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Antitumor activity *in vitro* and volatile components of metabolites from myxobacteria *Stigmatella* WXNXJ-B

Da-Hong Wang[1] and Wen-Yi Tao*

Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China.

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Myxobacteria can generally produce large families of secondary metabolites with various bioactivities, such as antifungal and antitumor activities. In this paper, the metabolites absorbed by the resin, XAD16 from *Stigmatella* WXNXJ-B exhibited a high level of antitumor activities *in vitro*. The IC_{50} values were 14.34, 4.11, 16.36 and 19.46 g/ml on mouse melanoma cell lines, murine colon carcinoma cells, human liver carcinoma cells and human breast cancer cells, respectively and the value of IC_{50} on mouse spleen cells was above 750 g/mL. The volatile components of *Stigmatella* WXNXJ-B were analyzed with gas chromatography- mass spectrometry. The main volatile compound in the fermentation broth consisted of alcohols (24.3%), esters (14.3%), ketones (14.3%), alkyls (10.0%), hetero-cyclic (17.1%), aromatic (5.7%), acids and aldehydes (8.6%) and others (5.7%) . Of these, 2,5-dimethylpyrazine,2,3-dimethyl-5-ethyl-pyrazine and 2-ethyl-6-methyl-pyrazine were assessed to be present.

Key words: *Stigmatella* WXNXJ-B, antitumor activity, volatile components.

INTRODUCTION

Myxobacteria are gram-negative unicellular rod shaped bacteria that can be suitably cultured at pH between 5.0 and 8.0. Myxobacteria can be frequently isolated from soil, dung of herbivorous animals, bark, rotting wood and other decaying organic material (Reichenbach and Dworkin, 1992). They are common, but unusual bacteria characterized by gliding behavior and fruiting body formation; and they are not obtained by the routine method used in culturing bacteria due to their complicated life cycle and thus require special techniques for their isolation (Ahn et al., 2007). Myxobacteria are also known to produce a wide variety of secondary metabolites which often show high pharmacological or anti-fungicidal activity, such as aromatics, heterocycles, alkaloids, quinoids, peptides, isoprenoids, macrocyclic and polyenic compounds, etc. Most substances derived or isolated from myxobacteria were completely new structures (Reichenbach and Hofle, 1993). A myxobacterial antibiotic of ambruticin was first isolated from a strain of *Polyangium cellulosum* in 1977 (Connor et al., 1977). The first structure of myxothiazol was then reported by Gerth et al. (1980). Epothilones, a new class of 16-membered macrolides, are from *Sorangium cellulosum.* So ce90 is about to be approved for breast cancer treatment because it stabilizes microtubulin in the cell, disables the assembly of functional mitotic spindles required for cell proliferation, and thus results in the induction of apoptosis. Its selective antitumor properties resemble Taxol. Significantly, these new antitumor agents exhibit selective cytotoxicity and are particularly effective against certain drug-resistant tumor cell lines, even in cases where Taxol fails (Höfle and Reichenbach, 2005; Reichenbach, 2001). Within the last two decades, myxobacteria have increasingly gained attention as producers of natural products with biological activity (Gerth et al., 2003).

In contrast, the analysis of volatile compounds from myxobacteria has received relatively little attention. The widespread bacterial product, geosmin has been identified in *Nannocystis exedens* (Trowitzsch et al., 1981) and is present in many other species, detectable by its characteristic odour. Stigmolone has been isolated from fruiting *Stigmatella aurantiara* and is believed to be the pheromone responsible for the attraction of the cells in the fruiting body formation process of this species (Wulf et al., 1998; William et al., 1998). In this study, the antitumor bioactivity of the metabolites from *Stigmatella* WXNXJ-B strain was evaluated using B16, CT-26, HepG2 and MDA-MB231 tumor cell lines by MTT method *in vitro*. In addition, in an attempt to further characterize the metabolic

*Corresponding author. E-mail: wytao1946@163.com. Tel: +86-510-85918205.

metabolic spectrum of the myxobacteria and identify the most important ketone, heterocyclic and aroma active compounds, the volatile substances released from cultures of *Stigmatella* WXNXJ-B were investigated by gas chromatography and mass spectrometry (GC-MS). The strain produced a very characteristic odor unlike that of any other myxobacterium, which allowed identification of the bacterium, simply by smelling.

