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Application of Microbial Fermentation Products Extracted from Maotai Daqu in Tobacco Sheets

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To improve the flavour of traditional tobacco sheets, aroma-producing bacterial including *Bacillus subtilis* MT-1, *B. amyloliquefaciens* MT-3, and *B. licheniforms* MT-2 isolated from Maotai Daqu (a kind of distiller's yeast for brewing Moutai spirit, a type of Chinese spirit) were cultured on wheat medium at 37 °C, 46 °C, and 55 °C respectively for 2 days. The authors prepared tobacco sheets by using the extracted fermentation products, tobacco waste, and an adjunct (Sheet 1). Afterwards, the tobacco sheets were compared with those tobacco sheets to which no fermentation products had been added (Sheet 2) in terms of physical and chemical properties and sensory quality. The substances present in Sheets 1 and 2 were extracted by using supercritical CO_2 and the extracted products analysed by gas chromatography-time of flight mass spectrometry (GC-TOF(MS)). The results showed that microbial fermentation extracts manifested little effect on the physical properties of tobacco sheets; the content of total sugars and the sugar:nicotine ratio of Sheet 1 were higher than those of Sheet 2; besides, the sensory quality score of the tobacco in Sheet 1 increased by 1.4 compared to Sheet 2; moreover, twelve aromatic substances were detected in both the fermentation extracts and Sheet 1. They were proved to be the main factor which contributed to the significant improvement in the sensory quality of Sheet 1.

Key words: Microbe, *Bacillus*, Fermentation, Tobacco Sheets, Sensory Quality.

Tobacco sheets are also known as reconstituted tobacco and refer to the tobacco made at certain proportion from waste tobacco powder, tobacco stem, tobacco leaf fragments, *etc*. yielded in harvest, production, and the re-drying process. They are mainly used as a filler in medium and low-end cigarettes. On one hand, the addition of reconstituted tobacco in cigarettes re-uses waste tobacco and thus reduces cigarette cost; on the other hand, it improves the burning and filling capacities of tobacco and reduces tar release[1,2]. However, tobacco sheets show many shortcomings, such as: a dry smoke taste, insufficient aroma, irritating or offensive odours, *etc*.[3]. Therefore, it is worthwhile to undertake research into the improvement of such tobacco sheets.

At present, microbial fermentation technology for tobacco, mainly targeted at shortening the fermentation period and increasing tobacco flavour[4,5], has been widely used around the world. In China, research concerning the use of microbial technology to improve tobacco sheet quality has just started[3]. Related studies mainly concentrate on improving the physical-additive quality of tobacco sheets produced by the rolling method. For example, Xiao et al.[6] improved the fibre-adding process to solve such quantity

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problems as "flowering" and "whitening" of the tobacco sheets produced in the rolling process. They firstly dispersed wood pulp fibre uniformly into the medium solution and then added tobacco powder. The sheets produced by their method showed excellent physical properties and the utilisation rate of cut sheets was thereby improved. Han[7] tried to enhance the physical and sensory qualities of tobacco sheets produced by the rolling method. Han mixed the powders of some fungal fruit-body bearing high aroma and low lignin content (X20051) with fragmented tobacco stems, tobacco powder, and tobacco picks. The mixtures obtained were further mixed with the fungal fermentation colloid extraction and chitin. Then using an original process, they produced a new type of tobacco sheet. Compared with the control, the new type was displayed a water resistance increase from 0.63 min to 5.76 min, a tensile strength improvement from 2.75 N/mm^2 to 4.02 N/mm^2, and a sensory quality increase of 2.18 points. So far, research concerning an increase in sheet aroma has rarely been reported. Moreover, there has been no research into the production of tobacco sheets using microbial fermentation extracts and waste tobacco. This study used three strains of bacillus isolated from the Moutai Daqu (a kind of distiller's yeast for brewing Moutai spirit, a type of Chinese spirit) for solid fermentation at high temperature by simulating the starter-making process of Maotai spirit[8,9], the fermentation products were then extracted using edible alcohols. They were then added to the tobacco sheets during production to improve the sheet aroma and as-smoked taste of cheap cigarettes made from tobacco sheets, and furthermore, produce sheet cigarettes bearing the characteristic local terroir of Guizhou (a province in China).

