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Solid-state Fermentation of Cottonseed Meal with *Saccharomyces cerevisiae* for Gossypol Reduction and Nutrient Enrichment

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ABSTRACT

Background: An experiment was conducted to study the effect of solid-state fermentation (SSF) of cottonseed meal (CSM) with *Saccharomyces cerevisiae* on anti-nutritional factors and amino acid profile.

Methods: A finely ground CSM was fermented with baker's yeast for 48 h at room temperature with periodic observations of pH, temperature and moisture. The fermented substrate was partially sun-dried and nutritional values were observed.

Result: After the SSF process on dry basses, the protein content of CSM increased from 8.74% to 12.67%. Besides increasing the nutritional value, the SSF showed a clear significant effect in reducing the anti-nutritional factors content of raw CSM like total gossypol from 0.28% to 0.21%, phytic acid from 3.3% to 0.3% and total tannin from 1.42% to 0.68%. CSM fermented with *S. cerevisiae* improved concertations of essential amino acids *viz*., histidine, isoleucine, valine, methionine and phenylalanine. The protein quality evaluation showed a significant increase in its nutritional value. Based on the present result, it can be concluded that the fermentation of CSM with *S. cerevisiae* decreases the anti-nutritional factors and improves essential nutrients.

Key words: Anti-nutritional factor, Cottonseed meal, *Saccharomyces cerevisiae*, Solid-state fermentation.

INTRODUCTION

Growing demand and cost and reduced availability of quality fishmeal in the required quantity have emphasised in search for alternative protein sources in the diets of fish and farmed animals (Fournier *et al*., 2004; Ramachandran and Ray, 2007). Therefore, research is directed towards finding quality alternative protein sources that are ideally less expensive and readily available natural resources (Currie, 2000). Such research work has been intensified in the last decade to determining the efficiency of alternative ingredients in terms of growth and production with better feed supply (Adeniji, 2007). In the current scenario, using non-conventional feed ingredients has been reported with good growth and economic benefits. Various by-products from agro-industries are gaining interest to use as feed ingredients in feed formulations since these by-products possess significant amounts of bioactive compounds. These processed end products are considered promising source of protein and energy for formulating economic and environment-friendly fish diets (Herrero *et al*., 2013).

Among the plant protein sources, oil seed cakes/meals are predominant choices for feed formulations. After oil extraction from the seeds, the leftover meals form the best by-products. Oil cakes are available in two forms, such as edible and non-edible (Sarker *et al*., 2015) and are the primary source of protein in animal feeds (Elzubeir *et al*., 1990; Mohmmed *et al*., 1995). The high content of protein in the oil cake meal offers an array of rich essential amino acids (Smith *et al*., 1959; Patel *et al*., 1970). These industrial by-products are used extensively in fish, livestock and poultry feed to provide protein worldwide (Ensminges, 1980; Tekeli, 2014; Zhao *et al*., 2016). These plant-based agro-industrial waste have limited use as feed ingredients owing to their high fibre content and presence of anti-nutritional factors (ANFs). These anti-nutritional factors negate the growth and other physiological responses of the organism during high rate of inclusion. Further, these are also deficient in certain essential amino acids like lysine and methionine (Eyo, 2003), have lower biological values, high indigestibility rate and experience loss of quality during storage (Lji *et al*., 2017).

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Cotton is the backbone of the textile industry, supporting 70% of the country's total fibre production. The cotton plant contributes to more food for humans and feed for animals than as a fibre source (Dinesh *et al*., 2003). Among all the plant protein sources, cottonseed a by-product of the textile industry, provides a significant quantity of edible oil and protein-rich meal for livestock (Munro, 1987) . Cottonseed meal (CSM) is a by-product generated from decortications of the seed after oil extraction, (Paiano *et al*., 2006). CSM is the second-largest protein source used in animal feed (Smith, 1972) due to its relatively higher content of protein and balanced amino acid profile. CSM is nutritionally valuable feed that contains 51.20% crude protein, 7.02% crude fiber, 1.6% ether extract, 9.3% ash and 2.71 ME (kcal/kg) Obioha, (1992).

