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Biosynthesis of acetate esters by dominate strains, isolated from Chinese traditional fermented fish (Suan yu)

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ABSTRACT

Biosynthesis of acetate esters by *Lactobacillus plantarum* 120, *Staphylococcus xylosus* 135 and *Saccharomyces cerevisiae* 31, isolated from Chinese traditional fermented fish (Suan yu) were studied. A buffer system containing acyl donors (acetic acid/glyceryl triacetate/acetyl-CoA) and aliphatic alcohols (C2-C6) was established, inoculated with intracellular and extracellular extracts of the three strains. The results showed that the biosynthesis pathway of *L. plantarum* 120 was esterification and alcoholysis, while the biosynthesis pathway of *S. xylosus* 135 and *S. cerevisiae* 31 was hydrolysis and esterification, rather than alcoholysis. The ester-synthesizing activity of *L. plantarum* 120 via alcoholysis was higher than that via esterification at high pH value, while an opposite result for each strain was observed at low pH value. Moreover, the ester-synthesis activity of *L. plantarum* 120 was higher than that of *S. xylosus* 135 and *S. cerevisiae* 31. In addition, microbial ester-synthesis activity increased with the increase of aliphatic alcohol carbon number.

1. Introduction

Ester compounds are common volatile components of flavour in fermented products, providing the desirable fruity aroma to the overall profile. There are two pathways for esters synthesis: spontaneous formation (chemically) and enzymatic synthesis (Liu, Holland, & Crow, 2004). As we known, esters usually originate from the alcoholic fermentation carried out by yeast, especially in wine. Secondary fermentation carried out by lactic acid bacteria (LAB) also produces esters in dairy products, especially cheese (Carr, Chill, & Maida, 2002; Liu, Holland, et al., 2004). Thus, microbiota plays an important role in the formation of esters in fermented products.

Esterification (reaction of an acid and an alcohol) is the most common pathway for microbial synthesis of esters catalyzed by esterases or lipases and has been reported in many food microorganisms. Lactic acid bacteria isolated from goat's and ewe's milk and cheeses were evaluated for their ability to synthesize ethyl esters (C2-C10), mainly ethyl butanoate and ethyl hexanoate, via esterification in a buffer system containing free fatty acid (butanoic acid or hexanoic acid), ethanol plus cell free extracts, and a great variability in activity among strains was observed (Abeijón Mukdsi, Medina, Alvarez, & González, 2009). Nardi, Fiez-Vandal, Tailliez, and Monnet (2002) also evaluated the capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters via esterification *in vitro*, where *L. lactis* cells were inoculated in the phosphate buffer containing free fatty acid (acetic acid or butanoic acid) and ethanol. After the reaction, ethyl acetate and ethyl butyrate were detected in the buffer. Talon, Chastagnac, Vergnais, Montel, and Berdague (1998) reported that the cells and extracellular extracts of staphylococci (*Staphylococcus carnosus*, *Staphylococcus xylosus*, *Staphylococcus warneri* and *Staphylococcus saprophyticus*) could synthesize ethyl esters in the presence of ethanol and short chain acids, including straight- chain and branched-chain fatty acids. In addition, aromatic esters formed from aromatic alcohols and short chain fatty acids catalyzed by arylesterases via esterification were reported in LAB (Fenster, Parkin, & Steele, 2003; Fernandez et al., 2000).

Alcoholysis is a transferase reaction of an ester and an alcohol in an aqueous environment, normally catalyzed by alcohol acyltransferases (Holland et al., 2005). Liu, Holland, and Crow (2003) reported the ability of 19 dairy lactic acid bacteria to synthesize ethyl butyrate in the presence of tributyrin and ethanol, and that *Streptococcus thermophilus* ST1 could transfer fatty acyl groups from tributyrin and on di- or monoglycerides of up to C10 to ethanol. Actually, lipases can exhibit acyltransferase activities transferring fatty acyl groups from

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acylglycerols to alcohols in an aqueous medium. A lipase produced by *Aeromonas hydrophila* was actually an acyltransferase that preferentially catalyzed ester synthesis via alcoholysis in an aqueous environment (Robertson, Hilton, & Buckley, 1992). Thus, it can be envisaged that strains that catalyze ester synthesis by alcoholysis may also catalyze ester synthesis by esterification. Moreover, alcohol acetyltransferases can also catalyze alcoholysis in which acetyl groups are transferred from acetyl-CoA to alcohols (including mercaptoalcohol) (Sumby, Grbin, & Jiranek, 2010), which is the primary synthesis pathway for esters in yeasts (Bardi, Crivelli, & Marzona, 1998).

Acidolysis is the reaction of an ester and an acid in which the acyl group is transferred to an acid, and transesterification is the reaction between two esters, which results in two new esters after the exchange of two acyl acceptors (Paiva, Balcao, & Malcata, 2000). Lipases can also synthesize esters through acidolysis and transesterification pathways (Liu, Holland, et al., 2004). However, acidolysis and transesterification are commonly used to produce structured lipids with a nutritional function, and little to do with flavour ester (Willis, Lencki, & Marangoni, 1998). In addition to above pathways, dehydrogenation for methyl formate catalyzed by methyl formate synthase was found in methylotrophic yeasts (Murdanoto et al., 1997; Sakai, Murdanoto, Sembiring, Tani, & Kato, 1995).

Lactobacillus plantarum 120, *Staphylococcus xylosus* 135 and *Saccharomyces cerevisiae* 31 are dominate strains, isolated from Chinese traditional fermented freshwater fish (Suan yu). They were used as starter culture to improve the overall aroma of Suanyu and proved to be associated with the formation of esters (unpublished). However, it was still unclear whether these strains were able to synthesize esters directly. The aim of the present work was to evaluate the ability of these spontaneous strains to synthesize acetate esters, and to develop appropriate starter cultures to improve the overall aroma of Suan yu.