MATERIALS AND METHODS

Strains and raw materials

Bacillus subtilis MT-1, *B. amyloliquefaciens* MT-3, and *B. licheniformis* MT-2 were isolated from Moutai Daqu; the tobacco sheets and rolling equipment were provided by China Tobacco Guizhou Industrial Co., Ltd.

Bacterial fermentation and extract detection

Bacterial activation

The strains of the three bacteria preserved on a test-tube slant were inoculated on sterilised PDA plates respectively and then cultured at 50 °C for 20 h. The strains obtained were inoculated into the PDA liquid media and cultured on a shaker at 120 rpm for 20 hours at 50 °C.

Solid fermentation

Under sterile conditions, some 3.5 mL of cultured bacterial suspensions of each of the three bacterial strains were removed respectively. The suspensions were then inoculated into the wheat media and cultured for 2 days at 37 °C, 46 °C, and 55 °C respectively[10]. In the later fermentation phase, sauce flavours were significant. The production process of the wheat medium was as follows: 100 g wheat was moderate1y fractured and infiltrated by 40 mL ultra-pure water. Then they were put into culture bags (24 × 7 × 3.5 cm) and sealed, in a knot, with special shrink rings (3.5 cm diameter, Yanxinglong Plastic Products Factory). Finally, they were sterilised at 121 °C for 30 min.

Detection of extract

After fermentation, the wheat media bearing cultured bacterial suspensions were extracted in 250 mL 55 % vol edible alcohol for 2.5 h. Then 20 mL extracted solutions were removed and centrifuged at 3000 rpm for 8 min (Xiangyi L-530 multi-frame automatic balancing centrifuge, Shanghai HuYueming Scientific Instrument Co., Ltd). The supernatants obtained were treated overnight using a proper amount of anhydrous sodium sulphate and then concentrated to 2 mL by rotation (rotary evaporator RE-52A, Shanghai Yarong Biochemical Instrument Factory). The solutions obtained were filtered using a 0.22 ìm organic membrane (Haining Shenghua Filter Equipment Co., Ltd). The filtrates were detected using GC-TOF (MS) (USA Waters Company) under the following conditions: GC: DM-FFAP(30 m×0.25 mm, 0.25 ìm); inlet temperature: 230 °C; sample size: 1 ìL; split ratio: 50:1; temperature programming: maintain the initial temperature of 60 °C for 1 min; then increase the temperature to 240 °C at 5 °C/min and maintain this elevated temperature for 5 min; carrier gas: He; flow rate: 1.0 mL/min; TOF (MS): EI source electron energy of 70 eV and an ion source temperature of 250 °C; and a mass scan range: 10 to 800 amu. The mass spectra collected were retrieved in the NIST08 spectral library.

Test for aroma increase of tobacco sheets

Process

The tobacco sheet was manufactured

using the process used by the China Tobacco Guizhou Industrial Co., Ltd: before mixing, the fermentation extracts were added to the materials in the proportions of material:fermentation extract of 1000 g to 1 mL (Figure 1) to make tobacco Sheet 1. Moreover, tobacco sheets without fermentation extracts were prepared as a control (Sheet 2).

Detection of the physical properties of the tobacco sheets

The physical properties of the tobacco sheets were analysed in accordance with the method proposed in "Part one: rolling" of "Reconstituted tobacco"(YC/T16.1-2002) (China Tobacco Monopoly Bureau).

Detection of the chemical indices of tobacco sheets

The chemical indices were detected according to the following standards: water-soluble sugar and reducing sugar-YC/T159-2002, total plant alkaloid-YC/T160-2002, total nitrogen-YC/T 161-2002, chlorine-YC/T162-2002, and potassium-YC/T217-2002.