MATERIALS AND METHODS

Strains and cultivation conditions

The isolation of the strain, *Stigmatella* WXNXJ-B from soil has been described previously (Guo et al., 2007). The strain was grown on CY medium (Zhang et al, 2003) at 30°C for 1 week. It was then inoculated in 250 mL Erlenmeyer flasks containing 30 mL seed medium. This medium consisted of (w/v), potato starch 1%; glucose, 0.8%; defatted milk powder, 0.2%; yeast extract, 0.2%; Fe(III)-Na-EDTA, 0.008 mg/L; $CaCl_2$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.1%; 4-(2-hydroxyethyl) -1- piperazine-ethanesulfonicacid (HEPES), 1.15% and pH 7.4 at 30°C; 160 r min^{-1} for 3 days. Culture broth was inoculated in 250 ml Erlenmeyer flasks containing 50 ml fermentation medium with 2% XAD- 16 adsorbent resin (Rohm and Haas, USA); it was cultivated at 30°C and shaken at 150 r min^{-1} for 7 days.

Secondary metabolites extraction

A 10 L fermentation of the producing strain was performed with XAD-16 adsorber resin. At the end of the cultivation, the resin was separated from the culture broth by sieving, and then rinsed with water to remove cells and fermentation broth. The resin was first extracted with 1 L of methanol, then with 500 ml of methanol, three times. The methanol was removed by rotary evaporation at 45°C to 10 ml and stored at 4°C for activity test.

Evaluation of the bioactive effect on tumor cell lines *in vitro* (MTT assay)

Various cancer cell lines, such as B16, CT-26, HepG2 and DMA-MB231 were used to evaluate the antitumor effects of the metabolites produced by the strain. Tumor cells were cultured in RPMI- 1640 medium (Gibco, USA) with 10% inactivated fetal bovine serum (Gibco, USA), penicillin (100 U/mL) and streptomycin (100 g /mL) at 37°C in a 5% CO_2 incubator. For MTT survival testing, cells were harvested with trypsin; they were counted, diluted and seeded into 96-well plates at a density of approximately 7,000 cells/well. After 24 h, the sample was dried at 45°C and dissolved in dimethylsulfoxide (DMSO). Five microliter of the sample was added to 1 mL cell medium, then, 200 l of the medium was added to each well. Cell medium containing 0.5% DMSO was used as a negative control. Incubation was carried out for another 48 h. The viability of cells was assessed in the MTT assay as described elsewhere (Yamaguchi et al., 2002). Twenty microliter of MTT solution (5 mg ml^{-1}) was added to each well and incubated at 37°C for additional 4 h. The formazan product was solubilized by adding 200 uL DMSO and was gently shaken for 5 min. The absorption was measured at 570 nm, with a microplate reader (Multiskan MK3, Labsystems, Finland). The inhibiting rate was calculated using the equation below:

$$\text{Inhibiting rate} = (1 - OD_{\text{treated}}/OD_{\text{negative control}}) \times 100\%$$

Scanning electron microscopy analysis and fluorescence microscopy observation of B16 cells

As described previously (Cui et al.,2007), B16 cells treated without or with10 g·ml^{-1} of the extract for 48 h were washed twice with PBS; they were centrifuged and pre-fixed with 2.5% glutaraldehyde at 4°C for 2 h. The cells were then rinsed thoroughly in PBS and post-fixed in 1% OsO4 at 4°C for 1 h and scanning electron microscopy was applied to investigate B16 cell surface morphology.

The cells, plated onto glass cover slips in 6-well plates and treated with 0 and 10 g·ml^{-1} of the extract for 48 h, were washed twice with PBS and stained with Hoechst dye 33342 (Sigma, USA) for 15 min at room temperature. After washing with PBS, cover slips were mounted onto microscope slide and nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan) at 400×.

