Solid-State Fermentation for Gossypol Detoxification and Nutritive Enrichment of Cottonseed Cake: A Scale-Up of Batch Fermentation Process

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Gossypol, a toxic polyphenolic compound, limits the use of cottonseed cake (CSC) in animal feed. Different approaches have been employed to detoxify gossypol and improve the nutritive properties of feed. Microbial fermentation improves the nutritive quality of CSC by increasing lysine content and reducing free and bound gossypol. In this study, microbial fermentation was scaled up under batch conditions using a prototype device at the capacity of 40 kg per day. The mixed fungal culture *C. tropicalis* + *S. cerevisiae* was used for fermentation. An industrial trial was taken to ascertain the gossypol detoxification efficiency. The fermented CSC obtained under scale-up process had 60 to 80% and 40 to 60% reduction of free and bound gossypol, respectively, compared with raw CSC. The fermented CSC demonstrated an increase in crude protein (4 to 12%) and lysine (0.3 to 0.4%) and decrease in crude fibre (3 to 11%). The fermented CSC met the standards of US Food and Drug Administration in terms of its nutritional property. Thus, the simple method described in this study could be adopted for the production of detoxified CSC for use in the animal feed industry.

DOI: 10.15376/biores.19.1.1107-1118

Keywords: Animal feed; Cottonseed cake; Degossypolization; Gossypol; Solid-state fermentation

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INTRODUCTION

Gossypol is a toxic polyphenolic yellow pigment that is produced in the cotton plant as a naturally occurring toxin that deters insect pests. Cottonseed cake (CSC) contains 0.1 to 1.5% of gossypol (Gadelha *et al.* 2014). Feeding diets containing gossypol to animals causes negative effects such as growth depression, reproductive disease, and intestinal and other organ abnormalities (Randel *et al.* 1992). The recommended levels of gossypol in feed materials are no more than 0.05% for cattle, sheep, and goats, 0.01 % for poultry, and 0.006 % for rabbits and pigs (EFSA 2008). Another important limiting factor of CSC in animal feed is low level of lysine content. At higher temperature, the aldehyde group of gossypol binds with the free epsilon group of lysine and forms bound gossypol, thereby reducing the nutritive value of cottonseed protein (Fernandez *et al*. 1994). Lysine is a limiting and essential amino acid that is vital for animal growth. The average lysine content in CSC is 1.0%, whereas soybean meal contains 2.0%. Meanwhile, the average requirement of lysine for growing swine or poultry is 0.5 to 1% of the total diet.

Protein is the most expensive ingredient in the animal's diet. According to the Food and Agricultural Organization (2017), the protein requirements in the feed of different non-ruminant animals are 25 to 35% (poultry), 40 to 45% (fish), and 15 to 25% (pig). Soybean is the major protein source for non-ruminant feed. Due to soaring market prices of soybean, feed industries are looking for alternative protein to supplement the non-ruminant feed ration. Cottonseed cake is an excellent source of protein and essential amino acids. The annual production of CSC in India is 9 million tonnes. In spite of its huge production, its use in non-ruminant feed is limited due to the presence of gossypol and deficiency in lysine. CSC is majorly available in many forms such as de-oiled cake (DOC), cottonseed meal (CSM), and undecorticated cake (UDC). The spectrophotometric analysis of gossypol as described by American Oil Chemists Society (AOCS)) is widely employed to estimate gossypol in CSC or any other cottonseed extractions. The other techniques used are High-Performance Liquid Chromatography (HPLC), and the Chemiluminescence and Enzyme-Linked Immunosorbant Assay (ELISA) (Mageshwaran 2017, 2021).

