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Full Length Research Paper

Identification of volatile compounds released by myxobacteria *Sorangium cellulosum* AHB103-1

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Secondary metabolite ethyl acetate extract (EAME) produced by *Sorangium cellulosum* AHB103-1 had good antitumor bioactivity. The results of MTT assay showed that IC_{50} value of EAME on human liver cancer cell line (HepG2), mouse melanoma cell line (B16), human breast cancer cell line (MDA-MB231) and human gastric cancer cells (SGC7901) were 5.29, 1.43, 8.77, 6.21 µg/ml respectively. Moreover, the IC_{50} value of EAME on normal mouse spleen cell was 278.87 µg/ml. After treatment with the EAME, significant changes in morphology of HepG2 cells were observed, such as disappearance of microvilli and formation of apoptotic bodies, etc. The volatile compounds released by *S. cellulosum* AHB103-1 were analyzed by gas chromatography-mass spectrometry. The ratio of various volatiles at the end of fermentation (7 days) was listed as follows: esters 11.41%, alcohols 13.40%, ketones 16.36%, fatty acids 29.60%, aroma compounds 9.0% and miscellaneous ring compounds 20.22%. The formation of some volatile compounds including isoamyl acetate, acetic acid, benzaldehyde, 4-(benzoyloxy)-2H-pyran-3-one, 1-methyl-indan, methyl-pyrazine, geranylacetone and n-methyl-3-pyridinecarboxamide was related to anti-tumor substances. Acetic acid and 4-(benzoyloxy)-2H-pyran-3-one were the most important among the above compounds because of relatively higher content. The change of their content could be used as an indicator of fermentation control.

Key words: *Sorangium cellulosum*, volatile compounds, secondary metabolite, antitumor bioactivity.

INTRODUCTION

Myxobacteria are unique Gram-negative bacteria which are famous for their gliding behavior and ability to form fruiting bodies upon starvation conditions (Reichenbach, 2001). In addition, in recent years increasing attention has been paid to myxobacteria as producer of much novel secondary metabolites with high pharmacological activities. Many novel bioactive substances such as ambruticin, myxothiazols, spiroketals, epothilones have emerged during the last 30 years, of which epothilone and its analogues are the most typical representative (Connor et al., 1977; Gerth et al., 1980; Gerth et al., 1996). But volatile compounds from myxobacteria receive relatively little attention. In fact, different myxobacteria have its unique odour that can even be used to distinguish it from others (Jeroen et al., 2005). Geosmin, the widespread bacterial volatile compound, is the first volatile identified in the myxobacterium *Nannocystis exedens* (Trowitzsch et al., 1981). It is also identified from the volatiles of *Myxococcus xanthus, Chondromyces crocatus* and *Stigmatella aurantiaca* (Dickschat and Wenzel, 2004; Schulz et al., 2004; Wenzel et al., 2005). Pyrazines are another important volatiles often found in the myxobacterium (Jeroen et al., 2005). Stigmolone isolated from fruiting *S. aurantiaca* is believed to be the pheromone responsible for the attraction of the cells in the fruiting body formation process of this species (William et al., 1998; Wulf et al., 1998).

Secondary metabolites EAME of *Sorangium cellulosum* AHB103-1 also had good antitumor activity, however, no epothilones were found in the secondary metabolites of *S. cellulosum* AHB103-1. The production of antibiotic by Streptomycetes in a fermentor is often accompanied by the biosynthesis of volatile odorous substances, and experienced technologists can estimate the type of the

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Table 1. Antitumor activity of EAME against various cell lines.

Cell lines	HepG2	B16	MDA-MB231	SGC7901	Mouse spleen cell
IC_{50} value (μg/ml)	5.29 ± 0.19	1.43 ± 0.17	8.77 ± 0.33	6.21 ± 0.08	278.87 ± 6.36

streptomyces culture and the phase the given culture is in only from the intensity of the odor spectrum (ezanka et al., 2007, 2008) . But the relationship between volatile compounds and secondary metabolites of myxobacteria has not been reported so far. During the culturation process of myxobacteria AHB103-1, whether volatile substances and antitumor secondary metabolite also have some links, which is the objective of this work.