Gas chromatography-mass spectrometry (GC-MS)

The fermentation broth in which resin XAD16 was not added at fermentation was centrifuged at 8000 r min^{-1} for 10 min and the supernatant was stored at -20°C for GC-MS analysis. The solid phase micro-extraction methods (SPME) were used. The 85 m PA fiber (Supelco, USA) was used to extract the volatile components at 50°C for 40 min. Volatile components were analysed by GC-MS, using a Finnigan Trace GC/MS(Thermo Quest Finnigan Co., USA) equipped with a DB-WAX capillary column (30 m × 0.25 mm × 0.25 m). Helium (flow-rate 0.8 mL min^{-1}) was used as carrier gas. The column temperature program was as follows: initial temperature, 30°C for 3 min; 90°C at 5°C min^{-1}; 240°C at 10°C min^{-1} and maintained for 8 min. Transfer line temperature was 250°C. The ionic source temperature was set at 200°C and the electronic energy was 70 eV. The ejection current was 200 uA and the scanning quality scope was recorded as 33~450 amu. The metabolites were identified by comparing their mass spectra with the NIST (National Institute of Standards and Technology) and Wiley libraries.

RESULTS AND DISCUSSION

The growth inhibit potency of the extraction from *Stigmatella* WXNXJ-B was validated with B16, HepG2 and MDA-MB231 tumor cell lines. MTT-assay result was that the extraction showed high bioactivity to these cancer cell lines. The IC_{50} values on B16, CT-26, HepG2 and MDA-MB 231 cell lines were 14.34, 4.11, 16.36 and 19.46 g/mL, respectively (Figure 1). The IC_{50} value on the mouse spleen cells was above 750 g/ml and the proliferation of the cells was slightly influenced at the same treated time and at the dose of 600 g/ml. This indicated that the extract was relatively safe for the mouse spleen cells. So, the extract could selectively inhibit the growth of tumor and normal cells because it probably led to the apoptosis of tumor cells.

The cell morphological analyses of the extract-treated B16 cells showed the apoptotic characteristics, such as appearance of apoptotic bodies, in contrast to control cells (Figures 2a and b). The changes of nuclear morphology of B16 with the extract treatment for 48 h were analyzed under a fluorescence microscope by Hoechst-staining (Figure 3). The nuclei of the treated cells

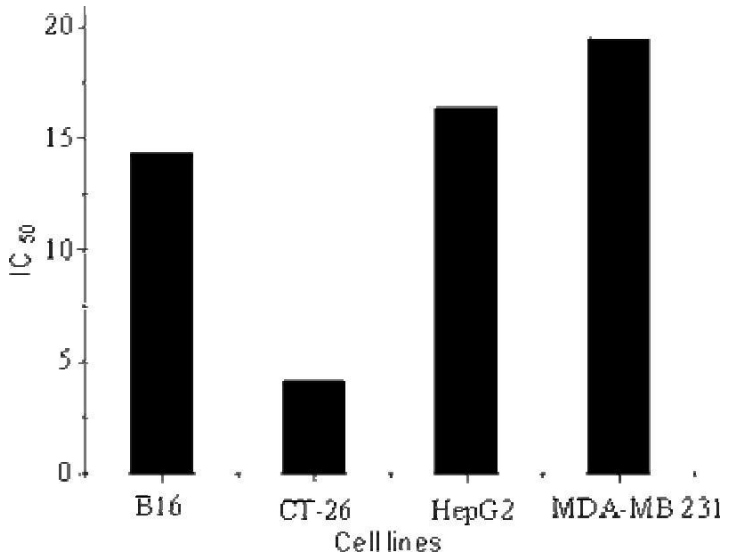

Figure 1. *In vitro* growth inhibition potency of the extract from *Stigmatella* WXNXJ-B to different tumor cell lines (IC_{50} g·ml^{-1}).

