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Effectiveness of the toxin-degrading strain screening strategy: Deficiency and improvement in screening the gossypol-degrading microorganism Aspergillus niger

Chen Zhang
Anhui Agricultural University
Gangqin Shu
Anhui Agricultural University
Hui Wang
Anhui Agricultural University
Daojie Li
Anhui Agricultural University
Yuan Xü
Anhui Agricultural University
Yunhua Zhang
Anhui Agricultural University
Maoji Cheng Cheng
Anhui Agricultural University
Lijuan Chen (zhangchenlijuan@163.com)
Anhui Agricultural University

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Effectiveness of the toxin-degrading strain screening strategy: Deficiency and improvement in screening the gossypol-degrading microorganism *Aspergillus niger*

Chen Zhang[1], Gangqin Shu[1], Hui Wang[1], Daojie Li[1], Yuan Xü[1] Yunhua Zhang[2], Maoji Cheng[1, 3], Lijuan Chen[1*]

[1] *The Biological Feedstuff Lab*, *College of Animal science and Technology*，*Anhui Agricultural University*, *Hefei*, *Anhui 230036*, P.R. *China*

[2] *Anhui Province Key Lab of Farmland Ecological Conservation and Pollution Prevention, College of Resources and environment, Anhui Agricultural University*, *Hefei*, *Anhui 230036*, P.R. *China*

[3] *Fisugarpeptide Blology Engineering CO., LTD, Luan, Anhui 237000,* P.R. *China*

[*] Correspondence: zhangchenlijuan@163.com; Tel.: +86-0551-65786328

ABSTRACT

The effectiveness of the classic screening strategy was verified, by duplicating and verifying the degradation of gossypol by the *Aspergillus niger*. It can reduce the free gossypol content through biosorption but has no effect on the total gossypol content and cannot effectively degrade gossypol. And the most interesting thing we found the strain can secrete agarase, utilise agar as carbon source. In this case, that will mislead researchers and lead them to make wrong judgments. That turns out the usual methods of previous screening strategies are not rigorous enough, the classic screening method has defect in screening toxin-degrading strain, so agar control group should be added. In this study, some suggestions are put forward to

optimise the same type of experiments and broaden the idea of detoxification by microorganisms and provide reference for screening effective toxin-degrading microorganisms.

Keywords: Gossypol, Degrade, *Aspergillus niger*, Agarase, Screening strategy

Introduction

Toxic substances in soil, water, food and feed can be degraded by microorganisms (Ghazali et al., 2004; Hazrat et al., 2010; Ji et al., 2010;). Compared with physical and chemic methods, the biodegradation technology has the advantages of safety, efficiency, moderate, environmental friendliness, absence of secondary pollution and low cost and is the most promising treatment method that has been paid attention by researchers. The classic screening method (Fig.1 A) is that (Lewis et al., 2020): (1) Samples are usually collected from a variety of specific areas where degrading microorganisms may exist. (2) A certain toxic substance is used as the sole carbon (or nitrogen) and energy sources, and microorganisms are inoculated in an agar solid medium. (3)The degradation effect is judged using the diameter of the hydrolysis circle or the growth state of the microorganism, and the strains that can utilise or degrade the toxin are screened and isolated. Through further study, targeted degrading enzymes are obtained, and this strategy has achieved many successes (Cheng et al., 1969; Dai et al., 2015; Liu et al., 2016).

The gossypol, as an antinutritional factor (ANF) or natural plant toxin, has antifertility effects on humans and animals (Randel et al., 1992). The gossypol exists in cottonseed oil and meal (CM), and is a typical food or feed contaminant (EFSA Panel on Contaminants in the Food

Chain (CONTAM) et al.,2017). Through the classic screening method, many researchers have discovered that some microorganisms, such as *Aspergillus niger* (AN) (Yang et al., 2012), *Candida tropicalis* (Mageshwaran et al., 2018), *Aspergillus flavus* (Yang et al., 2010) and *Bacillus subtilis* (Zhang et al., 2018), can degrade gossypol, and AN is the most reported microorganism. On the basis of previous screening strategies (Fig.1 B), AN that can grow in an agar solid medium with gossypol as the sole carbon source is isolated from cattle rumen liquid. The transcriptome of AN is obtained, and relevant genes or degrading enzymes involved in the degradation of gossypol in AN are attempted to be discovered. However, results have not met our expectations. Therefore, the rigor of the traditional screening strategy for toxin-degrading strains is questioned (Fig.1 C).

