

# *Cottonseed: a sustainable contributor to global protein requirements*

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## Highlights

- Physical, chemical, and biological methods for improving utilization of cottonseed as protein source.
- Solvent extraction remains the method of choice.
- Functional properties and amino acid profile for evaluation of quality of cottonseed protein are well discussed.
- Application of cottonseed protein as direct and indirect source of supplement in human nutrition.
- Food safety and regulatory issues for application of cottonseed protein/flour in foods is outlined in the review.

## Structured Abstract

*Background:* Cottonseed is a sustainable source of plant protein, producing ~10 million metric tons of protein globally. This protein has the potential to fulfil the annual protein requirement of more than half a billion people globally. Its functional properties have established the potential of cottonseed protein (CSP) as a candidate for alleviating malnutrition in the **Asian and African continents**. Regardless of these quality attributes, the inherent association of gossypol with CSP makes it unsuitable for direct human consumption due to its toxicity.

*Scope and Approach:* The present review elaborates on physical, chemical and biological methods for enhancing the quality and suitability of CSP for human nutrition by reducing the gossypol content to permissible limits (450 ppm) per the U.S. Food and Drug Administration and World Health Organization. Amino acid profiling, functional property (water holding capacity, oil holding capacity, foaming properties, emulsification characteristics, and protein solubility), *in vitro* protein digestibility and molecular weight analyses are the parameters considered important for the application of CSP in foods. This review also highlights the diverse applications of CSP directly in human nutrition or indirectly as animal protein.

*Key Findings and Conclusions:* Degossypolization is mainly performed by solvent extraction, although gamma irradiation and the use of microorganisms are gaining momentum. CSP is a good candidate for use in food and feed formulations, with a balanced amino acid composition and functional properties comparable to those of soy protein. Integration of both chemical and biological methods might prove to be more efficient for degossypolization and improving the utilization of CSP for human nutrition.

## 1 **Cottonseed: a sustainable contributor to global protein requirements**

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32 inherent association of gossypol with CSP makes it unsuitable for direct human consumption  
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38 capacity, oil holding capacity, foaming properties, emulsification characteristics, and protein  
39 solubility), *in vitro* protein digestibility and molecular weight analyses are the parameters  
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45 composition and functional properties comparable to those of soy protein. Integration of both  
46 chemical and biological methods might prove to be more efficient for degossypolization and  
47 improving the utilization of CSP for human nutrition.

48 **Keywords:** Cottonseeds; malnutrition; application; plant protein; food safety and regulation;  
49 protein isolate

50

## 51 **1. Introduction**

### 52 **1.1. Problem of malnutrition in the African and Asian continents**

53 Malnutrition is an ever-increasing pandemic throughout Africa and Asia. India accounts for  
54 43% of malnourished children under the age of five years, accounting for 61 deaths per  
55 thousand births. Bangladesh (41%) is closest-ranked to India, followed by Afghanistan (33%),  
56 Pakistan (31%), Nepal (29%), Myanmar (23%), Sri Lanka (22%), Thailand (7%), and China  
57 (3%) (Black and Sesikiran, web source: [https://www.nestle.com/sites/default/files/asset-](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf)  
58 [library/documents/creating%20shared%20value/expert-opinions.pdf](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf) accessed on 25/12/2020)  
59 (Fig. 1). Similarly, African countries also show a similar problem of malnutrition in children  
60 under the age of 5 years. Malnourished children in most other developing and underdeveloped  
61 nations range from high (30–39%) to very high (>40%) (United Nations Children’s Fund  
62 (UNICEF), World Health Organization, International Bank for Reconstruction and  
63 Development/The World Bank, 2020). This problem does not end in childhood, as it also  
64 persists in the elderly populations of these countries. There is an urgent need to address the  
65 dire problem of malnutrition in the Asian and African continents. Consequently, the UN  
66 Sustainable Development Summit in 2015 in New York adopted the 2030 agenda for  
67 sustainable development, which consists of 17 sustainable development goals (SDGs), out of  
68 which 2 SDGs, i.e., goal 2 ‘Zero hunger’ and goal 3 ‘Good health and well-being’, are  
69 directly related to alleviating malnutrition. The current review addresses the possible  
70 contribution of CSP to accomplishing these goals and alleviating malnutrition.

71

72

**Fig. 1.**

73

### 74 **1.2. Cottonseed as a sustainable protein source**

75 The global cottonseed production in 2019/2020 is estimated to be ~44.84 metric million tons  
76 (MMT) (Statista, 2020 accessed on 25/07/2020). Cottonseeds contain 17–22% oil, and after  
77 oil extraction, cottonseed meal (CSM) is obtained as a coproduct (Hernandez, 2016) (Fig. 2).  
78 CSM has the potential to produce ~10 MMT proteins, which could fulfil the annual protein  
79 requirements of more than half a billion people globally (Wedegaertner & Rathore, 2015).  
80 The cottonseed protein fraction contains the highest content of salt-soluble protein (globulins:  
81 33–63.7%), followed by water-soluble (albumins: 20.8–32.2%) and alkali-soluble (glutelins:  
82 9.2–28%) proteins (Balandrán-Quintana et al., 2019). As evidenced by a recent review, most  
83 African and Asian countries have a higher severity of malnutrition and are ranked 90–119 in  
84 the Global Hunger Index, 2018 (Rathore et al., 2020). Nevertheless, these are the highest  
85 cotton-producing nations of the world, producing more than 1000 tons of cottonseed annually  
86 (Rathore et al., 2020). Hence, the successful and efficient utilization of cottonseed as a protein  
87 source could be a game changer in mitigating **malnutrition in the most severely affected**  
88 **countries.**

89

### 90 **1.3. Problem of gossypol in cottonseed or cottonseed meal**

91 Gossypol is a toxic polyphenolic compound present throughout cotton (*Gossypium hirsutum*)  
92 plants, with the highest concentration (up to 2.4% on a dry weight basis) in cottonseeds. The  
93 presence of gossypol in cottonseeds or CSM limits its use both as feed for small ruminants  
94 and nonruminants and as a supplement in the human diet. The presence of this toxin in diets  
95 has several deleterious effects on the growth, development and reproductive health of  
96 animals, limiting the use of CSM as a feed in ruminants (Zhang et al., 2006a). Increasing  
97 prices of soy or animal-based proteins in Asian and African countries make CSM a more  
98 competitive and sustainable protein source for human nutrition. This protein source can be  
99 feasibly utilized if the gossypol content in CSM is reduced to a safe consumption level. The



100 United States Food and Drug Administration (USFDA) and the World Health Organization  
101 (WHO) have set the limits for free gossypol in CSM-based protein products for safe  
102 utilization by nonruminants to 450 ppm.

103

#### 104 **1.4. Strategies for efficient utilization of cottonseed meal (CSM) as a source of protein**

105 Initially, trials for the removal of gossypol from CSM were executed through traditional  
106 physical approaches, e.g., the gland flotation technique, heat treatment, pressure cooking, and  
107 the liquid cyclone process. These processes were further found to be non-feasible due to their  
108 high processing costs (Rathore et al., 2020). Gamma and electron beam irradiation are some  
109 of the recent physical techniques for reducing the gossypol present in the CSM. Both of these  
110 irradiations caused similar effects in reducing both free and total gossypol contents in a dose-  
111 dependent manner. Further molecular biology tools have been employed to reduce gossypol  
112 from cottonseeds, and recently, a group of researchers working at Texas A & M University  
113 achieved ultralow gossypol concentrations in cottonseeds using molecular biology tools  
114 (Rathore et al., 2020). However, there are several regulatory issues in almost all countries  
115 surrounding ensuring the safe release of transgenic products (transgenic cottonseed protein)  
116 into the environment and for animal and human health, making the utilization of CSM in  
117 human nutrition more difficult. In another work, researchers from the New Mexico  
118 Agricultural Experiment station developed a glandless cotton cultivar, ‘Numex COT 15 GLS’,  
119 through back crossing (Zhang et al., 2016).

120 The chemical-based removal of gossypol from CSM is more feasible for its use as a  
121 feed for nonruminants and as a supplement in human nutrition. The application of solvent-  
122 based techniques also reduced the gossypol levels in CSM, making it appropriate as feed for  
123 fisheries, poultry farms and piggeries (Rathore et al., 2020). Solvents such as ethanol and  
124 acetone acidified with phosphoric acid were found to effectively reduce 90–95% of the total

125 gossypol from the CSM (Pelitire et al., 2014). In another study, an acidified polar solvent  
126 containing 2-propanol and water in a ratio of 95:5 v/v with oxalic acid was used to remove  
127 gossypol from defatted CSM (Singh et al., 2020). The authors achieved a 95.43% reduction.  
128 Both acid and solvent synergistically assist in gossypol extraction. The acids assist in the  
129 hydrolysis and release of the bound gossypol, while the solvents solubilize the liberated  
130 gossypol. On the other hand, a biological approach using various microbial strains and  
131 enzymes is a greener approach for reducing the gossypol content in CSM (Kumar et al.,  
132 2019a).

133

## 134 **1.5. Application of cottonseed protein as a food supplement: functional characterization** 135 **and functional properties**

136 The potential of CSP for use in human nutrition was further established by various researchers  
137 through protein quality analysis. Amino acid profiling and functional property {(water  
138 holding capacity (WHC), oil holding capacity (OHC), foaming capacity (FC), foaming  
139 stability (FS), emulsification activity (EA), emulsification stability (ES), and protein solubility  
140 (PS)}, *in vitro* protein digestibility (IVPD), and molecular weight analyses are a few  
141 parameters that are considered important for the application of CSM in foods.

142 This review will assess the suitability of using CSP in human food by a) summarizing  
143 the currently available methodologies (physical, chemical, and biological) for reducing  
144 gossypol levels to legal limits, b) providing updated information on cottonseed protein  
145 nutritional and functional properties, and c) reviewing CSP applications as direct and indirect  
146 sources of supplements in human nutrition.

147

## 148 **2. Approaches for the efficient utilization of cottonseed meal (CSM) as a protein source**

### 149 **2.1. Physical methods for the degossypolization of CSM**

150 Cottonseed contains 0.002–6.64% gossypol on a dry weight basis depending upon the cotton  
151 variety and the region and climatic factors in which the cotton crop is cultivated (Gadelha et  
152 al., 2014). The cotton plant has resin glands ranging from 50–400  $\mu\text{m}$  in size. These glands  
153 are present in plant petals, leaves, root bark, bolls and seeds and secrete gossypol (Gardner et  
154 al., 1976). Gossypol is primarily found in cottonseed kernels at concentrations ranging from  
155 0.8–2% on a dry weight basis. It is biosynthesized in cotton plants and is classified as a  
156 dimeric sesquiterpenoid since it is synthesized by dimerization of hemi-gossypol moieties  
157 (Cai et al., 2010).

158 In the past, the extraction/removal of gossypol from cottonseed was achieved using  
159 gland flotation (Boatner et al., 1949), liquid cyclone (Smith, 1971), and air classification  
160 (Decossas et al., 1982) physical techniques. These methods were based on physical properties  
161 such as a difference in density and physical forces such as gravitational force. In both of these  
162 method types, the glands containing gossypol were separated from the CSM (Singh et al.,  
163 2015). In the gland flotation technique, a density difference between glands containing  
164 gossypol and kernel tissues was exploited, and the flakes were agitated in a slurry of solvents  
165 having a density less than that of the pigment glands. The glands were then separated by  
166 flotation and obtained as a top layer, while the slurry was allowed to stand. In the liquid  
167 cyclone process, the cottonseeds were suspended in a mixed solvent with low moisture and  
168 then passed through a colloidal mill, where the glands containing gossypol were dispersed  
169 without being broken. These resin glands were separated from CSM using gravitational force  
170 or sedimentation and/or flotation, depending upon the gravity of the suspending liquid (Smith,  
171 1971). The liquid cyclone process was reported to produce edible CSM with less than 400  
172 ppm free gossypol and more than 65% protein (Gardner et al., 1976). After approval by the  
173 USFDA, the liquid cyclone process commercially started production in 1973 in Lubbock,  
174 Texas. Despite its merits, this process had limited financial feasibility and thus was non-

175 operational after a short time period. The air classification process of gossypol separation was  
176 developed as an advanced version of the liquid cyclone process. The air classification  
177 technique was found to have advantages over the liquid cyclone process and was financially  
178 more feasible but was never used for commercial production (Decossas et al., 1982).  
179 Numerous patents were granted for the production of edible flour from cottonseeds using  
180 physical methods (Rathore et al., 2020; Rathore et al., 2019).

181 Heat and pressure conditions (Gribbins, 1951) have also been evaluated for reducing  
182 the gossypol concentrations in cottonseed kernels. It was established that pressure cooking  
183 decreased the gossypol concentration by up to 91.1% in CSM (Gad & El-Zalaki, 1980).  
184 However, this method decreased the protein content in the kernels and fatty acid content in  
185 the oil, limiting its application for the detoxification of CSM. Later, supercritical CO<sub>2</sub>  
186 extraction was also applied to degossypolization. Supercritical CO<sub>2</sub> extraction was employed  
187 for the extraction of oil with less than 0.045% gossypol from cottonseeds (Bhattacharjee,  
188 2007). Supercritical CO<sub>2</sub> extraction is the preferred technique because it is highly efficient,  
189 needs a shorter extraction time and reduces the requirement for refining, as it reduces the  
190 gossypol content in the oil.

191 Recently, gamma and electron irradiation have been found to be effective in reducing  
192 antinutritional factors from various plant-based sources (Nayefi et al., 2014; Shawrang et al.  
193 2011; Ebrahimi-Mahmoudabad & Taghinejad-Roudbaneh, 2011; Fatehi et al., 2020). Bahraini  
194 et al. (2017) studied the effects of gamma and electron irradiation (10, 20 and 30 kGy doses)  
195 on the protein quality, chemical composition and digestibility of protein from CSM. The  
196 results showed that electron irradiation at 30 kGy resulted in a higher reduction in the free  
197 gossypol content and total gossypol content compared to gamma irradiation. In another study,  
198 the application of 40 kGy electron irradiation was found to be more effective in reducing the  
199 free gossypol content (82.37%) compared to gamma irradiation (59.16%) (Nayefi et al.,

200 2014). A similar reduction was also registered when applying 25 kGy gamma and electron  
201 irradiation, making the resultant CSM fit for poultry feeding (Shawrang et al., 2011). The  
202 reduction in the gossypol concentration due to irradiation may be due to the formation of  
203 bonds between the gossypol units, their crosslinking with other compounds or the oxidation or  
204 fragmentation of the gossypol structure (Shawrang et al., 2011). Irradiation presents several  
205 advantages over the aforementioned traditional methods, such as the absence of negative  
206 environmental effects, the elimination of microbial and fungal contaminants, and the fact that  
207 the treatments cause no damage to nutrients or formation of undesirable products (Ghanbari et  
208 al., 2012). It is evident that recent physical technologies, including gamma and electron  
209 irradiation, can be effectively used to reduce the antinutritional factor gossypol to improve the  
210 nutritional profile of cottonseeds or CSM. The operation of this high-energy radiation is  
211 simple and economical compared to economically nonviable traditional approaches (air  
212 classification and gland flotation). However, gamma and electron irradiation techniques are  
213 not efficient enough to decrease the gossypol content to safer levels. In addition, the higher  
214 establishment cost and threats associated with gamma and electro-irradiation are major  
215 disadvantages of these techniques. Furthermore, the WHO has set a safer dose for gamma  
216 irradiation treatment, which is <10 kGy, as no toxicological hazard has been reported at this  
217 level. These lower levels, however, improve the overall quality of the foods but are not  
218 sufficient for decreasing antinutritional factors from oilseeds or pulses. Hence, more focused  
219 research is needed to optimize the conditions for reducing gossypol in cottonseeds or CSM.  
220 Recently, solvent extraction has been the most commonly employed method, which is  
221 discussed in section 2.2.

