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Cottonseed protein concentrate as an alternate protein source for fishmeal replacement in aquafeeds: Production optimisation and its nutritive profile

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ABSTRACT

Cottonseed protein concentrates (CPC) was produced from extruded cottonseed meal (CSM) through iso-electric pH precipitation method and the production protocol was optimised for maximum yield of CPC. The processing characteristics such as yield, protein content and key functional properties were investigated. The average yield of CPC obtained was 28.35%. Protein content of CPC was 69.47±1.28% which showed 64.97% increase in protein content of CSM used for production. Amino acid profile of CPC was on par with fishmeal for most of the essential amino acids expect for lysine and methionine. Functional properties such as water holding capacity and oil holding capacity of CPC was not statistically different from fishmeal ($p>0.05$) and other properties of CPC such as foaming capacity, foaming stability, heat coagulated protein and bulk density were significantly different ($p<0.05$) from fishmeal. The CPC production process significantly reduced the free gossypol content of CSM to the extent of 79.73% which will help to alleviate the detrimental effects of CSM. Pepsin digestibility of CPC was significantly higher than CSM and slightly lower than FM. The results of this study revealed that CPC could be a suitable alternative protein source feed ingredient for replacement of fishmeal in the diet of marine fishes due to its higher digestible protein, superior amino acid profile and lower gossypol content.

Keywords: Bulk density, Cottonseed protein concentrate, Foaming capacity, Free gossypol content, Pepsin diigestibility

Introduction

Aquafeeds contain higher amount of fishmeal (FM) than feeds of terrestrial animals. The inclusion level of fishmeal in aquafeed varies according to the feeding nature whether carnivorous or omnivorous and the pattern of utilisation of nutrients (Martin, 1999). Fishmeal is one of the most indispensable ingredients in marine fish feeds. As FM is the scarcest ingredient due to its decreased supply, high demand and price; there is a search for an alternate ingredient to replace FM in marine fish diets (Fournier *et al*., 2004). Among the several feed ingredients available in the market, agricultural byproducts or plant protein sources are widely available, renewable and reasonably priced ingredients (Chakraborty *et al*., 2019). Therefore, the use of plant protein sources in aquafeeds is considered as potential alternatives to fishmeal (Rana *et al*., 2009). Unfortunately, plant source proteins are often deficient in certain essential amino acids and boast high amounts of fibre content, complex carbohydrates, non-starch polysaccharides and presence of anti-nutritional factors (ANFs), which cause adverse effects on feed intake, digestion and absorption leading to reduced growth (Hixson, 2014). The ANFs may also have adverse effects on both their nutritional value and palatability (Kaushik *et al*., 2004). Hence, an elucidation to ameliorate the above mentioned issues to an acceptable level for inclusion of agricultural byproducts in place of fishmeal in marine fish feeds is warranted.

Among the different plant source proteins, cottonseed meal (CSM) is one of the most promising alternatives to marine source ingredients due to its high protein content (42-45%), wide availability, palatability and reasonably low price (Li and Robinson, 2006). Like other ingredients of plant origin, cottonseed meal also contains some ANFs such as gossypol, cyclopropene fatty acids, phytic acid and tannin. In aquafeeds, cottonseed meal is used to replace soybean meal and fishmeal due to its better palatability and nutritional quality (Li and Robinson, 2006). Some studies stated that cottonseed meal has deleterious effect on feed intake, growth, feed efficiency, gut health and general physiology of fish (Lee *et al.,* 2006; Anderson *et al.,* 2016; Liu *et al.,* 2020). Therefore, it is necessary to ameliorate the ill effects of cottonseed meal. Removal of anti-nutritional factors from feed ingredients is a cumbersome work and most of the methods employed for eradication of anti-nutritional factors in small scale

are not suitable or less appropriate or least effective in commercial scale. Therefore, it is a strategy to separate the protein content from the agricultural byproducts rather than amelioration of anti-nutritional factors. The use of plant-based protein concentrates is a promising alternative to the use of plant-based protein source ingredients as such (Deng *et al.,* 2006). In this context, production of protein concentrate from cottonseed meal seems to be a viable option which will increase its protein content to the tune of 70% (Martinez *et al.,* 1970), improve amino acid profile, protein digestibility and reduce anti-nutritional factors and fibre content (Gerasimidis *et al.,* 2007).