This study has confirmed the deficiencies of previous screening strategies for gossypol-degrading microorganisms (Fig.1 C) and expects to design a scientific and rigorous screening method for toxin-degrading microorganisms on the basis of existing methods.

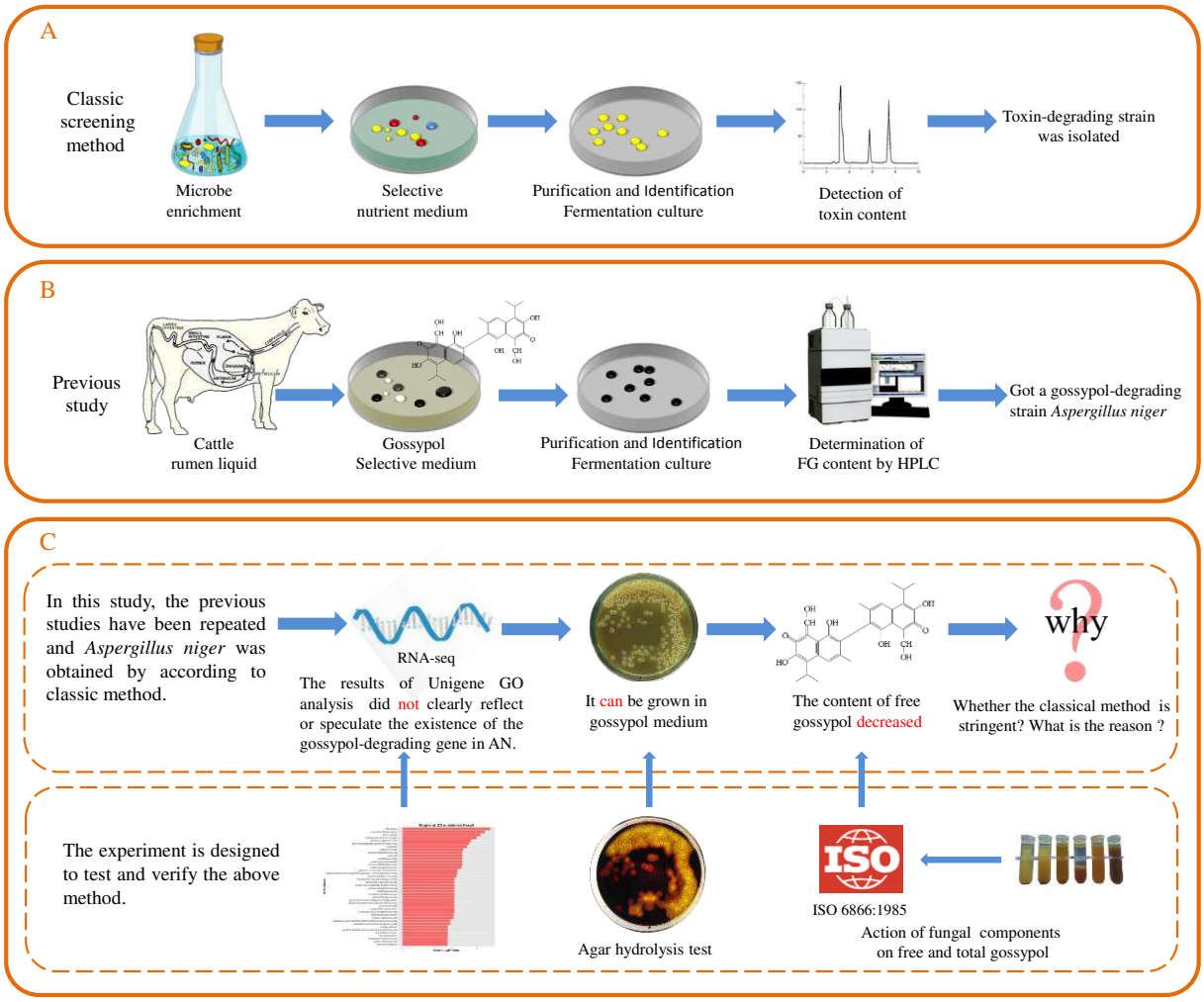


Fig. 1 Validation of the effectiveness of the classic toxin-degrading strain screening strategy

A: Classic screening method; B: Previous study; C: The starting point of the study and the design of verification.

