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GROWTH CHARACTERISTICS OF AROMA-ENHANCING BACTERIA IN RECONSTITUTED TOBACCO EXTRACTS USING ISOTHERMAL MICROCALORIMETRY

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Abstract

Microorganisms, particularly those contributing to aroma development, play a vital role in the aging of flue-cured tobacco. In this study, heat release by five aroma-enhancing bacteria were measured during their growth in reconstituted tobacco extracts using isothermal microcalorimetry (IMC). The growth of the strains H4 (*Pantoea*), H8 *(Klebsiella*), H9 (*Acinetobacter*), H11 (*Staphylococcus*), and H12(*Enterobacter*) was detected under the conditions of 3%(v/v) inoculations, temperature of 26, 30, 34 and 37℃, pH 6 and 2.6 baume degree. Additionally, the growth of H8 was analyzed under the conditions of 30℃, pH of 4, 5, 6, 7, 8, baume degrees of 2.6, 5.2, 10.4, 15.6, 20.8 and 26. The number and contents of aroma components in reconstituted tobacco extracts significantly increased compared to the control groups after fermentation by H8, with resulting in significant enhancement of aroma. Specifically, the total content of aroma components in the reconstituted tobacco extracts was 9.245 mg/mL. Further, important aroma components significantly increased compared to controls, with 4,7,9-megastigmatrien-3-one increasing by 0.43 mg/mL, (Z)-6,10-Dimethyl-5, 9-undecadien-2-one by 0.144 mg/mL, (E)-6,10-Dimethylundeca-5, 9-dien-2-one by 0.072 mg/mL, (E)-5-isopropyl-8-methylnona-6,8-dien-2-one by 0.059 mg/mL, and (2, 6, 6-Trimethyl-2-hydroxycyclohexylidene) acetic acid lactone by 0.044 mg/mL. The content of Benzyl alcohol changed the most, increased by 943.86%, Benzaldehyde increased by 93.75%, and (E)-5-isopropyl-8-methylnona-6,8-dien-2-one increased by 84.29%. These findings lay the groundwork for optimizing aroma enhancement through H8 and comprehending the underlying mechanisms. Furthermore, they serve as a foundation for optimizing microbial growth characteristics within opaque reconstituted tobacco extracts.

Key words: Isothermal microcalorimetry; Aroma-enhancing bacteria; Growth characteristics; Reconstituted tobacco extracts

Introduction

Microorganisms inhabiting the surfaces of tobacco plants (Reid *et al*., 1937) which those have effects on aroma, play important roles in reducing biological macromolecules (Yang *et al*., 2018) within plants and forming aromatic components (Zhao *et al*., 2015) during tobacco aging (Gopalam & Gopalachari, 1979; Su *et al*., 2011). However, there is a lack of convenient methods for studying growth characteristics and metabolic processes within tobacco environments.

Reconstituted tobacco sheet, which improves processing ability and can effectively reduce the tar and nicotine deliveries in mainstream smoke (Wang *et al*., 2005). Reconstituted tobacco extracts contain almost all of the tobacco chemical components within a liquid state and can be used as a good medium for investigating tobacco-derived aroma-enhancing microorganisms. Nevertheless, reconstituted tobacco extracts contain too many pigments, are black and opaque, such that conventional methods cannot readily be used to determine the growth characteristics and metabolic processes conducted by tobacco-derived microorganisms.

Isothermal microcalorimetry (IMC) is the real-time measurement of thermal power and heat at constant temperature and is a general technique that applies to measure diverse processes by providing reliable basic thermochemical data (Wads, 2001). The thermodynamic characterization of ligand binding processes is an important IMC application (Wadso, 1997). Microorganisms produce little heat during their metabolism (about 1–3 pW / cell) (James, 1987), but their exponential replication during cultivation enables their detection by microcalorimeter within several hours, even when initial cell numbers are low (Braissant *et al*., 2009). Thus, a microcalorimeter with sufficient sensitivity can identify relevant thermal spectra through on-line detection of thermal effects generated by microbial metabolism. Further, these data can be used to establish a thermodynamic equation that models microbial growth (Winkelmann *et al*., 2009) to investigate microbial growth and metabolism. IMC also features many advantages including in situ use, a lack of measurement interference, and high fidelity of results. Consequently, IMC has been widely used to study the kinetics of bacterial growth and metabolism. For example, bacterial growth-related heat flow patterns observed with IMC can allow rapid discrimination of medically important microorganisms (von Ah *et al*., 2008). Further, evaluation of bacterial growth with IMC has been used to evaluate the potential of high sensitivity calorimetry for monitoring bacterial contamination of drinking water (Maskow *et al*., 2012). Skoczowski *et al*., (2020) used it to estimate microbiological contamination of maize seeds. Likewise, IMC has been used to measure the growth and heat of bacterial adsorption on human mouth surfaces (Hauser-Gerspach *et al*., 2008). Moreover, Solokhina *et al*., (2019) used it to determine the metabolic

