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Isolation and Characterization of a Novel Ochratoxin A-degrading *Acinetobacter sp.strain* 2-9B

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Abstract

Ochratoxin A (OTA) is a polyketide mycotoxin that commonly contaminates agricultural products and causes significant economic losses. In this study, we investigated the OTA degradation ability of *Acinetobacter* sp. strain 2-9B which was isolated from the soil. The effects of temperature, incubation time, and pH were studied. Furthermore, it was examined whether the washed cell pellet and the culture supernatant of the fermented culture (SFC) of the bacterial cell components could detoxify OTA. The results showed that strain 2-9B, which achieved 100% degradation of 2.5 μg/mL OTA, was identified as *Acinetobacter* sp. strain 2-9B. It exhibited optimal degradation activity at 2.5 μg/mL under pH 6.0 and 51℃ incubation for 48 hours. The results also suggest that enzymes present in the supernatant of *Acinetobacter* sp. strain 2-9B were involved in OTA degradation. The biodegradation of OTA is a very feasible method for food and feed purification, as physical and chemical methods may not only eliminate mycotoxins but also nutrients and sensory-related compounds.

Keywords

OTA; Remove; *Acinetobacter sp. strain* 2-9B; Degradation Product.

1. Introduction

Mycotoxins contamination is a global problem that cannot be solved easily in the field, even with the introduction of highly efficient proper agricultural practices. Environmental conditions affected by climate change and improper storage conditions may facilitate mold growth and secondary metabolite production [1]. These fungi can infect many plants and a wide array of food and agricultural products, and they can also produce a broad spectrum of harmful secondary metabolites, such as carcinogenic Ochratoxin[2].

Ochratoxin is another mycotoxin that has attracted worldwide attention after aflatoxin. Ochratoxin A is a group of important mycotoxins that pollute food and are produced by 7 species of *Aspergillus* and 6 species of *Penicillium*. Among them, ochratoxin A is the most toxic, widely distributed, produces the highest amount of toxin, pollutes agricultural products the most, and has the closest relationship with human health[3]. It is a potent nephrotoxin that also displays teratogenic and immunotoxic properties, as well as being classified as a group 2B human carcinogen by the International Agency for research on Cancer (IARC) [4].

OTA occurs predominantly in various food raw materials, such as grapes and grape juice, coffee beans, and coffee drinks, is common worldwide, potentially causing further downstream contamination in processed foods and feed stuffs [5–6], leading to recalls and great economic losses.Finding economical, green, and efficient ways to control or eliminate OTA contamination

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in food is extremely important for food manufacturers, consumer health, and national food safety strategies due to the highly carcinogenic properties of this toxin[7] .

Therefore, it is important to find economical, green, and efficient ways to control or remove OTA contamination in food , which is extremely important for food manufacturer, consumer health, and national food safety strategies due to the highly carcinogenic properties of this toxin. Up to now, methods to control OTA with the above characteristics have been extensively studied, which can be classified as physical, chemical and biological methods, have been developed for OTA detoxification. Ultraviolet radiation, heat treatment, and X-ray treatment methods exhibited some potential in practical application, however, the narrow applicability, nutrient loss, and chemical residue problems that come with these methods have prevented their broader application[8]. In contrast, biological degradation has the potential to dissipate high degradation efficiency against OTA.

According to literature, biological degradation emanated from four areas: bacterial degradation, fungal degradation, enzyme-based degradation and selection of disease resistant cultivars. These have proven to prevent humans from the risk of exposure to chemical-based agents used against mycotoxins degradation[9]. OTA degradation has been reported to be successful by using many species of bacteria and fungi, *such as Acinetobacter pittii* AP19 [10], *Bacillus subtilis* CW14 [11], *Bacillus licheniformis* YB9[12] *Pediococcus* parvulus strains [13], In the present work, finding microorganisms with high OTA-eliminating capacity and studying their adsorption mechanism are the key steps to solve OTA contamination in food. Therefore, the purpose of this study is to screen the strains that can effectively degrade OTA and conduct preliminary research on the probiotic properties of the strains, so as to lay a foundation for biodegradation of OTA in feed grains and other products by biological methods.