Several methods such as liquid cyclone process, solvent extraction at higher temperature, calcium hydroxide, and ferrous sulphate treatment have been reported for gossypol detoxification in CSM. These methods either inactivate or bind the gossypol with meal. Mageshwaran (2021) reviewed the different methods for detoxification of gossypol for non-ruminants feed application. Microbial fermentation is an emerging method in which gossypol is biodegraded. In addition, microbial fermentation increases the protein content and other nutritive parameters in CSC. The solid-state fermentation (SSF) is one of the viable tools to enrich the amino-acids content in agro-residues (Pandey 1991). Yeast and mycelia fungal cultures are used for gossypol detoxification and lysine enrichment. The most commonly reported fungal cultures for gossypol detoxification are *Candida tropicalis*, *Saccharomyces cerevisiae*, *Pleurotus spp.*, *Aspergillus niger*, and *A. oryzae* (Khalaf and Meleigy 2008; Yang *et al.* 2011; Mageshwaran and Kathe 2013a; Shaikh *et al.* 2014). CSM fermented with *C. tropicalis* ZD-3 shows the maximum reduction of free gossypol (Zhang *et al.* 2007). During SSF, *Paecilomyces variotii* increases protein and lysine content in olive mill waste (Giannoutsou *et al.* 2012). In fermented CSC, free and bound gossypol is reduced, and lysine content is increased (Mageshwaran and Parvez 2016). The optimized solvent process employing acetone and isopropanol also reduces the free and total gossypol in CSM. The free and total gossypol reduction is similar to the level of microbial degossypolization (Satankar *et al.* 2021; Varsha *et al.* 2021).

Previously, a solid-state fermentation process was used for gossypol reduction and nutritive quality improvement in CSC using mixed fungal cultures *viz.*, *C. tropicalis* + *S. cerevisiae* and *P. sajor-caju* + *S. cerevisiae* (Shaikh *et al*. 2014). Eight new gossypol degrading soil fungal isolates were identified, of which *Fusarium thapsinum* F-8 showed higher gossypol detoxification efficiency in CSM. The fungus produced laccase in gossypol containing minimal medium for its biodegradation. The fermented CSC had improved nutritional properties such as 80% reduced free gossypol, 60% reduced bound gossypol, 15 to 25% increased lysine content, 40 to 50% improved protein content, and 25 to 30% reduced fibre content compared with untreated CSC (Vellaichamy 2016; Mageshwaran *et al.* 2017a, b). The evaluation of fermented CSC in poultry feed indicated that it is a safe and effective substitute for soybean meal in poultry ration. The replacement of 20 to 40% soybean meal with fermented CSC (fermented by *C. tropicalis* + *S. cerevisiae*) had positive effects on growth performance broilers, *i.e.*, body weight gain, feed consumption, feed conversion ratio, and mortality. The study revealed the potential of fermented CSC as a cheaper protein substitute in broiler ration (Kakde *et al.* 2020).

Considering the promising results obtained on gossypol detoxification and nutritive quality improvement in CSC by SSF and its positive impact on poultry ration, the present study aimed to improve the strains of *C. tropicalis* and *S. cerevisiae* and to scale-up the batch SSF (40 kg/ day) for production of detoxified and nutritive enriched fermented CSC.

EXPERIMENTAL

Microorganisms

The yeast strains *Saccharomyces cerevisiae* and *Candida tropicalis* were obtained from the Department of Microbiology, Ginning Training Center, ICAR-CIRCOT, Nagpur. The cultures were grown in malt extract broth at 30 ℃ under shaking conditions for 48 h and maintained at in malt agar slant at 4 ℃.

Cottonseed Cake

The CSC extractions viz., UDC and DOC were obtained from M/s Clean Cotton Impex, Tirupur, Tamil Nadu, India. The CSC obtained in flakes were grounded using pulveriser and used for SSF.

Improvement of Gossypol Detoxification Efficiency of Yeast Strains

The potential gossypol degrading yeast strains (*C. tropicalis* and *S. cerevisiae*) were repeatedly grown in medium containing 1000 mg/L of gossypol. The individual yeast strain (1%) was grown in gossypol-containing minimal medium (pH 5.5), which was composed of (g/L): $NaNO_3$—0.5; K_2HPO_4—0.65; KH_2PO_4—0.2; and $MgSO_4$—0.1. Gossypol acetic acid (MP Biomedicals, USA) dissolved in dimethyl sulfoxide (Fisher Scientific, USA) was added to 100 mL of sterile minimal medium for attaining the final concentration of 1000 mg/L of gossypol as the sole carbon and energy source. To evaluate the gossypol degradation ability, the improved yeast strains alone (2%) and in combination of mixed culture, *C. tropicalis*+ *S. cerevisiae* (each 1%) was inoculated in minimal medium containing 100 mg/L of gossypol. An uninoculated sample was maintained as the control. The control and inoculated flasks were incubated at 30 °C and 150 rpm. Ten mL of broth was sampled from the flasks at different incubation periods (0, 24, 48, 72 and 96 h) and centrifuged at 12000 × *g* for 5 min. The supernatants were analyzed for their residual gossypol level by AOCS Ba 7-58. The spectrophotometric method for determining gossypol involved reaction of gossypol with aniline to form a yellow dianilino-gossypol (AOCS 1989). The percent reduction of residual gossypol level in the supernatants was determined using the formula (Control-Treated)/Control × 100.

