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REGULAR ARTICLE

Isolation and identification of an extracellular enzyme from *Aspergillus niger* with Deoxynivalenol biotransformation capability

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ABSTRACT

In this study, the purification and characterization of an extracellular enzyme form Aspergillus niger was performed. With an optimized protocol, it was conducted a 42.6-fold purification with a yield of 26.2%. The purified lipase had a monomeric molecular weight of 40.5kDa and an isoelectric point of 6.01, and its maximum enzyme activity could be achieved at 40°C and pH 7.5-9.0. The enzyme could be activated by Ca^{2+}, Mg^{2+} and Fe^{2+}, while its activity could be inhibited by Zn^{2+} and Cu^{2+}. Additionally, organic compounds exerted an inhibitory effect on the enzyme activity in a descending order of methanol, ethanol, DMSO, EDTA, acetone. Meanwhile, the specificity analysis of the enzyme indicated a preference to tributyrin and vegetable oils as well as long-chain fatty acid methyl esters (C12-C18). Most importantly, this enzyme could successfully transform deoxynivalenol (DON). Using HPLC analysis, it was detected a biotransformation rate of more than 70%.The liquid chromatography-mass spectrometry (LC-MS) analysis showed that the molecular weight of the transformation product was 18.0 larger than that of DON, indicating that DON could be hydrolyzed by the enzyme. Overall, the proposed method here provides a new avenue for reducing the toxicity of DON, which appears to have a wide application outlook for DON biotransformation.

Keywords: Aspergillus niger; DON biotransformation; Enzyme; Identification; Purification

INTRODUCTION

Deoxynivalenol (DON), known as vomitoxin, is the most prevalent fusarium-mycotoxin among the type B group of trichothecenes worldwide. Asa member of the trichothecenes (secondary metabolites of fusarium), DON has been considered as one of the most common food contaminants (Papadopoulou-Bouraoui et al., 2004). Meanwhile, DON exhibits a strong cytotoxic activity, resulting in an impaired cell-cycle distribution and a significant anti-proliferative effect (Tiemann et al., 2003). Therefore, toxinexposure is a permanent health risk factor for both humans and farm animals. In addition, it is acknowledged that the toxic effects of DON are associated with its 12, 13-epoxy structure and removal of this epoxide group entails a significant loss of toxicity (Awad et al., 2010), and destroying its epoxy structure can largely reduce the DON toxicity. To date, several conventional methods including chemical, physical and microbiological treatments (Bretz et al., 2006; Abolmaali et al., 2008; Avantaggiato et al., 2004) have been attempted to reduce the toxicity of DON, and microbiological and enzymatic treatments may be the best choice for reducing the toxicity of DON (Reetz et al., 2004). Shim et al. (1997) isolated an *Agrobacterium-Rhizobium* strain E3-39 from soil samples, which could transform DON into3-ketoDON under aerobic conditions. Young et al. (2007) reported that *Bacterial isolates* LS100&SS3 was capable of transforming DON into DOM-1 (Deepoxy-4-deoxynivalenol), and the strain could be isolated from the chicken digesta. Using DON as the sole carbon source in inorganic salt medium, He et al. (2008) isolated an *Aspergillus tubingensis* strain (NJA-1) from soil and found that DON could be hydrolyzed by NJA-1. However, the effective product of strain NJA-1 has not been identified (Alassane et al., 2015; Taiana et al., 2015).

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Based on its substrate specificities and enzymatic properties, microbial enzymes are known as potential resources for food applications (Treichel et al., 2010). Importantly, microbial enzymes also play important roles in degraded resins as well as epoxy resins by breaking the ester bonds (Jaszcz, 2002). Tay et al. (2005) showed that polycaprolactone can be degraded by enzyme released from bacteria under the alkaline hydrolysis conditions. Additionally, Palomo et al. (2003) purified enzyme from *Mucor miehei* (MML) to immobilize and separate the epoxy resins from carboxylic acids under the catalytic hydrolysis conditions (Pinton and Isabelle, 2014). In present paper, a strain of *Aspergillus niger* (As-D.1) was isolated from soil samples. The paper mainly reports the identification of the enzyme isolated from the strain and its efficacy on DON-transformation.

