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**RESEARCH PAPER**

# Optimization of Solid State Fermentation Process for Gossypol Detoxification in Heat Sterilized Cotton Seed Cake by Mixed Fungal Cultures

Mageshwaran Vellaichamy

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

*Corresponding author: mageshbioiari@gmail.com

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**Abstract**

A solid state fermentation (SSF) process was optimized for gossypol detoxification in cot onseed cake (CSC) using mixed fungal cultures viz., *Pleurotus sajor-caju* with *Saccharomyces cerevisiae* and *Candida tropicalis* with *S. cerevisiae*. The effect of fermentation conditions such as initial moisture content, inoculum level, incubation temperature and period were tested for gossypol reduction in heat sterilized (HS) CSC. The results showed optimized parameters for gossypol detoxification in HS CSC were 70% moisture content, 15% inoculum level, 30° C incubation temperature and 48 h incubation period. The free gossypol (FG) detoxification rate was higher (83.6 %) in *P. sajor-caju* with *S. cerevisiae* fermented HS CSC. The detoxification rate of bound gossypol (BG) was higher (63.3%) in *C. tropicalis* with *S. cerevisiae* fermented HS CSC. The maximum increase in crude protein (CP) (10.2 %) and decrease in crude fibre (CF) (10.7 %) was recorded in *C. tropicalis* with *S. cerevisiae* and *P. sajor-caju* with *S. cerevisiae* fermented HS CSC respectively.

**Keywords:** Crude fibre, crude protein, gossypol, heat sterilization, detoxification, solid state fermentation

Cot en seed cake (CSC) is commonly used as a feed for ruminant animals. However, the use of CSC is limited in non-ruminants due to the presence of toxic compound called gossypol. Gossypol is a polyphenolic binaphthyl dialdelhyde, and yellow pigment present in the entire cot on plant including its seed (Kenar, 2006). Gossypol is present in two forms viz., free gossypol (FG) and Bound hossypol (BG). The total gossypol constitutes FG and BG. But FG is more toxic than BG. The BG is formed by reaction between gossypol and epsilon-amino groups from lysine and arginine, and forms Schiff base (Fernandez *et al*., 1994). Feeding diets containing gossypol to animals would cause several negative effects such as growth depression, reproductive diseases and intestinal and other internal organ abnormalities (Berardi *et al*., 1994; Francis *et al*., 2001; Robinson *et al*., 2001). The tolerance of FG in chicks however, varies widely (90 to 1000 mg $kg^{-1}$ diets) (Nagalakshmi *et al*., 2007). To overcome this problem of toxicity, detoxification is needed. So, the detoxification of gossypol in cot on seed meal (CSM) through microbial fermentation is a promising method since unlike chemical methods, the biodegradation of gossypol occurs during fermentation process (Yang *et al*., 2011). The fermented cot on seed meal (CSM) usually contains some kinds of exoenzyme (secreted by microorganisms) such as cellulolytic enzyme, amylase, protease and lipolytic enzyme, vitamins and other active substances apart from the detoxification of FG (Shi *et al*., 1998; Wu and

Chen, 1989). The previous reports showed among twelve mixed culture combinations of seven strains, the maximum detoxification of gossypol and nutritive quality improvement of CSC was obtained in the culture combinations, *P. sajor-caju* with *S. cerevisiae* and *C. tropicalis* with *S. cerevisiae* (Shaikh *et al*., 2014). The aim of the present paper is to optimize SSF process for FG and BG reduction in HS CSC using mixed fungal cultures and the results obtained are discussed here.

## Materials and Methods

### *Basal Substrate*

The undecorticated cotonseed cake was purchased from M/S Star Oil Mill, Tirupur (India). It was ground and passed through 10 mm mesh size sieve and stored at room temperature (25-30 °C), until used. The ground CSC was used as a basic fermentation medium. The moisture content (v/w), FG, BG, CP and CF contents present in CSC were 10 %, 2200 mg/kg, 2100 mg/g, 20 % and 37 %, respectively.

