Identification of Lysinibacillus Degradation Products of Zearalenone

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Abstract

Zearalenone (Zearalenone, ZEN) is a toxic secondary metabolite produced by Fusarium, which greatly threatens world food security and human health. Lysinibacillus (Lysinibacillus) is a strain screened in inorganic salt medium using ZEN as the sole carbon source, named L15. The active substance that degrades ZEN exists both inside and outside the cell, using liquid mass spectrometry Using technology to identify the product degraded ZEN presumed to be $C_{18}H_{24}O_6$.

Keywords

Zearalenone; Intracellular Enzyme; Lysobacterium.

1. Introduction

Mycotoxin ZEN (Zearalenone) is a natural compound produced by plants of the genus mold as a secondary metabolite, and is usually found on plants such as corn or wheat kernels in temperate climates. With the continuous understanding of mycotoxins, it has been discovered that many mycotoxins have teratogenic, carcinogenic, and mutagenic effects, and their chemical properties are very stable. Once grains, feed, etc. are contaminated, it is extremely difficult to remove under the existing technical conditions [1]. The mycotoxin pollution of these crops seriously affects the health of humans and animals. According to the World Food and Agriculture Organization, about 25% of grain crops in the world are infected by mycotoxins every year, and about 8% of grain crops lose their food and economic use due to their high toxin content [2]. ZEN has estrogen-like toxicity [3], and can compete with the estrogen receptor in the organism to cause a series of physiological reactions. ZEN can enter dairy products, beef, pork, mutton and other food chain products through moldy crops, causing human life and health problems and causing huge economic losses. Therefore, the prevention and control of zearalenone pollution to grain has become particularly important. However, ZEN has become a serious problem facing global food due to its wide distribution, difficult to remove easily and its complex migration and transformation capabilities in the food chain. Microbial remediation is a treatment method that utilizes living microorganisms or their degrading enzymes to effectively remove mycotoxin pollutants. It is a low-cost and environmentally friendly toxin elimination method.

2. Materials and Methods

2.1 Test materials

Source of sample soil: Taken from field soil in Yueyang, Hunan. Moist and dark soil under 5cm below the mud was taken, and soil specimens were collected to isolate degrading strains.

Zearalenone (Zearalenone, ZEN): purchased from Guangzhou Zhongshi Biological Technology Co., Ltd.

LB medium: bacteriological peptone 1%, Yeast Extract 0.50%, NaCl 1%, agar powder (solid) 1.50%. After the configuration is completed, adjust the pH to about 7.0, autoclave at 121 ℃ for 20 minutes, store the liquid medium at room temperature, and store the solid medium in the refrigerator at 4 ℃ after being poured into the plate.

MSN medium: ammonium sulfate 0.5 g/L, magnesium sulfate heptahydrate 0.2 g/L, disodium hydrogen phosphate 2.44 g/L, potassium dihydrogen phosphate 1.52 g/L, and calcium chloride 0.05 g/L. Adjust the pH to 6.8, sterilize at 121 ℃ for 20 min, and store at room temperature.

2.2 Test method

2.2.1 Separation and purification of strains

Weigh 5 g of the sample into a 50 mL screw-top centrifuge tube, and add 30 mL of physiological saline at the same time, close the cap of the screw-top centrifuge tube tightly, and shake on a vortex shaker for 5 minutes so that the sample is fully dispersed and suspended Then let it stand for 1 hour, use a 100 μL pipette to suck 100 μL of the bacterial suspension, add 900 μL of deionized water and dilute in sequence by 10-1, 10-2, 10-3, 10-4, and then dilute the diluted 10-3 , 10-4 bacterial suspension each draw 200 μL on the LB plate medium and spread it evenly with a spreader, place it in a 37 ℃ constant temperature incubator, place it on the front for 15 minutes and invert it for 24 hours. After the culture is completed, use an inoculating loop to pick out the colonies of different colors on the medium plate on the LB solid plate medium and streak them on the LB solid plate medium. Place them in a 37 ℃ constant temperature incubator, place them on the front for 15 minutes and invert them for 24 hours. h, then transferred to a new MSN (inorganic salt) medium containing ZEN (final concentration 2μg/ml) for culture. Continuously isolate and purify strains, while increasing the final concentration of ZEN to domesticate the strains. The calculation formula of ZEN degradation rate is as follows:

ZEN degradation rate (%) = (blank control group content-sample group content) ÷ blank control group content × 100%

2.2.2 Morphological observation of strains

Place the strains growing on the LB plate in a well-lit place to observe the size, shape, surface characteristics of the colony, edge shape, color, transparency and wetness of the single colony.