MATERIALS AND METHODS

Samples and materials

Soil samples were randomly collected from the region piled with epoxy resin, the region next to the gas station and the dining room next to the sewer, respectively. The *Aspergillus niger* As-D.1 was screened in Lab, other fungiand bacteria were obtained from Anhui Entry-exit Inspection and Quarantine Bureau. Standard DON with the purity ≥97.0% was purchased from Sigma Company (Sigma, St. Louis, MO, USA). DEAE-52 cellulose and Sephadex G-100 were supplied by a company of Beijing (Solarbio Science & Technology Co., Ltd., Beijing, China). All other chemical and biochemical reagents were obtained from Shanghai (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

Screeningand identification of strains

Aspergillus niger As-D.1wasincubated in the medium with glucose (1%), corn steep liquor (1%), KH_2PO_4 (0.1%), K_2HPO_4 (0.1%), $MgSO_4$ (0.05%), KCl (0.05%), agar (3%) and distilled water (pH 4.0). The seed culture medium was inoculated into a fermentation medium with glucose (1%), corn steep liquor (1%), phenyl epoxy ethane (10 mM), KH_2PO_4 (0.1%), $MgSO_4$ (0.05%), KCl (0.05%) and distilled water (pH 4.0). The inoculum (0.5%) (v: v) was added to the fermentation medium in a volume of 50 mL, and the As-D.1 was incubated for 72 h in a rotary shaker with 180 rpm at 32 °C. After 24 h, 5 mL of the samples were centrifuged for the enzymatic activity.

Enzymatic activity assays

Enzymatic activity was measured by spectrophotometric assay according to the method reported by Mateo (Mateo et al., 2003). A standard curve of phenyl epoxy ethane was established to calculate the enzyme activity. Additionally, the enzyme activity of intracellular, extracellular and membrane-bound fractions was tested based on the degradation effect phenyl ethylene oxides. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the reaction of 1 μL phenyl epoxy ethane per minute under conditions of pH 8.0 and 40 °C. All assays were performed in triplicate (three different wells for technical replication)to control the detected efficiency.

Determination of protein concentration

The protein concentrations were detected by the Bradford assay with the bovine serum albumin (BSA) as a standard. Each of standard solution and experiment was performed in triplicate. The mixture with an absorbance of λ595 nm was measured using a UV-visible spectrophotometer.

Crude enzyme extraction

The As-D.1 cultures were cultivated on arotary shaker (180 rpm) for 72 h at 32 °C, the suspension was squeezed through a double-layer muslin cloth and the cells were discarded. Then the filtrate was centrifuged at 12,000 rpm for 20 min at 4°C, the supernatant was collected and used as crude enzyme. The activity of crude enzyme was defined as 100% for the calculation of enzyme.

DEAE-52 chromatography

The crude enzyme (50 mg) was dissolved into phosphate buffer (5 mL, 50 mM, pH 8.0) and then loaded on a pre-equilibrated DEAE-52 column (1.8×100 cm). The enzymes were fractionated with a linear gradient of 0-1 M NaCl in the same buffer at a flow rate of 1.0 mL/min. Eluted fractions were collected into 60 tubes. The enzymatic activity and protein concentration of each tube were estimated, the fractions containing the enzyme were pooled and dialyzed against phosphate buffer (50 mM, pH 8.0). Dialyzed fractions were freeze-dried and stored at -70°C.

Sephadex G-100 chromatography

The freeze-dried enzyme was dissolved into phosphate buffer (5 mL, 50 mM, pH 8.0) and then loaded on a pre-equilibrated Sephadex G-100 column (1.6×80 cm). The enzyme was eluted at a flow rate of 1.0 mL/min and collected into 60 tubes. Similarly, the enzymatic activity and protein concentration of each tube were analyzed and the dialyzed fractions were freeze-dried to store at -70°C.

Gel electrophoresis

Molecular weight of the protein was determined by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)according to the reported method (Laemmli,1970).The gels (12%) were stained with Coomassie Brilliant Blue R-250 and then decolorized with a solution containing 5% methanol and 7% acetic acid. Molecular masses were estimated using the following protein standards, pork myosin (200 kDa),

E. coli β-galactosidase (116 kDa), rabbit phosphorylase b (97.2 kDa), BSA (66.4 kDa), hen egg white ovalbumin (44.3 kDa), cattle phosphoric acid glycosides enzyme (29 kDa), soybean trypsin inhibitor (20 kDa), hen egg white lysozyme (14.3 kDa) and cattle aprotinin (6.5 kDa).

Molecular identification of the enzyme

1D-LC-MS mass spectrometer (Thermo Finnigan, USA) was applied to detect the molecular weight, isoelectric point and amino acid sequence of the enzyme. The mass spectrometry analysis was performed with the following parameters, C18 reversed-phase capillary column; mobile phase, 0.1% formic acid in ddH_2O and 100% acetonitrile, ion type, positive ion mode, mass spectrometer method, Date-Dependent MS/MS.

