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Reduction of gossypol in cottonseed meal using halophilic archaeal fermentation for enhanced feed safety and nutritional value

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Abstract

This study investigated the capacity of halophilic archaea fermentation to detoxify gossypol, a poisonous polyphenolic compound present in cottonseed meal, a major protein source in animal feed. The objective of the study was to investigate the ability of the fermentation process of using halophilic archaeal fermentation to decrease the level of gossypol in cottonseed meal as well as to increase the protein content of the diets. Halophilic microorganisms are able to survive in harsh environmental conditions, offer a potential answer for difficult industrial operations. Cottonseed meal contains high quantities of gossypol, was subjected to fermentation with the halophilic archaeon *Halorubrum ezzemoulense* in order to reduce gossypol levels and enhance protein content. The study entailed the fermentation of cottonseed meal with *H. ezzemoulense*, and the amounts of gossypol in the feeds before and after fermentation were assessed using HPLC. Results showed that there was significant ($p<0.05$) reduction (5.59±0.17 mg/kg) in the gossypol level after microbial fermentation compared to the control which was (139.03±7.17 mg/kg). The protein and lipid content of substrate increased significantly ($p<0.05$). These analyses revealed modifications in the nutritional values as a result of the fermentation process. The findings indicated a substantial decrease in gossypol levels, coupled with a remarkable rise in protein content. This novel technology not only tackled the drawbacks linked to cottonseed meal but also highlighted the capacity of halophilic archaea fermentation as a sustainable and efficient technique for enhancing the nutritional value of animal feed. Further research could focus on optimizing fermentation, exploring scale-up possibilities, and evaluating broader implications for the livestock industry.

Keywords: Cottonseed meal, Fermentation, Halophiles, Archaea, Gossypol

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INTRODUCTION

Cottonseed meal (CSM) is an important feed source in animal husbandry due to its high protein and other nutritional components. However, gossypol, a polyphenol pigment found in high concentrations in CSM and toxic to animals, limits the amount of CSM used in feed (Kaya et al., 1995; Umur et al., 2019). By inhibiting the activity of pepsinogen, pepsin and trypsin in the gastrointestinal tract, free gossypol reduces the digestibility of protein and binds iron in the diet (Devanaboyina et al., 2007). The poultry's reproductive performance was compromised when the diet contained an excessive amount of free gossypol (Zeng and Peng, 2013). The variation in poultry tolerance to cottonseed meal, the age and species of broilers. For this reason, many studies have aimed at reducing the amount of free gossypol in CSM through fermentation and to improve the nutrient profile as another output of this process (Zhang et al., 2006; Zhang et al., 2007).

Halophilic microorganisms are microorganisms that can live in salty environments. For this reason, there is no risk of contamination by other microorganisms in processes where halophilic microorganisms are used. Since

halophilic microorganisms are microorganisms that can withstand extreme conditions such as high salinity, temperature and UV, their application areas in industrial processes are also quite wide (Kesbiç et al., 2023).

Several studies has shown that the amount of free gossypol in CSM subjected to fermentation with different microorganisms decreases and especially the protein ratio increases (Sun et al., 2015). During fermentation, microorganisms utilize gossypol as a carbon source, metabolizing it into less toxic compounds while enhancing the nutritional value of the substrate (Zhang et al., 2006; Zhang et al., 2007). Contamination is one of the most important risks that can negatively affect the fermentation process. Contamination of feed plants by unwanted microorganisms during the fermentation process means that the process is not carried out correctly and as a result, the product is spoiled (Queiroz et al. 2018). In studies on the fermentation of CSM, bacteria (Wang et al., 2012), fungi (Zhang et al., 2006) and yeasts (Zhang et al., 2007) were used in the fermentation process. In order to prevent contamination and to perform fully controlled microbial management, the substrate is autoclaved before microorganism inoculation in CSM fermentation studies (Zhang et al., 2007), but this process means high fixed investment and operating costs in industrial applications. In this study, the microorganism used for fermentation is a halophilic microorganism which are known to possess specialized enzymatic pathways which could facilitate the breakdown of gossypol (Le Borgne et al., 2008). The potential of using gossypol as a carbon source not only opens up new possibilities for its detoxification but also enhances the nutritional profile of CSM, making it a viable feed source. Given the high salinity environments where halophilic microorganisms thrive, these strains could offer a unique solution to reducing contamination risks and eliminating the need for autoclaving in fermentation processes, providing both economic and practical benefits for large-scale applications. In addition, no published study was found in the literature search on the fermentation of CSM in halophilic microorganisms. The study is original in this respect and is thought to be both a source of inspiration for new studies and an incentive for industrial applications.

