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Full Length Research Paper

Biodegradation of swainsonine by five types of plasmid-transformants from genomic library of *Arthrobacter* sp. HW08

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The aim of this study is to analyze the ability of swainsonine (SW) biodegradation by five types of transformants from the genomic library of *Arthrobacter* sp. HW08, explore preliminarily metabolites and deduce the metabolic pathway. Using 1000 mg·L^{-1} SW as the sole carbon source, mineral salts medium (MSM) containing five transformants at a proportion for 1:1(V:V) was incubated at 30°C, 180 r·min^{-1}. The degradation rate of SW was 99.78% in 48 h, and SW could be completely degraded in 72 h. Compared with samples containing SW in 0 h, two special spots of metabolites were visualized with iodine vapor by thin-layer chromatography (TLC). Two kinds of primary metabolites (stearic acid and palmitinic acid) and two kinds of intermediate metabolites appeared in the process of metabolism of SW by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) whose m/z was 388.2 and 314.3 amu respectively. Ultimately, four kinds of end-products appeared in the process of metabolism of SW. This work demonstrates the preliminary results on metabolites of degrading SW by five transformants from *Arthrobacter* sp. HW08. This work also provides further information on metabolic pathway of SW from strain HW08.

Key words: Swainsonine, biodegradation, metabolites, transformants, *Arthrobacter* sp. HW08.

INTRODUCTION

Locoweed is a common name for leguminous plant of the *Astragalus* spp. and *Oxytropis* spp. containing the toxic indolizidine alkaloid swainsonine (SW), (Figure 1) (Molyneux and James, 1982). SW is the main toxin causing locoism in animals (Colegate et al., 1979; Tulsiani et al., 1984; Cao et al., 1989). SW can inhibit the activity of α–mannosidase (Dorling et al., 1980), affect the synthesis of glycoproteins containing 'high manose' and hybrid asparagines-linked glycans in cultured cells (Elbein et al., 1981,1982) and the processing of cell glycoprotein *in vivo* (Abraham et al., 1983), cause oligosaccharide accumulation, depress cell function, and result in a series of poisoning symptoms (James et al., 1970; Tulsiani et al., 1985; Cao et al., 1989; Hartley et al., 1989; James and Panter, 1989; Stegelmeier et al., 1999). For the past decades, these poisonous plants have turned into dominant species on western rangelands in China and have cover up to 11 million hectares (Zhao et al., 2003). Aside from traditional manual eradication to decrease the amount of locoweed (Li, 2003), supplementation with bentonite or mineral elements in daily ration to be bound to SW and thus reduce

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Abbreviations: **SW,** Swainsonine; **TLC,** thin-layer chromatography; **GC,** gas chromatography; **GC-MS,** gas chromatography-mass spectrometry; **Me-Gal,** methyl-α-D-mannopyranoside; **BSTFA,** N,O-bis(trimethylsilyl) trifluoro-acetamide; **TMCS,** trimethylchlorosilane; **LB,** Luria-Bertani medium; **MSM,** mineral salts medium; **m/z,** mass-to-charge ratio.

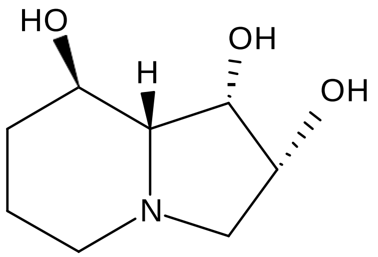

Figure 1. Structure of swainsonine.