activity of biofilms maintained on solid media, while Rong *et al*., (2007) used it to determine soil microbial activity. Among the above studies, solid media are especially useful for facilitating fungal growth in IMC ampoules and enable faster, more accurate studies (Wadsö *et al*., 2004).

In this study, IMC was used to measure the basic metabolic heat flow release of aroma-enhancing microorganisms in reconstituted tobacco extracts. Metabolic activity of the strains was measured by detecting heat changes in the environmental system, allowing the inference of growth curves. Moreover, optimal culture conditions and aroma-enhancing effects were evaluated, providing a basis for further optimization and clarification of aroma-enhancing mechanisms by bacterial populations.

Material and Methods

Preparation of the bacteria for microcalorimetry: The strains used in this study, H4 (*Pantoea*), H8 (*Klebsiella*), H9 (*Acinetobacter*), H11 (*Staphylococcus*), and H12 (*Enterobacter*), were isolated from soil and identified using phylogenetic tree method, and were kept in the ultra-low refrigerator (-80°C). After fermenting the tobacco leaf extract with the above microorganisms, a significant increase in aroma components can be detected. were screened by our laboratory and were kept in the ultra-low refrigerator (-80°C). Cultivated the five strains overnight at temperature 30°C, rotation speed 186g, inoculation amount 1% (v/v) in liquid lysogeny broth (LB) medium. Transferred the liquid cultures into 1.5 mL Eppendorf tubes, spun down in a benchtop centrifuge at 12400g, for 8 minutes and resuspended in phosphate-buffered saline (PBS). This washing step was carried out in triplicate. They were then adjusted using PBS to optical density at 600 nm (OD600) of 1 and used to inoculate the reconstituted tobacco extracts at 3% (v/v), respectively. All experiments were conducted in sterile environment and all materials were autoclaved at 121°C for 20min. The purity of the culture broth was determined using microscopic analysis.

Preparation of the reconstituted tobacco extracts: The tobacco leaf sample was the tobacco variety K326 from Sanmenxia, Henan Province, China in 2020.The sample was extracted with water at 50°C for 30 min at a solid-liquid ratio of 1: 4. Then concentrated to 26 baume degree (Baume degree(°Bé) is to represent the concentration of the solution. Immerse the Baume hydrometer in the measured solution, and the degree shown is called the baume degree) and stored at 4°C. Diluted it to different baume degrees: 2.6, 5.2, 10.4, 15.6 and 20.8 before use. The pH of reconstituted tobacco extracts at 2.6 baume degree is about 6. Adjusted the reconstituted tobacco extracts of 2.6 baume degree to different pH :4, 5, 7, 8 with diammonium phosphate. Autoclaved all the extracts at 121°C, 20min and stored in refrigerator at 4°C.

Isothermal microcalorimetry: The metabolic activity of strains was measured in a TAM Air (TA instruments, Lindon, US) isothermal calorimeter equipped with TAM Assistant Software. All experiments were conducted in 20mL glass ampoules. The ampoules and caps were autoclaved at 121°C, 20min. About 8mL of the reconstituted tobacco extracts with five strains was respectively put into the ampoules and tightly closed as the experimental group. The control group (Wadsö & Galindo, 2009) was filled with the reconstituted tobacco extracts without strains, to which sterile PBS was added with the same volume. The control groups were introduced to the microcalorimeter first to equilibrate for 4.5h. After the equilibration of the control groups were completed, the experimental group ampoules were put in the microcalorimeter. The measurement was started 40-50min (Corvec *et al*., 2020) after placing the experimental samples in it (full thermal stabilization of the sample). Experiments were carried out at different temperatures: 26°C, 30°C, 34°C, and 37°C, different baume degrees: 2.6, 5.2,10.4, 15.6, 20.8 and 26, and different pH:4,5,6,7,8. The signal of the calorimeter was expressed as heat flow (μW). Stop the experiment when the heat flow curve no longer changes with time, it indicates that the microbial growth metabolism ends.

