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ORIGINAL PAPER

Biodegradation of Swainsonine by *Acinetobacter calcoaceticus* strain YLZZ-1 and its isolation and identification

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Abstract Eight swainsonine (SW)-degrading bacteria were isolated from the soil where locoweed was buried for 6 months and one of the strains (YLZZ-1) was selected for further study. Based on morphology, physiologic tests, 16S rRNA gene sequence, and phylogenetic characteristics, the strain showed the greatest similarity to members of the order *Acinetobacters* and within the order to members of the *Acinetobacter calcoaceticus* group. The ability of the strain for degrading SW, as sole carbon source, was investigated under different culture conditions. The preferential temperature and initial pH for the strain were 25–35°C and 6–9, respectively. The optimal temperature for the strain was 30°C and the optimal pH was 7.0. There was a positive correlation between degradation rate and inoculation amount. The concentration of SW affected the degradation ability. When the concentration of SW was lower than 100 mg/l, SW decreased immediately after incubation, and when the concentration of SW was 200 mg/l, there was an inhibiting effect for bacteria growth and SW degradation. The strain could degrade SW completely within 14 h when the concentration of SW was 50 mg/l. These results highlight the potential of this bacterium to be used in detoxifying of SW in livestock consuming locoweed.

Keywords Swainsonine · Biodegradation · *Acinetobacter* sp. · Detoxification · Locoweed

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Introduction

Locoweeds are toxic plants of the genera *Astragalus* sp. and *Oxytropis* sp. containing the indolizidine alkaloid, swainsonine (SW, Fig. 1) (Molyneux and James 1982). Grazing of locoweeds for an extended period of several weeks may cause locoism (James et al. 1981) which is characterized by neurological changes, reproductive disturbance, emaciation, and eventually death. Neurological lesions or damage is permanent (Stegelmeier et al. 1999a, 2005). Locoweed toxicity can also occur in lambs and calves ingesting milk from mothers consuming large amounts of locoweed (Taylor and Strickl 2002). Locoweed poisoning is the most widespread poisonous plant problem in many countries including China (Li 2003), the United States (Ralphs and James 1999), Canada (Harries et al. 1972), Australia (Huxtable and

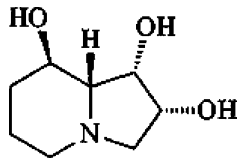

Fig. 1 The chemical structure of SW

Dorling 1982) and Brazil (Medeiros et al. 2003). A large range of animals including cattle (Pfister et al. 2003), sheep (Stegelmeier et al. 1998), horse (Pfister et al. 2002), and deer (Stegelmeier et al. 2005) have been poisoned by grazing locoweed and the toxin has caused tremendous economic loss annually to the livestock industry. In china, the distribution range of the locoweed is extending continuously and it has formed or is coming into dominant species gradually in some areas in recent years.

The toxicity of locoweed to livestock is attributed to the presence of SW (Tulsiani et al. 1984), which inhibits the lysosomal enzyme α-mannosidase (Dorling et al. 1980), and can result in accumulation of oligosaccharides processed incompletely, loss of cellular function, and cell death. Furthermore, SW inhibition of Golgi mannosidaseII (Tulsiani et al. 1985), alters glycoprotein synthesis, processing, and transport, leading to dysfunction in cellular adhesion molecules, circulating hormones (e.g., insulin) and various membrane receptors (Richards et al. 1999).

Up to now, although the toxicology and pathology of locoism have been described in detail (Obeidat et al. 2005; Stegelmeier et al. 1999b; Taylor and Strickl 2002), it remains a critical problem to protect animals from this poisonous disease. Employing special machines to remove locoweeds from fields has been a major approach to protect animals from locoweed in China (Li 2003). In America, bentonite was used to bind SW and mineral supplement or clinoptilotite as dietary treatment were also used to protect animals from SW poisoning (Pulsipher et al. 1994). These methods were neither effective nor affordable. Recently, SW and human serum albumin were conjugated (SW-HSA), and it was used to induce anti-SW antibody (Tong et al. 2007) which could protect animals from lesion when consuming locoweed.