MATERIALS AND METHODS

Microorganism and culture mediums

S. cellulosum AHB103-1 was screened and conserved in our lab. Both seed and fermentation mediums included (g/L): yeast extract 0.2%, defatted milk powder 0.4%, potato starch 1%, glucose 0.2%, $MgSO_4.7H_2O$ 0.1%, $CaCl_2.2H_2O$ 0.1%, 0.0008% Fe(III)- Na-EDTA. culture, that is, an extra 5% of glycerol and 2% of porous adsorbent resin XAD-16 were added to the medium (Gerth et al., 1996; Guo et al., 2008). The initial pH of two mediums were both adjusted to 7.2 before sterilization.

The pure strain was inoculated in 20 ml seed medium in a 250 ml conical flasks on a rotary shaker at 30°C and 160 rpm for 3 days, then 10 ml seed liquid was transferred into 100 ml fermentation medium in a 500 ml conical flask, subsequently, fermented for 7 days at 30°C and 160 rpm.

Solid-phase micro-extraction- gas chromatography-mass spectroscopy (SPME-GC-MS) analyse

10 ml fermented liquor was transferred to headspace bottle. By preheating for 30 min in 50°C water bath, a SPME syringe was inserted into the headspace of bottle, and the SPME fibre was exposed to the volatiles for 30 min. Then the SPME syringe was retracted and introduced into a GC-MS system, where the trapped volatiles were directly analyzed by desorption from the fibre. Qualitative analysis was carried out by coupled GC-MS, and 2-octanol was used as internal standard of quantitative analysis. The conditions for GC-MS were as follows: capillary column, fused silica DB-WAX (30 m, 0.25 mm inside diameter, 0.25 m film thickness), carrier gas, He; temperature program, isothermal for 4 min at 40°C, then change from 40 to 90°C at a rate of 6 oC/min, and from 90 to 230°C at a rate of 10°C/min, and isothermal for 6 min at 230°C, energy of ionization, 70 eV (Boland et al., 1984; Frauke et al., 1996). To determine the retention parameters, an aliquot of volatile compounds was supplemented with 1 l of a mixture of n-alkanes containing 6 to 18 carbon atoms and analyzed under the same conditions. The retention times of the volatile components of analyzed mixtures and normal alkanes were used to calculate the retention indices (RI). The volatiles were identified by comparing the retention indices and mass spectra obtained for the sample with the indices and mass spectra of the standards determined in the same column and taken from the literature as well as from the mass spectra libraries NBS and Wiley.

Secondary metabolites extraction and antitumor activity test

The absorbent resin was removed from the broth by centrifugation at the end of fermentation, and washed cleanly with distilled water. Then the resin was dried naturally and extracted three times with 3 x 500 ml of methanol for 24 h. After removing the methanol by rotary evaporator at 45°C, the remaining water/oil mixture was extracted with 3 x 100 ml of ethyl acetate. The ethyl acetate extract (EAME) was obtained by evaporation at 45°C and used to test antitumor activity on various cancer cell lines including B16, HepG2, SGC7901 and MDA-MB231 (Guo et al., 2008; Reichenbach and Hofle, 1993).

The antitumor activity was evalued by MTT assay. Cell medium containing 0.5% DMSO was used as a control. The inhibition rate was calculated as follows: Inhibition rate (%) = (1 – $OD_{sample}/OD_{negative\ control}$ X 100%, and the IC_{50} value was determined as described in literature (Satyanarayana et al., 2004; Yamaguchi et al., 2002). Data were obtained from four replicative experiments.

RESULTS AND DISCUSSION Anti-

proliferation of EAME to tumor cell lines

MTT assay result indicated that EAME had a good antitumor effect on four tumor cell lines *in vitro*, namely, HepG2, MDA-MB231, B16 and SGC7901 (Table 1). Compared with clinical drugs, antitumor activity of EAME on B16 was stronger than taxol (IC_{50} value, 2.03) by 29.56%, but weaker than Epothilone B (IC_{50} value, 0.86) by 39.86% (Wang and Tao, 2010). Moreover, IC_{50} value of EAME on normal mouse spleen cell and was 52.71 times as high as HepG2 cell, and 194.99 times as high as B16 cell. This showed EAME had selective inhibition on tumor cells in a certain concentration range, which was relatively safe.