Materials and Methods

Microbial strain

Aspergillus niger which can grow in an agar solid medium with gossypol as the sole carbon source is isolated from cattle rumen liquid was obtained by classic screening strategy (Yang et al., 2012; Zhang et al., 2018; Lewis et al., 2020). The cattle rumen liquid samples were

collected from Huangshan Junfeng ecological cattle farm.

Transcriptome sequencing (RNA-seq) and the trait identification of AN

AN cultured in potato dextrose agar (PDA, Hope Bio-technology Co., Ltd. Qingdao) was used as the control group, and AN cultured in PDA with 2% acetate gossypol (AG, Ci Yuan Bio-technology Co., Ltd. Shaanxi) was used as the experimental group. The Illumina Hiseq™ 2500 high-throughput sequencing technology was used to sequence the transcriptome of AN under normal and 2% AG conditions. Differentially expressed genes (DEG) were screened, and 2% AG response-related genes were analysed using the gene ontology (GO) and the KEGG (Kyoto Encyclopedia of Genes and Genomes) databases for annotation in AN, measured by Shanghai Biotechnology Co., Ltd.

The AN and spores were observed using scanning (SEM) and transmission (TEM) electron microscopy (Tizro et al., 2019).

Growth in different media

Solid medium were prepared as shown in Table 1, and an equal amount of the AN spore solution was inoculated and cultured at 30 °C for 24 h. The growth of AN was observed and recorded. And after growing AN for 30 h, the media were stained with Lugol's iodine solution (Fu et al., 2010) in the dark for 1 min, washed thrice with clean water and observed under a normal light source.

Table 1. Preparation of solid media for AN

No.	Medium components
1	Sugar-free CzA + 0.5% AG
2	Sugar-free CzA
3	CzA

CzA: Czapek–Dox Medium ($NaNO_3$ 2g, K_2HPO_4 1g, KCl 0.5g, $MgSO_4$ 0.5g, $FeSO_4$ 0.01g, Sucrose 30g, Agar 20g, distilled H_2O 1000mL, natural pH, 121°C, 20min); Sugar-free CzA: Czapek–Dox Medium without Sucrose.

Preparation of the crude enzyme and the cell wall pellet suspension

AN was inoculated in potato dextrose broth, and the crude enzyme and the cell wall suspension were prepared as follows.

(1) After 24 h, the AN fermentation broth was collected (before spore production), subjected to repeated freeze–thaw and crushed using ultrasonic wave (in ice water) until no intact cell structure was found using a microscope (Zhang et al., 2021).

(2) The fermentation broth was centrifuged at 4 °C and 8000 rpm for 10 min. The supernatant and the fungus precipitate were collected. After washing the fungus precipitate thrice with aseptic water, the precipitate was resuspended to the original volume, and the cell wall pellet suspension was obtained.

(3) The supernatant was filtered using the millipore filter (0.45 μm) (Sultana et al., 2018), and the crude enzyme was obtained and stored at −20 °C for later use.

Removal experiment of AG by AN components

The experiment was designed and carried out in accordance with Table 2. Experimental groups were restored to normal temperature, and AG was added to make the concentration reach 0.5% and shaken well. Samples 1, 2, 3 and 4 were used as experimental groups, and sample 5 was used as the blank control group. Samples were collected before and after standing at 30 °C in the dark for 24 h and stored at −20 °C for testing. Before testing samples

should been thawed, shaken and freeze dried. The free (FG) and the total (TG) gossypol were detected using the international standard ISO6866-1985, and the FG and TG removal rates were calculated according to the formula Eq. (1).

Eq. (1): $\mathrm{Rr} = \frac{C1-C2}{C1} \times 100\%$

Where, Rr: Removal rate, C1: Initial content of FG (or TG) and C2: Rest of FG (or TG)

Table 2. Experimental design of the gossypol removal

No.	Treatment methods
1	Crude enzyme
2	Crude enzyme inactivated using boiling water bath for 10 min
3	Cell wall pellet suspension
4	Cell wall pellet suspension inactivated using boiling water bath for 10 min
5	Aseptic pure water

Statistical analysis

All statistical analyses were performed using the SPSS version 19 (IBM, Armonk, NY) Statistical differences were determined using the Duncan's multiple range test, and significance was defined as $P < 0.05$.