The researchers Morgan and May were unsuccessful in their attempt to identify ruminant bacteria which can detoxify swainsonine (Sterling 1999). The objectives of this study were to find a bacterium which could degrade SW and investigate the SW biodegradation process. This bacterium then might be used to eliminate or decrease the concentration of SW in the body. Therefore, this could reduce SW effects on grazing animals, improve tolerance of locoweed, decrease revenue losses for ranchers from locoism, as well as provide sufficient forage at little extra cost.

Materials and methods

Materials and media

One species of locoweed, *Oxytropis kansuensis* Bunge identified by Professor Xueli Huo, College of Life Science, Northwest A & F University, Yangling, Shaanxi 712100, PR China, was collected at Tian zhu County, Gan su Province, China, in September 2006. Methyl-α-D-mannopyranoside (me-Gal) was purchased from SIGMA, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosil- ane (TMCS) from SUPELCO. Other chemicals used in this study were of analytical grade and were obtained from commercial sources. SW was supplied by the laboratory of Biotoxin and Molecular Toxicology of Northwest A & F University.

Two different media, an enrichment medium (EM) and a mineral medium (MSM) were used in both enrichment culture and testing of degrading ability. EM contains (in grams per liter) 5 g yeast extract, 1 g peptone, 0.5 g NaCl, and 0.1 g K_2HPO_4 (pH 7.0), SW dissolved in distilled water was added to the medium after autoclaving and adjusted to different concentrations. MSM contained (in grams per liter) 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, 1.5 g K_2HPO_4 (pH 7.0), and SW was added to the medium after autoclaving. Media were solidified, if necessary, by the addition of 17 g agar per liter.

Isolation of SW-degrading bacterium

One kilogram ground plant materials of *Oxytropis kansuensis* Bunge were buried with 10–20 cm underground for 6 months, after which a soil sample was collected from around these plants. A 10 g soil sample was collected around these plants, suspended in distilled water (90 ml), shaken, and allowed to settle. After 30 min, 10.0 ml of the supernatant solution was inoculated into 90 ml of enrichment medium. The culture was incubated at 30°C for 24 h

with shaking (180 rpm). Then 1.0 ml of this liquid culture was added to the enrichment medium with SW (30 mg/l) and kept under the same incubation conditions for 4 days. The procedure was repeated with SW concentration increasing to 50, 80, 100, 120, 150, 180–200 mg/l to enrich SW-degrading bacteria. After the last transfer, 1.0 ml of the liquid culture was serially diluted as a tenfold-dilution series into sterilized distilled water. Dilutions were spread onto solid plates of SW MSM containing SW 100 mg/l and incubated at 30°C for 3–5 days. Colonies were picked from dilution plates based on distinct colony morphology and transferred onto fresh plates several times to ascertain culture purity (Herigstad et al. 2001; Nishimori et al. 2000). Each isolation was then tested for its ability to degrade SW in MSM medium containing SW (50 mg/l) as sole carbon source. And the concentration of SW was measured regularly by gas chromatography (GC), as detailed below.

Analytic methods

The SW concentrations in all the liquid samples were determined by GC: 600 µl of the bacterial cultures was centrifuged at 6,000*g* for 10 min, and 500 µl of the supernatant was moved to a new tube. These samples were lyophilized. The dry samples were dissolved in 95 µl of pyridine, and well mixed with 80 µl of the internal standard (me-Gal) at a concentration of 0.25 g/l, and 25 µl of BSTFA + TMCS. The mixture was incubated at room temperature for 30 min to make sure that derivatization was completed. After that, 1 µl aliquots were injected into the gas chromatograph. For GC analysis of each derivative sample we used a Shimadzu model 14C gas chromatograph equipped with flame ionization detector (FID) and AT.SE-54 column. The column temperature was kept at 210°C, the injector port temperature was at 300°C and the detector block at 280°C. Purified dry nitrogen was used as carrier gas at a flow ratio of 2 ml/min; the split rate was 30:1 (Zhao et al. 2007).

Biochemical and phenotypic characteristics

Cell morphology of the isolated strain was observed by transmission electron microscope (TEM: JEM-1230) and scanning electron microscopy (JEOL JSM-6360LV).

Conventional physiological characteristics were determined according to the Manual of Identification for General Bacteriology (Dong and Cai 2001).

