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Research article

Instant catapult steam explosion combined with ammonia water: A complex technology for detoxification of aflatoxin-contaminated peanut cake with the aim of producing a toxicity-free and nutrients retention of animal feed

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ABSTRACT

Aflatoxin is one of the most toxic biotoxins found in contaminated agricultural products. It has strong mutagenicity, carcinogenesis and teratogenicity to humans and animals. In this study, instant catapult steam explosion combined with ammonia water was examined for its potential to degrade aflatoxin B_1 in peanut cake in order to improve its utilization as a toxic-free animal feed. Incubation of AFB_1-containing peanut cake followed by processing with Instant Catapult Steam Explosion (ICSE) led to approximately 79.03 % degradation of AFB_1, while the degradation of AFB_1 was up to 91.48 % under the treatment of ICSE combined with 4 % $NH_3 \cdot H_2O$ at 1.2 MPa in 200 s of process time. After treatment, nutrients in peanut cake were not significantly changed. The toxicity of AFB_1 degradation products was evaluated and the results showed that the toxicity of these products were found to be substantially less than that possessed by AFB_1. A low chemical pollution, efficient and toxic-free technology system of AFB_1 degradation was established, which detoxify aflatoxin-contaminated biomass for sustainable and safe utilization of agricultural biomass as animal feed.

1. Introduction

Approximately 25 % of agriculture products are contaminated with mycotoxins every year, of which 2 % food are inedible due to mildew [1,2] Crop pollution has brought great problems to the development of food industry and feed industry, which caused huge economic losses [3,4].

Many kinds of mycotoxins existed in nature, among which aflatoxin (AF) is the most toxic found in agricultural products, which has strong mutagenicity, carcinogenicity and teratogenicity to humans and animals [5]. The agricultural products such as corn, wheat, sorghum, peanut cake, soybean meal, cottonseed meal and corn stalk may be contaminated by AF because of improper storage or other

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reasons [6]. Among them, corn and peanut meal are the raw materials polluted seriously by AF. After these raw materials are contaminated, the concentration of AF can reach 80–100 µg/kg, which is far higher than the maximum limit of 20 µg/kg in China [7]. Contamination of animal production diets with AF can result in reduced animal weight, increased feed conversion rate, and may even lead to the development of tumors and cancer, ultimately resulting in mortality [8]. After AF is ingested by human, it is metabolized by cytochrome-P450 enzyme to form an active intermediate, which will interact with hepatocytes to cause liver cancer [9]. Therefore, it is necessary to prevent the raw materials from mildew in the production of food and feed. However, when the environmental conditions are conducive to the growth of toxic fungi, the removal of AF has become a major problem to be solved.

AF are secondary metabolites produced by filamentous fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*. They encompass a group of chemicals with similar structures, including AFB_1, AFB_2, AFG_1, and AFG_2. Among these, AFB_1 is considered the most toxic and is therefore used as the primary evaluation index for aflatoxin contamination [10]. In general, the degradation methods mainly include physical (heating, UV irradiation, gamma irradiation), chemical (chlorine dioxide, ammonization, formaldehyde, sodium bisulphate) and biological methods (microorganism, enzyme and insect) [11–16]. The physical adsorption method is simple and low-cost, which is a relatively mature detoxification method at present. However, it has been reported that this method will affect the absorption and utilization of nutrients in feed [17–19]. The chemical detoxification method will not only affect the nutritional quality and palatability of feed, but also bring pollution to the environment, which is not suitable for wide application in actual production [20,21]. In recent years, a variety of microorganisms have been found to degrade or adsorb aflatoxin B_1 [22–25]. However, most of the reported virus-free strains have the following two common problems in practical application: (1) the detoxification mechanism of some strains belongs to physical adsorption, which does not realize biodegradation; (2) the detoxification time is long, and the degradation efficiency is rarely more than 90 %, so the practical significance and application value are not ideal.

