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Applications of Solid State Fermentation for Value Addition of Cottonseed Deoiled Cake

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Abstract: The objective of this study was to improve the nutritional quality of cottonseed deoiled cake (CSDOC) necessary for animal feeding in a cost effective approach as a competitor to soyabean doc. CSDOC was undergone fermentation with different microbial strains like *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pseudomonas*, *Rhodosporium*, *Lactobacillus* out of which *Saccharomyces cerevisiae* had shown good results when grown on molasses medium for detoxification of CSDOC and to enhance its nutritive qualities like protein 48.14%, moisture 13.2%, lignin 42.3%, lysine 0.084% (g lysine/100g protein) fiber 25%, total gossypol levels range between 2000-3000 (mg/kg) and free gossypol 800-1000 (mg/kg). After solid state fermentation with *Saccharomyces cerevisiae* under maintenance of optimal condition at temperature of 30°C, time 48hrs, complete anaerobic type of fermentation, moisture 47% maintenance, 800µl yeast two days incubated inoculums in its YPD medium shows nutritive parameters change with increase in protein content 65.2%, moisture 46.6%, lysine 2.34% (glysine/100g protein) and decrease in fiber content 17%, lignin 23%, total gossypol 597.8 (mg/kg), free gossypol 149.3 (mg/kg). HPLC gossypol analysis was performed for 17 different Bt and non-Bt cotton seed varities, revealing a result that Bt plant variety RCH 779 BG-II shows less amount of gossypol levels which is highly recommended to farmers. CSDOC fermented by *S. cerevisiae* could substantially improve its nutritional value to an extent.

Keywords: Nutritive characters, gossypol, Saccharomyces cerevisiae, solid state fermentation.

INTRODUCTION

Due to increase in demand and high costs of soybean meal, there is an immediate requirement to find an alternate protein supplement in poultry feed. Nutritional quality of cottonseed protein is on par with soybean protein. The potential of CSDOC protein for animal nutrition is limited by the presence of gossypol ($C_{30}H_{30}O_8$) a toxic polyphenolic pigment [1] which is naturally produced in seeds that deters insect pests. Feeding diets containing gossypol to animals would cause negative effects such as growth depression, reproductive diseases, intestinal and other internal organ abnormalities [1,3]. Gossypol is released out into free form from the glands due to high shear force applied during the process of extraction of oil from cottonseeds. Gossypol reacts with protein (€- amino group of lysine) and with alkyl and phenylamines to form bound gossypol which is not toxic as that of free form [4,5]. Gossypol content exits more in Non-Bt plants compared with Bt plants [6] Solid state fermentation is the growth of microorganisms under controlled conditions in the absence of free water for the production of desired products [7].

Low availability of water reduces the possibilities of contamination by bacteria and Saccharomyces cerevisiae, higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism [8]. The inoculation with spores facilitates its uniform dispersion through the medium solid state fermentation in most cases characterized with low energy requirements which may likely reduce the production cost [9]. A nutritive value of CSDOC is lowered by the presence of bound gossypol revealing that both free form and bounded form of gossypol are useless [10]. The objective of this work is to study the nutritive characteristics of CSDOC like protein, fiber, lignin, lysine, moisture content and optimise them using *Saccharomyces cerevisiae* by screening from *Bacillus subtilis*, *Pseudomonas, Rhodosporium, Lactobacillus* solid state fermentation so that gossypol free protein rich CSDOC could be obtained, which has an equal demand with soyabean doc in a cost effective approach. HPLC gossypol analysis was performed between Bt and Non-Bt varities to recommend gossypol free and nutritive rich cotton plants for farmers.

MATERIALS AND METHODS

Sample collection: The CSDOC used as a sample for the analysis were obtained from kallam agro & oil pvt. lmt Andrapradesh and from CICR(Central institute of cotton research) Maharastra Bt varities (JK durga Bt, fusion Bt express, RCH 779 BG-II, Sampada BG-II, Maha sangram BG-II, Commando BG-II, Jadeo ganga kavari BG-II, RCH2C BG-II, KCH 14K59 BG-II,Bullet KCH 707 BG-II, VICH-311 BG-II,PRCH703 BG-II,JKCH2245 BG-II. Non-Bt varities (RCH-735, JKCH2245, MRC6301).