Extraction and Identification of flavor components in reconstituted tobacco extracts: The flavor components in reconstituted tobacco extracts were obtained by simultaneous distillation extraction method and analyzed by Agilent GC/MS (8890A-5977B, USA) equipped with a fused silica capillary column HP-5ms (60 m × 0.25 mm × 0.25μm). Separation was carried out in an Agilent 8890 GC/MS system using capillary column (60 m×250 μm ×0.25 μm), with the injector set at 280°C and using Helium gas (purity ≥99.9999 %) as the carrier gas for the splitless injection at a flow rate of 1.0 mL/min. The temperature was maintained at 50°C for 4 min, after which it was increased to 240°C at 2°C/min. The mass selective detector was operated in full scan mode. The mass spectrometer recorded the entire spectrum in range from 10 to 550 m/z, using electronic ionization energy at 70 eV. Each of the five extract samples obtained by simultaneous distillation extraction was injected three times, and the results were averaged. All experiments were performed in the triplicates.

Results and Discussion

Bioheat results based on IMC: 3.1.1. Growth characteristics of aroma-enhancing bacteria in reconstituted tobacco extracts at different temperatures.

The thermal profiles of H4, H8, and H12 exhibited diauxie (Zaharia *et al*., 2013), which showed the partly anaerobic bacterial growth characteristics (Fig. 1A). The diauxie showed that in the process of growth, there are two lag periods, exponential periods and stable periods to form a growth curve with two peaks. The first exponential growth period of H8 occurred during 0-30h due to aerobic metabolism. At 30–100 h, the heat production of H8 rapidly decreased and then significantly increased, indicating that H8 entered the secondary growth stage via delayed anaerobic respiration. These results suggested that H8 growth resulted in sustainable fermentation of reconstituted tobacco extracts at 26°C, with roughly equivalent thermal efficiency and heat production between anaerobic and aerobic metabolism stages. After 100h, heat production power rapidly decreased and entered a

declining period. The heat flow curve for H4 and H12 were roughly the same, with 0–60 h as the stagnation (lag) period, 60–100 h as the aerobic respiration stage, and 100–140 h as the anaerobic respiration stage. The thermal efficiency and heat production of H4 and H12 during anaerobic metabolism were greater than during aerobic metabolism. The single peaks within heat flow curves of H9 and H11 corresponded to their strictly aerobic metabolic characteristics. The first 15h comprised the exponential growth period of H9, while the exponential growth period of H11 comprised the first 25 h, after which the decay phase ensued.

The total heat released from the metabolism of the five strains at 26°C was significantly different (Fig. 1B). H4 and H11 produced the most heat and thus metabolized most vigorously, suggesting that H4 and H11 are better adapted to reconstituted tobacco extracts at a cultivation temperature of 26°C. H4, H8, and H12 conducted facultative anaerobic respiration at 30°C (Fig. 1C), while the heat production efficiency and heat production during anaerobic metabolism were much higher than during aerobic metabolism. H9 and H11 performed aerobic metabolism at 30°C, while only H8 exhibited facultative anaerobic respiration at 34°C (Fig. 1E), although the heat production efficiency and heat production during anaerobic metabolism were slightly lower than during aerobic metabolism. H4, H9, and H11 only conducted aerobic activity. H8 and H12 conducted facultative anaerobic respiration at 37°C (Fig. 1G), wherein the heat production efficiency and heat production during aerobic metabolism were much higher than during anaerobic metabolism. Thus, lower temperatures were conducive for facultative anaerobic bacteria to conduct anaerobic metabolism, while higher temperatures favored aerobic metabolisms. Specifically, H4, H9, and H11 only performed aerobic metabolism at 37°C. The heat flow curves of the five strains at 26°C, 30°C, 34°C, and 37°C indicated that the strains required at least 20–80 h to complete the exponential growth period at 26°C and only 10–20 h at 30°C, 34°C, and 37°C. Thus, lower temperatures led to longer active metabolic periods, and increased cultivation temperatures led to accelerated overall microbial metabolic rates.

The total heat of the five strains at 26°C, 30°C, 34°C, and 37°C are shown in (Fig. 2). H8 exhibited the highest heat production and the most vigorous metabolism, so it is better adapted to the reconstituted tobacco extracts environment, resulting in higher metabolic activities. Taking the time of growth period into account, 30°C is the most suitable temperature for H8 to growth and metabolism.

