COMPLETE REPORT

EFFECT OF SECRETED ENZYMES EXTRACTED FROM *Bacillus subtilis* TN51 AND *Saccharomyces cereviceae* CULTURING MEDIA ON AROMATIC CONSTITUENTS IN TEA (*Camellia sinensis*)

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Executive Summary

Tea (*Camellia sinensis*) has been one of the most popular drinks in the world for over 4,000 years. More than three million hectares worldwide have been used for planting tea. Nowadays, tea is also used in pharmaceutical and industrial applications. Tea is manufactured in many parts of the world. There have been many attempts to develop new tea products especially those with distinct aromas. One simple method is to include edible essential oils into the tea product to improve its aroma. Other approaches include modification of the tea production process (i.e., withering, rolling, and fermentation) which result in aroma changes by promoting and/or inhibiting the enzymes in the tea leaves. Key odor compounds detected from these experiments showed that monosaccharide or disaccharide flavorless glycoside precursors were present in fresh tea leaves. Free aroma constituents are then released by hydrolysis of glycoside precursors by β-D-glycosidase enzymes. In addition, the addition of external enzymes (i.e., pectinase and glucosidase) may improve tea aromas. However, there is no report describing the application of *B. subtilis* on tea. In order to develop and improve aroma quality in tea product, the aim of the present study is to investigate volatile odor components of teas fermented with different microbes. Five strains of *Bacillus subtilis* were used in this present study including *B. subtilis* TN51 isolated from *thua nao*, a Thai fermented soybean, *B. subtilis* ASA and *B. subtilis* BEST195 isolated from Japanese *natto*, *B. subtilis* S1-13 isolated from *terasi*, an Indonesia shrimp paste, *B. subtilis* TISTR008 obtained from Thailand Institute of Scientific and Technological Research (TISTR) and *Saccharomyces cerevisiae* obtained from TISTR. The supernatant was then collected to a sterile media bottle and was used as crude enzymes for tea fermentation. For extraction, tea sample was ground into very small particles (almost a powder) using an electric grinder. For each fermentation process, one hundred grams of powdered tea was inoculated with 100 ml of the various *B. subtilis* supernatant. For mixture of *B. subtilis* TN51 and ASA, 100 ml of each strain was added into 100 g of various tea samples. All samples were fermented with different *B. subtilis* strains for 2 h prior to extraction by

SPME. As results, at least 54 components were identified in all samples. Linalool, hotrienol and γ-terpinene were found to be the major components in dry Green Oolong tea while *B. subtilis*-fermented teas provided 2-pentylfuran and limonene in higher amounts. The contents of most major volatiles increased remarkably in the fermented tea samples. Superior quantity of volatile components was related to the use of *B. subtilis* culture supernatants compared to other cultures and pure enzyme whereas 2-pentylfuran and limonene were responsible for the special odor of *B. subtilis*-fermented teas. Some microbes will be added to instant tea to increase aromatic quality of tea which may be applied to tea manufacturing process next future. In addition, results from this study have been accepted for publication by Chiang Mai Journal of Science since 2013.

Abstract

The volatile components of Green Oolong tea No. 12 fermented with culture supernatants of five *Bacillus subtilis* strains, one strain of *Saccharomyces cerevisiae* and β-D-glycosidase enzymes were investigated. Initially, the culture supernatants of all different strains were prepared and subsequently used as crude enzymes to ferment tea samples while pure enzyme was prepared by adding in distilled water. After 2 h-fermentation, the volatile components were extracted using solid phase microextraction (SPME) technique and determined by gas chromatography-mass spectrometry (GC-MS). At least 54 components were identified in all samples. Linalool, hotrienol and γ-terpinene were found to be the major components in dry Green Oolong tea while *B. subtilis*-fermented teas provided 2-pentylfuran and limonene in higher amounts. The contents of most major volatiles increased remarkably in the fermented tea samples. Superior quantity of volatile components was related to the use of *B. subtilis* culture supernatants compared to other cultures and pure enzyme whereas 2-pentylfuran and limonene were responsible for the special odor of *B. subtilis*-fermented teas. Some microbes will be added to instant tea to increase aromatic quality of tea which may be applied to tea manufacturing process next future.

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Tea (*Camellia sinensis*) has been one of the most popular drinks in the world for over 4,000 years. More than three million hectares worldwide have been used for planting tea. Nowadays, tea is also used in pharmaceutical and industrial applications. Tea is manufactured in many parts of the world. Tea processing methods are classified by the degree of fermentation. Green tea is non-fermented, oolong and red tea are partially fermented, and black tea is completely fermented. Teas from different manufactures have their own characteristic color, taste, odor, and aroma. Tea is applied in pharmaceutical products. Green tea production does not involve fermentation whereas Oolong and red tea are produced through semi-fermentation. Black tea is obtained though a complete fermentation process. The odors and flavors of tea result from important components such as terpenes, caffeine, organic acids and polyphenols. There have been many attempts to develop new tea products especially those with distinct aromas. One simple method is to include edible essential oils into the tea product to improve its aroma. Other approaches include modification of the tea production process (i.e., withering, rolling, and fermentation) which result in aroma changes by promoting and/or inhibiting the enzymes in the tea leaves. Key odor compounds detected from these experiments showed that monosaccharide or disaccharide flavorless glycoside precursors were present in fresh tea leaves. Free aroma constituents are then released by hydrolysis of glycoside precursors by β-D-glycosidase enzymes. In addition, the addition of external enzymes (i.e., pectinase and glucosidase) may improve tea aromas.