2.2.3 Molecular identification of strains

(1) DNA extraction

In this experiment, the total DNA of the strain was extracted by the alkaline cleavage method, and the bacterial genomic DNA was extracted by the following operation using the bacterial genomic DNA extraction kit.

(2) 16SrDNA PCR amplification of strains

The 16S rDNA full sequence clone of this strain uses the total DNA of the strain as a template, using 16S rDNA universal primers: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-TACCTTGTTACGACTT-3', and the PCR amplification reaction system is 25 μL; strain total DNA template 1 μL; upstream and downstream primers 27F and 1492R each 1.25 μL; PrimeSTAR 12.5 μL, dd H2O 9 μL. The PCR amplification procedure is: 98°C, pre-denaturation for 30s, then 98°C, 10S, 56°C annealing for 5S, 72°C extension for 35S, 30 cycles, 72°C extension for 2min, and storage at 4°C.

(3) Electrophoresis detection and sequencing of PCR products

After the amplification reaction is over, carry out the detection of the PCR products: take 5μL of the PCR products and add 2 loading buffers, and add the samples to the 1% agarose gel spot wells. The electrophoresis conditions are voltage: 120 V, electrophoresis solution: 1xTAE, electrophoresis Time: 25 minutes, after the electrophoresis is over, check the results through the gel imaging system and take pictures. The purification and sequencing of PCR amplified products was completed by Shanghai Bioengineering Company.

2.2.4 Analysis of strain degradation

After the strain was cultured in the fermentation medium (LB) for 48 hours, the bacterial solution was divided into 50 mL sterilized centrifuge tubes, and the tube caps were tightened and centrifuged in a 4°C refrigerated centrifuge at a speed of 8000 for 10 min. After centrifugation, take the supernatant through a 0.22 μm organic phase filter membrane in a vacuum filtration pump to remove impurities and bacteria in the supernatant as much as possible, and then combine the filtered supernatant and pour it into a sterilized large petri dish. Seal with plastic wrap; Resuspend the bacterial pellet in PBS buffer, centrifuge and wash 3 times and then discard the buffer, add 50 mL of

MSN in a graduated cylinder, and after resuspension in an ice bath, use an ultrasonic cell disruptor to disrupt the bacterial cells, sonicate for 5 seconds, Interval 3s, 35% power ultrasonic breaking, the time should not exceed 30 minutes. Collect the broken intracellular fluid after centrifugation, take the supernatant (intracellular fluid) through a 0.22 μm organic phase filter membrane in a vacuum filtration pump, try to remove impurities and bacteria in the supernatant (intracellular fluid), and then pump The filtered supernatant (intracellular fluid) is combined and poured into a sterilized large petri dish, and sealed with plastic wrap. Use sterilized toothpicks to evenly insert small holes in the cling film, and freeze them overnight at -80°C. The fermentation supernatant and intracellular fluid after freezing overnight were quickly lyophilized to a viscous state using a freeze dryer, and the viscous active substances were collected on the ultra-clean bench. Add the freeze-dried degradation active substances, namely the fermentation supernatant and the intracellular fluid to 20 mL MSN medium without ZEN, and then divide them into a 10 mL system. Add one part to the ZEN mother liquor to make the final concentration 4 mg/L. The other part was boiled in a boiling water bath for 15 minutes to inactivate the active substance, and then the ZEN mother liquor was added to make the final concentration 4 mg/L as a control group. After mixing, quickly take 200 μL of the reaction solution as a control group for the initial ZEN concentration. After adding ZEN, place the reaction system in a shaker at 30°C in the dark, and react at 200 rpm. Samples are taken every 6 hours. Mix 200 μL with an equal volume of methanol each time and store in a refrigerator at 4°C. After sampling for 36 hours, all samples are collected, processed according to the liquid-phase sample processing method, and then tested by liquid-phase instruments.

