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# Volatile Compounds and Lactic Acid Bacteria in Spontaneous Fermented Sourdough

(Sebatian Meruap dan Bakteria Asid Laktik daripada Doh Masam melalui Fermentasi Spontan)

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ABSTRACT

*The aim of this study is to identify the predominating lactic acid bacteria (LAB) in a spontaneous fermented wheat sourdough. At the same time, an investigation towards volatile compounds that were produced was also carried out.* Lactobacillus plantarum *has been identified as the dominant species of lactobacilli with characters of a facultative heterofermentative strain. The generated volatile compounds that were produced during spontaneous fermentation were isolated by solvent extraction method, analysed by gas chromatography (GC), and identified by mass spectrophotometer (MS). Butyric acid has been found to be the main volatile compound with relative abundance of 6.75% and acetic acid at relative abundance of 3.60%. Esters that were formed at relatively low amount were butyl formate (1.23%) and cis 3 hexenyl propionate (0.05%). Butanol was also found at low amount with relative abundance of 0.60%. The carbohydrate metabolism of* Lactobacillus plantarum *may contributed to the production of acetic acid in this study via further catabolism activity on lactic acid that was produced. However, butyric acid was not the major product via fermentation by LAB but mostly carried out by the genus* Clostridium *via carbohydrate metabolism which needs further investigation*

*Keywords: Lactic acid bacteria; lactobacillus; sourdough; spontaneous fermentation; volatile compound*

ABSTRAK

*Kajian dilakukan untuk mengenal pasti kehadiran spesies utama bakteria asid laktik yang terdapat dalam doh masam yang difermentasi secara spontan. Di samping itu, sebatian meruap yang terhasil semasa fermentasi juga dikaji.* Lactobacillus plantarum *dikenal pasti sebagai spesies utama yang bersifat jenis heterofermentatif fakultatif. Sebatian meruap yang terhasil semasa fermentasi spontan telah diasingkan secara kaedah pengekstrakan pelarut diikuti dengan analisis kromatografi gas (GC), dan pengenalpastian oleh spektrofotometer jisim (MS). Asid butirik merupakan sebatian meruap utama dengan kelimpahan relatif 6.75%. Asid asetik didapati terhasil dalam kelimpahan relatif 3.60%. Ester yang terhasil pada tahap kelimpahan relatif yang rendah termasuk butil format (1.23%) dan cis-3-heksil-propionat (0.05%). Butanol juga dikesan dalam amaun yang rendah dengan kelimpahan relatif 0.60%. Metabolisme terhadap karbohidrat oleh* Lactobacillus plantarum *yang menghasilkan asid asetik juga berkemungkinan besar dipengaruhi aktiviti katabolisme terhadap asid laktik yang terhasil. Walau bagaimanapun, asid butirik bukan hasil utama daripada fermentasi oleh LAB tetapi lebih daripada fermentasi oleh genus* Clostridium *melalui metabolisme karbohidrat dan kajian yang lebih mendalam diperlukan.*

*Kata kunci: Bakteria laktik asid; doh masam; fermentasi spontan; lactobacillus; sebatian meruap*

## INTRODUCTION

The main purpose of using fermentation to process foods was to extend the product's shelf life e.g. beer, wine, bread and vegetables, while imparting to the final product a flavour distinct from that of the original food. The microorganisms employed play an important role in the development of complex mixtures of aromas through the catabolism of sugars, fats, and proteins. Sourdough plays an important role in improving texture and flavour of bakery products via fermentation. Vuyst and Heysens (2005) reported that sourdough microflora have primarily stable associations with lactobacilli and yeasts, having important metabolic interactions contributing towards production of flavour compounds. Two categories of flavour compounds produced during sourdough fermentation are volatile compounds and non-volatile compounds. The volatile compounds include alcohols, aldehydes, ketones, esters and sulphur.

The generation of volatiles in sourdoughs is influenced by the activity of the lactic acid bacteria (LAB) and the sourdough yeasts. Their activity mainly attributed by temperature and water content, which consequently influence the amounts of the metabolites formed. In general, LAB contribute in acidification process. Both LAB and yeasts are able to liberate aroma precursors, such as free amino acids, and it has been previously demonstrated that the concentrations of free amino acids increased significantly during sourdough fermentation (Hansen et al. 1989a). A sourdough wheat bread has richer flavour and contains more aromatic than in wheat bread due to the long fermentation time (Brümmer & Lorentz 1991).

Salim et al. (2006) reported that although yeasts have primary leavening role in sourdough fermentation, lactic acid bacteria (LAB), with trophic and non-trophic relationships, produce important flavour components. It is therefore the objective of this study to identify major volatile compounds in a spontaneous fermented sourdough, while establishing the predominant lactobacillus species found in the dough ecosystem. With the increasing consumer demand for natural additives in food, biotechnological production of flavour offers an alternative source of supply and may offer many advantages such as low production cost, continuous availability and stereospecific pure products.

## Materials and Methods

### MATERIALS

High protein wheat flour (Red Horse brand) for breadmaking was supplied by Interflour Sdn. Bhd. The protein content of the flour was 12.7% of dry matter (d.m.), moisture content 14% and ash content 0.55% (d.m.).