Thua nao is a conventional fermented soybean generally used as a flavor enhancer in dishes mainly in the northern part of Thailand. Cooked soybean is fermented with *Bacillus subtilis* and related bacilli. It has been reported that *Bacillus* species are capable of synthesis a wide range of enzymes that can be used in industry. A dramatic increase of several volatile components was found in soybean fermentation when using this bacterial

strain as a starter culture. Owens and co-workers reported large amounts of 3-hydroxy-2-butanone, 2,5-dimethylpyrazine and trimethylpyrazine during fermentation of soy-*daddawa*. The highest contents of pyrazines in African *soumbala*, fermented by pure-starter *B. subtilis*, were detected significantly. It is therefore evident that enzymatic action from *B. subtilis* can increase the amounts of volatiles in different soybeans products.

The identification of odor and flavor volatile components of tea plays an essential role in the aroma characteristics and flavors of teas. Gas chromatography-mass spectrometry is a useful technique that has been used to investigate the volatile components of teas. Solid-phase microextraction (SPME) is a fast, efficient, solvent-free alternative to conventional volatile extraction techniques that has been recently developed. In SPME, the analytes establish equilibrium between the sample matrix, the headspace above the sample, and a polymer-coated fused fiber. The analytes are then desorbed from the fiber into an injection port of the gas chromatograph for analysis. The number of components extracted onto the fiber depends on the partition of the analytes from the sample onto the SPME fiber. Due to its sensitivity, reproducibility, and high concentration capability, SPME has been used for extracting the volatile components from teas. However, there is no report describing the application of *B. subtilis* on tea. In order to develop and improve aroma quality in tea product, the aim of the present study is to investigate volatile odor components of teas fermented with different microbes.

1.2 Scopes of study

In the present study, the chemical compositions of tea fermented with different bacteria, yeast and β-D-glycosidase enzymes were identified by using SPME-GC-MS, and confirmed by the linear retention indices.

1.3 Expected output

Manuscript has been accepted for publication by Chiang Mai Journal of Science entitled of Analysis of volatile constituents of fermented tea with *Bacillus subtilis* by SPME-GC-MS.

CHAPTER 2
MATERIALS AND METHODS

2.1 Tea samples

Green Oolong tea No. 12 (*Camellia sinensis* var. *sinensis*) samples obtained from Boonrod farm, Chiang Rai, Thailand was used in this study. The sample was stored below 5 °C prior to fermentation with culture supernatants of various *Bacillus* strains. Mixtures of C_8 to C_{19} *n*-alkanes were purchased from Merck (Darmstadt, Germany).

2.2 Microbial strains, culture conditions and crude extract preparation

Five strains of *Bacillus subtilis* were used in this present study including *B. subtilis* TN51 isolated from traditional thua nao and has been identified previously by Chukeatirote et al. (2006), *B. subtilis* ASA and *B. subtilis* BEST195 isolated from Japanese *natto*, *B. subtilis* S1-13 isolated from *terasi*, an Indonesia shrimp paste, *B. subtilis* TISTR008 obtained from Thailand Institute of Scientific and Technological Research (TISTR) and *Saccharomyces cerevisiae* obtained from TISTR. Each bacterial strain was routinely cultured on nutrient agar (NA) while fungal strain was cultured on potato dextrose agar (PDA) and, for stock culture, the 20% glycerol bacterial culture was prepared and stored at -20 °C. For inoculum preparation, a single colony of each bacterial strain was subcultured to a test tube containing 3 ml of nutrient broth (NB) and incubated at 37 °C for 24 h. One milliliter of the cell suspension was then transferred to a flask containing 250 ml of NB and then incubated by shaking (170 rpm) at 37 °C. After approximately 24 h of incubation (the A_{600} values were ~ 1.0), the bacterial cells were harvested from the culture media by centrifugation (8,500 rpm at 4 °C for 10 min). The supernatant was then collected to a sterile media bottle and was used as crude enzymes for tea fermentation. Alternatively, the crude culture supernatants were kept at 4 °C until required.

2.3 Preparation of enzymatic solution

The β-D-glycosidase enzyme (0.05 g) was added was added in distilled water to give a final volume of 100 mL. The solution was then stirred for 30 min at room temperature before using as enzymatic solution.

2.4 Fermentation of tea

Tea sample was ground into very small particles (almost a powder) using an electric grinder. For each fermentation process, one hundred grams of powdered tea was inoculated with 100 ml of the various *B. subtilis* supernatant. For mixture of *B. subtilis* TN51 and ASA, 100 ml of each strain was added into 100 g of various tea samples. All samples were fermented with different *B. subtilis* strains for 2 h prior to extraction by SPME. The experiment was carried out in triplicate.

2.5 Analysis of volatile constituents

- Solid-phase microextraction (SPME)

The SPME apparatus with a SPME fiber assembly holding 1.0 cm fused-silica fibers was purchased from Supelco, Bellefonte, PA, USA. A 50/30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) fiber was selected to extract the volatile components from tea leaf fermented with various *Bacillus* strains. The fiber was mounted in the manual SPME holder and preconditioned for 2 h in a GC injection port set at 250 °C. For each extraction, the sample bottle was equilibrated at room temperature around 25 °C for 2 h. By insertion through the septum of the sample bottle, the fiber was then exposed to the sample headspace for 30 min prior to desorption of the volatiles into the splitless injection port of the GC-MS instrument for 5 min.