intoxication (Pulsipher et al., 1994) and immunology to protect animals from lesion when consuming locoweed (Tong et al., 2007, 2008), there are no other reports on effective methods to resolve locoweed intoxication. Nowadays, seeking for a method of SW biodegradation is the hotspot in the world. Chinese scholars buried *Oxytropis kansuensis* Bunge in the soil, and six months later extracted and identified two strains of bacteria which were capable of degrading SW (Zhao, 2008; Zhao et al., 2009). These bacterial strains were further studied to optimize their conditions for degradation (Wang et al., 2010b), identify their SW degradation mechanism, and were hoped to be used in the elimination or reduction of SW concentration in animal bodies to reduce losses due to animal locoweed intoxication. However, the degradation performance of the isolated strains was unsteady after passage. Our research team collected *Oxytropis ochrocephala* Bunge from Nanhua Mountain, Haiyuan County, Ningxia Hui Autonomous Region in Oct, 2008 and buried them in the soil for 6 months. A strain of *Arthrobacter* sp. HW08 capable of SW degradation with high efficiency was obtained after enrichment culture and pure cultivation (Wang et al., 2010a). It was kept in China General Microbiological Culture Collection Center (CGMCC NO: 3313) with the Patent No. 200910218983.5 (Wang et al., 2010). Its 16S rDNA sequence has been included in Genbank under the Registry No. GQ921838. Under the optimized temperature (30°C) and pH value (7.0), HW08 (OD600＝0.3) could degrade about 2 mg SW in 5 ml degrading reaction within 4 h (Wang et al., 2010a). And then, we have constructed a genomic library of strain HW08, screened for five types of degradative plasmids. Based on this work, we have studied the effects on SW degradation by the mixture of transformants of these degradative plasmids and preliminarily analyzed the metabolites of SW. The findings of this study lay the foundation for the research on metabolic pathway or degradation mechanism of SW.

MATERIALS AND METHODS

Plasmids and strains

Plasmids pUCSW-1, pUCSW-2, pUCSW-3, pUCSW-4 and pUCSW-5 were obtained from genomic library of *Arthrobacter* sp. HW08 and constructed by us. It was proved that SW (400 mg· l^{-1}), as the unique carbon source, cultivated with the mixture of the five plasmid-transformants could be degraded within 6 h. The degrading capability was equivalent to that of strain HW08 (Hu et al., 2011). *Escherichia coli* DH5α [*supE44, ΔlacU169 (Φ80 lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*] was kept in the laboratory.

Culture medium

Luria-Bertani medium (LB) for enrichment culture contained (g· l^{-1}) 5 g yeast extract, 10 g peptone, and 10 g NaCl (pH 7.2). The mineral salts medium (MSM) used in degradation tests comprised (g· l^{-1}) 5.0 g NH_4NO_3, 1.5 g $MgSO_4$, 5.0 g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, 5.0 g NaCl, and 1.5 g K_2HPO_4 (pH 7.2); SW was added to the medium after autoclaving. Media were solidified, if necessary, by the addition of 15 g agar per liter.

Reagents

SW standard substance, Methyl-α-D-mannopyranoside (me-Gal) and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) ＋ trimethylchlorosilane (TMCS) were purchased from SIGMA. Other chemicals used in this study were of analytical grade and were obtained from commercial sources.

Plasmids transformation and cultivation

Plasmids pUCSW-1, pUCSW-2, pUCSW-3, pUCSW-4 and pUCSW-5 were transferred to *E. coli* DH5α and were grown in LB solid plate, respectively, at 37°C for 12~16 h, and ampicillin-resistant transformants of *E. coli* DH5α were inoculated into LB slant by steak cultivation at 37°C for 48 h (Sambrook et al., 2001).

The capability of degrading SW for transformants

Transformants of LB slant were washed off with MSM and mixed at a proportion for 1:1(V:V). SW was added to a final concentration of 1000 mg·l^{-1}, and the mixture was incubated at 30°C for 72 h. Samples were collected for detection of bacterial solution concentration and SW content within 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h, respectively.

Analyses to metabolites of SW

SW and their metabolites were determined by thin-layer chromatography (TLC) as described previously (Molyneux and Roitman, 1980; Molyneux et al., 1988, 1991; Wang et al., 2011). Briefly, supernatant of samples was lyophilized after centrifugation at 10,000 × g for 5 min, and the lyophilized powder was dissolved in 20 µl methanol and developed on silica gel plates in $CHCl_3$:CH_3OH:NH_4OH:H_2O (V:V=70:26:2:2).The plates were stained with iodine vapor or Ehrlich's reagent. Metabolites of SW were visualized comparing the spots color and retention factor values (R_f value) of samples with that of sample in 0 h.