Inoculum Preparation

The cultures, *C. tropicalis* and *S. cerevisiae* were individually grown in 250 mL malt extract broth (pH 5.5) for 48 hours at 30 °C under shaking conditions (150 rpm). The grown yeast culture in a conical flask was completely transferred to culture drum containing 25 liters of jaggery broth medium. The jaggery medium was prepared by addition of Jaggery-30 g and cottonseed meal – 10 g in one liter of pure water. The medium was pasteurized by boiling the broth for 1 h and then cooled to room temperature. Aeration was provided to each drum to enhance the growth of yeast cultures in culture drum. After continuous pass of air to the drum for three days, the complete growth of yeast cultures was observed in jaggery medium. The growth was visible by change of colour of medium

from dark to colourless and more viscous after growth. The yeast culture grown in jaggery medium was used as inoculum for SSF.

SSF- Batch Process

The sequence of different steps involved in the microbial process of degossypolization in batch process is given in Fig. 1.

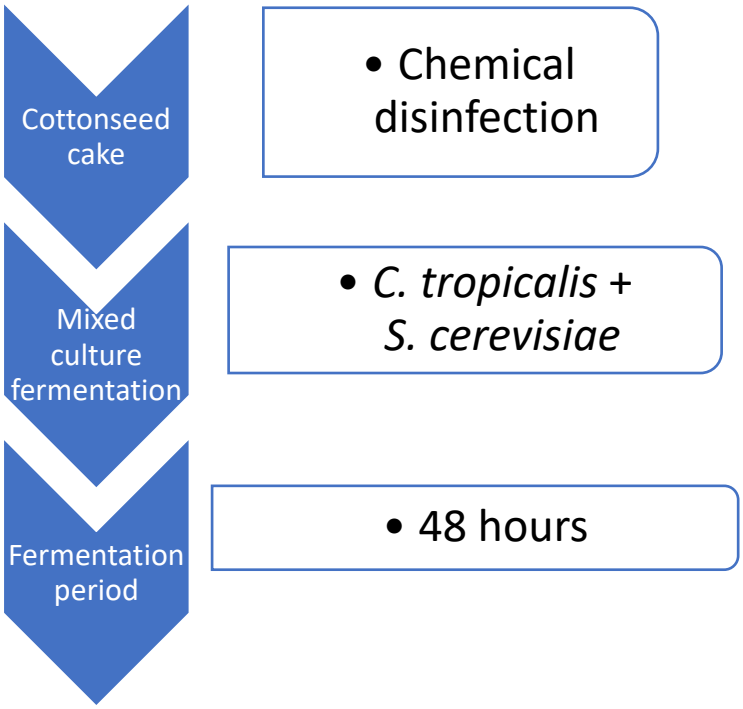

Fig. 1. Microbial degossypolization process

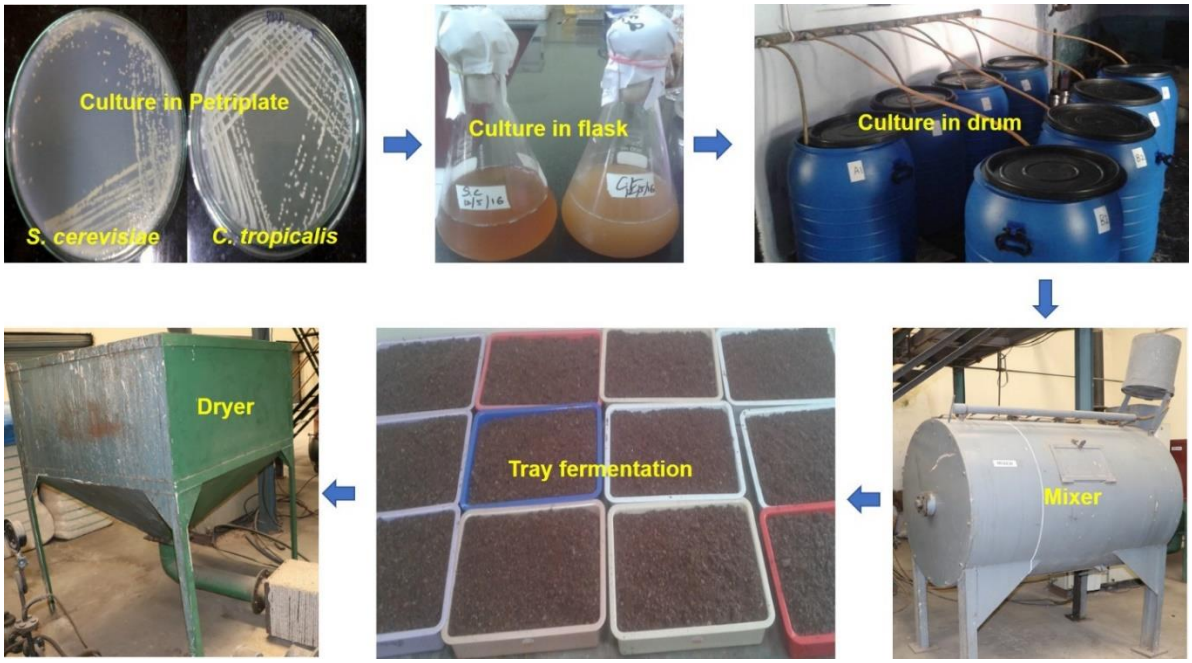

Fig. 2. Batch process for production of degossypolized and nutritive enriched CSC. The arrow indicates the sequence of steps involved in batch fermentation.

At Ginning Training Center (GTC), Nagpur, a facility was created for batch production of degossypolized and nutritive enriched CSC with the production capacity of 40 kg/day (Fig. 2). The developed microbial process of degossypolization is applicable to different extractions of DOC and UDC (Mageshwaran *et al.* 2014).

A scale-up of batch fermentation for microbial degossypolization was taken separately for 100 kg of DOC and UDC. For each batch, 20 kg of CSC was taken, and therefore five different batches were prepared to process 100 kg of CSC. The ground CSC was taken in mixer as given in Fig. 2 in which 0.5% lactic acid was sprayed in CSC and mixed for 30 min for chemical sterilization. After chemical sterilization, the culture from the drum was transferred to the mixer. The culture was added at the level of 7.5% of each culture, *i.e.