- Gas Chromatography-Mass Spectrometry (GC-MS)

The volatile constituents of tea leaves fermented with various *Bacillus* strains obtained from the SPME extracts with DVB-CAR-PDMS fiber were analyzed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies, Palo Alto,

CA, USA). It was equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Agilent Technologies, USA) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 40 °C and then increased by 2 °C/min to 220 °C. The injector and detector temperatures were 250 and 280 °C, respectively. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. EI mass spectra were collected at 70 eV ionization voltages over the range of *m/z* 29-300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Identification of volatile components was performed by comparison of their Kovát retention indices, relative to C_8-C_{19} *n*-alkanes, and comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST05 databases and 2007 with corresponding data of volatile flavor components in tea.

CHAPTER 3
RESULTS AND DISCUSSION

Results of the fingerprint in terms of odor volatile components of dry Green Oolong tea No. 12 from Boonrod farm and their fermentation with bacterium supernatants of various *B. subtilis*, *S. cerevisiae* strains and β-D-glycosidase enzymes analyzed by GC-MS and quantities determined with the compound to internal standard are summarized in Table 1. Similar characteristics of all fermented teas were illustrated. Fifty-four volatiles were identified among the Green Oolong tea No. 12 samples. Increased amounts of most volatile components occurred in different *B. subtilis*-fermented teas as compared to the dry tea sample. Linalool, hotrienol, γ-terpinene, 2-pentyl furan, δ-3-carene and endo-fenchol were found to be the major components in dry Green Oolong tea No. 12. Small amounts of terpinolene, 1,8-cineole, *cis*-linalool oxide (furanoid), limonene and *trans*-isolimonene were also detected. Tea fermented with culture supernatants of *B. subtilis* TN51 contained limonene, 2-pentyl furan, δ-3-carene, *E*-β-ocimene, hotrienol and linalool as the key odor constituents, while monoterpene components such as terpinolene, α-terpinene, *trans*-isolimonene, γ-terpinene, and allo-ocimene were minor components. The dominant components of *B. subtilis* ASA-fermented tea were 2-pentyl furan, limonene, linalool, hotrienol and δ-3-carene. They were accompanied by the small amounts of *E*-β-ocimene, terpinolene, *trans*-isolimonene, 1,8-cineole and caffeine. Green Oolong tea No. 12 fermented with *B. subtilis* BEST195 and S1-13 culture supernatants produced similar volatile profiles with the dominant components of 2-pentyl furan, limonene, linalool, hotrienol, δ-3-carene, *E*-β-ocimene, terpinolene and *trans*-isolimonene. Other components such as γ-terpinene, terpinolene, α-terpinene, endo-fenchol and allo-ocimene were detected in lower amounts. 2-pentyl furan was found to be the principle constituent in TISTR008-fermented tea followed by δ-3-carene, hotrienol, limonene, *E*-β-ocimene, caffeine, 1,8-cineole and terpinolene, respectively.

Table 1 Volatile odor compounds of Green Oolong tea No. 12 from Boonrod farm fermented with culture supernatants of various *B. subtilis* strains

Components	Relative peak area (mean±SD%)								
	RI[a]	Dry tea[c]	TN51[f]	ASA[e]	BEST195[g]	S1-13[d]	TISTR008[b]	*S. cerevisiae*[a]	β-D-glycosidase[de]
trans-Isolimonene	984	1.10±0.32	3.22±0.08	1.96±0.12	3.30±0.08	1.97±0.07	1.03±0.07	-	2.08±0.22
2-Pentyl furan	988	2.78±0.05	8.77±0.14	8.84±0.20	14.78±0.07	9.28±0.04	5.88±0.03	-	7.22±0.09
δ-3-Carene	1011	2.55±0.11	2.00±0.07	5.11±0.11	7.45±0.11	5.34±0.05	4.93±0.04	-	0.89±0.12
α-Terpinene	1017	0.53±0.20	3.96±0.14	1.31±0.08	2.43±0.05	1.72±0.04	0.71±0.05	-	2.56±0.11
p-Cymene	1024	0.40±0.12	0.20±0.09	0.88±0.09	1.65±0.07	0.93±0.08	0.66±0.08	-	-
Limonene	1029	1.16±0.06	20.43±0.27	7.14±0.10	11.68±0.10	8.23±0.07	3.27±0.13	5.24±0.11	15.11±0.18
1,8-Cineole	1031	1.67±0.13	0.24±0.08	1.73±0.25	2.96±0.08	1.48±0.05	1.19±0.07	-	-
Z-β-Ocimene	1037	0.25±0.24	2.74±0.09	0.83±0.11	2.07±0.04	0.84±0.07	0.37±0.10	-	1.15±0.09
E-β-Ocimene	1050	0.87±0.31	8.38±0.11	3.41±0.13	5.92±0.14	3.70±0.06	1.35±0.07	0.93±0.36	6.05±0.13
γ Terpinene	1059	2.93±0.11	3.08±0.09	1.33±0.17	2.61±0.04	3.10±0.08	0.85±0.05	-	2.08±0.25
cis-Linalool oxide (furanoid)	1072	1.33±0.22	0.40±0.17	1.16±0.09	2.26±0.08	0.97±0.05	0.04±0.02	4.43±0.17	-
Terpinolene	1088	1.70±0.08	5.70±0.15	3.32±0.08	5.91±0.17	2.87±0.02	1.17±0.04	-	3.70±0.15
Linalool	1096	4.27±0.07	7.15±0.09	6.45±0.41	9.94±0.11	5.78±0.09	0.95±0.04	-	5.44±0.27
Hotrienol	1108	3.63±0.17	7.28±0.05	5.28±0.14	9.43±0.08	4.30±0.10	3.46±0.03	1.13±0.22	5.08±0.15
endo-Fenchol	1116	1.96±0.23	2.26±0.17	1.07±0.21	2.37±0.05	1.15±0.11	0.83±0.09	-	1.14±0.07
allo-Ocimene	1128	0.21±0.14	2.80±0.12	1.25±0.09	2.23±0.06	1.12±0.09	0.53±0.04	0.15±0.32	0.98±0.12
Lavandulol	1181	0.34±0.14	1.02±0.11	0.90±0.11	1.14±0.04	0.62±0.08	0.56±0.02	-	0.42±0.11
Methyl salicylate	1191	0.21±0.32	1.59±0.17	1.09±0.22	1.73±0.08	0.75±0.05	0.13±0.04	-	0.68±0.17
Safranal	1196	0.64±0.15	1.25±0.08	0.61±0.12	1.25±0.04	0.66±0.04	0.39±0.03	-	0.54±0.08
2,6,6-Trimethyl cyclohexene carboxaldehyde	1212	0.51±0.11	1.29±0.07	0.79±0.11	1.41±0.09	0.46±0.02	0.37±0.04	-	0.57±0.16
Linalool formate	1216	0.23±0.17	0.14±0.09	0.06±0.07	0.13±0.05	0.16±0.04	0.14±0.06	0.08±0.09	-
E-Ocimene	1238	0.25±0.13	0.56±0.11	0.27±0.09	0.48±0.08	0.12±0.08	0.01±0.02	0.09±0.17	-
Isobornyl formate	1239	0.21±0.20	0.38±0.13	0.31±0.21	0.53±0.07	0.01±0.01	0.11±0.02	-	-
Car-3-en-2-one	1248	0.52±0.17	0.16±0.07	0.04±0.02	0.12±0.04	0.51±0.07	0.03±0.02	2.13±0.11	-
Linalool acetate	1257	0.12±0.16	0.34±0.07	0.30±0.08	0.42±0.08	0.09±0.04	0.10±0.03	-	-

