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Reduction of Free Gossypol Levels in Cottonseed Meal by Microbial Treatment

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ABSTRACT

Experiments were carried out to study the effect of some local selected fungi on the reduction of free gossypol (FG) levels during solid-state fermentation (SSF) of cottonseed meal (CSM) and evaluate the most effective strain for biodegradation of FG (BFG) through optimization the parameters of SSF process and also to determine the crude protein (CP) and amino acid (AA) content of CSM substrate fermented under optimal conditions. Results indicated that microbial fermentation could greatly decreased FG levels in CSM, but it differed among species of microorganisms with *Candida tropicalis* the most effective. The optimum fermentation conditions for BFG by *C. tropicalis* were incubation period of 48 h, incubation temperature at 30°C, inoculum level at 1 x 10^7 cells g^{-1} of solid substrate, moisture content of solid substrate 55% and pH in nature (5.2). The CP and AA content of the fermented substrate under optimizing conditions were improved markedly.

Key Words: Fungi; Free gossypol; Fermentation; Cottonseed meal

INTRODUCTION

Free gossypol (FG, polyphenolic binaphthyl dialdehyde, $C_{30}H_{30}O_8$), a toxic polyphenolic yellow pigment, produced in the seeds of the cotton plant as a naturally occurring toxin that deters insect pests. Its toxicity is a major concern for use of CSM as an animal feed (Roger *et al*., 1960). Feeding diets containing FG to animals would cause negative effects such as decrease of growth and feed conversion, depression of fertility, as well as intestinal and internal organ abnormalities (Berardi & Goldblatt, 1980; Robinson *et al*., 2001; Francis *et al*., 2001; Santos *et al*., 2003; Carruthers *et al*., 2007).

Negative effects of FG on animal health have long been recognized and toxic effects of FG are much greater in non-ruminants than ruminants due to binding of FG to soluble proteins in the rumen (Willard *et al*., 1995). Thus, if FG was transformed into BG (bound gossypol), it would not harm the animal because BG cannot be absorbed from digestive tract. Commonly, cottonseeds are processed into oil and meal, which may contain high concentrations of FG, thus, it is necessary for CSM to be further processed to reduce FG to permissible levels as animal protein feed resources.

Researchers studied for many years to find a way to detoxify FG and proposed a number of methods, such as, solvent extraction, by liquid cyclone and/or acetone (Pons & Eaves, 1971; Gardner *et al*., 1976); chemical treatment with iron sulfate (Barraza *et al*., 1991; Tabatabai *et al*., 2002) or calcium hydroxide (Nagalakshmi *et al*., 2003). Unfortunately, these methods affect the protein nutritive quality and are not in commercial use now. The reduction of FG using solvents suffers from the difficulty of totally removing residual solvents that may be potentially harmful to the animals that consume them, while calcium hydroxide often reduces the biological activity of vitamins and lowers detoxification efficiency (Zhang *et al*., 2006). Although, detoxification by iron sulfate was a convenient method, through the binding of FG, it did not block absorption of FG or BG (Barraza *et al*., 1991). It is vital to develop a new approach for degradation FG and prevention of its absorption into animal body.

It has been found that a few microorganisms are capable of degrading FG, including *Candida tropicalis, Torulopsis candida, Aspergillus flavus* and *A. niger* (Weng & Sun, 2006). CSM detoxicated by microorganisms not only reached safe criteria, but also highly enhanced the content of protein, amino acids, coenzymes (secreted by microorganisms) such as, cellulolytic enzymes, amylase, protease and lipolytic enzyme and some variety of vitamins (Wu & Chen, 1989; Brock *et al*., 1994; Jianyi,1997; Shi *et al*.,1998).