*, *C. tropicalis* and *S. cerevisiae* into the CSC. The initial moisture content maintained was 70%. The mixer was provided with shafts for continuous mixing of CSC. After mixing of CSC in mixer for 30 min, the treated CSC was transferred to the surface sterilized plastic trays and kept under room temperature (28 ± 2°C) for two days. After two days, the fermented CSC was dried at 70 °C for 2 h or until the moisture content of fermented cottonseed was less than 15%.

Industrial Trial

The industrial trial on batch fermentation of degossypolization and nutritive quality improvement of CSC was undertaken at M/s Clean Cotton Impex, Tirupur, Tamil Nadu, India. The trial was taken for one tonne each of DOC and UDC. The conditions of fermentation were followed as described earlier.

Sample Processing

After fermentation, the fermented substrates were 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 5 h. Free gossypol and total gossypol level were determined by the official method of the American Oil Chemist Society (AOCS 1989). The difference between total gossypol and free gossypol gave bound gossypol. 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 total lysine content was estimated according to Tsai *et al.* (1972). The detoxification percentage (%) of free and bound gossypol were calculated using the formula ((control – sample)/control) × 100. The increase and decrease of CP, lysine and CF contents were calculated from difference between the control and sample.

Statistical Analysis

The obtained data were 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 degossypolizing yeast cultures *viz*., *Saccharomyces cerevisiae* and *Candida tropicalis* were repeatedly grown in minimal medium containing gossypol at the level of 1000 mg/L. The improved yeast strains alone and its combination were grown in mineral medium containing 100 mg/L of gossypol. The results showed that combined culture, *S. cerevisiae* + *C. tropicalis* had higher degradation than individual culture. The residual gossypol present in the culture supernatant was 21 mg/L, which showed 79% of biodegradation of gossypol at 48 hours of incubation (Fig. 3).