Table 1 (continued)

Compound	RI[a]	Relative peak area (%)							
		Dry tea[c]	TN51[f]	ASA[e]	BEST195[g]	S1-13[d]	TISTR008[b]	*S. cerevisiae*[a]	β-D-glycosidase[de]
Geranial	1265	0.14±0.07	0.25±0.11	0.17±0.09	0.28±0.07	0.16±0.04	0.07±0.04	-	-
Dihydro-linalool acetate	1275	0.12±0.14	0.15±0.08	0.11±0.08	0.19±0.07	0.13±0.02	0.04±0.02	-	-
2-Ethyl menthone	1282	0.35±0.22	0.13±0.05	0.06±0.03	0.11±0.05	0.41±0.04	0.04±0.02	-	-
p-Cymen-7-ol	1290	0.08±0.14	0.23±0.07	0.23±0.07	0.26±.0.04	0.14±0.01	0.12±0.05	-	-
δ-Elemene	1325	0.48±0.31	0.66±0.10	0.61±0.04	1.21±0.09	0.48±0.07	0.22±0.14	-	1.28±0.16
α-Cubebene	1338	0.11±0.06	0.10±0.09	0.12±0.05	0.14±0.07	0.15±0.06	0.04±0.02	-	-
Calacorene	1342	0.12±0.07	0.15±0.06	0.09±0.02	0.09±0.08	0.15±0.02	0.03±0.02	-	0.09±0.07
α-Ionene	1348	0.11±0.05	0.13±0.07	0.08±0.04	0.10±0.07	0.12±0.04	0.03±0.04	-	0.08±0.07
α-Longipinene	1352	0.03±0.09	0.05±0.07	0.07±0.02	0.08±0.07	0.03±0.04	0.01±0.02	-	-
α-Copaene	1370	0.11±0.22	0.26±0.08	0.24±0.04	0.43±0.09	0.14±0.02	0.08±0.02	-	-
3Z-Hexenyl hexanoate	1380	0.03±0.15	0.27±0.09	0.14±0.05	0.24±0.07	0.11±0.02	0.07±0.02	-	-
β-Panasinsene	1382	0.06±0.07	0.12±0.06	0.08±0.04	0.15±0.02	0.15±0.04	0.02±0.03	-	-
Z-Jasmone	1392	0.12±0.31	0.33±0.13	0.19±0.07	0.31±0.05	0.60±0.03	0.10±0.04	-	0.19±0.13
α-Gurjunene	1409	0.64±0.25	1.53±0.17	1.42±0.09	2.72±0.10	0.60±0.05	0.52±0.05	-	0.28±0.17
2-epi-β-Funebrene	1412	0.06±0.05	0.17±0.08	0.15±0.08	0.19±0.08	0.07±0.03	0.04±0.03	-	-
β-Cedrene	1420	0.07±0.09	0.28±0.08	0.13±0.06	0.23±0.04	0.07±0.02	0.06±0.02	-	-
Neryl acetone	1436	0.05±0.05	0.16±0.06	0.14±0.05	0.21±0.07	0.07±0.03	0.06±0.03	-	-
γ-Elemene	1438	0.06±0.07	0.20±0.14	0.12±0.08	0.20±0.07	0.05±0.02	0.02±0.02	-	0.08±0.14
Z-Jasmonyl acetate	1455	0.05±0.11	0.14±0.08	0.08±0.08	0.12±0.08	0.05±0.04	0.05±0.02	-	0..5±0.08
9-epi-*E*-Caryophyllene	1466	0.02±0.00	0.12±0.09	0.09±0.04	0.17±0.11	0.02±0.03	0.01±0.02	-	0.06±0.09
γ-Muurolene	1479	0.02±0.08	0.21±0.05	0.23±0.05	0.41±0.09	0.03±0.02	0.05±0.03	-	0.07±0.05
Germacrene D	1485	0.03±0.24	0.13±0.05	0.12±0.04	0.21±0.07	0.29±0.04	0.02±0.02	-	1.14±0.05
E-β-Ionone	1489	0.43±0.05	0.72±0.16	0.47±0.09	0.88±0.05	0.05±0.02	0.28±0.08	-	1.89±0.11
α-Muurolene	1500	0.07±0.14	0.22±0.11	0.27±0.11	0.37±0.08	0.07±0.02	0.06±0.02	-	-
Germacrene A	1509	0.07±0.08	0.25±0.09	0.31±0.13	0.29±0.09	0.14±0.03	0.13±0.03	-	-
Cubebol	1515	0.02±0.02	0.21±0.07	0.17±0.09	0.18±0.07	0.03±0.04	0.02±0.03	-	-
trans-Calamenene	1521	0.12±0.05	0.42±0.25	0.39±0.08	0.71±0.04	0.18±0.05	0.16±0.04	-	1.52±0.35

