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A Study on the Reduction of Gossypol Levels by Mixed Culture Solid Substrate Fermentation of Cottonseed Meal

Wenju Zhang[1, 2], Zirong Xu[1, *], Jianyi Sun[1] and Xia Yang[1]
[1]Key Laboratory for Molecular Animal Nutrition of Ministry of Education, Feed Science Institute
Zhejiang University, Hangzhou, 310029, P. R. China

ABSTRACT : The objective of this work was to study the effect of mixed culture solid substrate fermentation of *C. tropicalis* ZD-3 with *A. niger* ZD-8 on detoxification of cottonseed meal (CSM), and to investigate the effect of fermentation period, proportion of CSM in substrate, sodium carbonate, minerals and heat treatment on the reduction of free gossypol levels during mixed culture solid substrate fermentation of CSM. Experiment 1: Three groups of disinfected CSM substrate were incubated for 48 h after inoculation with either of the fungi *C. tropicalis* ZD-3, *A. niger* ZD-8 or mixed culture (*C. tropicalis* ZD-3 with *A. niger* ZD-8). One non-inoculated group was used as the control. Levels of initial and final free gossypol (FG), CP and *in vitro* CP digestibility were assayed. The results indicated that mixed culture fermentation was far more effective than single strain fermentation, which not only had higher detoxification rate, but also had higher CP content and *in vitro* digestibility. Experiment 2: CSM substrates were treated according to experimental variables including fermentation period, proportion of CSM in substrate, sodium carbonate, minerals and heat treatment, Then, the treated CSM substrates were inoculated with mixed culture (*C. tropicalis* ZD-3 with *A. niger* ZD-8) and incubated at 30°C for 36 h in a 95% relative humidity chamber. After fermentation ended, FG and CP content of fermented CSM substrate was assayed. The results showed that the appropriate fermentation period was 36 h, and the optimal proportion of CSM in substrate was 70%. Addition of sodium carbonate to CSM substrate was beneficial for fermentative detoxification. Heat treatment could facilitate fermentative detoxification, and supplementation with minerals was instrumental in reducing gossypol levels during mixed culture solid substrate fermentation of CSM.
(**Key Words :** Fungi, Free Gossypol, Detoxification, Fermentation, Cottonseed Meal, Sodium Carbonate, Minerals)

INTRODUCTION

Gossypol, produced in the seeds of the cotton plant, is a naturally occurring toxin that deters insect pests. Feeding diets containing gossypol to animals would cause negative effects such as growth depression, reproductive disease and intestinal and other internal organ abnormalities (Berardi and Goldblatt, 1980; Francis et al., 2001; Robinson et al., 2001). Feeding gossypol to laboratory rodents would result in irregular estrous cycles and reducing pregnancy rates (Hahn et al., 1981; Lagerlof et al., 1985; Bender et al., 1988). In addition, Eisele (1986) reported that feeding sows and gilts diets containing 0.02% and 0.136% free gossypol (FG) had reduced conception rates by 72% and 77%, respectively, and failed to conceive; of those sows and gilts that did conceive, many aborted, produced stillborn piglets or had reduced litter sizes. An inhibition of ovarian steroidogenesis by gossypol in the monogastric female has been implicated as a possible cause for these detrimental effects on estrous cyclicity and the establishment and maintenance of pregnancy. The negative effect of gossypol on animal health has long been recognized, and the toxic effects of gossypol are much greater for non-ruminants than ruminants due to binding of FG to soluble proteins in the rumen (Willard et al., 1995). Therefore, FG will not do harm to animals if it is transformed into bound gossypol (BG), because BG cannot be absorbed through digestive tract. Commonly, cottonseeds are processed into oil and meal, which may contain high concentration of the toxin, thus, it is necessary for CSM to be further processed to reduce gossypol to permissible levels as animal protein feed resources.