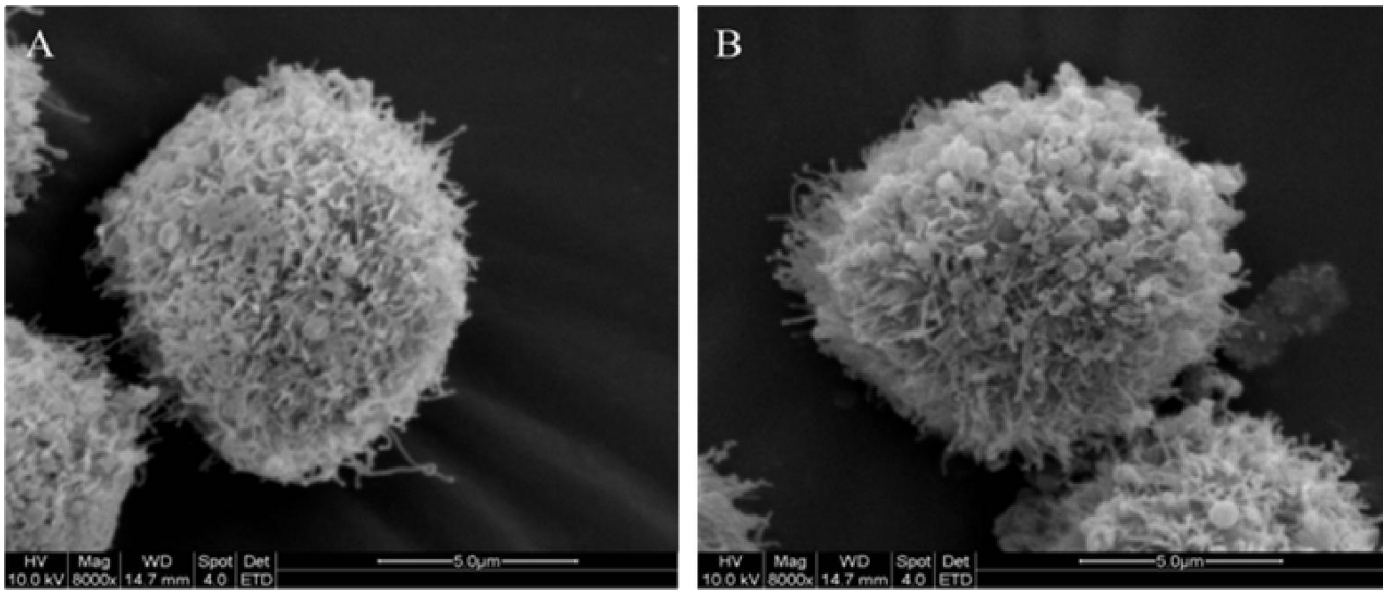

Figure 2. Scanning electron micrographs (×5000) of B16 cells treated; (a) without 10 g·mL^{-1} of the extract for 48 h; (b) with 10 g·mL^{-1} of the extract for 48 h.

had nuclear shrinkage and condensed chromatin. The apoptotic cells became brighter than the living cancer cells. Therefore, the results indicated the methanol extract proved to contain the broad-spectrum on tumor cell lines and made the cancer cells die, by probably apoptosis.

The results of the headspace analyses were summarized in Table 1, and total ion chromatograms of the volatile metabolites from *Stigmatella* WXNXJ-B were presented in Figure 4. Quantification of the volatile components produced by *Stigmatella* WXNXJ-B was carried out by using GC-MS analysis. Control experiments were performed with sterilized media without bacteria to exclude compounds emanating from the medium. The volatile components were identified by comparing the obtained mass spectra of the analysis with those of standards from the NIST and Wiley libraries. In the study, 69 compounds were identified. The chemical pattern consisted of alcohols (24.3%), esters (14.3%), ketones (14.3%), alkyls (10%), heterocyclic (17.1%), aromatic (5.7%), acids and aldehydes (8.6%), others (5.7%). Our