After treatment HepG2 cell with EAME for 24 h, the morphology of cells examined by scanning electron microscope changed significantly, as shown in Figure 1. The control group HepG2 cell surface was covered with filamentous microvilli. However, HepG2 cell membrane surface microvilli became sparse, apoptotic bodies appeared with treatment by 2.5 g/ml EAME for 24 h. After 10.0 g/ml EAME treatment for 24 h, most of cell surface microvilli disappeared, cell shrinkage, more apoptotic body formation. This indicated from the morphology that EAME had the activity of inducing tumor cell apoptosis (Ikeda et al., 2002). The curves of growth and antitumor substances formation were described in Figure 2. The exponential growth phase of *S. cellulosum* AHB103-1 occurred from 0 to 2 days, and peak biomass yield was obtained at the third day (OD value, 3.95). Subsequently, anti-tumor substances rapidly synthesized from the third day, and reached the maximum value at the

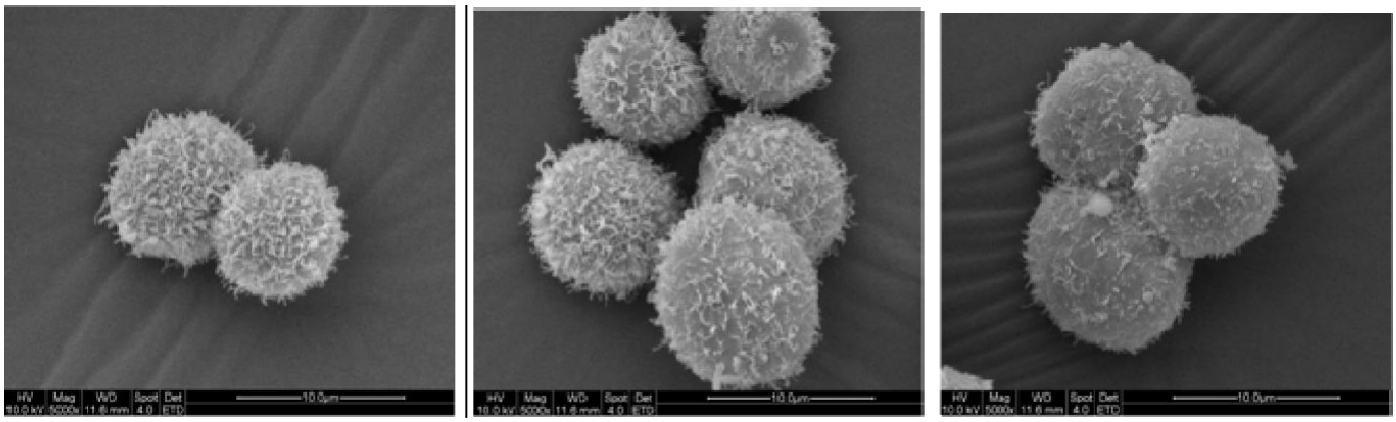

Figure 1. Morphological changes of HepG2 cells observed by SEM after treatment with different concentration of EAME for 24 h. (a) the control, (b) 2.5 µg/ml, (c) 10 µg/ml.

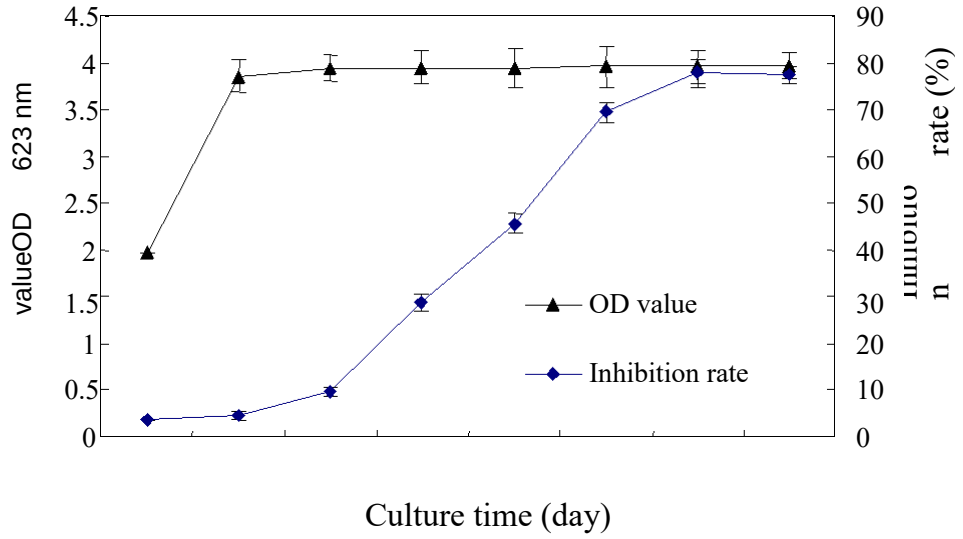

Figure 2. The change curve of inhibition rate and OD value of *S. cellulosum* AHB103-1.

seventh day.