The ICSE technique is a rapid and effective method that has ability to reduce toxic compounds and improve the digestibility of cellulose in lignocellulosic biomass [22]. T Application of ICSE resulted in approximately 100 % degradation of AFB_1 in corn stalk, demonstrating optimal degradation at 2.4 MPa (220 °C) for a processing time of 140 s. However, some potentially harmful and toxic compounds were still detected in the treated corn stalk following ICSE treatment. Therefore, it is crucial to establish an efficient and low toxicity or toxicity-free degradation method. Previous studies have shown that weak alkali treatment effectively removes AFB_1 from peanut raw materials under high temperature [26]. When peanut meal was treated at 121 °C and pH 10 for 60 min, the AFB_1 degradation rate reached as high as 84.50 %. Similarly, treating peanut oil with an alkali concentration of 23.42 % reduced AFB_1 concentration from 34.78 µg/kg to 0.37 µg/kg (98.94 % reduction) [26]. Ammonia and its derivatives have been extensively studied by scientists for their acceptable levels of mycotoxin reduction [27]. In a related study on exposing contaminated yellow corn to ammonia treatment under various conditions for 60 min, it was concluded that aqueous NH_4OH or NH_3 can effectively reduce AFB_1 in corn at high temperatures [28]. Additionally, they reported an undesirable brown coloration in the treated feed along with increased total nitrogen and non-protein nitrogen.

In this study, ICSE combined with ammonia water was performed and examined for its potential to treat and degrade AFB_1-containing peanut cake with the aim of producing a toxicity-free and nutrients retention of animal feed. Furthermore, the toxicity of degradation products of AFB_1 was tested through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with HepG2 cells, which is a commonly used method for toxicity test. Therefore, the use of ICSE combined with $NH_3 \cdot H_2O$ to treat aflatoxin-contaminated peanut cake could provide a new and rapid solution for the sustainable and safe utilization of agricultural biomass for animal production.

2. Materials and methods

2.1. Materials

The peanut cake used in this study was obtained from a local peanut field in Hebi, Henan. It had a moisture content of 90 %, which was adjusted with the addition of a distilled water solution. The nutritional analysis revealed that the protein content of the peanut cake was 45.42 %, while the fat content was 1.04 %. The peanut cake was then incubated at 30 °C and naturally developed mildew under these conditions. After 25 days of incubation, the total AFB_1 content in the peanut cake was measured to be 78.9 µg/kg. Subsequently, the peanut cake containing AFB_1 was dried to a constant weight at 75 °C and then stored at −20 °C before undergoing further analysis.

2.2. ICSE treatment

ICSE (QBS-80B) [29] treatment procedure as follows: 100 g of peanut cake sample containing 78.9 µg/kg AFB_1 was loaded into the chamber. The steam pressures were set at 1.2 MPa, 1.4 MPa, 1.6 MPa, 1.8 MPa, and 2.0 MPa respectively, according to the experimental requirements. Once the pressure reached the designated level, incubation of the peanut cake samples was conducted for different durations as required (50 s, 100 s, 150 s, 200 s, 250 s and 300 s). Then, the samples were collected when the temperature was dropped to around 50 °C. The collected samples were dried at a constant weight of 75 °C and crushed to pass through a sieve with an aperture size of 0 0.425 mm.

2.3. ICSE treatment and heat treatment combined with different ammonia concentration

100 g of peanut cake containing 78.9 µg/kg AFB_1 (5 samples) were mixed with 20 mL of different $NH_3 \cdot H_2O$ solutions (v/v, 1 %, 2 %,

3 %, 4 % and 5 %). ICSE treatment procedure as follows: 100 g of peanut cake sample containing 78.9 μg/kg AFB$_1$ with different NH$_3$·H$_2$O concentration was loaded to the chamber, the steam pressures and time were set up at 1.2 MPa and 200 s. Then, the collected samples were dried at 75 °C to constant weight and were crushed to pass through a sieve with an aperture of 0.425 mm. The heating treatment with varying concentrations of NH$_3$·H$_2$O was carried out as a control using the ICSE device. Unlike the ICSE method, no explosion occurred following the incubation of the peanut cake at the specified pressure and temperature conditions (1.2 MPa for 200 s). Instead, the ICSE device was switched off to naturally cool down the device till the pressure dropped to 0. Then, the treated peanut cake was taken out from charging tube, dried to a constant weight at 75 °C and stored −20 °C before further analysis.