222

## 223 **2.2. Chemical method for the degossypolization of CSM**

224 In this method, the solute CSM is dissolved in a liquid solvent, which facilitates the close  
225 association of gossypol with the solvent. The choice of solvent to be utilized is dependent  
226 upon the solubility of the solute in the desired solvent, effectiveness of the process,  
227 nontoxicity, reusability, and low cost (Gribbins, 1951, Smith, 1971; Batson et al., 1951;  
228 Thurber et al., 1954). The benefits of the solvent extraction technique include the good  
229 extraction efficiency of gossypol with very low economic inputs. The factors affecting the  
230 extraction technique are the particle size, moisture content, temperature, medium (acidic or  
231 neutral), solvent-to-seed ratio and extraction time (Zhang et al., 2018a).

232 The extraction or removal of gossypol from CSM/cottonseed or cottonseed  
233 supplemented with lysine can lead to improved protein quality with increased lysine  
234 availability (Gadelha et al., 2014; Saki et al., 2012). Gossypol acts as a toxic material in CSM,  
235 as it binds with the lysine of the protein and is converted from free to bound gossypol. The  
236 binding of gossypol to arginine and the lysine part of the meal protein occurs due to covalent  
237 bonds between the gossypol molecule and epsilon amino groups (Gadelha et al., 2014), as  
238 shown in Fig. 3 below.

239 This bound gossypol protein complex denatures the protein and imparts a dark  
240 brownish-black colour to the extracted CSM, which requires further detoxification (Berardi &  
241 Frampton, 1957). It has been reported in the literature that solvent extraction of CSM  
242 performed at elevated temperatures causes the binding of proteins with gossypol, degrading  
243 the nutritive value of the CSP (Hron et al., 1987). Harris et al. (1949) pointed out in their  
244 study that a number of useful byproducts of CSM, such as gossypol, fatty acids, protein and  
245 phospholipids, can be separated and used as marketable products.

246 Commonly employed solvents for extraction include light paraffinic petroleum  
247 fractions such as pentane (boiling range, 31–36 °C), hexane, heptane (boiling range, 90–99  
248 °C) and octane (boiling range, 102–129 °C). Nonpolar solvents such as hexane have been

249 found to perform better than polar solvents. Previously, mixed solvents including commercial  
250 hexane and ethyl alcohol (Liu et al., 1981), acetone-hexane (Kuk et al., 2005), acetone,  
251 cyclohexane and water (Lawhon, 1969), and methylene chloride and ethanol (Jhonson &  
252 Lusas et al., 1983) were used for the extraction or removal of gossypol from CSM.  
253 Researchers have also explored the removal of gossypol from CSM by multiple extractions  
254 with organic solvents, viz. 1-Butanol hydrochloride, dichloromethane, 2-propanol and 1-  
255 butanol (Liadakis et al., 1993). However, hexane is now considered a toxic solvent per  
256 environmental norms, so various alternative green solvents are currently being utilized. These  
257 green solvents include ethanol, methanol and isopropanol, butanol and their combinations  
258 (Byrne et al., 2016; Prat et al., 2016). In addition to solvents, the use of calcium hydroxide  
259 (2%) and pressure-cooking treatment also reduced the free gossypol content of CSM for  
260 application in poultry feed (Nagalakshmi et al., 2002). Furthermore, the addition of  
261 dehydrated ferrous-sulfate to CSM at an equal amount to that of free gossypol lowered the  
262 free gossypol levels to 0.0001%. Dehydrated ferrous-sulfate mixed CSM was found to be  
263 suitable for the consumption of broiler chickens up to 56 days of age. (Tabatabai et al., 2002).

264         Researchers have investigated the use of trichloroethylene as a solvent to minimise the  
265 free gossypol content with minimal denaturation of proteins (Arnold & Juhl, 1955). The  
266 solvent extraction yielded a higher soluble protein content from CSM compared to the earlier  
267 heating (cooking) method. A substantial reduction in free gossypol was obtained, with a  
268 minimum reduction in water-soluble protein fractions when the extraction was carried out at  
269 low temperatures of up to 50 °C. Two important factors that are influenced by solvent  
270 extraction of CSM are the free gossypol content and meal protein quality. The researchers  
271 agreed with the findings that extraction performed at low temperatures can considerably lower  
272 free gossypol contents in the residual oils and meals. Rao & Arnold (1958) utilized ethanol as  
273 a solvent to remove gossypol from cottonseeds in their pilot plant studies. They used four

274 different concentrations of ethanol (91.5%, 95.4%, 98% and 99.9%) and three different  
275 temperatures (65 °C, 70 °C, and 78.3 °C) with extraction times of 10 to 100 minutes. Dechary  
276 et al. (1952) employed ten different solvents, viz. methanol, ethanol, dioxane, butanone,  
277 acetone, isopropanol, chloroform, 1,2-di-chloroethane, perchloroethylene and  
278 trichloroethylene, for the extraction of gossypol from cottonseeds in their study. The percent  
279 removal of free gossypol using different solvent pairs ranged from 7.27% in the case of  
280 isopropanol to 79.54% for 90% aqueous butanone. Aqueous dioxane (90%) removed 70.54%  
281 of free gossypol, and aqueous butanone (95%) could remove 52.72% of free gossypol from  
282 the flakes, while chlorine-substituted hydrocarbons were least effective in removing free  
283 gossypol. The extraction temperature and amount of moisture in the extraction system  
284 affected the rate of gossypol extraction when butanone-water pairs were employed as  
285 solvents. This was attributable to the fact that a higher moisture content in the extraction  
286 system resulted in swelling of the flakes, which led to a decrease in the efficiency of solvent  
287 extraction. Baliga et al. (1957) found that removing the bound gossypol from meal resulted in  
288 an increase in the protein quality and its nutritive value. The bound gossypol was removed  
289 using 70% acetone and aniline without heat treatment to obtain a gossypol-CSP complex with  
290 3.25% bound gossypol. The insoluble, inert gossypol-protein complex reduced the loss of  
291 nutritive value of the protein. A method was reported for the preparation of cottonseed protein  
292 isolate (CSPI) using meal from commercial expeller press-solvent extraction (De. Buckle et  
293 al., 1979). Wan et al. (1995), in another study, employed alternate solvents for gossypol  
294 removal from cottonseeds, which posed a lower health risk than hexane. Five solvents, viz. n-  
295 heptane, neohexane, cyclopentane, and cyclohexane, were utilized in their study. All these  
296 solvents were able to reduce the gossypol levels by different rates. Gossypol was removed  
297 utilizing a solvent system consisting of isohexane and 5 to 25% ethanol or isopropyl alcohol  
298 (IPA) (Kuk & Hron, 1998). IPA as an extraction solvent was suggested as a promising



299 alternative to hexane (Lusas et al., 1991). Highly digestible cottonseed flour was obtained  
300 using solvent extraction with acidic ethanol, having a 53.8% protein content that makes it  
301 suitable for replacing fish meal (Anderson et al., 2016).

302         The extraction/removal of gossypol involves a two-stage process. In the first stage, the  
303 solvent comes into contact with the solid (cottonseed/CSM). The solvent interacts with the  
304 resin glands present in the solid, solubilizing gossypol. In the second stage, intense scrubbing  
305 of the solid occurs at the solid-liquid interface, causing efficient diffusion of the solute  
306 (gossypol) into the bulk liquid phase by molecular diffusion. The extraction now becomes a  
307 mass transfer process, which is controlled by the liquid film resistance and internal solid  
308 resistance. These resistances need to be countered by the solvent to allow it to penetrate into  
309 the solid, dissolve gossypol and diffuse back to the liquid phase. This internal solid resistance  
310 is the rate-limiting step. The whole process is explained below with the help of a schematic  
311 diagram (Fig. 3). The results of solvent-based degossypolization employed by different  
312 researchers are given in Table 1.

313         The generally recognized as safe (GRAS) status of solvents, e.g., acetone and ethanol  
314 (Kumar et al., 2019c), for the production of foodstuffs as per council directive 2009/32/EC  
315 (2009) makes them ideal solvents for the removal of gossypol. In addition, these solvents  
316 have a higher level of solubility of gossypol, making them suitable for the extraction or  
317 removal of gossypol from CSM. The solvents are also replenished during the process, making  
318 this method more economically feasible. Findings from the scientific community also suggest  
319 that acetone is a relatively better solvent than ethanol and methanol for the extraction or  
320 removal of gossypol from CSM. This may be due to the greater interaction of acetone with the  
321 hydrophobic structure of the gossypol present in the resin glands, leading to solubilization of  
322 gossypol in the solvent system, which ultimately reduces the gossypol content in the CSM.

323 However, more studies are required to optimize the process of degossypolization to safer  
324 limits.

325 **Fig. 3.**

326  
327 **Table 1**

### 329 **2.3. Biological method for degossypolization of CSM**

330 A number of physical and chemical methods have been developed by researchers for  
331 degossypolization, but there are several limitations associated with these methods. These  
332 methods result in an inferior active vitamin content, protein quality and feed palatability with  
333 high energy wastage (Zhang et al., 2018b). The residual solvent is difficult to remove in the  
334 case of chemical degossypolization from CSM. This residual solvent is also potentially  
335 harmful to both ruminants and nonruminants. During oil recovery from CSM, the use of low  
336 temperatures and short durations is crucial to maintaining the protein quality and acceptable  
337 concentration of free gossypol. Higher temperatures often resulted in a reduced lysine content  
338 in the protein. Therefore, it is necessary to develop an approach for degrading free gossypol  
339 and preventing its absorption in animal systems. Studies have indicated that some  
340 microorganisms belonging to the genera *Candida*, *Torulopsis*, *Aspergillus*, *Mucor*, *Rhizopus*  
341 and *Bacillus* could effectively degrade free gossypol. A brief overview of free gossypol  
342 detoxification by microorganisms with optimized parameters is presented in Table 2.  
343 Detoxification of CSM by microbes may follow two different hypotheses—first, the  
344 utilization of gossypol as a carbon source decreases the total gossypol in the CSM, and  
345 second, the transformation of free gossypol to bound gossypol decreases the overall toxicity  
346 of the CSM due to the action of microbes after the fermentation process (Zhang et al., 2018a).  
347 Microbial detoxification of CSM can not only help achieve the desired safety criteria but can

348 also enhance the protein and amino acid contents. Zhang et al. (2018a) found a significant  
349 reduction in free and bound gossypol contents in fermented samples compared to control  
350 (uninoculated) samples. A *Bacillus subtilis* strain found in the fluid of cow rumens was  
351 isolated and characterized and found to be involved in the biodegradation of gossypol (Zhang  
352 et al., 2018b). Short-term (4 days) and long-term (14 days) fermentation using yeast strains  
353 was evaluated to improve the overall quality of CSM. It was found that both fermentations  
354 caused an increase in both the total essential (highest increment in case of **M** = 44%) and total  
355 nonessential amino acid contents (16–18%). Furthermore, fermentation also resulted in the  
356 reduction of gossypol by 17%, which could be due to enzymatic or microbial degradation of  
357 the gossypol structure (Duodu et al., 2018).

358         Biodegradation of polyphenolic compounds is an oxidative process that is mediated by  
359 enzymes such as oxygenases, hydroxylases, peroxidases and laccases (Mageshwaran et al.,  
360 2018). Laccases secreted by wood-degrading fungi have received substantial attention due to  
361 their involvement in the transformation of phenolic compounds into their oxidized forms. The  
362 exact mechanism behind the biological degradation of gossypol is not yet clear, but  
363 Rajarathnam et al. (2001) observed the involvement of laccase in gossypol biodegradation.  
364 The authors found that an enzyme extract produced from *Pleurotus florida* on rice straw  
365 containing laccase was able to effectively degrade gossypol. An increase in the enzyme  
366 concentration caused an increase in gossypol decomposition. The enzyme blank containing  
367 boiled extract showed no gossypol degradation. Gossypol is a polyphenolic compound, and  
368 laccase, peroxidase and polyphenol oxidase are gossypol-degrading enzymes. A mixed fungal  
369 culture was grown on minimal medium containing gossypol, and its crude supernatant was  
370 extracted and tested for laccase, peroxidase and polyphenol oxidase activities. Workers  
371 observed higher laccase activity and lower polyphenol oxidase and peroxidase activity in the  
372 crude supernatant (Mageshwaran et al., 2018). The purified supernatant had 27–35-fold

373 higher specific activity of laccase. In the gossypol degradation experiment with crude and  
374 purified enzyme extracts, the authors found that residual gossypol levels were reduced by 30  
375 and 60%, respectively, which explains the role of laccase in gossypol degradation. The author  
376 also confirmed the identity of laccase by molecular mass determination using SDS-PAGE.  
377 FTIR analysis of degraded gossypol showed a considerable reduction in the toxic aldehyde  
378 stretch of gossypol.

379

### 380 **2.3.1. Factors affecting the degradation of gossypol in solid-state fermentation (SSF)**

381 Microorganism-mediated degradation of free gossypol in CSM has been well studied by many  
382 researchers using SSF. This method is affected by several input factors for reducing the  
383 gossypol concentration to a minimal amount. The initial moisture content in SSFs is one of  
384 the most important factors because it largely affects the physical properties of solid substrates.  
385 Low moisture levels during fermentation reflect poor solubility of nutrients in substrates with  
386 a lower degree of swelling, which ultimately results in poor microorganism growth (Murthy,  
387 1999). In contrast, a higher moisture level decreases the porosity of the substrate, which limits  
388 heat and oxygen transfer during fermentation, ultimately decreasing the efficiency of free  
389 gossypol degradation (Khalaf et al., 2008; Ohno et al., 1992). Several researchers have found  
390 that an initial moisture level of 50–55% is optimal for achieving maximum free gossypol  
391 degradation (Khalaf et al., 2008). The metabolic activities of microorganisms are largely  
392 affected by the pH value of the medium. For different fungi involved in this process, pH  
393 values ranging from 4–6 are optimal for the maximum degradation of free gossypol (Weng &  
394 Sun, 2006; Khalaf et al., 2008). A higher pH affects the enzyme activity and growth of the  
395 organism.

396 A favourable incubation temperature is the key for biological degradation of gossypol  
397 in a solid medium. The metabolic activities of organisms are markedly affected by

398 temperature fluctuations during the process. Khalaf et al. (2008) found a significant difference  
399 in the biodegradation of free gossypol, with a maximum of 86.5% at 30 °C compared to 81%  
400 at 35/25 °C and 57% at 40 °C. Therefore, the incubation temperature and its regulation during  
401 the process are important, as much heat production and accumulation occurs during SSF,  
402 resulting from the poor heat dissipation property of the solid substrate. The initial load of  
403 inoculum is another crucial factor for the biodegradation of free gossypol. A lower inoculum  
404 load may produce an insufficient amount of microbial biomass, which may result in poor  
405 efficiency of gossypol biodegradation, while a heavier inoculum may produce a much higher  
406 biomass, which may lead to poor gossypol-degrading enzyme secretion (Zhang et al., 2006b).  
407 Khalaf et al. (2008) investigated the effects of various inoculum levels ( $10^3$ – $10^9$  cells per g)  
408 on free gossypol reduction and found that a concentration of  $1 \times 10^7$  cell/g *Candida tropicalis*  
409 degraded a maximum of 88.6% free gossypol compared to  $1 \times 10^3$  cell/g, which could degrade  
410 only 56%. He found a decreasing trend in free gossypol as the inoculum level reached beyond  
411  $1 \times 10^7$  cell/g. Vellaichamy et al. (2016) used mixed cultures of fungi for degossypolization of  
412 cottonseed cakes and studied the effect of moisture, inoculum level, temperature, and time  
413 period during SSF. The optimum conditions, i.e., 70% moisture content, 30 °C temperature,  
414 15% inoculum level, and 48-h time period, resulted in the maximum detoxification of  
415 gossypol. Detoxification of free gossypol (83.6%) was observed in *Pleurotus sajor-caju* with  
416 *Saccharomyces cerevisiae*, and that of bound gossypol (63.3%) was observed in *Candida*  
417 *tropicalis* with *Saccharomyces cerevisiae*. In another work, a new strain of fungi isolated  
418 from soil was identified through molecular biology and morphological techniques. The  
419 *Aspergillus* genus demonstrated high degradation of gossypol at an optimum temperature of  
420 30 °C and incubation time of 72 h (Yang et al., 2011).

