-methylbutanoic acid, 2.28 g of $K_2HPO_4{\cdot}3H_2O$, 0.47 g of $NaH_2PO_4{\cdot}2H_2O$, 1.32 g of $(NH_4)_2SO_4$, 0.12 g of $MgSO_4$, 2.63 mg of $CaCl_2$, 0.72 mg of $FeSO_4{\cdot}7H_2O$, 0.46 mg of $ZnSO_4{\cdot}7H_2O$, and 0.22 mg of $MnSO_4{\cdot}H_2O$. The media were adjusted to pH 7.0 and then autoclaved for 20 min at 121°C.

For the first enrichment, 2 g of activated sludge from Liaohe Petrochemical Co. was added into 100 ml enrichment medium in a 500-ml Erlenmeyer flask and kept in a rotary shaker at 30°C with shaking at 150 rpm. After 2–5 days, the resulting culture was inoculated into a fresh enrichment medium (1%, v/v) and cultivated as described above. The enrichment process was repeated for 3–5 times. The final enrichment cultures were diluted using autoclaved saline and then streaked onto solidified Luria-Bertani medium (LB). After 1–3 days of incubation at 30°C, all the typical colonies (based on colony morphology, color, and size) appeared on the plates were picked and selected after several streaking processes.

16S rRNA genes of the isolates were routinely amplified using universal primers 27 F and 1492R, and sequenced by Shanghai Sangon (China). Searches for authentic homologous species were performed in the database EzTaxon-e (Kim *et al.*, 2012). Scanning electron microscope analysis was carried out using JSM-6390LV (JEOL, USA).

C_5-C_8 FA biodegradation experiments

Five different C_5-C_8 FAs (pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, and 2-methylbutanoic acid) were used in the biodegradation tests. They were supplemented individually (1,000 mg/L alone) or as a mixture (200 mg/L each, total 1,000 mg/L) into MM to serve as the carbon source.

The inoculum for the biodegradation process was prepared in two steps. Firstly, the new isolate was cultured in LB at 30°C with vigorous shaking. Cell growth reached late logarithmic phase at about 12 h and then the culture was used as a seed to be inoculated (2%, v/v) into MM supplemented with C_5-C_8 FA mixtures. About 60 h later, the MM preculture was harvested and used as an inoculum in the following procedure.

Biodegradation experiments were performed in triplicates using 250-ml Erlenmeyer flasks with loading volume of 50 ml each. The process was started with the inoculation of the above MM preculture (6%, v/v) and then controlled at 30°C with shaking at 150 rpm. Samples were collected at different stages for the analysis of cell growth and substrate consumption. Control experiments were carried out using autoclaved inoculums.

Analytical methods

Bacterial growth was measured by recording the optical density at 600 nm. FAs were quantified with an Agilent 7890A GC equipped with a flame ionization detector and a 30-m HP-FFAP (0.32 mm inside diameter, 0.25 μm film thickness) capillary column (19091F-413, Agilent). Butyric acid was used as the internal standard. The column oven was maintained at 40°C for 2 min and then programmed to increase to 280°C at a rate of 20°C/min, then maintained at 280°C for 2 min.

For the identification of metabolites, products in the culture broth were extracted with dichloromethane, concentrated and analyzed using the same GC system but with a 30-m HP-5 (0.32 mm inside diameter, 0.25 μm film thickness) capillary column (19091J-413, Agilent). The column oven was kept constant at 50°C for 2 min, and then programmed to 250°C with a temperature increase of 10°C/min and maintained at 250°C for 38 min. Metabolites were also matched with authentic standards using GC-MS (Agilent 6890 GC equipped with an Agilent DB-35 capillary column and a high resolution TOF-MS from GCT, Waters). The column oven temperature was initially maintained at 60°C for 3 min, then ramped to 250°C at 10°C/min, and finally maintained at 250°C for 2 min. MS in the electron impact mode were generated at 70 eV and scan mode in the range of 60 to 400 amu.