2. Materials and Methods

2.1. Chemicals and Culture Media:

OTA standard was purchased from Pribolab Biological Technical Company (Qingdao, China). High-performance liquid chromatography (HPLC) grade Acetonitrile and methanol were obtained from Merck Company (Darmstadt, Germany). Phosphate buffer saline(PBS) (pH 6.6; 1.74 g of sodium dihydrogen phosphate, 2.7 g of disodium hydrogen phosphate, and 1.7 g of sodium chloride in 400 mL of sterile distilled water [ddH_2O]) was prepared and used for the subsequent experiments.

Nutrient broth (NB) comprised 10 g/L peptone, 3 g/L beef extract, and 5 g/L NaCl. Lysogeny Broth (LB) was composed of 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract. All media were adjusted to a pH of 7.2 ± 0.1 and sterilized at 121°C for 20 minutes. Solid media were fortified with 20 g/L agar.

2.2. Methods

2.2.1. HPLC Detection

The extraction and detection of OTA in bacterial suspension broth is slightly modified by reference [11]. In brief, 300 uL of all samples were taken, then mixed with 1 mL of methanol for 1 min, centrifuged at 4℃ and 10000 rpm for 8 minutes, and finally filtered with 0.22 μm filter and loaded into the sample bottle. The concentration of OTA was detected by the HPLC.

HPLC (E2695) Waters (USA) using a C18 reversed-phase (250 × 4.6 mm and 5 um). A five-point calibration curve was prepared with standards of OTA (0.4, 0.6, 0.8, 1, 1.2 and 1.4 ug/mL). The mobile phase was acetonitrile, water, and acetic acid (99,99, 2: v: v: v). The flow rate was 1 mL/min. Loading volume was 10 μL. The fluorescence detector has an excitation wavelength of 330 nm and an emission wavelength of 460 nm. Lab-Solutions software was used to analyze the detection results [14].

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2.2.2. Isolation of OTA Degrading Strains

Soil samples(10g) were collected from the wet land of Foshan University. The soil samples were shifted to 200 mL of NB medium and incubated for 24 h at 37℃, 180 rpm. After 24 h, 200 μL of the culture was transferred to NA solid medium for one day. Single colonies on picked plates were re-streaked on NA solid medium. The plates were then incubated at 37℃ for 24 h. Select a single colony for three times of purification. After purification, the strain is stored at 4℃ for standby.

The primary screened strains were activated and inoculated with 5% inoculum in the medium containing OTA (2 μg/mL) in LB liquid medium, and the LB liquid medium containing the same content of OTA without inoculation was used as the control. It was placed on a shaking table at 37 ℃ and 180 r / min for 48 h, and the residue of OTA was detected by HPLC. After the detection, the strain with the highest degradation rate was selected as the target strain to identify and explore its biological characteristics.

The OTA degradation rate is calculated as follows:

OTA degradation rate (%) = [(OTA content in control group - OTA content in treated group) / OTA content in control group] ×100 %

2.2.3. Morphological and Physiological Characterization of OTA Reduced Bacterial Strains

Colonies and cells observation, 2-9B strain was spread on an NA plate and incubated in a 37 ◦C incubator for 1 day. The diameter of the colony was measured, and the cell morphology was observed by Gram-stain. The physiological and biochemical identification of strains was carried out according to the Common Bacterial System Identification Manual [10].

2.2.4. Identification of Strain 16S rDNA of OTA Descending Strain

The primers 27F (5′ -GAGTTTGATCCTGGCTCAG-3′) / 1492R (5′ - GTTACCTTGTTACGACT-3′) and 42F (5′ -CAGTCAGGAAATGCGTACGTCCTT-3′) / 1066R (5′ -CAAGGTAATGCTCCAGGCATTG CT-3′) were used to amplify the 16S rRNA and gyrA genes, respectively. The amplified DNA was subjected to direct sequencing by a sequencing service provider, and the resulted nucleotide sequence was compared by BLAST homology search (https://www. ncbi.nlm. nih. gov/blast). Phylogenetic tree was constructed based on the neighbor-joining algorithm of the 16S rRNA and gyrA gene sequences using MEGA software package version 11.0.

