Cottonseed: a sustainable contributor to global protein requirements

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: Degossypolyzation 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.

Cottonseed: a sustainable contributor to global protein requirements

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

1. Introduction

1.1. Problem of malnutrition in the African and Asian continents

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

Fig. 1.

1.2. Cottonseed as a sustainable protein source

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

1.3. Problem of gossypol in cottonseed or cottonseed meal

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

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

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

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

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

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

1.5. Application of cottonseed protein as a food supplement: functional characterization and functional properties

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

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

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

2.1. Physical methods for the degossypolization of CSM

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

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

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

Heat and pressure conditions (Gribbins, 1951) have also been evaluated for reducing the gossypol concentrations in cottonseed kernels. It was established that pressure cooking decreased the gossypol concentration by up to 91.1% in CSM (Gad & El-Zalaki, 1980). However, this method decreased the protein content in the kernels and fatty acid content in the oil, limiting its application for the detoxification of CSM. Later, supercritical CO_2 extraction was also applied to degossypolization. Supercritical CO_2 extraction was employed for the extraction of oil with less than 0.045% gossypol from cottonseeds (Bhattacharjee, 2007). Supercritical CO_2 extraction is the preferred technique because it is highly efficient, needs a shorter extraction time and reduces the requirement for refining, as it reduces the gossypol content in the oil.

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

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

2.2. Chemical method for the degossypolization of CSM

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

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

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

Commonly employed solvents for extraction include light paraffinic petroleum fractions such as pentane (boiling range, 31–36 °C), hexane, heptane (boiling range, 90–99 °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

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

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

Fig. 3.

Table 1

2.3. Biological method for degossypolization of CSM

A number of physical and chemical methods have been developed by researchers for degossypolyzation, but there are several limitations associated with these methods. These methods result in an inferior active vitamin content, protein quality and feed palatability with high energy wastage (Zhang et al., 2018b). The residual solvent is difficult to remove in the case of chemical degossypolization from CSM. This residual solvent is also potentially harmful to both ruminants and nonruminants. During oil recovery from CSM, the use of low temperatures and short durations is crucial to maintaining the protein quality and acceptable concentration of free gossypol. Higher temperatures often resulted in a reduced lysine content in the protein. Therefore, it is necessary to develop an approach for degrading free gossypol and preventing its absorption in animal systems. Studies have indicated that some microorganisms belonging to the genera *Candida*, *Torulopsis*, *Aspergillus*, *Mucor*, *Rhizopus* and *Bacillus* could effectively degrade free gossypol. A brief overview of free gossypol detoxification by microorganisms with optimized parameters is presented in Table 2. Detoxification of CSM by microbes may follow two different hypotheses—first, the utilization of gossypol as a carbon source decreases the total gossypol in the CSM, and second, the transformation of free gossypol to bound gossypol decreases the overall toxicity of the CSM due to the action of microbes after the fermentation process (Zhang et al., 2018a). 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

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

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

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

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

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

Furthermore, supplementation with minerals was found to increase the fermentation 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

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

3.2. Evaluation of functional properties of the cottonseed proteins

3.2.1. Functional properties

The functional properties of proteins are defined as the overall physicochemical behaviour of 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^2/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^2/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

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

3.2.3. Molecular weight

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

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 & Ibanoğlu, 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

components. Other CSPs by the same authors reflected flatter areas, tight surfaces and sharp angles (Kumar et al., 2021). EDS analysis of CSP reflected the presence of Ca, Cu, Na, Mg, K, and P, whereas C and O were detected as major elements due to the organic nature of the sample. The XRD pattern reflected the structure of CSP, which was reported by the same authors. XRD showed peaks at 2θ of approximately 9.4 and 20^0. This XRD pattern was found to be similar to that of soybean protein powder, reflecting α–helix and β–sheet structures of protein molecules, respectively (He et al., 2018). The water- and alkali-soluble CSPs were extracted sequentially, and mass spectrometric analysis showed the presence of 70 polypeptides with molecular weights ranging from 10 to 381 kDa. The most abundant peptides in the fraction were legumin A (58 kDa), B (59 kDa), vicilin C72 (70 kDa), vicilin GC72-A (71 kDa), and vicilin-like antimicrobial peptides (62 kDa) (He et al., 2018). This information is crucial for the application of CSP as a functional food additive.