### *Microorganisms*

The test microorganisms used in this study viz., *Pleurotus sajor-caju* MTCC 1806, *Candida tropicalis* MTCC 1406 and *Saccharomyces cerevisiae* MTCC 6933 were obtained from Microbiology Lab, ICAR-CIRCOT, Mumbai. The cultures were grown in malt extract broth at 30°±C under shaking conditions for 48 hours and maintained in malt agar slants at 4 °C.

### *Inoculum preparation*

The cultures mentioned above were grown in malt extract (1X) (Himedia, India, Mumbai) liquid medium (*p*H 5.5) for 48 h at 30 °C under shaking conditions (150 rpm). After growth, the biomass was separated by centrifugation at 10,000 rpm for 5 minutes, washed in sterile water for two times to remove any media residues. The pellet (biomass) was suspended in sterile water of the same quantity of broth culture and kept at 4 °C, until use. The inoculum of mixed fungal culture for SSF was prepared by mixing equal proportion of respective combination at the time of inoculation. The cell population maintained in inoculum was $10^6$ colonies forming units/ml.

### *Optimization of SSF in Heat sterilized (HS) Cot on seed cake (CSC)*

Five g of basal substrate with natural pH (6.0) was taken in 100 ml of erlenmeyer flask and autoclaved at 110 °C, 15 lb/in$^2$ for 20 min. The flask were cooled to room temperature and was used for SSF. The effect of various parameters such as moisture content, inoculum level, incubation temperature and period were optimized for gossypol detoxification in HS CSC. Triplicate flasks were set for each experimental variation. A control was maintained in which no culture was added.

### *Effect of initial moisture content*

To understand the effect of initial moisture content of the substrate, various initial moisture contents (40%, 50%, 60%, 70% and 80%) were adjusted using sterile distilled water and fermentation was carried out. The inoculum level was 15% and fermentation was carried out for 48 h at 30 °C. Since liquid inoculum was used, size of the inoculum was considered for fixing the initial moisture content. The optimum initial moisture content was fixed for the subsequent experiments.

### *Effect of level of inoculum*

The various inoculum levels (1%, 3%, 5%, 10%, 15%, 20% and 30%) were tested under optimum moisture content. The fermentation was carried out for 48 h at 30 °C. The optimum inoculum level was fixed for the subsequent experiments.

### *Effect of incubation temperature*

To study the effect of incubation temperature on gossypol reduction in CSC, the fermentation was carried out at various incubation temperatures (25, 30, 35 and 37 °C) under optimum moisture content and inoculum level for 48 h. The optimum incubation temperature was fixed for the subsequent experiments.

### *Effect of incubation period*

Various incubation periods (24, 48, 72 and 96 h) were tested and the fermentation was carried out with other parameters kept at their optimum levels.

***Sample processing***

After fermentation, the flask containing fermented substrates was dried in an oven at 60 °C for 24 h and weight loss was determined. Subsequently the samples were powdered for related analyses.

***Related index assay***

The moisture content was measured by drying using hot air oven at 105 °C for 5h. FG and total gossypol level was determined by the official method of the American Oil Chemist Society (AOCS, 1989). The difference between total gossypol and FG gave BG. The crude protein (CP) content assay was done by Kjeldahl's method (AOAC, 1999) and crude fibre (CF) was determined by auto fibre analyzer based on Weende method (Tecator, 1978). The percent changes in FG and BG were calculated using the formula ((control – sample)/control) × 100. The increase and decrease of CP and CF contents were calculated from difference between the control and sample.

***Statistical analysis***

The obtained data was analysed by Web Agri Stat Package (WASP) of ICAR Research Complex Goa. For all analysis, the differences were considered to be significant at $P < 0.05$.