Estimation of protein content: Method used for estimating protein was kjeldhal method (An industry service publication. Labconco) determination protein under nitrogen level by 3 different steps acid digestion, distillation and titration. To 2g of CSDOC sample 0.7g of copper sulphate and 15g of potassium sulphate and 40ml of concentration sulphuric acid and some glass beads were added, heat the flask for 1hr until a pale blue colour is observed now cool it to room temperature to which add 250ml of water; to this add 110ml of 45% NaOH. Distillation was performed from which 10ml of vapour was collected that was distillated of vapour was collected that was distillated. Titration was performed to this 10ml of sample with methyl red as indicator. Note volume of filtrate after distillation, base volume added into buret, Volume of colour obtained during titration. Moles of acid= molarity of acid* volume used in flask; Moles of base= molarity of base* volume used in flask; Moles of Nitrogen= moles of acid- moles of base; g of Nitrogen= moles of N *atomic mass; %protein= N*6.25.

Estimation of moisture content: Initial weight of the CSDOC was weighed before the moisture content was estimated [11] weight of petriplate was also noted. Now the sample was allowed to dry for overnight at 95°C. After the process of drying again weight of the sample was noted along with petriplate. %moisture = (wt. of dish with material before drying – wt. of dish with material after drying) / (wt. of dish with material before drying – wt. of empty dish) * 100.

Estimation of Lignin content: Weighed out 100mg of CSDOC in 150ml of erlenmayer flask to which added 3ml 72% H_2SO_4 and flask was sealed with Para film, incubated in water bath at 30°c for 30min [12-14]. This sample was diluted with 4% H_2SO_4 capped with the aluminium foil and autoclaved at 120°c for 1hr. Now a filter paper was weighed and sample which was autoclaved was filtered by using funnel. Dry filter paper in hot air oven for overnight at 90°c, later allowed paper to cool to room temperature and again its weight was taken into account. % of lignin = (initial wt. of filter paper – wt. of filter paper after filtering &drying in hot air oven)*100.

Estimation of fiber content: Weighed accurately 2.5-3g sample and transferred to a soxhlet extractor by using petroleum ether. Air dries the extracted sample and transfer to a dry 1lt conical flask. Transferred the whole of boiling acid to the flask containing the defatted material and immediately connect the flask with a water cooled reflux condenser & heat so that the contents of flask begin to boil within 1min,added few drops of octanol [15] after addition of sulphuric acid. Rotate the flask frequently & boil till 30min. Filter the sample through linen and wash with boiling water turning acid to litmus. Now wash the residue on the linen into the flask with 200ml of boiling NaOH solution. Now connect the flask to reflux condenser and boil for exactly 30min. After washing with water transfer to gooch crucible prepared with a thin layer of ignited asbestos now again wash with water & 15ml ethyl alcohol. Dry the gooch crucible along with its content at 105±2°c in an air oven until constant weight are achieved now cool and weight the contents and incinerate the contents of gooch crucible in muffle furnace until all carbonaceous matter is burnt. %Crude fiber= (wt. of gooch crucible and content before ashing – wt. of gooch crucible and content after ashing)/ wt. of sample in dry form *100

Estimation of Lysine content: To 2g of CSDOC 30ml of distillated water was added; since lysine is water soluble aminoacid it comes easily from sample dissolved in water. To this sample sonication was performed for 1hr followed by centrifugation collect supernant and added 15ml of 3% FDNB solution (FDNB in ethanol) and keep in shaker for 30min. Now transfer it to water bath at 95°c sample, perform strong agitation to prevent precipitation from this prepared sample collect 15ml and set p^H to 5.0 with 2M NaOH &add 10ml methanol [16, 19] 20µl of this sample was taken to perform HPLC analysis by setting up mobile phase of (35% methanol & 65% 0.01M sodium acetate p^H 4.5 with glacial acetic acid). Amount of lysine= (conc. of calibration curve * 8.33*10^-2)/ (%protein in sample * wt. of sample)