2.2.5 Degradation product analysis

After the sample is processed in a liquid phase, it is used for liquid-mass spectrometry to detect the molecular weight and possible molecular formula of the product. Detection basis: GB/T 6041-2020 General Principles of Mass Spectrometry Analysis Method [4]; Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis adopts high-resolution liquid chromatography-trapping ion mobility time-of-flight mass spectrometer (UltiMate3000 timsTOF), detection conditions: 1. Mass spectrometry related parameters:Ionization method:+ESI; Capillary voltage: 4.5 kV(pos)/3.5KV(neg); Drying gas temperature: 220 C; Atomizing gas: 1.5Bar; Drying gas: 8.0 l/min; Scan mode: primary full scan + secondary; 2. Liquid chromatography related parameters: Column: Waters ACQUITY UPLC BEHC18 column (100 mm*2.1 mm, Particle Sz.1.7 um); mobile phase: Phase A: Water; Phase B: Acetonitrile; Flow rate: 300 μL/min; Column temperature: 40°C.

Table 1. Degradation product

Time（min）	A（%）	B（%）
0.00	70	30
1.00	70	30
10.00	0	100
13.00	0	100
13.10	70	30
15.00	70	30

3. Results and Discussion

3.1 Isolation and identification of strains

According to the standard curve method established by the laboratory, y=146609x-192641, R2=0.9897, calculate the content of ZEN, and get the strain 15 with a degradation rate of 100% to 20μg/ml ZEN within 24h.

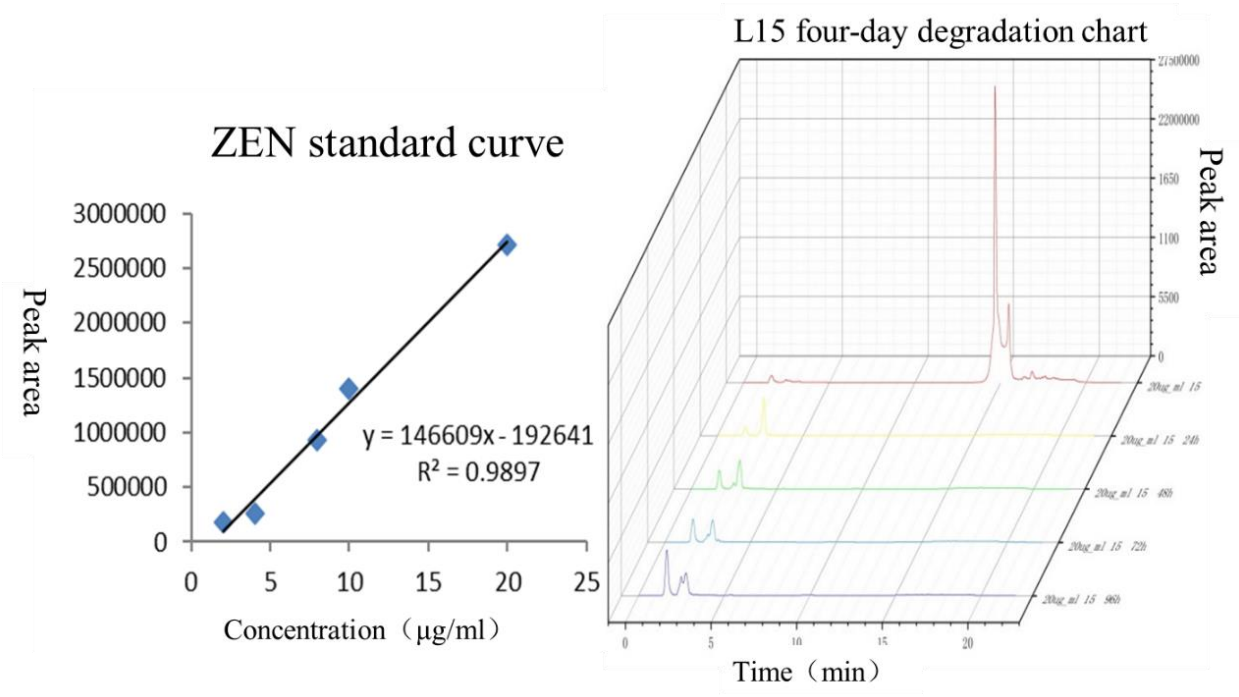

Figure 1. Isolated and purified strain 15

3.2 Morphological characteristics of the strain

The morphology of strain 15 on the LB solid medium is shown in Figure 2. The colony is white and nearly round, and the diameter of the colony is 2~5 mm after 2 days of growth.