4. Food and non-food application of cottonseed protein

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

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

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

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

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

Spadaro et al. (1979) reviewed numerous food applications of CSP concentrate, including meat products (such as beef burgers, meatballs, fresh sausages and frankfurters), 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

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

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

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

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

6. Conclusion and future perspectives

Gossypol chemically binds to cottonseed proteins during cottonseed processing and reduces their nutritive value. Different separation methods, e.g., physical, chemical and biological, have been used to reduce or extract gossypol from cottonseeds. Using biotechnological and breeding strategies for degossypolization strips the plants of their major defence against insect and pest predation. Thus, current efforts are focused on reducing gossypol levels in cottonseed products after harvesting and decreasing their oral bioavailability in feeds. The separation of gossypol from CSM can render a large amount of high-quality edible protein to be effectively utilized as animal rations and for human consumption. Toxicological studies have revealed that gossypol is fatal for animals and young ruminants if it is present in animal feed in large amounts beyond the permissible limit of 450 ppm. Gamma irradiation is emerging as a novel degossypolyzation physical methodology, replacing traditional approaches (gland flotation, cyclone, heat and pressure treatment and CO_2 supercritical extraction) burdened with some drawbacks (low protein yields and quality, inefficient detoxification, use of toxic reagents and environmental risks). Gamma irradiation is a greener technique that preserves the protein quality. However, gossypol contents below the permissible limit have not yet been reached, and therefore solvent extraction remains the

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

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

Conflict of interest

None

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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-
877 library/documents/creating%20shared%20value/expert-opinions.pdf accessed on
878 25/12/2020
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.

Cater, C. M., Mattil, K. F., Meinke, W. W., Taranto, M. V., Lawhon, J. T., & Alford, B. B. (1977). Cottonseed protein food products. *Journal of the American Oil Chemists' Society, 54(2),* A90–A93.

Cheng, H. N., Ford, C., Dowd, M. K., & He, Z. (2017). Effects of phosphorus-containing additives on soy and cottonseed protein as wood adhesives. *International Journal of Adhesion and Adhesives*, *77*, 51-57.

Cheng, H. N., He, Z., Ford, C., Wyckoff, W., & Wu, Q. (2020). A Review of Cottonseed Protein Chemistry and Non-Food Applications. *Sustainable Chemistry*, *1*(3), 256-274.

Cheng, H. N., Villalpando, A., Easson, M. W., & Dowd, M. K. (2017). Characterization of cottonseed protein isolate as a paper additive. *International Journal of Polymer Analysis and Characterization*, *22*(8), 699-708.

Council directive 2009/32/EC on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients. (2009). *Official Journal of the European Union,* L141/3.

De Buckle, T. S., Zapata, L. E., Silva, G., Cabrera, J. A., De Sandoval, A. M., Ben-Gera, I., …& Shomer, I. (1979). Two food applications of cottonseed flours and meals. *Journal of the American Oil Chemists' Society, 56(3Part2),* 297–299.

Dechary, J. M., Kupperman, R. P., Thurber, F. H., & Altschul, A. M. (1952). Removal of gossypol from cottonseed by solvent extraction procedures. *Journal of the American Oil Chemists Society, 29(8),* 339–341.

Decossas, K. M., Kadan, R. S., Spadaro, J. J., Ziegler, G. M., & Freeman, D.W. (1982). Edible cottonseed flour by air classification of glanded seed: cost analysis. *Journal of 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., & Ibanoğlu, 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). Ruminal 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

models for food applications. *Food Chemistry, 125(1),* 1–12.

Jia, G., Zhan, Y., Wu, D., Meng, Y., & Xu, L. (2009). An improved ultrasound-assisted extraction process of gossypol acetic acid from cottonseed soapstock. *AIChE Journal, 55(3),* 797-806.

Johnson, L. A., & Lusas, E. W. (1983). Comparison of alternative solvents for oils extraction. *Journal of the American Oil Chemists' Society, 60(2Part1),* 229–242.

Khalaf, M. A., & Meleigy, S. A. (2008). Reduction of free gossypol levels in cottonseed meal by microbial treatment. *International Journal of Agriculture and Biology*, *10(10),* 185-90.

Kim, S.-Y., Je, J.-Y., & Kim, S.-K. (2007). Purification and characterization of antioxidant peptide from hoki *(Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry, 18(1),* 31–38.

Kuk, M. S., & Hron, R. J. (1994). Supercritical carbon dioxide extraction of cottonseed with co-solvents. *Journal of the American Oil Chemists' Society, 71(12),* 1353–1356.