Solid-state fermentation (SSF) was used to produce industrial products including enzymes (Pandey, 1992) as well as microbial biomass and is an attractive process to produce valuable products due to its low capital investment and operating expenses (Deschamps *et al*., 1985). Therefore, it is attractive to explore the use of SSF as a

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process for BFG by microorganisms. The objectives of this study were to investigate the effect of some selected fungi on reduction of FG levels during SSF of CSM. We examined whether the selected fungi could reduce FG levels to an acceptable range and defined optimal fermentation conditions for detoxification to provide practical guidelines for using CSM as an animal feed protein source.

MATERIALS AND METHODS

Microorganisms. The strains *C. tropicalis*, *Saccharomyces cerevisae*, *Aspergillus oryzae*, *A. terreus* and *A. niger* were used as they are often used in feedstuff fermentation and have no known harmful effects on animals. They were part of cultures collected by the authors from different sources. Stock cultures were maintained on malt extract agar slants at 4°C and subcultured every month.

Basal substrate. The CSM was purchased from local market and stored at room temperature (25-30°C) until used.

Inoculum preparation. Yeasts inocula were prepared by transferring 2 mL suspension from 48 h old slant culture into 250 mL Erlenmeyer flasks containing 50 mL of sterile malt extract liquid medium at pH 5.5. The inoculated flasks were incubated on a rotary shaker at 200 rpm for 24 h at 30°C. Filamentous fungi spores were prepared by washing a 10 days old agar slants with sterile saline solution, containing 0.1% Tween 80. The harvested suspensions were collected until used.

SSF with the selected fungi. The experiments were conducted in 250 mL Erlenmeyer flasks containing 30 g CSM at natural condition of pH value (5.2). Distilled water was used in such away that the initial moisture content (IMC) in CSM was 55% (w/w). After sterilization by autoclaving at 121°C for 20 min, the flasks were cooled to room temperature and inoculated with an inoculum concentration of 10^6 cells or spores g^{-1} substrate, then incubated at 30°C for 72 h. Triplicate flasks were set up for each experiment variation. The best BFG strain achieved by this step was used in subsequent experiments.

Development of the SSF process by *C. tropicalis*. In order to optimize the fermentation conditions for BFG under SSF by *C. tropicalis*, the most effective strain, the optimum solid medium, CSM, was adopted. Various fermentation conditions (IMC, initial pH, incubation temperature, inoculum level & fermentation period) of the process of biodegradation were analyzed.

Effect of IMC. The fermentation was carried out under various IMC (40, 45, 50, 55, 60, 65, 70%). IMC was adjusted with distilled water. Other conditions were natural pH (5.2), inoculum level at 10^6 cells g^{-1} substrate, incubation temperature 30°C and incubation period for 72 h. The optimum IMC of solid substrate achieved by this step was fixed for subsequent experiments.

Initial pH. Different initial pH levels of the CSM, 3, 4, 5, 6, 7 and 8 adjusted with 1 N HCl or 1 N NaOH, were employed to investigate their effects on BFG. The fermentation was performed at 30°C for 72 and inoculum level 10^6 cells g^{-1} CSM in optimum IMC. The optimum initial pH achieved by this step was fixed for subsequent experiments.

Incubation temperature. The fermentation was carried out at various temperatures (20, 25, 30, 35 & 40°C) for 72 h to study their influence on BFG. All other conditions were kept at their optimum levels. The optimum incubation temperature achieved by this step was fixed for subsequent experiments.

Inoculum level. Various inoculum levels at 10^3, 10^5, 10^7 and 10^9 cells g^{-1} were used to evaluate their effects on BFG. The fermentation was carried out under optimum IMC and natural pH at 30°C for 72 h. The optimum inoculum level achieved by this step was fixed for subsequent experiments.

Fermentation period. Various incubation periods (24, 48, 72, 96 & 120 h) were used and the fermentation was performed with other parameters kept at optimum levels.

Sample processing. After fermentation was complete, fermented substrates were dried in an oven at 60°C for 48 h and weight loss determined. Samples were subsequently processed into flour for related analyses.