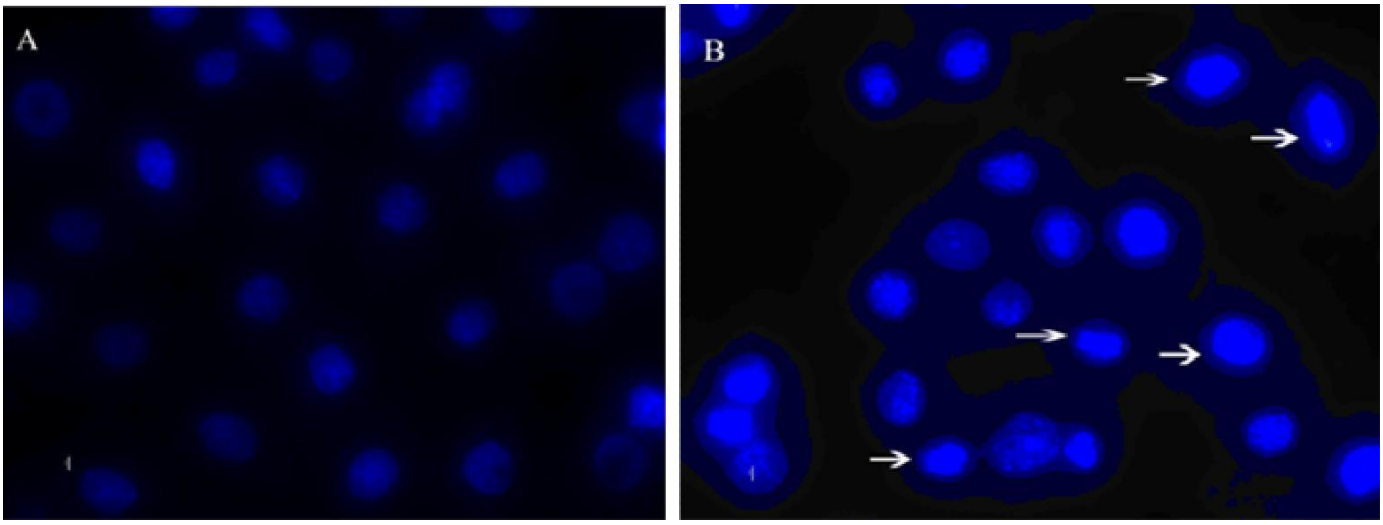

Figure 3. Fluorescence micrographs (×400) of B16 cells stained with Hoechst 33342. Cells were treated without 10 g·mL^{-1} of the extract for 48 h. They showed that the extract induced apoptosis in B16 cells, which is characterized by: (a) nuclear condensation or; (b) nuclear fragmentation (white arrows).