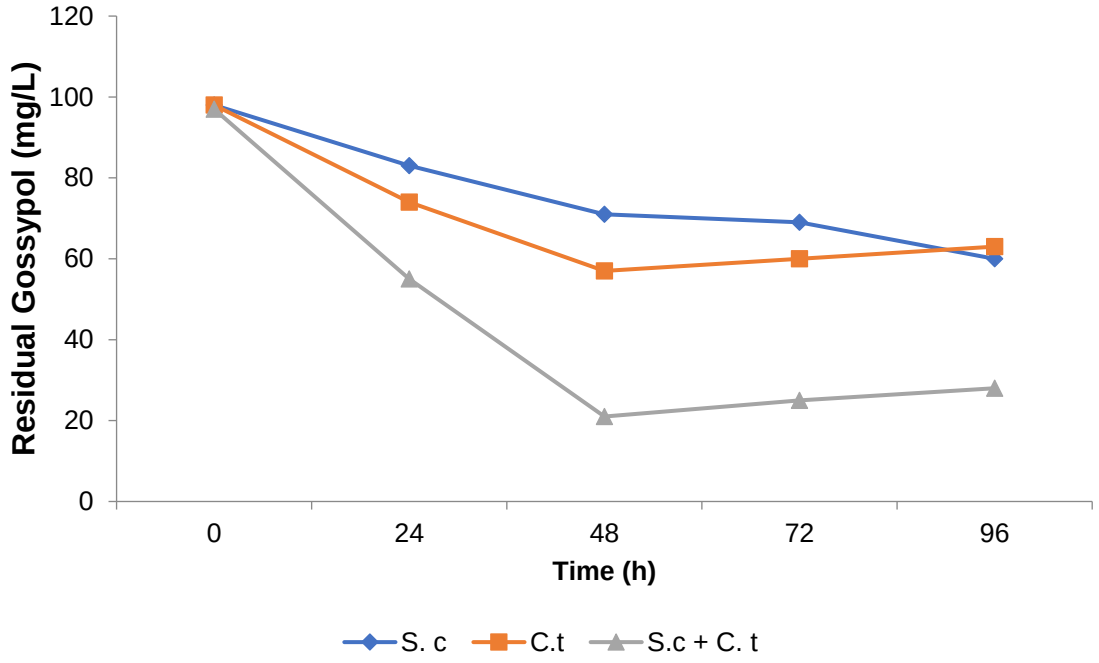

Fig. 3. Residual gossypol in fungal cultures grown in gossypol-containing minimal medium. Values are the means of three different experiments. ♦ *S. cerevisiae*, ■ *C. tropicalis*, ▲ *C. tropicalis* + *S. cerevisiae*

A slight increase in the residual gossypol level of the culture supernatant after 48 h might be due to the release of negligible levels of gossypol bound with fungal protein during the earlier stages of growth. Previously the mixed culture, *C. tropicalis* + *S. cerevisiae* had the gossypol degradation efficiency of 75% (Mageshwaran *et al.* 2018). However, the present study showed the marginal increase in gossypol degradation efficiency of 79%, after improvement of the strain after repeated growth of cultures in minimal medium containing 1000 mg/L of gossypol. The characterization of biodegraded gossypol using HPLC, FTIR, SDS-PAGE *etc.* showed that the laccase produced by mixed culture, *C. tropicalis* + *S. cerevisiae* in minimal medium, degraded the gossypol and biotransform gossypol from 517 g/mol into 474 g/mol (Krishnan and Vellaichamy 2017; Mageshwaran *et al.* 2018). Several studies attempted to isolate gossypol degrading strains from different sources such as soil, cotton rhizosphere, rumen, cottonseed industry, *etc*. The fungal isolates were obtained from the aforementioned sources using enrichment culture technique and further screened for gossypol detoxification efficiency under SSF. The native fungal isolate, F-8 obtained from rhizosphere of cotton plant, was found to be

efficient in gossypol detoxification efficiency and later the isolate was identified as *Fusarium thapsinum* (Mageshwaran and Kathe, 2013b; 2017a). In a similar study, attempt was made to isolate, screen, and identify efficient gossypol detoxifying microbes from the cotton rhizosphere as well as pink bollworm larvae. The study showed that the fungal strains *A. quadrilineatus*, *A. terreus*, *A. versicolor*, *P. griseofulvum* were efficient in reduction of free and bound gossypol under SSF (Santosh *et al.* 2020). A new fungal isolate, *Aspergillus niger* HQ-1 was obtained from cotton planted soil has the ability to grow in basal medium containing gossypol as the sole carbon and energy source (Yang *et al.* 2011).