Growth characteristics of H8 in reconstituted tobacco extracts in different baume degrees and pH at 30°C.

H8 exhibited the highest metabolic activity and released the most total heat from metabolism in reconstituted tobacco extracts with 2.6 baume degree (Fig. 3). In contrast, its metabolic activity was significantly lower in extracts in other baume degrees, accompanied by short life cycles, weak activity, and relatively minimal metabolism. The total heat released by metabolism decreased with increased baume degree. This result suggested that reconstituted tobacco extracts with high sugar and nicotine contents, in addition to high osmotic pressures, not being conducive for microbial growth. Thus, extracts with lower baume degree are more suitable for microbial metabolism.

Reconstituted tobacco extracts at pH 6 is most favorable for microbial activities.H8 exhibited the strongest metabolic activity and released the most total heat for the longest duration in reconstituted tobacco extracts at pH 6 (Fig. 4). The total heat flow from growth metabolism was slightly lower in reconstituted tobacco extracts at pH 7, with shorter metabolism that was not sustained. Metabolic activity was significantly weaker in reconstituted tobacco extracts at pH 4, 5, and 8, with relatively minimal overall activity.

The pH of the culture medium can induce changes in cell membrane charges, thereby affecting the absorption of nutrients by microorganisms and influencing enzyme activity during metabolic processes. It can also alter the availability of nutrients and the toxicity of harmful substances. Meanwhile, microbial metabolic activity can modify the pH value of the culture medium. The Baume degree mainly impacts the osmotic pressure of microorganisms.

GC-MS analysis and variation of aroma components: To evaluate the aroma-enhancing effects of the aroma-enhancing bacterial H4, H8, H9, H11, and H12 under fermentation conditions: temperature 30°C, pH 6 and 2.6 baume degree of the reconstituted tobacco extracts, the extracts fermented by H4, H8, H9, H11, and H12 and the control group (CK, extracts under the same conditions without being fermented) were chemically analyzed by GC/MS (Fig. 5).

The total ion chromatograms for fermented reconstituted tobacco extracts from H4, H8, H9, H11, and H12, in addition CK, were superimposed. Comparison of ion peaks and abundances of extracts fermented by different strains at the same time showed differences (Fig. 5). Thus, significant differences in aroma enhancement effects were apparent among five strains. The aroma components for the fermentation broths from H4, H8, H9, H11, and H12 were qualitatively and quantitatively analyzed using the normalization method (Table 1).

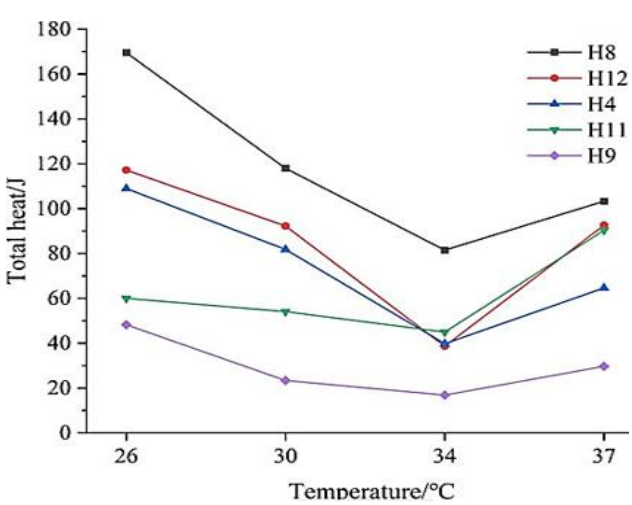

Fig. 2. Variation of total heat flow of five strains (H4, H8, H9, H11 and H12) at different temperatures of 26, 30, 34, and 37°C. Experiments were carried out in reconstituted tobacco extracts diluted to 2.6 baume degree at pH 6.

