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Gossypol Detoxification and Lysine Enrichment in Cottonseed Cake by Solid State Fermentation

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This study investigated the effect of mixed microbial cultures on gossypol and lysine levels in cottonseed cake (CSK) during solid state fermentation (SSF). The fermented CSK showed 71.4 % and 76.2 % free gossypol (FG) reduction and 77.0 % and 67.3 % bound gossypol (BG) reduction in *Pleurotus sajor-caju* + *Saccharomyces cerevisiae* and *Candida tropicalis* + *S. cerevisiae* treated samples respectively. The gossypol (FG and BG) reduction and lysine improvement in CSK was maximum after 36 h of incubation during SSF. The addition of crude enzyme extract containing protease activity of 500 and 400 U/g in CSK resulted in decrease of BG level. The High Performance Liquid Chromatography (HPLC) analysis of fermented CSK showed that lysine content was increased significantly from 0.92 to 1.36 in *P. sajor-caju* + *S. cerevisiae* and 1.54 in *C. tropicalis* + *S. cerevisiae*. The fungal count (cfu/g) was increased to 100 fold in fermented CSK. To our knowledge, this is the first report on SSF process for BG reduction and lysine enrichment in CSK.

Keywords: Bound Gossypol; Cottonseed cake; Free Gossypol; Lysine; Protease activity; Solid State Fermentation.

Researchers are looking for cheaper and alternative viable protein source for non-ruminants feed[1]. Cottonseed cake (CSK) is largely available particularly in well known cotton growing countries such as China, India, USA, Egypt etc.[2]. Currently, CSK is widely used as protein supplement for ruminant's animal. However, it's use in non-ruminants feed is highly limited due to the presence of gossypol and low in lysine content[3, 4]. The detoxification of gossypol and improvement of lysine content in CSK by SSF would bring as a potential protein supplement for non-ruminants[5].

Gossypol is a toxic polyphenolic compound present in cottonseed in two forms viz., free gossypol and bound gossypol. Feeding diets containing gossypol to animals would cause negative effects such as growth depression, reproductive disease and intestinal and other internal organ abnormalities[6]. The presence of even 100 ppm FG in layers diet causes egg yolk discolouration, mortality etc.[3]. The lysine is bound with gossypol in CSK thus become unavailable[7, 8]. The free epsilon group of lysine is bound with aldehyde group of gossypol forms schiff base[9]. The presence of BG decreases the digestibility of aminoacids during enzymatic digestion and thus reduces the nutritive value of cottonseed protein[10, 11].

Microbial fermentation is a viable method for detoxification of gossypol and improvement of nutritional properties of CSK in solid state especially on the improvement of amino-acids content in fermented CSK[2, 12, 13, 14]. The mixed fungal cultures *Saccharomyces cerevisiae* with

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Aspergillus niger and *Aspergillus niger* with *Aspergillus oryzae* were proved to be effective in gossypol detoxification and nutritive quality improvement in cottonseed meal[14, 15]. Though microbial fermentation for FG reduction was reported earlier, not much information is available on effect of microbial fermentation on BG level and lysine content in CSK. Considering the above, the present study was taken to evaluate the effect of SSF process on BG level and lysine content in CSK.

MATERIALS AND METHODS

Microorganisms and culture conditions

The test microorganisms used in this study viz., *Pleurotus sajor-caju* MTCC 1806, *Saccharomyces cerevisiae* MTCC 6933, *Candida tropicalis* MTCC 1406 were obtained from. Microbiology Lab, ICAR-CIRCOT. The cultures were grown in malt extract broth at 30°C under shaking conditions at 48 hours. The cultures were maintained in malt extract slants at 4°C.

Preparation of cottonseed cake substrate

Undecorticated cottonseed cake (CSK) sample was obtained from M/s star oil mills, Tirupur, India. The CSK was obtained in flakes were grounded using pulveriser and used for SSF.

Preparation of microbial culture for SSF

The cultures mentioned above were grown in malt extract broth (pH 5.5) for 48 hours at 30°C under shaking conditions (150 rpm). After growth, the biomass was separated using centrifugation at 10,000 rpm for 5 minutes and suspended in sterile water of the same quantity of broth culture at 4° C. The suspended biomass was used as inoculum for SSF.