421 Furthermore, supplementation with minerals was found to increase the fermentation  
422 efficiency (free gossypol detoxification) and protein content. Zhang et al. (2007) found an

423 increase in the free gossypol detoxification efficiency (from 93.47 to 96.67%) and an  
424 enhancement in the crude protein content in the fermented product with the addition of  
425 mineral additives. The researchers also found improved levels of lysine, methionine and  
426 threonine in the crude proteins compared to the control. The addition of urea resulted in  
427 decreased free gossypol levels and improved crude protein amounts. This could be due to the  
428 specific role of minerals such as phosphates, which help maintain the buffering capacity of the  
429 medium and act as structural components of phospholipids, nucleic acids and coenzymes. The  
430 addition of sodium and potassium ions is responsible for maintaining the osmolarity of the  
431 medium. Potassium is a major cation found in microbial cells that acts as a cofactor for  
432 phosphohexokinase (Caldwell et al., 1973; Durand & Kawashima, 1980). Other minerals,  
433 such as  $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Fe^{2+}$  and  $Mg^{2+}$ , also function as cofactors for several metabolic enzymes.  
434 The duration of fermentation is another important factor that is mainly decided by  
435 characteristics such as the growth rate of the organism and the efficiency of gossypol  
436 degradation. Identifying the optimum time of fermentation is crucial. A shorter fermentation  
437 time may result in incomplete utilization of the substrate and thus a reduced rate of gossypol  
438 degradation, while fermentation beyond the optimum range may result in denaturation and  
439 subsequent inactivation of the enzymes involved in gossypol degradation, which results from  
440 interaction with the other compounds/byproducts formed during the process (Zhang et al.,  
441 2006). Researchers have found that the optimum time of fermentation was 60 h for yeast and  
442 4-6 days for filamentous fungi (Wu & Chen, 1989; Shi et al., 1998; Weng & Sun, 2006).

443         Degossypolization by biological methods is considered a green strategy, as it employs  
444 beneficial microbes. The lower effectiveness of the method in minimising the level of  
445 gossypol is the only drawback. Although this method is cheaper and efficient for free  
446 gossypol degradation, this process is affected by several parameters, such as the initial  
447 moisture content, pH, incubation temperature, inoculum level, mineral additives and duration

448 of fermentation, which need to be optimized to achieve the minimum level of antinutritional  
449 factors in the resultant CSM. There is a need to discover novel strains of microbes and to  
450 optimize process conditions for the effective degradation of gossypol to improve the  
451 nutritional value of CSM. A combination of physical (gamma or electron irradiation) and  
452 solvent-based processes or physical and biological methods, a combination of biological and  
453 solvent-based processes, a combination of all three techniques may effectively remove  
454 gossypol to reach safer levels for effective utilization of CSM as a sustainable protein source.

455

456

## Table 2

457

### 458 **3. Evaluation of the quality and functional properties of cottonseed protein as a food** 459 **supplement**

460 It is important to scientifically establish the quality of CSP. The most important factor for the  
461 analysis of protein quality is its amino acid composition. The use of CSP in human nutrition  
462 not only depends on its amino acid profile but also relies on its ability to be incorporated in  
463 foods. Hence, evaluations of functional properties such as the WHC, OHC, FC, FS, EA, ES,  
464 PS, *in vitro* digestibility, and molecular weight are important to establish CSP compatibility in  
465 food matrices for different types of applications (Ma et al., 2018; Tsaliki et al., 2002). In the  
466 next section, the quality of the cottonseed flour/protein will be discussed in detail, followed  
467 by various functional properties.

468

#### 469 **3.1. Evaluation of the quality of cottonseed protein based on the amino acid profile**

##### 470 **3.1.1. Amino acid profile**

471 The amino acid profile of CSM was measured by He et al. (2015). Out of the 10 essential  
472 amino acids, the **R** content was the highest, amounting to 15-34% of the total protein. Other

473 essential amino acids represented approximately 5% of the total protein, while **M** and **C** had  
474 the lowest presence (1-2%). Of the nonessential amino acids, **E** amounted to 10% of the total  
475 protein content. Other nonessential amino acids ranged from 3 to 6% of the total protein.  
476 Similarly, the presence of **R** (12.47%) was the highest in essential amino acids, while **E** and **Q**  
477 (29.75%) were highest in nonessential amino acids in cottonseed protein hydrolysate obtained  
478 from digestion by Alcalase (Song et al., 2020). In another study, CSM was evaluated for the  
479 complete profile of both nonessential (**A**, **D**, **C**, **Q**, **G**, **P**, **S**, and **Y**) and essential amino acids  
480 (**R**, **H**, **I**, **L**, **K**, **M**, **F**, **T**, **W**, and **V**). The mean values were 1.87% (**A**), 4.51% (**D**), 0.79% (**C**),  
481 9.08% (**Q**), 1.99% (**G**), 1.32% (**P**), 2.09% (**S**), and 1.14% (**Y**) for nonessential amino acids  
482 and 5.70% (**R**), 1.34% (**H**), 1.48% (**I**), 2.95% (**L**), 2.15% (**K**), 0.72% (**M**), 3.0% (**F**), 1.55%  
483 (**T**), 0.64% (**W**), and 2.15% (**V**) for essential amino acids. The CSP was fed to swine, which  
484 showed a good ileal digestibility of 80%, and all the amino acids demonstrated more than  
485 60% digestibility (Ma et al., 2019). Delgado et al. (2019) evaluated salt and alkali-soluble  
486 protein fractions from glandless cottonseed. The authors found that essential amino acids,  
487 namely, **H**, **I**, **L**, **K**, **M**, **F**, **T** and **V**, constituted 30 and 28.1% of the total amino acids in  
488 alkali- and salt-soluble fractions, respectively. The total concentration of essential amino acids  
489 in glandless CSM was 26%, which was higher than that in soybean protein (17%) (Delgado et  
490 al., 2019). It is evident that cottonseeds have superior protein quality with an ideal balance of  
491 amino acids and hence could act as a sustainable alternative source of protein for human  
492 nutrition.

493

## 494 **3.2. Evaluation of functional properties of the cottonseed proteins**

### 495 **3.2.1. Functional properties**

496 The functional properties of proteins are defined as the overall physicochemical behaviour of  
497 foods during processing, storage and consumption. The WHC is a parameter that reflects the



498 ability of the protein to imbibe and retain water, whereas the OHC is the capacity of the fat  
499 particles to bind and integrate with the nonpolar side chain of the proteins. The WHC and  
500 OHC of the different samples of CSPI ranged between 1.6–2.9 g/g and 3.0–5.4 g/g,  
501 respectively. For soybean protein isolates, the WAC was 2.3 g/g, and the OAC was 4.5 g/g  
502 (Ma et al., 2018; Delgado et al., 2019). This implies that the WHC and OHC follow an  
503 overlapping pattern for soybean protein. These properties influence the texture and mouthfeel  
504 attributes of food products such as baked dough, comminuted meats and analogues  
505 (Adebowale et al., 2005).

506         The FC is the capacity of the continuous phase of protein to hold air, whereas the FS is  
507 its ability to retain air in the continuous phase for a 30-minute duration (Tsaliki et al., 2002).  
508 The FC and FS are preferred functional properties for whipping and aeration in food systems.  
509 The FC and FS values of CSPI were evaluated for a pH range of 4–7. The FC and FS were  
510 minimal at pH 5 and ranged from 15.1–31.1% and 38.8–89.0%, respectively, since this pH  
511 was close to the isoelectric points of the proteins in CSPI. The highest FC (50.0–81.5%) and  
512 FS (73.3–96.9%) were observed at pH 7.0 (Ma et al., 2018; Delgado et al., 2019). Tsaliki et  
513 al. (2002) also reported the highest values of FC and FS at pH 7.0. The ability to form foam  
514 with good foaming stability makes CSP an excellent ingredient for application in ice cream,  
515 mousses, and marshmallows.

516         The hydrophilic and hydrophobic constituents of proteins act as effective surface-  
517 active agents, making them suitable for use as emulsifiers in food colloids. The potential of  
518 cottonseed proteins to develop and maintain emulsions is considered important for their  
519 multifaceted application in food systems. The emulsification properties of the proteins are  
520 evaluated on the basis of the EC and ES (Tsaliki et al., 2004). The EC is the maximum  
521 amount of oil that is emulsified under controlled conditions by a specific amount of protein.  
522 The ES is measured in terms of the amount of oil and/or cream separated from an emulsion

523 during a certain period of time at a specific temperature and gravitational field (Pearce &  
524 Kinsella, 1978). The CSPI showed the EC varying from 13.3–23.1 m<sup>2</sup>/g, whereas the ES  
525 ranged between 17.3–29.6 minutes. These values were higher than the peanut protein isolate  
526 EC (14.8 m<sup>2</sup>/g) and ES (15.2 minutes). Due to the high EC and ES values, CSPI has been  
527 successfully used to manufacture bakery products, sausages, sweetmeats and other emulsified  
528 products (Ma et al., 2018).

529         The PS is the amount of protein that is dissolved in a solution under specific  
530 conditions. It is the chief determinant for use in food systems. The solubility of cottonseed  
531 protein at various pH values (3–11) is the measure of its performance when added to food  
532 matrices. Furthermore, it is also an important indicator of protein denaturation under heating  
533 and chemical processing (Horax et al., 2006). CSPI displayed maximum and minimum PS  
534 values at pH 11 and pH 5, respectively. Soybean protein isolates also showed a similar  
535 solubility profile, with a maximum at pH 11. It was suggested that the weakened interaction  
536 between water and protein resulted in increased protein-protein interactions, which ultimately  
537 caused aggregation and precipitation of CSPI (Ma et al., 2018).

538

### 539 **3.2.2. *In vitro* protein digestibility (IVPD)**

540 Protein digestibility is an important factor in determining protein availability for absorption in  
541 the intestinal tract. IVPD is a universally accepted assay to estimate the parameters related to  
542 protein digestibility. The IVPD assay mimics conditions similar to those of the human  
543 digestive tract by using different proteolytic enzymes (e.g., the papain system or pepsin-  
544 pancreatin enzyme system). This helps analyse the amount of hydrolysed proteins (Hur et al.,  
545 2011). This assay is more rapid, affordable and equivalently efficient than other *in vivo*  
546 assays. The existing literature is focused on animal digestion of CSM (Can et al., 2011; Heim  
547 & Krebs, 2018; Yue et al., 2007). There is, therefore, a research gap for future work that could

548 address the digestibility of CSM or cottonseed protein from the perspective of human  
549 digestion.

550

### 551 **3.2.3. Molecular weight**

552 The most commonly used technique for molecular size analysis of cottonseed protein is  
553 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This technique  
554 separates protein polypeptides on the basis of their molecular size. CSPIs from seven different  
555 sources were compared with those of peanut and soybean protein isolates through molecular  
556 weight profiling using SDS-PAGE under reducing and nonreducing conditions. Under  
557 nonreducing conditions, CSPI showed two high-intensity bands at 50 and 45 kDa, implying  
558 that these two fractions are the main constituents (Delgado et al., 2019; Ma et al., 2018).  
559 Under nonreducing conditions, these protein subunits include the salt soluble fraction  
560 (globulins), i.e., globulin 9S (Sun et al., 2012). In contrast, reducing conditions showed that  
561 many minor protein bands appeared between 35 and 14 kDa, suggesting the existence of  
562 disulfide bonds between the peptides of the CSPIs (Delgado et al., 2019; Ma et al., 2018).  
563 These protein subunits include both salt- (globulins 5S: 24 and 22 kDa) and water-soluble  
564 fractions (albumins; albumin 2S: 20, 15 and 14 kDa) (Sun et al., 2012). In another study, out  
565 of 4 protein fractions, globulins reached a maximum (33–63.7%), followed by albumins  
566 (20.8–32.2%), glutelins (9.1–28%), and prolamins at the lowest concentration (Singh & Kaur,  
567 2019). The molecular weights of polypeptides in cottonseed protein from *Gossypium*  
568 *arboreum* and *Gossypium hirsutum* lines were found to be in the range of 10–122 kDa (Singh  
569 & Kaur, 2019). The molecular weight of the alkali-soluble protein fraction varied from 10–54  
570 kDa in both species. Water- and alkali-soluble cottonseed protein profiling was performed by  
571 He et al. (2018). They found molecular weights of CSP in the range of 10–381 kDa.

572

#### 573 **3.2.4. Rheological properties**

574 The rheological behaviour of protein helps with the modelling and design of products, the  
575 development of food products, sensory evaluation, quality control, acceptability to consumers  
576 and long-term stability (Erçelebi & Ibanoglu, 2009). Various protein extraction processes  
577 disrupt chemical bonds, such as electrostatic interactions, hydrogen bonds, hydrophobic,  
578 hydrophilic and covalent bonds, and structure and aggregations of protein molecules and  
579 affect the viscosity and rheological behaviour of proteins (Turker Saricaoglu, 2019). He et al.  
580 (2016) studied the rheological behaviour of CSM, CSPI and water-washed CSM at different  
581 pH values. The adhesive strength of CSPI decreases with storage time and is ineffective in  
582 water-washed CSM at pH 6, 7.5 and 9. The viscosity depends on the shear rate, and the  
583 highest viscosity was obtained at pH 9 for water-washed CSM and pH 7.5 for CSPI. The  
584 viscosity remained unaltered in CSPI, but the viscosity of water-washed CSM increased with  
585 time. The storage and loss modulus values are two viscosity parameters that indicate the  
586 strength of gels. A high storage modulus increases the aggregation of CSPI molecules due to  
587 repulsive force reduction. A high pH and low ionic strength form a gel with thick strands and  
588 large aggregates and pores; however, a low pH and high ionic strength form a gel with a fine  
589 structure and small aggregates and pores. The ionic strength (NaCl) and pH affected the gel  
590 formation and rheological behaviour of CSPI (Zhou et al., 2015). The rheological behaviour  
591 of CSPIs provides many applications in food, such as protein-based gels and edible packaging  
592 films.

593

#### 594 **3.2.5. Surface hydrophobicity (SH)**

595 The SH represents the number of hydrophobic groups present on the surface of a protein. The  
596 surface hydrophobicity shows the subsequent aggregation and partial denaturation of  
597 hydrophobic groups of protein molecules. The SH increases with the number of hydrophobic

598 groups largely exposed on the surface. Ma et al. (2018) studied the SH of CSM and CSPI  
599 extracted with different methods. The maximal SH was obtained in the proteins isolated using  
600 subcritical extraction from CSM (727.5), followed by insect-resistant CSM (615.7), colour  
601 CSM (561.7), XinLiang CSM (128.1), YiHai CSM (120.5), JingGu CSM (110.5) and  
602 Tiankang CSM (103.7). A higher SH was found in soybean protein isolates than in proteins  
603 isolated from CSM with different extraction methods. The results suggested that the hot-  
604 pressing method of protein extraction unfolds CSP aggregations, and other methods of CSP  
605 extraction cause conformational changes. The SH of protein is closely related to its  
606 emulsifying properties and protein solubility. The SH of CSP enhances the emulsifying  
607 activity of CSP due to strong bonding between oil droplets and emulsifiers. CSPIs with better  
608 surface hydrophobicity have improved uses in the food industry.

609         The amino acid profile of the CSP showed a balanced ratio of essential amino acids,  
610 with the highest content of **R** amino acids. The evaluation of various food functional  
611 properties of CSP established the application of CSP in numerous food products as a  
612 preservative, foaming agent, and emulsifier. The predominance of salt and water-soluble  
613 proteins with excellent rheological properties further suggests the potential application of  
614 CSPs in foods.