Characterization of enzyme

To determine the optimal temperature, enzymatic activity was measured in a range of 20-70°C at pH 7.0 in 50 mM phosphate buffer. For the thermost ability, the purified enzyme was incubated at different temperatures, ranging from 20°C to 70°C for 2 h in 50 mM phosphate buffer, pH 7.0.Furthermore, the purified enzyme was incubated at pH 5.0-10.0 in different buffers for 2 h at 40 °C to further validate the pH stability of enzyme, and the activity was measured by spectrophotometric assay (Solarbio Science & Technology Co., Ltd., Beijing, China).

The chemicals were included as follows: metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fn^{2+} and Cu^{2+}) at different concentrations (0.1, 1 and 10 mM), organic compounds (methanol, ethanol, acetone, DMSO and EDTA) with different volume proportions (1%, 5% and 10%) to test the enzymatic activity, and the activity was measured by spectrophotometric assay.

For substrate specificity analyses, 5% (v/v) of soybean oil, palm oil, olive oil, tributyrin and different fatty acid methyl esters including methylbutyrate (C4),caprylate (C8), laurate (C12), palmitate (C16), stearate (C18)were used as substrates for the activity measurement. The enzymatic activity was measured by titrimetric assay according to an olive oil emulsion (Pignede et al.,2000).

Effect of the lipase on DON transformation

The effect of crude enzyme on DON transformation was quantitatively analyzed by HPLC. Fifty microliter of standard DON solution at different concentrations (20 μg/mL, 50 μg/mL and 100 μg/mL) was added into the tubes incubated at 40 °C for 5 min, respectively. The lipase (10 mg) was added into each centrifuge tubes. Only crude enzyme was added into the tubes containing the same phosphate buffer as negative control, the inactivated enzyme and DON were added as positive control. Subsequently, the DON extract was tested by HPLC analysis (Zou et al., 2012). The HPLC conditions were as follows: Agilent zorbax sb-Aq C18 column (4.6×250 mm, i.d., 5 μm), injection volume 20 μL, column temperature 29 °C, mobile phase acetonitrile-water (15:85 v/v), flow rate 0.6 mL/min, and UV detection wavelength 218 nm. Each experiment was conducted in triplicate. Data was analyzed by SPSS 12.0 with One-Way ANOVA mode.

Analysis of DON biotransformation products

HPLC-MS was performed to qualitatively analyze biotransformation products. The purified enzyme (1 mg) was dissolved in phosphate buffer (10 mL, 50 mM, pH 7.2), and 100 μL of the enzyme solution was added into three centrifuge tubes, respectively. HPLC-MS (Hewlett Packard, Palo Alto, CA, USA) conditions were as follows (Zou et al., 2012):ion source temperature 200 °C, scanning range m/z 150-350, APCI detection temperature, 450 °C.

RESULTS

Isolation and Identification of F-1

Only three strains were isolated from 53 single colonies and 15 strains and named B-1, F-1 and F-2. In addition, only the intracellular fluid of F-1 had a 56.5U/L enzyme activity in degradation of phenyl ethylene oxide. The purified strains had radial villus and white mycelia simultaneously in early culture stages (5d). After seven days, Diaphragms in hypha of this colony were visible with microscope. Electron microscope scanning image showed that the spore-chains and the top structure were clearly visible (Fig. 1). The ITS gene of F-1 was amplified using PCR and the results of PCR were shown in File S1. Meanwhile, the DNA sequence had 99% similarity to the *Aspergillus tubingensis*. The results of the sequence analysis and the morphology showed that the strain F-1 belonged to *Aspergillus tubingensis*.

Enzymatic purification

The culture filtrate of *Aspergillus niger* As-D.1 was concentrated and purified with 26.2% yields. As shown in Fig. 2, two elution curves were obtained. Of which, one was

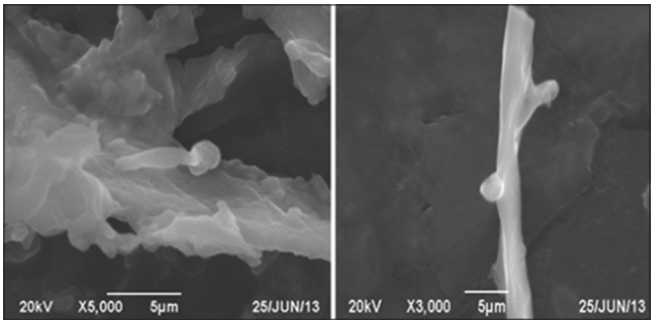

Fig 1. Electron microscope scanning photo of F-1.
Note: The structure of spore chains and sporangium.

a protein concentration curve including three symmetrical peaks, and another was an enzymatic activity curve with only one main peak. Moreover, the eluted enzyme was further fractionated by the gel filtration on Sephadex G-100 (Fig. 3), which was consistent with that of Fig. 2. Accordingly, it wascollected the fractions from the main peak for further analyses. The summarized results were shown in Table 1. SDS-PAGE results indicated that there was a clear target band between 29.0 kDa and 44.3 kDa (Fig. 4).