2. Material and methods

2.1. Preparation of cell extracts

L. plantarum 120 and *S. xylosus* 135 were separately subcultured twice according to the method of Hu, Xia, and Ge (2008). *S. cerevisiae* 31 was subcultured twice in YPD broth at 30 °C for 24 h. Supernatants were harvested as the extracellular extracts (EE), by centrifugation at 10,000 × *g* for 10 min at 4 °C. At the same time, the left cells were washed twice with potassium phosphate buffer (100 mM, pH 6.8), and sonicated in the same buffer in an ice bath using a JY88-II ultrasonic homogenizer (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) at 300 W (*L. plantarum* 120 for 5 min, *S. xylosus* 135 for 5 min and *S. cerevisiae* 31 for 10 min, respectively). Whole cells and cellular debris were separated by centrifugation (Sigma Laborzentrifugen, Model 4K15, Osterode, Germany) at 15,000 × *g* for 20 min at 4 °C. The supernatant was obtained as the intracellular fraction (Cell Free Extracts, CFE), used either immediately or stored at −18 °C. The concentrations of protein in the cell extracts were determined by a BCA Protein Assay Kit (Bradford, 1976). The results were expressed as mg protein/mL.

2.2. Analysis of ester synthesis pathway

Ester synthesis pathway was analyzed according to the method of Abeijón Mukdsi et al. (2009) with some modifications. For esterification, the assay mixture contained 100 mM potassium phosphate buffer (pH 6.8), 100 mM acetic acid, 100 mM alcohol (ethanol or isoamyl alcohol) and cell extracts (ca. 0.08–0.1 mg/mL). For alcoholysis, the assay mixture contained 100 mM potassium phosphate buffer (pH 6.8), 100 mM alcohol (ethanol or isoamyl alcohol), 33 mM triacetin or 0.6 mM acetyl-CoA, and cell extracts (ca. 0.08–0.1 mg/mL). 3 M sulfuric acid was added to stop the reaction after the assay mixture was incubated statically at 30 °C. Then the mixture was immediately transferred to the vial for headspace SPME-GC–MS analysis to determine the content of ethyl acetate, isoamyl acetate and acetic acid. The determination was conducted at 20 min intervals. Controls lacking cell extracts or substrates were also included.

2.3. Determination of ester-synthesizing activity for each strain

The reaction mixture was processed according to the above method. Ester-synthesizing activity for each strain was detected at different fermentation conditions by adjusting the buffer to pH 6.8, 5.0 and 3.6. Controls mentioned above were also included. A unit of ester- synthesizing activity was defined as the amount of enzyme that formed 1 nmol of ester in 1 h. Specific ester- synthesizing activity was defined as units per milligram of protein (U/mg protein).

2.4. Effect of chain length of alcohols on ester synthesis

A buffer system was established according to the above method. The assay mixture contained 100 mM potassium phosphate buffer (pH 6.8), 100 mM acetic, 100 mM alcohol (ethanol, propanol isoamyl alcohol, pentanol or hexanol) and EE (ca. 0.08–0.1 mg/mL). Next, 3 M sulfuric acid was added to stop the reaction after the assay mixture was incubated statically at 30 °C for 1 h. Ester-synthesizing activity was detected according to the above method. Controls lacking cell free extracts or substrates were also included.

2.5. GC–MS analysis

The following esters were determined by Solid Phase Microextraction (SPME) and GC–MS (TSQ Quantum XLS, Thermo Fisher Scientific, USA). It was carried out as the method of Fratini, Lois, Pazos, Parisi, and Medina (2012). The GC conditions were as follows. Column: DB-WAX, 30 m × 0.25 mm × 0.25 μm; Initial Temperature: 40 °C; Initial Time: 3 min; Rate1: 5 °C/min; Final Temperature: 90 °C; Hold Time: 0 min; Rate 2: 10 °C/min; Final Temperature 2: 230 °C; Hold Time 2: 7 min. The MS conditions were as follows. Ionization Mode: EI +; Emission Current: 80 μA; Electron Energy: 70 eV; Scan range: 30–500 *m/z*; Interface Temperature: 250 °C; Source Temperature: 200 °C; Carrier: He, 0.90 ml/min; Detector Voltage: 1000 V. The volatile flavours were identified according to NIST2005 and Willey 7 standard libraries and Retention Index (RI), and were semi-quantified by using 2,4,6-trimethyl pyridine as an internal standard. The concentration of esters in the samples was calculated by comparing the peak area of each compound with that of the internal standard, expressed as μg/mL.

2.6. Statistical analysis

The experiments were designed and analyzed statistically by ANOVA, and mean differences were evaluated by Duncan's multiple range test ($P < 0.05$). Statistical analysis was performed using the SPSS statistic program (Version 19.0, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Ester-synthesizing ability of each strain

The results for biosynthesis of the two esters by esterification and alcoholysis including three types of reactions in each strain are shown in Table 1. Both extracellular and intracellular fractions of all strains exhibited the ability for acetate esters synthesis. The reaction of acetic acid and alcohols had a corresponding ester compound, indicating that the esterification existed in each strain, which suggested that esterification by dominant strains might contribute to esters formation in Suan yu. Esterification has been reported in lactic acid bacteria (LAB) isolated from goat's and ewe's milk and cheeses (Abeijón Mukdsi et al., 2009), staphylococci in fermented sausages (Talon et al., 1998), and saccharomyces in wine (Sumby et al., 2010).