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Effect of selected fungi on the reduction of gossypol levels and nutritional value during solid substrate fermentation of cottonseed meal*

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Abstract: The objective of this work was to investigate the effect of six individual strains of fungi on the reduction of gossypol levels and nutritional value during solid substrate fermentation of cottonseed meal (CSM). Six groups of disinfected CSM substrate were incubated for 48 h after inoculation with either of the fungi *C. capsuligena* ZD-1, *C. tropicalis* ZD-3, *S. cerevisae* ZD-5, *A. terricola* ZD-6, *A. oryzae* ZD-7, or *A. niger* ZD-8. One not inoculated group (substrate) was used as a control. Levels of initial and final free gossypol (FG), crude protein (CP), amino acids (AA) and in vitro digestibility were assayed. The experiment was done in triplicate.

The experimental results indicated that microbial fermentation could greatly decrease ($P<0.05$) FG levels in CSM. The detoxification efficiency differed between the species of microorganisms applied. From the perspective of reducing CSM potential toxicity, *C. tropicalis* ZD-3 was most successful followed by *S. cerevisae* ZD-5 and *A. niger* ZD-8. They could reduce FG levels of CSM to 29.8, 63.07 and 81.50 mg/kg based on DM (dry matter), respectively, and their detoxification rates were 94.57%, 88.51% and 85.16%, respectively. If crude protein, amino acids content and their in vitro digestibility were also taken into account, *A. niger* ZD-8 may be the best choice. The CP content of CSM substrate fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 were improved by 10.76% and 22.24%; the TAA (total amino acids) contents were increased by 7.06% and 11.46%, and the EAA (essential amino acids) were raised by 7.77% and 12.64%, respectively. Especially, the levels of methionine, lysine and threonine were improved greatly ($P<0.05$). The in vitro CP digestibility of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 was improved by 13.42% and 18.22%, the TAA were increased by 17.75% and 22.88%, and the EAA by 16.61% and 21.01%, respectively. In addition, the in vitro digestibility of methionine, lysine and threonine was also improved greatly ($P<0.05$).

Key words: Fungi, Free gossypol, Solid substrate fermentation, Cottonseed meal, Detoxification, Nutritional value
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INTRODUCTION

The potential of cottonseed meal (CSM) protein for animal nutrition is limited by the presence of gossypol ($C_{30}H_{30}O_8$), a toxic polyphenolic pigment (Francis *et al*., 2001). It is produced in the seeds of the cotton plant, and feeding diets containing gossypol cause negative effects such as growth depression and intestinal and other internal organ abnormalities (Berardi and Goldblatt, 1980; Robinson *et al*., 2001). Its negative effect on animal health has long been recognized, and the toxic effect of gossypol is much greater for non-ruminants than ruminants due to binding of free gossypol (FG) to soluble proteins in the rumen (Willard *et al*., 1995). Thus, if FG was transformed into bound gossypol (BG), it would not

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harm animals, because BG cannot be absorbed through the digestive tract. Cottonseeds are commonly processed into oil and meal, which may contain high concentrations of the toxin, and further processing is necessary to reduce it to permissible levels. The development of glandless cottonseed by plant breeders to overcome this problem was limited due to the poorer yields and the increased susceptibility of this crop to insects and diseases. Consequently glandless cottonseed accounts for less than 0.5% of the total crop in the world. A number of methods have been developed for removing gossypol from cottonseed including solvent extraction of free gossypol (Damaty and Hudson, 1975; Canella and Sodini, 1977; Cherry and Gray, 1981; Rahma and Narasingo Rao, 1984), ferrous sulfate treatment (Tabatabai *et al*., 2002; Barraza *et al*., 1991), calcium hydroxide treatment (Nagalakshmi *et al*., 2002; 2003), microbial fermentation (Wu and Chen, 1989; Shi *et al*., 1998) and so on. These methods play an important role in detoxification of CSM, but the reduction of gossypol using solvent system suffers from the difficulty of totally removing residual solvents which have potentially harmful effects, as adversely affect the flavor. Ferrous sulfate treatment causes feed to become black which affects feed quality, and calcium hydroxide treatment often leads to the problem of reduced vitamin content. Microbial fermentation should be a kind of promising detoxification method, but literature on CSM fermentation is scarce, and the microbial species for investigation are limited.