GC-FID analysis was used for quantitative detection by our previous study (Zhao et al., 2009). Briefly, the culture supernatant was lyophilized after centrifugation at 10,000 × g for 5 min, and the lyophilized powder was used for derivatization with BSTFA + TMCS. SW concentration was analyzed by injecting 2 µl of derivatization sample into GC-14C gas chromatography spectrometer (Shimadzu, Japan) with a data handling system and FID, using high purity nitrogen (99.999%) as the mobile phase

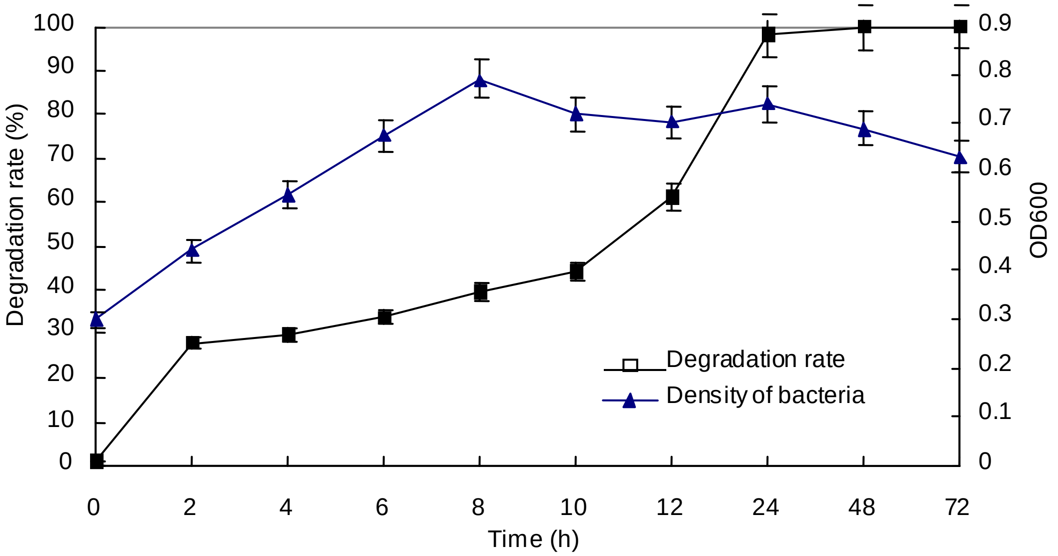

Figure 2. The curve of SW degradation and growth of 5 types of transformants in 72 h.

at a rate of 2 ml·min^{-1}. The split ratio was adjusted to 30:1. AT.SE—54 quartz capillary column(30 m × 0.25 mm × 0.25 μm), injector, and FID temperatures were 210, 280 and 300°C, respectively.

GC-MS analysis was performed on a Finnigan Trace GC ultra coupled to a quadrupole mass selective detector Finnigan Trace DSQ (ThermoFinnigan). The MS interface temperature was set to 320°C and the source temperature to 250°C. Full-scan mass spectra were recorded at an electron energy of 70 eV within a scan range of 40-700 mu at a scan rate of 2.5 scans·s^{-1}. Helium was used as the carrier gas at a constant flow rate of 1 ml·min^{-1}. The chromatographic conditions were as described for GC-FID analysis. Metabolite constituents were identified by comparing retention times with those of silylated reference compounds and by comparing mass spectra with the entries of the mass spectral library (NIST, 2002).

RESULTS

SW degradation ability of transformants

Figure 2 shows the curve of SW degradation and the growth of the mixture with five types of transformants in 72 h. It was found that SW could be degraded in MSM and could promote transformants physiology activity as unique carbon source. With the coculture time lasted, SW slowly degraded, transformants swiftly grew and reached plateau phase in 8 h. And the rate of degradation of 1000 mg·l^{-1} SW was 98.01% in 24 h, 99.78% in 48 h, 100% in 72 h, respectively.

Detection of metabolites by TLC

Fifty microlitre of samples in 0, 6, 12, 18, 24, 48 and 72 h were stained with Iodine vapor or Ehrlich's reagent (Figures 3A and B). R_f value of SW was 0.612, and R_f value of metabolites I, II was 0.388, 0.125, respectively, which the plate stained with Iodine vapor (Figure 3A). The spots color of SW was purple and its metabolite had no spots color appeared which stained with Ehrlich's reagent (Figure 3B). The results showed that SW contents clearly decreased with the extension of reaction time and spots of SW disappeared after 48 h. But 2 kinds of spots (I and II) of metabolite were clearly visualized during the process of cultivation in 48 h compared with the samples in 0 h, indicating that at least 2 kinds of metabolites produced in the process of SW biodegradation by 5 transformants. The spot I and II became lighter in 72 h, indicating that they were probably intermediate metabolites.