2.2.5. Effect of Time, Temperature and pH on OTA Degrading *Acinetobacter sp. strain* 2-9B

The activated *Acinetobacter* sp. strain 2-9B (100 μL, 9 × 10^8 CFU/mL) was inoculated into 1 mL of fresh LB liquid medium containing 2.5 μg/mL OTA. The groups were incubated at 30 ◦C, 37 ◦C, 44 ◦C, 51 ◦C, 58 ◦C and 65 ◦C, for 24 h. OTA was detected at 0,12,24,36and 48 h post-incubation under the abovementioned conditions. Subsequently, 100 μL of the activated*Acinetobacter sp*. strain 2-9B was inoculated into 1 mL of freshLB liquid medium (pH 3, 4, 5, 6, and 7, which was adjusted with H_2SO_4 or NaOH aqueous solution) containing 2.5 μg/mL OTA. A medium containing only OTA (50 ng/mL) was used as the control. The samples were incubated at 37 ◦C for 24 h, and the OTA content was detected. OTA extraction and detection of the above samples were performed as described in section 2.2.1 Each group had three replicates, and the experiment was conducted twice.

2.2.6. Reduction of OTA by Different Components of *Acinetobacter sp. strain* 2-9B

OTA degradation by cell pellets and cell free supernatant were determined according to the method of Sun et al[15]. and Sangare et al[16]. The isolated bacterium was incubated in LB at 37 ◦C on a rotatory shaker at 180 rpm for 24 h. Cell pellets and supernatant were collected by centrifugation at 9000 g for 10 min and tested for OTA degradation, respectively. After wash twice with sterile phosphate buffer (50 mM, pH 7.0), the harvested cell pellets were

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resuspended in phosphate buffer. OTA degradation by the fractions of cell pellets and cell free supernatant were analyzed by HPLC method. To evaluate the effect of dead cell pellets on OTA degradation activity, the cell pellets and cell free supernatant were heated in boiling water for 30 min before OTA degradation analysis. All tests were performed in triplicate. Furthermore, to evaluate the effect of cell extracellular protein on OTA degradation activity ,the cells were washed twice with 20 mM Tris-HCl (pH 8.0) buffer, collected by centrifugation, and re-dissolved in 10 mL 20 mM Tris-HCl (pH 8.0). Took the cell suspension and sonicated it in an ice bath until the solution was clear and translucent (working conditions: 200 W, work 3 s, pause 4 s, 25 min of total time). Then the broken cell liquid was centrifuged at 12,000 g for 10 min (4℃), and the supernatant was filtered with a sterile filter membrane with a pore size of 0.22 μm to obtain an intracellular solution.

3. Result

3.1. Morphological and Physiological Characterization of OTA Reduced Bacterial Strainss

A white bacterial strain was isolated through multiple streaking on LB agar medium. After picking a single colony and streaking on a plate, incubation at 37℃ for 24 hours resulted in a circular colony with neat edges and a smooth, shiny surface, as shown in Figure 1 (left). Following Gram staining and microscopic observation at 400 times magnification, as depicted in Figure 1 (right), the bacterial cells appeared elliptical, stained purple, with a few unstained circular bright spots, indicative of a rod-shaped morphology resembling *bacilli*.

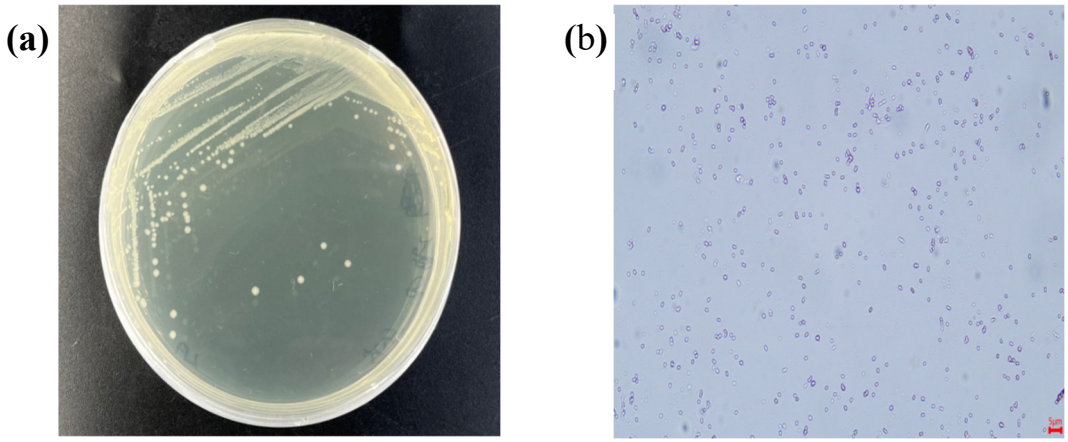

Fig 1. Strain morphology culture (a) Gram stain (b)