The objective of this work was to study the effect of selected fungi on the reduction of gossypol levels during solid substrate fermentation of CSM. Several representative fungal strains are bred by Zhejiang University, China. We examined whether the selected fungi through fermentation leads to reduction of the gossypol levels; which strain is more effective; and whether the amino acids (AA) profile and their in vitro digestibility are changed, especially essential amino acids (EAA) such as lysine and methionine.

MATERIALS AND METHODS

Substrate treatment

CSM was obtained from Shihezi district, Xinjiang autonomous region, China. This material was mixed with corn flour and wheat bran, the ratio of CSM:corn flour:wheat bran was 7:2:1. Then the mixture was moistened and the ratio of mixture versus water was 1:0.8. Afterwards, it was autoclaved at 112.6 °C for 20 min.

Microorganisms and inoculum

The strains *Candida capsuligena* ZD-1, *Candida tropicalis* ZD-3, *Saccharomyces cerevisae* ZD-5, *Aspergillus terricola* ZD-6, *Aspergillus oryzae* ZD-7, and *Aspergillus niger* ZD-8 were used in this work. They were all bred and collected by the Feed Science Institute, Zhejiang University. Among them, *C. capsuligena* ZD-1 and *A. oryzae* ZD-7 (used as reference research) could not be grown on Czapek's medium containing 0.5% of gossypol instead of sucrose as carbon source, but the other four strains grew well. The four strains were bred by a screening method of increasing gossypol concentration in Czapek's medium without sugar step by step, ultraviolet irradiation, chemical mutagen treatment and so on, at last rejuvenation by malt extract medium. Stock cultures were maintained on malt extract agar slants. Yeasts inocula were grown in 50 ml malt extract (5 °Bè) in 150 ml conical flasks at 30 °C for 48 h at 200 r/min. Filamentous fungal spores were washed from a 7-day agar slant culture with 10 ml sterile distilled water, and 5 ml aliquots were added to 100 g of sterilized solid substrate consisting of soybean meal and wheat bran (6:4, w/w), with adjusted moisture of 50% in 500 ml conical flasks, and incubated at 30 °C for 3 d in a 95% relative humidity chamber, then oven dried at 45 °C for 24 h, and processed into flour for the experimental inoculation.

Solid substrate fermentation

The treated substrate 100 g in each 500 ml conical flask was then inoculated with 5 ml either of yeast inocula, or 1% (w/w) mycelial inocula of filamentous fungi, and incubated at 30 °C for 48 h in a 95% relative humidity chamber. Triplicate flasks were set up for each experimental variation.

Sample processing

After fermentation was completed, every flask of fermented substrate was dried in an oven at 60 °C for 48 h respectively, and dry weight loss was determined. Then they were processed into flour for re-

lated index determination.

Related index assay

The dry matter (DM) content was measured by dying at 105 °C for 5 h. The crude protein (CP) assay was by Kjeldahl method (AOAC, 1999). FG was determined by the official method of the American Oil Chemists Society (AOCS, 1989). Amino acids assay was performed by the Center of Analysis and Measurements of Zhejiang University according to the AOAC (1984) method, using Beckman 6300 (Beckman Instrument, USA).

In vitro digestibility determination

Analysis procedure used was after the method of in vitro digestibility determination published by Sakamoto *et al*.(1980) and with little changes. Fermented or non-fermented CSM (10 g, exactly weighed) were put into 250 ml conical flasks. Then 30 ml 0.1 mol/L HCl and 30 mg pepsin were added and blended evenly and incubated at 39 °C at 150 r/min for 4 h. Then pH was adjusted to 7.0 and 30 ml of 40 U/ml trypsin solution was added, and blended again, then incubated at 39 °C and 150 r/min for 4 h. After digestion was completed, the digested suspension was centrifuged at 4000 r/min (1200×g) for 15 min. The sediment obtained was oven dried for nutrient assay.