### SAMPLES PREPARATION

A dough was prepared by manually mixing wheat flour with distilled water at a ratio 1:1, giving dough yield (DY) of 200. The dough sample was fermented at 30°C for 48 h.

### ISOLATION AND IDENTIFICATION OF LACTOBACILLUS SPECIES

For isolation and identification of lactobacillus species, 25 colonies from the highest dilution plate were isolated and streaked continually on MRS agar till pure colonies were obtained. All isolates that were Gram-positive, rod shaped and tested catalase negative, were preliminarily identified based on the phenotypic properties such as carbon dioxide production from glucose, ammonia production from arginine, and growth at different temperatures of 37°C and 45°C, respectively. Sugar fermentation patterns were determined by assaying cultures in API 50 CHL galleries (Bio Merieux SA, France) using API 50 CHL medium (Bio Merieux).

### IDENTIFICATION OF VOLATILE COMPOUNDS

For volatile compound extraction, a 70 g sample with 20% w/v of sodium chloride (Merck, Darmstadt, Germany) was centrifuged at 4500 g and 2°C for 5 minutes. 25 ml of the supernatant were mixed in a proportion at 1:1 with ethyl acetate (Sigma-Aldrich, USA) and stirred for 40 minutes. Then the mixture was centrifuged again (2000 g, 2°C, 5 min). The organic phases was transferred to a round-bottom flask and concentrated to 1.5 mL at 40°C using a vacuum rotary evaporator (Buchi, Switzerland).

The extracted volatile compound were analysed and identified by GC-MS analysis. This was carried out with a Shimadzu model GC-17A gas chromatograph coupled to a GC-MS-QP5050A mass spectrometer. A 30 m × 0.25 mm (ID) HP-5MS capillary column (Agilent Technologies), with 0.25 μm film thickness, was used. The sample (1 μL) was injected in split mode (1:50) and the injector temperature was 230°C. Helium was used as carrier gas at a constant flow of 1.1 mL/min. The oven temperature was programmed as follow 40°C for 7 min, increased at 5°C/min to 220°C, and then held constant for 17 min. The data acquisition was performed in scan mode. The following MS parameters were applied: m/z range 29-350; scans/s 4.44; EI source temperature 230°C; quadrupole temperature 150°C; EM voltage + 294 V relative to the value established by the tune programme (2788). The identification of volatile compounds was performed by comparison of the MS data obtained with those in NIST21 and WILEY229 libraries.

## Results and Discussion

### LACTOBACILLUS SPECIES IDENTIFICATION

Twenty-five isolates were phenotypically characterized based on growth at temperatures (37 and 45°C), production of gas from glucose and hydrolysis of arginine. (Table 1). All 25 isolates that were considered as presumptive lactobacillus species were rod shaped, Gram-positive, catalase-negative bacteria, did not form spores and were non-motile. Based on phenotypic characteristics and interpretation of the API database, 23 lactobacillus species isolated from wheat sourdough were tentatively identified as *Lactobacillus plantarum*, and 2 isolates were tentatively identified as *Lactobacillus brevis*. Our results are in agreement with Corsetti and Settani (2007) who reported *Lb. sanfrancisensis*, *Lb. brevis* and *Lb. plantarum* are the lactobacilli are most frequently isolated from sourdough. Stolz (2003) reported that LAB of fermented spontaneous sourdoughs are homofermentative lactobacilli and pediococci that includes *Lb. casei, Lb. delbrueckii, Lb. farciminis, Lb. plantarum, Pc. acidilactici* and *Pc. pentosaceus*. Vogel et al. (1999) also reported lactic acid bacteria as the dominant organisms in sourdoughs and in many cases they co-exist with yeasts. Several species of yeasts associated with LAB in sourdough (Rossi 1996) that were frequently isolated were *Saccharomyces cerevisiae* and *Candida milleri*.

### VOLATILE COMPOUNDS IDENTIFICATION

Volatile compounds in spontaneous fermented sourdough after 48 h of incubation are listed in Table 2 with details of peak identities and relative abundances of the volatile compounds expressed as ($compound_{peak\ area}$ /

TABLE 1. Some characteristics of the isolated strains of rod-shaped, gram positive and catalase negative lactic acid bacteria

| Strains Coding | $CO_2$ from Glucose | $NH_3$ from Arginine | Growth | | Species identity | ID % |
|---|---|---|---|---|---|---|
| | | | 37°C | 45°C | | |
| L.B. RH001 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH002 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH003 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH004 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH005 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH006 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH007 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH008 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH009 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH010 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH011 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH012 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH013 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH014 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH016 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH017 | - | - | + | - | *Lb. brevis* | 93 |
| L.B. RH018 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH019 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH021 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH022 | - | - | + | - | *Lb. plantarum* | 94 |
| L.B. RH025 | - | - | + | - | *Lb. brevis* | 77 |
| L.B. RH026 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH028 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH029 | - | - | + | - | *Lb. plantarum* | 98 |
| L.B. RH030 | - | - | + | - | *Lb. plantarum* | 93 |