Fig. 1. The improved production process for tobacco sheets

Table 1. Tobacco sheet properties

Sample	Moisture content %	Width/ mm	Length /mm	Cutting and drawing rate %	Rate of connected cut tobacco %	Rate of integrat cut tobacco/ %	Filling ability cm/g	Combusti-bility/S	Ash colour	Thickness/ mm	Tensile strength
Sheet 1	13.64	1.0	54	1.1	0.3	95.06	3.51	10	grey	0.126	2.54 N
Sheet 2	13.60	1.0	55	0.9	0.3	95.09	3.90	10	grey	0.130	2.61 N

Table 2. Chemical properties of tobacco sheets

Sample	Total sugar %	Reducing sugar %	Chlorine %	Nicotine %	Potassium %	Total nitrogen %	The ratio of total sugar to nicotine
Sheet 1	22.1167	17.5873	0.9661	1.5391	3.4130	1.7664	14.3699
Sheet 2	21.4872	17.5391	0.9615	1.5252	3.3464	2.0171	14.0881

Table 3. Sensory quality data

Sample	Aroma quality(10)	Aroma quantity(10)	Taste (12)	Offensive odour(10)	Irritation (10)	In total
Sheet 1	7.8	8.0	9.3	7.7	8.0	40.8
Sheet 2	7.5	7.5	9.0	7.5	8.0	39.4

Note: combustibility and ash colour, which are irrelevant to the aroma, were excluded.

Evaluation of the sensory quantities of tobacco sheets

Cut tobacco Sheets 1 and 2 were made into cigarettes which were then balanced for 24 hours in an environment with a relative humidity of 62 % at a temperature of 22 °C to maintain a gravimetric moisture content of approximately 12 %. Then they were scored by evaluating experts from China Tobacco Guizhou Industrial Co., Ltd in a dedicated smoking room.

Detection of the aroma substances of tobacco sheets

Extraction of the aroma substances by supercritical CO_2

Sheets 1 and 2 (at a certain mass) were baked at 50 °C for 2 hours in a constant temperature box. After cooling, they were pulverised using a plant smasher. Then 100 g of Sheet 1 powder was added to a 500 mL extracting axe after being infiltrated by 100 mL ethanol. Then a CO_2 cylinder, capable of providing a flow rate of 4Nm^3/h, was opened. After both the extracting axe and pressure axe reached their required temperatures, the system was pressurised by high-pressure pump. When the extraction conditions (extraction axe: 60 °C, 25 MPa; analytical axe: 55 °C, 5.5 MPa) were satisfied, sheet powder was extracted in a static state for 30 min and then in a dynamic state for a further 90 min under the same conditions. Extraction of Sheet 2 powder also followed this procedure.

Rotary evaporation

Some 20 mL of the extract of Sheets 1 and 2 were placed in separate triangular flasks respectively. After overnight treatment using an appropriate amount of anhydrous sodium sulphate: they were then moved into 50 mL centrifuge tubes and centrifuged for 8 min at 3000 rpm. The supernatants collected were concentrated to 2 mL by rotating and then filtered using 0.22 ìm organic membranes. Finally, they were analysed using GC-TOF(MS) (detection conditions were the same as those mentioned in Section 1.2.3).

Results and analysis

Analysis of the physical properties of the tobacco sheets

The physical properties of the tobacco sheets were analysed in accordance with the method proposed in "Part one: rolling" of "Reconstituted tobacco"(YC/T16.1-2002), as shown in Table 1.

As shown in Table 1, the physical properties of the both sheets were similar and met standard YC/T16.1-2002. This proved that the addition of the three strains exerted no influence on the physical properties of these tobacco sheets.

Analysis of the chemical indices of tobacco sheets

As an important index for evaluating the qualities of tobacco, total sugar content is positively correlated with the sensory quality evaluation results of tobacco sheets[11]. In addition, the ratio of total sugar to nicotine is positively correlated with smoking quality[12]. Table 2 shows the comparison of the chemical properties of the two tobacco sheets. As shown, Sheet 1, with

Table 4. The aroma-inducing substances of tobacco sheets and microbial fermentation extracts