Related assays. The dry matter (DM) and moisture content of solid and/or fermented substrate were measured by drying at 105°C to constant weight. FG was determined by quick method described by Hron *et al.* (1996). Crude protein (CP) assay was by Kjeldahl method (AOAC, 1999). Amino acids (AA) assay was performed by the Center of Analysis and Measurements of NCRRT according to the AOAC (1999) method number 994.12 (Llames & Fontaine, 1994) using Biochrom 20 (Biochrom Instrument, Swede).

***In vitro* digestibility determination.** *In vitro* digestible CP and AA in the CSM substrate fermented by *C. tropicalis* under optimal conditions was determined following the method of Sakamoto *et al.* (1980) and its minor modifications published by Zhang *et al.* (2006).

Statistical analysis. Each experiment was performed in three replicates and analyses were carried out in duplicate. Data given here are the averages of the measurements. The standard deviation of the duplicate never exceeded ± 6% of the mean through out present work.

RESULTS AND DISCUSSION

BFG in CSM by selected fungi. Residual FG levels of fermented CSM substrate due to fungal treatments were lower than the control (non-treated), indicating fermentation decreased the FG content of CSM (Table I). The local isolate of *C. tropicalis* fermented CSM had the lowest level of FG, with BFG of 86.18% followed by *S. cerevisae* and *A. niger*. Although the effect of *A. oryzae* and *A. terreus* was least, their FG contents were also reduced (Table I).

Microbial fermentation greatly decreased FG levels in CSM. This decrease may have been caused by binding of

FG to proteins or amino acids secreted by microorganisms and/or by microbial secreted enzymes that degraded gossypol. Detoxification results of CSM by *C. tropicalis* were consistent with Shi *et al.* (1998) and Zhang *et al.* (2006).

Whereas fermentation of *C. tropicalis* was more efficient, *S. cerevisae* had a high detoxification, although not more than *C. tropicalis*, but decreased the FG level in CSM substrate to 91 mg kg^{-1}, which is lower than legislated level 100 mg kg^{-1} in swine feed (Zhu & Xia, 2003). *A. niger* was far more effective in reducing FG than the other two filamentous strains. A character is at *A. niger* was that it had a faster growth rate during CSM fermentation and was suitable for fermenting CSM to reduce FG. The detoxification rate of *A. terreus* was much lower, making it unsuitable for FG detoxification (Table I).

Optimum IMC. Moisture content of solid medium is one of main factors, which determines the success of the SSF process. High BFG efficiency (86.2%) in CSM treated with *C. tropicalis* was attained when the initial moisture level was 55% in comparison with that at low or high moisture levels (Fig. 1). The critical importance of moisture level in SSF media and its influence on the BFG can be attributed to the interference of moisture in the physical properties of the solid particles. The low moisture content reduced the solubility of nutrients of the substrate and degree of swelling (Murthy, 1999) and increased water loss due to quick volatilization during fermentation and hence inhibited the growth of microorganism. Higher moisture content reduce the porosity of substrate, thus limiting heat and oxygen transfer, which was beneficial for growth of microorganism among particles in solid substrate (Ohno *et al.*, 1992; Balakrishna & Pandey, 1996).

Optimum initial pH. The effect of initial pH on BFG by SSF was shown in Fig. 2. Because the metabolic activities of microorganism were very sensitive to pH changes, the BFG by *C. tropicalis* was found to be affected when pH level was deviated from the optimum value (Fig. 2). BFG did not vary significantly within an initial pH range of 5-6. There was poor growth of *C. tropicalis* and low BFG at pH 3.0. The BFG increased to 86.8% and 82% at pH 5.0 and 6.0, respectively. Further increasing pH, BFG and the growth of *C. tropicalis* declined. Weng and Sun (2006) found that the maximal BFG in solid substrate occurred by *C. tropicalis* ZAU-1 at initial pH 4-6. Our results indicated there was no need to adjust the pH of the CSM medium, since its inherent pH 5.2 at which was optimum for *C. tropicalis* growth and BFG.