TABLE 2. Relative abundances* of volatile compounds detected in liquid sourdough extractive

| Peak No. | Volatile compound | Relative abundances (%) |
|---|---|---|
| 1 | 4 Methyl Pentanol | 0.30 |
| 2 | Acetic acid | 3.60 |
| 3 | Aldehyde C8 | 0.02 |
| 4 | Aldehyde C10 | 0.05 |
| 5 | Butanol | 0.60 |
| 6 | Butyl Formate | 1.23 |
| 7 | Butyric Acid | 6.75 |
| 8 | cis 3 Hexenyl Propionate | 0.05 |

* Relative abundance of the volatile compund is expressed as (compound$_{peak\ are}$/total compounds$_{peak\ area}$) × 100. Results are average of duplicates.

*total compounds*$_{peak\ area}$) × 100. A total of 9 compounds were detected and identified as 2 organic acids (Butyric acids and acetic acids), 4 esters (Butyl formate, aldehyde C8, aldehyde C10, cis 3 hexenyl propionate), 1 alcohol (Butanol), 1 ester alcohol (4 methyl propanol) and 1-heptyne.

Butyric acid (6.75%) was observed as having the highest relative abundance followed by acetic acid (3.60%), butyl formate (1.23%), butanol (0.60%) and 4 methyl propanol (0.30%). Other volatile compounds detected were 1-heptyne, aldehyde C10 and cis 3 hexenyl propionate with relative abundances of 0.05%, respectively. Aldehyde C8 was identified with the lowest relative abundance of 0.02%.

The primary metabolic products of LAB are lactic acids and acetic acids. Liu (2003) commented that lactic acid can be catabolised under aerobic conditions by lactate oxidase or $NAD^+$-independent LDH in some LAB such as *Lb. curvatus*, *Lb. sake*, *Lb. casei* and *Lb. plantarum* to produce pyruvate, which is further catabolised. Under anaerobic conditions, lactate can also be catabolised via $NAD^+$-independent LDH by some lactobacilli such as *Lb. brevis*, *Lb. buchneri* and *Lb. plantarum*. These lactate-degrading lactobacilli use other electron acceptors (e.g. shikimate or oxaloacetate derived from citrate and 3-hydroxypropionaldehyde derived from glycerol) for anaerobic catabolism of lactate as below : lactate → pyruvate → acetate + carbon dioxide or lactate → pyruvate → acetate + formate. The products generated via pyruvate degradation are dependent upon the presence of a particular enzyme(s) in a particular LAB, pyruvate oxidase or pyruvate-formate lyase or both. This could be one of the possibilities that had happened where long hour fermentation (48 h) in this study had offered sufficient time of lactate degradation, and consequently production of more acetic acid, instead of lactic acid. Besides, the absence of lactic acid could also be mainly due to non-volatility of this compound and therefore poor traceability in GC-MS analysis. Lactic acid belongs to the non-volatile compound, which will be analyzed and identified using high pressure liquid chromatography (HPLC).

Between the two organic acids that were identified, butyric acid was the predominant volatile organic acid detected. Butyric acid is commonly found in well-ripened cheeses and in butter which is produced by the activity of anaerobic bacteria e.g. *Clostridium butyricum*, *Bacillus butyricus* via butyrate fermentation. Stolz (2003) explained that microflora in a spontaneous fermented dough depends greatly on the microflora of the raw materials used e.g. flour / cereal and the prevailing hygienic conditions, including storage conditions of the flour as well as the technological parameters of the fermetnation process applied. *Bacillus* and *Clostridium* are among the detectable bacterial in cereals and flours thereof. In anaerobic fermentation, the genus *Clostridium* will produce butyric acid, acetic acid, $H_2$ and $CO_2$ during their exponential growth. When the culture enters stationary phase, the acids are converted to butanol, acetone and ethanol (White 2007). This is possibly contributing to the presence of butyric acid, acetic acid and butanol in this study.

Meanwhile, Schieberle (1996) reported that alcohols can be produced by yeast via the *Ehrlich* pathway by transamination of certain amino acids into the corresponding α-keto acids, followed by a decarboxylation into the aldehyde and finally reduction into the alcohol, it is therefore the identification of aldehyde C8, aldehyde C10, butanol and 4 methyl pentanol in this study.

## CONCLUSION

The results of this study indicated that during spontaneous sourdough fermentation, butyric acid and acetic acid are the major volatile compounds produced. Meanwhile, *Lactobacillus plantarum* was found the major LAB in the sample. The carbohydrate metabolism of *Lactobacillus plantarum* may have greatly contributed to the production of acetic acid in this study via further catabolism activity on lactic acid that was produced. However, butyric acid was not the major product via fermentation by LAB but mostly carried out by the genus *Clostridium* via carbohydrate metabolism. Nevertheless, it is also imperative to investigate the amino acids content as well as dominant species of yeast that are found in the sourdough sample. This would assist to identify further the role of the respective microorganisms in producing specific volatile compound in a spontaneous fermented sourdough.

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