16S rRNA gene sequence determination and phylogenetic analysis

Genomic DNA was isolated according to standard procedures described by Singh et al. (2003). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using *Taq* polymerase. The following pair of universal primers for bacteria was used, the forward primer SF8/20:5′-AGAGT TTGAT CCTGG CTCAG-3′ and the reverse primer BSR1541/20:5′-AAGGA GGTGA TCCAG CCGCA-3′ (Yang et al. 2005). PCR consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, with the last cycle followed by a ten-minute extension at 72°C. Sequences of the product were determined by Invitrogen Biotechnology Company Limited (Shanghai) and compared with 16S rRNA gene sequence data from type strains available in GenBank (http://www.ncbi.nlm.nih.gov/) using the BLASTN sequence match routines. The sequences were aligned using multiple sequence alignment software CLUSTALW Version 1.81 (Thompson et al. 1994). A phylogenetic tree was then constructed by the neighbor-joining method using Mega 3.1 software based on the 16S rRNA gene sequences of 16 strains phylogenetically close to the isolated strain.

Inoculum preparation for degradation studies

Unless otherwise stated, the inoculants for this experiment were bacteria growing on slant on MSM culture medium containing SW at 100 mg/l for 3 days. The cells were washed off with 5 ml MSM and then pelleted by centrifugation at 6,000*g* for 10 min. Cells then were washed three times with 5 ml MSM and quantified by the dilution plate count technique. For all experiments 10^6cells/ml were used and samples were incubated at 30°C and shaken at 180 rpm unless otherwise stated.

Degradation of SW by the isolated strain

The degrading ability of the isolated bacterium was assayed in MSM. SW was added into MSM to

achieve a concentration of 50 mg/l as the sole source of carbon. SW concentration in MSM was analyzed by GC as previously described. All experiments used a non-inoculated group as a control to eliminate the possible effect of evaporation. Effects of incubation temperature, initial pH, SW concentration, and inoculum density on degradation were examined using three parallel treatments.

Results

Isolation and characterization of a SW degrading strain

With the enrichment culture procedure used, eight isolates were obtained that grew on MSM plates containing 100 mg/l as the sole carbon source. All isolates were tested for their degrading capability under the conditions previously described. Strain YLZZ-1 was remarkable because it could degrade SW (50 mg/l)completely within 14 h. Therefore, further study was done with this strain. Strain YLZZ-1 is a Gram negative coccobacillus, non-encapsulated, non-motile, and 0.3–0.5 × 0.5–1.0 μm in size. The morphologic characteristics are shown in Figs. 2 and 3. The colony morphology of strain YLZZ-1 on plain agar plate was lacte, smooth, wet, eminentia and non-transparent.

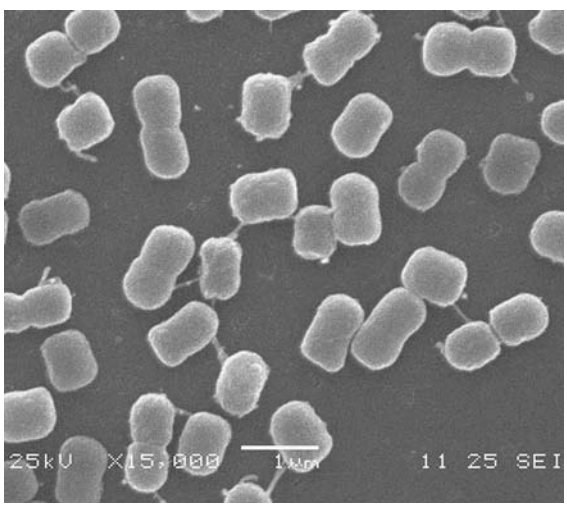

Fig. 2 Transmission electron micrograph of strain YLZZ-1 (50,000×)

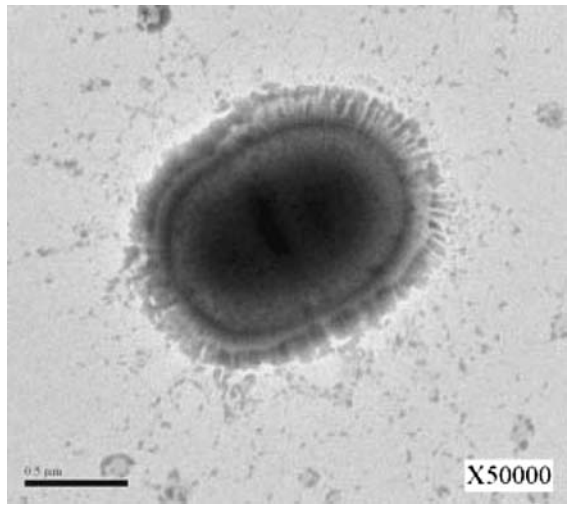

Fig. 3 Electron micrograph of strain YLZZ-1 (15,000×)