Incubation temperature. The maximum BFG efficiency (86.5%) was attained at 30°C (Fig. 3), indicating this temperature being favorable for stable cultivation in solid medium. A slight decrease in the BFG (81%) was observed when the incubation temperature was higher than the optimum incubation temperature (35°C). The BFG was markedly lower at 20-25°C than 30°C. When the incubation temperature rose to 40°C, the BFG declined to 57% (Fig. 3).

Table I. Effect of selected fungi on FG and BFG levels in treated CSM

Fungi	FG (mg kg^{-1} DM)	BFG (%)
Substrate, CSM (Control)	550	-
C. tropicalis	76	86.18
S. cerevisae	91	81.04
A. niger	125	73.95
A. oryzae	172	64.16
A. terreus	236	50.83

Table II. Crude protein (CP) and amino acid (AA) content (g kg^{-1} DM) of CSM non-fermented (control) or fermented by *C. tropicalis*

Nutrients	Control CSM	Fermented CSM
CP	204.6	235.8
Asp	17.2	19.4
Thr*	12.6	13.8
Ser	8.5	10.0
Glu	43.2	46.3
Pro	7.0	10.5
Gly	8.3	9.1
Ala	9.0	9.8
Cys*	3.6	4.0
Val*	9.1	9.7
Met*	1.8	2.8
Ile*	6.2	6.5
Leu*	14.4	16.3
Try	5.2	6.1
Phe*	9.5	10.9
His*	4.7	5.6
Lys*	7.6	8.3
Arg*	20.4	21.6
Total AA	188.3	210.7
*Essential AA	79.9	89.5

Table III. *In vitro* digestible crude protein (CP) and amino acid (AA) content (g kg^{-1} DM) of CSM non-fermented (control) or fermented by *C. tropicalis*.

Nutrients	Control CSM	Fermented CSM
CP	86.2	115.6
Asp	7.4	11.8
Thr*	3.3	5.1
Ser	3.5	4.7
Glu	18.2	23.5
Pro	2.4	3.0
Gly	2.8	4.4
Ala	3.5	4.6
Cys*	1.4	1.9
Val*	3.6	4.8
Met*	1.2	2.5
Ile*	2.7	3.7
Leu*	5.2	6.8
Try	1.8	2.1
Phe*	3.5	4.9
His*	2.2	2.8
Lys*	4.2	5.1
Arg*	14.4	16.7
Total AA	81.3	108.4
*Essential AA	41.7	54.3

Higher temperature had some adverse effect on the metabolic activities of the microorganisms and it has been reported earlier (Zhang *et al.*, 2006; Weng & Sun, 2006).

Fig. 1. Effect of initial moisture content (IMC) on BFG by *C.tropicalis* during SSF of CSM