Results

GO enrichment analysis of differentially expressed genes

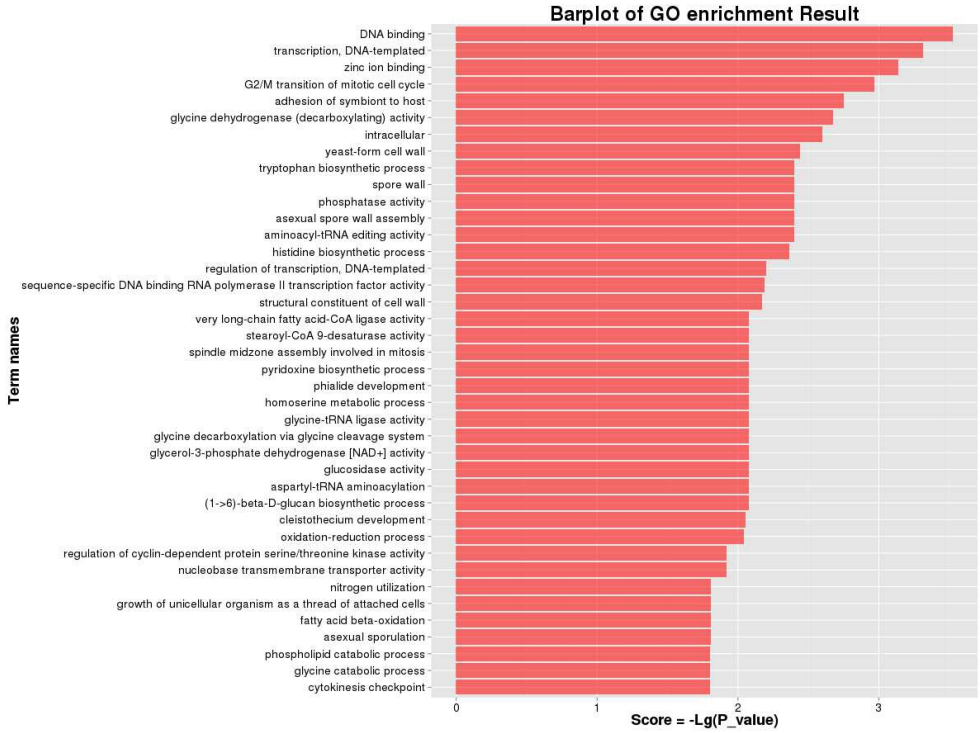


Fig. 2 GO functional annotation of differentially expressed genes under the gossypol stress

The Unigene GO analysis (Fig. 2) showed that 25 112 results were annotated and divided into three functional groups, namely, biological process (33.52%), molecular function (28.65%) and cell component (37.83%). The 10 most significant differences were DNA binding, transcription DNA-templated, zinc ion binding, G2/M transition of mitotic cell cycle, adhesion of symbiont to host, tryptophan biosynthetic process, asexual spore wall assembly, differentially expressed genes are closely related to cell stress resistance, proliferation, tissue repair, adhesion and cell structure (Zámborszky, 2013; Swirnoff et al., 1995; Sharma et al., 2020). Results did not clearly reflect or speculate the existence of the gossypol-degrading gene in AN.

Growth status in different media

AN could grow on all media. At the same culture time, the degrees of growth and maturity followed the order: CzA > sugar-free CzA > sugar-free CzA + 0.5% AG > agar, and spores were produced except in agar (Fig. 3A). The AN in the agar medium was the weakest (Fig. 3 A4) followed by sugar-free CzA added with 0.5% AG (Fig. 3 A1). Results showed that screening AN by using agar medium added with AG as the sole carbon source was not rigorous.