Myxobacteria AHB103-1 produced a wide variety of volatile compounds including esters, ketones, alcohols, fatty acids, and aroma miscellaneous ring compounds (Table 2 and Figure 3). When fermentation terminated (7 days), the ratio of various volatiles were as follows: esters 11.41%, alcohols 13.40%, ketones 16.36%, fatty acids 29.60%, aroma compounds 9.0% and miscellaneous ring compounds 20.22%. Among the aliphatic compounds, low-molar-mass substances below six carbon atoms dominated. The vast majority of esters was butanoic acid methyl ester, its peak content (6.35 g/L) was obtained at the third day, then gradually decreased until the termination of fermentation. Isoamyl acetate, octanoic acid ethyl ester and decanoic acid ethyl ester were also detected in the culture liquid. Octanoic acid ethyl ester reached the maxium content at the fifth fermentation day, and the other two at the sixth fermentation day. Trace oleic acid methyl ester was detected from the third fermentation day, then gradually increased till fermentation termination, which was possibly produced by cell autolysis.

Aldehydes and 1- alkanols were formed by reduction of the carboxy group. Another possibility -oxidation via -hydroxy acids could lead to aldehydes lacking one carbon, which could also be reduced to 1-alkanols (Croes et al., 1997; Stefan and Jeroen, 2007). Among aliphatic alkanols, butanol and its homologue such as

Table 2. Content of volatile compounds (g/L) during fermentation period.

Volatile compounds	1 day	2 day	3 day	4 day	5 day	6 day	7 day	8 day	9 day
Esters									
butanoic acid methyl ester	4.27	6.30	6.35	5.81	3.87	3.64	2.28	2.12	2.01
Isoamyl acetate	0	0	0.02	0.05	0.09	0.22	0.9	0.51	0.31
Octanoic acid ethyl ester	0.08	0.16	0.28	0.30	0.33	0.29	0.17	0.13	0.11
Decanoic acid ethyl ester	0	0.03	0.13	0.15	0.21	0.35	0.24	0.16	0.13
Oleic acid methyl ester	0	0	0.02	0.02	0.02	0.05	0.07	0.17	0.18
Alcohols									
1-butanol	1.59	3.12	3.18	2.33	1.94	1.56	1.39	1.08	1.08
2-methyl-1-butanol	0.29	0.56	0.66	0.48	0.36	0.17	0.11	0.09	0.06
3-methyl-1-butanol	2.52	3.79	3.94	3.81	3.70	3.51	2.80	2.37	2.19
Ketones									
3-hydroxy-2-butanone	0.69	0.84	0.97	0.95	0.93	0.91	0.55	0.49	0.41
2,3-pentanedione	0.36	0.47	0.59	0.41	0.19	0.29	0.38	0.54	0.31
1-hydroxy-2-propanone	1.16	1.32	1.63	1.50	1.36	1.10	0.81	0.44	0.23
2-pentanone	1.73	2.56	3.41	3.43	3.65	3.59	3.51	3.19	2.73
Acids									
Acetic acid	0.41	1.38	1.60	1.84	2.10	3.05	5.62	3.25	1.57
Propanoic acid	0.26	0.61	0.70	0.43	0.29	0.28	0.27	0.25	0.18
2-methyl-propanoic acid	1.98	2.67	2.79	2.68	2.62	2.42	1.81	1.30	1.06
Butanoic acid	0.98	1.11	1.24	1.23	1.18	1.11	0.59	0.53	0.49
2-methyl-butanoic acid	0.86	1.21	1.30	1.48	1.44	1.41	0.64	0.63	0.59
Octanoic acid	0	0.0	0.54	0.98	1.44	0.66	0.22	0.19	0.15
Decanoic acid	0	0.0	0.31	0.88	0.99	0.69	0.35	0.23	0.20
Aromatic									
1,4-diphenylbut-3-ene-2-ol	0.14	0.18	0.23	0.22	0.22	0.21	0.21	0.17	0.13
phenethyl alcohol	0	0	0.14	0.31	0.67	0.28	0.14	0.11	0.12
benzaldehyde	0	0	0.11	0.18	0.27	0.38	0.54	0.83	0.95
B-phenylethyl acetate	0	0	0.33	0.69	1.45	0.72	0.27	0.15	0.13
2-hydroxy-benzaldehyde	0	0	0.12	0.15	0.17	0.17	0.14	0.11	0.08
Heterocyclic									
4-(benzoyloxy)-2H-pyran-3-one	0	0	0	0.59	1.31	2.91	3.80	3.76	3.71
1-methyl-indan	0	0	0	0	0.08	0.16	0.28	0.27	0.25
Methyl-pyrazine	0	0.13	0.17	0.26	0.28	0.54	0.72	0.70	0.64
Pyridine	1.97	2.16	3.34	2.38	1.16	0.75	0.49	0.34	0.29
[E]-Geranylacetone	0	0	0.11	0.19	0.39	0.41	0.62	0.53	0.49
Methoxy-phenyl-oxime	3.21	4.77	5.05	4.65	3.22	1.74	1.59	1.15	1.11
N-methyl-3-pyridinecarboxamide	0	0	0.09	0.09	0.10	0.12	0.15	0.13	0.13
[E]-3-[4,8-dimethyl-3,7-nonadienyl]-Furan	0.18	0.26	0.33	0.32	0.30	0.28	0.24	0.21	0.20
2-methoxy[1]benzothieno[2,3-C]quinolin-6[5H]-one	0.21	0.36	0.43	0.37	0.28	0.21	0.19	0.18	0.16