In vitro nutrient digestibility (%)=(original nutrient amount–residual nutrient amount)/original nutrient amount×100%.

Statistical analysis

One-way analysis of variance was performed using the General Linear Models Procedures of the SAS software (SAS, 1999). Differences among means were tested using Duncan's multiple range tests. A significance level of 0.05 was used.

RESULTS AND DISCUSSION

Residual free gossypol and detoxification efficiency

Residual FG levels of fermented CSM substrate from different treatments were significantly lower ($P<0.05$) than control (substrate), indicating fermentation could decrease the FG content of CSM (Table 1).

The effect from the microbial species on FG contents could be differentiated statistically ($P<0.05$) as follows: *C. tropicalis* ZD-3 fermented CSM had lowest level, the amount of FG being found to be 29.8 mg/kg based on DM, detoxification efficiency reaching up to 94.57%; followed by *S. cerevisae* ZD-5, *A. niger* ZD-8 and *A. terricola* ZD-6, of which the FG levels in fermented substrate were up to 63.07, 81.50 and 93.87 mg/kg in DM respectively. Their detoxification efficiency reached 88.51%, 85.16% and 82.91% respectively. Although the effect of *C. capsuligena* ZD-1 and *A. oryzae* ZD-7 treatments was not better than those of other microflora, FG contents were reduced significantly ($P<0.05$) to safety level. The effect of this decrease may have been caused by the binding of FG to protein or amino acids secreted by microorganisms, or by introducing microbial enzymes degrading gossypol, or by both.

Crude protein determination

Table 1 presents the results of CP determination. It is evident from the results that CSM fermentation by different microbial strains improved CP content significantly ($P<0.05$), CP increased from 8.95% to 22.24%. Of all fungal treatments, *A. niger* ZD-8 fermentation efficiency was highest, CP content was increased by 22.24%. However, the contribution to CP increase of *C. capsuligena* ZD-1, *C. tropicalis*

Table 1 Effect of selected fungi on the reduction of gossypol levels during solid substrate fermentation of CSM

Treatment	FG (mg/(kg DM))	Detoxification efficiency (%)	CP (%DM)	CP increase percentage (%)
Substrate (control)	549.06±9.87^{a}	–	23.79±0.11^{c}	–
C. capsuligena ZD-1	145.51±10.02^{c}	73.50	25.92±0.23^{b}	8.95
C. tropicalis ZD-3	29.80±5.28^{f}	94.57	26.35±0.34^{b}	10.76
S. cerevisae ZD-5	63.07±7.38^{e}	88.51	26.43±0.09^{b}	11.10
A. terricola ZD-6	93.87±9.93^{d}	82.91	26.21±0.17^{b}	10.17
A. oryzae ZD-7	178.39±8.86^{b}	67.51	26.04±0.14^{b}	9.46
A. niger ZD-8	81.50±4.77^{d}	85.16	29.08±0.21^{a}	22.24

Means in a column without common superscript differ significantly ($P<0.05$)

ZD-3, *S. cerevisae* ZD-5, *A. terricola* ZD-6 and *A. oryzae* ZD-7 did not differ significantly from each other.

The additional amount of CP in CSM substrate was mainly due to the growth of microflora. Microbes converted substrate protein and other nutrients into microbial cell protein, secreted enzymes, and other biological products, consumed carbohydrate to supply energy, and meanwhile released CO_2 and H_2O, as well as some volatile materials, thus leading to a CP content increase per unit.

Among the six fungal strains used in this study, were three strains belonging to yeast, i.e., *C. capsuligena* ZD-1, *C. tropicalis* ZD-3 and *S. cerevisae* ZD-5; and three strains belonging to filamentous fungi, i.e., *A. terricola* ZD-6, *A. oryzae* ZD-7 and *A. niger* ZD-8.