GC analysis

Figure 4 shows the gas chromatogram of SW and its metabolites when 1000 mg·l^{-1} SW was utilized by the mixture of five transformants in 72 h compared with the samples in 0 h. It found that the retention time of me-Gal (internal standard) and SW was 4.64 and 5.03 (or 5.02) min, respectively. SW peak gradually decreased with the extension of culture time and the peak of intermediate metabolite A appeared in 2 h. Metabolite A peak gradually increased with the progress of reaction, and intermediate metabolite B and C peak appeared in 10 h. At the same time, D and E peak appeared while A peak reached the highest and B peak disappeared in 12 h. Then, A peak stepped down gradually with the peak of SW striking decrease, and F, G peak appeared while D peak disappeared in 24 h. It deduced that metabolite F, G came from D and became end-products because their peak heights had no change until SW completely

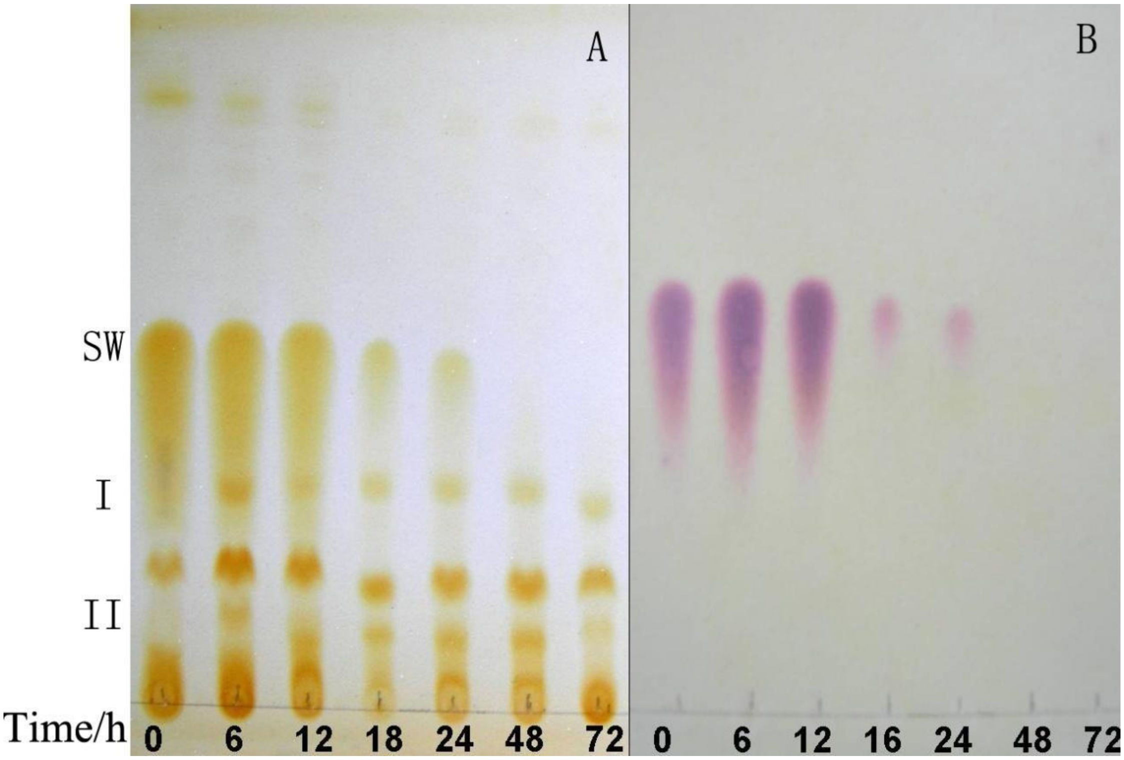

Figure 3. Thin-layer chromatography analysis of SW degradation stained with iodine vapor (A) and Ehrlich's reagent (B). The spots of I and II are visualized as intermediate metabolites of SW.

degraded. C, E peak increased gradually with the decrease of peak A. After that, H, I peak appeared when C reached the highest peak height in 48 h. In 72 h, the peaks of SW, A and C were all disappeared, and E, F, G, H peak existed. The results indicate that compound A, C are main intermediate metabolites and compound E, F, G, H are end-products in the process of metabolism of SW by 5 transformants.

