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An overview of gossypol and methods of its detoxification in cottonseed meal for non-ruminant feed applications

Vellaichamy Mageshwaran*

ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM),
Kushmaur, Mau Nath Bhanjan, Uttar Pradesh 275103, India

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Cottonseed is a by-product of the cotton crop rich in protein and oil. The products obtained from cottonseed are meal, oil, linters and hulls. The defatted cottonseed meal (CSM) is an important ruminant feed. However, its use in small animals is limited due to the presence of gossypol. Gosssypol is a toxic polyphenolic compound present in the entire cotton plant including its seed. The amount of total gossypol in CSM varies with the species and it ranges between 1.0 to 1.5%. Gossypol causes reproductive diseases and its toxicity level varies with the animals. Also, this substance is a compound of interest for pharmaceutical and medical applications. According to US-Food and Drug Administration, the food and feed products should contain less than 0.045% of free gossypol (FG). Researchers developed various methods, pre-processing (glandless cotton) and post-processing (physical, chemical and biological) for detoxification of gossypol in CSM to obtain FG level within this limit. The detoxified CSM finds application in the feed of dairy, poultry, piggery, aquaculture etc. In this review; chemistry, toxicity, bioactivity, and methods of estimation of gossypol and various strategies undertaken so far to detoxify gossypol in CSM for non-ruminant feed applications have been discussed.

Keywords: Bioactivity, Cottonseed meal, Detoxification, Gossypol, Non-ruminant feed, Toxicity.
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Introduction

In India, cotton is grown in an area of around 12 million hectares with a production of about 360 lakh bales in 2018-19[1]. Cottonseed forms a 2/3rd portion of the seed cotton. Its production during 2018-19 was 12 million tonnes[2]. Cottonseed is otherwise called "Golden goose" since all the parts are used as food, feed, and other valuable products. It contains 18% oil and 25% protein. The cottonseed meal (CSM) is rich in essential amino-acids and it is an important animal feed. The American Heart Association (AHA) recognized cottonseed oil as nutritive and healthy edible oil. The other products obtained from cottonseed are linters and hulls. The composition of cottonseed products obtained while processing cottonseed is given in Table 1.

A small portion of the whole cottonseed is used for direct feeding to animals while the major portion is used for seed crushing for oil extraction. Conventionally, the cottonseed is subjected to direct crushing for oil extraction. In India, more than 90% of industries follow a conventional method of processing. In the scientific method, cottonseed is subjected to series of processing to recover linters and hulls along with a meal rich in protein and oil. An illustrative description and comparison of the conventional and scientific methods of cottonseed processing are given in Fig. 1.

The cottonseed products production uses and value (USD) is given in Table 2. Cottonseed oil is used for cooking, salad dressings, and other industrial uses. The linters are rich in cellulose and used for the preparation of cellulose/microcrystalline cellulose (MCC) powder and cellulose derivatives. The high protein CSM has applications in animal feed especially for small animals if the CSM has a low level of gossypol. The hulls are lignocellulosic material used as roughage in animal feed. The undecorticated cottonseed cake has been used as animal feed especially for higher animals (goats, cows, and buffaloes). The total value of cottonseed products (oil, linters, meal, hulls and cake)

Table 1 — Cottonseed products composition

Cottonseed composition	Percentage (%)
Waste	2
Linters	8
Oil	18
Hulls	27
Meal	45

*Correspondent author
Email: mageshbioiari@gmail.com
Mob.: 919769941511

Table 2 — Cottonseed products- Indian scenario

Cottonseed products	Production (million tonnes)	Value (million USD)	Uses
Oil	1.35	990	Cooking, salad dressings
Linters	0.025	10	Cellulose, MCC, Cellulose derivatives
Meal	0.5	133	Animal feed, High protein source
Hulls	0.15	20	Roughage, Animal feed
Undecorticated cake	9.0	2400	Animal feed

Table 3 — Comparison of nutritive quality of soybean meal and cottonseed meal

Parameters (%)	Soybean meal	Cottonseed meal
Crude protein	44-48	30-38
Crude fibre	5-7	12-16
Lysine	2.5 -3.5	0.8-1.2
Gossypol	Nil	1.0-1.5

is 3553 million USD (Table 2). With the large availability of under-utilized undecorticated cottonseed cake (9 million tonnes) and CSM (0.5 million tonnes) annually, the detoxified CSM prepared from these extractions could be a huge protein supplement for small animals and humans.