MATERIALS AND METHODS

Supply of Cottonseed Meal

The cottonseed was supplied from a local producer and cottonseed meal was obtained in a cold press oil machine in order to ensure that the meal did not undergo any processing.

Culture Conditions of Halophilic Archaea

In the study, a halophilic archaeon *Halorubrum ezzemoulense* DSM 19316 (Genbank accession number: AM048786) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), which is stored at -20^{0}C in glycerol-added media, was used for fermentation. The microorganism was passaged into MAM JCM 168 medium and incubated at 39^{0}C for 3-7 days to have a fresh culture (Kesbiç and Gültepe, 2022).

Fermentation Process

A basal substrate containing cottonseed meal and wheat germ (3:2) was prepared. For the fermentation process, 100 g of substrate was inoculated with 10^8 concentration microorganism culture prepared with the salt-containing part of MAM JCM 168 medium (Trisodium citrate, $MgSO_4.7H_2O$, NaCl, KCl) in a way that it was moistened by 80 %. Fermentation continued at 39^{0}C for 21 days, and was kept at -20^{0}C to determine free gossypol level and the moisture, lipid, ash and protein percent of the product before fermentation and on the 21st day of fermentation.

Determination of Protein Amount of Cottonseed Meal

The Kjeldahl method was used to determine the protein amount of the meal (AOAC, 1975) and the analysis was performed in 3 replicates. Protein analysis were performed before and after the meal is subjected to fermentation and the change in the percentage protein amount of fermentation was calculated with the obtained data.

Determination of Free Gossypol Amount of Cottonseed Meal

The dried sample was weighed and extracted with acetonitrile (20 g/ 100 mL), according to the method of Ricci et al. (2015). The gossypol values of the cottonseed meal before and after fermentation and the effect of fermentation on gossypol were determined by High Performance Liquid Chromatography (HPLC, Shimadzu LC 20-A Prominence, Japan) with PDA detector (SPD-M20A) at 380 nm, and Agilent C18 (5 μm; 4.6 x 150 mm) column. The mobile phase A was acetonitrile (100 %), and mobile phase B was 0.3 % methanoic acid at the flow rate of 1.0 mL/min (Cheng et al., 2018).

Moisture Analysis

Before analysis, samples were weighed and placed in tared aluminum foil containers and dried in an oven at 50^{0}C until their weight remained constant.

Lipid Analysis

1 gram of dry sample was weighed for lipid analysis. After, the samples were kept in methanol-chloroform mixture in capped test tubes and filtered. They were taken into the first weighed flasks and lipid extraction was

done in the evaporator. Afterwards, the flasks placed in the desiccator were weighed after reaching a constant weight. The amount of crude oil was calculated using the following formula (Folch et al., 1957).

% Crude Oil Amount = Weight change of the volumetric flask (g) / Sample weight (g) x 100

Ash Analysis

For ash analysis, a homogeneous 0.5 g of sample was taken and placed in porcelain crucibles that have been previously tared. Then, the crucibles were burnt in the incineration furnace at 525^{0}C for 12 hours. The ash content of the samples were calculated according to the weight change of the crucibles according to the method of (AOAC, 1998). Formula is stated below:

% Raw Ash Content = Weight change of porcelain crucible (g) / Sample weight (g) x 100

Imaging by Scanning Electron Microscope

The strain was imaged by the scanning electron microscope (SEM, FEI, Quanta FEG 250, United States) before inoculating the substrate and after 21 day of fermentation on CSM (Kesbiç and Gültepe, 2022).