GC-MS analysis

Samples of SW degradation by five transformants in 0, 12, 24 and 48 h were determined by GC-MS. Figure 5 shows the gas chromatogram by Trace GC. Six kinds of compound peaks appeared. According to mass spectrum scanogram (Figures 6A and B) with the entries of the mass spectral library (NIST, 2002), the retention time of Me-Gal, SW, palmitinic acid and stearic acid was 2.90, 3.20, 6.10 (or 6.09), 11.22 (or 11.23, 11.24, 11.26), respectively. Two special compound peaks (retention time: 5.72 or 5.73, 10.20 or 10.22) appeared after 12 h, named as compound α (Figure 6A) and β (Figure 6B), respectively. Their peak heights increased with the extension of culture time, and reached the top in 24 h. Then, the peak heights gradually decreased even disappeared with the SW peak change, indicating that they were main intermediate metabolites of SW degradation. Their molecular ion (M+H) peaks were very abundant and m/z was 388.2 and 314.3 amu respectively.

DISCUSSION

Arthrobacter strains are metabolically diverse and are capable of catabolizing a variety of chemical and environmentally relevant compounds because they contain genes or pathways for the catabolism of any of these compounds (Mongodin et al., 2006). We have proved that *Arthrobacter* sp. HW08 is particularly well-endowed genetically to metabolize SW by selecting for degradative plasmid-transformants from genomic library of strain HW08 and have screened for 5 types of degradative plasmids which laid the foundation of this study.

TLC is the method currently most used for the analysis of polyhydroxy alkaloids (Molyneux et al., 1991). SW may be visualized as a pyrrolidine ring fused to a piperidine ring moiety, yielding a bicyclic five/six ring system because it is a toxic polyhydroxy indolizidine alkaloid which stained with Ehrlich's reagent producing a purple color (Molyneux et al., 2002). Figure 2B shows the effect on degradation of SW by the mixture of 5 types of transformants. The purple color spots of samples become smaller with the extension of culture time. And they disappeared in the sample of 48 and 72 h, indicating that

