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Reduction of Gossypol and Increase in Crude Protein Level of Cottonseed Cake using Mixed Culture Fermentation

*Asim Shaikh, A. A. Kathe and V. Mageshwaran**

Central Institute for Research on Cotton Technology, Matunga (E), Mumbai-400019, India

**Corresponding author: mageshbioiari@gmail.com*

Abstract

Cottonseed is a good source of high quality meal and edible oil. However it contains a toxic polyphenolic compound called gossypol, an anti-nutritious factor that limits the use of cottonseed cake (CSK) in non-ruminants feed. The aim of this paper was to study the effect of seven selected microbial strains and their combination on free gossypol (FG) and total gossypol (TG) detoxification, reduction in fibre content and also to evaluate the crude protein (CP) improvement during solid state fermentation of CSK. Initially, CSK contained 20.6% CP, 37.8% crude fibre, 0.28% FG and 2.37% TG. The microbial strains used were *Saccharomyces cerevisiae, Pleurotus flabellatus* MTCC 1799*, Candida tropicalis, S. cerevisiae* MTCC 6933*, Monascus purpureus* MTCC 1090*, Pleurotus sajor-caju* MTCC 1806 and *Aspergillus oryzae* MTCC 3782. The cultures were individually and in combinations inoculated in sterile CSK and incubated for 48hrs at 30 ^{o}C, after incubation, the level of FG and TG, crude fibre and CP were determined. The results showed that individually, *C. tropicalis* could reduce FG by 82.1% and *M. purpureus* MTCC 1090 could reduce TG by 59%. The significant gossypol detoxification was observed in culture combinations *P. sajor-caju* MTCC 1799 and *S. cerevisiae* MTCC 6933 (FG, 91.6% and TG, 65%) followed by *C. tropicalis* and *S. cerevisiae* (FG, 75% and TG, 67.7%). The combination of *P. sajor-caju* MTCC 1799 and *S. cerevisiae* MTCC 6933 showed 6.5% CP improvement and 10.8% fibre reduction while *C. tropicalis* and *S. cerevisiae* exhibited excellent CP improvement (9.3%) and fibre reduction (11.3%). The study revealed that the mixed culture fermentation of CSK was more effective than single strain fermentation, which not only had higher gossypol reduction but also improved CP and decreased crude fibre contents.

Keywords: Cottonseed Cake, Detoxification, Fungi, Gossypol, Solid state fermentation

1. Introduction

Cottonseed cake (CSK) is widely used as feed for ruminant animals. However, CSK is not recommended to non-ruminants due to the presence of toxic compound called gossypol and higher fibre content. Gossypol is a polyphenolic compound that is an integral part of the cotton plant's self-defence system against insect, pests and possibly some diseases (1). Some amount of gossypol tends to react with epsilon group of amino-acids in cottonseed and forms the bound gossypol that is non-harmful. Heating of cottonseeds during oil extraction binds gossypol to proteins, thus reduces protein availability from cottonseed meal (2). However the unbound gossypol known as "free gossypol" (FG) is toxic. The FG and bound gossypol constitutes TG.

Gossypol toxicity is a major concern for use of CSK as an animal feed (3). 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 (4-7), thus FG is an anti-nutritional factor that limits the use of cottonseed cake and its product. Negative effects of gossypol on animal health have long been recognized and toxic effects of FG are much greater in non-ruminants than ruminants due to the binding of FG to soluble proteins in the rumen (8). Thus it is necessary for CSK to be further processed to reduce free and total gossypol to permissible level as animal protein feed resources. A number of methods have been developed for removing gossypol from cottonseed including solvent extraction of free gossypol (9 & 10), ferrous sulfate treatment (11 &12), calcium hydroxide treatment (13) and microbial fermentation (14).

The microbial fermentation might be a kind of promising detoxification method, because fermented CSK usually contains some kinds of exoenzymes (secreted by microorganism) such as cellulolytic enzymes, amylases, proteases and lipolytic enzymes, vitamins, and other active substances (15). Solid-state fermentation (SSF) was used to produce industrial products including enzymes (16) as well as microbial biomass and is an attractive process to produce valuable products due to its low capital investment and operating expenses (17). Therefore, it is meaningful to explore the use of SSF as a process for gossypol detoxification in CSK by microorganisms. The objective of this study was to investigate the effect of some selected fungi individually and in combination to detoxify FG and TG levels during SSF of un-decorticated cotton-seed cake (UDCSK).