Estimation of gossypol content: Gossypol is a trepenoid aldehyde & exists in two different forms total &free form. Total gossypol is bounded with protein and free gossypol is readily available so estimation protocol varies for both the forms of gossypol [20-23]. Gossypol is detected by HPLC in gossypol acetic acid form [24, 25].

Estimation of total gossypol: To 1g of CSDOC, 25ml of complex reagent (2ml of 3-amino proponal + 10ml glacial acetic acid make up to 100ml with N, N dimethyl formamide) was added & placed in water bath at 100°c for 2hrs. After cooling filtration was performed from which 1ml of filtrate was collected to this 4ml of N, Ndimethyl formamide was added out of which 20µl of sample was taken for HPLC analysis with mobile phase of 80:20 (Acetonitril: 0.01M KH_2PO_4) [24, 26].

Estimation of free gossypol: To 1g of CSDOC, 50ml of 70% acetone and 10g of glass beads were added and placed in a rotator shaker for 1hr at 35°c. After shaking filtration was performed, 1ml of filtrate was collected and added 1mlof N, N dimethyl formamide & 1ml of complex reagent and placed in water bath for 100°c for 30min. After cooling it to room temperature add 3ml of N, N dimethyl formamide from which 20µl of sample was collected to perform HPLC analysis [24, 26]. Area of sample gossypol peak (y) >18lakh: X=(y+80500/202301)*dilution factor*volume left over. Area of sample gossypol peak (y) <18lakh: X=(y-16670/174402)*dilution factor*volume left over.

Inoculum preparation for solid state fermentation: 5g of Saccharomyces cerevisiae was activated in YPD(yeast extract, peptone, dextrose) medium [9], after growth kinetics was observed these yeast cells were transferred into 10%molasses medium and incubated for 24hr [27]. 20ml of this solution was sonicated allowing cells to disrupt, later centrifuge and pellet was collected. Yeast cells that releases extracellular enzymes were transferred on to cottonseed doc in a circular disc plates under strict anaerobic condition with supplement of N_2 gas to remove all excess CO_2 to perform solid state fermentation for 48hrs.

Statistical Analysis: The total gossypol, free gossypol, protein, lignin, lysine response used for statistical analysis was the mean± standard deviation (SD) in at least three independent experiments. All statistical analyses were carried out using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA).one way analysis of variance (ANOVA) was used to compare the significant level of variation in means between groups. Statistical values of $P<0.05$ were considered significant, which rejected the null hypothesis between groups.

RESULTS AND DISCUSSION

Value addition of CSDOC is observed through the process of application of solid state fermentation [27]. Improvement of nutritive characteristics is seen after doc is fermented by a extracellular enzymes released by strain [28]. Microbial strain selection is performed on the basis of reduction in gossypol levels **Table-1**.

Initially all these microbes were activated in their growth medium for 24hr later they were transferred on to molasses medium where some of the strains like *lactobacillus, Pseudomonas, Rhodosporium* could not grow due to insufficient supply of sugars and imbalance of p^H in molasses medium; *Saccharomyces cerevisiae and Bacillus subtilis* had shown their growth in the medium, **Table-2** even though *Bacillus subtilis* had shown reduction in gossypol levels with solid state fermentation in doc, it is not recommended because some of the extracellular enzymes released by bacterial strain might cause effect on ruminant intestine when this fermented doc is consumed [20]. Suitable microbial strain that shows reduction in gossypol levels is *Saccharomyces cerevisiae.* [9, 29]

Table-1: Microbial strain selection on the basis of gossypol reduction.