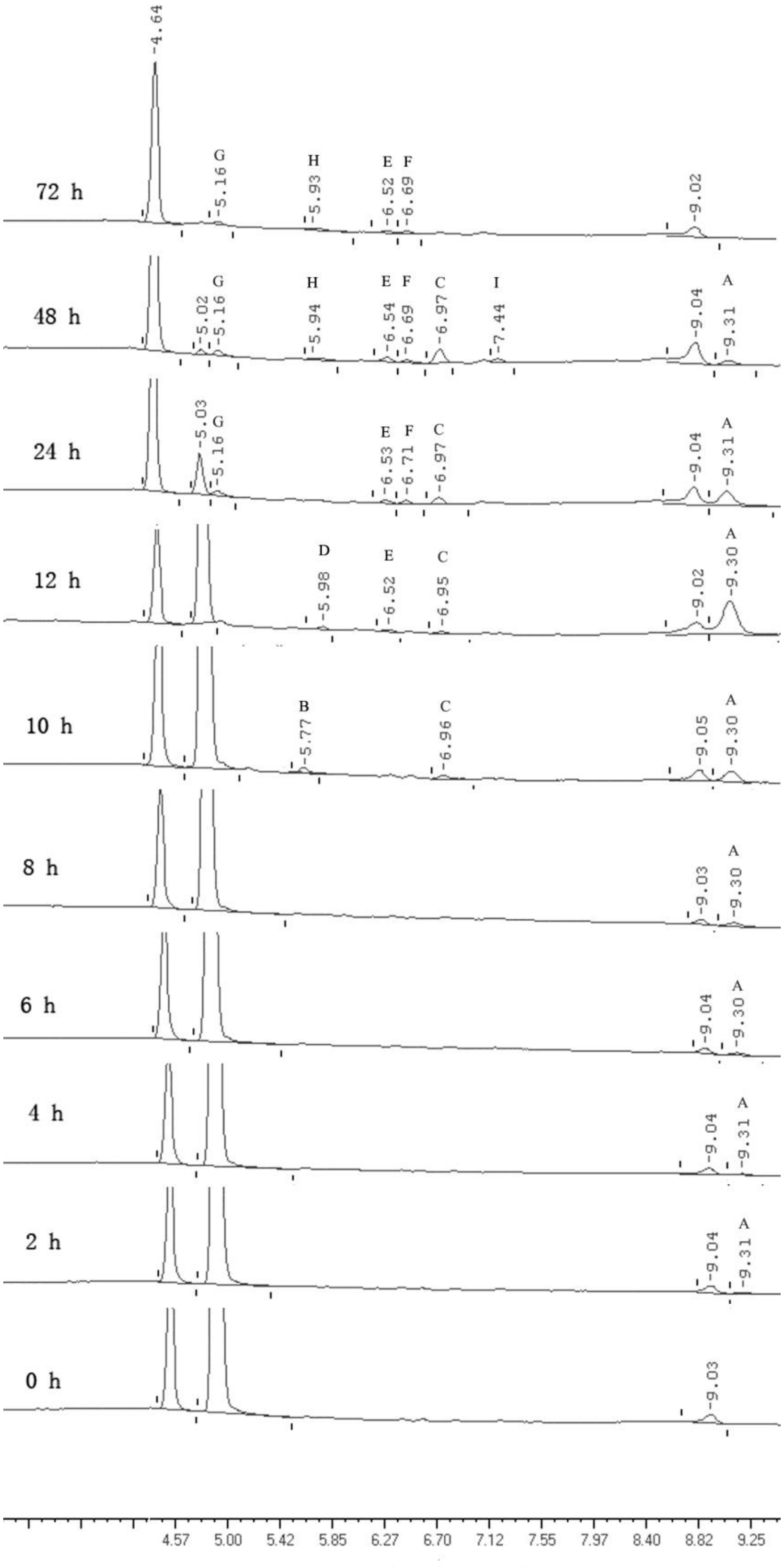

Figure 4. The gas chromatography of 1 g· l^{-1} SW degradation by the mixture of 5 types of transformants in 72 h.