2.4. AFB$_1$ extraction and determination by LC-MS from the treated peanut cake

AFB$_1$ in the treated peanut cake was extracted and determined as the protocol described by Xie [22]. In brief, a subsample of the dried peanut cake sample after ICSE treatment was added to a 500 mL shake flask containing 200 mL of methanol (v/v, 70.0 %). The sample was subjected to ultrasound at 35 °C for 30 min and then underwent three rounds of repeated ultrasound. The resulting mixed filtrate solution was dried below 35 °C under vacuum conditions for 24 h to evaporate the solvent. After being dissolved in a solution of acetonitrile-ammonium acetate (5 mL), the residue was prepared using a mixture of ammonium acetate solution (10 mmol/L, pH 6.0) and acetonitrile (1:1 v/v). Subsequently, the solution was transferred into a measuring flask with a capacity of 10 mL. The flask containing the residue was washed three times using acetonitrile-ammonium acetate solution following dissolution. The wash solutions were combined with the primary dissolution solution in the measuring flask and later subjected to filtration and analysis. AFB1 content was determined using an Agilent HPLC-MS system (Agilent 1290e6460 series, CA, USA) equipped with an Agela C18 column (2.1 mm × 100 mm, 3 mm).

2.5. Determination of crude protein, fat and ash content

The content of crude protein in 0.300 g treated and untreated peanut cake was determined according to the national standard GB/T 6432-1994 of the People's Republic of China [30]. The content of crude fat in 0.5000 g treated and untreated peanut cake was determined accordance to the national standard GB/T 6433-1994 of the People's Republic of China [31]. The content of ash in 1.000 g treated and untreated peanut cake was determined according to the national standard GB/T 6438-1992 of the People's Republic of China [32].

2.6. Determination of amino acid content

0.300 g treated and untreated peanut cake was weighed to the hydrolysis tub with adding 10 mL of 6 mol/L hydrochloric acid, then the hydrolysis tube was sealed by the alcohol burner. The mixture was hydrolyzed at 110 °C for 24 h and was transferred into a beaker after cooling. The mixture was washed three times by the ultrapure water and was adjusted to pH 9 by 6 mol/mL NaOH solution. Afterwards, the solution was transferred into a 50 mL measuring flask and the beaker was washed three times using the ultrapure water again. Finally, the wash solutions were mixed with the primary solution in the 50 mL measuring flask.

0.4 mL of the hydrolysate was transferred into the centrifuge tube with adding 0.2 mL of the derivative A and derivative B which were diluted by 5 times. Then the centrifuge tube was heated at 50 °C for 45 min after shaken. When the temperature was reduced to the room temperature, 0.4 mL of hexane was added to the centrifuge tube. After placed for 30 min, the lower layer was filtrated by organic membrane with a pore size of 0.45 μm. The filtrate was analyzed by a high-performance liquid chromatography HPLC system (Agilent 1290–6460 series, CA, USA) with an Ultimate Amino acid (4.6 mm × 250 mm, 5 μm). The injection volume was 5 μL, the column temperature was 37 °C, and the detection wavelength was 254 nm. The mobile phase (A) was 0.1 mol/L sodium acetate solution (pH 6.5) and acetonitrile with the ratio of 93 to 7 and the mobile phase (B) was H$_2$O and acetonitrile with the ratio of 93 to 7, respectively, at 0.3 mL/min. Gradient elution program was as follows (Table 1).