Results

Characterization of the newly isolated microorganism *Myroides* sp. ZB35

ZB35 was one of the isolated FA-degrading strains using 2-methylbutanoic acid as the sole carbon source. It forms smooth surfaced, round regular edged, and yellow pigmented colonies on LB plates (Fig. 1A). Cells are gram-negative rods that are 0.4 to 0.6 μm in diameter and 0.7 to 1.6 μm in length (Fig. 1B). Strain ZB35 grows well aerobically at mesophilic temperatures in neutral media. It produces a fruity odor in LB. The partial 16S rRNA gene sequence accession number in GenBank is KF874659. It shares high pairwise similarities with 16S rRNA gene sequences from 3 *Myroides*

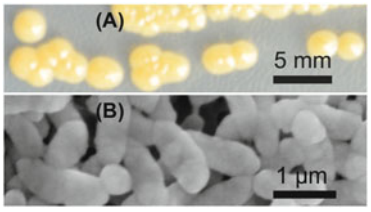

Fig. 1. ***Myroides*** **sp. ZB35 colonies (30°C, 12 h) on LB plates (A) and its scanning electron image (B).**

type strains: 99.38% with sequence AJ854059 from *M. odoratimimus* CCUG 39352, 98.99% with sequence EU204978 from *M. profundi* D25, and 97.36% with sequence GQ857652 from *M. marinus* JS-08. Then the new isolate was named as *Myroides* sp. ZB35 and has been deposited in China Center for Type Culture Collection under the deposition number CCTCC M 2013024.

Biodegradation process of FAs by *Myroides* sp. ZB35

In order to obtain a large amount of cells efficiently, ZB35 was firstly cultured in a rich medium, LB. Then it was transferred into MM (supplemented with the FA mixture) to adapt to the harsh environment before the formal biodegradation test. However, even having been precultured in the harsh medium, bacterial growth suffered obvious lag phases (at least 24 h) when individual FA was supplied as the sole carbon source (Fig. 2A). The FA cleavage curves (Fig. 2B) corresponded to these processes with slow substrate removal rates at early hours. The lag phase was prolonged with increasing FA molecular carbon atom numbers among the 4 straight chain FAs in this study. This phenomenon suggests that as the acyl chains became longer the bacterium appeared to encounter greater difficulties in the biodegradation of the C_5-C_8 straight FAs.

In contrast, an opposite phenomenon was displayed when the mixed C_5-C_8 FAs were fed to strain ZB35. FAs with longer acyl chain were assimilated more quickly (Fig. 3). The total C_5-C_8 FA degradation and bacterial growth curves (Fig. 2, the two curves with stars) are also quite different with those obtained from the use of individual FAs as the carbon source. Without growth and degradation lag phase, *Myroides* sp. ZB35 showed much higher average degradation efficiency. Thus, the depletion of 1000 mg/L of the mixture of the 5 FAs was 85 h; but the average time for the depletion of 1000 mg/L of the 5 individual FAs was 120 h.

Under both conditions, the branched chain FA 2-methylbutanoic acid was always the last one to be depleted, indicating that this molecule was the most difficult one to be degraded among the 5 FAs. Substrate concentration effect on ZB35 growth was then tested in MM with a variable amount of 2-methylbutanoic acid as the sole carbon source. 2-Methylbutanoic acid not only showed no substrate inhibition effect on bacterial growth, but also the biomass yield positively correlated with substrate concentration in the test range from 200 to 1,000 mg/L.

Identification of volatile metabolites

To investigate the possible origins of these volatile FAs emerged in the biologically processed wastewater, the metabolites of *Myroides* sp. ZB35 were extracted from LB culture. Of course, the compositions of LB are extremely far from that of activated sludge. However, both LB and activated sludge contain components needed for bacterial cell growth and LB can produce more repeatable results. Thus tentatively, the pure culture of ZB35 and LB were used to imitate the activated sludge system with great simplicity.