No.	Compound	Molecular formula	Relative percentage		
			Sheet ck	Sheet 1	Microbial fermentation extract
	Ketones				
1	2,5-dimethyl-2,4-dyhydroxy-3-(2H)-furan-3-ketone	$C_6H_8O_4$	-	0.05	0.10
2	2(5H)- furanone	$C_4H_4O_2$	-	0.05	0.12
3	4,5-dimethyl -1,3- dioxole-2-ketone	$C_5H_6O_3$	-	0.20	0.38
4	3-5-dyhydroxy-2- methyl -4H- pyran -4-ketone	$C_6H_6O_4$	-	0.23	0.29
5	4,5-dimethyl -3- hydroxy-2(5H)-furanone(Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one)	$C_6H_8O_3$	-	0.09	0.32
6	2- cyclopentene -1,4- diketone	$C_5H_4O_2$	-	-	0.83
7	Solanone	$C_{13}H_{22}O$	0.46	0.29	-
8	1,2- Cyclopentane diketone	$C_5H_6O_2$	0.02	0.21	0.44
9	Pineapple ketone(4- hydroxy-2,5-dimethyl -3(2H) furanone)	$C_6H_8O_3$	0.03	0.09	0.19
10	α- pyrrolidone	C_4H_7NO	0.14	0.24	0.07
11	Alloy cyclopropanone	$C_{18}H_{36}O$	0.07	-	-
12	4 - (2,4,4 – trimethyl hexamethylene-1,5 - dialkylene) - butone-3 - alkene-2 - ketone	$C_{13}H_{18}O$	0.15	0.07	-
13	5,6- dihydro-6 -amyl-2H-pyran-2-ketone	$C_{10}H_{16}O_2$	1.53	1.82	-
14	2,3 - dihydro-3,5 - dyhydroxy-6 - methyl-4H-pyran-4 - ketone	$C_6H_8O_4$	0.50	6.30	9.56
15	megastigmatrienone	$C_{13}H_{18}O$	0.57	0.45	-
16	(3E)-4 - (5 - hydroxy-2,6,6 - trimethyl-1 - cyclohexene -1 - radical) -3 - butene-2 - ketone	$C_{13}H_{20}O_2$	0.19	0.20	-
17	3,5-Dimethoxy acetophenone	$C_{10}H_{12}O_3$	0.30	0.43	1.88
18	4 - (3 - hydroxy-1 - butenyl) -3,5,5 - trimethyl-2 - cyclohexene-1 - ketone	$C_{13}H_{20}O_2$	0.66	1.04	-
19	1,2,3,3a,4,5,6,8,9, 9a,10,10a- N-Cyclohexylcyclo - 7- (1 - Methyl ethyl) -1,9 -dimethyl -4 - methylene, Cyclopentadiene [AÿD] cyclooctene -5 - ketone	$C_{20}H_{30}O$	1.29	0.33	-
	alcohols				
20	5-methyl-2- furfuryl alcohol	$C_6H_8O_2$	-	0.24	0.80
21	β- phenethyl alcohol	$C_8H_{10}O$	-	0.14	0.34
22	2,3- butanediol	$C_4H_{10}O_2$	0.04	0.40	0.71
23	(2R,3R)-(-)-2,3- butanediol	$C_4H_{10}O_2$	0.08	0.34	0.46
24	1,2- propylene glycol	$C_3H_8O_2$	6.94	9.41	0.07
25	furfuryl alcohol	$C_5H_6O_2$	0.03	0.25	0.56
26	oxo benzoic acid methyl alcohol	$C_8H_6O_3$	0.49	0.51	0.05
27	(3a,17E) - progesterone -5, 17 (20)- diene -3 - alcohol	$C_{21}H_{32}O$	1.69	0.14	-
28	4,8,13 - pine triene -1,3 - diol	$C_{20}H_{34}O_2$	1.69	-	-
	Esters				
29	methyl acetate	$C_3H_6O_2$	-	0.09	0.21
30	L- ethyl lactate	$C_5H_{10}O_3$	0.05	3.30	0.20
31	diethyl succinate	$C_8H_{14}O_4$		0.05	-
32	D(-)-Pantoyl lactone	$C_6H_{10}O_3$	0.07	0.07	-
33	methyl palmitate	$C_{17}H_{34}O_2$	0.35	0.05	-
34	ethyl palmitate	$C_{18}H_{36}O_2$	0.27	0.03	0.10
35	mono-ethyl succinate	$C_6H_{10}O_4$	0.04	0.18	0.59
36	4 - Methoxy carbonyl -4 - butyrolactone	$C_6H_8O_4$	-	-	0.58
37	(S)-3- hydroxy-ã- butyrolactone	$C_4H_6O_3$	0.25	0.35	0.31