615

### 616 **3.2.6. Surface, structure, and peptide characterization of cottonseed protein (CSP)**

617 The functional properties of protein isolates depend on the chemical composition and other  
618 surface characteristics. The surface properties of CSM and water-soluble and alkali-soluble  
619 CSP were evaluated by He et al. (2017) using scanning electron microscopy (SEM), energy  
620 dispersive spectrometry (EDS), and X-ray diffraction. Alkali- and water-soluble proteins  
621 showed similar surface properties. SEM analysis of CSP showed irregular wrinkling with  
622 light spongy porous structures, which was attributed to the presence of polysaccharide

623 components. Other CSPs by the same authors reflected flatter areas, tight surfaces and sharp  
624 angles (Kumar et al., 2021). EDS analysis of CSP reflected the presence of Ca, Cu, Na, Mg,  
625 K, and P, whereas C and O were detected as major elements due to the organic nature of the  
626 sample. The XRD pattern reflected the structure of CSP, which was reported by the same  
627 authors. XRD showed peaks at  $2\theta$  of approximately  $9.4^\circ$  and  $20^\circ$ . This XRD pattern was found  
628 to be similar to that of soybean protein powder, reflecting  $\alpha$ -helix and  $\beta$ -sheet structures of  
629 protein molecules, respectively (He et al., 2018). The water- and alkali-soluble CSPs were  
630 extracted sequentially, and mass spectrometric analysis showed the presence of 70  
631 polypeptides with molecular weights ranging from 10 to 381 kDa. The most abundant  
632 peptides in the fraction were legumin A (58 kDa), B (59 kDa), vicilin C72 (70 kDa), vicilin  
633 GC72-A (71 kDa), and vicilin-like antimicrobial peptides (62 kDa) (He et al., 2018). This  
634 information is crucial for the application of CSP as a functional food additive.

635

#### 636 **4. Food and non-food application of cottonseed protein**

637 **Plant based-proteins are gaining importance due to their renewable and sustainable nature.**  
638 **Kumar et al., (2021a) summarized various methods of extraction of plant proteins for possible**  
639 **utilization as food supplement.** Cottonseeds are traditionally used as the chief ingredient in a  
640 widely popular Indian ethnic beverage popularly known as Paruthi Paal (Kumar, 2019b).  
641 Owing to the presence of superior-quality proteins, essential fatty acids and sugars, CSP is  
642 regarded as a 'triple nutrient'. It has been reported to have many health benefits, such as  
643 healing stomach ulcers, preventing polycystic ovary syndrome, regulating the menstrual  
644 cycle, modulating blood pressure, and improving neurological health. Food products made  
645 from cottonseeds were served to soldiers at the time of World War II due to the unavailability  
646 of various other nutritional sources. Additionally, cottonseed soup has been traditionally used  
647 as food in India, Pakistan, and Bangladesh in the **Asian continent** and in Nigeria, Uganda,

648 Burkina Faso, Zambia, Tanzania, and ten other countries in **the African continent**. These  
649 countries are chief cotton producers but at the same time face the menace of protein calorie  
650 malnutrition (Kumar, 2019b). The use of CSP either directly or indirectly in place of animal  
651 protein can help overcome the problem of malnutrition. Apart from food applications, there  
652 are numerous non-food applications of CSP or CSM. Applications such as packaging (films  
653 and coating) of agriculturally based products, adhesives, bioplastic, interfacial and  
654 emulsifying applications, hydrogels, and other applications are discussed in section 4.2.

655

#### 656 **4.1. Food applications of cottonseed meal (CSM) and cottonseed protein (CSP)**

##### 657 **4.1.1. CSP as a direct source of protein for human nutrition**

658 CSPI and flours have been widely used to develop various food products and are prevalently  
659 accepted as nutritional and functional ingredients in baked foods and meat products (Zhuge et  
660 al., 1988). In the past, Cater et al. (1977) established the potential of CSPs in a variety of food  
661 systems. The authors reported that CSP had been used in the US as a food additive since the  
662 1930s. Less than 5% of defatted cottonseed flour was added to cookies, doughnuts and  
663 chocolate candies due to the functional properties of the CSP rather than its nutritional  
664 attributes. This was also confirmed by Spadaro et al. (1979), who referred to a product named  
665 “Proflo”, which was mainly used to give functional characteristics to bakery and  
666 confectionery products. Both Spadaro et al. (1979) and Cater et al. (1977) reported in the late  
667 1950s and the 1960s that a low-cost and highly nutritious food product called INCAP  
668 vegetable mixture or “Incaparina” was popular in Guatemala and Colombia and used 2  
669 million pounds of cottonseed in 1964.

670 Spadaro et al. (1979) reviewed numerous food applications of CSP concentrate,  
671 including meat products (such as beef burgers, meatballs, fresh sausages and frankfurters),  
672 extruded cereal-type products (such as snacks and textured vegetable proteins), and baked

673 goods (such as cookies, doughnuts, cakes and breads). De Buckle et al. (1979) also reported  
674 that cottonseed flours could be texturized in a way similar to that of soybean flour,  
675 simultaneously reducing the free gossypol content to safe levels. The same authors also  
676 produced a CSPI with 90% protein, which had a white creamy colour and a bland flavour.  
677 Zhuge et al. (1988) processed CSM through extrusion, drying, grinding and air classification  
678 and obtained a low-gossypol product. The final coarse product had a particle size larger than  
679 84 µm and a protein content of 36.6–45.2%. Alford et al. (1996) reviewed several human  
680 studies using dietary CSPs and concluded that these proteins assist in ameliorating the health  
681 of malnourished or undernourished children and adult women. In addition to CSM, glandless  
682 cottonseed kernels were used for the development of the nut-like snack ‘Tamunuts’ using a  
683 dry roasting technique standardized by Texas A & M University, Texas, USA. These nuts  
684 were preferred over ‘soy nuts’ by the tasting panel (Dowd, 2015; Lusas et al., 1978; Rathore  
685 et al., 2020).

686         Although much research was carried out in the late 1970s and 1980s on CSP and its  
687 incorporation into foods, interest in this protein gradually declined. Only a few studies have  
688 been conducted on cottonseed as a food since the early 2000s, and a few of these studies on  
689 beef and extruded snacks are elaborated upon below. CSM was incorporated into ground beef  
690 at 0–3% (Rhee et al., 2001). The 3% CSM added to beef served as a highly effective  
691 antioxidant, decreasing 2-thiobarbituric acid-reactive substances (TBARS) by 77–91%  
692 compared to the cottonseed-free control beef. Reyes-Jáquez et al. (2012) produced extruded  
693 snacks using CSM. The optimal inclusion level was found to be 10% CSM, and the resulting  
694 snack had less fat and more protein than the control snacks. In another study, the peptide  
695 fractions obtained from the **alcalase** enzyme treatment of CSP demonstrated novel properties.  
696 It was found that the higher content of positively charged amino acids (**K, R & H**) and lower  
697 content of negatively charged amino acids (**D, E**) resulted in higher antioxidant and



698 antibacterial activities. Later, cottonseed protein hydrolysates underwent *in vitro* digestion  
699 (Song et al., 2020). These isolates showed no antibacterial activity before or after *in vitro*  
700 digestion, although they exhibited some antioxidant activities. One peptide retained high  
701 natural antibacterial activity even after 6 h of digestion (Song et al., 2020). He et al. (2020)  
702 evaluated water-soluble fractions of lab scale-produced CSP from glandless, glanded, and  
703 pilot-scale-produced glanded CSP. Pilot scale-produced protein showed the maximum  
704 antioxidant activity using the DPPH assay (70.6%), while both lab scale-processed fractions  
705 exhibited comparable antioxidant properties. The superior antioxidant activity of pilot scale-  
706 produced protein fractions was higher due to the presence of an increased number of peptide  
707 fragments with exposed hydrophobic amino acids (**W**). The improved hydrophobic nature of  
708 the pilot scale-produced protein peptides allowed them to act as antioxidants by improving  
709 their solubility in nonpolar solvents, subsequently enhancing their interactions with free  
710 radicals (Kim et al., 2007). These findings are valuable, as they show the potential to develop  
711 peptides that could be used in functional food formulations from low-value cottonseeds. In a  
712 study, detoxified CSP protein obtained from alkali-salt based method was evaluated critical  
713 quality standards as per the guidelines of Food Safety and Standards Regulation, 2011  
714 (Kumar et al., 2021b). It was concluded that lyophilised protein powder obtained at optimised  
715 conditions have higher crude protein content (93.1%), lower free (0.03%) and total gossypol  
716 (0.27%) with no presence of food borne pathogens. It is evident that cottonseeds have a  
717 balanced ratio of amino acids and can be a sustainable source of protein in countries where  
718 cotton is grown as the predominant crop.

719

#### 720 **4.1.2. Cottonseed/cottonseed meal (CSM) as an indirect source of protein for human** 721 **nutrition**

722 In addition to having direct applications as a food supplement in human nutrition,

723 degossypolized CSM can indirectly be used for alleviating protein energy malnutrition. Low-  
724 gossypol CSM can be effectively used as feed in both the poultry and aquaculture industries.  
725 These animals can effectively convert feed protein into edible animal protein (Rathore et al.  
726 2020). Several protein conversion ratios (PCRs: feed protein used/edible animal protein  
727 produced) have been established for the conversion of ultralow gossypol CSP into animal-  
728 based protein for human nutrition. For example, poultry for egg production is reported as the  
729 most efficient means of converting plant protein to edible animal protein with a PCR of 2.6.  
730 Chicken meat production with a PCR of 4.7 is superior to that of other animal-based protein  
731 sources, such as pigs (5.7), fish (4.6–5.7) and prawns (7.7) (Rathore et al., 2020). Several  
732 authors have recently investigated the effect of CSP, CSF, CSC and CSPI on feed diets in  
733 aquacultural species (Anderson et al, 2016; Wang et al, 2020; Ye et al, 2020; Yin et al, 2018),  
734 pigs (Li et al, 2019), and lambs (Moretti et al, 2019). Delgado et al., (2021) studied the  
735 functional properties of the extruded shrimp feed containing CSM with ultra-low gossypol  
736 content. It was concluded that use of ultra-low gossypol CSM as fishmeal substitute is a  
737 feasible alternative to reduce the costs of the shrimp feed while showing a balanced content of  
738 minerals, amino acids, protein, and essential fatty acids. In an independent study,  
739 degossypolised CSP was evaluated for the energy and nutrient digestibility and as a source of  
740 supplement in the nursery pigs (Wang et al., 2019). It was concluded that degossypolised CSP  
741 can be utilised as a supplement in nursery pig diets at the proportion of 5 and 10% within two  
742 and after two-weeks of weaning, respectively. CSP can be effectively metabolized by poultry,  
743 fish, and pigs to convert plant protein into animal protein. Hence, cottonseed may serve as an  
744 indirect source of protein for nonvegetarians. The conversion of CSP into animal protein also  
745 reduces the risk of gossypol, which is otherwise associated with direct consumption of CSP.

746

#### 747 **4.2. Nonfood application of cottonseed meal (CSM) and cottonseed protein (CSP)**

748 The mechanical properties, water solubility, plasticizing characteristics, crosslinking  
749 behaviour, and 3D structure of cottonseed protein contribute to many non-food applications.  
750 These applications include packaging, adhesive, bioplastic, hydrogel, interfacial material, and  
751 emulsifying applications (Cheng et al., 2020). The application of CSP as a wood adhesive has  
752 been established in the recent past, and it has been reported that the combination of CSP and  
753 phosphoric acid is even more effective as a wood adhesive than soy protein (Cheng et al.,  
754 2017). Li et al. (2019) evaluated the effects of dipotassium hydrogen phosphate, phosphoric  
755 acid, calcium oxide and calcium hydrogen phosphate on the adhesive strength of CSP at 20 to  
756 80 mM concentrations. The authors found that CSP with 40 mM phosphoric acid treatment  
757 resulted in higher adhesive strength with high water resistance, indicating CSP as an eco-  
758 friendly adhesive in the wood industry. CSM in combination with urea formaldehyde is also  
759 used as a green and environmentally friendly adhesive in wood-based composites (Liu et al.,  
760 2018). Cheng et al. (2017) used CSP in combination with different acids as a paper additive  
761 and studied the resulting characteristics by Fourier transform-infrared (FTIR) spectroscopy  
762 and SEM analysis. The results suggested that CSP interacted with acids and paper fibres to  
763 enhance the strength. Similarly, CSP was evaluated by thermogravimetric analysis and SEM  
764 and FTIR analysis to study its characteristics as a strength enhancer. The results suggest that  
765 CSPs interact with cotton fibres and increase the strength in nonwoven fabric (Villalpando et  
766 al., 2018). Vigorous blending enhances water- and alkali-soluble CSP recovery and quality,  
767 providing good characteristics and enabling industrial applications (He et al., 2013).

768 Films and coatings are used in the packaging of Agri-based products such fruits and  
769 vegetables to enhance their shelf life. Glycerol, urea and aldehyde were used as additives in  
770 bioplastic preparations. These crosslinking agents enhance the mechanical strength, water  
771 absorption resistance, and thermal stability of the products (Cheng et al., 2020). CSP was  
772 plasticized with glycerol and embedded with biodegradable material through extrusion and

773 thermosetting. The study established that glycerol plays a crucial role in making CSP  
774 thermoplastic through a 54 °C increase in the thermal denaturation temperature. Attention to  
775 the development of edible protein films has increased recently surrounding applications in  
776 food packaging (Chen et al., 2019).

777

## 778 **5. Food safety and regulatory issues**

779 Food Safety and Standards Regulations (FSSR), 2011 (version IV, published on 9/11/2017),  
780 suggests that cottonseed flour or protein can be utilized as a food ingredient if it follows  
781 certain standards. These quality parameters include the moisture content (< 8%), crude protein  
782 (> 47%), free gossypol content (< 0.06%), total gossypol content (< 1.2%), ash insoluble  
783 content (< 0.35%), total ash content (< 5.0%), available lysine content greater than 3.6 g/100  
784 g of crude protein, crude fibre content (< 5.0%), and fat content less than 1.5% dry weight. In  
785 addition to these parameters, the microbial count, i.e., the total bacterial count, should be less  
786 than 50000/g. The *Coliform* bacterial count should be less than 10/g, and the *Salmonella*  
787 bacterial count should be nil in 25 g. The European Union has provided specific guidelines on  
788 the concentration of gossypol for applications as feed for ruminants and nonruminants. The  
789 maximum free gossypol concentration for cottonseed is 5,000 ppm, that for CSM or cake is  
790 1,200 ppm, that for laying hens and piglets is 20 ppm, that for rabbits and pigs is 60 ppm, that  
791 for poultry and calves is 100 ppm and that for cattle, sheep, and goats is 500 ppm. According  
792 to the **USFDA**, CSPs and their food products can be considered edible if they contain less  
793 than 0.045% and 0.8% free gossypol and bound gossypol, respectively (Ma et al., 2018).  
794 Numerous pulse and oilseed proteins can act as potent allergens, causing an undesirable  
795 immune response in susceptible individuals. To address this concern, the **USFDA** has  
796 identified 8 major allergens, **e.g.**, eggs, milk, shellfish, crustaceans, peanuts, tree nuts,  
797 soybean, and wheat, that cause 90% of allergic reactions in susceptible individuals (Rathore et

798 al., 2020). These rules are followed by most countries, and allergens need to be compulsorily  
799 mentioned by food manufacturers on food labels. In contrast, cottonseed is not reported to  
800 instigate any allergic or hypersensitive immune response. This is because its protein profile is  
801 very similar to the proteins present in a variety of legumes, peanuts, and tree nuts. All the  
802 mentioned quality parameters are achievable by following eco-friendly and cost-effective  
803 methods for the extraction of protein from CSM. The protein recovered can potentially be  
804 used as a supplement in human nutrition.