Table 1
Gradient elution program.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.01	100	0
11	93	7
13.9	88	12
14	85	15
29	66	34
32	30	70
35	0	100
42	0	100
45	100	0
60	100	0

2.7. Cell culture

The HepG$_2$ cells were cultivated in the RPMI-1640 complete medium at 37 °C, with 5 % CO$_2$ in the air. When fusion degree reached 90 %, the medium was abandoned, and the culture plate was washed twice by phosphate buffer (PBS) (Shanghai GenechemCo., Ltd., China). After cells were digested by trypsin for 3 min, digestion was stopped by adding the complete medium with 10 % fetal bovine serum (FBS) (Shanghai GenechemCo., Ltd., China). Then the cells were inoculated in a new culture plate and cultivated at 37 °C with 5 % CO$_2$ in the air.

2.8. MTT assay

Prior to MTT assay, ICSE treated peanut cake sample and ICSE combined with NH$_3$·H$_2$O treated peanut cake sample (25.0 g, containing 5.0 µg AFB$_1$ equivalent) were extracted using the method described previously, respectively. The extraction solution was concentrated to 1.0 mL by evaporation through purging nitrogen gas to achieve a solution with 5.0 µg/mL AFB$_1$ equivalent. This concentrated extraction solution was diluted to several concentrations (AFB$_1$ equivalent, 1.25 µg/mL、2.5 µg/mL、5.0 µg/mL) for MTT assay. For control trial, AFB$_1$-free peanut cake (25.0 g) with ICSE treatment was extracted and concentrated to 0.9 mL. 0.1 mL of AFB$_1$ solution (50 µg/mL, diluted from 500 µg/mL stock solution with water) was mixed with the 0.9 mL solution and was further diluted to several AFB$_1$ concentrations (1.25 µg/mL、2.5 µg/mL、5.0 µg/mL) for MTT assay. In addition, AFB$_1$ solutions (0.39–100 µg/mL) diluted from AFB$_1$ stock solution (500 µg/mL) in water were directly used for MTT assay. For MTT assay, HepG$_2$ solutions were spread into a 96-well plate (100 µL per well with a total of 10,000 cells in each well), followed by the addition of 10 µL of extraction solutions with different AFB$_1$ or AFB$_1$ equivalent concentrations. The plate was incubated at 37 °C for 48 h. The incubation at each concentration was conducted in five replicates.

Following the cultivation, 20 µL of 5 mg/mL MTT in PBS solution was added to each well of the plate. The plate was incubated at 37 °C for 4 h. After incubation, the medium was discarded, and the formed crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance of each DMSO solution in the microplate was measured at 490 nm by a micro plate reader (Infinite, Austria). The fatality rate was calculated.

Fatality rate (%) = 1-(OD of exprimental wall)/(OD of control well) × 100%

3. Results and discussion

3.1. Effect of ICSE treatment on AFB$_1$ degradation in peanut cake

Fig. 1 showed AFB$_1$ degradation results at different treatment conditions in peanut cake. Under the same explosion pressure, the degradation of AFB$_1$ gradually increased with the increase of the treatment time. Under the pressure of 1.2 MPa for 200 s, the degradation of AFB$_1$ reached 79.03 % after ICSE. Further increase in treatment time to 300 s led to 87.72 % degradation following ICSE. However, peanut protein was vulnerable to thermal denaturation, which resulted in its surface gelatinized with the increasing of
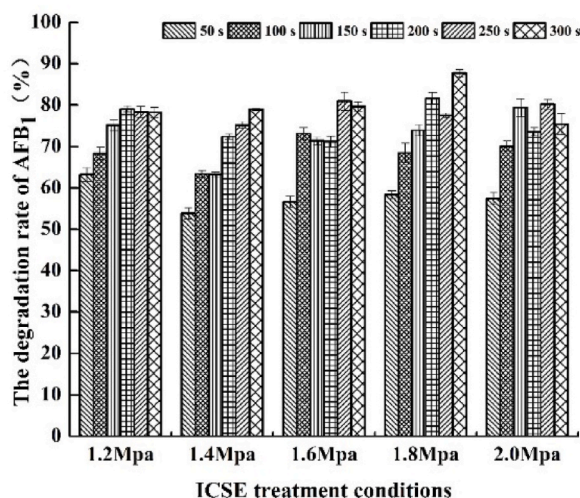