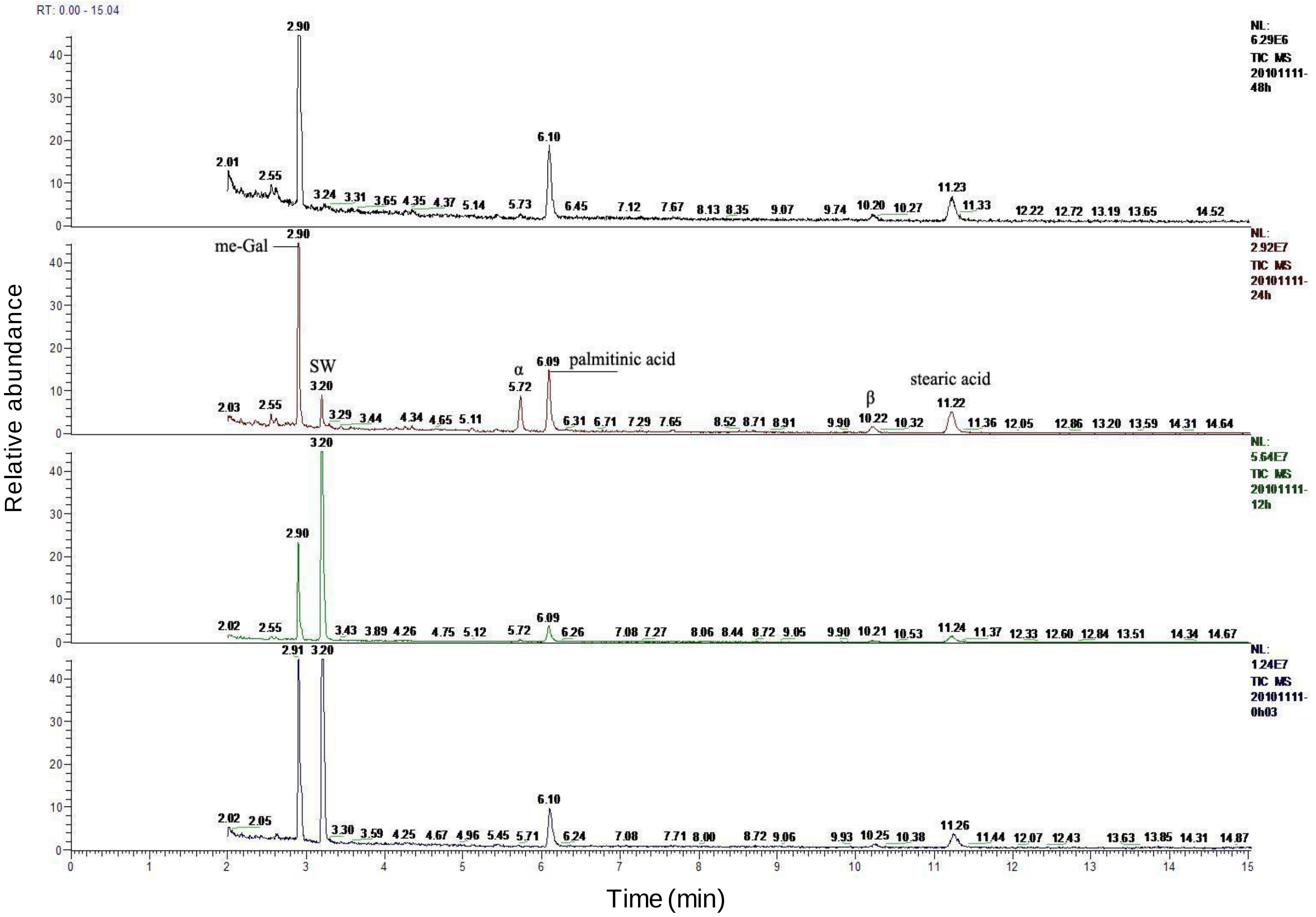

Figure 5. The gas chromatography of 1 g· l^{-1} SW degradation by 5 transformants with Trace GC.

the indole ring system of SW was changed. That is to say, the, metabolites of SW by five transformants probably not contain indole ring. Whereas metabolites can be stained with Iodine vapor which are applies to most of organic compounds containing heteratom, double or triple bound, aromatic ring, polyalcohols, etc (Akhrem and Kuznetsova, 1963; Sherma, 2000).

According to TLC, GC and GC-MS analysis, the results illustrated that five types of transformants co-cultured with 1000 mg.l^{-1} SW as sole carbon source, and SW could be completely degraded in 72 h. SW had no inhibit bacterial growth and several metabolites produced in the metabolism of SW. Palmitinic acid and Stearic acid existed in the sample of 0 h, too. And their peak areas obviously increased with the extension of reaction time, inferring that they were produced as primary metabolites of transformants by continuous stimulation of SW (Wu, 2006).

Because of the structural relationship of polyhydroxy alkaloids to sugars, SW must be derivatized with BSTFA and TMCS in pyridine which provides hydroxyl groups with the necessary volatility and stability for GC and GC-MS analyses (Nash et al., 1986). But the sample must be maintained at 60°C for 1 h to ensure that all of the alkaloids give single peaks. In the course of mass spectrometric analysis of the derivative of SW, the characteristic fragment ions at m/z 120, m/z 185, m/z 260, m/z 299 and m/z 374 are generated (Yu, 2009). But it is difficult to analyze the mass spectra of intermediate metabolite α and β because of their derivatization.

Metabolites pathway of SW would be deduced by gas chromatogram (Figure 4). Figure 7 shows the deduced result, indicating that SW being degraded by five types of transformants at least need two kinds of enzymes (Enzyme 1 and 2) to complete. Intermediate metabolite B, C, D, I are probably converted into end-products E, F, G, H by more than 2 kinds of enzymes. Further studies are needed to clarify the structures of metabolites by preparative TLC chromatography, silica gel column chromatography and NMR so as to prove the metabolism pathway of SW and explore SW degradation mechanism by 5 types of transformants.