Solid state fermentation (SSF)

Fifty g of grounded CSK was taken in 500 ml conical flasks and mixed with lactic acid @ 0.5 % (v/w) and incubated for 30 minutes. The disinfected CSK was inoculated with 20 % of microbial cultures (v/w) and incubated for 48 hours at room temperature (28 ± 2° C). Mixed microbial cultures were used for SSF. There were two treatments viz., *P. sajor-caju* + *S. cerevisiae* (each 10%) (T1) and *C. tropicalis* + *S. cerevisiae* (each 10%) (T2). A control was maintained in which no microbial treatment was given. The initial moisture content of 70% (including microbial cultures) was maintained using sterile water. Five g of samples were taken during solid state fermentation at 6 hours interval viz., 0, 6, 12, 18, 24, 30, 36, 42 and 48 respectively and analyzed for microbial count, free gossypol, total gossypol and lysine content as described below.

Sample preparation and analysis

The wet sample was used for estimation of microbial count using standard serial dilution technique. The samples for other analysis were subjected for drying at 70° C for 24 hours. The processed samples were analyzed for free and total gossypol[16] and lysine content[17]. The bound gossypol (BG) was calculated from difference between the free and total gossypol. The percentage reduction of BG and FG was calculated using the formula [{(Initial value -Final value)/

Table 1. Effect of protease on lysine, free gossypol and bound gossypol in CSK

Protease (U/g)	*P. sajor-caju* + *S. cerevisiae*			*C. tropicalis* + *S. cerevisiae*		
	Lysine (%)	Free Gossypol (%)	Bound Gossypol (%)	Lysine (%)	Free Gossypol (%)	Bound Gossypol (%)
0 (control)	0.39	0.22^{B}	1.13^{A}	0.39	0.22^{B}	1.14^{A}
100	0.40	0.22^{B}	1.13^{A}	0.39	0.22^{B}	1.14^{A}
200	0.40	0.22^{B}	1.13^{A}	0.40	0.21^{B}	1.14^{A}
300	0.40	0.21^{B}	1.11^{A}	0.39	0.22^{B}	1.14^{A}
400	0.40	0.30^{A}	1.06^{B}	0.41	0.32^{A}	1.03^{B}
500	0.41	0.30^{A}	1.05^{B}	0.40	0.32^{A}	1.04^{B}
CD (P = 0.05)	NS	0.069	0.035	NS	0.069	0.035

Treatment values followed by same alphabet do not differ significantly at P = 0.05. Values are the means of three different experiments. CD: Critical Difference; NS: Non Significant

Table 2. Microbial count (colony forming units/g) during solid state fermentation of CSK

Treatment	0 hr			12 hr			24 hr			36 hr			48 hr		
	F	B	A	F	B	A	F	B	A	F	B	A	F	B	A
Cake fermented with *Psc* + *Sc*	$5.1x10^6$	$2.1x10^2$	0	$8.3x10^6$	$7.4x10^2$	0	$4.0x10^7$	$4.6x10^2$	0	$7.7x10^7$	$7.6x10^2$	0	$4.0x10^8$	$5.2x10^3$	0
Cake fermented with *Ct* + *Sc*	$5.8x10^6$	$3.7x10^2$	0	$8.4x10^6$	$6.7x10^2$	0	$5.8x10^7$	$7.1x10^3$	0	$8.6x10^7$	$6.6x10^3$	0	$4.9x10^8$	$4.2x10^3$	0
Control	2.4x10	$4.1x10^3$	2.1x10	$7.8x10^1$	$7.1x10^3$	2.6x10	$5.8x10^2$	$3.6x10^3$	3.1x10	$7.5x10^2$	$6.5x10^3$	4.3x10	$3.1x10^3$	$4.2x10^4$	$2.1x10^1$

F: Fungi; B: Bacteria; A: Actinomycetes; P.sc – *P. sajor-caju*; C. t – *C. tropicalis*; S. c- *S. cerevisiae*

Initial value} × 100].

Estimation of protease activity

One g of fresh fermented sample of different intervals as mentioned earlier was taken in 0.1% Tween 80 (10 ml) and extraction was done at shaking conditions for two hours. The enzyme extract (supernatant) was separated by centrifugation at 10,000 rpm for 5 minutes. The protease activity in the supernatant was determined by Universal protease activity assay by taking casein as a substrate as described by Sigma Aldrich and expressed in U/g.