As shown in Fig. 4, ZB35 produces branched chain C_5 FAs, alcohols, and their esters in LB culture. Control experiments with dead cells did not yield any kind of these products. Isoamyl isovalerate and 2-methylbutyl 2-methylbutanoate share not only the exactly same GC retention time, but also highly similar electron ionization MS. The slight MS difference between them only exists in the mass peak at m/z 129

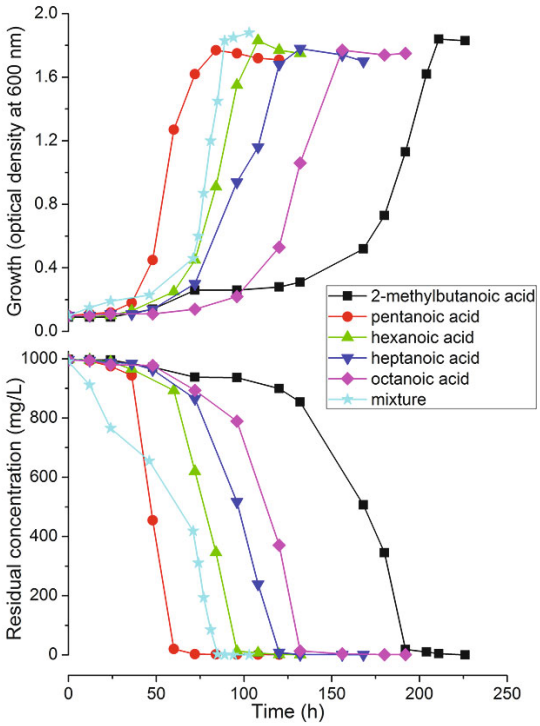

Fig. 2. Process trends of ***Myroides*** **sp. ZB35 growth (A) and the biodegradation profiles of C_5-C_8 FAs in MM (B).** The average relative deviation was less than 11%.

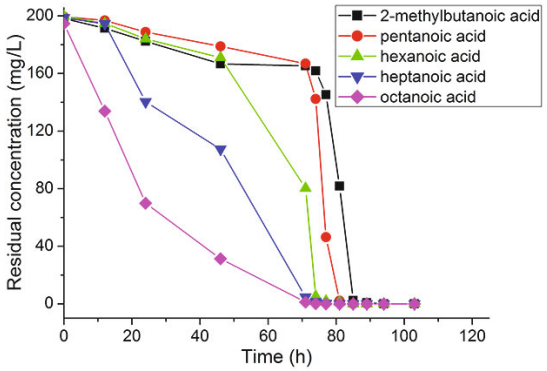

Fig. 3. Sequential biodegradation of C_5-C_8 FAs when ***Myroides*** **sp. ZB35 was cultured in MM supplemented with C_5-C_8 FA mixtures.** The average relative deviation was less than 3%.

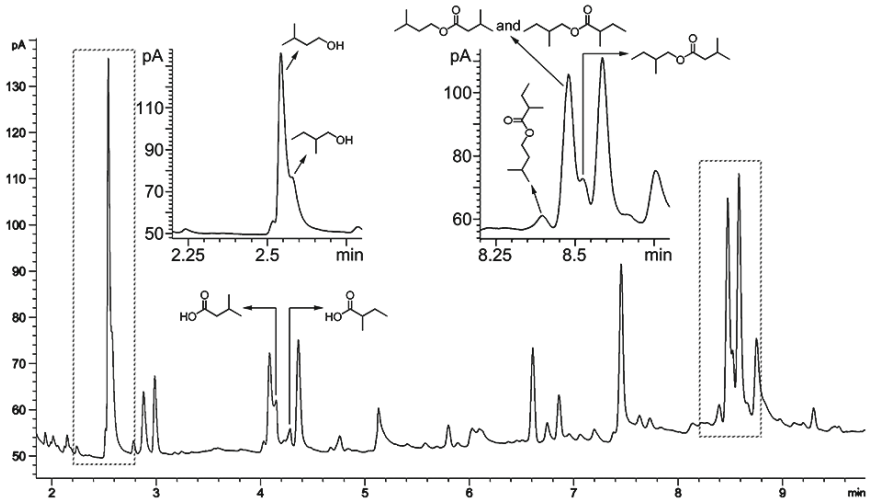

Fig. 4. **The GC profile of volatile metabolites from *Myroides* sp. ZB35 cultured in LB. Areas in the rectangles are enlarged.**