3.2. Identification of Strain 16S rDNA of OTA Descending Strain

A 16S rDNA sequence alignment using BLAST on NCBI showed that the strain 2 -9B sequence shares 99% sequence identity with *Acinetobacter sp*. Combined with the phylogenetic tree analysis shown in Figure 2, strains 2-9B were identified as *Acinetobacter sp*. strain 2-9B.

3.3. Effect of Temperature on the Activity of OTA Bacterial Strains

According to figure 3 (a), the degradation rate of OTA by *Acinetobacter sp*. strain 2-9B increases with the increase of temperature within the range of 30℃ ~ 51℃ within 24 h ($p<0.05$). When the temperature increases from 30℃ to 51℃, the degradation rate increases from 31.92% to 100%. However, under the condition of 51℃ ~ 65℃, the degradation rate decreased with the increase of temperature ($p<0.05$), and the degradation rate of OTA at 65 ℃ was only 29.03%. This shows that *Acinetobacter sp.* strain 2-9B has good heat resistance and the optimal incubation temperature of the strain is 51℃.

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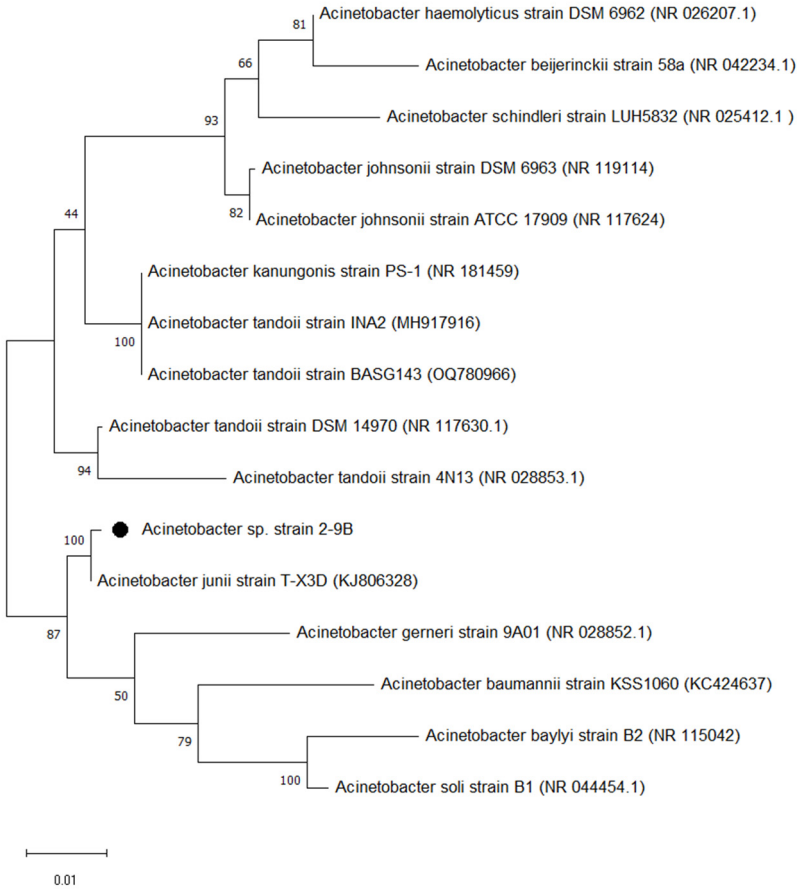

Fig 2. Phylogenetic tree of 16S rDNA of *Acinetobacter sp*. strain 2-9B

3.4. The pH Effects on the Degradation of OTA Activity

According to figure 3 (b), within 24 hours, the degradation rate of OTA by *Acinetobacter sp*. strain 2-9B has little change ($p>0.05$) with the increase of pH value in the range of pH 3.0 - 5.0, and the highest degradation rate can reach 49.22% at pH 6.0. However, the degradation rates at pH values of 3.0 and 7.0 were only 28.89% and 34.97%. This shows that the degradation effect will be affected in the over acid or over alkali environment. The degradation effect is the best in the environment with pH value of 6.0, and the degradation rate reaches the highest. Therefore, the initial pH value of 6.0 was selected as the optimal initial pH value for the degradation of OTA.