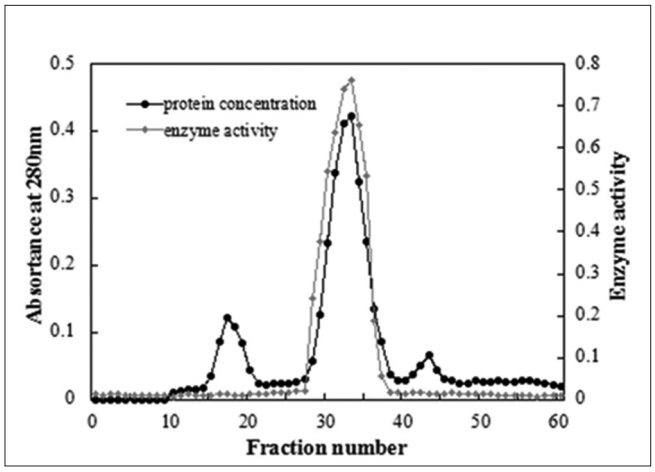

Figure 2. Weak anion exchange chromatography on DEAE-52.
Note: Protein concentration curve (black line); Enzymatic activity curve (gray line). The protein concentration of each tube was determined at a wavelength of 280 nm.

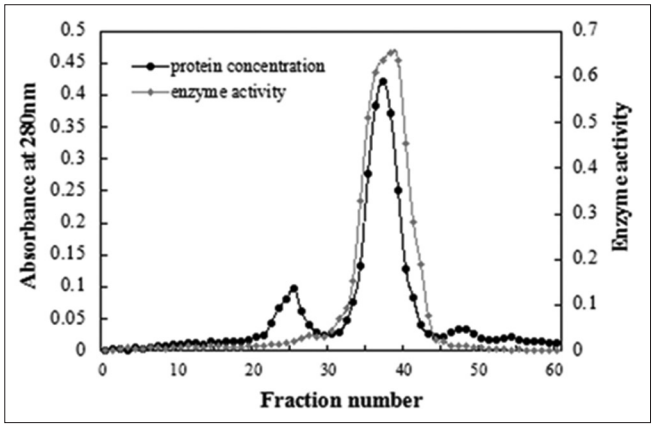

Figure 3. Weak anion exchange chromatography on Sephadex G-100.
Note: Protein concentration curve (black line); Enzymatic activity curve (gray line). The protein concentration of each tube was determined at a wavelength of 280 nm.

Properties of purified enzyme

Effects of temperature and pH on enzymatic activity

The purified enzyme exhibited its maximum activity at pH 8.5, and it was stable in a pH range of 7.5-9.0. Only about 55.0% of the initial activity could be detected (pH<7.0 or pH> 9.5), indicating that the enzyme was only stable under a slightly alkaline condition (Fig. 5b).The maximum enzymatic activity was observed at 40°C as is shown in Fig. 5a. The enzymatic activity was decreased with the temperature increasing while the activity was only about 36.0% at 60°C. Nonetheless, a relatively higherstability was observed in the range of 20-45 °C. The enzymatic activity was rapidly decreased at the temperature of 50°C, and only approximately 14.0% relative activity was detected at 70 °C.

Effects of ions and organic compounds on enzymatic activity

The effects of ions and organic solvents on enzymatic activity were depicted in Fig. 6 and Fig. 7. The results showed that the enzymatic activity could be inhibited by Zn2+ and Cu2+, while Ca2+, Mg2+ andFe2+displayed no inhibitory effects on this enzyme. As shown in Fig. 6, it was notable that the enzymatic activity was decreased by 46.43% and 27.95% in the presence of 0.1 mM Cu2+and 10.0 mM Cu2+, respectively. Herein, we speculated that Cu2+ was a strong inhibitor for the enzyme activity. Furthermore, the enzymatic activity was negatively correlated with the concentration of ions except for Mg2+ and Zn2+. Thus,

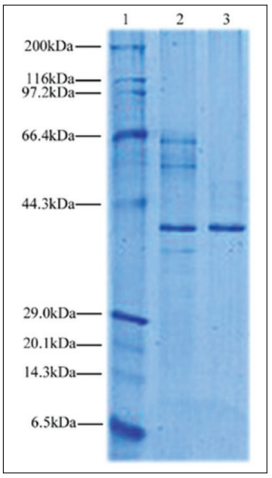

Fig 4. SDS-PAGE analysis of the purified enzyme from *Aspergillus niger*.
Note: Lanes: 1, markers; 2, crude enzyme; 3, purified enzyme.