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

* Corresponding Author: Zirong Xu. Tel: +86-571-86091820, Fax: +86-571-86994963, E-mail: zhang-wj1022@tom.com
[2] College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, P. R. China.
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(Barraza et al., 2002; Tabatabai et al., 2002), calcium hydroxide treatment (Nagalakshmi et al., 2002, 2003), microbes fermentation (Shi et al., 1998; Wu and Chen, 1989) and so on. These methods have played an important role in detoxification of CSM, especially, microbial fermentation might be a kind of promising detoxification method, because fermented CSM usually contains some kinds of exoenzyme (secreted by microorganism) such as cellulolytic enzyme, amylase, protease and lipolytic enzyme, some variety of vitamins, and some unknown active substance (Brock et al., 1994), but there are few literatures about CSM fermentation, detoxification efficiency was not very high and there was scarcely report about CSM fermentation by mixed cultures.

The objective of this work is to study the effect of mixed culture solid substrate fermentation of *C. tropicalis* ZD-3 with *A. niger* ZD-8 on detoxification of CSM, and investigate the effect of fermentation period, proportion of CSM in substrate, sodium carbonate, minerals and heat treatment on the reduction of FG levels during mixed culture solid substrate fermentation of CSM, meanwhile, study the morphology of strains in the fermented CSM substrate through the environmental scanning electron microscope (ESEM) technology.

MATERIALS AND METHODS

Basal substrate treatment

CSM was obtained from Xinjiang autonomous region, China. This material was mixed with corn flour and wheat bran, the ratio of CSM:corn flour:wheat bran is 7:2:1, then the mixture was moistened, and the ratio of mixture to water is 1:0.8, after that, autoclaved at 112.6°C for 20 min.

Microorganisms and inocula

The strains *Candida tropicalis* ZD-3 and *Aspergillus niger* ZD-8 were used in this study. They were bred and collected by Feed Science Institute of Zhejiang University. The two strains were bred by the screening method, that is, increasing gossypol concentration in the Czapek's medium without sugar step by step, ultraviolet radiating, chemical mutagen treating and so on, at last rejuvenated by malt extract medium. Stock cultures were maintained on malt extract agar slants. Yeast inocula was grown in 50 ml malt extract (5° Bè) in 150 ml conical flasks at 30°C for 24 h at 200 rpm. Filamentous fungi 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, which consists of soybean meal and wheat bran (6:4, w/w), adjusted moisture 50% in 500 ml conical flasks, and incubated at 30°C for 3 days in a 95% relative humidity chamber, then oven dried at 45°C for 24 h, processed into flour for trial mycelial inocula.

Solid substrate fermentation

Single strain fermentation: The treated substrate 60 g in each 500 ml conical flask was inoculated with 3 ml either of *C. tropicalis* ZD-3 inocula, or 1% (w/w) of mycelial inocula of *A. niger* ZD-8, then incubated at 30°C for 48 h in a 95% relative humidity chamber. Triplicate flasks were set up for each experimental variation.

Mixed culture (two strains) fermentation: The treated substrate 60 g in each 500 ml conical flask was inoculated with 3 ml of *C. tropicalis* ZD-3 inocula, and 1% (w/w) of mycelial inocula of *A. niger* ZD-8, evenly blended, then incubated at 30°C for 36 h in a 95% relative humidity chamber. Triplicate flasks were set up for each experimental variation.

ESEM photo

The treated basal CSM substrates were inoculated with *C. tropicalis* ZD-3 inocula, mycelial inocula of *A. niger* ZD-8 and mixed culture, respectively, the control was not inoculated. After fermentation ended, fresh samples were sent to Center of Analysis and Measurements of Zhejiang University for ESEM photo.

Sample processing

After fermentation ended, every flask of fermented substrate was dried in an oven at 60°C for 48 h respectively, then processed into flour for related index analysis.

Chemical index assay

The dry matter (DM) content was measured by dying at 105°C for 5 h. The CP assay was by Kjeldahl method (AOAC, 1999). FG was determined by the official method of the American Oil Chemists Society (AOCS, 1989).