Kuk, M. S., & Hron, R. J. (1998). Cottonseed extraction with a new solvent system: Isohexane and alcohol mixtures. *Journal of the American Oil Chemists' Society, 75(8),* 927–930.

Kuk, M. S., & Tetlow, R. (2005). Gossypol removal by adsorption from cottonseed miscella. *Journal of the American Oil Chemists' Society, 82(12),* 905–909.

Kumar, M. (2019b). Paruthi Paal, a nutrient-rich healthy drink from cottonseed: an Indian delicacy. *Journal of Ethnic Foods*, 6(1), 1-6.

Kumar, M., Dahuja, A., Sachdev, A., Kaur, C., Varghese, E., Saha, S., & Sairam, K. V. S. S. (2019). Evaluation of enzyme and microwave-assisted conditions on extraction of anthocyanins and total phenolics from black soybean (*Glycine max* L.) seed 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., Haghighat, M., & Salary, J. (2012).
1105 Effects of cottonseed meal supplemented with lysine and enzyme (*Hydroenzyme* XP) on

egg quality and performance of laying hens. *Revista Brasileira de Zootecnia, 41 (10),* 2225-2231.

Saricaoglu F. T. (2019). Application of high-pressure homogenization (HPH) to modify functional, structural and rheological properties of lentil (*Lens culinaris*) proteins, *International Journal of Biological Macromolecules, 144*, 760–769

Saxena, D. K., Sharma, S. K., & Sambi, S. S. (2012). Kinetics and thermodynamics of gossypol extraction from defatted cottonseed meal by ethanol, *Polish Journal of Chemical Technology, 14,* 29–34.

Shawrang, P., Mansouri, M. H., Sadeghi, A. A., & Ziaie, F. (2011). Evaluation and comparison of gamma- and electron beam irradiation effects on total and free gossypol of cottonseed meal. *Radiation Physics and Chemistry, 80(6),* 761–762.

Shi, A. H., Zhang, Y.,Qu, P., Yan, J. G., & Xiao, H. J. (1998). Screening and breeding of highly-effected degrading cotton-phenol strains and study on detoxification technology and conditions. *Acta Microbiologica Sinica, 38,* 318–20.

Singh, A., & Kaur, A. (2019). Comparative studies on seed protein characteristics in eight lines of two *Gossypium* species. *Journal of Cotton Research*, *2(1),* 6, 1–12

Singh, S., Sharma, S. K., & Kansal, S. K. (2019). Batch extraction of gossypol from cottonseed meal using mixed solvent system and its kinetic modeling. *Chemical Engineering Communications, 206(12),* 1608-1617.

Singh, S., Sharma, S. K., & Kansal, S. K. (2020). Extraction of Natural Pigment Gossypol from Defatted Cottonseed Using 2-Propanol-Water Green Solvent, Its Kinetics and Thermodynamic Study. *Arabian Journal for Science and Engineering, 5***,** 7539–7550.

Singh, S.,Sharma, S. K., & Kansal, S. K., (2015). Extraction of Gossypol from Cottonseed. *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.

(2006b). Optimization of process parameters for reduction of gossypol levels in cottonseed meal by Candida tropicalis ZD-3 during solid substrate fermentation. *Toxicon, 48(2),* 221–226.

Zhang, Y., Zhang, Z., Dai, L., Liu, Y., Cheng, M., & Chen, L. (2018a). Isolation and characterization of a novel gossypol-degrading bacteria *Bacillus subtilis* strain Rumen Bacillus Subtilis. *Asian-Australasian journal of animal sciences*, *31*(1), 63.

Zhou, J., Zhang, H., Gao, L., Wang, L., & Qian, H.-F. (2015). Influence of pH and ionic strength on heat-induced formation and rheological properties of cottonseed protein gels. *Food and Bioproducts Processing, 96,* 27–34.

Zhuge, Q., Posner, E. S., & Deyoe, C. W. (1988). Production study of a low-gossypol protein product from cottonseed meal. *Journal of Agricultural and Food Chemistry*, *36(1)*, 153–155.

Figures:

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

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.

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

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, comminated 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.

Table 1. Use of solvent extraction and other methods for the extraction/removal of gossypol 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_2 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)

The table enumerates amounts of reduction in free (FG) and total gossypol (TG) from cottonseed materials using different solvents/methods

1339 **Table 2.** Brief overview of free gossypol detoxification by microbes using solid-state
1340 fermentation.