**Results and Discussion**

The initial moisture content is one of the factors determining the efficiency of SSF. The heat sterilization of CSC was done with autoclaving at 110 °C for 20 minutes at its original moisture content (10%). During fermentation, the required moisture content in CSC was adjusted using sterile water after autoclaving. This was done to avoid cooking of CSC, since cooking converts free to BG and affects the protein value in CSM (Atia and Rahim, 2009; Wang *et al*., 2012). The results showed that the moisture content had positive effect on gossypol detoxification. The optimum moisture content for gossypol detoxification in SSF was found to be 70% in HS CSC fermented with fungal cultures, *P. sajor-caju* with *S. cerevisiae* and *C. tropicalis* with *S. cerevisiae* ($P < 0.05$) (Fig. 1a & 2a). The increase in moisture content substantially increased the solubility of nutrients in the substrate thereby effecting the growth of microorganisms. However, the excess moisture may reduce the oxygen transfer which limits the microbial growth (Zadrazil and Brunnert, 1981; Ohno *et al*. 1992; Murthy *et al*. 1999). In a similar study, the optimum moisture content for gossypol detoxification in CSM was 50 to 55% (Zhang *et al*. 2006b; Weng and Sun, 2006). The difference in the results might be due to absorption of higher moisture by high fibre content in CSC. The fibre content is higher in undecorticated CSC among various cotonseed extractions (Mageshwaran *et al*. 2014).

Among different inoculum level tested (1, 3, 5, 10, 15, 20 and 30%), the addition of inoculum size of 15 % was found to be optimum in HS CSC fermented with *P. sajor-caju* with *S. cerevisiae* 6933 and *C. tropicalis* with *S. cerevisiae* ($P < 0.05$) (Fig. 1b & 2b). The results showed that with the increase in inoculum up to certain level, the detoxification rate increases beyond which not much change occurs. The results are in agreement with previous reports where 5 % inoculums level was found to be optimum for gossypol detoxification in CSM (Khalaf and Meleigy, 2008; Weng and Sun, 2006). The high protein level in the substrate accelerates the initial growth of microbes. The higher inoculum requirement for gossypol detoxification in CSC might be due to low protein content in CSC than CSM.

The detoxification rate was almost similar at incubation temperatures 25 and 30 °C. The optimal temperature for gossypol detoxification by *P. sajor-caju* with *S. cerevisiae* 6933 and *C. tropicalis* with *S. cerevisiae* culture combinations in HS CSC was 30 °C ($P<0.05$). The results are presented in Fig. 1c &2c. The results are in agreement with the previous reports where the optimum temperature for biodegrdation of gossypol reported was 30 °C (Zhang *et al*. 2006 a, b, c). The FG detoxification rate at incubation periods 24 and 48 hours did not differ significantly ($P > 0.05$) in fermented HS CSC.