Table 1: Purification of the enzyme from *Aspergillus niger*

Purification	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude enzyme	1879.3±21	9735.4±127	5.2	100.0	1.0
$(NH_4)_2SO_4$ precipitation	796.6±13	8452.7±106	10.6	86.8	2.0
DEAE-52	145.8±7	5986.1±48	41.1	61.5	7.9
Sephadex G-100	11.5±0.1	2549.8±25	221.7	26.2	42.6

Values are mean±SD of three experiments

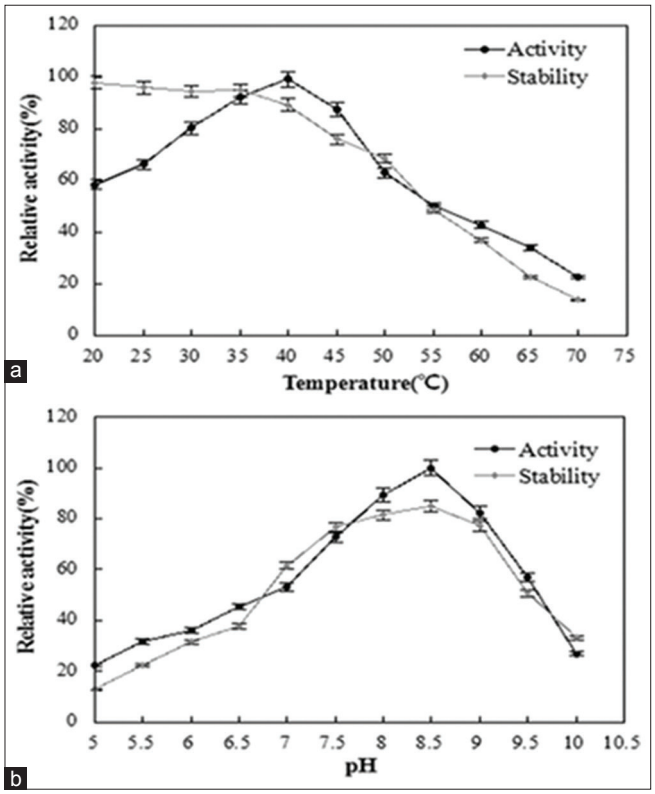

Fig 5. Effects of temperature and pH on the activity and stability of the enzyme.
Note: a. Effect of temperature on enzyme activity and stability, the activity at 40 °C was set as 100%; b. Effect of pH on enzyme activity and stability, the activity at pH 8.5 was set as 100%. Values represent mean ± SD.

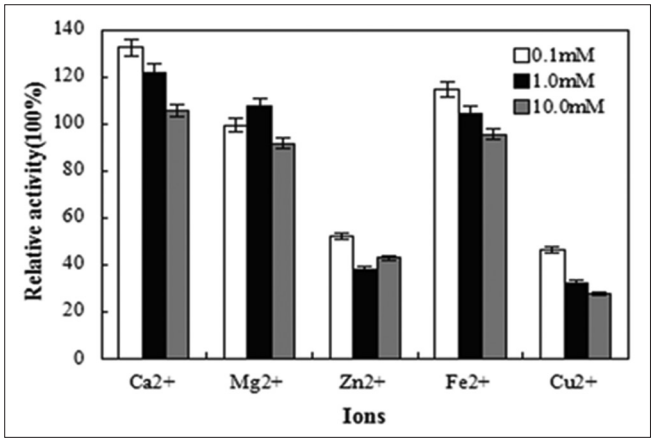

Fig 6. Effects of various metal ions on the enzyme activity.
Note: The activity without metalions was set as 100%.

all above suggested this enzyme was a metal enzyme, and its activity was obviously influenced by ions.

Different organic compounds exerted certain effects on the enzymatic activity. The enzymatic activity could be slightly inhibited by acetone, DMSO and EDTA, while no inhibition by Methanol and ethanol. As shown in Fig. 7, the enzymatic activity was also negatively correlated

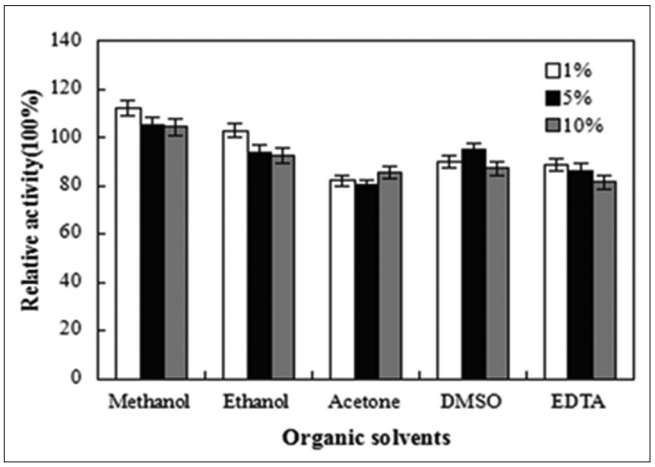

Fig 7. Effects of various organic solvents on the enzymatic activity.
Note: The activity without organic solvents was set as 100%.