Soybean meal is commercially used as a protein source in non-ruminant feed industries. The comparison of nutritive parameters of CSM and soybean meal is presented in Table 3. The nutritive composition of CSM is comparable to soybean meal. Though amino-acid composition of CSM is comparable to soybean meal except for lysine (data not shown), the other nutritive parameters of CSM is deficient than soybean meal. The range of crude protein, fibre content and lysine present in soybean meal and CSM are 44-48 and 30-38, 5-7 and 12-16, 2.5-3.5 and 0.8-1.2% respectively. The anti-nutritional factor, gossypol is absent in soybean meal while it is present in CSM in the range of 1.0 to 1.5%. Thus, the limitations of CSM in non-ruminant feed applications are the presence of a toxic compound, gossypol, low level of essential amino-acid, lysine and high fibre content[3].

Gossypol – Natural occurrence and chemical nature

Gossypol is a toxic polyphenolic yellow pigment, produced in the cotton plant including its seeds as a naturally occurring toxin that deters insect pests. It is concentrated in the cottonseed but can also be found in other parts of the cotton plant such as hulls, leaves, and stem. It is a phenolic aldehyde that permeates cells and acts as an inhibitor for several dehydrogenase enzymes. It has a molecular formula of $C_{30}H_{30}O_8$ with a molar mass of 518.6 g/mol and IUPAC name 2, 2'-bis-(formyl-1, 6,7 trihydroxy-5-isopropyl-3-methylnaphthalene) (Fig. 2). It has two naphthalene rings (with identical constituents), and restricted rotation about the bond connecting the rings, forming two identical structures that have no plane of symmetry and a differing optical properties ((+) - and (−) - enantiomers)[4]. Gossypol content and the proportion of (+) - and (−) - enantiomers in cottonseed are under genetic control, and vary within and between species of cotton[5].

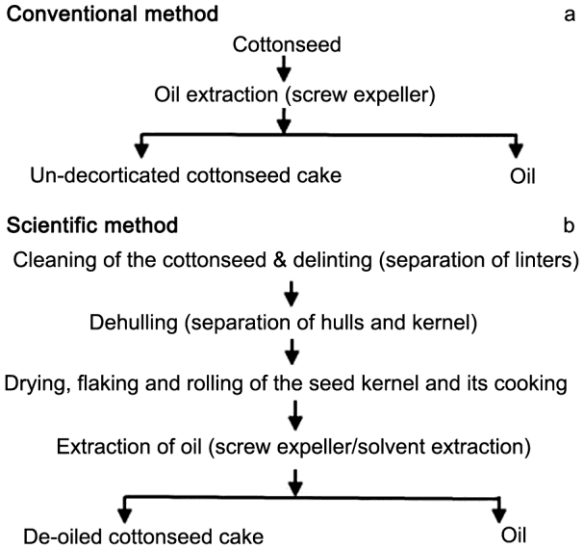

Fig. 1 — (a) conventional and, (b) scientific processing of cottonseed.

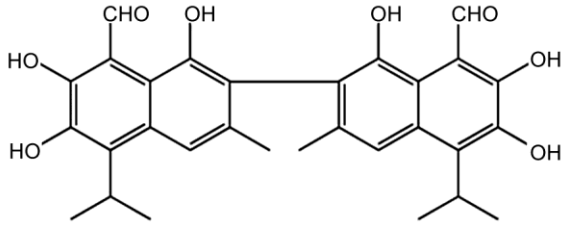

Fig. 2 — Chemical structure of gossypol.

Gossypol exists in two forms viz., free and bound. The free form is toxic, whereas gossypol that binds to proteins is in the "bound" or non-toxic form. The reaction between gossypol and protein results in "bound gossypol" during processing at a higher temperature. The free epsilon-amino group of lysine

and arginine is highly reactive with an aldehyde group of gossypol and forms a Schiff base called browning or Milliard reaction[6-8]. The free and bound gossypol constitutes total gossypol. The amount of total gossypol in the cottonseed could be quite variable. Many factors influence gossypol content such as species of the cotton plant, climatic conditions, soil conditions, fertilizer, etc. The seed of upland cotton (*G. hirsutum*), which represents about 98% of the cotton grown in the United States, has a total gossypol content ranging from 0.50 to 0.76% with (-) gossypol of 33.8 to 43.2%[9]. Seed of Pima cotton (*G. barbadense*) grown in the United States has a gossypol content of 0.67 to 1.25%, with the (-) enantiomer ranging from 35.2 to 55.2%[10]. Under Indian conditions, the free and total gossypol present in defatted CSM in the range of, 0.08 to 0.12 and 1.0 to 1.5% respectively[11].