(with relative intensity about 2% and 0%, respectively. However, the measurement of relative intensities is often inaccurate, especially to the mass peaks in low contents. Then if both compounds coexist, GC or/and GC-MS are inadequate to distinguish them. Isoamyl alcohol, 2-methylbutanol, isovaleric acid, and 2-methylbutanoic acid, which can form four esters together, were identified in this study. Isoamyl 2-methylbutanoate and 2-methylbutyl isovalerate were also identified by authentic standards. Both isoamyl isovalerate and 2-methylbutyl 2-methylbutanoate were eluted perfectly in the same position (as shown in Fig. 4). Mass spectrum matched isoamyl isovalerate and 2-methylbutyl 2-methylbutanoate very well too. Therefore, the peak between isoamyl 2-methylbutanoate and 2-methylbutyl isovalerate should be isoamyl isovalerate plus 2-methylbutyl 2-methylbutanoate (Fig. 4).

The typical yields of isoamyl alcohol, 2-methylbutanol, isovaleric acid, 2-methylbutanoic acid, isoamyl 2-methylbutanoate, isoamyl isovalerate plus 2-methylbutyl 2-methylbutanoate, and 2-methylbutyl isovalerate (Fig. 4, sorted by retention time) were about 2.0, 0.6, 0.2, 0.06, 0.08, 1.0, 0.2 mg/L, respectively, by *Myroides* sp. ZB35 cultured at 30°C in LB within 24 h. When the same amounts of pure chemicals were added into fresh LB, the same fruity odor appeared, supporting the qualitative and quantitative consistency from these results.

To investigate whether the four esters as unraveled in Fig. 4 were generated spontaneously with the acid and alcohol substrates produced by ZB35 in the culture medium, additional control experiment was carried out as follows. Isoamyl alcohol, 2-methylbutanol, isovaleric acid, and 2-methylbutanoic acid of 10, 100, 1000 mg/L were added into LB and kept shaking at 30°C for 24 h. No ester products were detected at the 10 and 100 mg/L substrate levels. Substrates at 1000 mg/L yielded only trace amounts of ester products, much less than the natural yield of *Myroides* sp. ZB35. This control experiment showed that the esterification efficiency was very high with rather low substrate levels in the presence of *Myroides* sp. ZB35, indicating that the esterification was catalyzed by certain enzyme(s) in *Myroides* sp. ZB35.

Discussion

The mechanisms of sequential biodegradation of FAs in *Myroides* sp. ZB35

The breakdown of linear or straight chain FAs is mediated by the β-oxidation cycle and the intermediates are used as an energy/carbon source via central metabolic networks (Kazakov *et al.*, 2009). Typically, long-chain FAs are transported across the cell membrane using the outer membrane transporter and the inner membrane-associated acyl-CoA synthetase (Fujita *et al.*, 2007). Acyl-CoA products are then converted to enoyl-CoAs catalyzed by acyl-CoA dehydrogenase, which is followed by hydration, oxidation, and thiolytic cleavage performed by the FA oxidation complex (Kazakov *et al.*, 2009).

Fatty acyl-CoA synthetase (EC 6.2.1.3) plays a central role in intermediary metabolism by catalyzing the formation of fatty acyl-CoA. *Escherichia coli* can use FAs with various chain lengths as sole carbon and energy sources (Fujita *et al.*, 2007). Although eukaryotic systems possess multiple acyl-CoA synthetases with different FA chain-length specificities, *E. coli* has a single acyl-CoA synthetase, FadD, of broad substrate specificity (active on FAs with chain lengths of 6 to 18 carbon atoms) (Kameda and Nunn, 1981). Maximal activity of *E. coli* FadD was observed with lauric acid (12 carbon atoms). Then the enzyme activity (preference) decreased according to the change of chain length (preference $C_6 < C_7 < C_8 < C_9 < C_{10} < C_{11} < C_{12}$, then $C_{12} > C_{13} > C_{14} > C_{15} > C_{16} > C_{17} > C_{18}$) (Kameda and Nunn, 1981). Although *Pseudomonas aeruginosa* possesses multiple FadD homologues, its medium chain acyl-CoA synthetase FadD2 follows similar substrate preferential pattern but with optimum chain length at C_8 (preference $C_6 < C_8$, then $C_8 > C_{10} > C_{12} > C_{14} > C_{16}$) (Kang *et al.*, 2010).