S. No	Microbial strain	Total gossypol (mg/kg)	Free gossypol (mg/kg)
1	Control (Doc)	3071.94	1004.45
2	*Rhodosporium*	2638.7	789.3
3	*Saccharomyces cerevisiae*	1246.06	123.5
4	*Pseudomonas*	3484.64	569.3
5	*Bacillus subtilis*	1975.8	235.6
6	*Lactobacillus*	1932.7	435.7

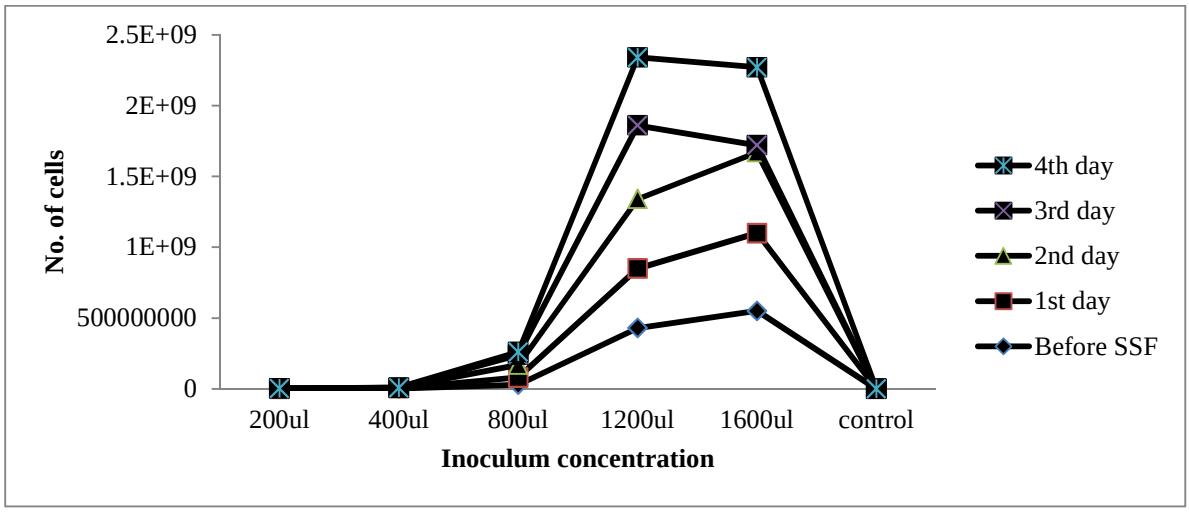

Figure 1: Growth kinetic of yeast at different inoculum concentration time as a factor.

Table-2: Selection of microbial strain between *Saccharomyces cerevisiae and Bacillus subtilis* in double recovery of gossypol.

Batch-I	**Strain**	**1st recovery**		**Sample**	**2nd recovery**	
		TG(mg/kg)	FG(mg/kg)		TG(mg/kg)	FG(mg/kg)
	Yeast	801.45	430.93	Yeast	337.10	129.89
	Bacillus	1604.37	295.18	Bacillus	396.79	23.5
	Control	2517.16	1129.8	control	151.67	-
Batch-II	Strain	1st recovery		Sample	2nd recovery	
		TG(mg/kg)	FG(mg/kg)		TG(mg/kg)	FG(mg/kg)
	Yeast	703.26	147.43	yeast	112.92	18.87
	Bacillus	1496.41	259.6	Bacillus	165.4	139.04
	control	2795.6	853.29	control	82.11	-

After selection of microbial strain suitable for degradation of gossypol its nutritive qualities has to be enhanced by application of solid state fermentation [28]. Parameters that has to be maintained during fermentation has a key role, there should be a continuous monitoring check on these parameters until a desired condition of product is reached [30]. Optimization of fermentation parameters is necessary for nutritive change in cottonseed deoiled cake [27, 31].

Table-3: Optimization of moisture content based on inoculums concentration supply before and after solid state fermentation.