Several methods such as iso-electric precipitation, aqueous-enzymatic process and aqueous-alcoholic protein recovery are currently employed for protein isolate or concentrate preparation. Commercially, protein concentrates are prepared by removing soluble carbohydrate fraction as well as some flavour compounds from defatted meal at the iso-electric pH (pH 4.5) of carbohydrates (Guo, 2009). Thus, protein concentrate preparation involves insolubilisation of protein to remove soluble sugars and separation of solid mass by centrifugation which contain mainly proteins and insoluble carbohydrates (Wang *et al.,* 2004). Other than soybean meal, the preparation of protein isolate and concentrate were reported from jatropha (Kumar *et al.,* 2011; Shamna *et al.,* 2015) rubber seed (Fawole *et al.,* 2016), red seaweed *Kappaphycus alvarezii* (Kumar *et al.,* 2014), sunflower seed meal (Lovatto *et al.,* 2018) and Crambe meal (Lovatto *et al.,* 2018) to enhance its nutritive profile and reduce the anti-nutritional effect. In this context, cottonseed protein concentrate preparation was demonstrated from cottonseed meal to enhance its nutritional quality and reduce the level of free gossypol content. Thus, the objective of this study was to determine the feasibility of preparing cottonseed protein concentrate (CPC) from extruded cottonseed meal to evaluate the nutritional and functional properties of CPC in comparison with extruded cottonseed meal (CSM) and fishmeal.

Materials and methods

Procurement of CSM and particle reduction

Extruded expelled cottonseed meal (CSM) was procured from Abhaycottex India Pvt., Mumbai. The cotton seed meal was milled in a kitchen mixer grinder (Preeti Blue Leaf, India) to reduce the particle size to about 100 µm for protein concentrate preparation and determination of functional properties and nutritive profile.

Standardisation of CPC preparation

The protein concentrate preparation from CSM through isoelectric pH precipitation was done by modified method described by Lusas and Rhee (1995), which was standardised for optimum yield of CPC. Briefly, to 100 g of cottonseed meal (CSM) 1000 ml of water was added, adjusted the pH of the slurry to 11 with 10 M NaOH, the mixture was stirred for 2 h and pH was monitored at 30 min interval and stored in refrigerator (4°C) overnight. The refrigerated slurry was centrifuged at 7500 rpm for 20 min, collected the supernatant and removed the solid material. Adjusted pH of the supernatant to 4.5 (isoelectric) with 6 M HCl to precipitate the protein. Stirred the mixture for 30 min, left in refrigerator overnight and centrifuged the refrigerated solute at 9000 rpm for 20 min to separate the protein and the supernatant was discarded. The resultant pellet was dried at 60°C in hot air oven. The dried CPC was crushed in mixer grinder to a fine powder of 100 µm particle size and labeled prior to storage in air tight container.

Yield estimation

The yield of CPC and cotton seed spent (SP) material prepared from CSM was calculated as follows:

$$\text{Yield of CPC (\%)} = (\text{Weight of CPC/Weight of CSM}) \times 100$$

$$\text{SP material (\%)} = 100 - \% \text{ Yield of CPC}$$

Proximate composition of CSM and CPC

The proximate composition CSM, CPC and SP materials were analysed. The crude protein (Kjeldahl nitrogen × 6.25) was determined by the Kjeldahl method after acid digestion using a Kjeldahl System (FOSS Kjeltec 2300), total fat was estimated using a Soxhlet System (FOSS Soxtec 2043), ash content was determined by incinerating the samples in muffle furnace (Kemi, India) at 600ºC for 4 h (AOAC, 2000a; method 942.05) and moisture content was estimated using hot air oven (AOAC 2000b; method 934.01). The amount of nitrogen free extract (NFE) was estimated as:

$$\text{NFE (\%)} = 100 - (\text{Moisture} + \text{Total Ash} + \text{Crude Protein} + \text{Crude Fat} + \text{Crude Fibre})$$

The gross energy content was estimated using the formula:

$$\text{Gross energy (kcal kg}^{-1}) = \text{Protein} \times 5.4 + \text{Lipid} \times 9.3 + \text{Carbohydrate} \times 4.1$$

Estimation of free gossypol content

The free gossypol content of cotton seed meal, cotton seed protein concentrate and the spent materials were assessed by HPLC (Waters, USA). Fifty millilitre of 70% acetone was added to 1 g cottonseed meal to which 10 g glass beads was added and placed in a shaker at 150 rpm for 1 h. It was then filtered using filter paper (Whatman No. 1) and 1 ml of filtrate was collected to which 1 ml of

N, N di-methylformamide and 1 ml of reagent complex (2 ml of 3-amino proponol and 10 ml of glacial acetic acid were added and made up to 100 ml using N,N-di-methylformamide) were added. This complete setup was placed in a water bath for 30 min at 100°C. Solution was allowed to cool to room temperature and diluted to 5 ml by adding 3 ml of N,N di-methylformamide from which 20 µl of sample was analysed by HPLC (Karishma *et al*., 2016).