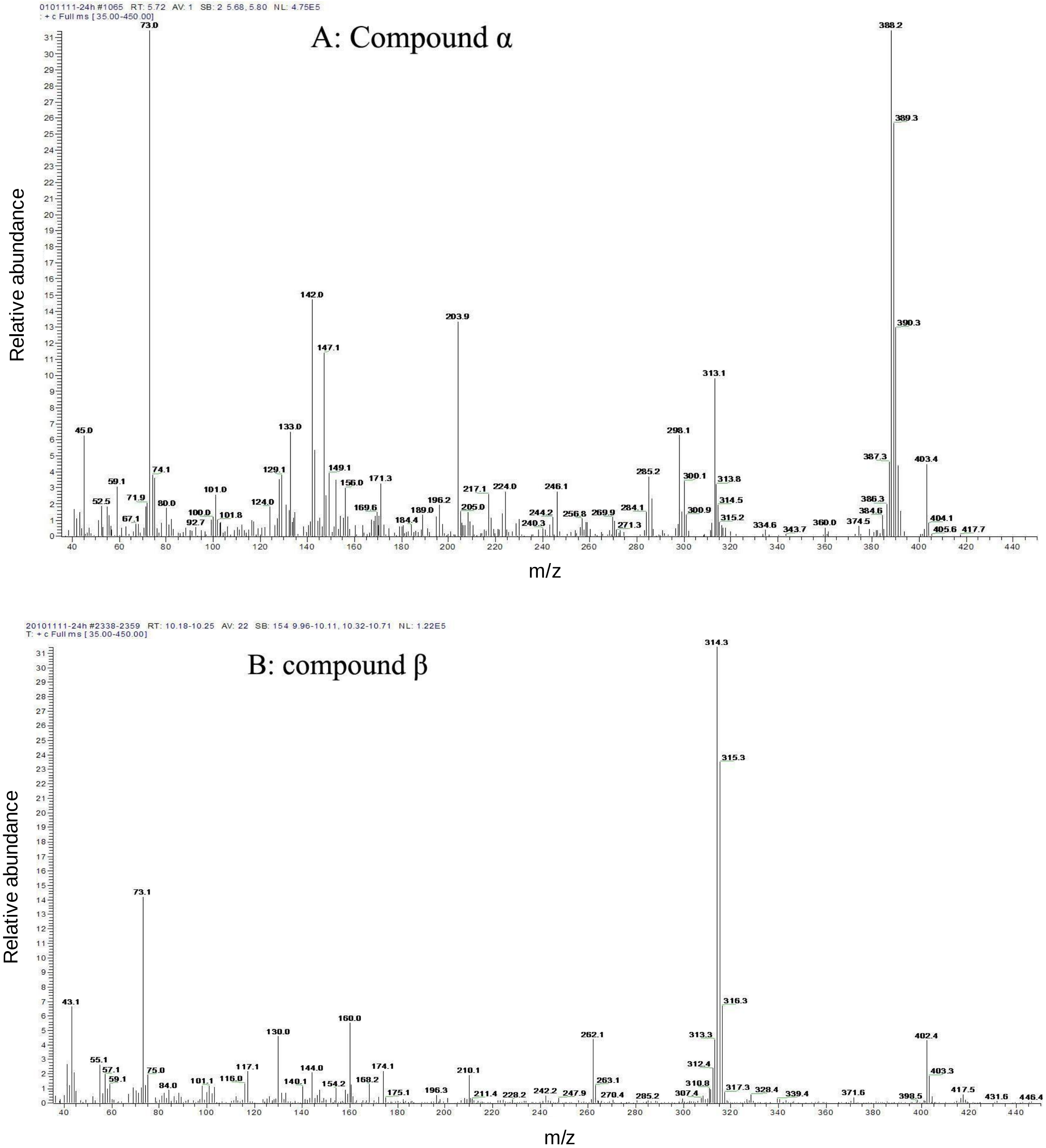

Figure 6. The mass spectrogram of metabolites for SW degradation by 5 types of transformants detected by Trace DSQ. Intermediate metabolite: (A) compound α and (B) compound β.

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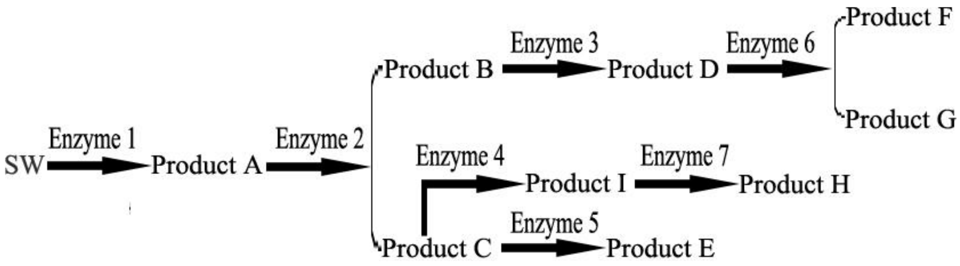

Figure 7. The deduced metabolic pathway of SW degradation with 5 types of transfomants by GC-FID analysis of Figure 4.

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