S. cerevisiae concentration	**% moisture before solid state fermentation**	**%moisture after solid state fermentation**
200µl	22.2%	17.2%
400µl	39.8%	35.6%
800µl	52%	47%
1200µl	103.2%	103%
1600µl	109%	111%
Control	13.2%	13.2%

Faster growth rate of *S. Cerevisiae* on CSDOC during solid state fermentation **Figure 1** and also due to good smell of ethanol production characteristic, so it was fit for fermenting CSDOC to reduce gossypol levels and increase nutritive value [27].

Table-4: Type of fermentation suitable by *S. Cerevisiae* for reduction of gossypol levels. Effect of Total gossypol content in seed varities, which have shown statistically significant difference in the means were selected. One way annova: Between groups (F=4.798; P<0.05). Free gossypol content in seed varities, which have shown statistically significant difference in the means were selected by one way annova: Between groups (F=5.625; P<0.05)

%Moisture	Inoculums supply	No. of cell count/ml	Complete anerobic		Complete aerobic		Microaerofilic	
			TG(mg/kg)	FG(mg/kg)	TG(mg/kg)	FG(mg/kg)	TG(mg/kg)	FG(mg/kg)
17.2%	200µl	5*10^5	5916.4	516.1	2307.5	779.5	1574.5	293.7
35.6%	400µl	25*10^5	3056.6	834.6	3483.4	800.1	1039.8	200.8
47%	800µl	3*10^7	1835.2	125.3	4069.1	590.1	809.1	183.3
103.0%	1200µl	43*10^7	2098.6	90.3	2252.1	891.2	810.3	149.3
111%	1600µl	55*10^7	2437.5	463.3	3888.5	29.2	948.71	119.9
13.2%	control	6*10^3	7675.2	935.2	2975.6	1025.6	3020.5	565.4

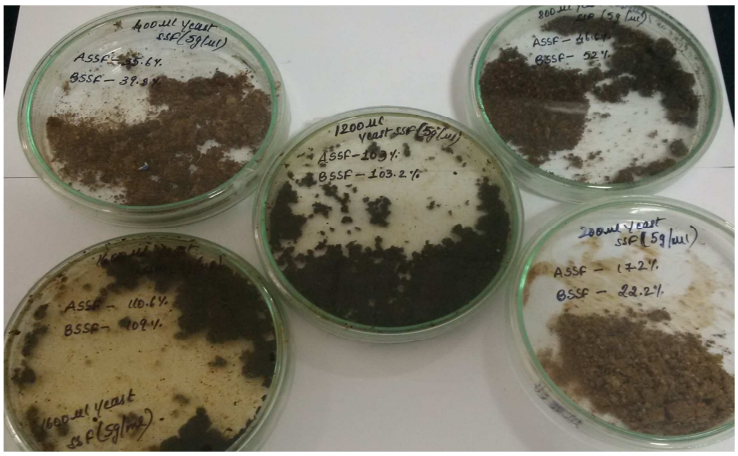

Figure 2: Solid state fermentation occurring in a circular disc (5g sample/ µl) at different inoculums concentrations

Parameters necessary to maintain during solid state fermentation is time required for fermentation necessary for reduction of both total gossypol and free gossypol was at 48hrs (**Table-6**). Inoculum concentration of *S. Cerevisiae* required for reduction of gossypol levels *is* 800µl depending on the cell count total gossypol found to be degraded upto 49% and free gossypol to 8.37% (**Table-5**). Type of fermentation required for *S. Cerevisiae* for major amount degradation of total gossypol 1835.2 (mg/kg) and free gossypol 125.3(mg/kg) at 800µl inoculum concentration in complete anaerobic condition were all the respiratory gases are removed by N_2 supply (**Table-4**). Percentage of moisture required for best reduction of gossypol is 47% (**Table-3**).

Table-5: Degradation/conversion of free gossypol/total gossypol at different inoculums concentrations.