Effect of protease on FG, BG and lysine levels in CSK

The extraction of protease from 24 h old fermented CSK (50 g) was done as described above and subjected for concentration using lyophilisation. The concentrated protease extract (500 U/ml) was added in different concentrations such as (500, 400, 300, 200 and 100 U/g) to CSK treated with lactic acid 0.5%. The initial moisture content maintained was 70%. The protease treated CSK was incubated and the samples were taken after two hours for analysis such as FG, BG and lysine content.

Amino acid analysis

The amino acid profile of CSK (fermented and control) was determined as per AOAC 994 0.12 with HPLC fluorescence using OPA derivitization[18].

Scanning electron microscope (SEM) analysis

Morphology of fungal cells in 48 h old fermented CSK fresh sample was examined by an SEM. The fermented sample was mounted on the SEM stub with double-sided adhesive tape then sputter coated with gold or palladium using SPI-Module TM sputter coater (SPI Supplies Division of Structure Probe, Inc.). The sample surface was imaged using a Philips® XL30 scanning microscope operated at 10 kV with a tilt angle of 45 .

Statistical analysis

Data of study analysed in the completely randomized design (CRD) using one way analysis of variance (ANOVA) (WASP.1; ICAR research complex, Goa). Three replications were kept for each experiment. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

The CSK samples were taken at different intervals during solid state fermentation and analyzed for FG, BG and lysine contents. The fresh sample was used for microbial count estimation. The results showed that the FG (%) content in fermented CSK reduced from 0.21 to 0.06 in *P. sajor-caju* + *S. cerevisiae* and 0.05 in case of *C. tropicalis* + *S. cerevisiae* which corresponds to 71.4 and 76.2 % respectively at 48 h of incubation (Fig. 1). The BG (%) was reduced from 1.13 to 0.26 in *P. sajor-caju* + *S. cerevisiae* and 0.37 in *C. tropicalis* + *S. cerevisiae* which corresponds to 77.0 and 67.3% respectively (Fig. 1). At 48 h of incubation, the lysine content (%) was increased (0.38 to 0.76) in *P. sajor-caju* + *S. cerevisiae* and (0.38 to 0.66) in *C. tropicalis* + *S. cerevisiae* treated samples respectively (Fig. 2). The significant decrease in gossypol (FG and BG) and increase in lysine content was started at 18 h of incubation and reached maximum after 36 h of incubation (Fig. 1 &2).

The protease activity was higher at 24 h of incubation which corresponds to 93.5 U/g and 88 U/g in *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* respectively (Fig. 3). A decline in activity was observed after 36 h of incubation. The experiment on the effect of protease activity on BG and lysine levels showed that the addition of crude protease of 500 and 400 U/g in CSK results in change in FG and BG levels (Table 1). The addition of crude protease (500 U/g) to CSK results in increase in FG (0.22 to 0.30) and (0.22 to 0.32) and reduction of BG (1.13 to 1.05) and (1.14 to 1.04) in *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* treated samples respectively (Table 1). However, the addition of protease at these concentrations did not have any change in lysine level in CSK.

The microbial count (bacteria, fungi and actinomycetes) (cfu/g of CSK) at different incubation time was determined by serial dilution technique. The results showed that in control (CSK treated with lactic acid alone), the microbial count observed was bacteria (4.2×10^3), fungi (24) and actinomycetes (21) at 0 h of incubation while at 48 h, population estimated was, bacteria (4.2×10^4), fungi (3.1×10^3) and actinomycetes (21) (Table 2). In the treated CSK, the microbial load observed was 5.1×10^6 and 5.8×10^6 (fungi), 2.1×10^2 and 3.7×10^2 (bacteria) in *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* respectively at 0 h of incubation. While at 48 h, the fungal count estimated was 4.0×10^8 and 4.9×10^8 cfu/g, which corresponds to hundred fold (10^2) increase in fungal