Statistical Analysis

Data was tested using one-way variance analysis with a statistical package program. Differences between values was tested using the Tukey multiple range test and a significant level of 0.05 was accepted as an indicator of the difference.

RESULTS AND DISCUSSION

Nutritional and gossypol content of experimental groups is presented in Table 1. According to the results, after 21 day fermentation with a halophilic archaeon *Halorubrum ezzemoulense* DSM 19316, the gossypol level of cottonseed meal were significantly decreased ($p<0.05$). While the gossypol level was 139.03±7.17 mg/kg in the control sample, it was reduced to 5.59±0.17 mg/kg after microbial fermentatiton. In addition, the protein and lipid content of CSM substrate were increased from 17.48±0.06 to 23.10±0.62 %, and from 10.68±0.16 to 13.65±0.26 %, respectively. The amount of ash in the fermentation group was found to be significantly higher than the control groups due to the salt content added for both sanitation and the growth of the target microorganism.

Table 1. Nutritional and gossypol content of experimental groups.

	Control	Storage control	Halophilic fermentation	p value
Protein (%)	17.48±0.06^b	15.18±0.05^c	23.10±0.62^a	<0.001
Lipid (%)	10.68±0.16^b	10.75±0.30^b	13.65±0.26^a	<0.001
Ash (%)	4.67±0.09^b	4.68±0.12^b	19.49±0.16^a	<0.001
Gossypol (mg/kg)	139.03±7.17^a	52.67±1.53^b	5.59±0.17^c	<0.001

Figure 1 shows the calibration curve of gossypol standards. Calibration points were prepared as 1, 5, 10, 20, 50 mg/kg. (r^2=0.999).

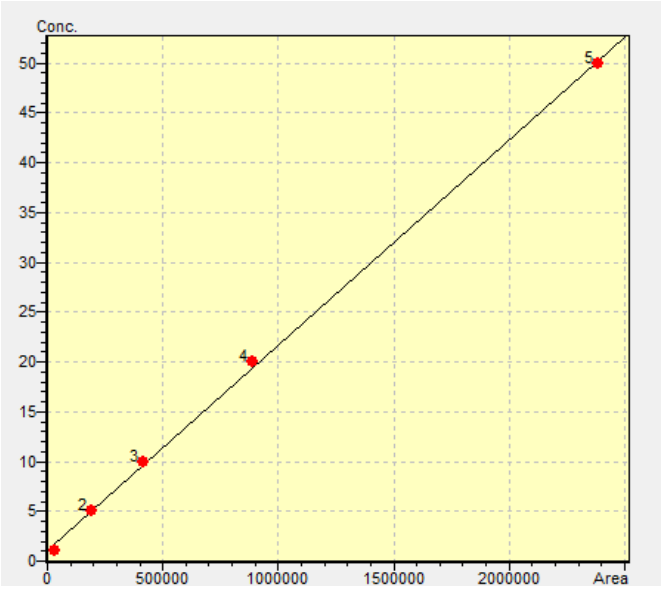

Figure 1. The linear calibration curve of gossypol standards.

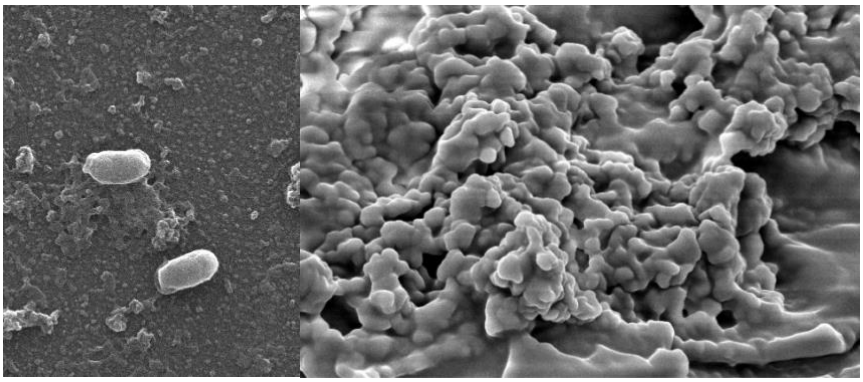

Figure 2. a) Images of *Halorubrum ezzemoulense*, b) after 21 day fermentation, *H. ezzemoulense* colonies on the CSM substrate.