3.5. Effect of Time on the Degradation of OTA Activity

According to figure 3 (c), within 24h, the degradation rate of OTA by *Acinetobacter sp*. strain 2-9B will gradually increase with the extension of culture time ($p<0.05$). The degradation rate increased from 42.64% to 53.38% when the incubation time was from the first 6h to 12h. When the incubation time reached 48h, the degradation rate reached 97.23%. After that, there was no significant difference in the degradation rate of OTA between the strains as time went on ($p>0.05$), which indicated that the degradation rate at 48h was the highest and tended to be stable. This may be because the best growth time of Acinetobacter baumannii is 48h, when the vitality of the strain is the strongest and the degradation rate of OTA reaches the maximum. Even if the culture time is extended, the degradation rate of Ota will not change significantly..

3.6. Effect of Cell Components on the Degradation Activity of OTA

As shown in Figure 3 (d), different cell components of *Acinetobacter sp.* strain 2-9B had significant differences in the degradation effect of OTA within 48 hours ($p<0.05$). The highest

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degradation rate of OTA was 99.42% in the cell free supernatant and 83.05% in 2-9B cells. However, the lowest degradation rate of OTA in cell extracellular protein solution and dead cells was only 23.08%. It can be seen that the remove effect of *Acinetobacter sp.* strain 2-9B on OTA is not a single way, but a combination of multiple pathways. The main remove effect is the degradation of OTA by the cell free supernatant, and the bacterial cells also have a certain adsorption effect on OTA.