	acids				
38	butyric acid	$C_4H_8O_2$	-	0.28	0.14
39	acetic acid	$C_2H_4O_2$	3.89	9.44	14.51
40	formic acid	CH_2O_2	0.47	1.03	0.13
41	2- methylbutyric acid	$C_5H_{10}O_2$	0.07	0.22	-
42	pentanoic acid (valeric acid)	$C_5H_{10}O_2$	-	0.02	-
43	Ethoxyacetic acid	$C_4H_8O_3$	4.54	4.46	-
44	furoic acid	$C_5H_4O_3$	0.08	0.87	2.36
45	nicotinic acid	$C_6H_5NO_2$	0.20	0.55	0.69
46	cetylic acid (palmitic acid)	$C_{16}H_{32}O_2$	11.92	2.66	4.44
47	Phenylmalonic acid	$C_9H_8O_4$	1.3	1.58	0.30
	phenols				
48	ethyl maltol	$C_7H_8O_3$	0.02	0.25	0.53
49	2- methoxyl -4- vinylphenol	$C_9H_{10}O_2$	0.02	0.52	1.28
	aldehydes				
50	phenylacetaldehyde	C_8H_8O	-	0.04	0.04
51	5- xymethylfuraldehyde	$C_6H_6O_2$	0.17	0.88	2.53
52	DL- glyceraldehyde	$C_3H_6O_3$	5.16	6.73	8.90
53	5- hydroxymethyl-furaldehyde	$C_6H_6O_3$	3.63	-	2.77
54	vanillic aldehyde (vanillin)	$C_8H_8O_3$	0.34	0.41	-
55	3- furaldehyde (3- furan formaldehyde)	$C_5H_4O_2$	0.11	0.25	0.68
	furan				
56	2- methoxyl furan (2- Cefuroxime methyl ether)	$C_5H_6O_2$	-	-	0.15
57	3- acetamido furan	$C_6H_7NO_2$	-	-	0.14
	alkaloid				
58	nicotine	$C_{10}H_{14}N_2$	21.74	21.96	-
59	á- nicotine	$C_{10}H_{14}N_2$	13.70	0.18	-
60	Isonicoteine	$C_{10}H_8N_2$	0.85	1.30	-
	other		-	-	-
61	glutaconic anhydride	$C_5H_4O_3$	-	0.62	3.21
62	ethylâ-D- ribonucleotide	$C_7H_{14}O_5$	-	16.68	35.74
63	2- methylpyrazine	$C_5H_6N_2$	-	-	0.13
64	Dimethyltrisulfide	$C_2H_6S_3$	-	-	0.14
65	3- eicosane acetylene	$C_{20}H_{38}$	8.86	-	-
66	2- acetyl pyrrole	C_6H_7NO	0.10	0.17	0.06
67	succinimide(succimide)	$C_4H_5NO_2$	0.10	0.13	0.04
68	bis [6,6 - dimethyl =ÿ- 2, 2'- (1, 2 - piperazinyl) bicyclo [3.1.1] enanthine -2 - alkene	$C_{20}H_{30}$ -	1.02	-	-
69	1,4:3,6 - dianhydrog – ± - D - glucopyranose	$C_6H_8O_4$	0.16	-	-
70	Unknown compound 1	$C_{20}H_{30}$	0.62	-	-
71	Unknown compound 2	-	0.08	0.90	1.93
72	Unknown compound 3	$C_{20}H_{30}$	2.42	0.23	-
73	Unknown compound 4	$C_{20}H_{30}$	0.51	-	-

Note: "-" represented undetected compounds

microbial fermentation extract, presents a higher total sugar content possibly due to the sugars in fermentation liquors; the ratio of total sugar to nicotine of Sheet 1 exceeded that of Sheet 2; contents of chlorine, reducing sugar, nicotine, and potassium were not significantly different between Sheets 1 and 2; the total nitrogen content was lower in Sheet 1. Preliminary analysis of the chemical indices revealed that the tobacco sheets with added microbial fermentation extract displayed an improved smoking quality. However, analysis and evaluation of aroma-enhancing substances should be further evaluated.

An evaluation of the sensory quality of the tobacco sheets

According to evaluation requirements, the two sheets were judged by a panel of seven experts from China Tobacco Guizhou Industrial Co.,

Ltd: data are shown in Table 3.

Table 3 showed that the internal quality of the tobacco sheet was improved by the addition of fermentation extracts of the three strains during production. The total evaluation score of Sheet 1 was 1.4 points higher than that of Sheet 2. Moreover, the aroma of the cigarettes made from Sheet 1 wass more favorable, present in sufficient intensity, soft, delicate, and moderately-concentrated. This result agreed with the positive correlations of the sugar content and the ratio of total sugar to nicotine with smoking quality.