CSM can fill the scarcity of conventional feed ingredients for fed aquaculture systems. However, the utilisation of these plant proteins in fish diets is limited due to their low levels of digestible protein and anti-nutritional factors, which interfere with nutrient bio-availability and utilisation in unprocessed form (Abowei and Ekubo, 2011; Kumar *et al*., 2021). CSM contains anti-nutritional factors *viz*., gossypol (Withers and Carruth, 1915), phytic acid and tannin (Tacon, 1990), leading to limitations of its use as a feed ingredient. Gossypol is available in either bound gossypol (BG) or free gossypol (FG) form, the bound form being non-toxic and of little significance, since it is unavailable and passes through the gastrointestinal tract unabsorbed (Tanksley, 1990). FG binds with protein (amino group of lysine) and hinders its availability to animals (Mahmood *et al*., 2011). FG of CSM has anti-nutritional properties (Romano and Scheffler, 2008); it affects growth (Wan *et al*., 2018) and causes infertility in fish (Liu *et al*., 2020). Therefore, lowering the effect of gossypol from CSM is necessary to improve the quality of protein for the fish.

Solid-state fermentation (SSF) of agro-industrial residues has become a suitable pre-treatment that could allow their use as biologically active secondary metabolites, especially as animal feed (Singhania *et al*., 2009). SSF is defined as the fermentation involving solids in the absence (or near absence) of free water; however, the substrate must hold enough moisture to maintain the microorganism's growth and metabolism. (Pandey *et al*., 1995 and 2000). In recent years SSF has gained importance due to its simple design, reduced energy requirements and minimum wastewater discharge (Manzanares *et al*., 2012; Andreaus *et al*., 2016; Meghavarnam and Janakiraman, 2017). Microbial degradation of the residues improves the substrate value as animal feed (Pandey, 2003) by increasing the probiotics content in the feed (Dawood and Koshio, 2020). SSF of agro-industrial by-products and plant ingredients reduces these products' crude fibre content and increases the nutrients' bio-availability (Onyimba *et al*., 2015; Meshram *et al*., 2018). The present work was conducted to study the utilisation of the CSM as an alternative feed ingredient by SSF to reduce the effect of the anti-nutritional factor gossypol and nutrient enrichment with *S. cerevisiae*.

MATERIALS AND METHODS

Cottonseed meal

Commercial cottonseed meal (CSM) was finely ground in a pulveriser and sieved to get a uniform particle size for solid-state fermentation.

Inoculum preparation

Commercial baker's yeast (*Saccharomyces cerevisiae*) was used to ferment CSM. The baker's yeast was activated on potato dextrose agar (PDA, Himedia) and the culture was maintained at 4°C. Spores from six-day-old cultures were grown at room temperature (28°C) to use as inoculum for SSF of CSM (Costa *et al*., 1998).

Solid-state fermentation (SSF)

SSF was conducted in a customised tray fermenter (Viesturs *et al*., 1987) with 2 kg dried (moisture<10%) and powdered cotton seed meal. 20 ml of water was sprayed at each tray to adjust the final moisture content of the fermentation mixture by about 50%. The mixed substrate was then sterilised in an autoclave at 121°C for 15 min. Trays were inoculated with 60 mg of *S. cerevisiae* and thoroughly mixed using a sterile glass rod. The mixture was allowed to ferment for 48 h at room temperature. The variations in pH, temperature and moisture monitored the fermentation process. The standardisation of the SSF of CSM was carried out until complete fermentation was achieved. After fermentation, the fermented substrate was partially sun-dried for 12 h to obtain a homogenous material (Hassaan *et al*., 2015). The dried substrate was ground and kept in the refrigerator.

Evaluation of anti-nutritional components

Gossypol

Before and after fermentation, the anti-nutritional factors of free and bond gossypol in the CSM were analysed at Central Institute for Research on Cotton Technology (ICAR), Matunga, Mumbai, India.

Phytic acid

The phytic acid content of CSM was estimated by using the spectrophotometric procedure (Gao *et al*., 2007). The assay comprises of addition of 10 ml, 3.5% HCl to 0.5 g sample followed by shaking for one h at 200 rpm (ORBITEK shaker; Scigenics, India). Then this sample extract was centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, USA) at 1600 g for 10 min and the supernatant was collected and mixed with 1 ml, wade reagent (0.03% $FeCl_3.6H_2O$ + 0.3% sulphosalicylic acid) and centrifuged at 1600 g for 10 min. The absorbance of the collected supernatant was recorded at 500 nm using a UV-visible spectrophotometer (Thermo Scientific, USA) and a blank with each sample was run.