2. Materials and Methods

2.1 Basal Substrate

The UDCSK was purchased from Star Oil Mill, Tirupur (India), grounded and passed through 10 mm mesh size sieve and stored at room temperature (25-30^{o}C) until used.

2.2 Microorganisms

In this study, the cultures *S. cerevisiae, P. flabellatus* MTCC 1799*, C. tropicalis, S. cerevisiae* MTCC 6933*, M. purpureus* MTCC 1090*, P. sajor-caju* MTCC 1806 and *A. oryzae* MTCC 3782 were used, as they are often used in feedstuff fermentation and have been known for harmless nature to animals. The cultures were obtained from Microbiology lab, CIRCOT, Mumbai, India. The cultures were maintained on Malt Extract Agar slants at 4^{o}C.

2.3 Inoculum Preparation

The yeast and fungal inocula were prepared by transferring 1% suspension of 48h grown malt extract broth cultures into 250ml erlenmeyer flask containing 50 ml of sterile malt extract (1X) liquid medium at pH 5.5. The flasks were incubated on a rotary shaker at 150 rpm for 48 h at 30 ^{o}C.

2.4 Solid State Fermentation

The solid state fermentations were carried out in 100 ml erlenmeyer flask containing 5g UDCSK which were natural condition of pH 6.0. After sterilization by autoclaving at 110^{o}C for 20 min, the flask were cooled to room temperature and inoculated with respective culture inoculum in such a way that the initial moisture content in UDCSK was 80% (v/w). The flasks were then incubated at 30 ^{o}C for 48 h. The cultures which were shown better FG and TG reduction were combined in pair and subjected for mixed culture fermentation. The other conditions such as pH, moisture content, temperature and incubation period were maintained similar to single culture fermentation. However in mixed culture fermentation, 40% (v/w) of respective culture inoculum was added to maintain initial moisture content of 80% and then they were mixed under aseptic conditions and incubated. Duplicate flasks were set for each treatment.

2.5 Related Index Assay

After the incubation period, every flask of fermented UDCSK was kept at 60^{o}C for 3 h to stop the enzymes activity. The fermented UDCSK were mixed properly using sterile glass rod and the samples were taken for analysis. The moisture content was measured by drying at 105 ^{o}C for 5hrs. FG and TG level was determined by the official method of the American Oil Chemist Society (18). The FG and TG levels were expressed in percentage as per the method. The crude protein (CP) content assay was done by Kjeldahl's method (19) and crude fibre was determined by auto fibre analyzer based on Weende method (20). The percent reduction in FG and TG was calculated using the formula ((control – sample)/ control) x 100. The increase and decrease of CP and fibre contents were calculated from difference between the control and sample.

2.6 Statistical analysis

The obtained data was analysed by Web Agri Stat Package (WASP) of ICAR Research Complex Goa. A significant level of 0.05 was used.

3. Results and Discussion

The fermented UDCSK substrate by single and mixed cultures was assayed for their gossypol detoxification efficiency, protein content improvement and reduction in crude fibre. The free and total gossypol level of fermented UDCSK was significantly lower than uninoculated cake (control), indicating that fermentation could decrease the gossypol content of UDCSK.

The results showed that *C. tropicalis* treated samples had lowest level of FG. The amount of free gossypol reduction was found to be 82.1%, followed by *P. flabellatus* MTCC 1799 *and S. cerevisiae* MTCC 6933 of which FG reduction level in fermented substrate were found to be 67.9% and 46.4 % respectively. The total gossypol detoxification efficiency was reached up to 59.1% in *Monascus purpureus* MTCC 1090 fermented UDCSK, whereas the TG reduction in *P. sajor-caju* MTCC 1806 and *S. cerevisiae* treated cake were found to be 56.1% and 52.7% respectively (Table 1). The comparative gossypol reduction in single culture fermented UDCSK is shown in figure 1.