Analysis of the aroma-inducing substances

According to design requirements, the total ion chromatographic flows of the microbial fermentation extract, Sheet 1, and Sheet 2 were obtained using GC-TOF (MS) analysis (as shown in Figures 2 to 4 respectively).

Table 4 lists the 73 types of aroma-inducing substances obtained by retrieving the mass spectrum from the NIST08 spectrum library.

The aroma-inducing substances in Table 4 mainly include: ketones, acids, alcohols, esters, aldehydes, phenols, alkaloids, furans, *etc*., which are compared as follows:

1) Ketones: a total of 19 types of ketone were detected in Sheet 1, Sheet 2, and in the microbial fermentation extracts. With the exception of alloy cyclopropanone which was not detected in Sheet 1, 12 types were found both in Sheet 1 and Sheet 2. The fermentation extract contained 11 types of ketone, five kinds therein, including 2,4-dyhydroxy-2,5-dimethyl-3dihydrofuran-3-ketone, 2(5H)-furanone, 4,5-dimethyl-1,3-dioxole-2-ketone, 3-5-dyhydroxy-2-methyl-4H-pyran-4-ketone, and 3-hydroxyl-4,5-dimethyl -2(5H)-furanone(Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one) were also present in Sheet 1 rather than Sheet 2. This result suggested that Sheet 1 was provided with specific aroma-related substances by microbial fermentation extract.

2) Alcohols: a total of nine alcohols were detected in Sheet 1, Sheet 2, and the microbial fermentation extracts. With the exception of 4,8,13 -pine triene -1,3 – diol, Sheets 1 and 2 contained traces of seven common alcohols. Of the seven types of alcohols detected in the fermentation extracts, the 5-methyl-2-furfuryl alcohol and β-phenethyl alcohol were also detected in Sheet 1 but not in Sheet 2. This outcome suggested that the addition of microbial fermentation extract increased the alcohol aroma-related substances in Sheet 1. It was notable that β- phenethyl alcohol is also one of the characteristic aroma-inducing substances in Moutai wine[14-16]. The content of a- phenethyl alcohol in Moutai wine is three times higher than in Fenjiu and Luzhou Tequ wines[17]. Moreover, β- phenethyl alcohol is also found to be characteristic aroma-related substance in 10 different types of Moutai-flavoured liquors[18] and also a characteristic flavour substance in various

Table 5. Prominent aroma-related substances in both Sheet 1 and microbial fermentation extracts

	Aroma-related characteristics	Reference
4,5-dimethyl -3-hydroxy -2(5H)-furanone	It shows strong persistent cane molasses-like aroma and a slightly burnt fruit fragrance	[20]
β-phenethyl alcohol	This compound has a rose flower fragrance. It has been reported as the important metabolite of the three bacillus strains of Moutai Daqu	[21]
2,5-dimethyl -2,4-dyhydroxy-3-(2H)-furan-3-ketone	Aroma characteristics were undetermined. According to molecular structure, it was judged that this compound contributed to aroma improvement.	[22]
methyl acetate	This compound showed undetermined aroma characteristics and formed the basis for the tobacco's perfume	[23]
Butyric acid	This compound had a cream flavour and had fruit fragrances with its synthesis into esters with low alcohols.	[24]
Phenylacetaldehyde	This compound had a sweet smell and a Moutai-flavour	[25]

sauces[19]. The presence of β- phenethyl alcohol in Sheet 1 suggested that the β-phenethyl alcohol produced during microbial fermentation had access to Sheet 1. This provided basic data for the improvement of the smoking taste of cigarettes using Moutai wine microorganisms and related metabolic products.

3) Esters: a total of nine types of ester were detected in Sheet 1, Sheet 2, and the microbial fermentation extracts. Except for diethyl succinate, Sheet 1 and Sheet 2 presented traces of six common esters and of the six esters detected in the microbial fermentation extracts, methyl acetate was also detected in Sheet 1 but not in Sheet 2. This result suggested that the addition of microbial fermentation extract increased the amount of esteric aroma-inducing substances in Sheet 1.