In vitro digestibility determination

Analysis procedure was referred to the methods of *in vitro* digestibility determination by Sakamoto et al. (1980) and Ando et al. (2005), and made a little amendment. Briefly, fermented CSM 10 g, exactly weighed, were put into 250 ml conical flasks, added with pH 2.0, 10,000 μ/ml pepsin solution 30 ml, blended evenly, incubated at 39°C and 150 rpm for 4 h, then adjusted pH to 7.0, added with 625 U/ml trypsin solution 30 ml, and blended again, then incubated at 39°C and 150 rpm for 4 h. After digestion completed, digesta suspension was centrifuged at 4,000 rpm for 15 min, then sediment was collected, and in an oven dried for nutrient assay.

In vitro nutrient digestibility(%)
= (origin nutrient amount-residual nutrient amount)
/origin nutrient amount×100%

Table 1. Fermentation results of C. tropicalis ZD-3 or /with A. niger ZD-8[1] (based on DM)

	FG content (mg/kg)	Detoxification rate (%)	CP content of substrate (%)	*In vitro* CP digestibility (%)
CSM substrate (control)	549.06[a]	0	23.79[c]	44.79[c]
C. tropicalis ZD-3	29.80[d]	94.57	26.35[b]	50.80[b]
A. niger ZD-8	81.50[b]	85.16	29.08[a]	52.95[a]
Mixed fermentation	45.92[c]	91.64	30.41[a]	54.15[a]
SEM[2]	143.92	-	1.70	2.40

[1] Values are presented as means, n = 3 per treatment. Means in a column with no common superscript differ significantly ($p<0.05$).
[2] Standard error of the mean.

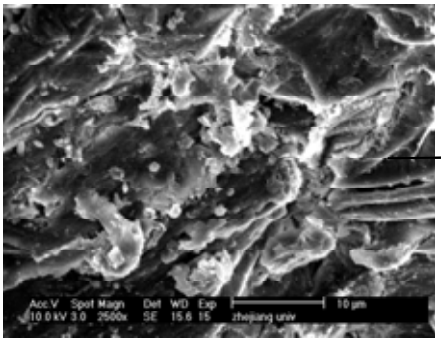

Figure 1. ESEM photo of CSM substrate not fermented (control) (2,500×).

Statistical analysis

The data was analysed by one-way ANOVA Procedures of the SAS software (1989). Differences among means were tested using Duncan's multiple range tests. A significant level of 0.05 was used.

RESULTS AND DISCUSSION

Effect of CSM substrate fermented by *C. tropicalis* ZD-3 or /with *A. niger* ZD-8

Three groups of disinfected CSM substrate were incubated at 30°C for 48 h after inoculation with either of the fungi *C. tropicalis* ZD-3, *A. niger* ZD-8 or mixed culture (*C. tropicalis* ZD-3 with *A. niger* ZD-8). One non-inoculated group was used as the control. Levels of initial and final FG, CP and *in vitro* CP digestibility were assayed.

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

The effect from the microbial species on FG contents could be differentiated statistically ($p<0.05$) as follows: the CSM substrate fermented by *C. tropicalis* ZD-3 had the lowest FG level, the amount of FG was 29.8 mg/kg DM, and detoxification efficiency reached up to 94.57%; followed by mixed culture (*C. tropicalis* ZD-3 with *A. niger* ZD-8) and *A. niger* ZD-8, which FG content was 45.92 and 81.50 mg/kg DM respectively, and detoxification rate was 91.64% and 85.16%, respectively.

The reason of FG decrease was due to binding FG to protein and/or amino acids secreted by microorganisms, or due to introducing microbial exoenzyme of gossypol degradation, or by both (Risco et al., 1992; Willard et al., 1995). Detoxification characteristics of *C. tropicalis* ever studied by Shi et al. (1998) and Yang et al. (2000), but the fermentation of *C. tropicalis* ZD-3 was more efficient.

The results of CP determination are shown in Table 1. Of all fermentation treatments, fermentation efficiency of mixed culture was most advanced, CP content increased by 27.83%, followed by *A. niger* ZD-8 and *C. tropicalis* ZD-3, CP content increased by 22.24% and 10.76%, respectively. It was evident from the results that CSM fermentation by different microbial strains could improve CP content significantly ($p<0.05$).