Note: Compounds found in control run are not shown. All values were means of three experiments.

3-methyl-1-butanol and 2-methyl-1-butanol were major components. It was noteworthy that 3-methyl-1-butanol proved to be an attractant of the Caribbean fruit fly Anastrepha suspense (Epsky et al., 1998; Stefan and

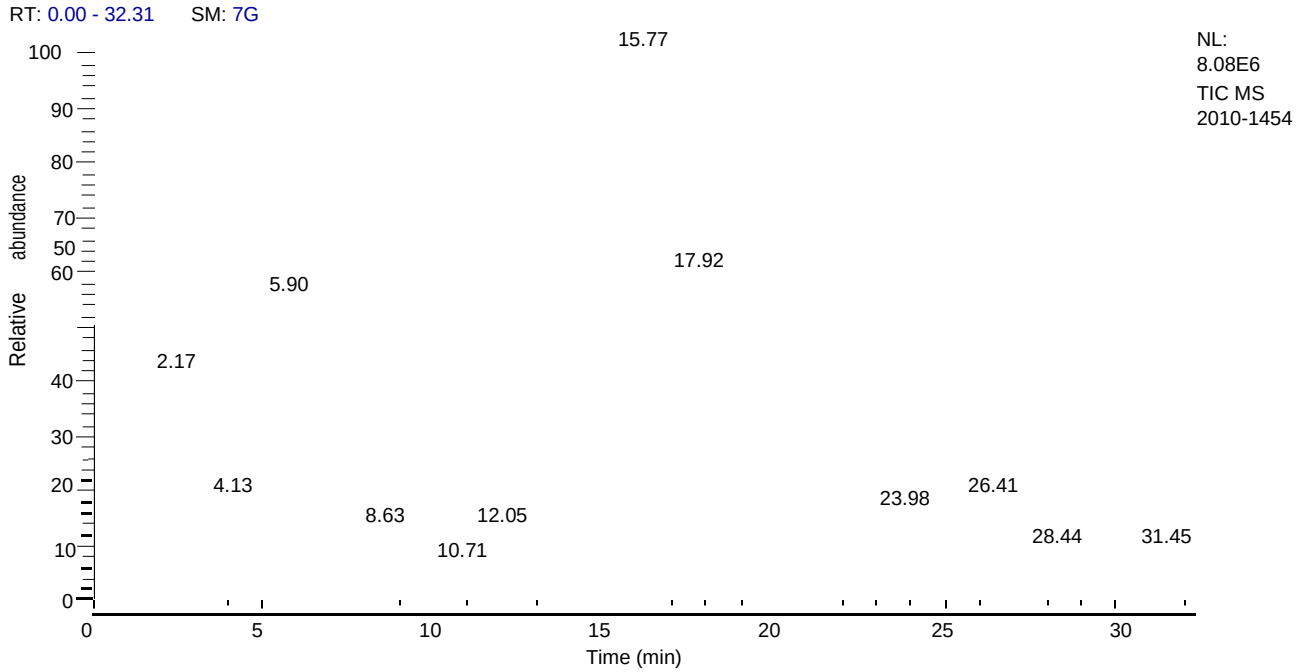

Figure 3. Total ion chromatograms of volatile metabolites collected from myxobacteria AHB103-1.

Jeroen, 2007).

Acetic acid and butanoic acid homologues accounted for the vast majority of fatty acids. All fatty acids except 2-methyl-butanoic acid and propanoic acid emitted by the bacteria contained an even number of carbons. Their biosynthesis typically starts with acetyl- CoA, which is extended with malonate units to ultimately assemble fatty acids containing an even number of carbons. Reverse reactions with similar intermediates take place during the degradation of the fatty acids through the -oxidation pathway (Bhaumik et al., 2005), and 2-methyl-butanoic acid may occur by incorporation of methylmalonate instead of malonate in the elongation steps (Stefan and Jeroen, 2007). The peak level of propanoic acid, 2-methyl-propanoic acid and butanoic acid were obtained at the third day of fermentation period, subsequently, dropped slowly. Obviously, they were produced in the bacterial growth phase. Octanoic acid and decanoic acid reached the peak level at the fifth day. In contrast, acetic acid content increased gradually, reached the highest content at the seventh day.

All aliphatic ketones were methyl ketones, those with an odd number of carbon atoms (2- pentanone, 1-hydroxy-2-propanone, 2, 3-pentanedione) were possibly derived from even-numbered -keto acids by decarboxylation like many bacteria (Bruce et al., 2004; Jeroen et al., 2005). Even numbered methyl ketones arose from fatty acids with an odd number of carbons, and were therefore rarer. However, 3-hydroxy-2-butanone was released by the bacteria.