Total tannin

The concentration of tannin in the CSM and FCSM was determined by following by Folin-Denis method (Schanderi, 1970). The crude extract (0.2 ml) was diluted with 8.3 ml of distilled water and then mixed with 0.5 ml of Folin-Denis

reagent. The reaction mixture was alkalinised by adding 1 ml of 15% (w/v) sodium carbonate solution and kept in the dark for 30 min at room temperature. The absorbance of the solution was read at 700 nm using a spectrophotometer (Shimadzu UV-1800) and the concentration of tannin in the extracts was determined using pure tannic acid (MERCK, India) as standard.

Amino acid profile analysis

The amino acid profile was carried out using 5 g dry powder of CSM and FSFM of feed with HPLC, LACHROM L-7000 ATOZ Pharmaceuticals PVT.LTD., Chennai, Tamil Nadu. The amino acid profile was done with the ChromNAV software system from JASCO-HPLC analysis.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 25.0 (IBM Corp.) was used to test the differences between various treatments by one-way analysis of variance (ANOVA). Duncan's multiple range test ($p<0.05$) was used to find the significant difference between treatments.

RESULTS AND DISCUSSION

Effect of fermentation on anti-nutritional factors present in CSM

Cottonseed meal utilisation in mono-gastric animals is generally limited. In the present study, fermentation of CSM with commercial baker's yeast (*S. cerevisiae*) has resulted in a significant decrease in the anti-nutritional factors ($p<0.05$) shown in Table 1. The results showed that the gossypol (%) content in CSM was reduced from 0.28% to 0.21% by SSF. Feeding diets containing gossypol causes adverse effects, such as growth depression and intestinal and other internal organ abnormalities in feed animals (Berardi and Goldblatt, 1980). Further, the gossypol in the pigment glands of the cottonseed is released during the mechanical process. It reacts with the amino groups of lysine, rendering its non-availability to the fish (Jackson *et al*., 1982). Tang *et al*. (2012) reported the fermentation of CSM with *Bacillus subtilis* BJ-1 had reduced the gossypol content from 0.82 to 0.21 g/kg. A similar finding showed decreased gossypol level from 90 to 30 mg/kg when fermented with *Candida utilis* (Xiong *et al*., 2016). Microbes or microbial enzymes growing during SSF utilise or bind the undesirable anti-nutrients like gossypol, reducing their availability in the free form. The optimum temperature and incubation period of CSM by yeast during fermentation are responsible for the biodegradation of gossypol in CSM. Similar observations at 30°C and 24 to 48 h fermentation have shown the detoxification of gossypol in CSM (Zhang *et al*., 2007; Khalaf *et al*., 2008; Zhang *et al*., 2022).

Oilseeds contain 3-6% phytic acid (Graf, 1983). They exhibit their anti-nutritional property by binding phosphorous and other essential nutrients, thereby decreasing their availability in feed for most monogastric animals, including fish (Canibe *et al*., 1999). In the present study, fermentation of CSM with *S. cerevisiae* resulted in a significant decrease ($p<0.05$) in the phytate activity (Table 1). The phytic acid content was reported as 2.55 and 1.05 mg/kg in CSM and FCSM, respectively and a 58.8% reduction was observed. Apparently, SSF of CSM by *S. cerevisiae* could reduce the phytic acid. The decrease in phytic acid was mediated through phytate degrading yeast phytase, preventing the formation of protein-phytate complexes during the SSF process, making nutrients and minerals bio-available (Hirabayashi *et al*., 1998). In corroboration to our finding, *S. cerevisiae* fermentation effectively removed phytic acid in Mutuo plant tubers (*Icacina mannii*), a West African tropical plant (Antai and Nkwelang, 1998). *S. cerevisiae* also decreased phytic acid in de-oiled soybean meal after SSF (Hassaan *et al*., 2015). Similar reports revealed that fermentation of black gram seed meal with *Bacillus* sp. (Ramachandran and Ray, 2007), rice bran with *S. cerevisiae* reduced its phytic acid content (Geetha *et al*., 2015) and gossypol reduction of CSM with *Bacillus coagulans* (Zhang *et al*., 2022).