805

## 806 **6. Conclusion and future perspectives**

807 Gossypol chemically binds to cottonseed proteins during cottonseed processing and  
808 reduces their nutritive value. Different separation methods, e.g., physical, chemical and  
809 biological, have been used to reduce or extract gossypol from cottonseeds. Using  
810 biotechnological and breeding strategies for degossypolization strips the plants of their major  
811 defence against insect and pest predation. Thus, current efforts are focused on reducing  
812 gossypol levels in cottonseed products after harvesting and decreasing their oral  
813 bioavailability in feeds. The separation of gossypol from CSM can render a large amount of  
814 high-quality edible protein to be effectively utilized as animal rations and for human  
815 consumption. Toxicological studies have revealed that gossypol is fatal for animals and young  
816 ruminants if it is present in animal feed in large amounts beyond the permissible limit of 450  
817 ppm. Gamma irradiation is emerging as a novel degossypolyzation physical methodology,  
818 replacing traditional approaches (gland flotation, cyclone, heat and pressure treatment and  
819 CO<sub>2</sub> supercritical extraction) burdened with some drawbacks (low protein yields and quality,  
820 inefficient detoxification, use of toxic reagents and environmental risks). Gamma irradiation  
821 is a greener technique that preserves the protein quality. However, gossypol contents below  
822 the permissible limit have not yet been reached, and therefore solvent extraction remains the

823 method of choice, despite the presence of residual solvent in CSP. The use of gossypol-  
824 degrading microorganisms appears promising but is still in need of process optimization.

825         Recent advances in feed technology have resulted in lower gossypol levels in feed and  
826 higher awareness of acute gossypol poisoning. Thus, the development of sustainable  
827 detoxification techniques and the isolation of edible high-quality proteins should be the focus  
828 of future research. A number of recent techniques, such as ultrafine grinding and pulsed  
829 magnetic field treatment, achieved an 83% reduction in the free gossypol content. Other  
830 nonconventional technologies, including microwave-assisted, ultrasound-assisted, subcritical  
831 or supercritical removal of gossypol, can be used in the future to further improve the  
832 utilization of CSM for human nutrition. Functional characterization of CSP indicates a  
833 superior amino acid profile with excellent functional properties, making it a suitable candidate  
834 for application in the food industry. Nevertheless, interest in this protein has decreased since  
835 the 2000s. Emerging greener degossypolization strategies seem to justify future revisitation of  
836 the use of CSP as a direct or indirect source of protein for the alleviation of hunger, which  
837 will also assist in achieving the UN sustainable development goals.

838

#### 839 **Conflict of interest**

840 None

841

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846 **References**

- 847 Adebowale, Y. A., Adeyemi, I. A., & Oshodi, A. A. (2005). Functional and physicochemical  
848 properties of flours of six *Mucuna* species. *African Journal of Biotechnology*, 4(12),  
849 1461–1468.
- 850 Alford, B. B., Liepa, G. U., & Vanbeber, A. D. (1996). Cottonseed protein: What does the  
851 future hold? *Plant Foods for Human Nutrition*, 49(1), 1–11.
- 852 Anderson, A. D., Alam, M. S., Watanabe, W. O., Carroll, P. M., Wedegaertner, T.C., &  
853 Dowd, M. K. (2016). Full replacement of menhaden fish meal protein by low-  
854 gossypol cottonseed flour protein in the diet of juvenile black sea bass *Centropristis*  
855 *striata*, 464, 618–628.
- 856 Arnold, L. K., & Juhl, W. G. (1955). The reduction of free gossypol in cottonseed flakes  
857 during solvent extraction. *Journal of the American Oil Chemists' Society*, 32(3), 151–  
858 152.
- 859 Bahraini, Z., Salari, S., Sari, M., Fayazi, J., & Behgar, M. (2017). Effect of radiation on  
860 chemical composition and protein quality of cottonseed meal. *Animal Science*  
861 *Journal*, 88(9), 1425-1435.
- 862 Balandrán-Quintana, R. R., Mendoza-Wilson, A. M., Montfort, G. R. C., & Huerta-Ocampo,  
863 J. Á. (2019). Plant-Based Proteins. In *Proteins: Sustainable Source, Processing and*  
864 *Applications*, 97-130. Academic Press.
- 865 Baliga, B. P. & Carl, M. L. (1957). *Journal of the American Oil Chemists' Society*, 34 (1), 21-  
866 24.
- 867 Batson, D. M., Thurbur, F. H., & Altschul, A. M. (1951). The effect of screw-press and  
868 hydraulic-press processing conditions on pigment glands in cottonseed, *Journal of the*  
869 *American Oil Chemists' Society*, 28(11), 468–472.

- 870 Berardi, L. C., & Frampton, V. L., (1957). Note on gossypol and its relation to color fixation  
871 in cottonseed oil. *Journal of the American Oil Chemists' Society*, 34 (8) 399–401.
- 872 Bhattacharjee, P. (2007). Supercritical carbon dioxide extraction of cottonseed oil. *Journal of*  
873 *Food Engginering*, 79, 892–898.
- 874 Black and Sesikiran, The Double Burden of Childhood Undernutrition and Overweight  
875 Globally and the Situation in South Asia, web source:  
876 [https://www.nestle.com/sites/default/files/asset-](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf)  
877 [library/documents/creating%20shared%20value/expert-opinions.pdf](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf) accessed on  
878 [25/12/2020](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf)
- 879 Boatner, C.H., Hall, C. M., & Merifield A. L. (1949). Inventors; Heavy gravity liquid  
880 separation of cottonseed, U S Patent 2,482,141, September 20.
- 881 Byrne, F. P., Jin, S., Paggiola, G., Petchey, T. H. M., Clark, J. H., Farmer, T. J., Hunt, A. J.,  
882 McElroy, C. R., & Sherwood, J. (2016). Tools and techniques for solvent selection:  
883 green solvent selection guides. *Sustainable Chemical Processes*, 4(7), 1-24.
- 884 Cai, Y., Xie, Y., & Liu, J. (2010). Glandless seed and glanded plant research in cotton. A  
885 review. *Agronomy for Sustainable Development*, 30(1), 181–190.
- 886 Caldwell, D. R., Keeney, M., Barton, J. S., Kelley, J. F., (1973). Sodium and other inorganic  
887 growth requirements of *Bacteroides amylophilus*. *Journal of Bacteriology*, 114, 782–  
888 789.
- 889 Can, A., Hummel, J., Denek, N., & Südekum, K.-H. (2011). Effects of non-enzymatic  
890 browning reaction intensity on *in vitro* ruminal protein degradation and intestinal  
891 protein digestion of soybean and cottonseed meals. *Animal Feed Science and*  
892 *Technology*, 163(2-4), 255–259.



- 893 Cater, C. M., Mattil, K. F., Meinke, W. W., Taranto, M. V., Lawhon, J. T., & Alford, B. B.  
894 (1977). Cottonseed protein food products. *Journal of the American Oil Chemists'*  
895 *Society*, 54(2), A90–A93.
- 896 Cheng, H. N., Ford, C., Dowd, M. K., & He, Z. (2017). Effects of phosphorus-containing  
897 additives on soy and cottonseed protein as wood adhesives. *International Journal of*  
898 *Adhesion and Adhesives*, 77, 51-57.
- 899 Cheng, H. N., He, Z., Ford, C., Wyckoff, W., & Wu, Q. (2020). A Review of Cottonseed  
900 Protein Chemistry and Non-Food Applications. *Sustainable Chemistry*, 1(3), 256-274.
- 901 Cheng, H. N., Villalpando, A., Easson, M. W., & Dowd, M. K. (2017). Characterization of  
902 cottonseed protein isolate as a paper additive. *International Journal of Polymer*  
903 *Analysis and Characterization*, 22(8), 699-708.
- 904 Council directive 2009/32/EC on the approximation of the laws of the Member States on  
905 extraction solvents used in the production of foodstuffs and food ingredients. (2009).  
906 *Official Journal of the European Union*, L141/3.
- 907 De Buckle, T. S., Zapata, L. E., Silva, G., Cabrera, J. A., De Sandoval, A. M., Ben-Gera, I.,  
908 ...& Shomer, I. (1979). Two food applications of cottonseed flours and meals.  
909 *Journal of the American Oil Chemists' Society*, 56(3Part2), 297–299.
- 910 Dechary, J. M., Kupperman, R. P., Thurber, F. H., & Altschul, A. M. (1952). Removal of  
911 gossypol from cottonseed by solvent extraction procedures. *Journal of the American*  
912 *Oil Chemists Society*, 29(8), 339–341.
- 913 Decossas, K. M., Kadan, R. S., Spadaro, J. J., Ziegler, G. M., & Freeman, D.W. (1982).  
914 Edible cottonseed flour by air classification of glanded seed: cost analysis. *Journal of*  
915 *the American Oil Chemists' Society*, 59, 488-494.

- 916 Delgado, E., Valles-Rosales, D. J., Flores, N. C., & Reyes-Jáquez, D. (2021). Evaluation of  
917 fish oil content and cottonseed meal with ultralow gossypol content on the functional  
918 properties of an extruded shrimp feed. *Aquaculture Reports*, 19, 100588.
- 919 Delgado, E., Valverde- Quiroz, L., Lopez, D., Cooke, P., Valles- Rosales, D., & Flores, N.  
920 (2019). Characterization of Soluble Glandless Cottonseed Meal Proteins Based on  
921 Electrophoresis, Functional Properties, and Microscopic Structure. *Journal of Food*  
922 *Science*, 84(10), 2820-2830.
- 923 Dowd, M. K. (2015). Seed. In Cotton, Agronomy Monograph 57; Fang, D. D. & Percy, R.G.,  
924 Eds. American Society of Agronomy, Inc., Crop Science Society of America, Inc.,  
925 and Soil Science Society of America, Inc.: Madison, WI, 745–782.
- 926 Duodu, C. P., Adjei-Boateng, D., Edziyie, R. E., Agbo, N. W., Owusu-Boateng, G., Larsen,  
927 B. K., & Skov, P. V. (2018). Processing techniques of selected oilseed by-products of  
928 potential use in animal feed: Effects on proximate nutrient composition, amino acid  
929 profile and antinutrients. *Animal Nutrition*, 4(4), 442-451.
- 930 Durand, M., & Kawashima, R. (1980). Influence of minerals in rumen microbial digestion. In  
931 ‘Digestive physiology and metabolism in ruminants’.(Eds Y Ruckebush, P Thivend)  
932 pp. 375–408.
- 933 Ebrahimi-Mahmoudabad, S. R., & Taghinejad-Roudbaneh, M. (2011). Investigation of  
934 electron beam irradiation effects on anti-nutritional factors, chemical composition and  
935 digestion kinetics of whole cottonseed, soybean and canola seeds. *Radiation Physics*  
936 *and Chemistry*, 80(12), 1441–1447.
- 937 Erçelebi, E. A., & Ibanoglu, E. (2009). Rheological properties of whey protein isolate  
938 stabilized emulsions with pectin and guar gum. *European Food Research and*  
939 *Technology*, 229(2), 281–286.

- 940 Fatehi, P., Assadi-Alamouti, A., Behgar, M., & Norouzian, M. A. (2020). Effect of electron  
941 irradiation on some physical, chemical and digestion properties of pistachio by-  
942 products. *Radiation Physics and Chemistry*, 108921.
- 943 Gad, T. E., & El-Zalaki, E. M. (1980). Effect of various pressure cooking conditions on  
944 gossypol content of cottonseed meal and oil. *Fette, Seifen, Anstrichmittel*, 82(11),  
945 450–453.
- 946 Gadelha, I. C. N., Fonseca, N. B. S., Oloris, S. C. S., Melo, M. M., & Soto-Blanco, B.  
947 (2014). Gossypol Toxicity from Cottonseed Products. *The Scientific World Journal*,  
948 1–11.
- 949 Gardner, H. K., Hron, R. J., Vix, H. L. E. (1976). Removal of pigment glands (gossypol) from  
950 cottonseed. *Cereal Chemistry*, 53, 549-60.
- 951 Ghanbari, F., Ghoorchi, T, Shawrang, P., Mansouri, H., & Torbati-Nejad, N. M.  
952 (2012). Comparison of electron beam and gamma ray irradiations effects on ruminal  
953 crude protein and amino acid degradation kinetics, and *in vitro* digestibility of  
954 cottonseed meal. *Radiation Physics and Chemistry*, 81, 672–678.
- 955 Gribbins, G. H. (1951). The reduction of free gossypol in cottonseed by pressure cooking,  
956 *Journal of American Oil and Chemist's Society*, 28, 41–45.
- 957 Harris, W. D., Hayward, J. W., & Lamb, R. A. (1949). Isopropanol as a solvent for extraction  
958 of cottonseed oil. II. Separation of purified oil from miscella. *Journal of the American*  
959 *Oil Chemists' Society*, 26(12), 719–723.
- 960 He, Z., Chapital, D. C., & Cheng, H. N. (2016). Effects of pH and storage time on the  
961 adhesive and rheological properties of cottonseed meal-based products. *Journal of*  
962 *Applied Polymer Science*, 133(27).

- 963 He, Z., Cheng, H. N., Olanya, O. M., Uknalis, J., Zhang, X., Koplitz, B. D., & He, J. (2017).  
964 Surface characterization of cottonseed meal products by SEM, SEM-EDS, XRD and  
965 XPS analysis. *JMSR*, 7(1), 28-40.
- 966 He, Z., Zhang, D., & Cao, H. (2018). Protein profiling of water and alkali soluble cottonseed  
967 protein isolates. *Scientific reports*, 8(1), 1-9.
- 968 He, Z., Zhang, D., & Modesto Olanya, O. (2020). Antioxidant activities of the water-soluble  
969 fractions of glandless and glanded cottonseed protein. *Food Chemistry*, 126907.
- 970 He, Z., Zhang, H., & Olk, D. C. (2015). Chemical Composition of Defatted Cottonseed and  
971 Soy Meal Products. *PLOS ONE*, 10(6), e0129933.
- 972 Heim, R. A. L., & Krebs, G. L. (2018). Ruminant protein digestibility of Australian produced  
973 oilseed meals. *Animal Production Science*.
- 974 Hernandez, E. (2016). Cottonseed: Overview. In C. Wrigley, H. Corke, K. Seetharaman & J.  
975 Faubion (Eds.), *Encyclopedia of Food Grains (Second Edition)* (pp. 242-246).  
976 Oxford: Academic Press.
- 977 Horax, R., Hettiarachchy, N. S., Chen, P., & Jalaluddin, M. (2006). Functional Properties of  
978 Protein Isolate from Cowpea (*Vigna unguiculata* L. Walp.). *Journal of Food Science*,  
979 69(2), 119–121.
- 980 Hron, R. J., Koltun, S. P., Pominski, J., & Abraham, G. (1987). The potential commercial  
981 aspects of gossypol. *Journal of the American Oil Chemists' Society*, 64(9), 1315–  
982 1319.
- 983 Hron, R. J., Kuk, M. S., Abraham, G., & Wan, P. J. (1994). Ethanol extraction of oil,  
984 gossypol and aflatoxin from cottonseed. *Journal of the American Oil Chemists'*  
985 *Society*, 71(4), 417–421.
- 986 Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion

- 987 models for food applications. *Food Chemistry*, 125(1), 1–12.
- 988 Jia, G., Zhan, Y., Wu, D., Meng, Y., & Xu, L. (2009). An improved ultrasound-assisted  
989 extraction process of gossypol acetic acid from cottonseed soapstock. *AIChE Journal*,  
990 55(3), 797-806.
- 991 Johnson, L. A., & Lusas, E. W. (1983). Comparison of alternative solvents for oils extraction.  
992 *Journal of the American Oil Chemists' Society*, 60(2Part1), 229–242.
- 993 Khalaf, M. A., & Meleigy, S. A. (2008). Reduction of free gossypol levels in cottonseed meal  
994 by microbial treatment. *International Journal of Agriculture and Biology*, 10(10),  
995 185-90.
- 996 Kim, S.-Y., Je, J.-Y., & Kim, S.-K. (2007). Purification and characterization of antioxidant  
997 peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion.  
998 *The Journal of Nutritional Biochemistry*, 18(1), 31–38.
- 999 Kuk, M. S., & Hron, R. J. (1994). Supercritical carbon dioxide extraction of cottonseed with  
1000 co-solvents. *Journal of the American Oil Chemists' Society*, 71(12), 1353–1356.
- 1001 Kuk, M. S., & Hron, R. J. (1998). Cottonseed extraction with a new solvent system:  
1002 Isohexane and alcohol mixtures. *Journal of the American Oil Chemists' Society*,  
1003 75(8), 927–930.
- 1004 Kuk, M. S., & Tetlow, R. (2005). Gossypol removal by adsorption from cottonseed miscella.  
1005 *Journal of the American Oil Chemists' Society*, 82(12), 905–909.
- 1006 Kumar, M. (2019b). Paruthi Paal, a nutrient-rich healthy drink from cottonseed: an Indian  
1007 delicacy. *Journal of Ethnic Foods*, 6(1), 1-6.
- 1008 Kumar, M., Dahuja, A., Sachdev, A., Kaur, C., Varghese, E., Saha, S., & Sairam, K. V. S. S.  
1009 (2019). Evaluation of enzyme and microwave-assisted conditions on extraction of  
1010 anthocyanins and total phenolics from black soybean (*Glycine max* L.) seed  
1011 coat. *International journal of biological macromolecules*, 135, 1070-1081.