Amino acid profile of CSM and CPC

The amino acid profile of CSM and CPC samples in triplicates were analysed by reverse-phase high-performance liquid chromatography (HPLC) (Waters, USA). Samples were hydrolysed with 6 M HCl at 110◦C for 24 h and hydrolysates were added to a C-18 column and amino acids were separated *via* reverse phase HPLC. Amino acids were quantified with a photodiode array detector following post-column derivatisation with ninhydrin as described in Prabu *et al*. (2020).

Characterisation of CSM and CPC

Water and oil holding capacities were determined by the method of Childs and Park (1976). Foaming capacity and foaming stability were measured according to Lawhon and Cater (1971). The volumes before and after whipping were recorded and the percentage volume increase calculated as follows:

Foaming capacity (% Volume increase) = [(Volume after whipping - Volume before whipping) / (Volume before whipping)] ×100

Foaming stability (% Volume decrease after 30 min) = [(Volume immediately after whipping - Volume after 30 min of whipping)/ Volume immediately after whipping] ×100

Heat-coagulated protein was measured according to Kramer and Kwee (1977) and expressed as percentage of crude protein. The percentage of coagulated protein was calculated as follows:

Percentage coagulated protein = [(Absorbance before heating - Absorbance after heating) /Absorbance before heating] × 100

pH of the products

The pH of the CSM, CPC, SP and FM were assessed using a pH pen (Hanna, India) after dissolving 10 g of the respective sample in 100 ml distilled water.

Bulk density

A 100 ml graduated beaker was filled with cotton seed meal or protein concentrate and then weighed to calculate the bulk density as follows:

Bulk density (g ml^{-1}) = Weight of the sample/Volume of sample

In vitro pepsin digestibility

The *in vitro* pepsin digestibility was assessed by the AOAC (2005) method 971.09.

Statistical analysis

The data were analysed using the statistical package SPSS version 16. Comparison between products was made using Duncan's Multiple Range Test (DMRT) (Duncan, 1955). Comparison among all the samples was done by oneway ANOVA at 5% probability level.

Results and discussion

Cottonseed oil is extracted by pressing the kernel of cotton seed (with yield of 17%), which is ranked sixth among the world's edible oils and the residual cotton seed meal (47%) is used as a good protein source for fish and other animals (Cheng *et al.,* 2020). Even though cotton seed meal is rich in protein, presence of anti-nutritional factors especially free gossypol limits the utility of cotton seed meal in the animal feeds. In this context, production of protein concentrate from cottonseed meal seems to be a viable option which will increase its protein content, improve amino acid profile, protein digestibility and reduce free gossypol and fibre content (Gerasimidis *et al.,* 2007; Makkar *et al.,* 2008; Marrufo-Estrada *et al.,* 2013).

The method of CP preparation through isoelectric precipitation was standardised and the protocol witnessed the yield of 28.35±3.44% (Mean±SE) of CPC from the processed CSM. This is in accordance with the inference of Zhang *et al*. (2009) who extracted cottonseed protein concentrate by using an alkaline medium at varying pH values and obtained cottonseed protein concentrate with protein content of 70%. Alkali extraction followed by isoelectric precipitation is a suitable method for isolate/ concentrate preparation from canola (Drew *et al*., 2007). Fawole *et al*. (2016) reported a protein recovery of 31.19-36.81% of rubber seed protein isolate at different pH levels.

The proximate composition of cottonseed products and fishmeal as a reference material is listed in Table 1. The nutritive profile in terms of crude protein content was assessed and the protein content of CPC was 69.47%. The protein content of CSM and SP materials were 42.11 and 21.53% respectively. The protein content showed an increase of 64.97% in CPC over CSM. Generally, protein concentrate and isolate preparation reduce the anti-nutritional factors, crude fibre contents and carbohydrate levels in the plant source ingredients; thus, enhance the protein content of the source ingredients. This is in

Table 1. Proximate composition analysis of cotton seed products and commercial fishmeal