The most important factor restricting the use of plant resources as a nutrient, in animal nutrition is the antinutrient contents of plants (Soetan and Oyewole, 2009). With the development of technology, the unwanted content of nutrients is being eliminated. However, disposal of the separated content adds additional costs to the process. For these reasons, fermentation is a long-standing method for conserving, improving and reducing antinutrient content of plant sources. (Jeyakumar and Lawrence, 2022).

The primary function of fermentation used as a feed technology is to extend the shelf life of the product and make it available for consumption. Its secondary importance is to improve the nutritional profile of the source. The most common use of the fermentation process in plant sources is silage production. In this process, wild fermentation is used, that is, the product is taken into a closed environment, an anaerobic atmosphere is provided and the plant is subjected to fermentation (Carvalho et al., 2014).

Another version of this process is the fermentation of plants with inoculants. In this case, the substrate should be sanitized to protect it from undesirable microorganisms. This leads to additional costs for the fermentation process. In our research, we tested the nutritional performance of silage of cottonseed meal, which is a common source of protein in livestock, using a halophilic archaea species. Our findings showed that gossypol, the most important antinutritional factor in cottonseeds, decreased by 96 % after 21 days of fermentation. This ratio is consistent with the boundaries of national feed declarations and international authorities in the field of animal feeding. According to the study of Zhang et al. (2006), fermentation of CSM with selected fungus species. The result of this study supports the findings of Zhang et al. (2006). This study achieved gossypol inhibition with a higher performance.

The plant's nutrient profile may change as a result of the fermentation of plant sources. During the fermentation process, microorganisms metabolize the vegetable source content and thus reproduce. As a result, a decrease in the content of cellulose, gossypol, etc. in the plant may result in a proportional increase in nutrient content, such as protein and fat. The findings of our current research support this statement. After 21 days of fermentation, an inverse ratio of gossypol and protein-fat content was observed in the cottonseed meal. Cottonseeds are among the most frequently used sources, in animal feeding. However, due to the toxicity caused by the gossypol content, it can be included in rations in a limited proportion. Therefore, cottonseed meal should be depurated before being used as a ration. The easiest method used in this cleansing process was to heat the substrate. In this case, the gossypol will deteriorate. However, as seen in our study, there are many advantages in purifying gossypol by microbial fermentation. Gossypol may decrease when the temperature is applied to the cottonseed meal, while denaturation-related protein may be decreased. In addition, heat-induced processes can cause the oil in the meal to oxidize. Xu et al. (2022) reported that heating decreased the protein in cottonseed meal (2022). Another study found that high-temperature treatment triggered fat oxidation in the meal (Waheed et al., 2004). In our study, the protein and fat ratio of the group fermented with *Halorubrum ezzemoulense* showed a significant ($p<0.05$) increase while the gossypol levels decreased.

This change is not believed to be due solely to the metabolism of the contents in the plant by the microorganism. It is known that plant resources, especially industrial waste, are used as a source of carbon and as a culture vessel to produce single-cell proteins. During fermentation, microorganisms produce proteins and lipids using carbon and nitrogen sources. This is an efficient way to support both the zero waste process and to generate added value from waste. As we did in our study, microorganisms reproduce using plant source content as a source of carbon. As a result, the protein and lipid content of the multiplied microorganisms contributed to the nutritional content in the total product. And the research we've done has some evidence that supports this

process. While the fat content of products stored at operating temperature did not change and the protein content showed reduction, fermentation group resulted in a significant ($p<0.05$) increase in these rates.

CONCLUSION

A halophilic archaeon, *H. ezzemoulense* is efficient microorganism for fermentation, which reduces the gossypol ratio of the cottonseed meal without requiring sanitation, and increases the protein-lipid ratio. Halophilic archaea are advantageous over other microorganisms because they can bypass the sanitation process in industrial applications. It is recommended to test the gossypol reduction and fermentation performance of different species in future.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The author has no conflict of interest to declare.

Author contribution

FIK designed the study and performed it. Wrote the paper and reviewed it.

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