- 1012 Kumar, M., Mageshwaran, V., Saxena, S., D'Souza, C., Patil, S., Mahapatra A., ... & Tak, Y.  
1013 (2019a). Value addition of cottonseed meal for feed industries by degossypolization  
1014 employing papain and solvent. *Research Journal of Biotechnology Vol, 14*, 11.
- 1015 Kumar, M., Potkule, J., Patil, S., Mageshwaran, V., Saxena, S.,..... & D'Souza, C. (2021).  
1016 Evaluation of detoxified cottonseed protein isolate for application as food  
1017 supplement. *Toxin reviews*.
- 1018 Kumar, M., Potkule, J., Patil, S., Saxena, S., Patil, P. G., Mageshwaran, V., ... & D'Souza, C.  
1019 (2021). Extraction of ultra-low gossypol protein from cottonseed: Characterization  
1020 based on antioxidant activity, structural morphology and functional group  
1021 analysis. *LWT*, 110692.
- 1022 Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., ... & Kennedy, J. F.  
1023 (2021). Advances in the Plant Protein Extraction: Mechanism and  
1024 Recommendations. *Food Hydrocolloids*, 106595.
- 1025 Lawhon, J. T. (1969). Processing methods to preserve quality and color of cottonseed flakes.  
1026 *Journal of the American Oil Chemists' Society*, 46(7), 380–383.
- 1027 Li, J., Pradyawong, S., He, Z., Sun, X. S., Wang, D., Cheng, H. N., & Zhong, J. (2019).  
1028 Assessment and application of phosphorus/calcium-cottonseed protein adhesive for  
1029 plywood production. *Journal of Cleaner Production*, 229, 454-462.
- 1030 Li, R., Hou, G. F., Song, Z. H., Zhao, J. F., Fan, Z. Y., Hou, D. X., & He, X. (2019).  
1031 Nutritional value of enzyme-treated soybean meal, concentrated degossypolized  
1032 cottonseed protein, dried porcine solubles and fish meal for 10- to -20 kg pigs. *Animal  
1033 Feed Science and Technology*, 252, 23-33.
- 1034 Liadakis, G. N., Floridis, A., Tzia, C., & Oreopoulou, V. (1993). Protein isolates with reduced  
1035 gossypol content from screw-pressed cottonseed meal. *Journal of Agricultural and  
1036 Food Chemistry*, 41(6), 918–922.

- 1037 Liu, F.-K., Jou, S. Y., & Jung, L. Y. (1981). A new method of detoxification of cottonseed by  
1038 means of mixed solvent extraction. *Journal of the American Oil Chemist's Society*,  
1039 58(2), A93–A96.
- 1040 Liu, M., Wang, Y., Wu, Y., He, Z., & Wan, H. (2018). “Greener” adhesives composed of  
1041 urea-formaldehyde resin and cottonseed meal for wood-based composites. *Journal of*  
1042 *Cleaner Production*, 187, 361-371.
- 1043 Lusas, E. W., Lawhon, J. T., Clark, S. P., Matlock, S. W., Meinke, W. W., Mulsow, D.  
1044 W., Rhee, K. C., & Wan, P. J. (1978). Potential for edible protein products from  
1045 glandless Potential for edible protein products from glandless cottonseed.  
1046 Proceedings, glandless cottonseed: its significance, status and prospects. USDA-ARS,  
1047 Beltsville, MD, 31-43.
- 1048 Lusas, E. W., Watkins, L. R., & Koseoglu S. S., (1991). Isopropyl alcohol to be tested as  
1049 solvent. *INFORM*, 2, 970-976.
- 1050 Ma, M., Ren, Y., Xie, W., Zhou, D., Tang, S., Kuang, M., & Du, S. (2018). Physicochemical  
1051 and functional properties of protein isolate obtained from cottonseed meal. *Food*  
1052 *Chemistry*, 240, 856–862.
- 1053 Ma, X., Hu, J., Shang, Q., Liu, H., & Piao, X. (2019). Chemical composition, energy content  
1054 and amino acid digestibility in cottonseed meals fed to growing pigs. *Journal of*  
1055 *Applied Animal Research*, 47(1), 280–288.
- 1056 Mageshwaran, V., Sharma, V., Chinnkar, M., Parvez, N., & Krishnan, V. (2018).  
1057 Biodegradation of Gossypol by Mixed Fungal Cultures in Minimal Medium. *Applied*  
1058 *Biochemistry and Microbiology*, 54(3), 301-308.

- 1059 Moretti, D. B., Jimenez, C. R., Trinca, H. M., Machado-Neto, R., & Louvandini, H. (2019).  
1060 Effect of maternal cottonseed feed on the immune and antioxidant status of Santa Ines  
1061 lambs. *Comparative Immunology, Microbiology and Infectious Diseases*, 62, 58-63.
- 1062 Murthy, M. V. R., Mohan E. V. S. & Sadhukhan, A. K. (1999). Cyclosporin A production by  
1063 *Tolypocladium inflatum* using solid state fermentation. *Process Biochemistry*, 34,  
1064 269–80
- 1065 Nagalakshmi, D., Sastry, V. R. B., & Agrawal, D. K. (2002). Detoxification of undecorticated  
1066 cottonseed meal by various physical and chemical methods. *Animal Nutrition and*  
1067 *Feed Technology*, 2(2), 117-126.
- 1068 Nayefi, M., Salari, S., Sari, M., & Behgar, M. (2014). Treatment by gamma or electron  
1069 radiation decreases cell wall and gossypol content of cottonseed meal. *Radiation*  
1070 *Physics and Chemistry*, 99, 23–25.
- 1071 Ohno, A., Ano T., & Shoda, M. (1992). Production of antifungal antibiotic, iturin in solid  
1072 state fermentation by *Bacillus subtilis* NB 22 using wheat bran as substrate.  
1073 *Biotechnology Letters*, 14, 817–822.
- 1074 Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a  
1075 turbidimetric technique. *Journal of Agricultural and Food Chemistry*, 26(3), 716–  
1076 723.
- 1077 Pelitire, S. M., Dowd, M. K., & Cheng, H. N. (2014). Acidic solvent extraction of gossypol  
1078 from cottonseed meal. *Animal Feed Science and Technology*, 195, 120–128.
- 1079 Pons, W. A., Pominski, J., King, W. H., Harris, J. A., & Hopper, T. H. (1959). Recovery of  
1080 gossypol from cottonseed gums. *Journal of the American Oil Chemists' Society*,  
1081 36(8), 328–332.



- 1082 Prat, D., Wells, A., Hayler, J., Sneddon, H., Mcelroy, C. R., Abou-shehada, S., & Dunn, P. J.  
1083 (2016). CHEM21 selection guide of classical- and less classical-solvents. *Green*  
1084 *Chemistry*, 18, 288–296.
- 1085 Rajarathnam, S., Shashirekha, M. N., & Bano, Z. (2001). Biodegradation of gossypol by the  
1086 white oyster mushroom, *Pleurotus florida*, during culturing on rice straw growth  
1087 substrate, supplemented with cottonseed powder. *World Journal of Microbiology and*  
1088 *Biotechnology*, 17(3), 221-227.
- 1089 Rao, R. K., & Arnold, L. K. (1958). Alcoholic extraction of vegetable oils. V. Pilot plant  
1090 extraction of cottonseed by aqueous ethanol. *Journal of the American Oil Chemists*  
1091 *Society*, 35(6), 277–281.
- 1092 Rathore, K. S. Pandeya, D. Campbell, L. M., Wedegaertner, T. C., Puckhaber, L., Stipanovic,  
1093 R. D., Thenell, J. S., Hague, S., & Hake, K. (2020) Ultra-Low Gossypol Cottonseed:  
1094 Selective Gene Silencing Opens Up a Vast Resource of Plant-Based Protein to  
1095 Improve Human Nutrition. *Critical Reviews in Plant Sciences*, 39, 1, 1-29.
- 1096 Rathore, K. S., Pandeya, D., Campbell, L. M., and Palle, S. R. 2019. Cotton transgenic event  
1097 TAM66274. US patent Application 2019/0008113 A1.
- 1098 Reyes-Jáquez, D., Casillas, F., Flores, N., Andrade-González, I., Solís-Soto, A., Medrano-  
1099 Roldán, H., ...& Delgado, E. (2012). The Effect of Glandless Cottonseed Meal  
1100 Content and Process Parameters on the Functional Properties of Snacks during  
1101 Extrusion Cooking. *Food and Nutrition Sciences*, 03(12), 1716–1725.
- 1102 Rhee, K. S., Ziprin, Y. A., & Calhoun, M. C. (2001). Antioxidative effects of cottonseed  
1103 meals as evaluated in cooked meat. *Meat Science*, 58(2), 117–123.
- 1104 Saki, A. A., Pournia, K., Tabatabaie, M. M., Zamani, P., Haghghat, M., & Salary, J. (2012).  
1105 Effects of cottonseed meal supplemented with lysine and enzyme (*Hydroenzyme* XP) on

- 1106 egg quality and performance of laying hens. *Revista Brasileira de Zootecnia*, 41 (10),  
1107 2225-2231.
- 1108 Saricaoglu F. T. (2019). Application of high-pressure homogenization (HPH) to modify  
1109 functional, structural and rheological properties of lentil (*Lens culinaris*) proteins,  
1110 *International Journal of Biological Macromolecules*, 144, 760–769
- 1111 Saxena, D. K., Sharma, S. K., & Sambhi, S. S. (2012). Kinetics and thermodynamics of  
1112 gossypol extraction from defatted cottonseed meal by ethanol, *Polish Journal of*  
1113 *Chemical Technology*, 14, 29–34.
- 1114 Shawrang, P., Mansouri, M. H., Sadeghi, A. A., & Ziaie, F. (2011). Evaluation and  
1115 comparison of gamma- and electron beam irradiation effects on total and free  
1116 gossypol of cottonseed meal. *Radiation Physics and Chemistry*, 80(6), 761–762.
- 1117 Shi, A. H., Zhang, Y., Qu, P., Yan, J. G., & Xiao, H. J. (1998). Screening and breeding of  
1118 highly-effected degrading cotton-phenol strains and study on detoxification  
1119 technology and conditions. *Acta Microbiologica Sinica*, 38, 318–20.
- 1120 Singh, A., & Kaur, A. (2019). Comparative studies on seed protein characteristics in eight  
1121 lines of two *Gossypium* species. *Journal of Cotton Research*, 2(1), 6, 1–12
- 1122 Singh, S., Sharma, S. K., & Kansal, S. K. (2019). Batch extraction of gossypol from  
1123 cottonseed meal using mixed solvent system and its kinetic modeling. *Chemical*  
1124 *Engineering Communications*, 206(12), 1608-1617.
- 1125 Singh, S., Sharma, S. K., & Kansal, S. K. (2020). Extraction of Natural Pigment Gossypol  
1126 from Defatted Cottonseed Using 2-Propanol-Water Green Solvent, Its Kinetics and  
1127 Thermodynamic Study. *Arabian Journal for Science and Engineering*, 5, 7539–7550.
- 1128 Singh, S., Sharma, S. K., & Kansal, S. K., (2015). Extraction of Gossypol from Cottonseed.  
1129 *Review in Advance Science and Engineering*, 4, 301–318.

- 1130 Smith, A. K. (1971). Practical considerations in commercial utilization of oilseeds. *Journal of*  
1131 *American Oil and Chemist's Society*, 48, 38–42.
- 1132 Song, W., Kong, X., Hua, Y., Li, X., Zhang, C., & Chen, Y. (2020). Antioxidant and  
1133 antibacterial activity and *in vitro* digestion stability of cottonseed protein  
1134 hydrolysates. *LWT*, 108724.
- 1135 Spadaro, J. J., & Gardner, H. K. (1979). Food uses for cottonseed protein. *Journal of the*  
1136 *American Oil Chemists' Society*, 56(3Part3), 422–424.
- 1137 Statista, (2020). Accessed on 25/07/2020.  
1138 <https://www.statista.com/statistics/259489/worldwide-production-of-cottonseed/>
- 1139 Sun, H., Jiang-Wu, T., Xiao-Hong, Y., Yi-Fei, W., Wang, X., & Feng, J. (2012).  
1140 Improvement of the nutritional quality of cottonseed meal by *Bacillus subtilis* and the  
1141 addition of papain. *International journal of Agriculture and Biology*, 14(4).
- 1142 Tabatabai, F., Golian, A., & Salarmoeini, M. (2002). Determination and detoxification  
1143 methods of cottonseed meal gossypol for broiler chicken rations. *Agricultural*  
1144 *Sciences and Technology*, 16(1), 3-15.
- 1145 Thurber, F. H., Vix, H. L. E., Pons, W.A. Jr., Crovetto, A. J., & Knoepfler, N. B. (1954).  
1146 The effect of processing conditions on the properties of screw-press cottonseed meal  
1147 and oil. *Journal of the American Oil Chemists' Society*, 31(9), 384-388.
- 1148 Tsaliki, E., Kechagia, U., & Doxastakis, G. (2002). Evaluation of the foaming properties of  
1149 cottonseed protein isolates. *Food Hydrocolloids*, 16(6), 645–652.
- 1150 Tsaliki, E., Pegiadou, S., & Doxastakis, G. (2004). Evaluation of the emulsifying properties of  
1151 cottonseed protein isolates. *Food Hydrocolloids*, 18(4), 631–637.
- 1152 United Nations Children's Fund (UNICEF), World Health Organization, International Bank  
1153 for Reconstruction and Development/The World Bank. Levels and trends in child  
1154 malnutrition: Key Findings of the 2020 Edition of the Joint Child Malnutrition

- 1155 Estimates. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0  
1156 IGO.
- 1157 Vellaichamy, M. (2016). Optimization of Solid State Fermentation Process for Gossypol  
1158 Detoxification in Heat Sterilized Cotton Seed Cake by Mixed Fungal  
1159 Cultures. *International Journal of Food and Fermentation Technology*, 6(1), 97-102.
- 1160 Villalpando, A., Easson, M., Cheng, H. N., & Condon, B. (2019). Use of cottonseed protein  
1161 as a strength additive for nonwoven cotton. *Textile Research Journal*, 89(9), 1725-  
1162 1733.
- 1163 Wan, P. J., Pakarinen, D. R., Hron, R. J., Richard, O. L., & Conkerton, E. J.  
1164 (1995). Alternative hydrocarbon solvents for cottonseed extraction. *Journal of the*  
1165 *American Oil Chemists' Society*, 72(6), 653–659.
- 1166 Wang, J., Clark, G., Ju, M., Castillo, S., & Gatlin, D. M. (2020). Effects of replacing  
1167 menhaden fishmeal with cottonseed flour on growth performance, feed utilization and  
1168 body composition of juvenile red drum *Sciaenops ocellatus*. *Aquaculture*, 523,  
1169 735217.
- 1170 Wang, Q. Y., Zhang, G., Zhao, J. B., Zhou, X. J., Dong, W. X., Liu, L., ... & Zhang, S.  
1171 (2019). Energy and nutrient digestibility of degossypolized cottonseed protein and its  
1172 utilization as a protein source in nursery pigs. *Livestock Science*, 223, 53-59.
- 1173 Wedegaertner, T. & Rathore, K. (2015). Elimination of gossypol in cottonseed will improve  
1174 its utilization. *Procedia Environmental Sciences*, 29, 124–125.
- 1175 Weng, X. Y. & Sun, J.Y. (2006). Kinetics of biodegradation of free gossypol by *Candida*  
1176 *tropicalis* in solid-state fermentation. *Biochemical Engineering Journal*, 32, 226–32.
- 1177 Wu, X.Y. & Chen, J.X. (1989). The utilization of microbes to break down FG in cottonseed  
1178 meal. *Scientia agricultura Sinica*, 22, 82–6