The additional amount of CP in CSM substrate is mainly due to the growth of microflora. Microbes converted substrate nutrients such as protein into microbial cell protein, secreted enzymes and other biological substance to outside of cells, consumed carbohydrate to construct cell component and supply energy for cell metabolism, meanwhile released CO_2 and H_2O, and some volatile materials, thus led to CP content increase per unit (Prescott, et al., 2002). Secondly, fermented CSM contained lots of volatile fatty acids and other volatile substance that were lost when dried in an oven for sample, thus led to increase of proportion of protein in substrate. In addition, the inocula of *C. tropicalis* ZD-3 and *A. niger* ZD-8 contained high concentration of CP by 40-60% based on DM, which could also result in crude protein content increase per unit when inoculated to substrate for fermentation.

The results of *in vitro* CP digestibility of fermented CSM are presented in Table 1. The *in vitro* CP digestibility of CSM fermented by mixed culture, *A. niger* ZD-8 and *C. tropicalis* ZD-3 alone were improved by 20.90%, 18.22% and 13.42%, respectively. The results demonstrated that *in vitro* CP digestibility of fermented CSM was increased significantly ($p<0.05$) compared with the control, and the effect of mixed culture fermentation was the best.

From the present results of detoxification efficiency, CP content and *in vitro* CP digestibility, it could be concluded that mixed culture fermentation was far more effective than other two strains fermentation alone, which not only had

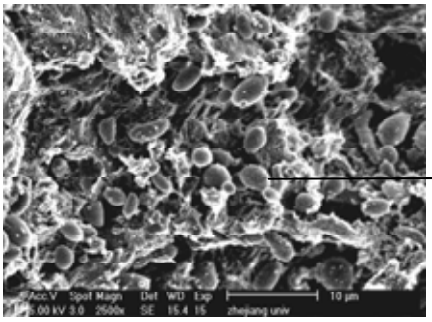

Figure 2. ESEM photo of CSM substrate fermented by *C. tropicalis* (2,500×).

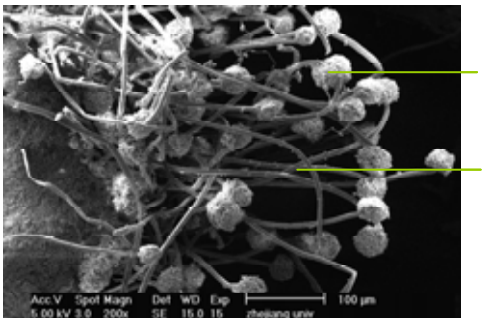

Figure 3. ESEM photo of CSM substrate fermented by *A. niger* (200×).

higher detoxification rate, but also had higher CP increase and *in vitro* digestibility during solid substrate fermentation of CSM. In addition, characteristics of mixed culture were with good smell during CSM fermentation, and with faster growth rate, so it was fit for fermenting CSM to reduce FG concentration and increase nutritive value.

ESEM photo of CSM substrate fermented by *C. tropicalis* ZD-3 or /with *A. niger* ZD-8

ESEM photo of CSM substrate, not fermented (control), is given in Figure 1. Figure 1 showed that there was not microorganism on substrate. ESEM photo of CSM substrate fermented by *C. tropicalis* is presented in Figure 2. There were a number of spheres shape of *C. tropicalis* on substrate, which size in diameter was from around 2 μm to 5 μm. Figure 3 shows the ESEM photo of CSM substrate fermented by *A. niger*. It was clearly observed that there were lots of mycelia of *A. niger* on substrate. And conidial head and conidiophore could be seen on the photo. The length of mycelium was around 500 μm and the size of sporangium in diameter was about 50 μm. ESEM photo of CSM substrate fermented by mixed culture, *C. tropicalis* with *A. niger*, is given in Figure 4. Oval shape of yeasts and filamentous mycelia could be observed clearly on substrate, and mixed together. The size of mycelia in diameter had been reduced to around 2 μm, less than the mycelia in the substrate fermented by *A. niger* alone, which was about 10 μm. On the photo, conidial head could not be observed and only mycelia could be seen, but morphology of *C. tropicalis* ZD-3 was found not being transformed.