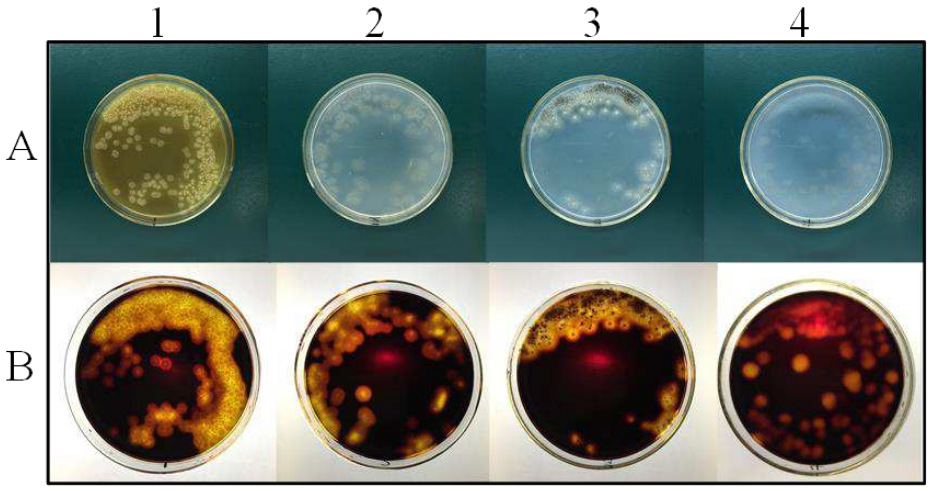


Fig. 3 Growth status of AN in media and results of Lugol's iodine staining

A: Before and B: after staining with Lugol's iodine

1: Sugar-free CzA + 0.5% AG, 2: Sugar-free CzA, 3: CzA, 4: agar medium

Lugol's iodine staining

Four media were stained with Lugol's iodine after growing AN for 30 h. Results are shown in Fig. 3B. After staining, corresponding bright spots were observed on the growth position of AN, and the surface of media were slightly dented. The result showed AN could secrete agarase to hydrolyse agar and utilise agar as carbon and energy sources.

Morphological changes in AN

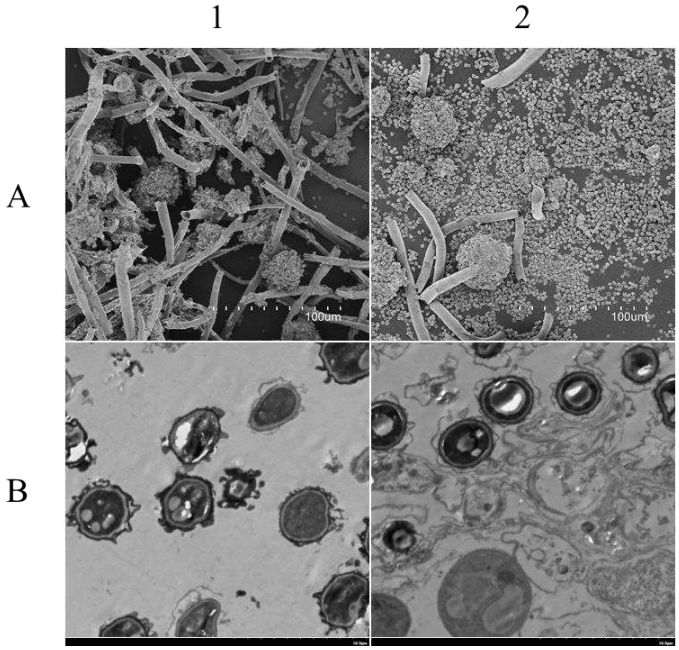


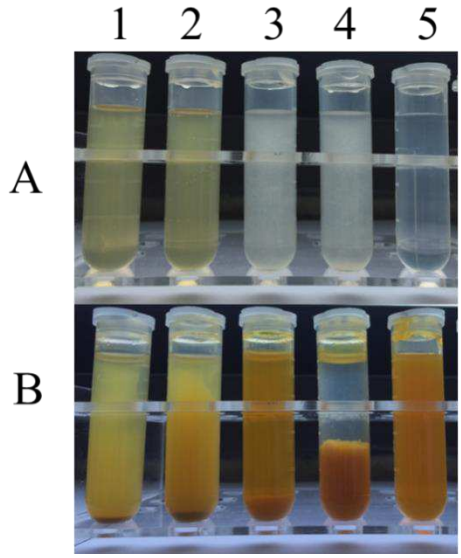
Fig. 4 Micrographs of AN and spores

A: SEM image of AN, B: TEM image of spores;

1: Growth of AN on PDA added with 2% AG, 2: Growth of AN on PDA.