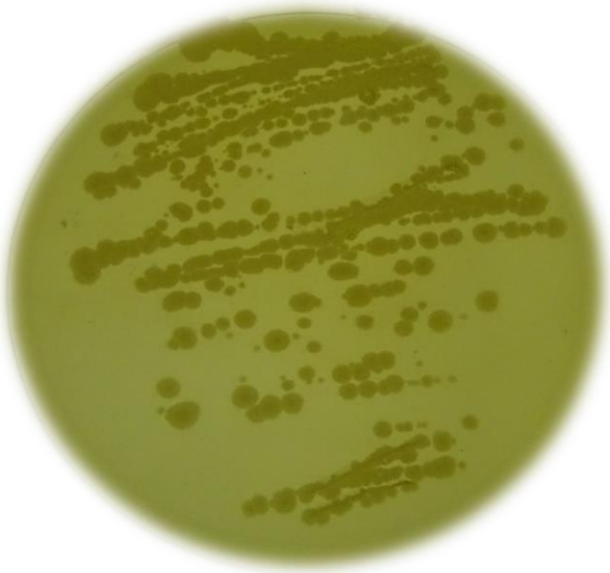

Figure 2. Schematic diagram of the purified strain

3.3 Molecular identification of strains

After extracting the whole genomic DNA of the bacteria by alkaline lysis method, the 16S rDNA sequence was amplified by using bacterial universal primers. The band is shown in Figure 3, and the result of the sequencing part is shown in Figure 4. The result is compared with the existing DNA sequence in NCBI Compare. The data shows that the strain with higher homology is Lysobacterium, the similarity reaches 100%.

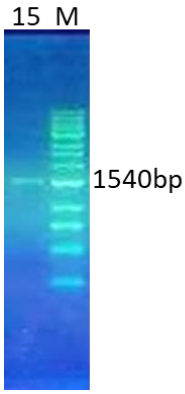

Figure 3. Results of electrophoresis of strain 16SrDNA

ATCTAATTCCACCTTTTCTGCGGCTGGCTCCAAAGGTTACCTCACCGACTTCG
GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGTAGGCG
AGTTGCAGCCTACAATCCGAACTGAGAACGACTTTATCGGATTAGCTCCCTCTCG
CGAGTTGGCAACCGTTTGTATCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATA
AGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGT
CACCTTAGAGTGCCCAACTAAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC

Figure 4. Sequencing results of strain 15

3.4 Analysis of strain degradation

In some studies, the reduction of toxins is partly attributed to the adsorption of bacterial or fungal cells [5,6]. Therefore, the mechanism of strain L15 to eliminate ZEN needs further study. In this study, the culture fermentation supernatant and the catalytic degradation experiment of the degradable active substances in the cells were carried out, and the cell debris was removed. The degradable active material obtained after freeze-drying and concentration was reacted with ZEN. After 36 hours, the residual amount of ZEN was detected, and the degradation rate of the active material to ZEN was calculated.