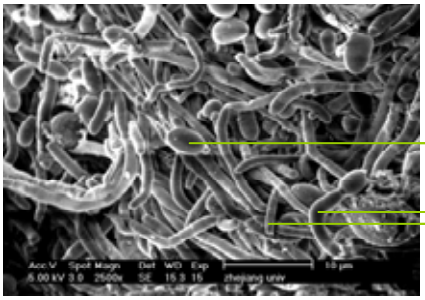

Figure 4. ESEM photo of CSM substrate fermented by *C. tropicalis* with *A. niger* (2,500×).

Effect of fermentation period on mixed culture solid substrate fermentation of CSM

The treated basal substrate was inoculated with *C. tropicalis* ZD-3 inocula and mycelial inocula of *A. niger* ZD-8 (mixed culture), then incubated at 30°C for 24, 36 or 48 h in a 95% relative humidity chamber. Triplicate flasks were set up for each experimental variation.

The results are presented in Table 2. The FG content of CSM substrate fermented for selected periods (24, 36, 48 h) was decreased significantly ($p<0.05$) compared with the control (0 h). Among different treatments, the fermentation period of 48 h was with the lowest gossypol level (45.44 mg/kg), which detoxification rate reached up to 91.72%, but which had not significant difference ($p>0.05$) compared with the other two treatments (24 and 36 h). The CP contents of 24, 36 and 48 h fermentation period were improved by 14.47%, 19.42% and 21.06%, respectively,

Table 2. The effect of fermentation period on mixed culture fermentation[1] (based on DM)

	FG content (mg/kg)	Detoxification rate (%)	CP content of substrate (%)	CP increase percentage (%)
CSM substrate (control)	549.06[a]	-	23.79[b]	-
24 h	50.48[b]	90.81	27.23[a]	14.47
36 h	46.83[b]	91.47	28.41[a]	19.42
48 h	45.44[b]	91.72	28.80[a]	21.06
SEM [2]	144.77	-	1.32	-

[1] Values are presented as means, n = 3 per treatment. Means in a column with no common superscript differ significantly ($p<0.05$).
[2] Standard error of the mean.

Table 3. Ingredient of substrates with different proportion of CSM (%)

Groups	Cottonseed meal	Corn flour	Wheat bran
CSM100 *	100	0	0
CSM90	90	6	4
CSM80	80	12	8
CSM70	70	20	10
CSM60	60	25	15
CSM50	50	30	20

* The number behind the character "CSM" refers to the proportion of CSM in substrate.

compared with the control. The results suggested that CP content of different fermentation periods could be increased significantly ($p<0.05$) compared with the control.

Fermentation period is an important factor for fermentation efficiency. But the results of previous experiments about the effect of fermentation period on fermentation detoxification were generally inconsistent. There was 36 h period (Yang et al., 2000), 4-6 d period (Wu et al., 1989), and 48 h period (Shi et al., 1998). The present results indicated that detoxification efficiency was slightly increased with the fermentation period prolonged, and the fermentation period of 36 h might be a better selection for mixed culture solid substrate fermentation of CSM.

Effect of proportion of CSM in substrate on detoxification of CSM

Substrates ingredient are presented in Table 3. Individual substrate was moistened and autoclaved. Then the treated substrates were fermented by mixed culture.

The results are presented in Table 4. The proportion of CSM in substrate had not significant influence on detoxification of CSM among different treatments, but detoxification rate had slight increase with the decrease of proportion of CSM in substrate. The detoxification rates of different proportion of CSM were all more than 90%, especially, the detoxification efficiency of 50% proportion of CSM reached up to 94.91%.

The proportion of CSM in substrate in the previous investigation was 100%, adding nothing to substrate (Shi et al., 1998; Wu et al., 1989; Yang et al., 2000). However, CSM contained higher concentration of CP, crude fiber and crude fat, lower levels of fermentable carbohydrates.