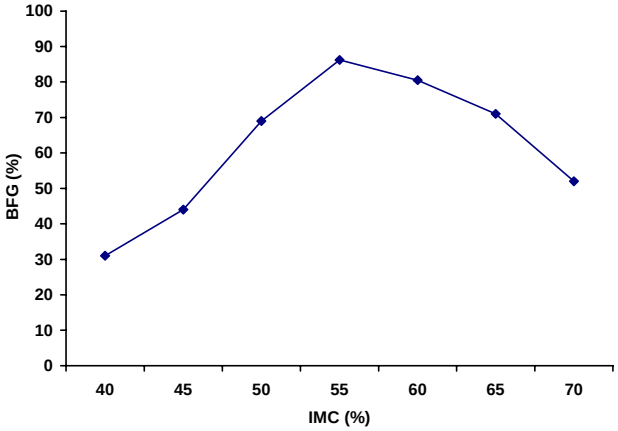


Fig. 2. Effect of initial pH on BFG by *C. tropicalis* during SSFof CSM

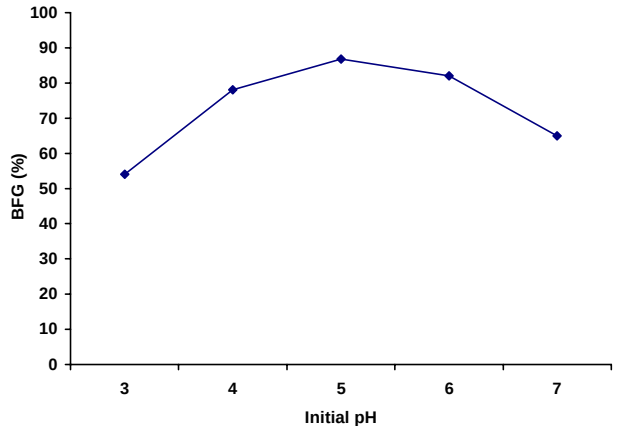


Fig. 3. Effect of incubation temperature on BFG by *C. tropicalis* during SSFof CSM

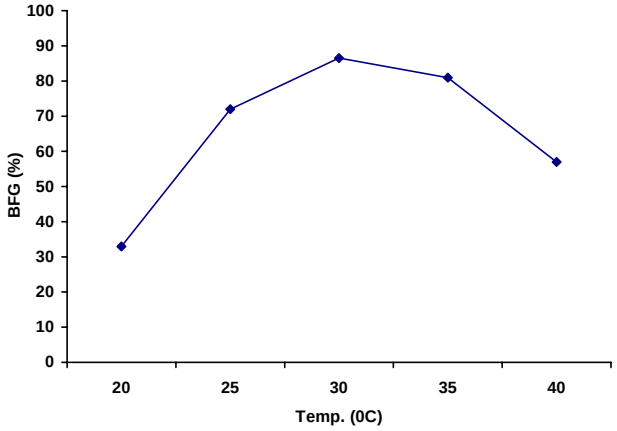
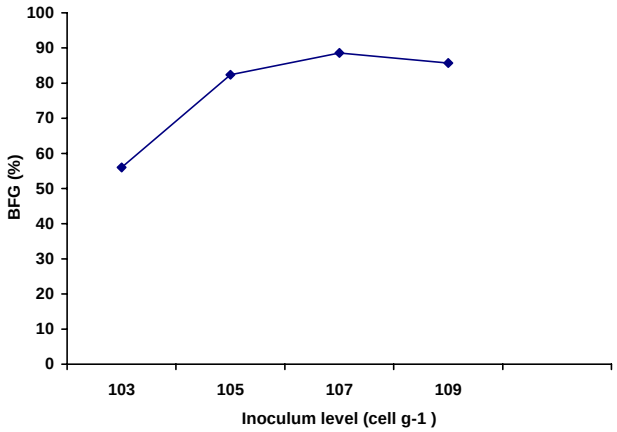


Fig. 4. Effect of inoculum level of *C. tropicalis* on BFG during SSFof CSM



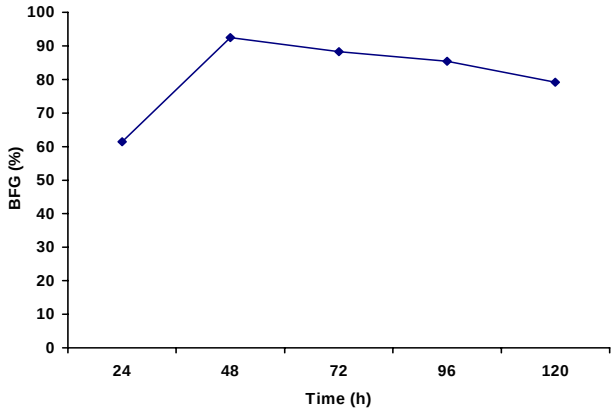
Hence, incubation temperature and its control in SSF process is crucial as the heat generated during SSF processes is accumulated in the medium due to poor heat dissipation in solid substrate. This results in reduced microbial activity, thereby decreasing the BFG efficiency.