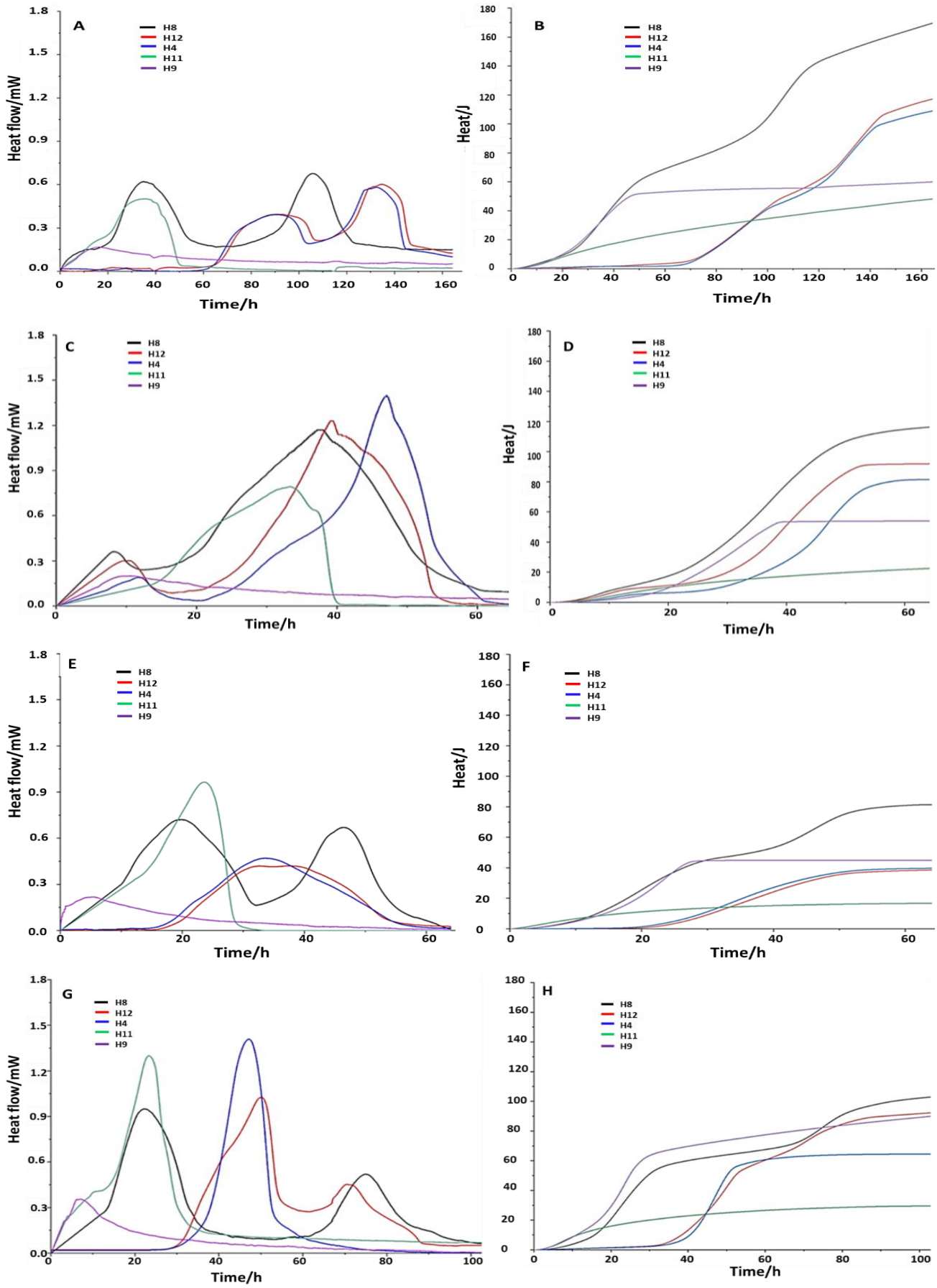

Fig. 1. Growth characteristics of five strains (H4, H8, H9, H11, and H12) followed by IMC. Heat flows were measured at various temperatures of 26, 30, 34, and 37°C as shown in panels A, C, E, and G, respectively. Corresponding total heats are presented in panels B, D, F, and H. Experiments were carried out in reconstituted tobacco extracts diluted to 2.6 baume degree at pH 6.