Table 4. The effect of proportion of CSM in substrate on the reduction of gossypol levels (based on DM)[1]

Groups	FG content of Pre-fermentation (mg/kg)	FG content of Post-fermentation (mg/kg)	Detoxification rate (%)
CSM100*	784.37	71.17	90.93
CSM90	705.93	65.92	90.66
CSM80	627.50	61.52	90.20
CSM70	549.06	40.60	92.61
CSM60	470.62	36.37	92.27
CSM50	392.19	19.96	94.91

[1] Values are presented as means, n = 3 per treatment.

* The number behind the character "CSM" refers to the proportion of CSM in substrate.

Therefore adding fermentable carbohydrate such as maize flour and wheat bran to CSM substrate benefit fermentation. The present results suggested that the substrates containing different proportion of CSM fermented by mixed culture *C. tropicalis* ZD-3 with *A. niger* ZD-8 all could acquire superior detoxification effect, but 70% CSM in substrate might be an appropriate proportion. The proportion of CSM in other experiment of this study accounted for 70% in substrate.

Effect of sodium carbonate on the reduction of gossypol levels

Basal CSM substrate was added with 0, 0.5% and 1.0% of sodium carbonate, then moistened and autoclaved. The treated substrates were fermented by mixed culture.

The results are presented in Table 5. The FG contents of 0, 0.5% and 1.0% sodium carbonate treatment were 46.99, 39.28 and 33.16 mg/kg, respectively, and their detoxification efficiency reached up to 91.44%, 92.85% and 93.96%, respectively. Therefore, the FG content of different treatments was significantly lower ($p<0.05$) than the basal substrate. The CP content of fermented CSM substrates were also increased significantly ($p<0.05$), and the CP increase percentage was from 19.13% to 21.06%. The effects of sodium carbonate treatments were better than the control, the detoxification rate increased slightly with the rise of sodium carbonate concentration, and the FG contents of sodium carbonate treatments were significantly lower ($p<0.05$) than the control, but there were no significant differences in CP content between the control and the

Table 5. The effect of sodium carbonate on the reduction of gossypol levels[1] (based on DM)

Treatment	FG content (mg/kg)	Detoxification efficiency (%)	CP content (%)	CP increase percentage (%)
Basal substrate	549.06	-	23.79 [b]	-
Control	46.99 [a]	91.44	28.34 [a]	19.13
0.5 % sodium carbonate	39.28 [b]	92.85	28.80 [a]	21.06
1.0 % sodium carbonate	33.16 [c]	93.96	28.29 [a]	18.92
SEM [2]	4.00	-	1.36	-

[1] Values are presented as means, n = 3 per treatment. Means in a column with no common superscript differ significantly ($p<0.05$).

[2] Standard error of the mean.

Table 6. The effect of minerals and heat treatment on the reduction of gossypol levels[1] (based on DM)

Treatment	FG content (mg/kg)	Detoxification efficiency (%)	CP content (%)	CP increase percentage (%)
Basal substrate	549.06 [a]	-	23.79 [b]	-
Control (heated)	46.99 [c]	91.44	28.34 [a]	19.13
Unheated treatment	147.12 [b]	73.21	27.67 [a]	16.31
minerals	29.44 [d]	94.64	28.52 [a]	19.88
SEM[2]	140.22	-	1.28	-

[1] Values are presented as means, n = 3 per treatment. Means in a column with no common superscript differ significantly ($p<0.05$).
[2] Standard error of the mean.

sodium carbonate treatments.

The experimental effects showed that the addition of sodium carbonate to CSM substrate decreased significantly ($p<0.05$) FG levels, and was beneficial for fermentation detoxification of CSM. The results suggested that supplementation with 0.5% sodium carbonate to substrate was appropriate during mixed culture solid substrate fermentation of CSM.

Sodium carbonate is an alkaline substance. The action of sodium carbonate for the CSM substrate was mainly caused by the pH effect of the carbonate ion (Fodor, 1999). For this reason the effect of sodium carbonate on the substrate depends on the buffer capacity of fermentation ecosystem. The pH of CSM substrate would decrease when fermented by the mixed culture, and lower pH value did not benefit fermentation because the strains growth was inhibited. However, adding optimum quantity of sodium carbonate to substrate could increase pH value to appropriate range due to buffer capacity of sodium carbonate/bicarbonate, and therefore could facilitate fermentation of CSM.