Under the gossypol stress, AN had increased hyphae and decreased number of spores compared with AN grown on PDA alone (Fig. 4A). The spores grown on AG were deformed; had blisters on the surface; thin, uneven and damaged cell wall and small ratio of organelles to cell volume (Fig. 4B). Results showed that gossypol had a negative effect on the AN growth and could cause a bubblelike structure to form on the spores' surface and structural injury in the cell wall. However, AN had a certain tolerance to gossypol injury.

Removal efficiency of fungal components on AG



158

159 **Fig. 5 Flocculation and sedimentation of fungal components in AG**

160 A: Before and B: after adding AG;

161 1: Crude enzyme, 2: Inactivated crude enzyme, 3: Cell wall pellet suspension,

162 4: Inactivated cell wall pellet suspension, 5: Blank control (aseptic pure water).

163 AG was added to each group, and the mixture was placed at room temperature for 1 h (Fig. 5).

164 Flocculation and sedimentation were observed in all experimental groups. The influence of

165 the inactivated cell wall pellet (Fig. 5 B4) was most notable followed by that of the

166 un-inactivated cell wall pellet (Fig. 5 B3).

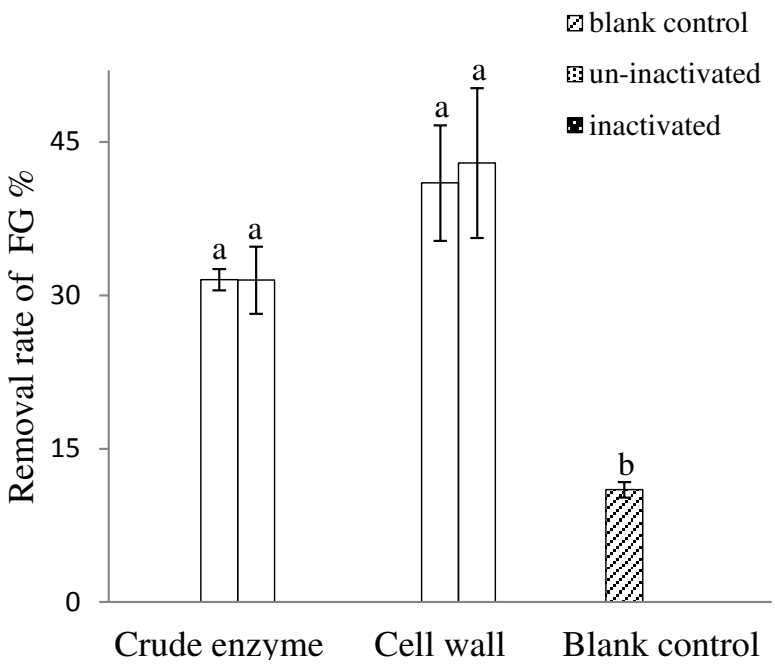
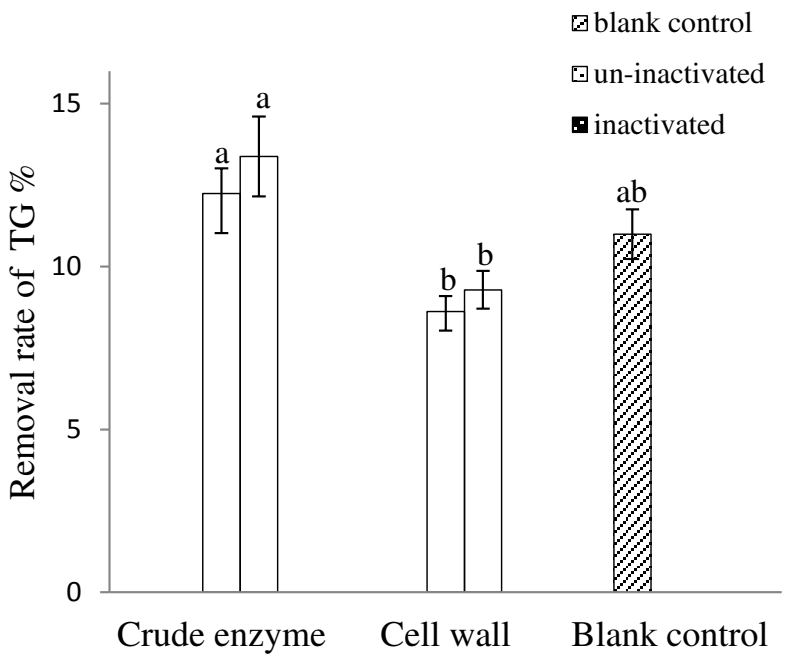