Gossypol has an ultraviolet (UV) absorption maximum at about 385 nm (solvent dependent). It melts at temperatures around 200°C depending on its polymorphic form. Many polymorphic forms and crystalline solvates of gossypol exist depending on the solvent of crystallization. As racemic gossypol forms a 1:1 crystalline complex (solvate) with acetic acid that is less soluble in some organic solvents than gossypol itself, thus, acetic acid is commonly used when isolating gossypol. Nomeir and Abou-Donia[12], reviewed the history of extraction of gossypol. As early as 1860's, the yellow pigment present in the cottonseed was recovered from the "foots" of cottonseed oil after the removal of fatty acids. The product obtained was quite impure, a mixture of oxidation products, bluish-green to brown colour, having pungent and powerful dyeing properties. Later in 1890's, Marchlewski named the compound, gossypol by a combination of the two words, gossypium and phenol. Withers and Carruth[13] first obtained pure gossypol and gave some indication of the difficulties involved in obtaining a pure product without oxidation.

Methods of gossypol estimation

Gossypol is estimated in CSM or any other cottonseed extractions by techniques such as spectrophotometric, High-Performance Liquid Chromatography (HPLC), chemiluminescence and Enzyme-Linked Immunosorbant Assay (ELISA). Of the different methods, spectrophotometric method of analysis of gossypol[14] is officially and widely used. In this method, FG is estimated by following the protocol of AOCS Ba 7-58 while the total gossypol is estimated by AOCS Ba 8-78. In these methods, the extracted gossypol is made complex with aniline to form dianilinogossypol and is estimated spectro-photometrically. A modified AOCS method of analyzing FG in cottonseed products was developed by Hron *et al.*[15] in which the authors claimed that the time required for each sample was 25 min in place of 2 h in a conventional method. The sensitivity of the spectrophotometric method is up to 0.01% of gossypol. Thus, it is difficult to estimate gossypol in the sample containing less than 0.01%. To overcome this problem, researchers developed highly sensitive techniques such as HPLC, chemiluminescence, ELISA etc. for detection and quantification of gossypol at a low level. Hron *et al.*[16] developed HPLC method to quantify gossypol enantiomers. The method involves the derivatization of gossypol with 2-amino-1-propanol followed by HPLC separation with reversed-phase column eluted with 80% acetonitrile and 20% 10 mM KH_2PO_4 adjusted to pH 3.0.

In a similar study, gossypol in trace levels was detected by chemiluminescence method[17]. Iron has a strong affinity towards the aldehyde group in gossypol and thus helps to detect gossypol in the sample. In this method, gossypol was sensitized by reaction of luminol with ferricyanide in sodium hydroxide medium. Still, a more sensitive method was reported by Wang *et al.*[18] in which a competitive direct ELISA was developed for the analysis of gossypol at a low level. A good correlation (R^2 = 0.96, P <0.05) was obtained between the competitive direct ELISA and AOCS official method of FG analysis. The methods such as HPLC, chemiluminescence, and ELISA are highly specific, more sensitive and capable of the quantification of gossypol in cottonseed meal or any other cottonseed products having a low level (<100 ppm) and an ultra low level of gossypol (<1 ppm) which cannot be determined by conventional AOCS method. The high sensitive methods have application in the analysis of cottonseed flour from glandless cotton or degossypolized cottonseed meal. It is essential to understand that the quantification result of gossypol by the AOCS method is always on a higher side than other methods because AOCS determines gossypol and gossypol derivatives in the extract[19] while the other methods are highly specific and estimate only the gossypol compound. Hence, utmost care should be taken while comparing the quantification results of gossypol in the samples analyzed by other methods

with the AOCS method. Though the AOCS method is simple and commonly used, it consumes large time and chemicals especially solvents for analysis. Hence, the factors such as type of sample (level of gossypol in the sample), availability of resources and time have to be considered before selection of any method for analysis of gossypol.

Toxicity of gossypol

The toxicity symptoms, anti-growth properties and lethal dose (LD 50) value of gossypol in different animals have been previously reported[8,20]. The most important toxicity of gossypol to animals is the malfunctioning of male and female reproductive organs. The other clinical symptoms of gossypol toxicity in animals are anorexia, difficulty in breathing, metabolic disorders and high concentration cause even death. Generally, (–) - gossypol is more biologically active than (+) - gossypol. Gossypol severely inhibits spermatogenesis in males while in females, it disrupts estrous cycles, pregnancy, and embryo development. Monogastric animals are more susceptible to gossypol toxicity than ruminants[21-24]. Early in the 1950's, lower childbirth was noticed among the people in Jiangsu province of China because people consumed unheated cottonseed oil contaminated with gossypol. It gave considerable interest for the Chinese to use gossypol as a male anti-fertility and therapeutic agent for the treatment of some gynaecological diseases[25,26]. Later, the efforts were withdrawn due to the development of a side effect (hypokalemia) among 10% of users[24,27,28].