Fig. 1. The degradation of AFB$_1$ in peanut cake under different ICSE treatments. The steam pressures were set up at 1.2 MPa, 1.4 MPa, 1.6 MPa, 1.8 MPa and 2.0 MPa, respectively and the time was counted for 50 s, 100 s, 150 s, 200 s, 250 s and 300 s. Under the pressure of 1.2 MPa for 200 s, the degradation of AFB$_1$ reached 79.0 % after ICSE. Further increase in treatment time to 300 s led to 87.7 % degradation following ICSE.

the explosion pressure and the prolonging of the treatment time.

In recent years, many studies have reported degradation of AFB_1 in peanut and peanut oil, but the removal of AFB_1 from peanut cake has not been reported in detail. The degradation products and the degradation pathways of AFB_1 in corn stalk under the conditions of 1.2 MPa for 200 s were determined. By comparing the curves of a, b and c of UPLC chromatogram (Fig.S1), it can be concluded that there are three degradation products, namely degradation products D, E and F. A new degradation product F ($C_{16}H_{16}O_9$, m/z 352.2) appeared compared with 2.4 MPa for140 s [22]. The UPLC-Q-TOF-MS/MS spectra and proposed fragmentation of the degradation products of AFB_1 were shown in Fig.S2. Its structural formula is similar to AFB_1 (Fig. 2), and the changes of the product F mainly occurred on the double bonds of the furan rings and the structures of lactone. Based on the structural profiles of the degradation products of AFB_1, two AFB_1 degradation pathways were proposed (Fig. 3). The first degradation pathway is the same as that of the condition of 2.4 MPa for 140 s [22]. The second degradation pathway is a new degradation pathway, in which ether bond of the intermediate product $C_{17}H_{18}O_9$ was hydrolyzed to produce degradation product F.

The C_8=C_9 double bond of the furofuran moiety, the lactone ring, and the cyclopantenone in AFB_1 structure were crucial to its toxicity and carcinogenicity. It has been demonstrated that the product D (68 %, proportion of the product D in total degradation products) and E (4.5 %, proportion of the product E in total degradation products) are non-toxic [27,33]. Although the C8=C9 double bond of the product F (6.3 %, proportion of the product F in total degradation products) were not destroyed, the lactone ring and the cyclopantenone were both fractured. Consequently, under conditions of 1.2 MPa for 200 s, the toxicity of these degradation products was found to be significantly lower than that exhibited by AFB_1. Furthermore, 1.2 MPa for 200 s was the best explosion condition, and the degradation of AFB_1 in peanut cake reached 79.03 % under this condition (78.9 μg/mL to 15.5 μg/mL).

3.2. Effect of ICSE combined with $NH_3·H_2O$ and heat treatment with $NH_3·H_2O$ on AFB_1 degradation in peanut cake

The degradation rate of AFB_1 (79.03 %) in peanut cake at 1.2 MPa for 200 s was very low compared with the degradation rate of AFB_1 (100.0 %) in corn stalk at 2.4Mpa for 120 s. To future increase the degradation rate of AFB_1 and hardly reduce the toxicity of the degradation products, ICSE combined with $NH_3·H_2O$ to degraded AFB_1 in peanut cake was investigated under the pressure of 1.2 MPa for 200 s (Fig. 3a). With the increase of the $NH_3·H_2O$ concentration, the degradation of AFB_1 in peanut cake gradually increased, which was consistent with Weng' research results [28]. When the $NH_3·H_2O$ concentration was up to 4 %, 91.48 % of AFB_1 was degraded in peanut cake which was higher than the degradation rate of AFB_1 only by ICSE (79.03 %). However, heating treatment with $NH_3·H_2O$ for 200 s alone without ICSE only led to 84.6 % degradation (Fig. 3b). The high shear force generated during ICSE, resulting from the sudden release of pressure, was significantly amplified due to the considerably shorter explosion time. Consequently, this led to a more efficient degradation of AFB_1.