Optimum inoculum level. Inoculum level was also an important factor for BFG. Various inoculum levels (10^3 – 10^9 cell g^{-1}) were tried to investigate their effect on the reduction of FG (Fig. 4). When the CSM medium was mixed with the inoculum of *C. tropicali*s at the level $1x10^3$ cell g^{-1}, the BFG was low (56%). With the cell concentration increasing the BFG was enhanced. As the cell concentration of *C. tropicalis* was up to 10^7 cell g^{-1}, the BFG reached the maximum of 88.6% (Fig. 4). A further increase in cell concentration have inhibitory trend in BFG. It is important to provide an optimum inoculum level in fermentation process. A lower inoculum density way give insufficient biomass and cause decreased BFG efficiency, whereas a higher inoculum may produce too much biomass and lead to the poor secretion of the enzyme of gossypol degradation (Zhang *et al.*, 2006).

Optimum fermentation period. The incubation time is governed by characteristics of the culture and is based on growth rate and BFG efficiency. In present study and after 24 h incubation, *C. tropicalis* biodegraded 61.4% of FG (Fig. 5). With the fermentation course prolonged, the BFG increased. High BFG efficiency (92.5%) was obtained at 48 h incubation period (Fig. 5). It was observed that the strain produced better fermentation efficiency in the range of 48-96 h. In some studies, the incubation time employed for gossypol degradation was 60 h or 4-6 days for yeast or filamentous fungi (Wu & Chen, 1989; Shi *et al.*, 1998; Weng & Sun, 2006). The decline in BFG rate observed before 36 h might be due to CSM substrate fermented incompletely, while the decline in BFG rate observed after 60 h might be due to denaturation and/or decomposition of the enzyme of gossypol degradation as a result of interactions with other compounds in the fermented substrate (Zhang *et al.*, 2006).

Crude protein and AA assay of CSM substrate fermented by *C. tropicalis*. It was evident that fermentation efficiency due to optimization of fermentation conditions

Fig. 5. Effect of incubation periods on BFG by *C. tropicalis* during SSFof CSM



was best during SSF of CSM. Thus the CP and AA content of fermented CSM were determined. The CP content of the fermented substrate was improved markedly by 31.2 g kg^{-1}, compared to the control and the total amino acids (TAA) and essential amino acids (EAA) of fermented substrate also increased by 22.4 and 9.6 g kg^{-1}, respectively (Table II). Levels of lysine, methionine and theronine were improved greatly by 0.7, 1.0 and 1.2 g kg^{-1}, respectively compared to the control (Table II).

The CP value difference before and after fermentation in CSM substrate was mainly due to growth of *C. tropicalis*, which synthesized cellular protein, enzymes and other cellular components. The present results are consistent with previous experiments (Wu & Chen, 1989; Yang *et al.*, 2000; Zhang *et al.*, 2006).

***In vitro* digestible CP and AA of CSM substrate fermented by *C. tropicalis*.** The *in vitro* digestible CP and AA content of fermented substrate were higher compared to the control, with the *in vitro* digestible CP improved by 29.4 g kg^{-1} and the *in vitro* digestible TAA and EAA content increased by 27.1 and 12.6 g kg^{-1}, respectively (Table III). In addition, the *in vitro* digestible lysine, methionine and threonine were also increased greatly by 0.9, 1.3 and 1.8 g kg^{-1}, respectively (Table III). Data on *in vitro* digestible CP and AA in fermented CSM is scarce to date. However the digestible CP increase was probably mainly associated with the increase in protein degradation enzymes, which the microorganism secreted extra-cellulary.

In conclusion, Microbial fermentation can effectively reduce FG levels in CSM, although the effectiveness differed among species of microorganisms. From the perspective of reducing CSM potential toxicity due to FG, *C. tropicalis* (local isolate) was most effective. The optimum fermentation conditions for BFG are 55% IMC of solid substrate, incubation temperature at 30°C, inoculum level $1x10^7$ cells g^{-1} of solid substrate, incubation time 48 h and pH in nature (5.2.). It could be concluded that the SSF may help in efficiently decomposing some anti-nutritional factors in feed resources and consequently improving their nutritional values.

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