The toxicokinetic property of the gossypol such as absorption, distribution, biotransformation, and elimination of gossypol varies with the species and strains of animals for example mice eliminate gossypol faster than rats[29-32]. The other metabolic disorders caused by gossypol to animals are inhibition of DNA synthesis, oxidative stress, liver damage etc.[33-35].

Bio-activity of gossypol

Gossypol has therapeutic/pharmaceutical properties such as anti-fertility, anti-oxidant, anti-cancerous, anti-viral and anti-microbial properties[24,36,37]. Gossypol and its methylated forms, 6-methoxygossypol and 6,6'-dimethoxygossypol, showed greater anti-oxidant, anti-cancer and anti-trypanosomal effects. The methylated form, 6,6'- dimethoxygossypol had stronger inhibition against the growth of *Trypanosoma brucei* cells[38]. In this review, the anti-microbial activities of gossypol have been discussed in little detail.

Fig. 3 depicts the antimicrobial spectrum of gossypol against gram-positive bacteria, gram-negative bacteria, yeast and filamentous fungi. The minimum inhibitory concentration (MIC) of pure gossypol against aerobic spore formers and lactobacilli, yeasts, filamentous fungi and gram-negative bacteria range from 10 to >200 µg/mL[39,40]. The gram-positive bacteria were more sensitive to gossypol than gram-negative bacteria. Gossypol at 100 µg/mL inhibited almost all the gram-positive bacteria tested whereas only 1/3 of the gram-negative bacteria were inhibited at 200 µg/mL. It was observed that the anti-bacterial activity of gossypol was related to the gram character of the organisms. Gram-negative bacteria have an outer membrane that acts as a barrier for transport of big molecule like gossypol while gram-positive bacteria doesn't have any outer membrane[37,41].

Yildrim-Aksoy *et al.*[42] reported that MIC of gossypol against *Edwardsiella ictaluri* was 3 µg/mL. *E. ictaluri* is the aetiological agent of enteric septicaemia of catfish (ESC). The gossypol and its derivatives, gossypolone and apogossypolone showed strong inhibition against pathogenic filamentous fungi *Aspergillus flavus*, *A. parasiticus*, *Penicllium chrysgogenum*, *Fusarium graminearum*, *F. moniliforme*, *Phythium irregulare*, *P. ultimum*, *Rhizoctonia solani*. The MIC of gossypol against cotton root fungal pathogens, *P. irregulare* and *P. ultimum* were 4.0 and 13.2 µg/mL respectively. These studies suggest gossypol and its derivatives could be used as a therapeutic agent for the control of economically important diseases in the plant and animals[43-46].

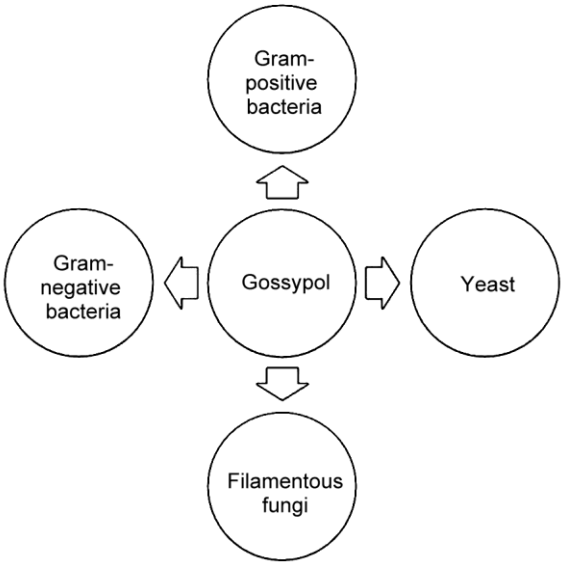

Fig. 3 — Anti-microbial spectrum of gossypol.

Gossypol detoxification strategies

The strategies involved in detoxification of gossypol CSM are divided into (i) pre-production and (ii) post-harvest processing. In pre-production, breeders attempted to develop lines of cotton plants having no gossypol glands, especially in seeds. The other aspect of detoxification lies in the post-harvest processing of cottonseed. In post-harvest processing, different physico-chemicals and biological methods were developed to eliminate gossypol in CSM. A schematic diagram representing the strategies involved in gossypol detoxification in CSM for its use in food and feed applications are given in Fig. 4.