Table 1 (continued)

Compound	RI[a]	Relative peak area (%)							
		Dry tea[c]	TN51[f]	ASA[e]	BEST195[g]	S1-13[d]	TISTR008[b]	*S. cerevisiae*[a]	β-D-glycosidase[de]
Caffeine	1842	0.32±0.09	0.27±0.11	1.48±0.04	0.33±0.04	0.84±0.04	1.32±0.05	-	-
2E,6E-Farnesyl acetate	1846	0.10±0.11	0.32±0.08	0.33±0.05	0.19±0.08	0.09±0.03	0.12±0.03	-	-

This study was statistically analyzed by using ANOVA (tukey test). The results obtained are shown in Table below.

Treatment	N	Subset						
		1	2	3	4	5	6	7
S. cerevisiae	162	0.2583						
TISTR008	162		0.6038					
Dry tea	162			0.6502				
S1-13	162				1.1346			
β-D-glycosidase	162				1.1444	1.1444		
ASA	162					1.1786		
TN51	162						1.7129	
BEST195	162							1.9297
Sig.		1	1	1	0.9895	0.0542	1	1

The statistical analysis of this study presented the differentiation among these treatments. As the results, volatile profiles obtained from TN51, ASA, BEST195, S1-13

and β-D-glycosidase were significantly greater than those found from dry tea. However, volatile profiles of *S. cerevisiae* and TISTR008 were presented significantly lower than those from dry tea.

It was noted that *S. cerevisiae* strain was not suitable for tea fermentation due to only few compounds were obtained. This was resulted from the different enzymes found inside of this yeast compared those found on Bacillus bacteria. Moreover, solution of β-D-glycosidase enzyme was used to ferment with tea sample. It was found that similar profile was obtained campared to those found on *B. subtilis* TN51 and ASA, respectively.

As the results, supernatants from Bacillus presented higher number of compounds than pure enzyme solution. This may be resulted from low concentration of pure enzyme. Moreover, some compounds were not detected by using pure enzyme. Although, pure enzyme provided similar to those obtained from Bacillus supernatants, prize of this enzyme is expensive. Therefore, the application of Bacillus can be used to increase concentration of aroma compounds of tea due to cheap and safety.

Dajanta et al. (2011) previously noted that *Bacillus subtilis* supernatants can cause a change in quality and quantity of odor volatile components in Green Oolong tea No. 12. As the results, caffeine, bitter xanthine alkaloid, impacted the higher value which was found in fermented tea with *B. subtilis* ASA, TISTR008 and S1-13 culture supernatants compared to original sample. In addition, greater amounts of some components including 2-pentyl furan, *E*-β-ocimene, limonene, δ-3-carene and hotrienol were detected in TN51- and BEST195-fermented teas, whereas Green Oolong tea No. 12 from Boonrod farm fermented by S1-13, ASA and TISTR008 culture supernatants showed significantly lower amounts. Lower amounts of γ-terpinene, p-cymene, *cis*-linalool oxide (furanoid), dihydro-linalool acetate and α-cubebene were present among all tea samples.

It was found that greater intensity of most volatile components was detected in *B. subtilis* TN51-fermented tea while tea fermented by *B. subtilis* ASA culture supernatants provided highest amount of caffeine. Volatile compounds of tea fermented with various supernatants of *B. subtilis* in this study were different from the study of Su et al. (2010)