Parameter	CSM	CPC	SP	FM	p value
Moisture (%)	9.71±0.52^{b}	7.77±0.41^{a}	6.85±0.17^{a}	6.76±0.29^{a}	0.001
Crude protein (%)	42.11±0.29^{b}	69.47±1.28^{d}	21.19±0.20^{a}	63.34±0.27^{a}	0.001
Crude lipid (%)	3.97±0.12^{b}	0.63±0.04^{a}	4.95±0.09^{c}	7.08±0.27^{d}	0.001
Total ash (%)	7.46±0.12^{b}	2.93±0.05^{a}	10.79±0.31^{c}	19.87±0.13^{d}	0.001
Crude fibre (%)	4.65±0.14^{b}	0.34±0.03^{a}	7.78±0.08^{c}	0.23±0.01^{a}	0.001
NFE (%)	32.10±0.47^{c}	18.85±0.85^{b}	48.42±0.60^{d}	2.68±0.28^{a}	0.001
Gross energy (kcal kg^{-1})	3959.1±9.23^{b}	4583.3±33.25^{d}	3590.3±17.50^{a}	4189.9±8.01^{c}	0.001
Free gossypol content (mgkg^{-1})	965.67±11.56^{c}	195.68±5.23^{a}	732.33±9.33^{b}	0.00	0.001

CSM: Cotton seed meal; CPC: Cotton seed protein concentrate; SP: Spent; FM: Fishmeal; NFE: Nitrogen free extract
The data is represented as arithmetic mean of three replicates±SE
The values in the same row with different superscripts differ significantly ($p<0.05$).

agreement with Martinez *et al*. (1970) who revealed that CSM through further processing, can be prepared as protein concentrate with about 70% protein and protein isolates with 90% crude protein. Similarly, Wang *et al*. (2004) found increased protein content in the soy protein concentrate (67.53%) and isolates (87.53%) prepared from soybean meal (54.34%) by the reduction of carbohydrates and fibre contents of source materials. Glencross *et al*. (2004) reported that the total protein content of soy protein concentrate is 65-67% and soy protein isolate is 89%. Canola protein concentrate with 69% protein and lysine content of 3.55% was reported by Drew *et al*. (2007). The free gossypol content of CSM was 965.67 mg kg^{-1} which was reduced to 195.68 mg kg^{-1} upon preparation of CPC. Karishma *et al*. (2016) studied the total and free gossypol content of various varieties of cotton seed extracts and found that the free gossypol content was in the range of 1153-2860 mg kg^{-1} except the higher level of 4140 mg kg^{-1} in one variety. The gossypol content showed a reduction of 79.73% in CPC than CSM.

The amino acid profile of CPC, CSM and SP materials were analysed and represented along with FM in Table 2.

Table 2. Amino acid profile of cotton seed products (g kg^{-1})

Parameter	CSM	CPC	SP	FM
Essential amino acids (EAA) (g kg^{-1})				
Arginine	36.26	58.45	12.23	32.26
Histidine	9.45	15.84	4.17	12.55
Lysine	15.44	20.35	3.89	49.85
Isoleucine	12.63	19.48	5.82	24.55
Leucine	21.11	34.19	6.92	44.76
Methionine	5.92	9.14	2.12	16.33
Phenylalanine	20.44	29.56	5.1	27.57
Threonine	12.57	19.27	3.3	29.45
Tyrosine	8.75	14.37	3.56	18.95
Valine	17.22	24.44	7.82	27.65
Cystine	6.58	8.74	2.12	5.34
∑EAA	166.37	253.83	57.05	289.26
Non-essential amino acids (NEAA) (g kg^{-1})				
Glycine	8.95	15.56	4.67	16.45
Glutamine	13.45	19.8	7.8	26.9
Glutamic acid	41.85	62.31	6.89	69.78
Asparagine	9.1	14.45	3.23	22.65
Aspartic acid	16.1	24.43	4.57	33.45
Hydroxyproline	21.46	35.89	5.29	38.78
Proline	10.2	16.78	3.82	23.32
Serine	22.11	36.78	5.4	43.56
∑NEAA	158.82	249.31	46.77	292.69

CSM: Cotton seed meal; CPC: Cotton seed protein concentrate; SP: Spent; FM: Fishmeal
Data represented as arithmetic mean of three replicates

The limiting amino acids, lysine and methionine levels of CPC showed an increase of 31.8 and 54.39% respectively than CSM. The lysine and methionine content of CSM and CPC were comparatively lower than FM. The arginine, histidine, phenylalanine and cystine content of CSM and CPC were higher than FM. Glycine, glutamic acid and valine content of CPC were on par with FM. This is in agreement with the results of Yuan *et al*. (2019) which reported similar essential amino acid profile in fishmeal as well as cotton seed protein hydrolysate. Similarly, Li *et al*. (2010) found same trend with slightly higher level of essential and non-essential amino acids in cottonseed meal and cottonseed protein isolate (Martinez *et al*., 1970). Lawhon *et al*. (1974) also reported similar trend of essential and non-essential amino acids in cotton seed whey protein concentrate.