Glandless cotton

Early in the 1950s, researchers made effort to develop breeding lines of cotton plants lacking gossypol pigment. The plant thus developed was highly susceptible to the pest and diseases. Gossypol is a natural pigment produced in cotton plants and plays a vital role in providing defense against the attack of pests and diseases. Later, breeders reoriented their efforts to develop breeding lines of cotton plants lacking gossypol pigments limited to seed[47]. The cotton plant was genetically modified using ribonucleic acid (RNA) interference technology to disrupt gossypol biosynthesis specifically in cottonseed tissue by engineering the endogenous terpene pathway[48]. The disruption of gossypol production was seed-specific. Some of the lines produced seeds with 0.2 g/kg as compared to wild type seeds with 10 g/kg of gossypol. Agrobacterium-mediated transformation was used to insert a construct containing a seed-specific promoter driving RNAi hairpin into the cotton nuclear genome that suppresses delta-cadinene synthase, a critical enzyme involved in building gossypol. Thus it is possible to produce a plant containing gossypol-free cottonseed while retains its natural defense mechanism through gossypol in the non-seed tissues[49]. Further, studies are required to establish whether the low gossypol trait in the plants is also stable under field conditions.

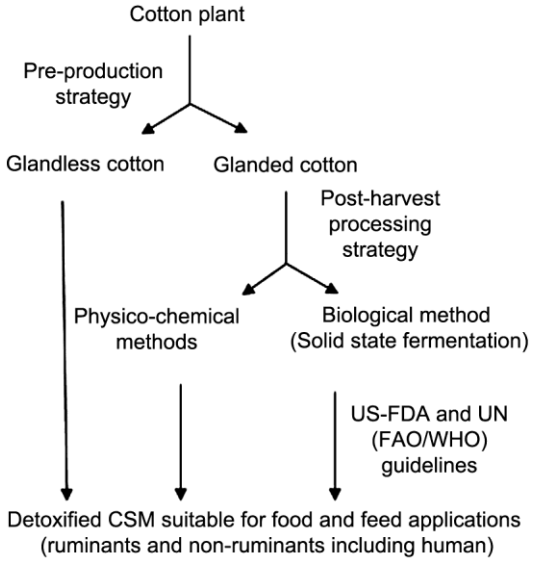

Fig. 4 — Strategies for gossypol detoxification in cottonseed meal.

Physico-chemical methods

Researchers studied for many years to find a way to detoxify FG and proposed a number of methods such as liquid cyclone process, solvent extraction, air classification, chemical treatment with iron sulfate or calcium hydroxide. A liquid cyclone process (LCP) was developed based on liquid classification using hexane in which the process yielded fine, edible and high-protein concentrate. The principle behind the process is separating the intact pigment glands from protein. The mechanical separation of grounded cottonseed kernel slurried in hexane resulted in two fractions, the underflow containing most of the pigment glands and the overflow containing most of the protein free of pigment glands[6,50]. The cottonseed flour produced by the LCP process had protein, 65-68%, total and free gossypol, less than 0.06 and 0.045% respectively, lipid less than 1 %, 2-3% crude fibre, 7% ash and 3.9 g available lysine /16 g nitrogen. The protein had more than 95% solubility and the product was having bland in flavour and light in colour[51]. The CSM was subjected to different milling methods such as fixed hammer disintegrating, pin milling and air gun pulverizing. The milled CSM was air classified at different rotor speeds (rpm) at different stages wherein in each air classification step a small coarse fraction is obtained and fines fraction was reclassified. The low gossypol edible cottonseed flour was obtained from CSM milled with a fixed hammer disintegrator. The important factors in obtaining low gossypol edible cottonseed flour were minimal hulls content, moisture (2%) and minimal residual lipids (2%)[52,53].

The grounded cottonseed flakes were extracted with 75-80% acetone in the water medium removed most of the free fatty acids and raffinose resulted in light-coloured meals, high in protein and low in gossypol content. Further in the process, the oil obtained from the cottonseed kernel extraction using hexane was light in colour due to the removal of most of the gossypol in the acetone extraction process. This

process was useful for the removal of toxic mould metabolites like aflatoxins from mould-damaged seed[54]. A two-stage solvent extraction process was developed using aqueous and anhydrous acetone to detoxify gossypol in CSM and during the process, cottonseed protein concentrate obtained was having 72.2% protein content[55]. The addition of 10 to 25% of acetone in n-hexane improved the extraction of free and total gossypol from cottonseed flakes. The CSM obtained after extraction had a minimal odour and 80–90% reduction of FG[56]. The addition of phosphoric acid in acetone and ethanol-based solutions, heated and refluxed was found effective in reducing total gossypol level in the meal between 5 to 10% in CSM[57].