In several studies, the fungal strains were found to be more effective in degradation of gossypol in CSM. The reason might be the presence of efficient enzymatic machinery in the fungal system for degradation of polyphenols and other complex organic compounds in the environment. The advantages of microbial degossypolization in CSM are maximum gossypol detoxification efficiency, higher bound gossypol reduction, and improvement in protein and lysine content in fermented CSM. The most efficient fungi for gossypol detoxification and nutritive quality improvement in CSM are *Candida tropicalis*, *Saccharomyces cerevisiae*, *Pleurotus spp.*, *Aspergillus niger*, and *A. oryzae* (Khalaf and Meleigy 2008; Yang *et al.* 2011; Mageshwaran and Kathe 2013a; Shaikh *et al.* 2014). Mixed fungal culture had higher gossypol detoxification efficiency than individual cultures. The mixed fungal cultures of *Pleurotus sajor-caju + Saccharomyces cerevisiae* and *Candida tropicalis + S. cerevisiae* showed higher gossypol detoxification efficiency than the other combinations tested (Shaikh *et al.* 2014). In a similar study, Atia and Rahim (2009) reported 90.2% reduction of free gossypol in CSM fermented with mixed culture, *Aspergillus niger + S. cerevisiae* combination. However, the authors described the effect of fermentation on bound gossypol level in CSM.

The optimization of process parameters of SSF for gossypol detoxification in CSC was carried out by employing mixed culture, *C. tropicalis + S. cerevisiae*, and it was found that that the chemical disinfected/ heat sterilized CSC with 70% moisture content, 15% inoculum level, 30 ℃ incubation temperature, and 48-hour incubation period had higher gossypol detoxification efficiency (Vellaichamy, 2016; Mageshwaran *et al.* 2017b). The fermented CSC showed improved nutritive quality parameters. The free gossypol, bound gossypol, CP, CF, and lysine content in initial and fermented CSM were (2200, 360 mg/kg), (2100, 770 mg/100g), (20, 33.5%), (35, 25%), and (0.4, 0.8%) respectively. The detoxification percentages of free gossypol and bound gossypol were 79.5% and 59.5%, respectively. The increase in CP (13.4%) and decrease in CF (11.4%) were recorded in fermented CSC. The feed conversion ratio and other growth performance of broilers was found to be at par in the treatment where 20 to 40% of soybean meal replaced with fermented CSC and commercial broilers diet (Kakde *et al.* 2020).

The chemical disinfection of CSC, culture addition, incubation, and drying are shown in Fig. 1. The chemical disinfection was done with 0.5% lactic acid. The mixed culture employed for SSF was *C. tropicalis*+ *S. cerevisiae*. Experiments were conducted for the preparation of 100 kg degossypolized and nutritive enriched DOC and UDC using the facility of batch process of degossypolization at GTC, Nagpur. The sample was taken from the degossypolized DOC and UDC and tested for various properties (%) (Free Gossypol, Bound Gossypol, CF, CP and Lysine). The properties of fermented DOC and UDC was compared with raw DOC and UDC (Table 1).

Table 1. Effect of Fermentation on Degossypolization and Nutritive Quality Improvement of CSC

Sample		Properties (%)				
		Free Gossypol	Bound Gossypol	CF	CP	Lysine
DOC	Raw	0.1[b]	1.2[b]	15.0[c]	32.0[b]	1.0[c]
	Fermented	0.02[c]	0.58[d]	12.0[d]	38.0[a]	1.4[a]
UDC	Raw	0.22[a]	2.32[a]	37.1[a]	20.1[c]	1.0[c]
	Fermented	0.045[c]	0.89[c]	25.6[b]	32.3[b]	1.25[b]

Note: Treatment values followed by the same alphabet do not differ significantly at P=0.05
DOC- deoiled cake; UDC-undecorticated cake