and Wang et al. (2001), they reported that major compound of geraniol, benzyl alcohol, phenylethanol and *Z*-3-hexenol appeared significantly in Oolong teas fermented with enzyme. *B. subtilis* cultures fermentation of tea could induce increasing amounts of various volatile components due to enzyme production of each strain. Total volatiles significantly increased from its original non-fermented sample. These occurrences happened could be explained that the existing form, amount and kind of aroma precursors were different in different tea explained by Su et al. (2010). Besides, the enzyme could show different substrate specificity to different aroma precursors. Increased amounts of 2-pentyl furan shown in all *B. subtilis*-fermented teas may be related to enzymatic production of soybean that Sugawara et al. (1985) reported that 2-pentyl furan was a key bean-like odor compound of the soybean. Several investigations also reported that 2-pentyl furan was detected in soybeans (Leejeerajumnean et al., 2001; Owens et al., 1997; Sugawara et al., 1985). It was found that *B. subtilis* generated 2-pentyl furan in different materials such as soybean and tea. Although *B. subtilis* culture supernatants increased contents of volatile components of ferment teas, some major compounds found in pure Bacillus-fermented *thua nao* and in naturally fermented soybean such as 2,5-dimethylpyrazine, 2-methylbutanoic acid, 2,3,5-trimethylpyrazine and 2-methylpropanoic acid (Dajanta et al., 2011) were disappeared in *B. subtilis*-fermented teas. It seems that 2-pentyl furan play important role in the special odor of *B. subtilis*-fermented teas especially in BEST195-fermented tea. Increased intensities of most components might be also affected from enzymatic production of *B. subtilis* which enzymatic activities produced by microbes, such as protease, amylase and galactosidase (Campbell-Platt, 1980; Odunfa, 1986) improved odor volatile components during fermentation. Furthermore, various isolated *B. subtilis* could produce several extracellular enzymes with the same function, such as nattokinase, protease, amylase, phytase, lipases and glutamyl hydrolase (Chantawannakul et al., 2002; Chukeatirote et al., 2006; Chunhachart et al., 2006; Dajanta et al., 2009; Visessanguan et al., 2005). Enzymatic degradation products might be generated further complex odorous compounds through other reactions. Culture supernatants of *B. subtilis* TN51 and ASA strains were selected to

ferment with various tea samples due to their effectiveness on increasing of volatile components and caffeine, respectively.

Furthermore, mixture of TN51 and ASA supernatants was also chosen to mix with various teas to compare their odor volatile components with obtained by TN51 and ASA supernatants. Fingerprints of odor volatiles detected in tea from Boonrod farm, Chuy Fong Company Limited and Wang Put Tan tea valley fermented with culture supernatants of TN51, ASA, and mixture of TN51 and ASA are illustrated in Fig. 1, 2 and 3, respectively.

As the results, similar profiles of odor volatiles were obtained. Caffeine was greater significantly in Boonrod tea fermented with mixture of *B. subtilis* TN51 and ASA supernatants than their present in ASA-fermented teas, respectively, while TN51 supernatants disappeared. High level of α-gurjunene and δ-elemene eluted at retention time of 27.13 and 30.16 min were also found in mixed *B. subtilis* supernatants. Chuy Fong fermented tea demonstrated similar fingerprints among these microorganism supernatants. Caffeine was detected only in TN51-fermented Chuy Fong tea and Wang Put Tan tea valley tea fermented by *B. subtilis* ASA supernatants. Various *B. subtilis* and their ratios affected on odor volatile components of teas. It was found that most volatiles detected from Boonrod tea were achieved by using mixture of *B. subtilis* while TN51 and ASA supernatants were successful on Chuy Fong and Wang Put Tan tea valley tea, respectively.