In this study, the phenomenon of prolonged or delayed lag phase with individual straight chain FA as the sole carbon source (Fig. 2) could be tentatively explained by different transmembrane movement abilities of the substrates. Or in other words, smaller molecules were easier to get into cells. However, when mixed FAs were fed to *Myroides* sp. ZB35

(Fig. 3), the dominant rate-limiting step would very likely become the activation of FAs catalyzed by a FadD homologue. Among the 4 straight chain FAs, this *Myroides* FadD was postulated to have similar preferences as *E. coli* FadD or *P. aeruginosa* FadD2: $C_5 < C_6 < C_7 < C_8$. Then these substrates competed with each other, just as the competitive inhibition of the hexanoyl-CoA synthetase activity with dodecanoate as competitor in *E. coli* (Samuel *et al.*, 1970). For the C_5-C_8 FAs, the spontaneous decrease at a rate of 0.063 ± 0.009 mg/L/h was observed in this study. With taking this effect into account, shorter FAs would be slowly degraded by *Myroides* sp. ZB35 whilst longer FAs were being assimilated, thus supporting the hypothesis that C_5-C_8 longer chain FAs competitively, but not completely, inhibit the biodegradation of shorter chain FAs.

The recalcitrant biodegradability of 2-methylbutanoic acid could be explained by the use of other pathways other than the β-oxidation cycle. The inclusion of methyl branches at different points along the alkyl chain necessitates the presence of new or modified catabolic pathways in order to degrade these materials (Ngo *et al.*, 2012). For example, 2-methylbutyric acid can be assimilated through the isoleucine or the α-oxidation pathway (Van Bogaert *et al.*, 2011). The enhancement of degradation efficiency for the FA mixture could be the result of the preculture procedure, where the cells had fully adapted to the same environment in the exactly same medium. The shortening of the lag phase favored the faster growth of cell population accompanied by efficient substrate removal rate.

Biosynthesis of novel aroma compounds in *Myroides* sp. ZB35

During the search for the possible origins of volatile FAs emerged in the biologically treated wastewater, novel metabolites were identified in *Myroides* sp. ZB35. To our knowledge, this is the first report on the natural production of the four aroma esters (as shown in Fig. 4) in bacteria.

Most *Myroides* strains can produce a characteristic fruity odor (*Myroides* resembling perfume, Vancanneyt *et al.*, 1996). However, what kinds of compounds endowing the aroma remained unknown. Fruity odor chemicals are usually produced by plants such as fruits and flowers. For example, 2-methylbutyl and 2-methylbutanoate esters are biologically synthesized in apples (Rowan *et al.*, 1996). In microorganisms, yeasts received most research interests because they are widely used in food and beverage industries and different yeasts can produce a variety of natural flavouring molecules (Buzzini *et al.*, 2003).

According to our current knowledge, methyl-branched alkanoic acids and alcohols can be generated via multiple metabolic pathways in bacteria. However, esterification is the rate-limiting step towards aroma ester production, especially when the acid and alcohol substrates exist in very low levels in cells. Alcohol acyltransferase (EC 2.3.1.84), which acts on an alcohol and an acyl-CoA, plays an important role in volatile ester biogenesis in yeasts and plants (Verstrepen *et al.*, 2003).