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

1341

Figure 1

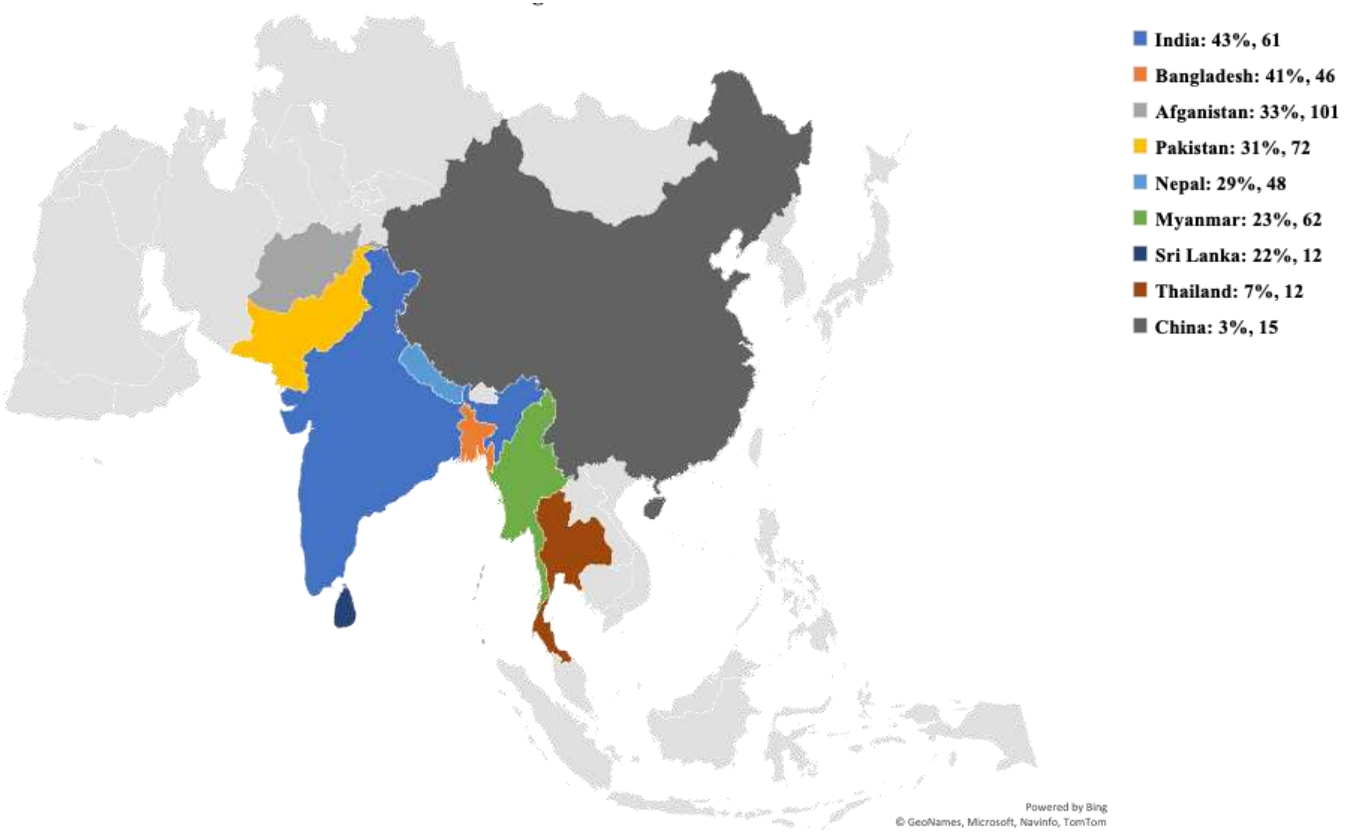
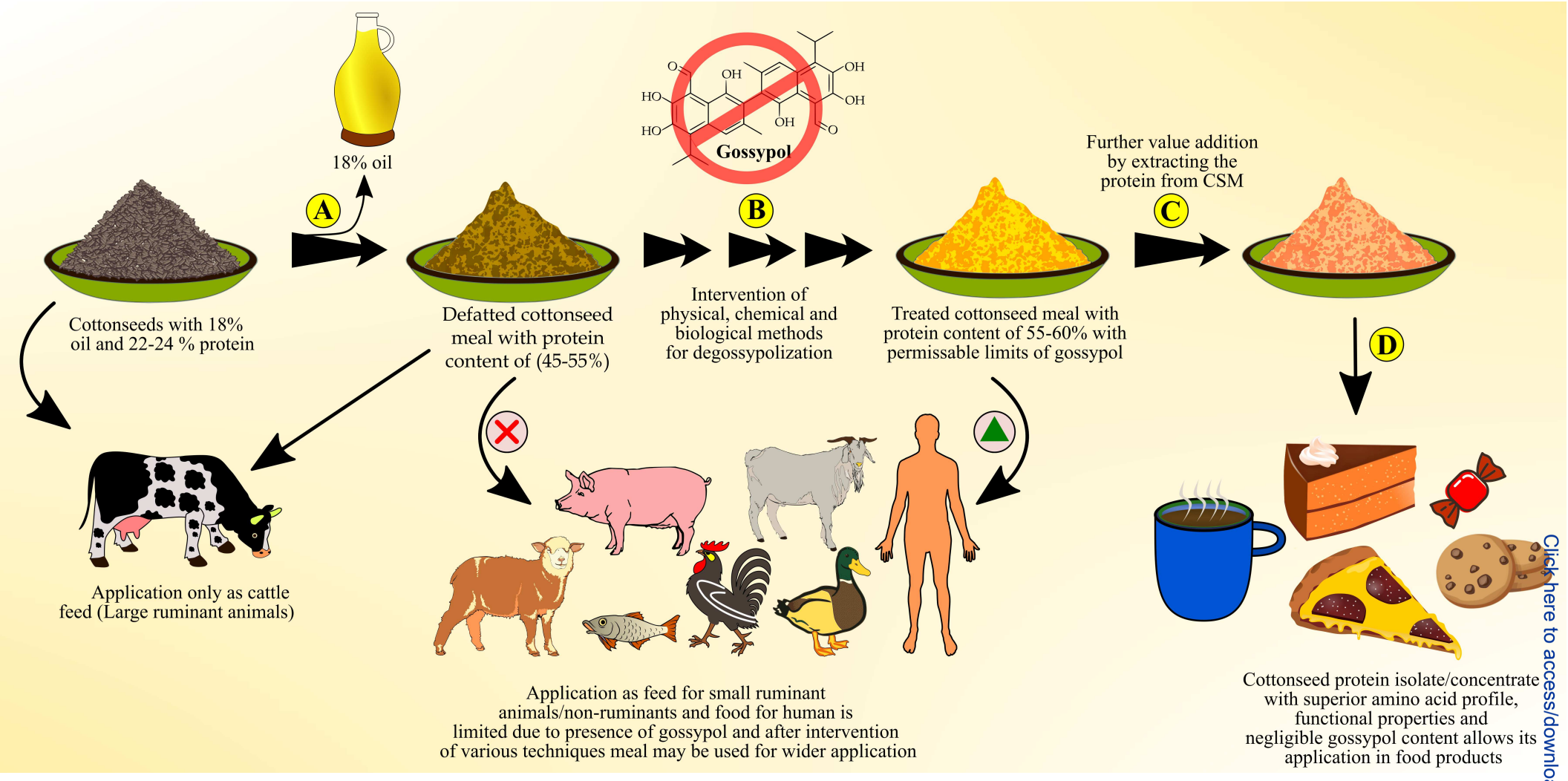