Table 2: Effect of theenzyme on DON biotransformation

No. of sample	Initial concentration (µg/mL)	Final concentration (µg/mL)	Percent conversion(%)
1	20	4.58±0.08	77.1%*
2	50	14.04±0.05	71.9%*
3	100	28.4±0.02	71.6%*
Negative control	0	0	0
Positive control	1	0.993±0.01	0.7%

Values are mean±SD of three experiments; *indicates a significant difference between samples and positive control (P<0.01)

with the concentration of organic solvents except for acetone and DMSO. Additionally, the effects of these organic compounds on the enzymatic activity were in a descending order as follows, methanol, ethanol, DMSO, EDTA, acetone. The substrate specificity of the enzyme on different methyl fatty acid esters and oils were determined by the titrimetric method. As shown in Fig. 8, higher activities were achieved with tributyrin and vegetable oils as substrates, indicating that the enzyme may be a true lipase.

Analysis of the DON biotransformation products

Using HPLC, the retention time of DON and biotransformation product were 6.67 min, 3.48 min and 2.60 min, respectively. Fig. 9a showed the peak of DON standard before the transformation, while Fig. 9b showed the peak of biotransformation product and the peak areas of DON were reduced after the transformation. These results indicated that the lipase exerted a strong effect on the DON transformation. The effect of the lipase on the DON transformation was shown in Table 2. A high conversion rate of above 70% was observed from all DON samples at different concentrations (20 µg/mL, 50 µg/mL and 100 µg/mL), and the difference between these samples and positive control was significant.

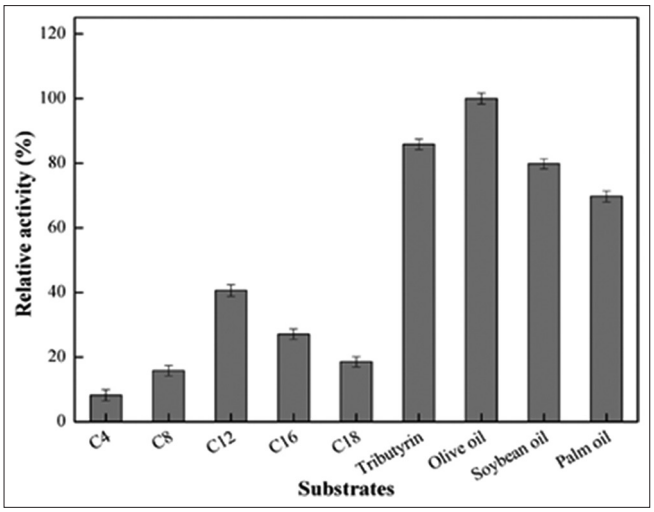

Fig 8. Substrate spectrum of purified enzyme.
Relative activity was expressed as the percentage of the activity towards olive oil (taken as 100%). Where C4 = methyl butyrate, C8 = methyl caprylate, C12 = methyl laurate, C16 = methyl palmitate, C18 = methyl stearate. Values are mean ± SD of three experiments.