3.3. Changes of the nutrients in peanut cake after ICSE combined with $NH_3·H_2O$ treatment

ICSE combined with $NH_3·H_2O$ can effectively degrade AFB_1 in peanut cake. By comparing the changes of nutrients in peanut cake before and after treatment, the effectiveness of ICSE combined with $NH_3·H_2O$ on AFB_1 was evaluated. The change of crude protein, true protein, crude fat and crude ash were shown in Fig. 4. The nutrients in peanut cake were not significantly changed. Due to the addition of $NH_3·H_2O$, the content of crude protein increased slightly by 10.67 % in the treatment process, which was consistent with Weng' research results [28], and there was no decrease in true protein content. Therefore, the contaminated peanut cake treated by ICSE
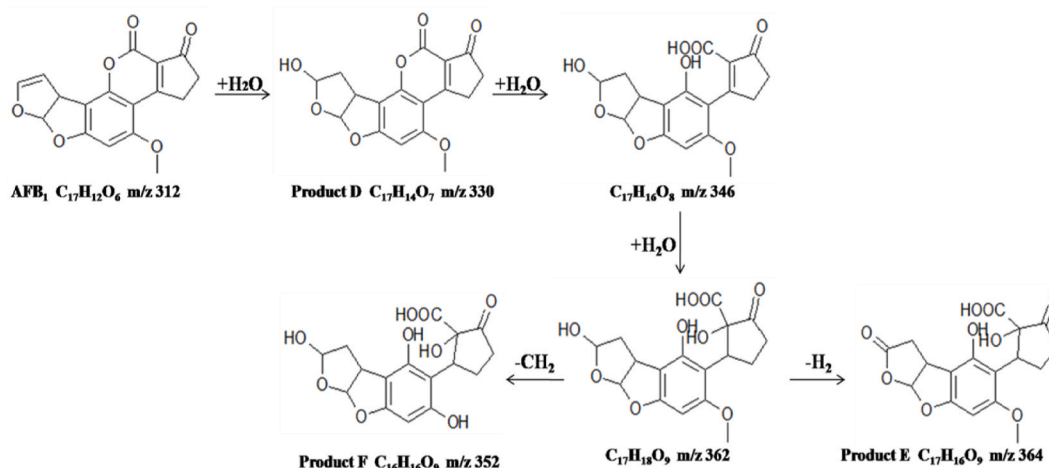

Fig. 2. The degradation pathway of AFB_1 by ICSE treatment at 1.2 MPa for 200 s.
Two AFB_1 degradation pathways were proposed. The first degradation pathway included addition of water molecule, hydration of lactone and dehydrogenation to produce degradation product D and E. The second degradation pathway is a new degradation pathway, in which ether bond of the intermediate product $C_{17}H_{18}O_9$ was hydrolyzed to produce degradation product F.
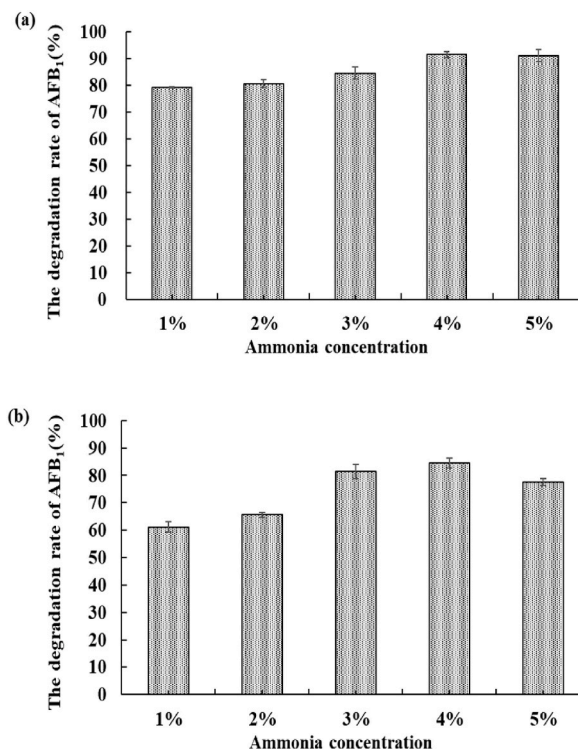
Fig. 3. Effect of ICSE combined with NH$_3$·H$_2$O and heat treatment with NH$_3$·H$_2$O on AFB$_1$ degradation in peanut cake. (a) ICSE combined with NH$_3$·H$_2$O treatment. The steam pressures and time were set up at 1.2 MPa and 200 s, and the concentration of different ammonia was 1 %, 2 %, 3 %, 4 % and 5 % (v/v), respectively; (b) heat treatment with NH$_3$·H$_2$O. Explosion was not applied and the ICSE device was switched off to naturally cool down till the pressure dropped to 0.
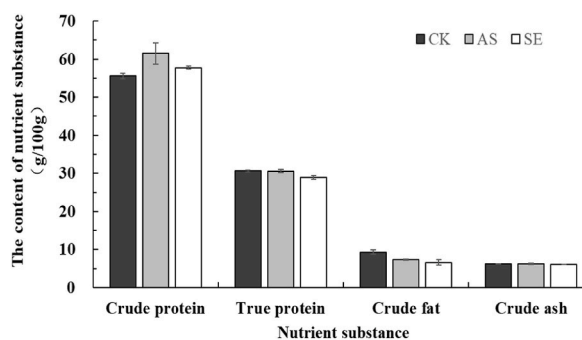