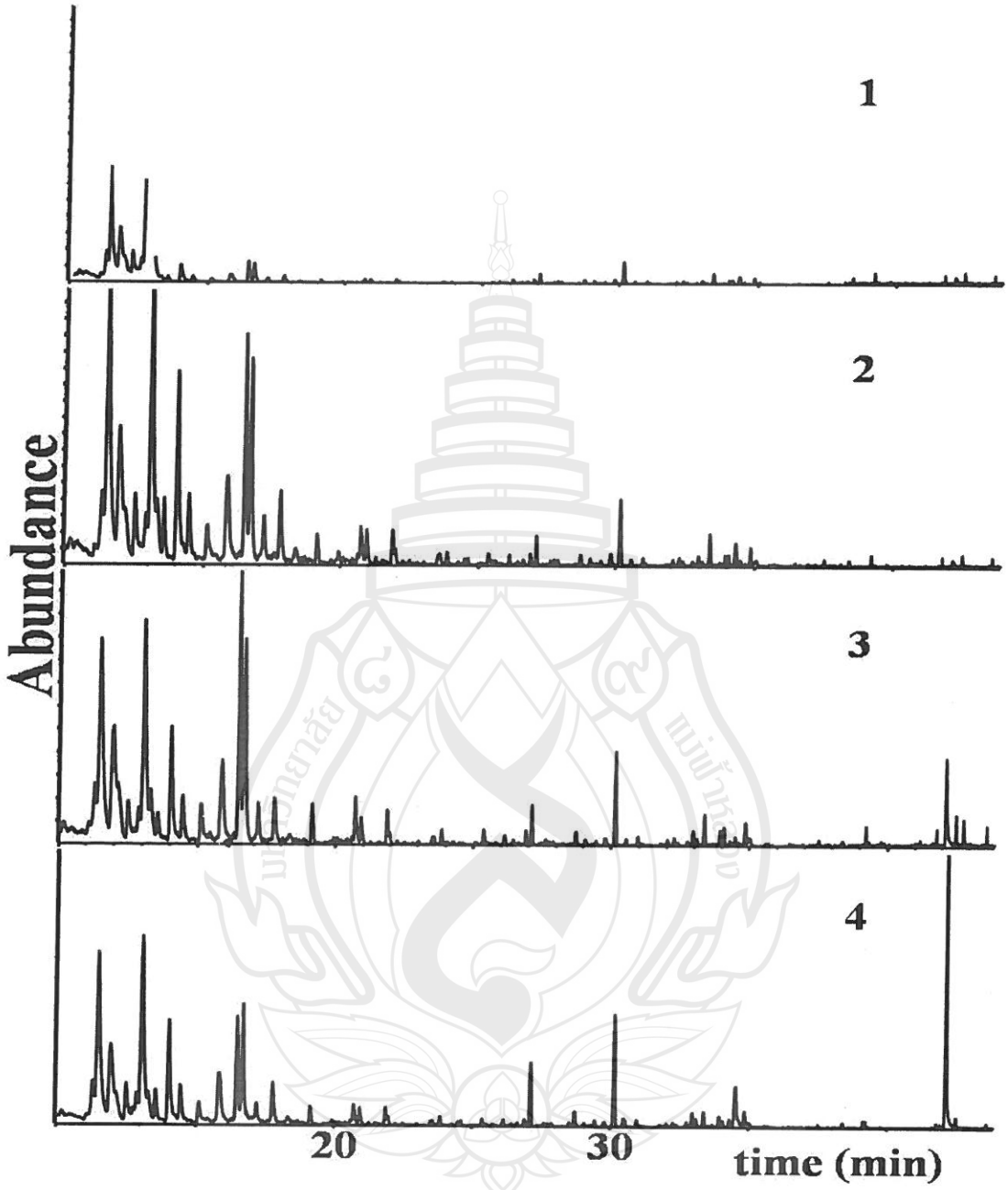

Fig. 1 GC-MS chromatograms of volatile odor compounds of dry Green Oolong tea No. 12 from Boonrod farm (1) and dry green Oolong tea No. 12 fermented with various *B. subtilis* culture supernatants. 2; TN51, 3; ASA and 4; mixed TN51 and ASA

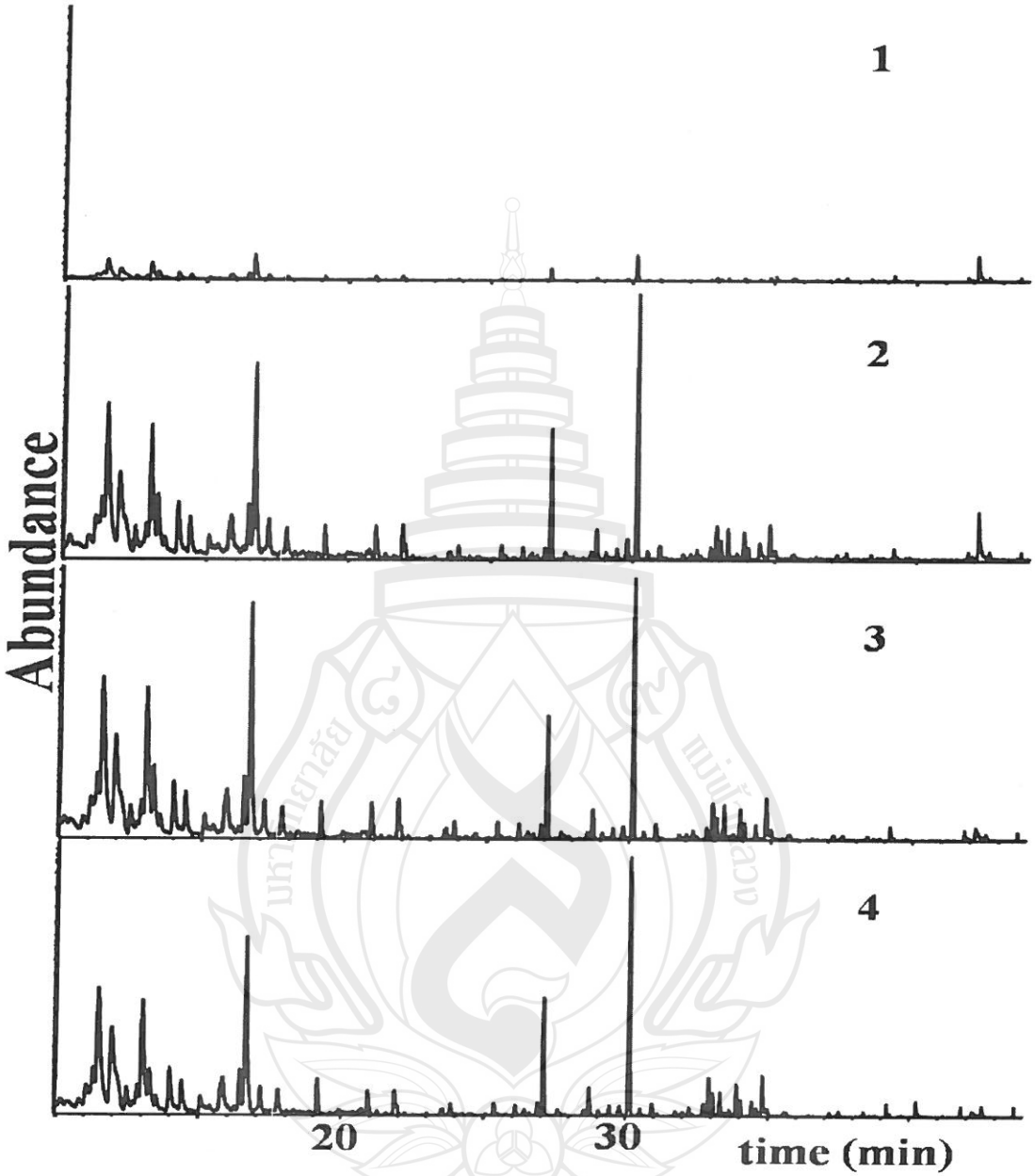

Fig. 2 GC-MS chromatograms of volatile odor compounds of dry Green Oolong tea No. 12 from Chuy Fong Company Limited (1) and dry green Oolong tea No. 12 from Chuy Fong Company Limited fermented with various *B. subtilis* culture supernatants. 2; TN51, 3; ASA and 4; mixed TN51 and ASA