The addition of ferrous sulphate at the rate of 580 mg of Fe /kg of fish (Nile Tilapia) diet containing 40% CSM significantly inactivated FG[58]. Similarly, the addition of $Ca(OH)_2$ at the rate of 0.5, 1.0 and 2.0% to CSM resulted in 21, 28 and 40% reduction of FG respectively[59]. Though the physico-chemical methods have capable of producing cottonseed flour containing > 65% protein and significant reduction of FG, they have certain drawbacks like effluent generation, high capital investment, high capacity processing to be economically viable. 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[60]. Although detoxification by iron sulfate was a convenient method, it merely binds with FG and doesn't block the conversion of bound gossypol into FG[61].

Biological method

To overcome the drawbacks of physico-chemical methods of detoxification, there was a need to develop a new approach for the degradation of FG and the reduction of bound gossypol in CSM. It has been found that a few microorganisms are capable of degrading FG, including *Candida tropicalis, S cerevisiae, Pleurotus flabellatus, P. sajor-caju, Torulopsis candida, Aspergillus oryzae* and *A. niger*[62-66]. CSM detoxicated by microorganisms not only reached safe criteria, but enhanced the nutritive value by improvement of protein, amino acids, and secretion of cellulolytic, amylase, protease and lipolytic enzymes and variety of vitamins[67,68].

Khalaf and Meleigy[64] evaluated some fungi (*C. tropicalis, S. cerevisiae, A. terreus, A. oryzae* and *A. niger*) for the reduction of FG levels during solid-state fermentation (SSF) of CSM. Results indicated that *Candida tropicalis* was more effective in biodegradation of FG in CSM among the different strains tested. The optimum fermentation conditions were incubation period at 48 h, incubation temperature at 30°C, inoculum level at 1 x 10^7 cells g^{-1} of solid substrate, moisture content 55% and pH (5.2). The crude protein and amino-acids content of the fermented substrate under optimized conditions were improved markedly. Mageshwaran and Kathe[65] reported solid state fermentation of CSM with *P. flabellatus* M-1 resulted in 70 and 50% reduction of free and bound gossypol respectively. The crude protein content in CSM was increased significantly in *P. flabellatus* M-1 fermented CSM. The optimized process parameters were 80% moisture content in the autoclaved CSM, 48 h incubation, 30 °C and inoculum level at 5%.

The different fungi, *Candida capsuligena ZD-1, C. tropicalis ZD-3, S. cerevisae ZD-5, A. terricola ZD-6, A. oryzae ZD-7* and *A. niger ZD-8* were evaluated for gossypol detoxification in feed ration containing 60-70% CSM[60,63,69,70]. The effect of the addition of carbohydrates, urea, minerals and heat treatment on reduction of FG during solid substrate fermentation was evaluated. Among the fungi tested, *C. tropicalis ZD-3* and *A. niger* ZD-8 were found most effective. The FG reduction in the substrate was 95%. The CSM supplemented with starch and sucrose enhanced the detoxification of gossypol. Heat treatment and minerals addition were also effective in reducing FG during solid substrate fermentation of CSM. Weng and Sun[62] studied the biodegradation of FG by *Candida tropicalis* ZAU-1 under solid state fermentation. Maximum biodegradation of FG (92.3%) occurred under optimal conditions such as 60 h incubation, 30-35°C and solid medium containing CSM 60%, wheat bran 20%, rice bran 18%, rice wine spent grain 2%, molasses 1%, $MgSO_4.7H_2O$ 0.5% and KH_2PO_4 1.5% including moisture content, 50-55%, pH 4.0-6.0 and inoculum level at 1 × 10^7 cells/g of solid substrate.

The effect of mixed culture solid substrate fermentation of *C. tropicalis* with *A. niger* on detoxification of CSM was studied[71]. Maximum gossypol detoxification efficiency (90.2%) occurred after 48h of incubation at 30 °C in a 250 mL conical flask containing 15 g of CSM supplemented with 1% (w/w) $(NH4)_2SO_4$ at the optimal conditions including the initial moisture content 55% (w/w) and inoculum level at 5% (v/w). Similar to CSM, gossypol detoxification was achieved in other cottonseed

extractions such as undecorticated cottonseed cake[72]. The detoxification of FG was a growth-associated process, which was highly correlated with dry matter weight loss. Moreover, high activities of hydrolytic enzymes produced in solid-state fermentation enhanced the nutritive value of the detoxified CSM.