4) Acids: a total of 10 types of acid were detected in Sheet 1, Sheet 2, and the microbial fermentation extracts. With the exception of valeric acid, Sheets 1 and 2 showed traces of eight common acids. The seven acids detected in the microbial fermentation extract were also found in

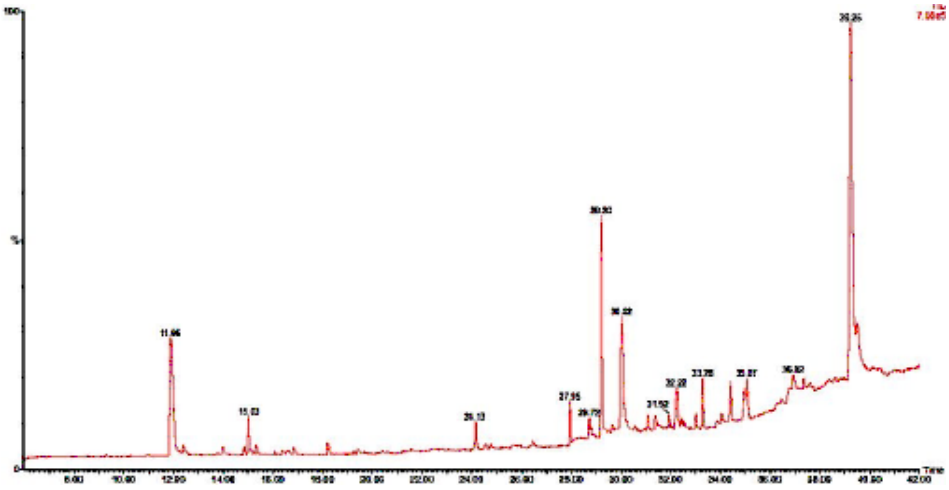

Fig. 2. The total ion concentrations of the microbial fermentation extracts

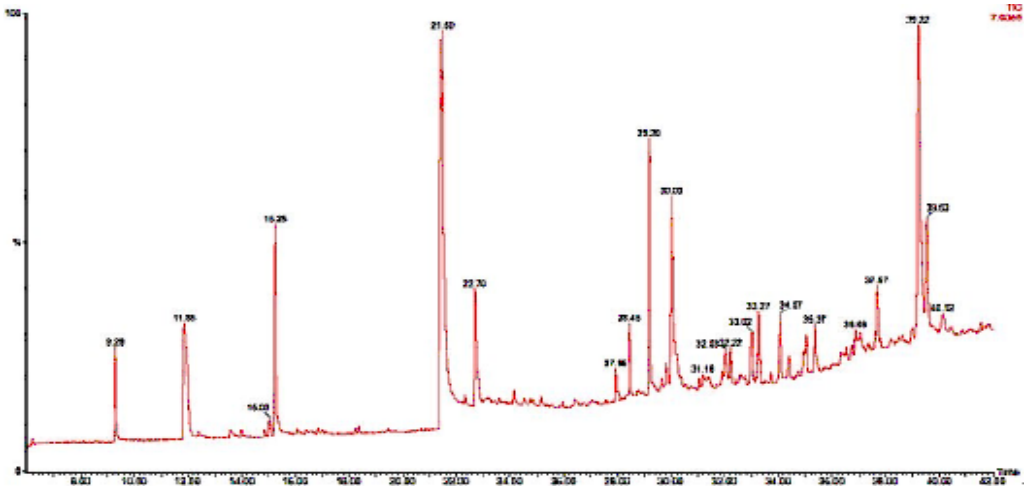

Fig. 3. The total ion concentrations of components in tobacco from Sheet 1 extracts