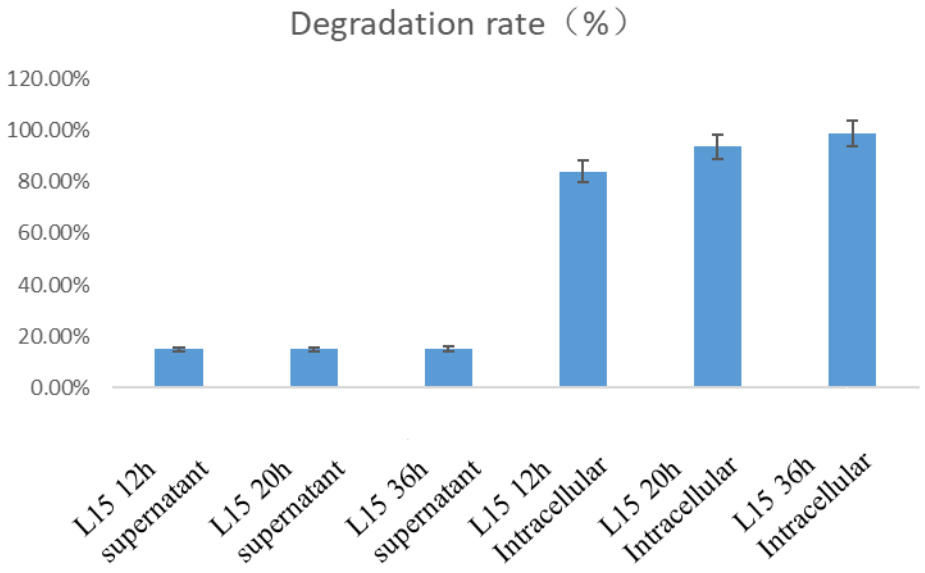

Figure 5. Plot of ZEN results for the degradation of fermentation supernatant and crushed bacterial solution degradation activity of L15

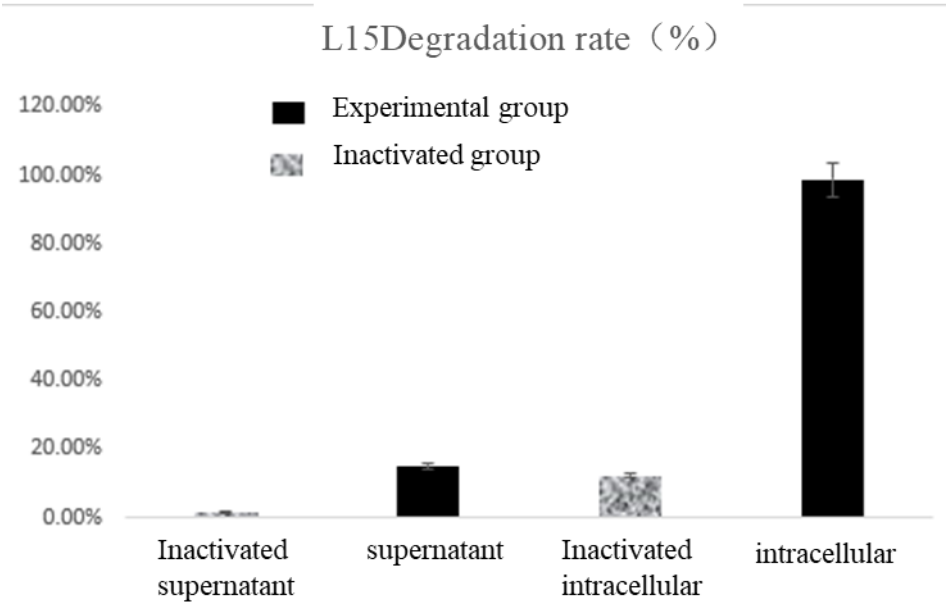

Figure 6. Comparison of inactivation of degrading active substances of strain L15 and active degradation of ZEN

After the crude enzyme protein extracted from the intracellular fluid of L15 was used for 36 hours, the degradation rate of ZEN reached 98.88%. After the crude enzyme protein of fermentation supernatant L15 acted for 36 hours, the degradation rate of ZEN reached 15.1%. Taking into account the problem of the enzyme concentration in the supernatant, it is inferred that the protein active substance that the strain can degrade ZEN is both intracellular and extracellular enzymes. Both the fermentation supernatant and the broken intracellular fluid's degradation ability to ZEN decreased significantly after inactivation, which means that the elimination of ZEN is mainly due to the degradation of the active substance rather than physical adsorption.

3.5 Degradation product analysis

In order to further prove the degradation effect of L15 on ZEN, its degradation products need to be identified [7,8]. The L15 strain, through the LC-MS total ion chromatography (TIC) data obtained in the positive enhanced full scan mode and the negative enhanced full scan mode (EMS), showed that a major ZEN conversion product was formed when the retention time Rt=1.0 minutes. After subtracting the culture control TIC, the full scan mass spectrum of the resultant putative product was evaluated in detail (Figure 3-15). After comparing these data with the control group, the signal at the negative ionization mode m/z 335[M-H]- is considered to be a deprotonated molecular ion.