Fig. 6 Removal efficiency on FG by fungal components

Different lowercase letters indicate significant differences amongst treatment groups ($P < 0.05$)

Compared with the control group, the crude enzyme and the cell wall of AN had significant removal effects on the FG in the sample ($P < 0.05$, Fig. 6), and the inactivated cell wall group had the highest removal rate (42.94%). The FG removal rate of each component after inactivation was higher than that before inactivation. The FG removal rates of cell wall groups were higher than that of the crude enzyme groups, but no significant difference was observed amongst experimental groups ($P > 0.05$).



177

178 **Fig. 7 Removal efficiency on TG by fungal components**

179 Different lowercase letters indicate significant differences amongst treatment groups ($P <$

180 0.05)

181 The components of AN had different removal effects on TG in the sample (Fig. 7). The

182 removal rate of each group was low (all < 15%), and the inactivated crude enzyme group had

183 the highest TG removal rate (13.38%). The FG removal rate of each component was higher

184 after inactivation, but no significant difference was observed amongst the same component

185 groups ($P > 0.05$). The crude enzyme group had significant differences amongst cell wall

186 groups ($P < 0.05$), but no significant difference was observed between the control and the

187 experimental groups ($P > 0.05$).

188 **Discussion**

189 In this experiment, AN can grow in four kinds of media, and screening the degrading strains

190 simply by using an agar medium added with toxin as the sole carbon source is not rigorous.

The Lugol's iodine solution can stain polysaccharides in agar into a dark brown colour and cannot stain the degraded oligosaccharides of agar (Fu et al., 2010). This solution has been used to visualise the agarase activity to screen the agarase production by microorganisms on a culture plate (Kawai, 2003). Similar to the AN in this study, some strains (Fu et al., 2010; Chi et al., 2014) can utilise agar to grow normally on the medium without any sugar, interfere with the expected experimental results and affect the experimenter to make a true judgment on the results.

Enzymes are metabolites secreted by living organisms and can be divided into primary (constitutive enzymes) and secondary (inducible enzymes) metabolites. This study first discovers that AN can secrete a constitutive enzyme that hydrolyses agar and utilise agar as sole carbon source. Therefore, we suspect that the gossypol-degrading enzyme of AN may not be a primary metabolite and that the gossypol cannot be directly utilised. This enzyme may be an inducible enzyme and can be secreted only under the condition of the gossypol as an inducer. In this study, the gossypol has a negative effect on the normal growth of AN. However, AN has a certain tolerance to the gossypol injury and produces active spores. Thus, AN may produce some special inducing enzymes under the induction of the gossypol. Given that the premise of the basic physiological metabolism of the cell is guaranteed, when exogenous stimuli interfere with the normal metabolism of cells or cause cell damage, certain proteins (such as Quinone oxidoreductase, Cytochrome p450， Catalase) are secreted abundantly by the cells, which are under the exogenous stimulus stress against exogenous substances and protect cells (Mcclain et al., 1999; Gu et al., 2020). Therefore, when screening

212 for a toxin-degrading strain by using a medium with the toxin as the sole carbon (or nitrogen)
213 and energy sources, the function of inducing enzymes may be overlooked by usual screening
214 methods of the predecessors.
215 For the removal effect of FG (or TG), no significant difference is observed before and after
216 inactivation in crude enzyme groups (or cell wall groups). The TG removal rate of each group
217 is lower than the FG removal rate (Fig. 6 and Fig. 7). For AN, the FG removal function and
218 metabolic enzymes may not be closely related. Heating promotes proteolysis and increases
219 the content of free amino acids (Jiang et al., 2013; Chang et al., 2014;). The crude enzyme
220 solution is composed of proteins, and the cell wall suspension also contains some little cell
221 wall proteins. After heat inactivation, the content of free amino acids in the solution increases.
222 The Maillard reaction occurs and is strengthened between the carbonyl group from the
223 gossypol and free amino acids under ISO detection conditions. As a result, the TG content
224 decreases, and the TG removal rates of the crude enzyme and the cell wall groups increase
225 when inactivated. Significant differences between the crude enzyme and the cell wall groups
226 are observed due to different protein contents. The gossypol is unstable at room temperature,
227 and a small amount of gossypol is degraded (Nomeir et al., 1982). No significant difference in
228 the TG removal rate is observed between the control and the crude enzyme or the cell wall
229 group ($P > 0.05$). Thus, we believe that the decrease in TG content is not due to AN.
230 Hyphae secrete enzymes that convert the organic material from media into small molecules
231 that can be taken up by the fungus to serve as nutrients (Vinck et al., 2005). Hyphae in
232 filamentous fungi also serve as store house for nitrogen and phosphorus in the form of DNA,