- 1179 Yang, X., Guo, J., & Sun, J. (2011). Biodegradation of free-gossypol by a new fungus isolated  
1180 from cotton planted soil. *African Journal of Microbiology Research*, 5(19), 3066-  
1181 3072.
- 1182 Ye, G., Dong, X., Yang, Q., Chi, S., Liu, H., Zhang, H., Tan, B., & Zhang, S. (2020). Low-  
1183 gossypol cottonseed protein concentrate used as a replacement of fish meal for  
1184 juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂):  
1185 Effects on growth performance, immune responses and intestinal microbiota.  
1186 *Aquaculture*, 524, 735309.
- 1187 Yin, B., Liu, H., Tan, B., Dong, X., Chi, S., Yang, Q., Zhang, S., & Chen, L. (2018).  
1188 Cottonseed protein concentrate (CPC) suppresses immune function in different  
1189 intestinal segments of hybrid grouper ♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus*  
1190 *lanceolatu* via TLR-2/MyD88 signaling pathways. *Fish & Shellfish Immunology*, 81,  
1191 318-328.
- 1192 Zhang, J., Wedegaertner, T., Idowu, O. J., Flynn, R., Hughs, S. E., & Jones, D. C. (2016).  
1193 Registration of ‘NuMex COT 15 GLS’ glandless cotton. *Journal of Plant*  
1194 *Registrations*, 10(3), 223-227.
- 1195 Zhang, Q.-W., Lin, L.-G., & Ye, W.-C. (2018b). Techniques for extraction and isolation of  
1196 natural products: a comprehensive review. *Chinese Medicine*, 13(1).
- 1197 Zhang, W. J., Xu, Z. R., Sun, J. Y., & Yang, X. (2006a). Effect of selected fungi on the  
1198 reduction of gossypol levels and nutritional value during solid substrate fermentation  
1199 of cottonseed meal. *Journal of Zhejiang University Science B*, 7(9), 690-695.
- 1200 Zhang, W. J., Xu, Z. R., Zhao, S. H., Sun, J. Y., & Yang, X. (2007). Development of a  
1201 microbial fermentation process for detoxification of gossypol in cottonseed  
1202 meal. *Animal Feed Science and Technology*, 135(1-2), 176-186.
- 1203 Zhang, W.-J., Xu, Z.-R., Zhao, S.-H., Jiang, J.-F., Wang, Y., & Yan, X.-H.

- 1204 (2006b). Optimization of process parameters for reduction of gossypol levels in  
1205 cottonseed meal by *Candida tropicalis* ZD-3 during solid substrate fermentation.  
1206 *Toxicon*, 48(2), 221–226.
- 1207 Zhang, Y., Zhang, Z., Dai, L., Liu, Y., Cheng, M., & Chen, L. (2018a). Isolation and  
1208 characterization of a novel gossypol-degrading bacteria *Bacillus subtilis* strain Rumen  
1209 *Bacillus Subtilis*. *Asian-Australasian journal of animal sciences*, 31(1), 63.
- 1210 Zhou, J., Zhang, H., Gao, L., Wang, L., & Qian, H.-F. (2015). Influence of pH and ionic  
1211 strength on heat-induced formation and rheological properties of cottonseed protein  
1212 gels. *Food and Bioproducts Processing*, 96, 27–34.
- 1213 Zhuge, Q., Posner, E. S., & Deyoe, C. W. (1988). Production study of a low-gossypol protein  
1214 product from cottonseed meal. *Journal of Agricultural and Food Chemistry*, 36(1),  
1215 153–155.
- 1216
- 1217
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1230 **Figures:**

1231 **Fig. 1.** The problem of malnutrition in South-East Asian countries in the age group of under 5  
1232 years. (Black and Sesikiran, web source: [https://www.nestle.com/sites/default/files/asset-](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf)  
1233 [library/documents/creating%20shared%20value/expert-opinions.pdf](https://www.nestle.com/sites/default/files/asset-library/documents/creating%20shared%20value/expert-opinions.pdf) accessed on 25/12/2020).

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**Fig. 2.** Flow chart describing the stages required for the use of cottonseed protein in food and feed. A) Deoiling of cottonseed to produce cottonseed meal (CSM) with 45–55% protein. CSM at this stage can only be utilized as feed for large ruminant animals due to the presence of the toxic polyphenol gossypol. B) Degossypolization of CSM by physical, chemical, and biological methods resulted in its improved utilization as feed for both small ruminants and nonruminants. C) Extraction of protein from CSM resulted in wider applications, as gossypol levels further reduced gossypol in the obtained protein. D) Applications of cottonseed protein as a supplement in various food products.



1279 **Fig. 3.** Schematic diagram showing the mechanism of physical, chemical and biological  
1280 methods for degossypolization. A) Physical techniques—the application of gamma irradiation  
1281 to cottonseed and cottonseed meal (CSM) resulted in a reduction in the overall gossypol  
1282 content due to degradation or dimerization or an unknown mechanism. B) Chemical methods  
1283 mainly use solvents that interact with the resin glands present in cottonseed or CSM,  
1284 solubilizing gossypol. Intense scrubbing of solids occurs at the solid-liquid interface, causing  
1285 efficient diffusion of the solute (gossypol) into the bulk liquid phase by molecular diffusion.  
1286 C) The biological method of degossypolization mainly involves the use of solid-state  
1287 fermentation by employing a microbial consortium. The exact mechanism of nullifying the  
1288 effect of gossypol in CSM is unknown; however, laccase action is correlated with  
1289 detoxification of the CSM by degrading or oxidizing the toxic aldehyde groups in the  
1290 gossypol structure.

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**Fig. 4.** Illustration showing the amino acid profile, functional properties, molecular size, food safety and regulatory issues, and application of cottonseed protein (CSP). A) The quality of the CSP is mostly determined by the amino acid composition. It can be seen from the figure that CSP has a nice balance of essential and nonessential amino acids. B) The functional properties of the CSP prompted its application in the preparation of baked dough, comminuted meat, marshmallows, mousses, ice creams, bakery products, and sausage. C) Based on the nutritional and functional properties of cottonseed, various products have been developed, such as Paruthi Paal, Proflo, Incaparina, and Tamunuts, and used as functional ingredients in food products. D) In addition to a direct source of protein, CSP is also utilized as an indirect source of protein. The protein conversion ratio of eggs (2.6) is the most efficient in converting cottonseed protein into animal protein for human nutrition. E) Molecular weight studies of CSP showed that salt-soluble proteins are found in maximum concentrations, whereas alcohol-soluble proteins are minimal. F) The application of cottonseed flour or protein in food products should follow food safety and regulatory issues. The most critical parameters considered for application in foods are presented in the illustration.

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1332 **Table 1.** Use of solvent extraction and other methods for the extraction/removal of gossypol

1333 from cottonseed meal/flakes

Cottonseed material used	Solvent utilized	% Gossypol removal (GR)/ % yield of gossypol	Remaining gossypol in seed/flakes/oil/Meal (%)	Reference
Defatted cottonseed flakes	90% aqueous butanone	79.54 % FG (flakes swell more)	0.225% gossypol in flakes	(Dechary et al., 1952)
Defatted cottonseed	Solvent extraction with pure ethanol	61.55 % gossypol	Less than 0.04% gossypol	(Saxena et al., 2012)
Cottonseed meal	Acidic Ethanol; Ethanol-Water [95:5] with 1.4 M phosphoric acid, 2hr (solvent wash only)	94.53% gossypol, Dry matter yield high	5.47% gossypol	(Pelitire et al., 2014)
Cottonseed meal	Acidic Ethanol; Ethanol-Water [95:5] with 1.4 M oxalic acid, 2hr (solvent and water wash)	93.07% total gossypol, Dry matter yield medium	6.92% TG	(Pelitire et al., 2014)
Cottonseed meal	Acidic Ethanol; Ethanol-Water [95:5] with 1.0 M sulphuric acid, 2hr (solvent and water wash)	95.7% gossypol but dry matter yields lowest	4.3% TG	(Pelitire et al., 2014)
Cottonseed meal	Gamma and electron radiation	Free gossypol reduction 59.16%, 82.37% TG reduction-40%	-	(Nayefi et al., 2014)
Cottonseed	Acidic solvent extraction using butanol-ethanol-water	94.73 % total gossypol	5.27 % TG	(Singh et al., 2019)
Cottonseed flour	SC CO <sub>2</sub> with 5% co-solvent iso- propanol/ Ethanol	-	0.02% gossypol in oil	(Kuk & Hron, 1994)
Cottonseed	Liquid cyclone process	-	0.04-0.07% FG & 0.30% TG	(Smith, 1971)
Cottonseed meal	Ethanol and hexane	TG reduced to 0.32 to 0.55 %	FG from 0.013-0.044%	(Liu et al., 1981)
Cottonseed meal	95% Ethanol	More than 50% TG and 90% aflatoxin	-	(Hron et al., 1994)
Cottonseed flakes	Solvent extraction with heptane	-	0.23% gossypol with heptane	[(Wan et al., 1995)
Cottonseed flakes	Solvent extraction with isohexane	-	0.29% gossypol with isohexane	(Wan et al., 1995)
Hexane miscella of Cottonseed	Solvent extraction miscella + adsorption with Mag. Silicate	96% gossypol	-	(Kuk & Tetlow, 2005)
Cottonseed meal	Iso-hexane 75% Ethanol 25%	89.3% FG 42.8% TG	0.11/1.03 FG 0.6/1.05 TG	(Kuk & Hron, 1998)
Cottonseed flakes	Solvent extraction with isohexane and alcohol	70% FG & 45% TG	0.41-0.72% FG & 0.11-0.30 % TG	(Kuk & Hron, 1998)
Cottonseed gums	MEK with phosphoric acid and acid hydrolysis	47% gossypol recovery from gums; Yield: 41% pure gossypol (98%)		(Pons et al., 1959)

1334 The table enumerates amounts of reduction in free (FG) and total gossypol (TG) from cottonseed materials using different solvents/methods  
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1339 **Table 2.** Brief overview of free gossypol detoxification by microbes using solid-state  
 1340 fermentation.

Microorganism	Optimum conditions				Inoculum level (cells/g)	Free gossypol removed (%)	Improvement in protein content (%)	Reference
	IMC (%)	pH	Temp (°C)	Fermentation time (h)				
<i>Bacillus subtilis</i> GH38	50	6.5	39	72	10 <sup>7</sup>	78.86	4.98	(Zhang et al., 2018a)
<i>Candida tropicalis</i>	55	5.2	30	48	10 <sup>7</sup>	88.6	15.24	(Khalaf et al., 2008)
<i>C. tropicalis</i> ZD-3	50		30	48	10 g mycelia/Kg substrate	94.6	10.76	(Zhang et al., 2007)
<i>C. tropicalis</i> ZAU-1	55	6.0	30	72	10 <sup>7</sup>	92.29	-	(Weng & Sun, 2006)
<i>Saccharomyces cerevisiae</i> ZD-5	50		30	48	5 mL yeast inoculum	88.51	11.09	(Zhang et al., 2007)
<i>Aspergillus niger</i> ZD-8	50		30	48	10 g mycelia/Kg substrate	85.15	22.23	(Zhang et al., 2007)