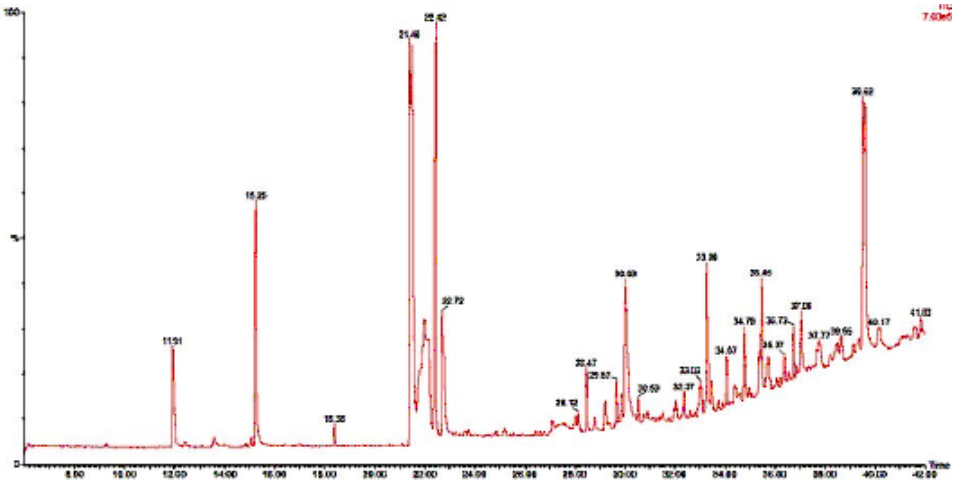

Fig. 4. The total ion concentrations of components in tobacco from Sheet 2 extracts

Sheet 1 but not Sheet 2. This result suggested that the addition of microbial fermentation extract increased the butyric acid content in Sheet 1.

5) Aldehydes: a total of six types of aldehydes were detected in Sheet 1, Sheet 2, and the microbial fermentation extracts. With the exception of hydroxymethylfurfural, Sheet 1 and Sheet 2 showed traces of four common aldehydes. Of the five aldehydes detected in microbial fermentation extracts, phenylacetaldehyde was also found in Sheet 1 but not Sheet 2. This result suggested that the addition of microbial fermentation extract increased the phenylacetaldehyde content of Sheet 1. Phenylacetaldehyde (also present in sauces as a flavour-related compound) has been detected in the distiller's grain used to produce Maotai-flavoured wine[15]. Thus it may have contributed to the increased aroma of Sheet 1.

6) Other compounds: glutaconic anhydride and ethyl β-D-ribonucleoside were also detected in Sheet 1 and microbial fermentation substances but were not detected in Sheet 2. It was speculated that these two substances may have contributed to the improved smoking evaluation effect of Sheet 1.

Table 5 lists those aroma-related substances detected in Sheet 1 but not Sheet 2 and their aroma-related characteristics. These substances, detected from the microbial fermentation extract, played an important role in improving the aroma and internal quality of the tobacco sheets.

In addition to the six common compounds above detected in Sheet 1 and the fermentation extract, 2(5H)-furanone and 5-methyl-2-fufuryl methanol were constituent parts of the composition of *Dictyophora indusiata*[26]; 4,5-dimethyl-1,3-dioxole-2-ketone was a constituent part of the flavour components of various sauces[27]; glutaconic anhydride arises in the composition of pomelo wine[28]; 3-5-dyhydroxy-2-methyl-4H-pyran-4-ketone and ethylβ-D-ribonucleotide remained undetermined. These chemical compounds were found simultaneously in Sheet 1 and the fermentation extract, but the aroma-related characteristics of these substances remained unclear, which required their contribution(s) to the aroma of Sheet 1 to be studied further in future research.

CONCLUSIONS

The metabolites of three types of bacillus strains extracted by high-temperature fermentation were added to reconstituted tobacco sheets. The cigarettes made from these tobacco sheets showed flavoured, sufficient, soft, delicate, and moderately-concentrated aromas. The smoking score of their sensory quality was increased by 1.4 points over those made from the control group tobacco sheets. The raw material and fermentation extract were mixed in a ratio of 1000 g to 1 mL. The extract was

insufficient to wet the raw material and thus showed no influences on the physical properties of the tobacco sheets. The sugars in the microbial fermentation extract increased the total sugar content of the tobacco sheet and thus resulted in an increase in the ratio of sugar to nicotine in Sheet 1, while the ratio of sugar to nicotine was positively correlated to the smoking quality of the resulting cigarettes[12]. GC-TOF(MS) detection of the aroma-related substances in Sheet 1, Sheet 2, and the microbial fermentation extracts revealed that Sheet 1 and the microbial fermentation extract presented 12 common aroma-related substances, which were not detected in Sheet 2. This outcome suggested that the 12 common aroma substances were sourced from the microbial fermentation extract. Since most of these substances had pleasant flavours, the sensory qualities of Sheet 1 were improved thereby.

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