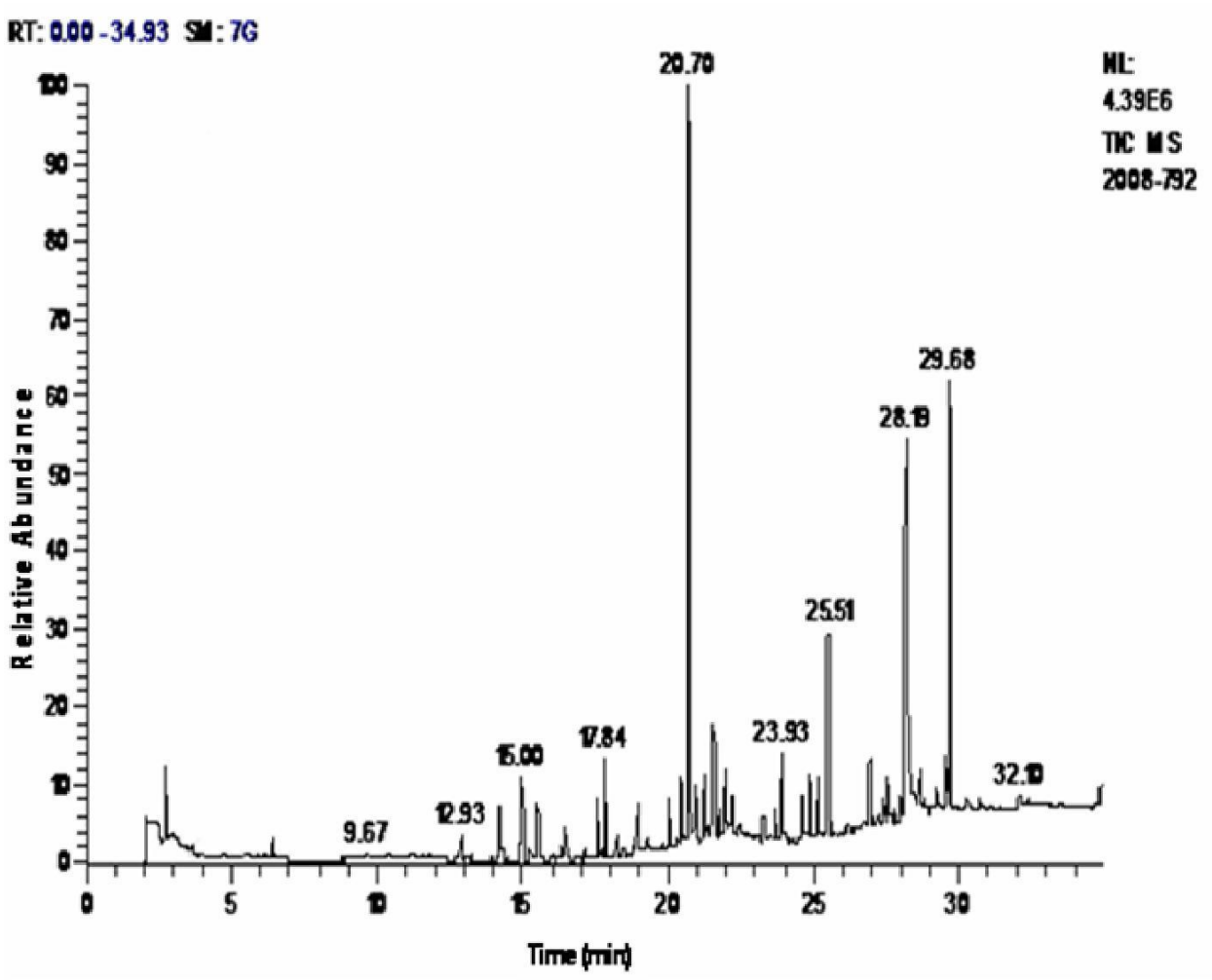

Figure 4. Total ion chromatograms of volatile metabolites collected from myxobacteria WXNXJ-B.

Table 1. The volatile compounds produced by *Stigmatella* WXNXJ-B.

Class	Metabolites	Class	Metabolites
Alcohols	Ethanol		2-dodecanone
	3-methyl-1-butanol		2,6-Cycloheptadienone
	1-nonen-3-ol		4-hydroxy-3-hexanone
	Butyl alcohol	**Alkyls**	1.4.7.10-tetraoxa-cyclododecane
	3-methyl-4-penten-2-ol		4,5-octanediol
	4-ethoxy-1-butanol		Methoxy ethane
	3-pentanol		2-ethyl-1-hexanol
	4-methyl-2,3-pentanediol		2,3,4-trithiapentane
	2-methoxy-1-propanol		Butane-1,1,2,2-tetracarbonitrile-3,4-dicyclopropylcyco
	2-methyl-3-hexanol		2-hydroxy-2-(p-methoxyphenyl)-3-hydroxycarbonyl-butane
	Acetol		Trans-3-hexenal
	2,3-butanediol	Heterocyclic	2-(5-chloro-2-methoxyphenyl) pyrrole
	2-(methoxymethoxy)propan-al		1-methyl-2-pyrrolidinone
	2-methyl-2,4-pentanediol		2-ethyl-6-methyl-pyrazine
	Dipropylene glycol		2,3-dimethyl-5-ethyl- pyrazine
	1,2-ethanediol		2,5-dimethylpyrazine
	Benzeneethanol		4-(benzoyloxy)-2h-pyran-3-one
	1,4- butanediol		Indole
Esters	1,4-benzenedicarboxylic acid dimethyl ester		3-methyl-2-oxatricyclo[6,6,0,0(4,8)] tetradeca-9,12,14-triene
	Hexadecanoic acid meyhyl ester		2-ethyl-1H-isoindole-1,3(2H)-dithione
	Octadecanoic acid methyl ester		2-pyrrolidinone
	Oleic acid methyl ester		2,3-dihydro-benzofuran
	3-nonenoic acid methyl ester		4,5-dimethylpyrimidine
	Elcosanoic acid methyl ester	**Acids and**	Benzaldehyde
	Butanoic acid methyl ester	**Aldehydes**	3-methyl-oxirane-2-carboxylic acid
	2-(2-ethoxyethoxy)ethyl acetate		Glyoxylic acid
	Benzeneacetic acid methyl ester		4-methyl-2-oxovaleric acid
	2-ethyl-benzoic acid methl ester		
	2-hydroxy-ethyl ester	**Aromatic**	4-methyl-phenol
Ketones	cyclopentanone		Methylnaphthalene
	4-acetyloxy-2-butanone		Phenol
	6-methyl-2-heptanone	**Others**	Propanamide
	3-hydroxy-2-butanone		Acetamide
	5-methyl-2-heptanone		N,N-dimethyl Acetamide
	4-hydroxy-4-methyl-2-pentanone		
	4-(1,3)dioxolan-2-yl-3,4-dimethyl-cyclohex-2-enone		