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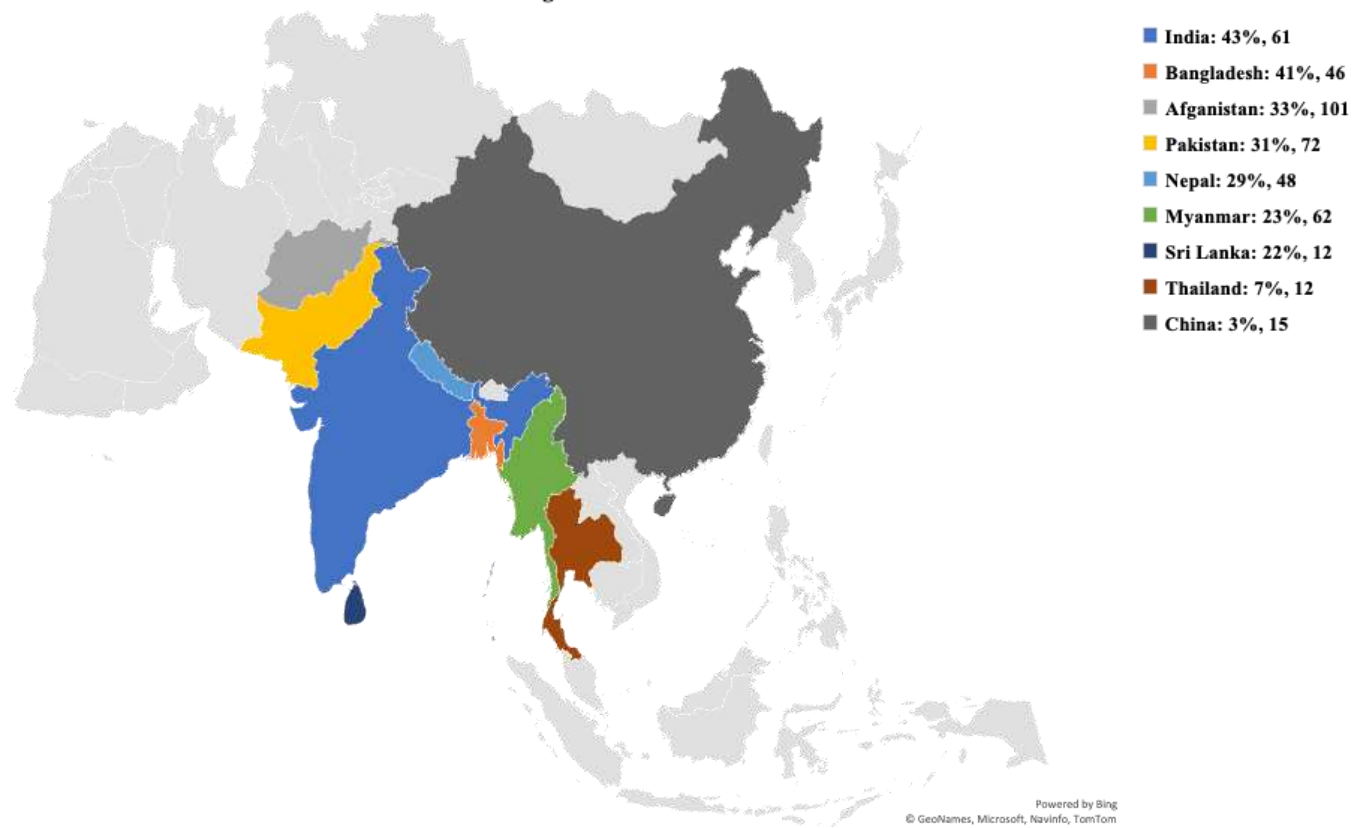
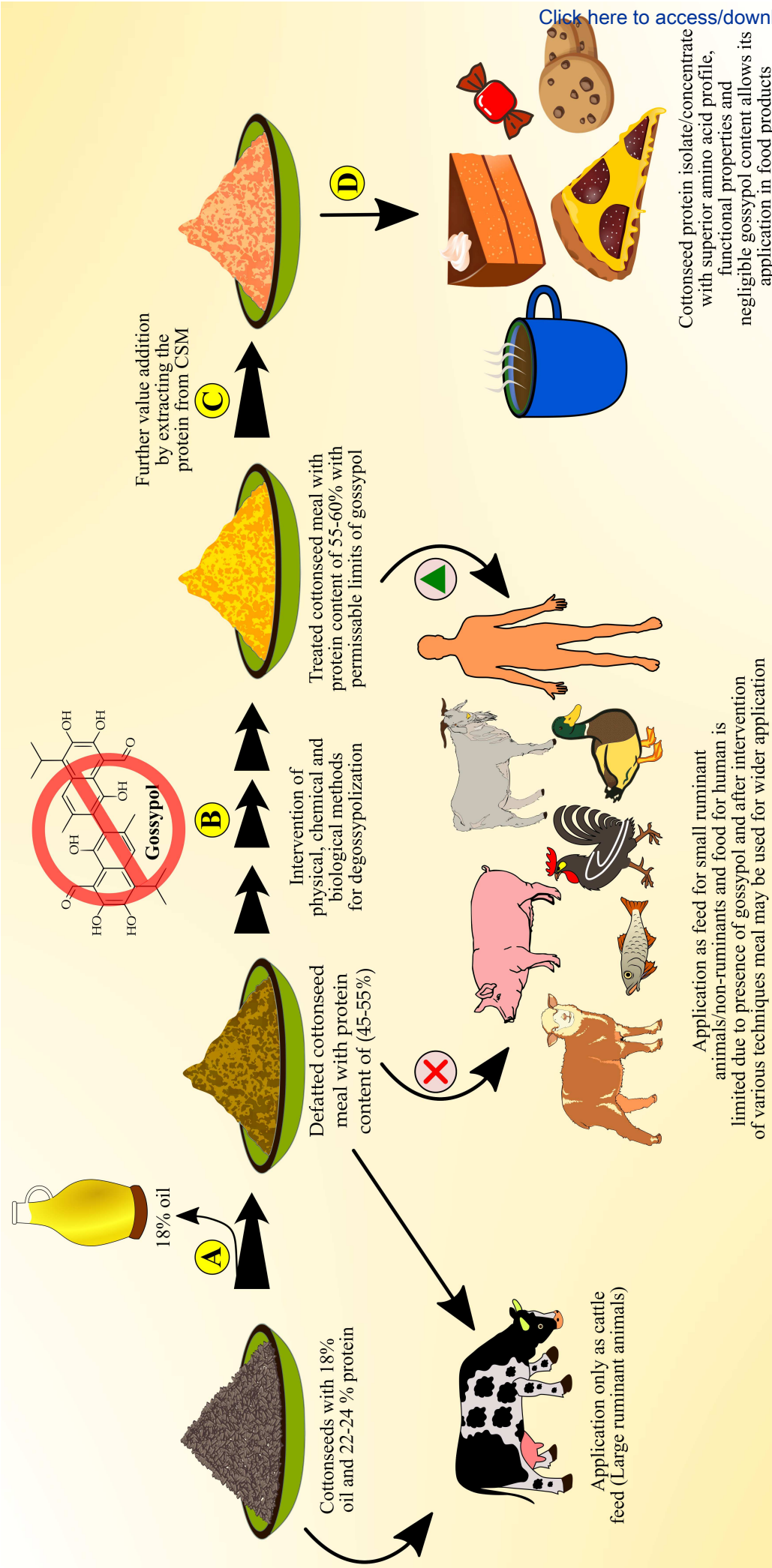
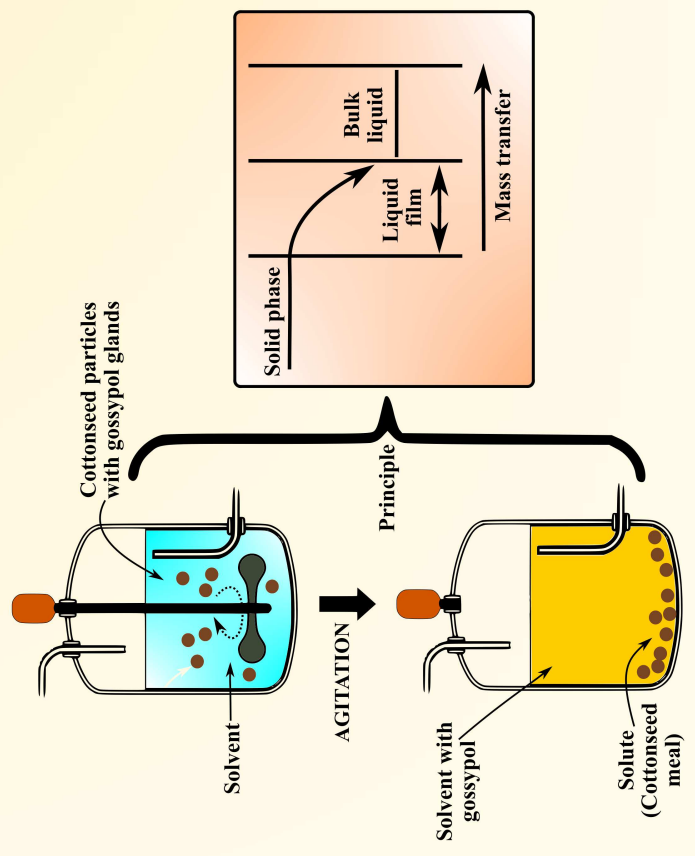
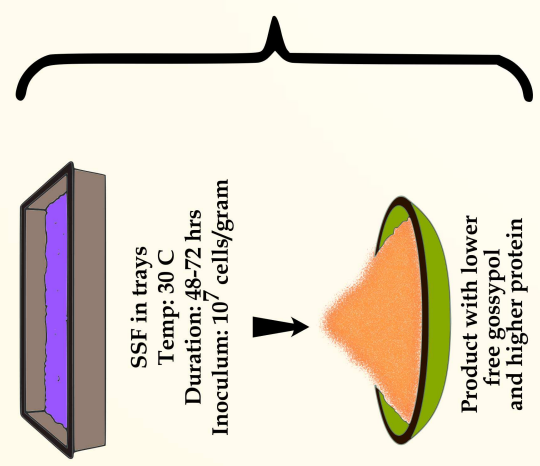
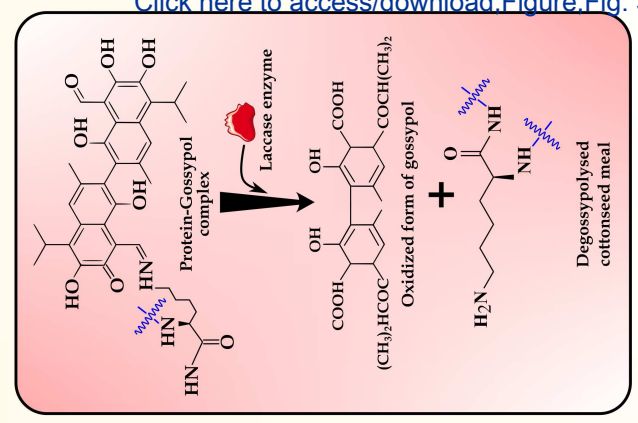


Figure 2

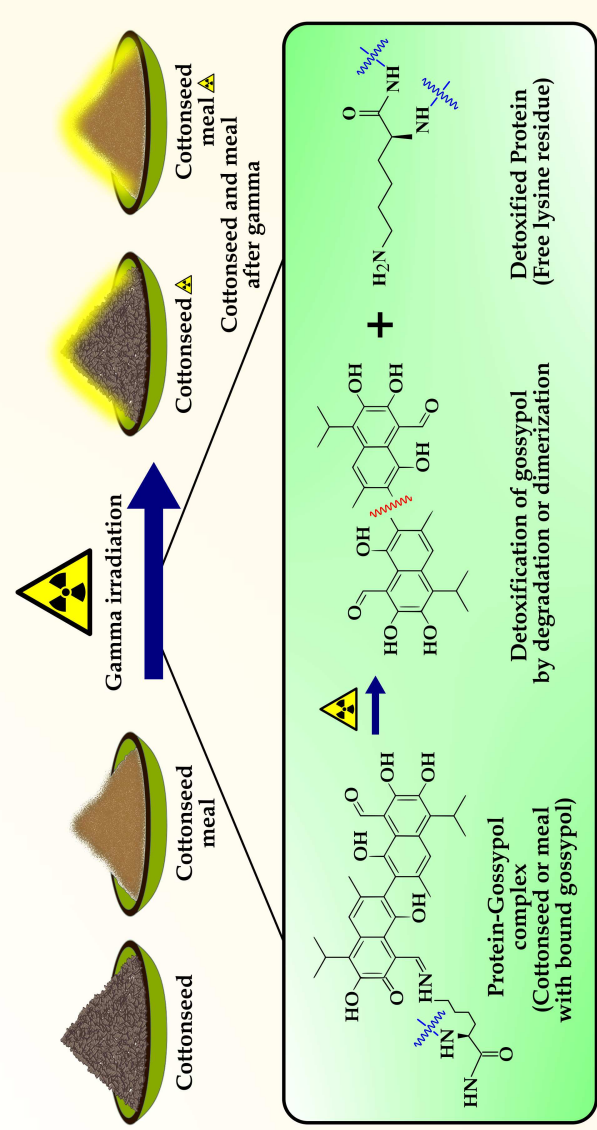




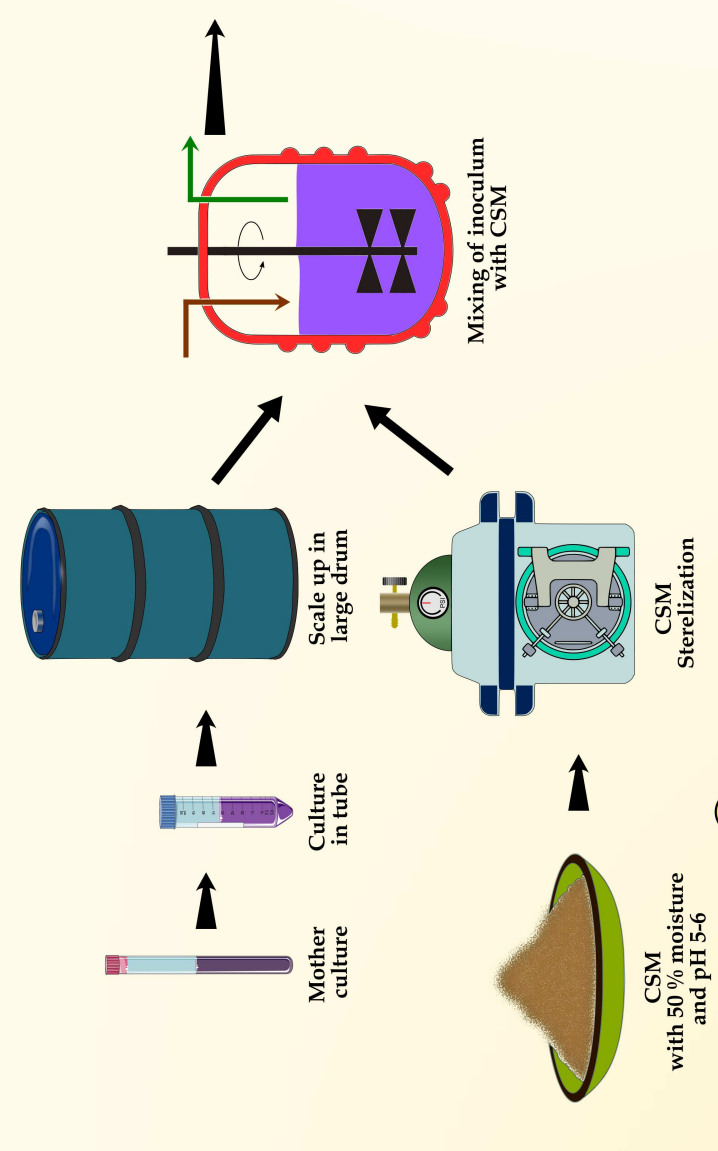
**B** Chemical method: Degossypolization using solvents

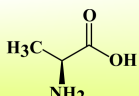
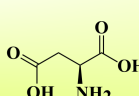
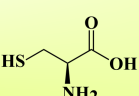
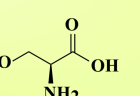
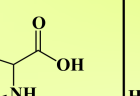
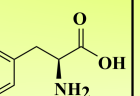
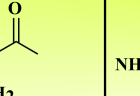
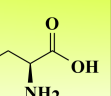
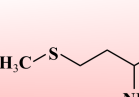
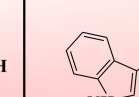
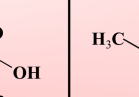
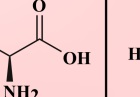
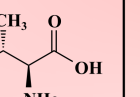
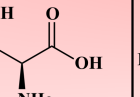
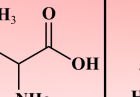
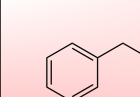
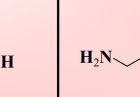
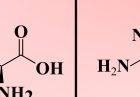









**C** Biological method: Degossypolization using fermentation

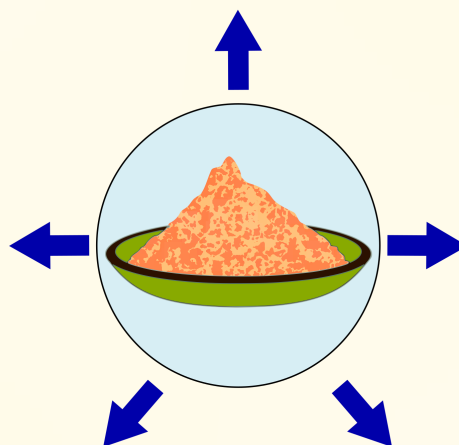


**A** Physical method: Degossypolization using gamma irradiation









A Amino acid profile of CSP							
 <b>Alanine (1.87%)</b>	 <b>Aspartate (4.51%)</b>	 <b>Cysteine (0.79%)</b>	 <b>Serine (2.09%)</b>	 <b>Proline (1.32%)</b>	 <b>Tyrosine (1.14%)</b>	 <b>Glycine (1.99%)</b>	 <b>Glutamine (9.08%)</b>
 <b>Methionine (0.72%)</b>	 <b>Tryptophan (0.64%)</b>	 <b>Leucine (2.95%)</b>	 <b>Isoleucine (1.48%)</b>	 <b>Threonine (1.55%)</b>	 <b>Valine (2.15%)</b>	 <b>Histidine (1.34%)</b>	
Key finding by He et al., (2015): Arginine was highest amounting to 15-34% of the total protein while Methionine and Cysteine were lowest with 1-2%. Glutamate was the most abundant non-essential amino acid amounting to 10% of total protein content				 <b>Phenylalanine (3.00%)</b>	 <b>Lysine (2.15%)</b>	 <b>Arginine (5.70%)</b>	

B Functional properties of the CSP (Ma et al., 2018, Tsaliki et al., 2002, 2004)	
Functional properties	Application
WAC: 1.6-2.9 g/g OAC: 3.0-5.4 g/g	  Baked dough    Comminated meat
FC <sub>5</sub> : 15.1-31.1% FC <sub>7</sub> : 50.0-81.5% FS <sub>5</sub> : 38.8-89.0%	   Marshmallow    Mousses    Ice cream
EC: 13.3-23.1 m <sup>2</sup> /g ES: 17.3-23.6 minute	  Bakery products    Sausage
PS: Highest at pH 11 & Lowest at pH 5	



E Food safety and regulatory issues
These quality parameters include Moisture content < 8% Crude protein > 47% Free gossypol content (FGC) < 0.06% Total gossypol content (TGC) < 1.2% Ash insoluble content < 0.35% Total ash content < 5.0% Available lysine > 3.6g/100g of crude protein Crude fibre content < 5.0% Fat content < 1.5% dry weight basis Microbial count Total bacterial count < 50000/g <i>Coliform</i> bacterial count < 10/g <i>Salmonella</i> bacterial count < nil in 25g Hexane < 10 ppm

C Cottonseed/ cottonseed protein as direct	
	Paruthi Paal
	Proflo
	Incaparina
	Tamunuts
	Food additive nutritional and functional ingredient

D Cottonseed/cottonseed meal as an indirect source	
	4.7
	2.6
	5.7
	20
	5.7-4.6
	7.7

E Molecular weight of CSP	
<ul style="list-style-type: none"> <li>● Non-reducing conditions: 50 and 45 kDa</li> <li>● Reducing conditions: Additional bands between 35 and 14 kDa</li> <li>● Type of protein: Protein contains salt soluble (globulins; 60-70%), water soluble (albumins), alkali soluble (gliadins), and alcohol soluble fractions (prolamines) with balanced ratio of essential amino acids</li> </ul>	