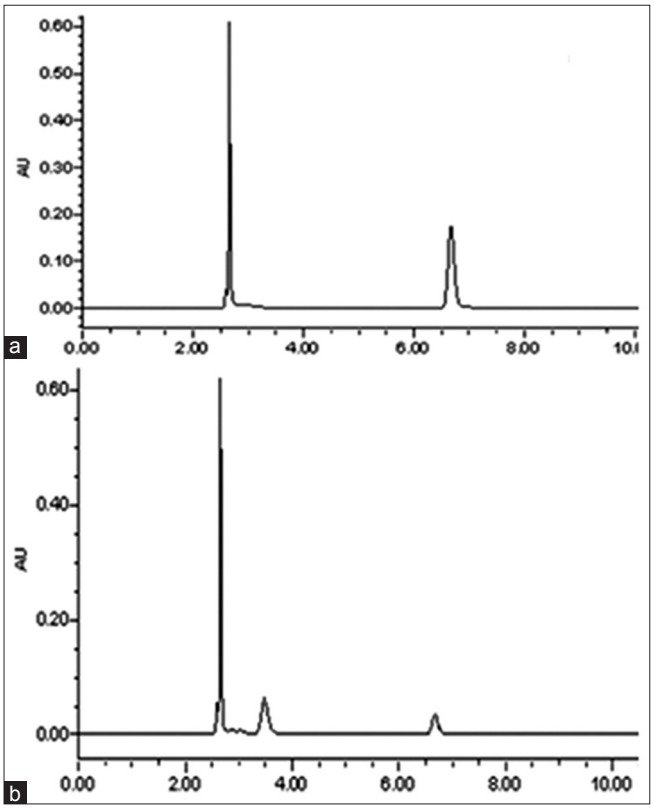

Fig 9. DON biotransformation by lipase.
Note: The X axes for time (min), the Y axes for Au. (a). Before the transformation, the retention time of DON was 6.67 min; (b). After the transformation, the time was 3.48 min.

HPLC-MS was used in qualitative analysis of the biotransformation products. Fig. 10a showed that the

HPLC-MS was used in qualitative analysis of the biotransformation products. Fig. 10a showed that the

molecular weight of DON was 296.00, whereas Fig. 10 b showed that the molecular weight of biotransformation product was 314.00 and the peak areas of DON were reduced after the transformation. In conclusion, HPLC and HPLC-MS analyses showed that the lipase could effectively transform DON with 70% conversion rate, and the molecular weight of the transformation product was 18.0 greater than that of DON.

DISCUSSION

The molecular weights of reported microbial lipases are variable, ranging from 12 kDa (Mase etal., 1995) to 76 kDa (Jurgens et al., 1981). In this study, the 1D-LS-MS revealed a molecular mass of 40.565 kDa, which was greater than the enzyme (37.0 kDa) reported by Sharma and smaller than that (45.0 kDa) reported by Ali et al.(2012).A further 1D-LS-MS analysis revealed that the amino acid number of the enzyme (354 AA, FileS2) from *Aspergillus niger* was different from that (297 AA) reported by Mhetras et al.(2009).The differences might be mainly due to the alteration of metabolism products under different metabolism conditions. Considering amino acid sequence of the enzyme was highly similar with protein YALI0A01441p in NCBI database, it can be deduced that the enzyme might be a lipase. Until recently, this deduced lipase has been reported by several studies. Kumar et al. (2005) found that the lipase had a maximum activity at 45 °C and pH 8.5. Yu et al. (2007) indicated that the lipase functions were normal at pH from7.0 to 8.5or at temperature from 35 °C to 45 °C. Wang et al. (2012) also suggested that the lipase activity was significantly decreased above 45°C or pH below 6.0 and above 9.0.Present data suggested that the enzyme had a strong activity at 45 °C and acidic pH below 7.0. Obviously, these results were mostly in agreement with above three investigations, thus providing support to the accuracy of the method applied in thiscurrent study.

Meanwhile, it was detected that the enzymatic activity was inhibited to some extent by metal ions and organic compounds, which was identical to the previous study (Yu et al., 2007). However, compared with previous study (Castro-Ochoaet al., 2005), the enzyme remained a little higher activity in EDTA. It was indicated that the enzyme could be slightly activated by some metal ions (Ca^{2+}, Mg^{2+} and Fe^{2+}) and organic solvents (methanol and ethanol). In a similar study, it was reported that the lipase from *Mucor hiemalisf. Corticola* was more stable in ethanol and methanol than the other tested organic solvents (Ulker and Karaoglu, 2012).One of the most important properties of the lipase was the substrate specificity towards triglycerides. Most importantly, tributyrin was a triglyceride of short chain

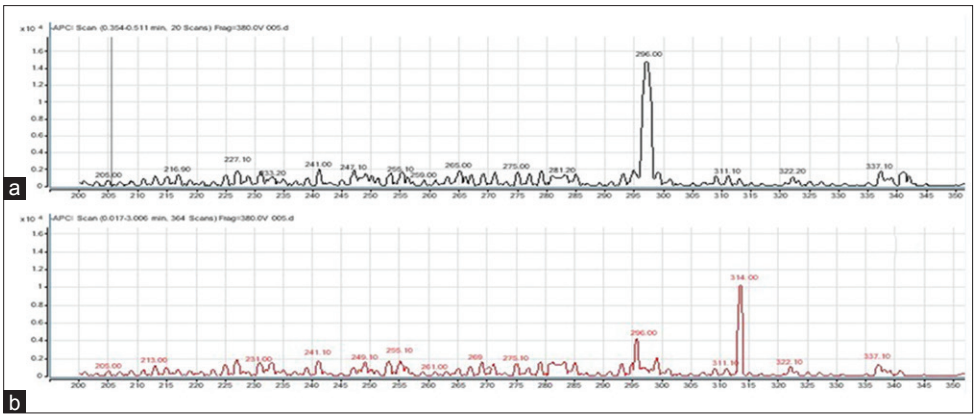

Fig 10. Detection of transformation products by HPLC-MS.
(a). Before the transformation, the molecular weight of DON standard was 296.00; (b). After the transformation, the molecular weight of transformation product was 314.00.