The free gossypol, bound gossypol, CF, CP, and lysine content (%) in raw DOC and fermented DOC were 0.1 and 0.02, 1.2 and 0.58, 15 and 12, 32 and 38, and 1.0 and 1.4, respectively (Table 1). Thus, the results showed there was a reduction ($P<0.05$) in the level of gossypol (both free and bound) in fermented DOC. The CF in the fermented DOC was reduced, while the CP and lysine contents were increased in fermented DOC when compared to raw DOC. Accordingly, the detoxification percentages of free and bound gossypol were found to be 80 and 51.6%, respectively (Table 2). The increase in CP (6%), lysine (0.4%) and decrease in CF (3%) were recorded in fermented DOC. The free gossypol, bound gossypol, CF, CP, and lysine content (%) in raw UDC and fermented UDC were 0.22 and 0.045, 2.32 and 0.89, 37.1 and 25.6, 20.1 and 32.3, and 1.0 and 1.25, respectively (Table 1). Similar to DOC, there was a reduction ($P<0.05$) in gossypol (free and bound) and CF content in fermented UDC, while CP content and lysine content was improved in fermented UDC in comparison to raw UDC. Accordingly, the detoxification rate of free and bound gossypol was found to be 79.5 and 61.6%, respectively (Table 2). The increase in CP (12.2%), lysine (0.25%), and decrease in CF (11.5%) was recorded in fermented UDC. The bulk density of fermented DOC was lower (503) as compared to raw DOC (600), while the there was no change in angle of repose (Table 3).

Table 2. Detoxification % of Free and Bound Gossypol in Fermented CSC

Sample		Detoxification (%)	
		Free Gossypol	Bound Gossypol
SSF- Batch Process	DOC	80.0	51.6
	UDC	79.5	61.6
Industrial trial	DOC	61.6	45.1
	UDC	66.6	56.1

DOC- deoiled cake; UDC-undecorticated cake

Table 3. Mechanical Properties of DOC

Sample	Properties		
	Moisture content (%)	Bulk Density	Angle of repose
Raw	14.56	600	38.1
Fermented	11.46	503	38.0

DOC- deoiled cake; UDC-undecorticated cake

The industrial trial confirmed the detoxification potential of yeast culture, *C. tropicalis* + *S. cerevisiae* even to further scale (one tonne of CSC). A similar trend was observed in the analysis of fermented DOC and UDC obtained from industrial trial in

which the free gossypol and bound gossypol were reduced while the protein and lysine content increased in the fermented CSC (Fig. 4). Accordingly, the detoxification rate of free and bound gossypol was found to be 61.6 and 45.1%, respectively, in fermented DOC. The increase in CP (7.5%) and lysine (0.4%) was recorded in fermented DOC. Meanwhile, the detoxification rate of free and bound gossypol was found to be 66.6 and 56.1%, respectively. In addition, an increase in CP (4%) and lysine (0.3%) was recorded in fermented UDC (Table 2). The fermented CSC improved degossypolization efficiency (reduced free and bound gossypol) and improved nutritive quality (increased protein and lysine content). Amongst the different methods of gossypol detoxification, microbial fermentation has been reported for bound gossypol reduction and lysine enrichment in fermented CSC (Mageshwaran and Parvez, 2016). The profiling of amino-acids using HPLC analysis showed the amino-acids *viz*., lysine, arginine, cystine, and phenyl alanine, were increased in fermented CSC as compared to raw CSC (Weng and Sun, 2006; Zhang *et al*. 2006). The bound gossypol reduction in fermented CSC was due to release of proteases by fungal cultures, while the increase in lysine content corresponds to increase in fungal biomass during SSF (Mageshwaran and Parvez 2016).