Tannins, like gossypol, are a diverse polyphenolic compound associated with toxic and anti-nutritional effects, including reduced feed intake and growth and impaired nutrient absorption (Butler *et al*., 1986). Several researchers reported the toxicity of tannin and interference with the digestive enzymes in fish (Krogdahl, 1989; Mukhopadhyay and Ray, 1999) and higher animals (Reddy and Pierson, 1994). Tannins have been found to interfere with digestion by displaying anti-trypsin and anti-amylase activity (Helsper *et al*., 1993). Tannin also inhibits the protein and dry matter digestibility by impending the protease and forming indigestible complexes that might lead to growth retardation (Krogdahl, 1989; Joye, 2019). Tannins also can be complex with vitamin B_{12} (Liener, 1980). Reduction in polyphenol compounds like tannins during the SSF might be due to microbial fermentation of phenolic oxidase (Tajoddin *et al*., 2014; Tian *et al*., 2019). Similar findings of fermentation by lactic acid bacteria showed a reduction in the tannin content of sesame seed meal from 20 to 10 g kg^{-1} was noticed by Mukhopadhyay and Ray (1999) and tannin degrading fungal

Table 1: Effect of solid-state fermentation of CSM and thier anti-nutritional factors.

Ingredients	Gossypol (mg/100 g)	Phytic acid (mg/100 g)	Total Tannin (mg/100 g)
CSM	0.28%	2.55 ± 0.07^{b}	2.66 ± 0.19^{b}
FCSM	0.21%	1.05 ± 0.05^{a}	0.29 ± 0.12^{a}
p-value		<0.05	<0.05

All values are Mean±SE (n=3). Values with different superscripts in the same column differ significantly ($p< 0.05$). CSM - Cottonseed meal; FCSM - Fermented Cottonseed meal.

Table 2: Amino acid profile of CSM and FCSM after 48 h fermentation with *Saccharomyces cerevisiae*.

Essential amino acids	CSM	FCSM	% Increase (↓)/Decrease (↑)
Amino acid (mg/100 g)			
Arginine	14.01	15.51	10.7 ↑
Histidine	6.50	6.61	1.7 ↑
Isoleucine	12.40	13.27	7.0 ↑
Leucine	22.28	21.63	3.0 ↓
Lysine	14.81	16.49	11.3 ↑
Phenylalanine	13.10	12.92	1.3 ↓
Methionine	2.61	2.98	14.2 ↑
Threonine	6.69	8.02	19.9 ↑
Tryptophan	1.31	1.45	10.7 ↑
Valine	4.91	4.16	15.3 ↓
Non-essential amino acids			
Alanine	7.90	7.90	25.3 ↓
Glycine	9.06	10.62	17.2 ↑
Aspartic acid	25.31	26.83	6.0 ↑
Glutamic acid	32.72	29.86	8.7 ↓
Serine	8.68	9.79	12.8 ↑
Asparagine	15.2	16.4	7.9 ↑

CSM - Cottonseed meal; FCSM - Fermented cottonseed meal arrow (↑↓) indicates % Increase/Decrease of amino acids of FCSM in comparsion with that of CSM.

enzymes during the fermentation process (Jacqueline and Visser, 1996). In the present study, fermentation of CSM with brewery's yeast significantly reduced ($p<0.05$) tannin activity, as shown in Table 1. A decrease of 89.1% in the tannin activity of CSM was recorded following solid-state fermentation with *S. cerevisiae*.

Amino acid content is also one of the significant factors in determining the quality of feeds. The requirements for amino acids in animals are well defined in various sets of recommendations such as those of NRC (1993). Amino acids requirements vary depending on the species and age of animals (Agbo, 2008). Fermentation of CSM with *S. cerevisiae* increased the lysine and methionine content (11.3% and 14.2%, respectively) of FCSM after 48 h of fermentation. Other essential amino acids in FCSM, like arginine, isoleucine and threonine, also increased compared to CSM. However, some essential amino acids like leucine, phenylalanine and valine decreased after the fermentation of CSM (Table 2). Microbial fermentation with brewery yeast and reduction of phytic acid might be shown an increased level of essential amino acids in the FCSM. Gossypol binds with the epsilon group of amino acids, primarily lysine, possibly arginine and cysteine, of proteins during heating in oil extraction and makes these amino acids unavailable to the animals (Fernandez *et al*., 1995).

CONCLUSION

Enzymatic and fermentative treatment with *S. cerevisiae* has enhanced the nutritional value of CSM are being explored in terms of cost, high product yield and efficient recovery. Solid-state fermentation of CSM has been demonstrated as a promising method since biodegradation of gossypol occurs during the fermentation process. It can reduce other anti-nutrients like phytic acid and total tannin. The nutritionally enriched with enzymes, proteins and other active substances like essential nutrients and amino acids in the CSM. SSF of the Agro-industrial waste can improve protein quality, increase essential nutrients like lysine and methionine and be an eco-friendly process to utilise the non-conventional feed ingredients as the best alternative protein supplement for non-ruminants.

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