Table 1. Effect of single culture fermentation on gossypol level in UDCSK

Cultures combination	FG *(%)	TG *(%)
P. flabellatus MTCC 1799	0.09 ^{d}	1.42 ^{c}
S. cerevisiae	0.25 ^{b}	1.12 ^{e}
C. tropicalis	0.05 ^{e}	1.36 ^{d}
S. cerevisiae MTCC 6933	0.15 ^{c}	1.32 ^{d}
M. purpureus MTCC 1090	0.28 ^{a}	0.97 ^{f}
P. sajor-caju MTCC 1806	0.27 ^{ab}	1.04 ^{e}
A. oryzea MTCC 3782	0.27 ^{a}	1.53 ^{b}
Control	0.28 ^{a}	2.37 ^{a}

*Treatment values followed by the same alphabet do not differ significantly at P=0.05

FG: Free Gossypol; TG: Total Gossypol

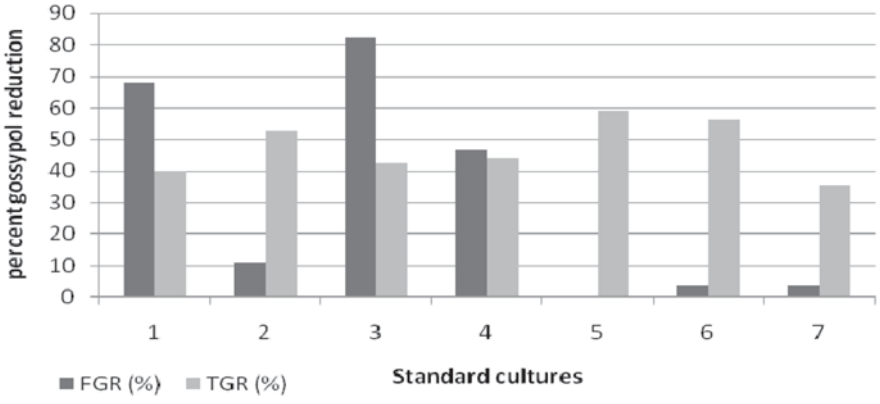

Figure 1. Percent reduction of gossypol in single culture fermentation on UDCSK

Note: FGR: Free Gossypol Reduction; TGR: Total Gossypol Reduction; 1- *P. flabellatus* MTCC 1799, 2- *S. cerevisiae,* 3-*C. tropicalis,* 4- *S. cerevisiae* MTCC 6933, 5-*M. purpureus* MTCC 1090, 6-*P. sajor-caju* MTCC 1806 and 7-*A. oryzae* MTCC 3782

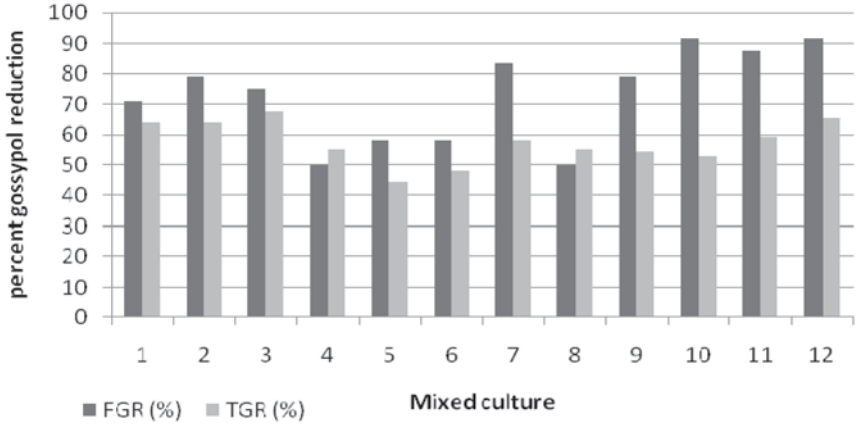

Figure 2. Percent reduction of gossypol in mixed culture fermentation on UDCSK.