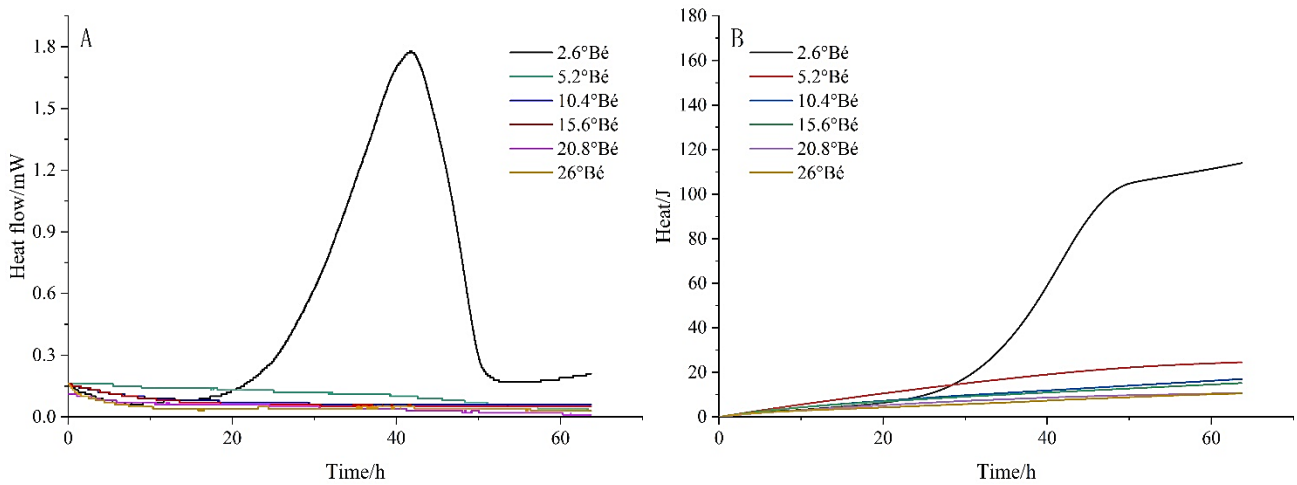

Fig. 3. Growth characteristics of H8 followed by IMC. Heat flows were measured at various baume degrees of 2.6, 5.2, 10.4, 15.6 and 26 as shown in panel A. Corresponding total heats are presented in panel B. Experiments were carried out in reconstituted tobacco extracts at temperature 30°C, pH 6.

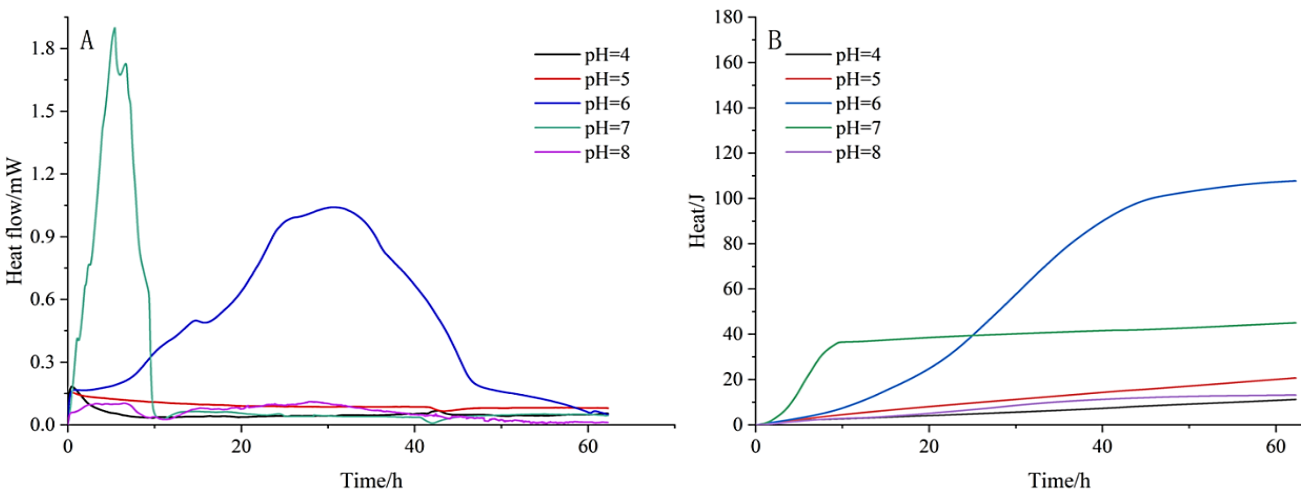

Fig. 4. Growth characteristics of H8 followed by IMC. Heat flows were measured at various pH of 4, 5, 6, 7 and 8 as shown in panel A. Corresponding total heats are presented in panel B. Experiments were carried out in reconstituted tobacco extracts diluted to 2.6 baume degree at temperature 30°C.