A: ZEN control group: Inorganic salt medium with ZEN (blank control)

B: L15 control group without substrate (12h): Total ion spectrum of L15 strain grown in inorganic salt medium without ZEN as substrate for 12 hours

C: L15 control group without substrate (24h): the total ion spectrum of L15 strain grown in inorganic salt medium without ZEN as substrate for 24 hours

D: L15 reaction group (12h): the total ion spectrum of L15 strain grown in inorganic salt medium with ZEN (20mg/L) substrate for 12 hours

E: L15 reaction group (24h): the total ion spectrum of L15 strain grown in inorganic salt medium with ZEN (20mg/L) substrate for 24 hours

According to Cmpd1 of A, the retention time of ZEN is RT=6.2min, and the molecular weight is 317.1390[M-H]-. Comparing the peaks of B, C, D, and E, it is found that D and E have a substance of m/z335.1503[M-H]- at Rt=1.0min. This substance should be a product produced by ZEN as a substrate. In the L15 strain no substrate control group, there is no peak at Rt = 1.0min.

F: ZEN control group: ZEN anion first-order mass spectrum

G: L15 reaction group (12h): L15 strain plus ZEN (20mg/L) 12 hours Rt=1.0min product m / z335.1503[M+Na]$^+$

H: L15 reaction group (24h): L15 strain plus ZEN (20mg/L) 24 hours Rt=1.0min product m / z335.1503[M+Na]$^+$