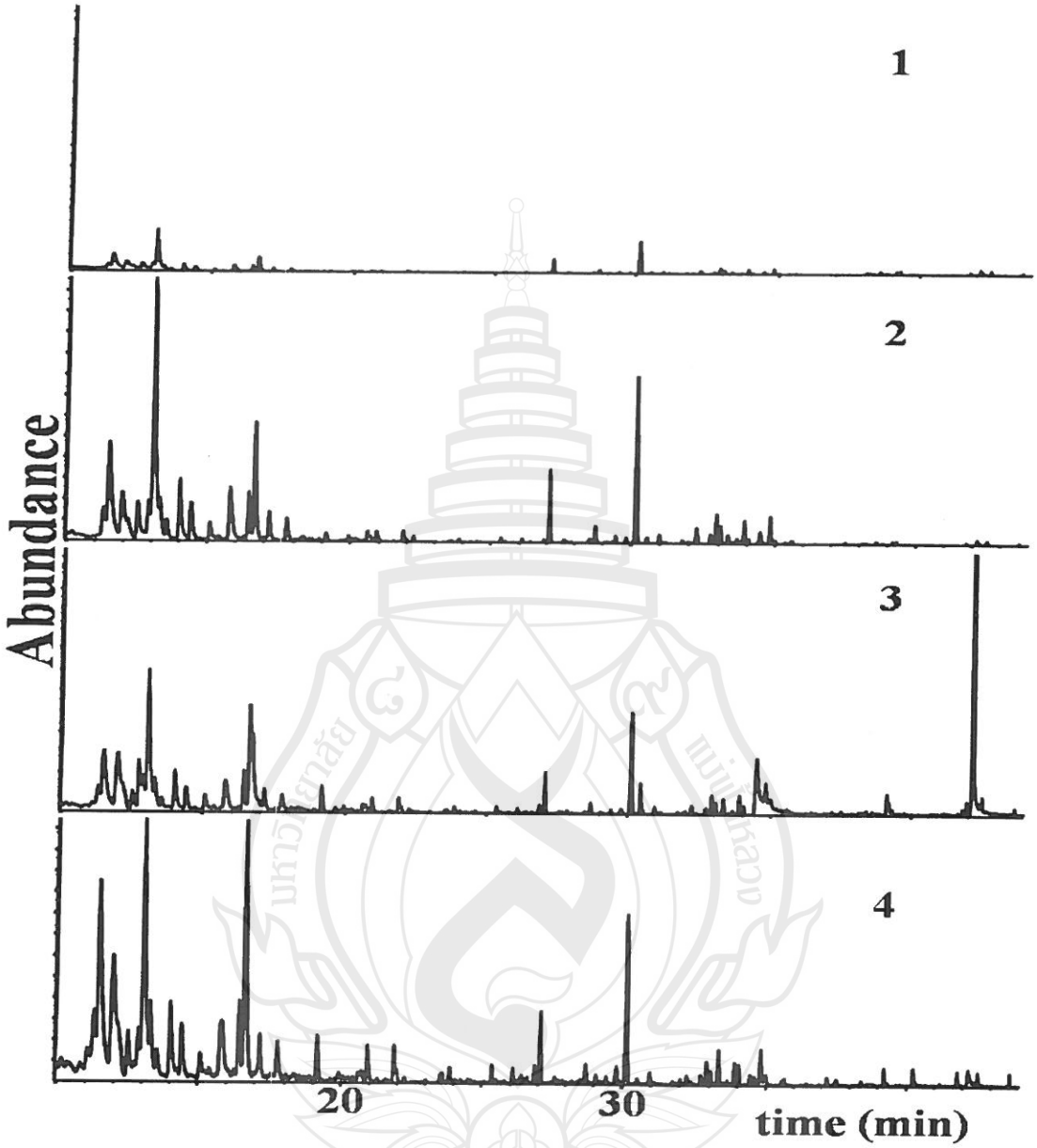

Fig. 3 GC-MS chromatograms of volatile odor compounds of dry Green Oolong tea No. 12 from Wang Put Tan tea valley (1) and of dry Green Oolong tea No. 12 from Wang Put Tan tea valley fermented with various *B. subtilis* culture supernatants. 2; TN51, 3; ASA and 4; mixed TN51 and ASA

CHAPTER 4
CONCLUSION

Increased contents of total volatiles were detected in all *B. subtilis* culture supernatants compared to other microbes, β-D-glycosidase and dry tea, respectively, being the major volatiles were 2-pentyl furan, limonene, linalool and δ-3-carene. Every Green Oolong tea No. 12 has similar volatile profiles whist their amounts were different according to the different origin, genotype breeding and ratio of supernatants of *B. subtilis*. Higher contents of most odor volatiles were detected in Boonrod tea fermented with mixture of TN51 and ASA culture supernatants whereas ASA culture supernatants provided greater amount of caffeine. Volatiles of Green Oolong tea No. 12 from Chuy Fong Company Limited and Wang Put Tan tea valley were generated from *B. subtilis* TN51 and ASA supernatants, respectively. Whether the significantly increasing of volatile profiles in fermented teas affected from exogenous enzymes secreted from *Bacillus* strains remained to be investigated. In addition, *Bacillus* strains may be added to improve aroma quality of tea in non fermentation tea processing near future.

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Transcriptomics: RT-PCR, Real time, PCR

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