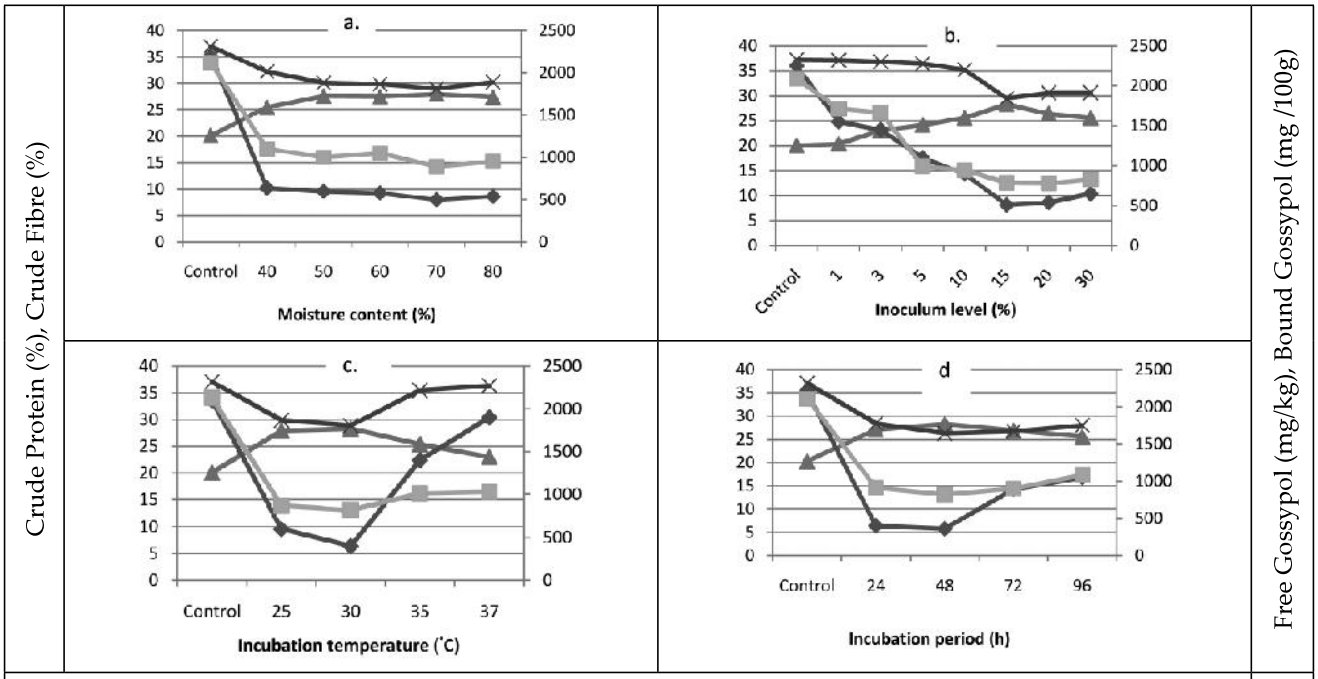


**Fig. 1: Effect of fermentation conditions on gossypol detoxification in heat sterilized (HS) CSC by *P. sajor-caju* + *S. cerevisiae*. (a) Moisture content (b) Inoculum level (c) Incubation temperature and (d) Incubation period. Crude protein (▲), Crude fibre (x), Free Gossypol (+), Bound gossypol(■)**