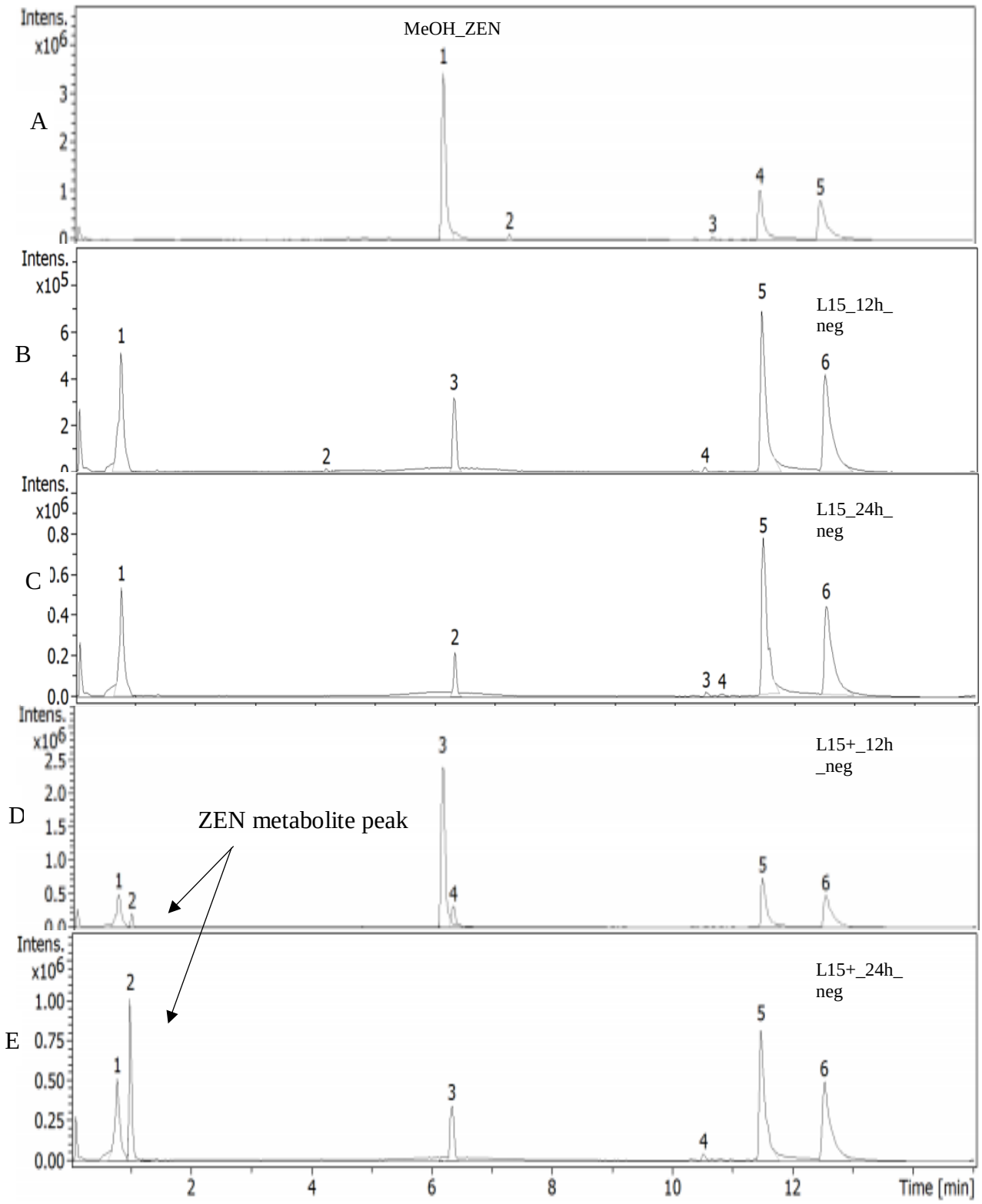

Figure 7. Total ion chromatogram of strain L15 degrading 20mg/L ZEN

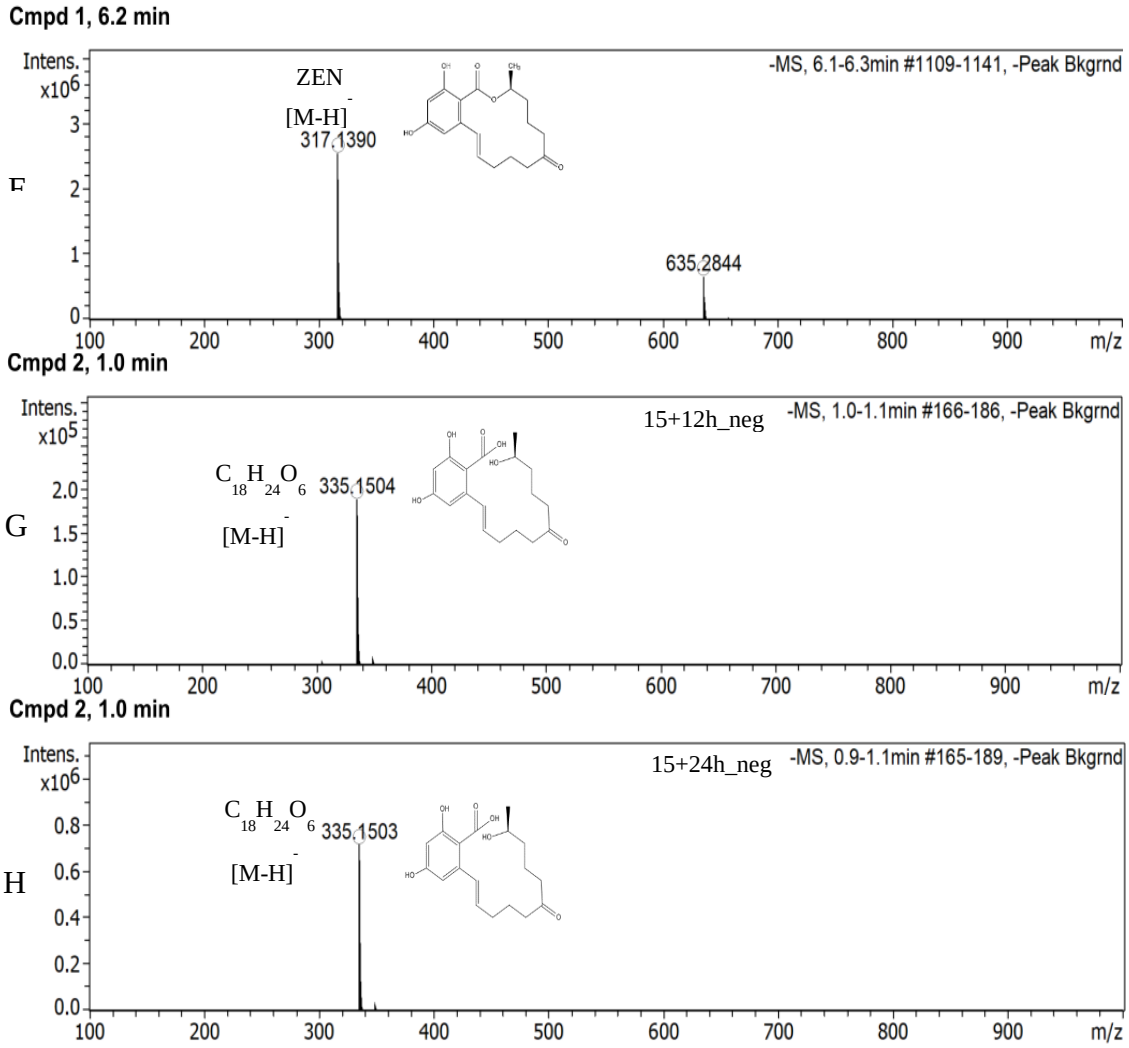

Figure 8. Primary mass spectra of the ZEN product $C_{18}H_{24}O_6$ degraded by strain L15

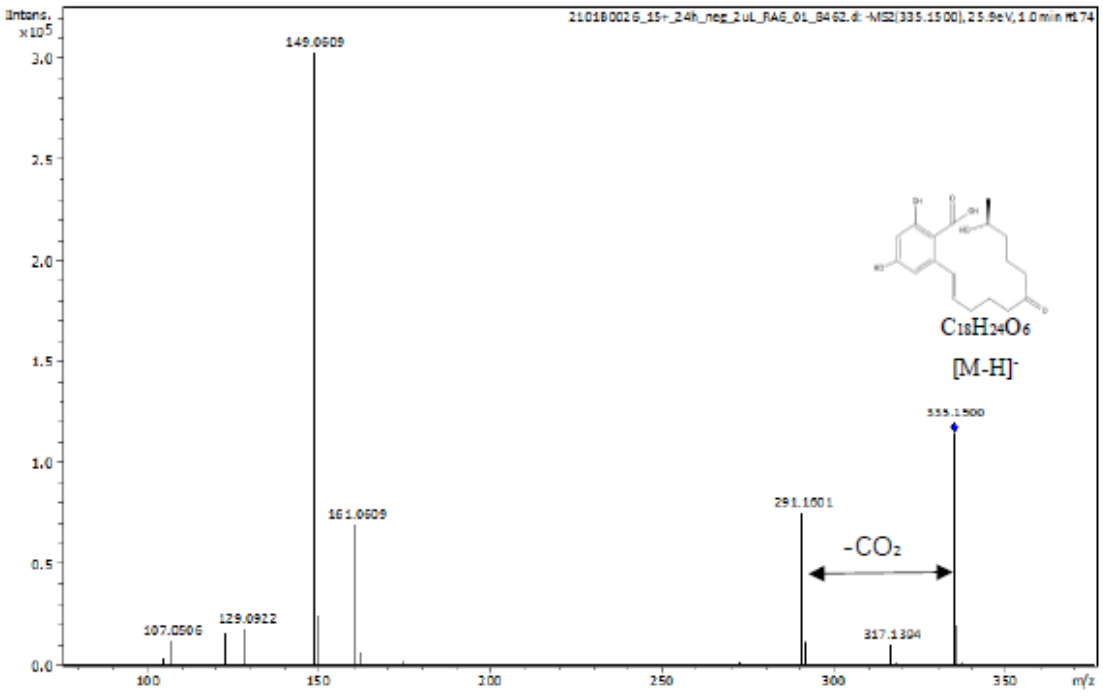

Figure 9. Secondary mass spectra of strain L15 degrading ZEN anions

The conversion process of the 335 compound with a molecular weight of 44, which is about 318.1467 different from ZEN's molecular weight, is predicted to be: after the C10' ketone carbonyl position of the ZEN lactone ring is opened, a $C_{18}H_{24}O_6$ compound with a carboxyl group is generated.

4. Conclusion

In this experiment, a strain of Lysobacterium that degrades ZEN was isolated and purified, and the type of degradation and possible products were studied. It was proved that the degradation of ZEN by Lysobacterium was mainly by enzymatic hydrolysis, which produced a product with the molecular formula $C_{18}H_{24}O_6$.

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