S. Cerevisiae concentration	**Total gossypol (mg/kg)**			**Free gossypol(mg/kg)**		
	Control	S. Cerevisiae	% degradation	Control	S. Cerevisiae	%degradation
200µl	2556.7	1842.6	72%	1004.4	315.9	31%
400µl	2840.8	2089.5	73%	1200.2	330.7	27%
800µl	2912.4	1400.7	49%	1123.3	94.1	8.37%
1200µl	2084.2	1343.5	64%	1002	174.5	17.4%
1600µl	2178.4	1005.5	46%	116.9	140.2	12.5%
2000µl	2279.4	976.7	42%	1123.5	115.3	10.2%

Table-6: Fermentation time required for reduction of total gossypol and free gossypol by *S. Cerevisiae.*

Time	**Total gossypol(mg/kg)**						**Free gossypol(mg/kg)**					
	Batch-I			Batch-II			Batch-I			Batch-II		
	Control	400µl	800µl	Control	400µl	800µl	Control	400µl	800µl	Control	400µl	800µl
24hr	2556.7	2089.5	1450.7	2746.7	2718.3	2187.7	1004	330.7	94.1	1041.5	220.2	110.2
48hr	2556.8	2820.2	2459.9	2746.7	3235.5	2542.3	1004	26.7	167.2	1041.5	129.1	150.1
72hr	2556.7	1877.8	1878.9	2356.1	1793.4	1678.5	1004	383.8	180.2	1041.5	259.6	173.2

Initial characteristics of CSDOC were found to be with protein content of 48.14%, moisture content 13.2%, lignin content 42.3%, fiber content 25%, total gossypol ranges between 2000-3000 (mg/kg), free gossypol ranges between 800-1000 (mg/kg), lysine content of 0.084% (g lysine/100g protein). Change in nutritive characteristics in CSDOC seen after solid state fermentation with *S. Cerevisiae* found to be increase in protein content 65.2%, moisture 46.6%, lysine 2.34% (g lysine/100g protein) and decrease in fiber content 17%, lignin 23%, total gossypol 597.8 (mg/kg), free gossypol 149.3 (mg/kg). *S. Cerevisiae* shows growth on CSDOC by utilising supply of sugars in 10% molasses medium and other nutrients available on CSDOC.

Mechanism of Change in nutritive parameters is achieved by action of extracellular enzyme of *S. Cerevisiae* degrading cottonseed proteins which act as a solid substrate during fermentation. CSDOC on fermentation with *S. Cerevisiae* shows degradation in protein **(Figure 3)**, already available free gossypol now plays an ideal role by forming a bond with interaction with degraded proteins i.e FG-lysine converting gossypol into bounded from, form a toxic state to non-toxic state[31] Simultaneously we could observe decrease in fiber content, lignin content and gossypol content enhancing protein and lysine content. Analysis of gossypol content between Bt and non-bt varities was performed revealing less amount of both total gossypol and free gossypol **(Figure 5)** is seen in Bt varities.