Note: FGR: Free Gossypol Reduction; TGR: Total Gossypol Reduction; 1- *C. tropicalis*+ *M. purpureus* 2-*C. tropicalis*+ *P. sajor-caju*, 3-*C. tropicalis*+ *S. cerevisiae,* 4-*P. flabellatus*+ *M. purpureus,* 5-*P. flabellatus*+ *P. sajor-caju,* 6- *P. flabellatus*+ *S. serevisiae,* 7-*S. cerevisiae* 6933 +*M. purpureus,* 8-*P. flabellatus*+ *S. cerevisiae 6933, 9-S. cerevisiae*+ *S. cerevisiae 6933, 10-C. tropicalis*+ *S. cerevisiae 6933, 11-S. cerevisiae*+ *M. purpureus* and *12-P.sajorcaju*+*S. cerevisiae6933*

Results of mixed culture fermented UDCSK showed the better gossypol detoxification than single culture fermentation process as given in Table 2. The percent reduction of FG and TG are presented in Figure 2. The combination of *C. tropicalis* and *S. cerevisiae* experiment showed highest gossypol detoxification efficiency. The reduction in FG was found to be 75% while total gossypol reduction was found to be 67.7%. The second combination *P. sajor-caju* MTCC 1806 and *S. cerevisiae* MTCC 6933 exhibited detoxification level for FG at 91.7% and TG at 65.1%, whereas fermentation of *C. tropicalis* + *S. cerevisiae* MTCC 6933 and *M. purpureus* MTCC 1090 + *S. cerevisiae* combination although had better FG reduction, TG reduction was not more effective.

Table 2. Effect of mixed culture fermentation on gossypol level in UDCSK.

Cultures combination	FG * (%)	TG * (%)
C. tropicalis+ *M. purpureus* MTCC 1090	0.07 ^{e}	0.84^{g}
C. tropicalis+ *P. sajor-caju* MTCC 1806	0.05 ^{fg}	0.84^{g}
C. tropicalis+ *S. cerevisiae*	0.06 ^{f}	0.75^{h}
P. flabellatus MTCC 1799 + *M. purpureus*	0.12 ^{b}	1.04^{e}
P. flabellatus+ *P. sajorcaju* MTCC 1806	0.10 ^{cd}	1.29^{b}
P. flabellatus MTCC 1799+ *S. cerevisiae*	0.10 ^{d}	1.21^{c}
S. cerevisiae 6933 +*M. purpureus* MTCC 1090	0.04 ^{g}	0.97^{f}
P. flabellatus MTCC 1799 + *S. cerevisiae* MTCC *6933*	0.12 ^{c}	1.04^{e}
S. cerevisiae+ *S. cerevisiae* MTCC *6933*	0.05 ^{fg}	1.06^{cd}
C. tropicalis+ *S. cerevisiae* MTCC *6933*	0.02 ^{h}	1.09^{d}
S. cerevisiae+ *M. purpureus* MTCC 1090	0.03 ^{h}	0.95^{fg}
P.sajorcaju MTCC 1806 + *S. cerevisiae* MTCC *6933*	0.02 ^{h}	0.81^{fg}
Control	0.24 ^{a}	2.32^{a}

*Treatment values followed by the same alphabet do not differ significantly at P=0.05

FG: Free Gossypol; TG: Total Gossypol

Table 3. Effect of single culture fermentation on crude protein and crude fibre content of UDCSK

Standard Cultures	Crude Protein*(%)	Crude Fibre* (%)
P. flabellatus MTCC 1799	23.9 ^{bc}	23.2 ^{bc}
S. cerevisiae	23.2 ^{d}	23.1 ^{bc}
C. tropicalis	23.0 ^{d}	22.2 ^{d}
S. cerevisiae MTCC 6933	23.8 ^{cd}	24.1 ^{b}
M. purpureus MTCC 1090	25.2 ^{a}	23.8 c
P. sajor-caju MTCC 1806	23.4 ^{d}	21.3 ^{e}
A. oryzea MTCC 3782	24.3 ^{b}	24.0 ^{bc}
Control	20.6 ^{e}	37.8 ^{a}

*Treatment values followed by the same alphabet do not differ significantly at P=0.05

The physical and chemical methods for gossypol detoxification reported earlier do only inactivate FG. Unlike the other methods, microbial fermentation is the effective method of gossypol detoxification, since, microorganisms secrete enzymes which releases bound gossypol and degrade FG thus reduces both FG and TG. FG detoxification results of CSK by *C. tropicalis* in this study were consistent with (14) and (21). However, the present study revealed that fungal cultures used could reduce both FG and TG during SSF. The results of crude protein and fibre contents in single culture fermented UDCSK and mixed culture fermented cake are shown in Table 3 and 4 respectively. It is evident from the results that UDCSK fermentation by mixed culture improved protein content and reduce crude fibre content compared to single culture fermentation.