Fig. 4. Change of nutrients in untreated peanut cake and treated peanut cake. CK: untreated peanut cake; AS: treated peanut cake by 4 % ammonia water combined ICSE; SE: treated peanut cake by ICSE at pressure 1.2 MPa for 200 s.

combined with NH$_3$·H$_2$O treatment was no loss in nutritional value and could still be used as feed material.

3.4. Changes of amino acid content in peanut cake after ICSE combined with NH$_3$·H$_2$O treatment

Peanut cake consists of 17 kinds of amino acids, including 8 kinds of essential amino acids. Changes of 17 kinds of amino acids in peanut cake before and after treatment were shown in Fig. 5. The total amount of amino acid in untreated peanut cake was 161.59 g/g, and the total amount of amino acid increased to 162.31 g/g by ICSE treatment, while the content of amino acid decreased slightly to 156.58 g/g by ICSE combined with NH$_3$·H$_2$O. Therefore, the amino acids in peanut cake before and after treatment were not seriously damaged, which indicated that this method of ICSE combined with NH$_3$·H$_2$O not only could effectively degrade AFB$_1$, but also greatly retained the nutrients in peanut cake.
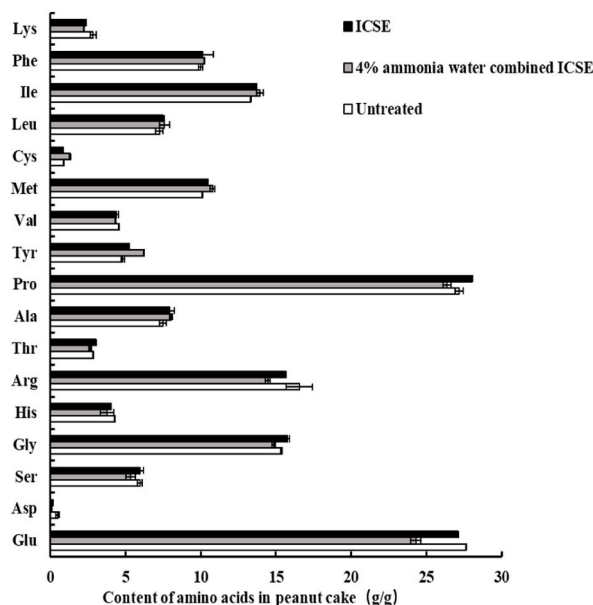
Fig. 5. The contend of amino acids in untreated peanut cake and treated peanut cake. The contend of 17 kinds of amino acids, including 8 kinds of essential amino acids in peanut cake before and after treatment have no significant difference.