16S rRNA gene sequence and phylogenetic analysis

After PCR amplification of 16S rRNA gene from strain YLZZ-1, a single fragment of 1,463 bp, GenBank accession No. EU022688, was obtained and completely sequenced. According to BLAST analysis the resulting sequence was similar to the 16S rRNA gene sequence of bacteria belonging to *Acinetobacter* group, and closely clustered with strain CAI-13 (GenBank accession No. DQ257421) and strain NCCB (GenBank accession No. AJ888983), with sequence

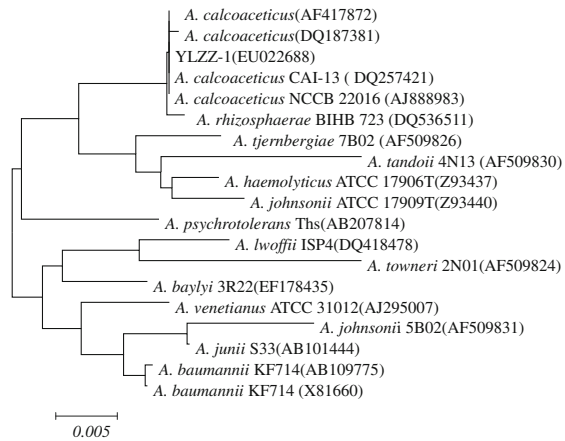

Fig. 4 Phylogenetic tree based on 16S rRNA gene sequence of strain YLZZ-1

identities of 100 and 98%, respectively. A phylogenetic tree was constructed based on the 16S rRNA gene sequences (Fig. 4) by the Neighbor-joining method using MEGA 3.1. The results again indicated that the isolate belongs to the *Acinetobacter calcoaceticus* group. Therefore, the isolate was designated as *A. calcoaceticus* strain YLZZ-1.

Degradation of SW by strain YLZZ-1

The time course of the degradation of SW by strain YLZZ-1 was followed by GC (Fig. 5). Peak 1 (3.86 min) corresponds to the internal standard, and peak 2 (4.30 min) corresponds to SW. After just 6 h of incubation at 30°C, peak 2 decreased as compared