Referencing the metabolic map of aroma compound production in yeast (Lilly *et al.*, 2006) and branched chain amino acid (BCAA) catabolism pathways in bacteria (for example, Beck *et al.*, 2004), the biogenesis pathways of aroma esters from leucine and isoleucine in *Myroides* sp. ZB35 were postulated as shown in Fig. 5. In this study, ZB35 produced a fruity aroma in LB but not in MM. LB contains a rich amount of amino acids including leucine and isoleucine. However, BCAA pathways are sophisticated in different species. For

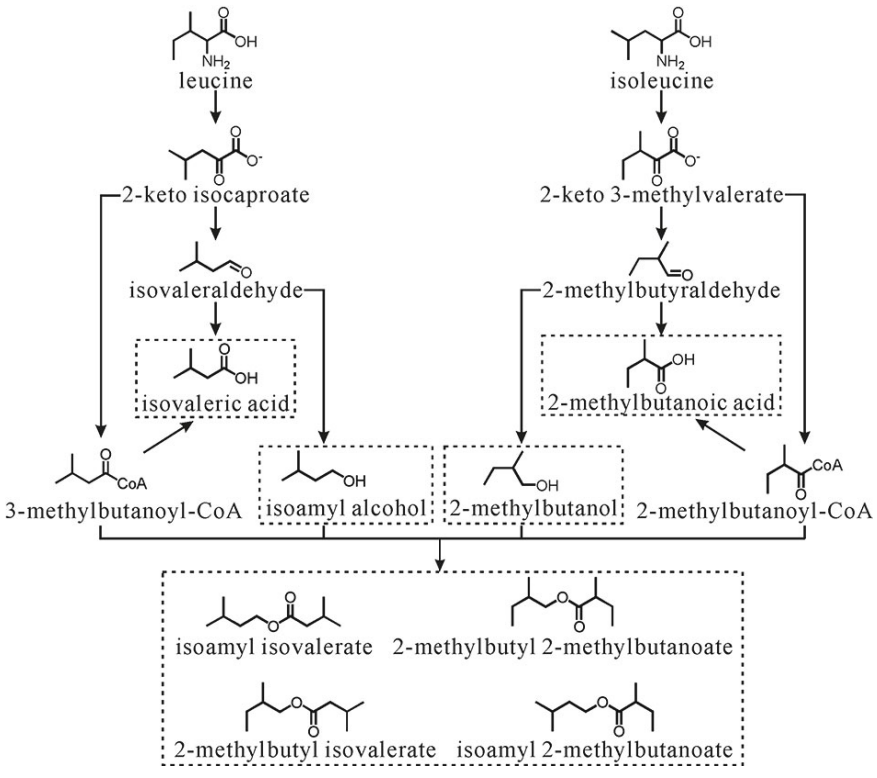

Fig. 5. Postulated biosynthesis pathways of aroma esters in *Myroides* sp. ZB35 with leucine and isoleucine as the substrates. Metabolites in the rectangles have been identified in Fig. 4.

example, 2-methylbutyric acid could be generated from leucine in *Lactococcus lactis* (Ganesan *et al.*, 2006), not from isoleucine as shown in Fig. 5. In addition to ester synthases, esterases (EC 3.1.1.1.) also play a role in ester accumulation. Esterases represent a diverse group of hydrolases catalyzing the cleavage, but in some cases also the formation, of ester bonds (Verstrepen *et al.*, 2003). Isoamyl isovalerate has been synthesized using an immobilized lipase by direct esterification (Chowdary *et al.*, 2000). In the case of esterase functioning, isovaleric acid and 2-methylbutanoic acid would react with the branched chain alcohols in Fig. 5. Furthermore, the intermediates could be biosynthesized via other metabolic networks if other substrates were supplied in the culture media. In brief, it is too early to provide a full understanding of the ester biosynthesis pathways in *Myroides* as each particular prediction or hypothesis requires further experimental validation. But from a biotechnological viewpoint, this study reveals the potential of *Myroides* species as a promising source of aroma esters that could be attractive to food and fragrance industries.

From the perspective of treating heavy oil wastewater, the FAs emerged in the biologically treated effluents most likely come from the metabolic intermediates of cellular constituents (e.g. lipids, BCAAs) or alkanes (abundant in oil refining wastewater) (Van Bogaert *et al.*, 2011). These FAs compete with each other during breaking down in bacterial cells such as those from *Myroides* sp. ZB35. Under certain circumstances (such as in the presence of certain microbial species and substrates), novel metabolites could be generated, making wastewater biotreatment mechanisms even more complicated.

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