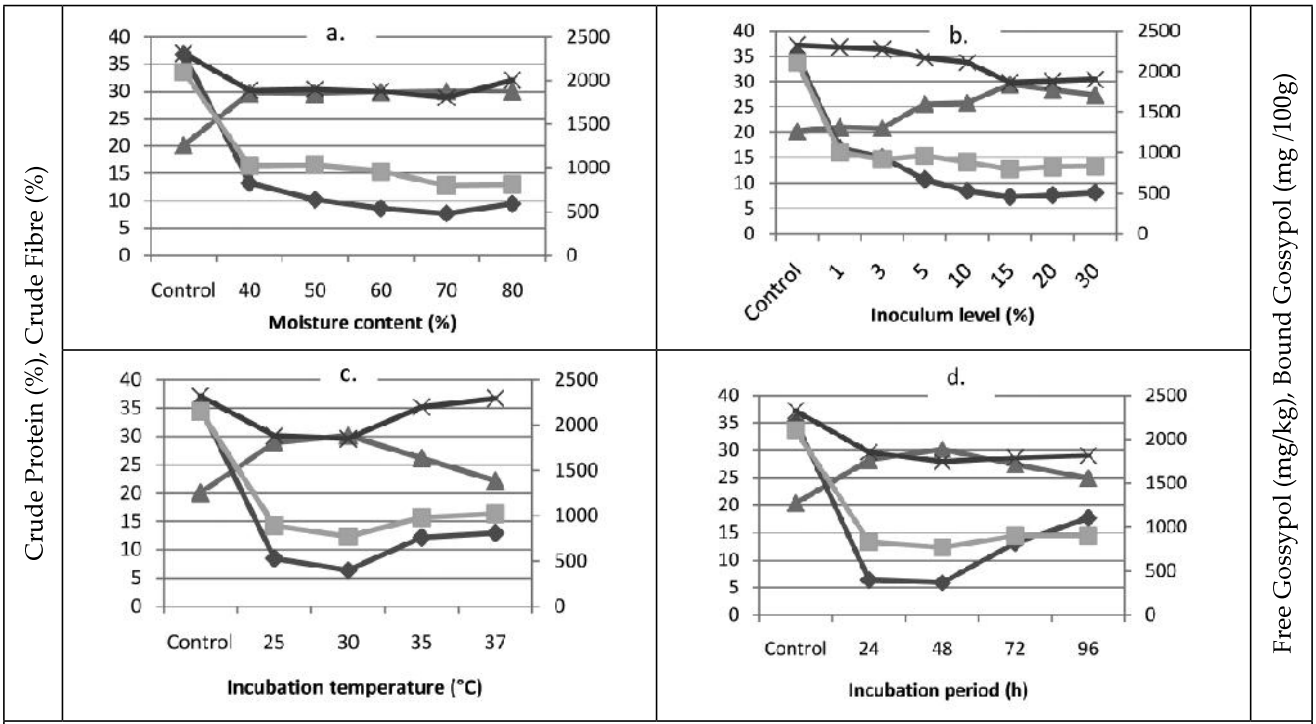


**Fig. 2: Effect of fermentation conditions on gossypol detoxification in heat sterilized (HS) CSC by *C. tropicalis* + *S. cerevisiae*. (a) Moisture content (b) Inoculum level (c) Incubation temperature and (d) Incubation period. Crude protein (▲), Crude fibre (x), Free Gossypol (+), Bound gossypol (■)**

The results showed that the optimal incubation period for detoxification of gossypol and other nutritive quality improvement was 48 h in HS CSC fermented with *P. sajor-caju* with *S. cerev*isiae and *C. tropicalis* with *S. cerevisiae* ($P < 0.05$) (Fig. 1d & 2d). In a similar study, the optimum incubation period for detoxification of gossypol in CSM reported was 24 to 48 h (Zhang *et al*., 2006 a,b, Zhang *et al*., 2007; Khalaf and Meleigy, 2008) and 60 to 72 h (Weng and Sun, 2006). The fermented CSC incubated for 48 hours had higher CP and low CF contents.

Hence, under optimized conditions, the maximum reduction of FG (2200 to 360 mg/kg) was observed in HS CSC fermented with *P. sajor-caju* with *S. cerevisiae*. The BG was recorded low (770 mg/100g) in HS CSC fermented with *C. tropicalis* with *S. cerevisiae*. The maximum CP (30.1%) and low CF content (26.3 %) was recorded in HS CSC fermented with *C. tropicalis* with *S. cerevisiae* and *P. sajor-caju* with *S. cerevisiae*. The US FDA limits the FG content in food products and ingredients to 450 mg/kg. While the protein advisory group of UN (FAO/WHO) has a guideline which limits FG to 600 mg/kg and BG to 1140 mg/100g (Rhee, 1992). In the present study, fermented CSC meets the gossypol level as set by the standards. The SSF significantly increased CP and decreased CF contents in fermented CSC. In a similar study, the fermented CSM had significantly reduced fibre content (Atia and Rahim, 2009; Tang *et al*., 2012). The SSF increased the protein content from 3 to 7% and improved aminoacids profile in CSM (Zhang *et al*., 2006 a, b, c).

Microbial fermentation of gossypol detoxification is the best alternative among different gossypol detoxification methods, since fermentation process enhance the nutritional value of CSM (Atia and Rahim, 2009). Previous work on SSF showed that fungal cultures *C. tropicalis*, *S. cerevisiae*, *A. niger* and *P. florida* greatly reduced the FG content in CSM (Rajarathnam *et al*., 2001; Zhang *et al*., 2006 a, b, c, Zhang *et al*., 2007; Khalaf and Meleigy, 2008). The mixed fungal cultures, *C. tropicalis* ZD-3 with *A. niger* ZD-8 and *S. cerevisiae* with *A. niger* had higher FG detoxification rate in CSM (Atia and Rahim, 2009; Zhang *et al*., 2006c). In this study, the mixed fungal cultures viz., *P. sajor-caju* with *S. cerevisiae* and *C. tropicalis* with *S. cerevisiae* were used for SSF.

The initial pH of basal substrate was 6.0 in HS CSC. Invariably, previous reports showed that gossypol detoxification in CSM were found to be bet er at its natural pH (5.5 to 6.0) (Atia and Rahim, 2009; Weng and Sun, 2006).

## Conclusion

The results of the study showed the optimal conditions for gossypol detoxification and nutritive quality improvement in CSC was 70% initial moisture content, 15% inoculum level, 30°C incubation temperature and 48 h incubation period. Under these optimal conditions, the fermented HS CSC had significantly lower level of FG, BG and CF contents and higher CP compared to the untreated sample. The FG and BG levels in fermented CSC meets the international standard requirements which show its potential to be used as a safer animal feed.

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