Figure 2



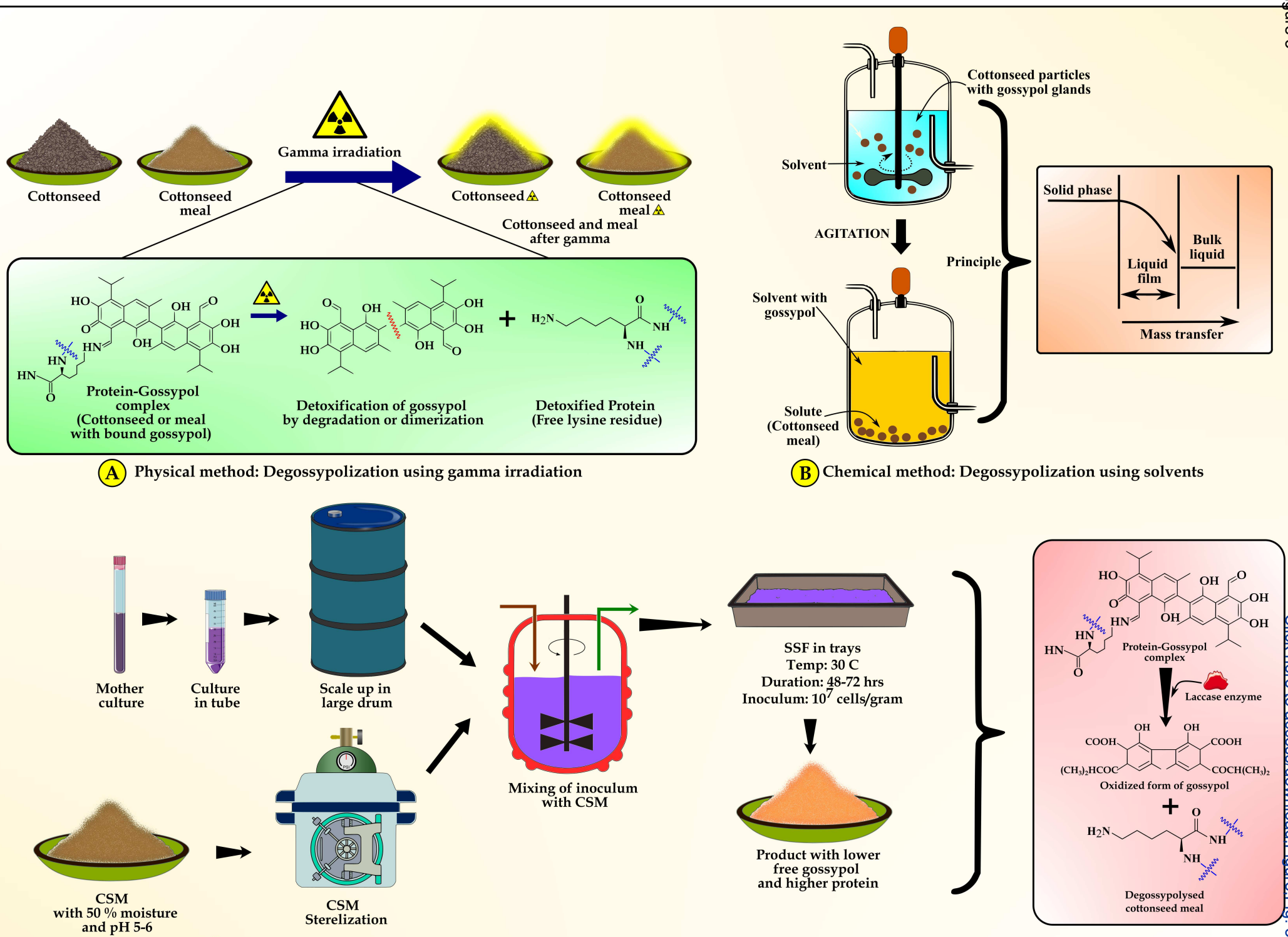


Figure 4

A Amino acid profile of CSP

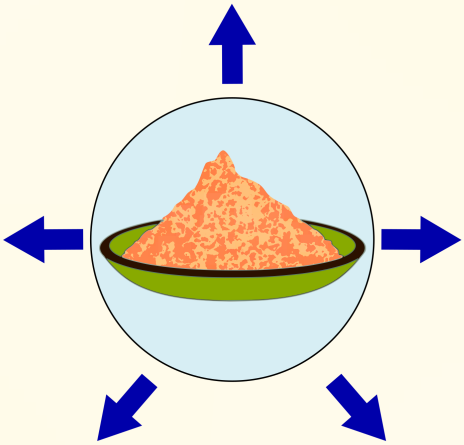
Alanine (1.87%)	Aspartate (4.51%)	Cysteine (0.79%)	Serine (2.09%)	Proline (1.32%)	Tyrosine (1.14%)	Glycine (1.99%)	Glutamine (9.08%)
Methionine (0.72%)	Tryptophan (0.64%)	Leucine (2.95%)	Isoleucine (1.48%)	Threonine (1.55%)	Valine (2.15%)	Histidine (1.34%)	
Phenylalanine (3.00%)	Lysine (2.15%)	Arginine (5.70%)					

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 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_5: 15.1-31.1% FC_7: 50.0-81.5% FS_5: 38.8-89.0%	Marshmallow; Mousses; Ice cream
EC: 13.3-23.1 m^2/g ES: 17.3-23.6 minute	Bakery products; Sausage

PS: Highest at pH 11 & Lowest at pH 5



F 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
Coliform bacterial count < 10/g
Salmonella 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

- Non-reducing conditions: 50 and 45 kDa
- Reducing conditions: Additional bands between 35 and 14 kDa
- 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