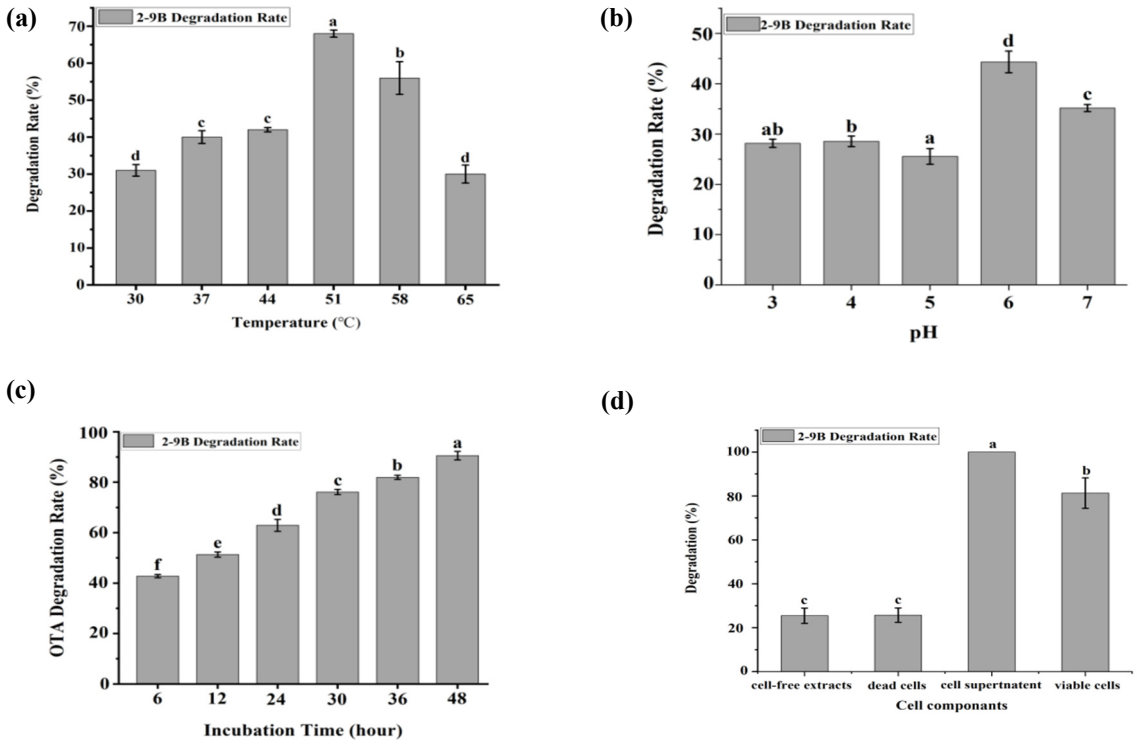

Fig 3. (A): Effect of temperature on OTA on degradation activity (B): Effect of pH on OTA on degradation activity (C): Effect of culture time on OTA on degradation activity (D): Effect of cell components on OTA on degradation activity

4. Discussion

The food security concern in developing countries is more severe than that in developed countries, and the latest technology and strategies must keep pace with the growing demand for food security to manage the contamination of hazardous materials. Biological detoxification technology has swiftly emerged as a research hotspot due to its strong specificity, environmental friendliness, high efficiency, and enhanced preservation of food or feed nutrition. At present, many fungi and bacteria that degrade OTA have been reported, and the degradation efficiency of bacteria is much higher than that of fungi Abrunhosa et al. screened many fungi that can degrade OTA from grapes, but the best strain of *Aspergillus niger* needs 6 days to completely degrade 1 μg/ml OTA[18]. Wei et al isolated a strain of bacteria *Lysobacter sp*.cw239 that can degrade low concentration of OTA from soil, and within 24 h, 30 μ g/L of OTA was completely degraded[19]. Luo et al. isolated *Stenotrophomonas acidaminiphila* cw117 strain can completely degrade 50 mg/L OTA within 60 h[20].A variety of bacterial strains belong to the species of *Acinetobacte*r [21], *Alcaligene*s[22], Bacillus [23]and *Streptomyce*s[24] have been reported with OTA removal ability. OTA removal efficiency achieved by the strain of *Acinetobacter sp. strain* 2-9B is higher than the results obtained in the study by El Khoury et al. , where a maximum 52.6% of OTA with an initial concentration of 0.95 μg/mL could be removed by the tested Streptomyces strains after 5 d incubation in liquid medium. Adsorption and

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degradation are known to be the two main ways involved in microbiological detoxification [25-26]. To further elucidate the basis for the OTA-reducing ability of the isolated *B. amyloliquefaciens* YL-1, cell-free culture supernatant and cell pellets before and after thermal inactivation were evaluated for their OTA removal ability.

In this experiment, an *Acinetobacter* strain was isolated from the soil of field, which can effectively remove OTA. Its detoxification ability reached 99%, and it grew well under the conditions of pH 6, 51℃ and 180 r/min shaking rate. T *Acinetobacter* species are abundant in the natural environment, including soil, freshwater, oceans, sediments, polar regions, and hydrocarbon-contaminated sites[27]. The *Acinetobacter sp. strain* 2-9B identified in this study is not a generally recognized as safe (GRAS) organism and cannot be directly applied to the degradation of OTA in food or feed. However, because the strain originates from soil, we envision it being applied as a mitigation and remediation agent for OTA-contaminated soil, wastewater, manure, sewage sludge and similar environmental matrices. Alternatively, the gene encoding the OTA degrading enzyme can be cloned from *Acinetobacter sp. strain* 2-9B and expressed in a GRAS organism to construct a food-grade genetically engineered strain capable of degrading OTA. In addition, the enzymes responsible for OTA degradation can be heterologously expressed and purified for applications in the food and feed industries.

5. Conclusion

In this study, a strain of *Acinetobacter sp*. strain 2-9B with OTA removal ability was isolated and identified. *Acinetobacter sp*. strain 2-9B showed 100% degradation of 2.5 μg/mL OTA. The temperature, time and acid tolerance in low pH and high temperature conditions. Exploring the detoxification effect of OTA in different cell components, we found that OTA was the best effect degraded by extracellular enzymes. In conclusion, this strain has great potential for future development as microecological agents in the feed industry.

Acknowledgments

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