In single culture treated UDCSK, CP was increased by 4.6% and 3.7% in *M. purpureus* MTCC 1090 and *A. oryzae* MTCC 3782 experiment respectively, while uninoculated control substrate contained 20.6% CP content. Similarly crude fibre content of control UDCSK was 37.8% while in single strain fermentation by *C. tropicalis* and *P. sajor-caju* MTCC 1806 was reduced by 15.6 % and 16.5 % respectively (Table 3). In mixed culture fermentation of UDCSK, combination of *C. tropicalis* and *S. cerevisiae* improved CP content by 9.3% followed by *C. tropicalis* + *S. cerevisiae* MTCC 6933 and *P. sajor-caju* MTCC 1806 + *S. cerevisiae* MTCC 6933 which was 8.8% and 6.5% respectively. The fibre content reduction was found to be maximum in the combination of *C. tropicalis* and *S. cerevisiae* followed by *P. sajor-caju* MTCC 1806 + *S. cerevisiae* MTCC 6933 at 11.3% and 10.8% respectively (Table 4).

The additional amount of CP in UDCSK substrate is mainly due to the growth of microorganisms. Microbes converted substrate nutrients such as protein into microbial cell protein, secreted enzymes and other biological substances to outside of the cells, consumed carbohy-

drate to construct cell component and supply energy for cell metabolism, meanwhile released CO_2 and H_2O, and some volatile substances, thus led to CP content increase per unit (22). Secondly, fermented UDCSK contained lots of volatile fatty acids and other volatile substances that were lost during drying in an oven for sample, thus led to the increase of proportion of protein in the substrate. The obtained result of CP improvement in fermented UDCSK is correlated with the study of (23) and (13). The results on gossypol reduction and CP improvement in mixed culture fermentation of UDCSK were in accordance with (21).

Table 4. Effect of mixed culture fermentation on crude protein and crude fibre content of UDCSK

Standard Cultures Combination	Crude Protein* (%)	Crude Fibre* (%)
C. tropicalis+ *M. purpureus* MTCC 1090	26.90 ^{dc}	27.6 ^{i}
C. tropicalis+ *P. sajor-caju* MTCC 1806	26.20 ^{e}	29.9 ^{efg}
C. tropicalis+ *S. cerevisiae*	30.36 ^{a}	25.8 ^{k}
P. flabellatus MTCC 1799 + *M. purpureus*	27.56 ^{d}	28.4 ^{h}
P. flabellatus+ *P. sajorcaju* MTCC 1806	27.91 ^{d}	28.8 ^{gh}
P. flabellatus MTCC 1799+ *S. cerevisiae*	28.26 ^{c}	30.2 ^{d}
S. cerevisiae 6933 + *M. purpureus* MTCC 1090	29.57 ^{bc}	29.8 ^{def}
P. flabellatus MTCC 1799 + *S. cerevisiae* MTCC *6933*	27.47 ^{d}	31.5 ^{c}
S. cerevisiae+ *S. cerevisiae* MTCC *6933*	29.83 ^{b}	32.3 ^{b}
C. tropicalis+ *S. cerevisiae* MTCC *6933*	29.86 ^{bc}	30.1 ^{de}
S. cerevisiae+ *M. purpureus* MTCC 1090	28.68 ^{c}	28.9 ^{fg}
P.sajorcaju MTCC 1806 +*S. cerevisiae* MTCC *6933*	27.54 ^{d}	26.3 ^{j}
Control	21.09 ^{f}	37.1 ^{a}

*Treatment values followed by the same alphabet do not differ significantly at P=0.05

To conclude, the results obtained in this study revealed that mixed culture fermentation of UDCSK was found giving better gossypol reduction and CP improvement compared to the single culture fermentation. The combination of *C. tropicalis* and *S. cerevisiae* and *P. sajor-caju* MTCC 1806 + *S. cerevisiae* MTCC 6933 resulted in high gossypol detoxification, FG at 75 % and 91.7 % and TG at 67.7 % and 65.1 % respectively. This combination also improved CP at 9.3 % and 6.5%and reduced crude fibre contents at 11.3% and 10.8 % respectively. The future study may focus on evaluation of the fungal culture treated UDCSK as protein supplement in non-ruminants feed.

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5. References

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