Among the aromatic alcohols and phenols, 2-phenylethanol is one of the most widespread volatile aromatic compounds, and is produced by diverse bacteria. It and its derivatives 2-phenylethyl acetate were also emitted by the bacteria. Methoxy-phenyl-oxime was the highest content of aromatic components, accounting for 55.02% of the total aromatic compounds (7 day). As shown in Table 2, methoxy-phenyl-oxime was formed during the growth process, reached the maximum content at the third day. 4-(benzoyloxy)-2H-pyran-3-one and pyridine were two relatively higher content of heterocyclic compounds. Similar to methoxy-phenyl- oxime, pyridine was also formed at the growth stage. Conversely, 4-(benzoyloxy)-2H-pyran-3-one was produced at the latter stage of fermentation. Among the remaining low levels of heterocyclic compounds, 1-methyl-indan, methyl-pyrazine, [E]-Geranylacetone, and N- methyl-3-pyridinecarboxamide began to produce when bacteria entered the stationary phase. In contrast, both [E]-3-[4,8-dimethyl-3,7-nonadienyl]-Furan and 2-methoxy[1]benzothieno[2,3-C]quinolin-6[5H]-one reached the maximum content at the third day. It was noteworthy that much aromatic heterocyclic compounds including 4-(benzoyloxy)-2H-pyran-3-one, 1-methyl-indan, Methoxy-phenyl-oxime, N-methyl-3-pyridinecarboxamide, [E]-3-[4,8-dimethyl-3,7-nonadienyl]-Furan and 2-methoxy[1]benzothieno[2,3- C]quinolin-6[5H]-one had so far not been reported from volatiles of other myxobacteria.

As shown in Table 2 and Figure 2, some volatile compounds, mainly heterocyclic compounds, their content change curves were similar to anti-tumor activity.

These volatiles included: isoamyl acetate, acetic acid, 4-(benzoyloxy)-2H-pyran-3-one, 1-methyl-indan, methyl-pyrazine, geranylacetone and n-methyl-3-pyridinecarboxamide. Acetic acid and 4-(benzoyloxy)-2H-pyran-3-one were the most important among the above compounds because of relatively higher content. Therefore, the change of their content could be used as an indicator of fermentation control.

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