giving hyphal tips the capability of persistent extension and foraging in new areas (Maheshwari, 2005). The inadequate nutrition in sugar-free medium prompts AN to increase absorbable nutrients indirectly by increasing the number of hyphae. The toxic effect of the gossypol prompts AN to extend away from the gossypol damage by increasing the number and the length of hyphae. The agar medium lacks minerals and is not enough to support the growth of AN. AN relies on stored nutrients in spore growth and grows weakly. The main components of the AN cell wall are chitin and glucan and used as biosorbent for heavy metals (Filipovic-Kovacevic et al., 2000). The removal effect of AN on FG may be due to the biosorption of fungi. The gossypol can destroy the structure of AN and increase the gene expression of fungus cell stress resistance, proliferation, tissue repair, adhesion and cell structure. The reduction in gossypol is more likely to be consumed by damaging AN or protein.

When screening toxin-degrading microorganisms, we usually regard the toxin as the sole carbon source, in the preparation of agar solid medium tend to ignore the agar could be a potential carbon source, moreover, in culturing, is likely to ignore the carbon dioxide (CO_2) (Peng et al., 2010 Steffens et al.,2021) in the air even light (Shi et al., 2008) may be a carbon or energy for some microorganisms. Thus, factors, such as agar, CO_2, illumination and inducible enzymes should be fully considered.

HPLC has previously been employed to detect gossypol content changes, and this method can only detect free gossypol, while total gossypol can only be effectively detected by ISO 6866:1985 (Animal feeding stuffs—Determination of free and total gossypol). The

degradation effect of microorganisms on toxin is an important basis to evaluate whether strains have further research value. Toxins can be absorbed by microbial adsorption, and free toxins are remarkably reduced. but no degradation, will free again under specific conditions, when evaluating the toxin degradation effect of a strain, the reduction binding toxins should be considered fully, basing on the change in total toxin to determine whether the target strain adsorbs or degrades toxins.

Besides, substances or toxins can be directly, exactly and efficiently analysed quantitatively and qualitatively by using a mass analyzer and other equipment (Zhou et al., 2016; Yan et al., 2018). The toxin can be more intuitive judged whether or not to be degraded or form a new substance. Yet the toxin degradation products by microorganisms usually have many characteristics, such as low content and mixed with a variety of complex substances, the composition is complicated and difficult to extract and purify. In addition, the mass analyser has high requirements on operating ambient detection conditions and is not suitable for use at the initial phases of screening degrading strains.

During the processes, such as agricultural production, the exploitation and the utilisation of resources, sewage treatment and food storage and processing. Various degrees of toxin pollution, including organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), dyes, mycotoxins and others, are available, and most of them are refractory organic pollutants, bioaccumulative, persistent and toxic with carcinogenic and mutagenic activities (Jones et al., 1999; Kelly et al., 2007; Huen et al., 2014). The successful screening and the domestication of microorganisms with degradation effect on a certain pollutant are important for environmental

protection and human health. When screening toxin-degrading strains by using a toxin as the sole external carbon source and the solid medium as gel, the selection strategy should be optimised, and control groups with agar, CO_2 and light should be established at the same time. The screening scope can be extended to toxin-resistant organisms, and the content can be reduced by the inducible enzyme or consumed by the injury of resistant organism. The screening of pollutant-degrading strains is a tedious task and contingent in nature. Therefore, screening strategies should be fully considered when conducting strain screening for improved accuracy.

Authors' contributions

C Z, G S, H W, D L, Y X, Y Z, M C, L C designed and analyzed the experiments. C Z and G S performed the experiments. C Z and L C interpreted the data and wrote the manuscript, and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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