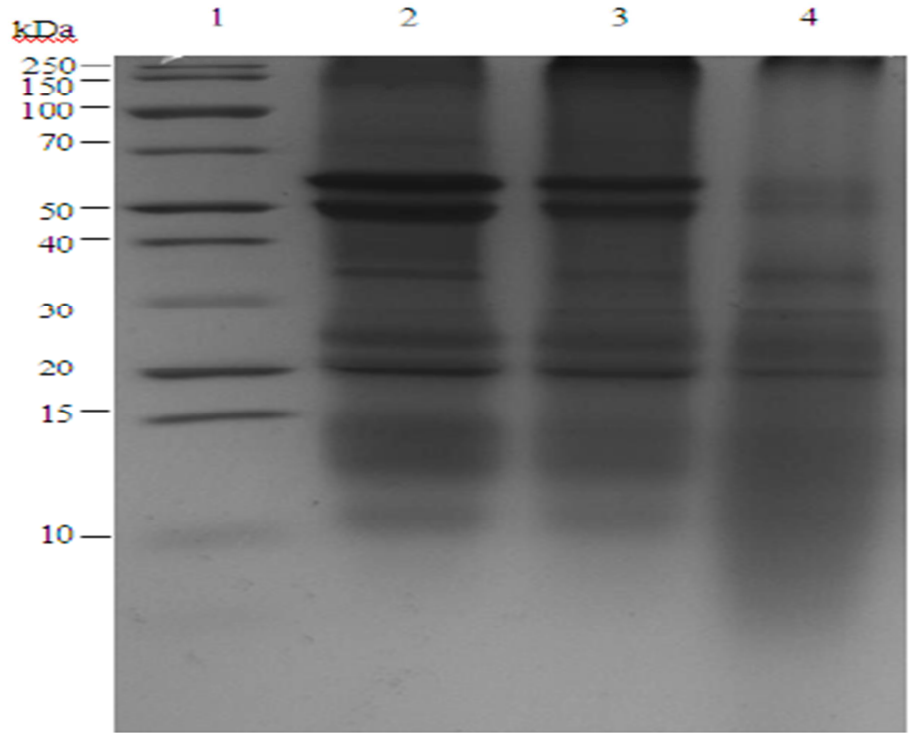

Figure 3: SDS-PAGE analysis of protein fractions of CSDOC Lane1: Molecular marker; Lane2: CSDOC; Lane3: Before SSF CSDOC; Lane 4: CSDOC fermented by *S. Cerevisiae.*

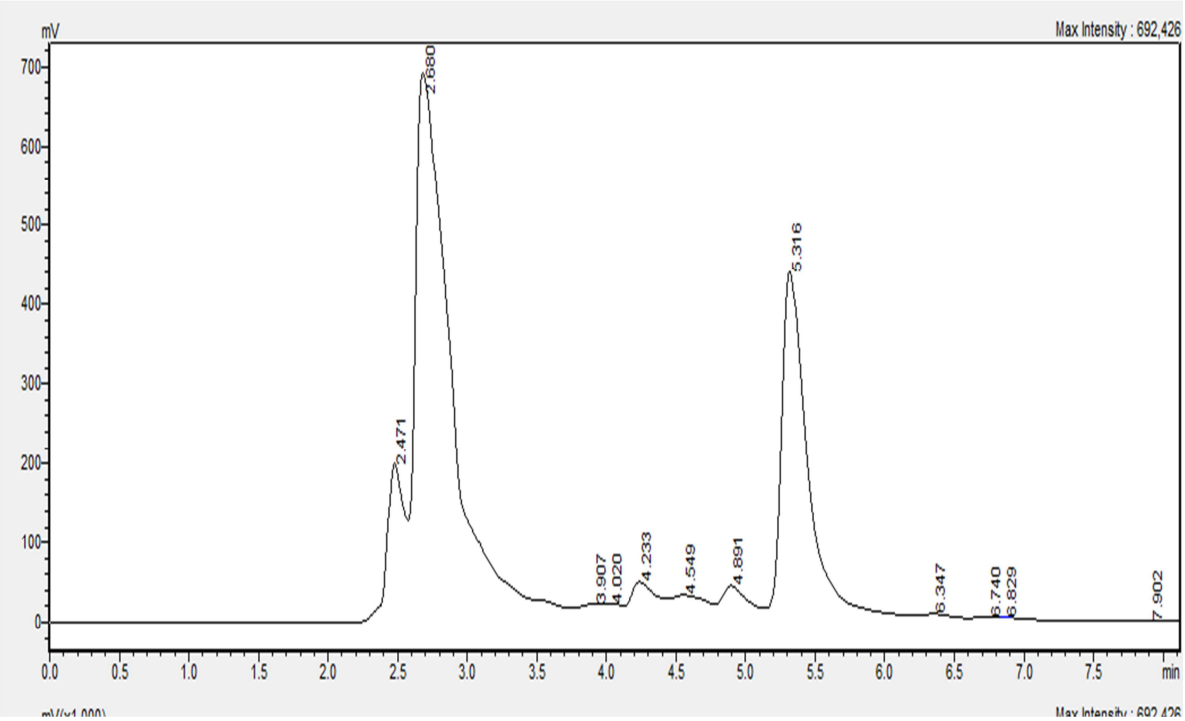

Figure 4: At maximum concentration standard gossypol peak is seen at retention time of 5.316min at a flow rate of 1ml/min.