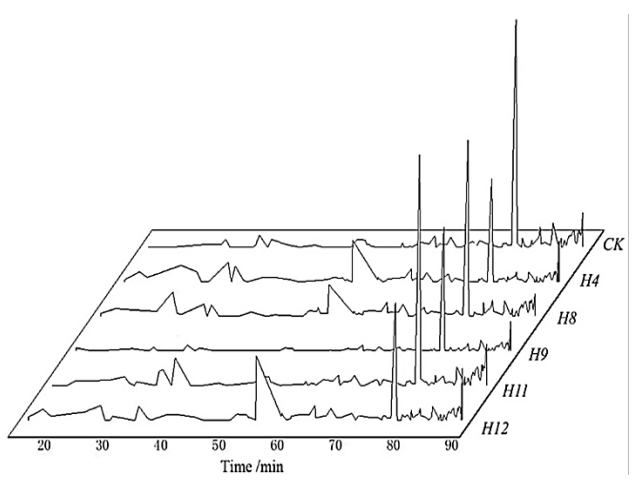

Fig. 5. Total ion chromatogram.

The total amount and quantity of aroma components in the extracts significantly increased after fermentation by H4, H8, and H12 (Table 1). Specifically, the contents of several compounds were enhanced in the extracts after fermentation including those of styrene, 2-Buten-1, 4-olide, benzaldehyde, benzyl alcohol, (E)-5-isopropyl-8-methylnona-6, 8-dien-2-one, 1-(2, 6, 6-Trimethyl-1-cyclohexen-1-yl)-2-buten-1-one, (Z)-6, 10-Dimethyl-5, 9-undecadien-2-one, 2-tridecanone, and 2, 4-di-tert-butylphenol. Among these, 1-(2, 6, 6-Trimethyl-1-cyclohexen-1-yl)-2-buten-1-one was the most enhanced, with 150% enrichment due to H4 and H8 fermentation, along with 250% enrichment after fermentation by H12. The total contents of aroma components were highest after fermentation by H8 (9.245 mg/mL), which was significantly higher than CK (4.325 mg/mL). Further, the number of aroma components was also highest for H8, being significantly higher than CK. Some typical aroma components in H8 were at the highest level in all samples, including 2-Buten-1, 4-olide, benzyl alcohol, 2-acetyl pyrrole, 2-Undecanone, 1-Cyclohexyl-2-buten-1-ol, (E)-6, 10-Dimethylundeca-5, 9-dien-2-one, (Z)-6, 10-Dimethyl-5, 9-undecadien-2-one, 2-tridecanone, 2,4-di-tert-butylphenol, (2, 6, 6-Trimethyl-2-hydroxycyclohexylidene) acetic acid

lactone, 4, 7, 9-megastigmatrien-3-one, Naphtho [1, 2-b]furan-2(3H)-one, decahydro-5a-methyl-3, 9-bis (methylene)-, (3aS, 5aR, 9aS, 9bS), (4aS, 7R)-1, 4a-Dimethyl-7-prop-1-en-2-yl-3, 4, 5, 6, 7, 8-hexahydronaphthalen-2-one, and (5E, 9E)-6, 10, 14-Trimethylpentadeca-5, 9, 13-trien-2-one. And 1-Cyclohexyl-2-buten-1-ol, and 2-undecanone were unique aroma components. In addition, fermentation by H8 led to increases of 4, 7, 9-megastigmatrien-3-one by 0.430 mg/mL, (Z)-6, 10-Dimethyl-5, 9-undecadien-2-one by 0.144 mg/mL, (E)-6, 10-Dimethylundeca-5, 9-dien-2-one by 0.072 mg/mL, (E)-5-isopropyl-8-methylnona-6, 8-dien-2-one by 0.059 mg/mL and (2, 6, 6-Trimethyl-2-hydroxycyclohexylidene) acetic acid lactone by 0.044 mg/mL. Thus, the aroma-enhancing effects of H8 were the best among the five strains. *Pantoea dispersa* was reported which has the ability of convertion lutein to 3-hydroxy-β-ionone and then β-ionone (Zhao *et al*., 2015). Several studies have shown that *Klebsiella (*Su *et al*., 2011*)*, *Acinetobacter* (Wu *et al*., 2022*)*, and *Enterobacteriaceae* (Huang *et al*., 2022) are involved in the catalytic transformation of aroma precursors during tobacco fermentation and aging processes.

Table 1. Content of aroma components in reconstituted tobacco extracts after fermentation by H4, H8, H9, H11, and H12.