Table 3. Amino acid profile of fermented CSK

Amino acid (g/100g)	Control	*P. sajor-caju* + *S. cerevisiae*	*C. tropicalis* + *S. cerevisiae*	CD (P= 0.05)
Cystine	4.53^{C}	5.12^{A}	4.70^{B}	0.09
Aspartate	0.76^{B}	0.78^{B}	0.89^{A}	0.09
Threonine	1.99^{B}	2.08^{B}	2.37^{A}	0.14
Serine	0.72^{C}	0.84^{B}	0.98^{A}	0.09
Glutamate	4.49^{A}	3.98^{B}	4.70^{A}	0.23
Glycine	0.72^{B}	0.79^{B}	0.91^{A}	0.09
Alanine	0.83^{B}	0.83^{B}	1.03^{A}	0.14
Valine	0.89	0.84	0.94	NS
Methionine	0.59	0.52	0.57	NS
Leucine + Isoleucine	2.35	2.40	2.37	NS
Tyrosine	1.89^{A}	2.05^{A}	1.27^{B}	0.18
Phenylalanine	1.04^{B}	1.54^{A}	1.16^{B}	0.15
Lysine	0.92^{C}	1.36^{B}	1.54^{A}	0.18
Histidine	0.46^{B}	0.59^{A}	0.64^{A}	0.09
Arginine	2.41^{B}	2.77^{A}	2.56^{AB}	0.23

Treatment values followed by same alphabet do not differ significantly at P = 0.05. Values are the means of three different experiments.CD: Critical Difference; NS: Non Significant

count (Table 2). The SEM analysis showed the dense presence of round globular yeast cells in *C. tropicalis* + *S. cerevisiae* treated CSK samples (Fig. 4).

The amino acid analysis of fermented CSK using HPLC method revealed that there is an increase in profile of aminoacids contents. The lysine content was increased significantly from 0.92 to 1.36 in *P. sajor-caju* + *S. cerevisiae* and 1.54 in *C. tropicalis* + *S. cerevisiae* treated samples respectively. The increase in other aminoacids observed were arginine, cystine, phenyl alanine etc. (Table 3).

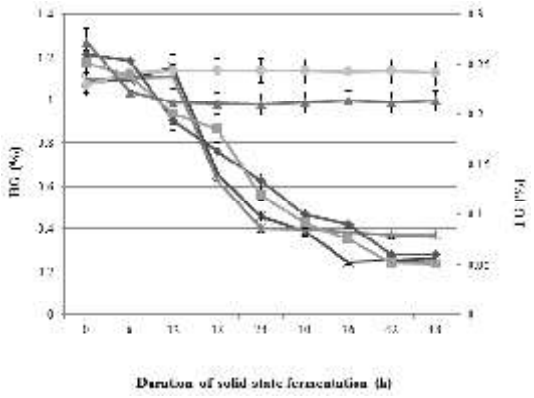

Fig. 1. Effect of solid state fermentation on Free Gossypol (FG) and Bound Gossypol (BG) levels in CSK. (• Control (BG), ▲ Control (FG), × *P. sajor-caju* + *S. cerevisiae* (BG), ♦ *P. sajor-caju* + *S. cerevisiae* (FG), × *C. tropicalis* + *S. cerevisiae* (BG), ■ *C. tropicalis* + *S. cerevisiae* (FG))

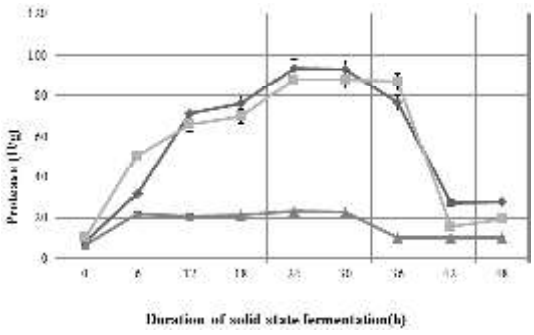

Fig. 3. Protease activity during solid state fermentation of CSK. (♦ *P. sajor-caju* + *S. cerevisiae*, ■ *C. tropicalis* + *S. cerevisiae*, ▲ Control)

DISCUSSION

The previous experiments on screening of microbial strains for gossypol detoxification in CSK showed that the mixed culture, *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* showed maximum detoxification of FG in CSK[19]. The present study was aimed to evaluate the effect of SSF on BG and lysine content in CSK. The analysis of samples at different intervals showed that the FG and BG reduction occurs from 18 h of incubation and recorded maximum after 36 h of incubation (Fig. 1). The results are in accordance with previous reports on FG reduction in cottonseed meal by SSF [2, 20, 21].