The functional properties of CSM, CPC and SP materials are given in Table 3. The functional properties such as water holding capacity and oil holding capacity of CPC were not significantly different from fishmeal ($p>0.05$) and other properties of CPC such as foaming capacity, foaming stability, heat coagulated protein and bulk density were significantly different ($p<0.05$) from fishmeal. The water and oil holding capacity of CPC was higher than FM as well as CSM in this study. The results are in accordance with Childs and Park (1976), who observed slightly higher water and oil holding capacity of glandless cottonseed flour and Patil *et al*. (1993) recorded comparable amount of water and oil holding capacity in defatted peanut kernel meal. In the present study, the foaming capacity and stability were found better in CPC followed by FM and CSM. The result is in line with that of Osman *et al*. (1987), who found better foaming capacity and stability of gossypol-poor cottonseed protein isolates at lower pH. This agrees with the present result where higher foaming capacity and stability were recorded at lower pH of the materials. Similarly, Patil *et al*. (1993) also reported same amount of foaming capacity and stability of defatted peanut kernel meal. The heat coagulated protein content of CPC was significantly higher than CSM and FM in this study. Osman *et al*. (1987) also found slightly higher percentage of coagulated protein content of cottonseed protein concentrate fractions produced by various methods. Similar result was also recorded by Kramer and Kwee (1977), in heat coagulated protein content of tomato protein concentrate.

The mean pH of CSM, CPC and SP were 6.03, 4.57 and 8.61 respectively. pH of CPC was lower due to the acidic pH (4.5) in which isoelectric precipitation of cottonseed protein was carried out. The bulk density of CPC was slightly low compared to CSM (Table 3) which was due to the reduction in fibre content of CPC. Kramer and Kwee (1977), reported significantly lower bulk density of tomato protein concentrate. The *in vitro* pepsin digestibility of the cottonseed protein products and fishmeal showed significant difference ($p<0.05$) among them. The pepsin digestibility of CPC increased compared to the CSM. This is in agreement with Osman *et al*. (1987) who reported that *in vitro* pepsin digestibility of cotton-seed protein concentrate produced by various methods was over 90%. Similarly, Shamna *et al*. (2015) found the digestibility of Jatropha seed cake protein concentrate to be around 93% which is very high as compared to unprocessed jatropha oil cake or meal.

The nutritive profile of cotton seed protein concentrate was enhanced in terms of protein and amino acid composition. The free gossypol content was significantly reduced through cottonseed protein concentrate production. Functional properties such as water holding capacity, oil holding capacity and foaming capacity of cottonseed protein concentrate were observed to be higher than cottonseed meal. Also the pepsin digestibility of cottonseed protein concentrate increased significantly than cottonseed meal. All these enhanced nutritional properties of cotton seed protein concentrate make it a potential alternative to fishmeal in the diet of marine fishes.

Table 3. Functional properties of cotton seed products (wet basis)

Parameter	CSM	CPC	SP	FM	p value
Water holding capacity (ml g^{-1})	1.84 ± 0.07^{b}	2.34 ± 0.06^{c}	1.53 ± 0.07^{a}	2.19 ± 0.02^{c}	0.001
Oil holding capacity (ml g^{-1})	1.45 ± 0.05^{ab}	1.74 ± 0.05^{c}	1.37 ± 0.07^{a}	1.64 ± 0.06^{bc}	0.007
Foaming capacity (% increase)	15.56 ± 0.23^{b}	19.25 ± 0.25^{c}	11.82 ± 0.29^{a}	15.33 ± 0.39^{b}	0.001
Foaming stability (% decrease after 30 min)	70.00 ± 1.15^{b}	54.33 ± 2.33^{a}	88.67 ± 1.45^{c}	72.33 ± 1.46^{b}	0.001
Heat coagulated protein (%)	20.69 ± 0.64^{b}	28.68 ± 0.57^{c}	12.37 ± 0.72^{a}	21.98 ± 1.15^{b}	0.001
pH	6.03 ± 0.12^{b}	4.57 ± 0.03^{a}	8.62 ± 0.06^{d}	6.21 ± 0.04^{c}	0.001
Bulk density (g ml^{-1})	0.78 ± 0.005^{b}	0.73 ± 0.010^{a}	0.94 ± 0.003^{c}	0.77 ± 0.008^{b}	0.001
Pepsin digestibility (%)	79.16 ± 0.63^{b}	88.56 ± 0.37^{c}	67.81 ± 1.32^{a}	96.35 ± 0.30^{d}	0.001

CSM: Cotton seed meal; CPC: Cotton seed protein concentrate; SP: Spent; FM: Fishmeal
Data represented as arithmetic mean of three replicates±SE
Values in the same row with different superscripts differ significantly ($p<0.05$).

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