The effect of mixed fungal cultures *viz*., *P. sajor-caju* with *S. cerevisiae* and *C. tropicalis* with *S. cerevisiae* on detoxification of gossypol in heat sterilized CSM was studied under solid state fermentation[73,74]. The optimized process parameters for gossypol detoxification in heat sterilized cottonseed cake were 70% moisture content, 15% inoculum level, 30 °C incubation temperature and 48 h incubation period. The FG and bound gossypol detoxification rate achieved was 83.6 and 63.3% respectively[74]. In another study, the cost of heat sterilization was reduced by chemical disinfection of cottonseed cake. The chemical disinfection was done by treatment with 0.5% lactic acid and subsequently inoculated with mixed fungal culture, *C. tropicalis* and *S. cerevisiae*. The detoxification rates of FG and bound gossypol were 79.5 and 59.5% respectively. The crude protein was increased to 13.4% and crude fibre was decreased to 11.4%[.66]. The study suggests that the reduction of bound gossypol was due to the release of protease by fermenting organisms while the improvement of protein and lysine in CSM was due to the increase in fungal biomass during fermentation[75].

Experiments were conducted to study the mechanism of biodegradation of gossypol. A new *Aspergillus* strain HQ-1 isolated from soil was able to biodegrade FG on agar plates. The analysis of intracellular proteins of *A. niger* AN-1 grown in gossypol containing medium showed the protein spots in the range of 25 to 66 kDa in two-dimensional gel electrophoresis[76,77]. An increase in laccase activity of *P. florida* was observed with respect to the increase in degradation of gossypol over the period of 5 to 25 days[78]. In another study, the gossypol-degrading enzyme was isolated from the culture supernatant of mixed fungal cultures, *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* grown in gossypol containing medium. The gossypol degrading enzyme was found to be the laccase group of enzyme and it was separated in the SDS-PAGE in the molecular weight range of 45 to 66 kDa. The characterization of residual gossypol (biodegraded) in the supernatant revealed the reduction of functional aldehyde stretches and the monoisotopic mass of the biodegraded gossypol was found to be 474 g/mol[79]. Attempts were also made to isolate and identify native microbial strains for gossypol detoxification. The native fungal strains, *Fusarium thapsinum* F-8 and *Alternaria alternata* F-3 isolated from cotton growing soil showed free and bound gossypol detoxification efficiency of 60-65 %[80, 81].

The detoxified CSM produced by biological method (solid state fermentation) had improved protein and amino-acid profile, FG and bound gossypol reduction. The other characteristics of solid state fermentation of CSM are zero effluent generation, low capital investment, and suitability for economically viable small & medium levels of processing. However, the drawback lies with the biological method are scale-up of fermentation and continuous production of detoxified CSM at the industrial scale. A comparison of different physico-chemical and biological methods, the process and product highlights are given in Table 4.

Potential of gossypol detoxified CSM for non-ruminant feed applications

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 that limits FG to 600 mg/kg and bound gossypol to 1140 mg/100 g[82]. CSM or cottonseed flour containing FG less than 450 mg/kg was considered for edible purposes especially for snack purposes in US[47]. The presence of bound gossypol reduces the availability of amino-acids especially lysine during enzymatic digestion and thus reduces the nutritive value of CSM. Also, FG can be released from bound form during enzymatic digestion[8,83]. Thus, CSM for feed/food purposes should have bound gossypol levels as minimal as possible. As discussed earlier, certain degossypolization methods have the potential to reduce bound gossypol as well in CSM. The other parameters to be taken into account to consider cottonseed flour/meal for edible purposes are (a) protein content should be more than 60%, (b) residual lipid in the flour should preferably be less than 1 %, (c) the flour should be free of mould contaminants and should not have an objectionable odour (d) the flour should be light in color and free of residual solvents.

In the early 1970's, the South Regional Research Center (SRRC), United States Department of Agriculture (USDA) in the USA had done lead work in the preparation of edible flour from cottonseed meal. The degossypolization technology such as solvent extraction, LCP etc. was employed to produce edible flours, concentrates and isolates from CSM.

Two edible protein products were prepared from CSM viz., "Proflo" and "Incaprina". "Proflo" was used to impart functional properties of baked and confectionery products while "Incaprina" was used to combat malnutrition in Latin America[84,85]. The inclusion of 10% CSM in the diet of *Labeo rohita* showed better performance in terms of growth, % survival, feed conversion ratio and protein efficiency ratio[86]. Feeding of the detoxified CSM by *S. cerevisae* and *A. niger* as a mixed culture did not show any adverse effects on rabbits[71]. The replacement of 8% soybean meal with fermented CSM in the diet of yellow-feathered broilers had higher (P <0.05) body weight gain than the other treatments[87]. The CSM fermented with the fungus, *Diplodia* was fed to swine and found to have improved daily gain, diet intake and feed efficiency and reduced mortality rate[88,89]. Thus, the reports strongly suggest the inclusion of gossypol detoxified CSM (within standard limit) in feed ration does not have any negative impact on the non-ruminants.