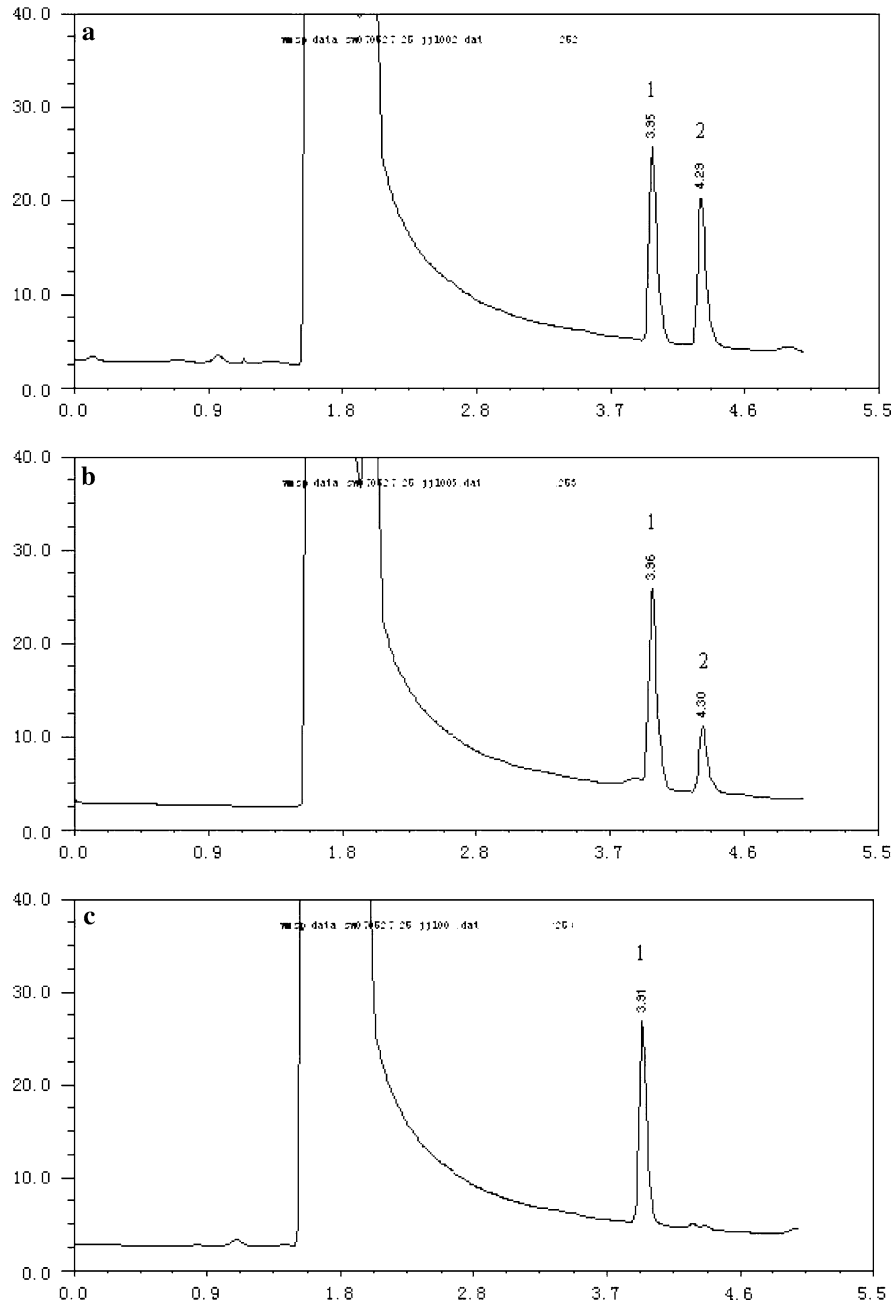

Fig. 5 Gas chromatogram of a culture of strain YLZZ-1 in MSM with SW (50 mg/l) incubated at 30°C for 0 h (**a**), 6 h (**b**), and 12 h (**c**). The two peaks were 186 identified as me-Gal (1) and SW (2)

to that seen at 0 h of incubation, and 12 h later there was no peak recorded.

Effect of the initial condition on degradation of SW

The growth of strain YLZZ-1 and the effect of SW degradation by strain YLZZ-1 were observed under different culture condition, such as different temperature, initial pH, initial SW concentration and inoculum amounts. As seen in Fig. 6, the growth and SW degradation of strain were good from 25°C to 35°C. The optimal temperature was 30°C. There was weak degradation at 20°C and 40°C. The effects of initial pH on the growth rate and SW degradation of strain YLZZ-1 are shown in Fig. 7. The degradation rate of the strain is higher between pH 6.0 and 8.0 than at pH 9.0 and 6.0. A value of 7.0 of initial pH was optimum for growth and degradation of SW by strain YLZZ-1.

Figure 8 show the dynamic curve of SW degradation of strain YLZZ-1 with different initial SW concentrations. When SW concentration was under 100 mg/l, the degrading rates were not obviously changed, but when SW concentration was 200 mg/l, the curve showed a lag phase. As time went on, after 6 h, the SW concentration was decreased rapidly, no SW residue was observed after 72 h. This indicated that at the experimental concentration levels, the degradation and growth of strain YLZZ-1 could partly be inhibited by SW of 200 mg/l; however, when the strain adapted to the environment, the degradation continued and SW could be degraded completely.

At the high inoculum density (>5%), SW was degraded completely within 12 h; however, degradation was slower at lower density, as shown in Fig. 9.