3.5. Toxicity analysis of the degradation products

According to the structure of the degradation products D, E and F under the conditions of 1.2 MPa for 200 s, the toxicity of the degradation products was found to be substantially less than that possessed by AFB_1. To further evaluate the cytotoxicity of AF-contaminated peanut cake by ICSE combined with $NH_3 \cdot H_2O$ treatment, the biological toxicity of AFB_1 and the degradation products of AFB_1 in peanut cake were evaluated by MTT assay. First, the correlation of fatality rate with the different concentrations of AFB_1 solutions was investigated. The correlation of fatality rate with the AFB_1 and AFB_1 equivalent concentrations in the treated peanut cake extraction solutions was studied (Fig. 6). The cell death rate of the untreated peanut cake increased with the concentration of the extract. However, the toxicity of peanut cake degradation product by ICSE combined with $NH_3 \cdot H_2O$ and ICSE was also far less than the untreated peanut cake, and the toxicity of the former is slightly higher than that of the latter. This possibly was due to (1) the increase of AFB_1 and degradation products concentrations to a threshold at which the difference of fatality rate was no longer obvious and/or (2) the synergistic effect from both AFB_1 (and AFB_1 degradation products) and $NH_3 \cdot H_2O$. Therefore, it may be that 4 % $NH_3 \cdot H_2O$ has toxic effect on cultured cells. Before MTT experiment, it is necessary to retreat the sample extraction and completely remove $NH_3 \cdot H_2O$ by treatment to reduce cytotoxicity.

4. Conclusion

The application of ICSE resulted in approximately 79.03 % degradation of AFB_1 in peanut cake, demonstrating optimal degradation at 1.2 MPa and processing time of 200 s. While the degradation of AFB_1 was up to 91.48 % under the treatment of ICSE combined with 4 % $NH_3 \cdot H_2O$ at the same condition. After treatment, nutrients in peanut cake were not significantly changed. The degradation product of the peanut cake by ICSE has no toxic effect on cells at all, while the toxicity of the products is significantly less than that possessed by AFB_1. This study demonstrates that a low chemical pollution, efficient and toxic-free technology system of AFB_1 degradation was established.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author upon reasonable request.
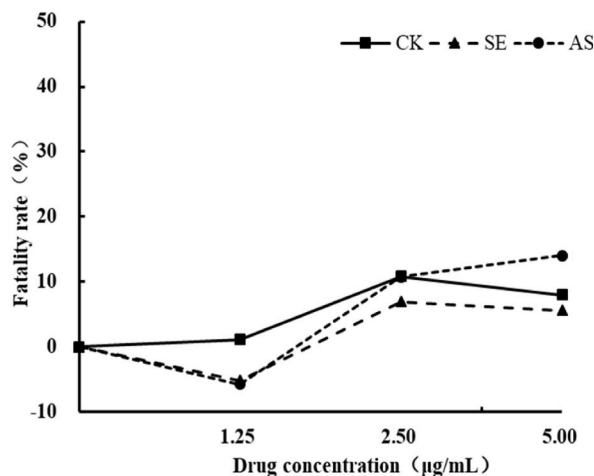
Fig. 6. The MTT test of untreated peanut cake and treated peanut cake. CK: untreated peanut cake; AS: treated peanut cake under 4 % ammonia water combined ICSE; SE: treated peanut cake under ICSE. AFB_1 concentrations and degradation concentrations were set up at 1.25 μg/mL、2.5 μg/mL、5.0 μg/mL for MTT assay.

CRediT authorship contribution statement

Hui Xie: Writing – original draft, Methodology, Data curation, Conceptualization. **Lei Gao:** Methodology, Data curation, Conceptualization. **Zhimin Li:** Data curation, Conceptualization. **Guotao Mao:** Methodology, Conceptualization. **Hongsen Zhang:** Investigation. **Fengqin Wang:** Resources, Conceptualization. **Su Shiung Lam:** Writing – review & editing, Supervision, Funding acquisition. **Andong Song:** Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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