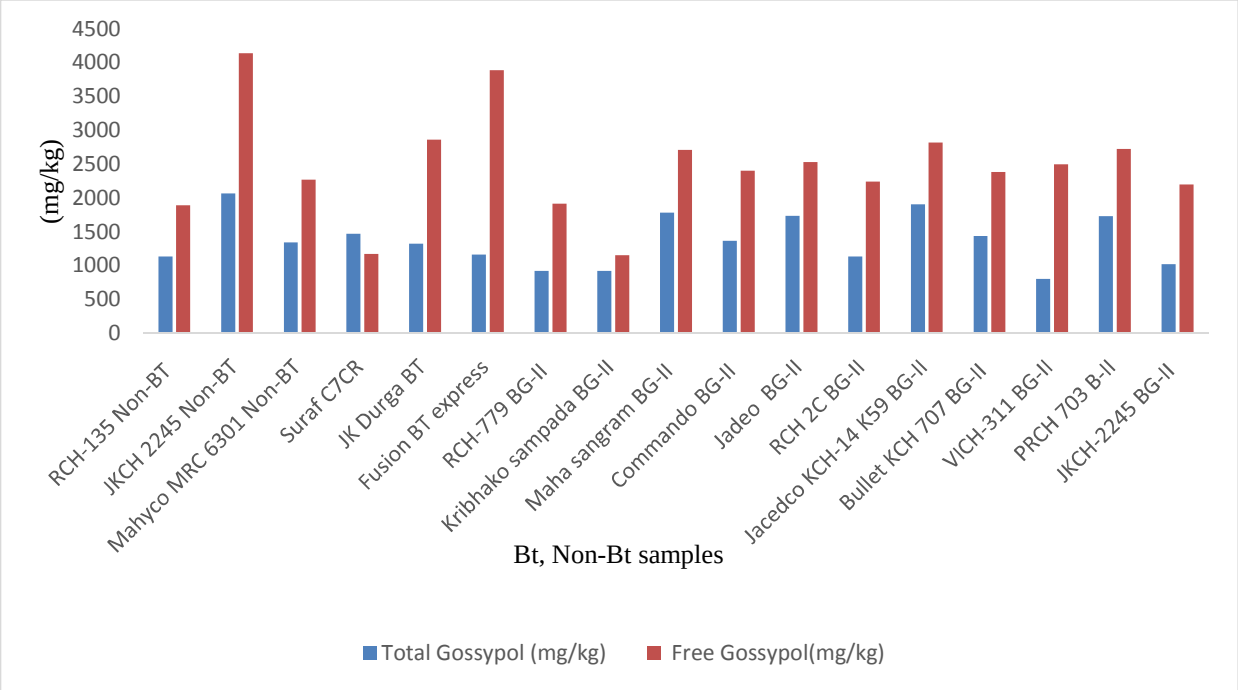

Figure 5: Distribution of Total gossypol (TG) content in different Cotton seed samples. Effect of Total gossypol content in seed varities, which have shown statistically significant difference in the means were selected. One way annova: Between groups ($F=38.95$; $P<0.05$). **:** Distribution of free gossypol in cottonseed varities. Effect of Total gossypol content in seed varities, which have shown statistically significant difference in the means were selected.One way annova: Between groups ($F=38.95$; $P<0.05$).

CONCLUSION

Solid state fermentation of cottonseed deoiled cake with *S. Cerevisiae* shows reduction of both total gossypol and free gossypol forms to an extent with enhancing protein content and lysine content with less fiber and lignin content in a cost effective approach giving a high demand with soyabean deoiled cake serving as an alternate protein rich feed in poultry animals.

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