Discussion

In this study, eight SW-degrading isolates were obtained from soil where locoweed plants had been buried materials for 6 months. One isolate (strain YLZZ-1) had high degrading capability for SW in MSM, and showing high degradation activity and

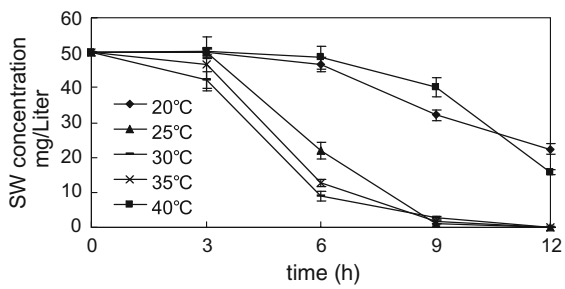

Fig. 6 Effect of temperature changes on degradation of SW by strain YLZZ-1

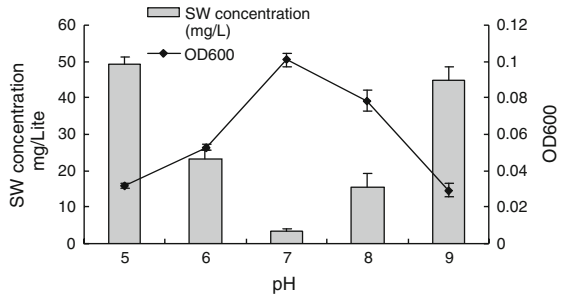

Fig. 7 Effect of initial pH changes on degradation of SW by strain YLZZ-1

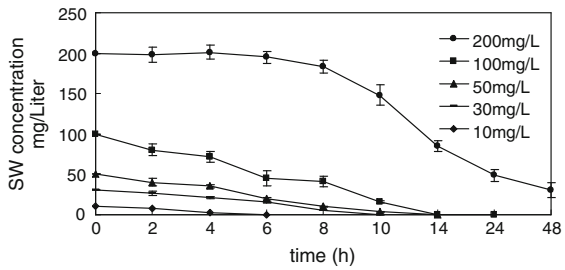

Fig. 8 Effect of initial SW concentration changes on the degradation of SW by strain YLZZ-1

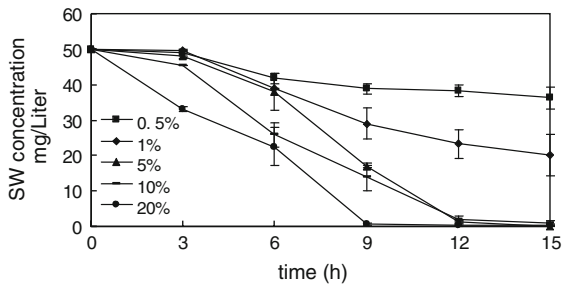

Fig. 9 Effect of inoculum changes on degradation of SW by strain YLZZ-1

resistance to SW. During a previous study in our lab we tried to find SW-degrading bacteria from soil where locoweed grows, using a traditional enrichment procedure (Wackett and Hershberger 2001), but only found several bacteria could resist high concentrations of SW, but could not degrade SW or grow in SW MSM. These results indicate that burying locoweed plants can acclimate environmental bacteria to obtain or enhance the ability to degrade SW, and this makes the later enrichment procedures more efficient.

Morgan et al. (1999) obtained several bacterial types from bovine rumen samples that could grow on the sugar mannose as a preliminary step toward growing them on SW since mannose and SW shared similarities in their chemical structures. SW was not used directly due to its cost and lack of availability. Two of the isolates were chosen for further experimentation, and they grew well when mannose was added, but growth on SW alone was insignificant, additionally, no loss of SW was detected in the cultures. The SW concentration in the experiment might have been toxic to organisms (Sterling 1999); however, the concentrations used in the present work were lower than those used by Morgan et al. (1999) and allowed us to isolate bacterial strains that can use SW a sole carbon source.

In this study, we showed that the *Acinetobacter calcoaceticus* strain YLZZ-1 is effective in degrading SW. The metabolic pathway for the transformation of SW remains unknown and continues to be studied in our laboratory, the identification of the degradation products (by GC-MS) together with the identification of proteins which are related to the degradation pathway should be examined in detail. From the point of environmental protection and economic value, the present observation of using bacterium for degrading SW may be exploited further in biotechnology for the effective detoxification of locoweed. However, many case studies show that results from laboratory studies can differ greatly from results in field studies due to many more variables with the latter, such as the potential for pathogenicity (Ebringer et al. 2005 of the isolated strain, therefore, careful must be given to the actual application of SW-degrading bacteria in the environment.

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