Among the three yeast strains, detoxification efficiency and CP increase were highest for *C. tropicalis* ZD-3. The detoxification characteristics were according to Shi *et al*.(1998) and Yang *et al*.(2000), whereas the fermentation of *C. tropicalis* ZD-3 was more efficient. *S. cerevisae* ZD-5 is widely used in the feed fermentation industry, however, our trial results showed that *S. cerevisae* ZD-5 had better detoxification efficiency, although not more efficient than *C. tropicalis* ZD-3. It could decrease FG level in CSM substrate up to 63.07 mg/kg, which is far less than the restricted level of 100 mg/kg in swine feed. Therefore, *S. cerevisae* ZD-5 could be also applied for detoxification of CSM. The strain ZD-1 of *C. capsuligena* is mainly used in feed yeast production employing decomposed cellulose. It had ordinary effect on gossypol detoxification. The results presented above suggest that strain ZD-3 of *C. tropicalis* would be the first choice for microfloral detoxification of gossypol followed by *S. cerevisae* ZD-5.

Concerning filamentous fungi, the present results showed that *A. niger* ZD-8 was far more effective than the other two strains. It not only had a higher detoxification efficiency, but also had higher CP increase during solid fermentation of CSM. A characteristic of *A. niger* ZD-8 was that it had a good smell during CSM fermentation, and had a faster growth rate, and so is fit for fermenting CSM to reduce FG concentration. The strain of *A. terricola* ZD-6 had relatively good detoxification ability, and improving protein quality was common, and it does not have a good smell during fermentation. It could grew well on Czapek's medium containing 0.5% gossypol instead of sucrose as carbon source, therefore, *A. terricola* ZD-6 was a good biological engineering species for investigating the enzyme needed for gossypol degradation. The detoxification efficiency of strain *A. oryzae* ZD-7 was lower and it was not fit for fermentation detoxification.

The results in the present study demonstrated that *C. tropicalis* ZD-3 and *A. niger* ZD-8 are two better strains for fermentation detoxification of FG in CSM.

Amino acids assay of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8

Amino acids content of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 is presented in Table 2. The results showed that the TAA (total amino acids) and EAA of fermented CSM were increased significantly ($P<0.05$) compared with negative control, the TAA of substrate fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 were improved by 7.06% and 11.46%, and the EAA were increased by 7.77% and 12.64%, respectively. Especially, the levels of methionine, lysine and threonine were improved greatly ($P<0.05$), and the effectiveness of *A. niger* ZD-8 was superior to that of *C. tropicalis* ZD-3.

In vitro CP and AA digestibility of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8

The results of in vitro CP and AA digestibility of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 are presented in Table 3. The results demonstrated that in vitro CP and AA digestibility of fermented CSM was increased significantly ($P<0.05$) compared with control, the in vitro CP digestibility of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 was improved by 13.42% and 18.22%, the TAA were increased by 17.75% and 22.88%, and the EAA by 16.61% and 21.01%, respectively. In addition, the in vitro digestibility of methionine, lysine and threonine was also improved greatly ($P<0.05$). The experimental results suggested that the fermentation efficiency and digestibility of *A. niger* ZD-8 was superior to that of *C. tropicalis* ZD-3.

Table 2 Amino acids content (%) of CSM fermented or non-fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 (based on DM)