No.	Aroma ingredient	Aroma ingredient concentration mg/mL					
		CK	H4	H8	H9	H11	H12
1.	Styrene	-	0.361	0.370	-	-	0.430
2.	2-Buten-1,4-olide	-	0.160	0.333	-	0.141	0.125
3.	Benzaldehyde	0.128	0.575	0.248	0.167	0.170	0.296
4.	Benzyl alcohol	0.114	0.342	1.190	-	0.285	0.584
5.	Phenylacetaldehyde	0.375	-	0.119	0.305	0.075	-
6.	2-Acetyl pyrrole	-	-	0.096	-	-	-
7.	Acetophenone	-	0.691	0.579	-	0.-	0.000
8.	Phenylethyl alcohol	0.088	-	-	-	0.496	-
9.	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	-	0.056	-	-	-	0.091
10.	5-(Hydroxymethyl) dihydrofuran-2(3H)-one	0.132	0.144	-	-	-	0.204
11.	Decanal	-	0.148	0.189	-	-	0.201
12.	2-Phenylethyl acetate	0.098	0.065	-	-	0.059	0.100
13.	2-Undecanone	-	-	0.069	-	-	-
14.	Nicotine	0.362	0.145	0.259	0.271	0.285	0.215
15.	(E)-1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	0.314	0.127	0.254	0.204	0.302	0.185
16.	(E)-5-isopropyl-8-methylnona-6,8-dien-2-one	0.070	0.067	0.129	-	0.071	0.078
17.	1-Cyclohexyl-2-buten-1-ol	-	-	0.150	-	-	-
18.	1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-2-buten-1-one	-	1.467	1.570	-	-	2.588
19.	(E)-6,10-Dimethylundeca-5,9-dien-2-one	0.179	0.143	0.251	0.122	0.135	0.181
20.	(Z)-6,10-Dimethyl-5,9-undecadien-2-one	-	0.080	0.144	-	0.045	0.124
21.	2-Tridecanone	-	0.076	0.104	-	-	0.098
22.	2,4-Di-tert-butylphenol	-	0.087	0.227	-	-	0.149
23.	(2,6,6-Trimethyl-2-hydroxycyclohexylidene) acetic acid lactone	0.221	0.111	0.265	0.177	0.196	0.204
24.	4,7,9-megastigmatrien-3-one a	0.179	0.143	0.251	0.122	0.135	0.181
25.	4,7,9-megastigmatrien-3-one b	0.512	0.447	0.700	0.513	0.354	0.575
26.	4,7,9-megastigmatrien-3-one c	0.145	0.095	0.190	0.000	0.134	0.166
27.	4,7,9-megastigmatrien-3-one d	0.454	0.329	0.579	0.387	0.352	0.443
28.	Naphtho[1,2-b]furan-2(3H)-one,decahydro-5a-methyl-3,9-bis(methylene)-, (3aS,5aR,9aS,9bS)-	0.209	0.111	0.238	0.185	0.166	0.128
29.	(4aS,7R)-1,4a-Dimethyl-7-prop-1-en-2-yl-3,4,5,6,7,8-hexahydronaphthalen-2-one	0.097	0.045	0.098	-	0.065	0.060
30.	(5E,9E)-6,10,14-Trimethylpentadeca-5,9,13-trien-2-one	0.456	0.189	0.468	0.369	0.306	0.259
31.	3,7,11,15-Tetramethyl-1-hexadecen-3-ol	0.139	-	0.100	-	0.091	-
32.	1,2-Benzenedicarboxylic acid dibutyl ester	0.080	-	0.075	-	-	0.050
	Total amount mg/mL	**4.325**	**6.204**	**9.245**	**2.822**	**3.863**	**7.715**

* Note:"-" means not detected

Conclusions

In this study, we used IMC to determine microbial growth in reconstituted tobacco extracts, leading to the identification of a bacterial strain (H8) with optimal aroma enhancement effects. H8 exhibited the most vigorous metabolism and the highest heat production in reconstituted tobacco extracts under the conditions of 3% (v/v) inoculation, temperature 30°C, baume degree 2.6 and pH 6. Eleven aroma substances were detected in reconstituted tobacco extracts after fermentation, which were not found in the control group. The content of Benzyl alcohol changed the most, increased by 943.86%, Benzaldehyde increased by 93.75%, and (E)-5-isopropyl-8-methylnona-6,8-dien-2-one increased by 84.29%. Further, the amounts and contents of aroma components in reconstituted tobacco extracts significantly increased after fermentation by H8, which also exhibited the best aroma enhancement effects.

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