and easily hydrolysable by lipases and esterases (Kim et al., 2007). In addition, the enzyme showed a better activity toward C12-C18 methyl esters, with the highest activity toward methyl laurate (C12), indicating a preference for long acyl chains, which was similar with the observed result for lipase from *Penicillium crustosum* (Rigo et al., 2012).

Similar with the study reported by He et al. (2008), the enzyme with the capacity to transform DON into another product was isolated. Shim et al. (1997)also isolated an *Agrobacterium-Rhizobium* strainE3-39 transformed DON into 3-keto DON under aerobic conditions and the molecular weight of biotransformation product was 294.0. Young et al. (2007) reported that *Bacterial isolates* LS100&SS3transformed DON into DOM-1,and the molecular weight was 280.3. As a natural extract from microbial sources, the enzyme was increasingly recognized as highly versatile biocatalysts for degrading epoxy resins (Jaszcz, 2002; Tay et al.2005). Moreover, based on the structure of DON and the LC-MS data, we inferred that DON could be hydrolyzed by the enzyme via opening an epoxy ring, resulting in two hydroxyl groups. Awad et al. (2010) had shown that the toxic effects of DON were associated with its 12,13-epoxy structure, and could be largely reduced by destroying the epoxy structure of DON. Based on thisrecent study, the proposed method herein provides a new method for DON biotransformation, which appears to have a wide application outlook for DON biotransformation.

CONCLUSIONS

From this study, it was purified and characterized an extracellular enzyme from *Aspergillus niger*. The enzyme exhibited the maximum enzyme activity at 40 °C and pH 7.5-9.0. The specificity analysis of the enzyme indicated a strong preference totributyrin and vegetable oils as the substrate, and the enzyme showed a good activity to long chain fatty acid methyl esters (C12-C18). Most importantly, this enzyme could successfully transform deoxynivalenol (DON). Using HPLC analysis, it was detected a biotransformation rate of more than 70%. This work also provided a method of enzyme purification and a possibility for DON biotransformation.

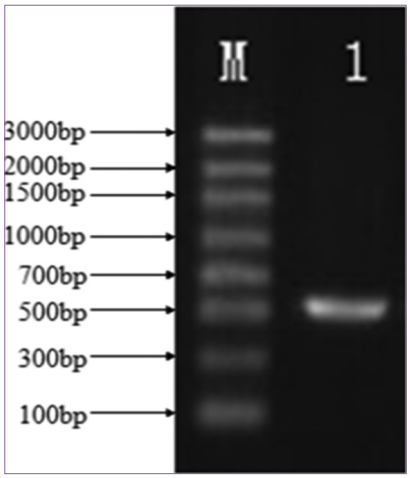

Figure S1. PCR amplification of ITS gene

Supporting Information

File S1 PCR amplification and sequencing of ITS gene

Fig. S1, M: DNA marker; 1: PCR product of its gene. Fig. S2, The detailed information of PCR product sequencing. (DOCX).

File S2 Results of amino acid sequence analysis and Similarity comparison.

Note: the AA in red means the new sequence detected in this study. (DOCX).

TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAG
GTCAACCTGGAAAGAATGGTTGGAAAACGTCGGCAGGCGCCGGCCAATCC
TACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCC
GCCGCTGCCTTTCGGGCCCGTCCCCCCGGAGAGGGGGACGGCGACCCAAC
ACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCC
CGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAA
TTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGG
AACCAAGAGATCCATCGTTGAAAGTTTTAACTGATTGCATTCAATCAACTC
AGACTGCACGCTTTCAGACAGTGTTCGTGTTGGGGTCTCCGGCGGGCACG
GGCCCGGGGGGCAGAGGCGCCCCCCCGGCGGCCGACAAGCGGCGGGCCC
GCCGAAGCAACAGGGTACAATAGACACGGATGGGAGGTTGGGCCCAAAG
GACCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAA

Figure S2. PCR product sequencing

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Authors' Contributions

Conceived and designed the experiments: Guoqing Liu, Shaohua Yang. Performed the experiments: Jun Yang. Analyzed the data: Yinghui Bao, Kaiping Wang, Wei Wang. Contributed to sample collection: Rong Yan. Wrote the paper: Shaohua Yang, Yu Wu.

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