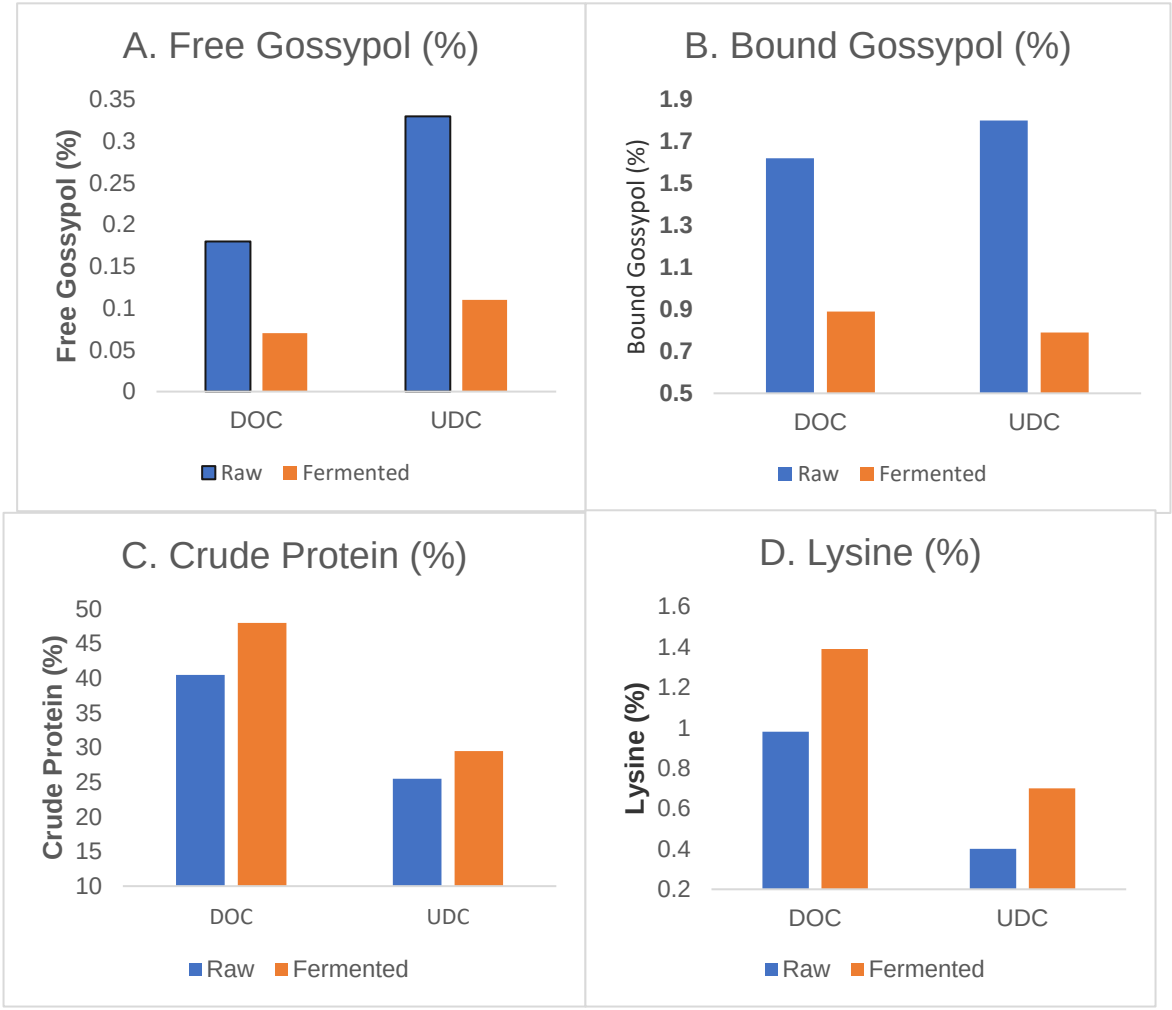

Fig. 4. Effect of fermentation on degossypolization and nutritive quality improvement of CSC under industrial trial

In the present study, the bound gossypol and lysine content was significantly decreased and increased, respectively, in fermented CSC as compared to the raw CSC. The nutritive properties of degossypolized DOC and UDC met the standards of US FDA and UN protein advisory group. It follows that the fermented CSC can be effectively used for protein feed supplement for poultry and other non-ruminant's ration.

CONCLUSIONS

1. The combination of improved yeast strains viz., *C. tropicalis* + *S. cerevisiae* resulted in 79% reduction of gossypol in minimal medium.
2. A scale-up of solid-state fermentation (SSF) was undertaken in which 100 kg of de-oiled cake (DOC) and undecorticated cake (UDC) was subjected for microbial degossypolization. The fermented cottonseed cake (CSC) had 60 to 80% and 40 to 60% reduction of free and bound gossypol, respectively. The protein and lysine content were increased while crude fibre (CF) decreased in fermented CSC.
3. The mechanical properties of DOC were improved after fermentation. Thus, the developed process would be helpful for application of CSC in animal feed.
4. Further evaluation of fermented CSC in different non-ruminants feed is warranted.

ACKNOWLEDGEMENTS

The funding received from ICAR-CIRCOT during the period 2018-19 to carry out this research work is highly acknowledged. The first author sincerely acknowledges the help and support rendered by Mr. C. Rangasamy, M/s Clean Cotton Impex, Tirupur, Tamil Nadu, India for carrying out an industrial trial at their facility.

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Article submitted: July 24, 2023; Peer review completed: October 21, 2023; Revised version received and accepted: November 21, 2023; Published: December 18, 2023.
DOI: 10.15376/biores.19.1.1107-1118