Nutrition composition	Substrate (control, *C*)	*C. tropicalis* ZD-3 (T_1)	$(T_1-C)/C\times100\%$	*A. niger* ZD-8 (T_2)	$(T_2-C)/C\times100\%$
DM	89.87	88.63	–	88.15	–
Asp	2.35[a]	2.35[a]	0	2.47[a]	5.28
Thr*	0.82[b]	0.95[a]	15.56	1.02[a]	24.31
Ser	1.05[b]	1.16[a]	10.32	1.22[a]	15.85
Glu	5.29[a]	5.58[a]	5.46	5.38[a]	1.74
Gly	1.04[b]	1.15[a]	10.40	1.24[a]	18.63
Ala	1.11[b]	1.27[b]	13.88	1.51[a]	35.68
Cys*	0.50[a]	0.52[a]	4.05	0.58[a]	16.78
Val*	1.03[b]	1.17[a]	13.54	1.28[a]	24.12
Met*	0.22[c]	0.30[b]	33.11	0.35[a]	57.36
Ile*	0.76[c]	0.89[b]	17.15	1.06[a]	40.43
Leu*	1.61[c]	1.82[b]	13.10	2.01[a]	24.80
Tyr	0.66[b]	0.73[a]	10.61	0.82[a]	24.20
Phe*	1.20[b]	1.40[a]	16.24	1.34[a]	10.91
Lys*	1.04[b]	1.16[a]	11.20	1.10[ab]	5.57
His*	0.60[a]	0.62[a]	3.25	0.64[a]	6.25
Arg*	2.65[a]	2.42[b]	–8.46	2.37[b]	–10.35
Pro	0.86[b]	0.93[a]	8.23	1.02[a]	18.79
TAA	22.80[b]	24.41[ab]	7.06	25.41[a]	11.46
EAA	10.44[b]	11.25[a]	7.77	11.76[a]	12.64

Means in a row without common superscript differ significantly ($P<0.05$); DM: Dry matter; TAA: Total amino acids; EAA: Essential amino acids; *The amino acid is an essential amino acid

Table 3 In vitro AA digestibility (%) of CSM fermented by *C. tropicalis* ZD-3 and *A. niger* ZD-8 (based on DM)

Nutrition composition	Substrate (control, *C*)	*C. tropicalis* ZD-3 (T_1)	$(T_1-C)/C\times100\%$	*A. niger* ZD-8 (T_2)	$(T_2-C)/C\times100\%$
CP	44.79[c]	50.80[b]	13.42	52.95[a]	18.22
Asp	41.38[b]	54.22[a]	31.03	56.83[a]	37.34
Thr*	65.74[b]	74.91[a]	13.95	77.06[a]	17.22
Ser	40.48[b]	52.11[a]	28.73	53.56[a]	32.31
Glu	38.76[b]	48.04[a]	23.94	49.94[a]	28.84
Gly	34.44[c]	49.17[a]	42.77	51.67[a]	50.03
Ala	52.58[a]	52.34[a]	–0.46	54.53[a]	3.71
Cys*	44.62[c]	49.96[b]	11.97	52.87[a]	18.49
Val*	49.08[a]	49.65[a]	1.16	50.88[a]	3.67
Met*	49.71[b]	65.38[a]	31.52	67.05[a]	34.88
Ile*	39.09[c]	49.33[b]	26.20	52.09[a]	33.26
Leu*	38.25[c]	46.89[b]	22.59	49.12[a]	28.42
Tyr	44.29[c]	49.28[b]	11.27	51.55[a]	16.39
Phe*	37.73[b]	47.97[a]	27.14	49.73[a]	31.80
Lys*	50.08[c]	58.04[b]	15.89	61.17[a]	22.14
His*	51.66[c]	62.80[b]	21.56	64.36[a]	24.58
Arg*	68.98[b]	72.13[ab]	4.57	74.54[a]	8.06
Pro	31.69[c]	34.62[b]	9.25	39.85[a]	25.75
TAA	45.80[c]	53.93[a]	17.75	56.28[a]	22.88
EAA	49.49[b]	57.71[a]	16.61	59.89[a]	21.01

Means in a row without common superscript differ significantly ($P<0.05$); DM: Dry matter; TAA: Total amino acids; EAA: Essential amino acids; *The amino acid is an essential amino acid

CONCLUSION

From the results of the present study it could be concluded that microbial fermentation is instrumental in reducing FG levels in CSM. The effectiveness differed between the species of microorganisms applied. From the perspective of reducing potential CSM toxicity, *C. tropicalis* ZD-3 was most successful followed by *S. cerevisae* ZD-5 and *A. niger* ZD-8. If crude protein, amino acids content and their in vitro digestibility are also taken into account, *A. niger* ZD-8 may be a good selection.

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