Table 4 — Comparison of different methods of gossypol detoxification in cottonseed meal

Method of gossypol detoxification	Process parameters	Product highlights	Reference
Physico-chemical methods			
Solvent extraction	Raw material- cottonseed flakes, Aqueous acetone extraction, Acetone and hexane mixture	Free and total gossypol reduction	54, 56
Liquid cyclone process	Raw material- cottonseed flakes, Liquid classification using hexane	FG (< 0.045 %), protein content – 65 %	6, 50
Air classification process	Raw material- CSM, Milled CSM air classified at different rotor speed (rpm) at different stages	Low gossypol edible cottonseed flour was produced from CSM milled with fixed-hammer disintegrator	52
Two stage solvent extraction process	Raw material – cottonseed flakes, Aqueous acetone and anhydrous acetone	Cottonseed protein concentrate (72.2 % protein content)	55
Ferrous sulphate treatment	Raw material- CSM, 500 to 600 ppm of Fe in animals diet	FG inactivation	58, 61
Calcium hydroxide treatment	Raw material- CSM, 0.5 to 2 % $Ca(OH)_2$	FG reduction (20 to 40 %).	59
Acidic solvent extraction	Raw material – CSM, Phosphoric acid in acetone/ethanol, heated & refluxed	Total gossypol reduction (5 to 10 %)	57
Biological method (solid state fermentation)			
Candida tropicalis ZAU-1	CSM- 60% in ration. 60 h incubation, 30-35°C, moisture content (50-55%), pH 4-6, inoculum level - 1×10^7 cells/g	FG detoxification efficiency – 92.3%	62
C. tropicalis ZD-3, *A. niger* ZD-8	CSM-70% in ration. 50% moisture content, 30°C for 48 h incubation, 1 -5% inoculum, 95% relative humidity	FG reduction – 95%, Increase in amino-acid composition	60, 63, 69, 70
Locally selected fungi (*C. tropicalis*, *S. cerevisiae*, *A. oryzae*, *A. terreus* & *A. niger*)	48 h incubation, 30° C, inoculum level - 1×10^7 cells/g, moisture content-55%, pH 5.2	Improvement in crude protein and amino-acids content	64
P. flabellatus M-1	CSM-100% in ration. Autoclaved CSM (110°C), 80% moisture content, pH in nature, 48 h incubation, 30°C, inoculum level – 5%	70% FG and 50% bound gossypol reduction	65
S. cerevisiae + *A. niger*	CSM-100% in ration. 48 h incubation, 30°C, addition of 1% $(NH_4)_2SO_4$, moisture content – 55%, inoculum level – 5%	FG detoxification efficiency – 90.2%	71
P. sajor-caju + *S. cerevisiae*	CSM-100% in ration. Heat sterilized CSM, 70% moisture content, 30°C, incubation period – 48 h	Free and bound gossypol detoxification rate – 83.6 & 63.6% respectively	68, 74
C. tropicalis + *S. cerevisiae*	CSM-100% in ration. Chemically disinfected CSM, moisture content – 70%, 15% inoculum addition, 30°C, incubation period – 48 h	Free and bound gossypol detoxification rate – 79.5 & 59.5% respectively. Crude fibre reduction – 11.4%, improvement in crude protein – 13.4%.	66
Native fungal isolates from cotton growing soil (*Fusarium thapsinum* F-8; *Alternaria alternata* F-3)	CSM-100% in ration. Heat sterilized CSM, 80% moisture content, 30°C, incubation period – 48 h	Free and bound gossypol detoxification rate, 60-65%.	80, 81

Conclusion

Gossypol is a unique compound present largely in cotton plants and its related species. According to an estimate, about 50,000 tonnes of gossypol could be obtained from cottonseed available annually in India even after considering half of its recovery. The purified gossypol and derivatives have enormous pharmaceutical applications as anti-fertility, anti-cancerous, anti-parasitic, anti-oxidant and anti-microbial agents. There is a large availability of CSM annually under Indian conditions. The different methods of detoxification of gossypol have widened the use of CSM in unconventional feed sectors such as piggery, poultry, aquaculture etc. while its use in human feed needs further detailed scientific confirmations and precautions. In future, research may be focussed on gossypol extraction from cottonseed kernel/flakes rather than CSM for effective separation and to produce degossypolized CSM having low bound gossypol. Thus, the valuable gossypol could be recovered during the process of obtaining detoxified cottonseed flour suitable for non-ruminant feed applications. In addition, a rapid and cost-effective method may be developed to quantify gossypol at lower concentrations in cottonseed meal/cottonseed products at an industrial site which could benefit traders, processors involved in cottonseed processing.

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Conflict of interest

The author declares that there is no conflict of interest.

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