Effect of heat treatment on the reduction of gossypol levels

CSM substrate was moistened, then the wet medium was autoclaved at 112.6°C for 20 min (heated), or not autoclaved (unheated). The treated substrates were fermented by mixed culture.

The results are presented in Table 6. The FG content of heated CSM was significantly ($p<0.05$) lower than unheated CSM, and detoxification rate was increased from 73.21% to 91.44%. The CP content of heated treatment was slightly higher ($p>0.05$) than unheated treatment, and heated and unheated treatment increased CP content by 19.13% and 16.31%, respectively. These results showed that heat treatment benefit fermentation and gossypol removal during mixed culture solid substrate fermentation of CSM.

High temperature favors the formation of stable bonds between gossypol and other molecules. Bound gossypol is generally considered to be physiologically inactive (Randel et al., 1992). Nagalakshmi et al. (2002) reported that CSM was cooked at 100°C for 30, 45 and 60 min; or pressure cooked at 5, 10 and 15 min. The raw CSM contained FG 2,700 mg/kg. The FG content was lowered ($p<0.01$) by the 30-min cooking and then with the 45-min cooking; however, a further increase in cooking time (60 min) did not reduce FG content. A linear reduction ($p<0.01$) of FG was observed as the time of pressure-cooking increased. The results of previous experiments on the effects of heat treatment on detoxification of CSM were generally consistent (Shi et al., 1998; Wu et al., 1989; Yang et al., 2000). On the present results, CSM substrate was firstly autoclaved, then inoculated and incubated, FG content was greatly decreased ($p<0.05$) compared with unheated treatment. These results suggested that heat treatment could facilitate microbial fermentation detoxification of CSM.

Effect of minerals on the reduction of gossypol levels

Mineral solution tested contains 2 g $NaNO_3$, 1 g $MgSO_4 \cdot 7H_2O$, 2 g K_2HPO_4, 1 g NaCl, 2 g $FeSO_4$, 0.6 g $MnSO_4$ and 0.4 g $CuSO_4 \cdot 5H_2O$ for 1 L solution. CSM substrate was moistened with this mineral solution and the ratio of substrate to solution is 1:0.8, then the wet medium was autoclaved. The control CSM substrate was moistened with distilled water. The treated substrates were fermented by mixed culture.

Table 6 showed the results. The results showed that supplementation with minerals could further decrease FG content in CSM substrate significantly ($p<0.05$), reduced FG content by 37.35% compared with the control, and increased detoxification efficiency from 91.44% to 94.64%. The CP content of minerals treatment was slightly higher than the control. It could be concluded that supplementation with minerals was instrumental in reducing gossypol levels during mixed culture solid substrate fermentation of CSM.

In this study, mineral elements had well defined metabolic roles. Phosphates contribute to the buffering capacity of the medium, and phosphorous is a constituent of nucleic acids, phospholipids and coenzymes. Supplying potassium or sodium ions mainly changes the osmotic pressure. Potassium is a major cation in microbial cells, especially as a cofactor for such enzymes as phosphohexokinases. Sodium is required by a number of microbial species (Caldwell et al., 1973; Durand and kawashima, 1980). It is well known that Cu^{2+}, Mn^{2+}, Fe^{2+} and Mg^{2+} are all cofactors of corresponding enzymes. The

present results suggested that adding minerals solution to CSM substrate benefited fermentation detoxification.

IMPLICATION

This study indicated that mixed culture fermentation was far more effective than single strain fermentation, which not only had higher detoxification rate, but also had higher CP content and *in vitro* digestibility. The proper fermentation period was 36 h, and the suitable proportion of CSM in substrate was 70%. Adding sodium carbonate to CSM substrate was beneficial for fermentation detoxification. Heat treatment could facilitate fermentation detoxification of CSM, and supplementation with minerals was instrumental in reducing gossypol levels during mixed culture solid substrate fermentation of CSM.

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