Compounds found in blank runs are not shown.

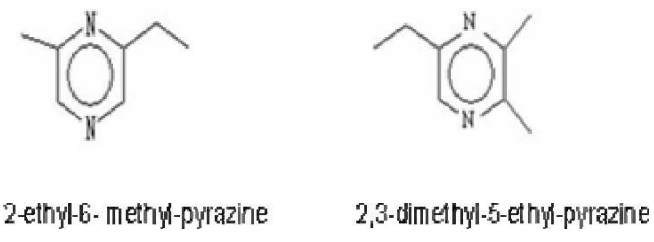

Figure 5. New pyrazine compounds from *Stigmatella* WXNXJ-B.

Our results indicated that two major compound classes: heterocyclics and ketones were produced by the strain.

Several heterocyclic compounds, for example, pyrazines, benzofuran and pyrrolidinone were present.

Pyrazines exhibiting strong odor properties are important aroma compounds and have been found abundantly in several foodstuff (Murray and Whitfield, 1975).They are important flavouring components and have been identified from several bacteria (Seitz, 1994). Methyl and ethyl derivatives like 2,5-dimethyl-pyrazine are particularly common and may serve a communicative role in plants, in some instances (Ryu et al., 2003; 2004) . Among the pyrazines produced from *Stigmatella* WXNXJ-B, 2,5-dimethylpyrazine,2,3-dimethyl-5-ethyl-pyrazine and 2-ethyl-6-methyl-pyrazine were identified and the predominant member was 2,3-dimethyl-5-ethyl-pyrazine (Figure 5). The compound, 2,5-dimethyl-pyrazine had been found in myxobacterium *Chondromyces crocatus* and *Myxococcus Xanthus* DK1622.2,3-dimethyl-5-ethyl-pyrazine, and 2-ethyl-6-methyl-pyrazine was first found in

myxobacteria. In addition, the other heterocyclic compounds, 2-(5-chloro- 2-methoxyphenyl) pyrrole,1 -methyl-2-pyrrolidinone, 2,3-dihydro-benzofuran and 4-(benzoyloxy)- 2h-pyran-3-one, were so far not reported from other myxobacteria.

Ketones were another kinds of compounds from the strain. Stigmolone,2,5,8-trimethyl -8-hydroxyl-nonan-4-one was isolated and purified from *Stigmatella aurantiaca* by Willam et al.(1998) and Wulf et al. (1998). In our research, the compound was not found by SPME, but, 4-(1,3) dioxolan-2-yl-3,4-dimethyl-cyclohex -2-enone,2-dodecanone and 2,6-cyclohe ptadienone were found for the first time in myxobacteria. The other ketones were the isomer and homologues of ketones reported by Dickschat et al. (2004).

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