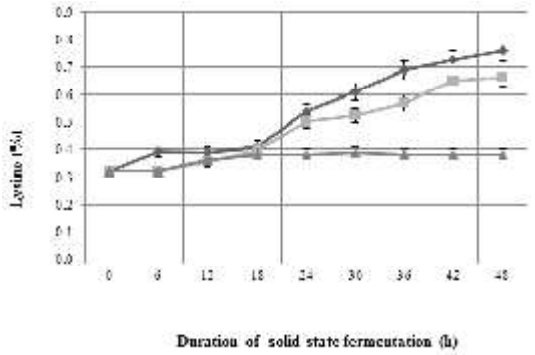

Fig. 2. Effect of solid state fermentation on lysine content in CSK. (♦ *P. sajor-caju* + *S. cerevisiae*, ■ *C. tropicalis* + *S. cerevisiae*, ▲ Control)

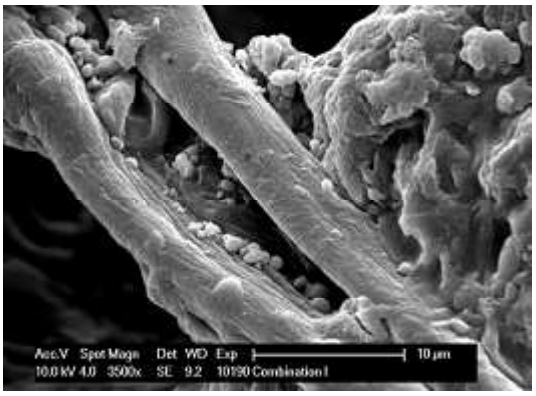

Fig. 4. Presence of yeast cells (white globule like) in *C. tropicalis* + *S. cerevisiae* fermented CSK

During the fermentation, the increase in lysine content was observed along with increase in incubation period. The analysis at different intervals showed that the increase in lysine content was observed after 18 h (Fig. 2). The SSF is one of the viable tools to enrich the amino-acids content in agro-residues[22, 23]. In a similar study, SSF of cottonseed meal improved the lysine content from 0.52 % to 0.68%[13]. The olive mill waste along with molasses fermented by *Paecilomyces variotii* showed lysine enrichment from 0 to 0.27 %[24]. In another study, cottonseed meal treated with microbial cultures showed increase in crude protein from 23.8 to 29.0% and lysine content from 1.04 to 1.16 %[12].

In our study, protease activity was estimated at different intervals during SSF and the results showed that the maximum activity was observed during the incubation period of 24 h (Fig. 3). The addition of protease (500 and 400 U/g) in CSK resulted in decrease in BG and increase in FG (Table 1). Thus the results suggests that release of FG by proteases is responsible for BG reduction in CSK. Khandeparkar et al. (1981)[25] reported the relationship between the release of FG and protease activity in *B. subtilis* inoculated medium containing cottonseed meal. The addition of proteases is generally practiced in animal feed industries to increase the digestibility of protein[26].

In this study, lactic acid @ 0.5 % was used as disinfectant only and hence the presence of certain microflora was observed in control (disinfected CSK) (Table 2). Lactic acid has been known for disinfectant and also as additive in animal feed to improve the palatability[27]. The increase in fungal count of 100 times in fermented CSK was observed at 48 h of incubation. The similar observation of increased microbial count was observed in SSF of palm kernel cake by *Rhizopus oryzae* Me01[28]. The dense presence of round globular yeast cells was observed in SEM analysis (Fig. 4). Thus, the results suggest the increase in lysine might be due to increase in microbial count in fermented CSK. The amino acids content analysis by HPLC showed that lysine content was increased from 0.92 to 1.36 and 1.54 in *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* treated samples which is ever higher than previous reports on SSF for lysine improvement in cottonseed meal (Table 3).

CONCLUSIONS

The present study revealed that mixed fungal cultures, *P. sajor-caju* + *S. cerevisiae* and *C. tropicalis* + *S. cerevisiae* reduced gossypol level (FG and BG) and increased the lysine content in CSK during SSF. The BG reduction in fermented CSK might be due to release of proteases by fungal cultures while the increase in lysine content corresponds to increase in fungal biomass during SSF. Thus the fermented CSK developed by SSF process would be a viable protein supplement for non-ruminants. In continuation, the mechanism of action of protease on gossypol-lysine complex may be studied.

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