

Optimization of Process Parameters for Gossypol Detoxification in Chemical Disinfected Cottonseed Cake by Mixed Fungal Culture during Solid State Fermentation

V Mageshwaran*, A Shaikh and A A Kathe

ICAR- Central Institute for Research on Cotton Technology, Matunga (East), Mumbai – 400019

Received 29 December 2016; revised 03 May 2017; accepted 5 August 2017

A solid state fermentation process was optimized for gossypol detoxification in cottonseed cake using mixed fungal culture, *Candida tropicalis* with *Saccharomyces cerevisiae*. The effect of fermentation conditions such as initial moisture content, inoculum level, incubation temperature and period were tested for gossypol reduction in chemical disinfected cottonseed cake. The results showed optimized parameters for gossypol detoxification in chemical disinfected cottonseed cake were 70% moisture content, 15% inoculum level, 30° C incubation temperature and 48 h incubation period. The detoxification rates of free gossypol and bound gossypol were 79.5 and 59.5 percentage respectively. The increase in crude protein (13.4 %) and decrease in crude fibre (11.4 %) was recorded in fermented cottonseed cake. This is the first report on solid state fermentation for bound gossypol and crude fibre reduction in cottonseed cake.

Keywords: Free Gossypol, Bound Gossypol, Crude Fibre, Crude Protein, Optimization, Solid State Fermentation

Introduction

Cottonseed cake (CSC) is widely used as feed for ruminant animals. However, CSC is not recommended for non-ruminants due to the presence of toxic compound called gossypol and higher fibre content¹. Gossypol is a polyphenolic binaphthyl dialdehyde, and yellow pigment present in entire cotton plant including its seed. Gossypol is present in two forms viz., free gossypol (FG) and bound gossypol (BG). The total gossypol constitutes FG and BG. FG is more toxic than BG. The BG is formed by reaction between gossypol and epsilon-amino groups from lysine and arginine and forms Schiff base². Feeding diets containing gossypol to animals would cause negative effects such as growth depression, reproductive disease and intestinal and other internal organ abnormalities³⁻⁶. The tolerance of FG in chicks varies widely (90 to 1000 mg kg⁻¹ diets)⁷. The detoxification of gossypol in cottonseed meal (CSM) through microbial fermentation is a promising method since unlike chemical methods, the biodegradation of gossypol occurs during fermentation process⁸. The fermented CSM usually contains some kinds of exoenzyme (secreted by microorganisms) such as cellulolytic enzyme, amylase, protease and lipolytic enzyme, vitamins and other active substances apart

from the detoxification of FG^{9,10}. Solid state fermentation (SSF) was used to produce industrial products including enzymes¹¹ and aminoacids¹² and is an attractive process due to its low capital investment and operating expenses¹³. The previous reports showed among twelve mixed culture combinations of seven strains, the maximum detoxification of gossypol and nutritive quality improvement of CSC was obtained in the culture combinations, *Pleurotus sajor-caju* with *S. cerevisiae* and *C. tropicalis* with *S. cerevisiae*¹⁴. The aim of the present paper is to optimize SSF process for FG and BG reduction in chemical disinfected (CD) CSC using mixed fungal culture, *C. tropicalis* with *S. cerevisiae*.

Materials and methods

Basal substrate

The undecorticated cottonseed cake was purchased from M/s Star oil mill, Tirupur (India). It was grounded and passed through 10 mm mesh size sieve and stored at room temperature (25 – 30°C) until used. The grounded CSC was used as basic fermentation medium. The moisture (v/w), FG, BG, crude protein (CP) and crude fibre (CF) contents in basal substrate were 10 %, 2200 mg/kg, 2100 mg/g, 20 % and 37 % respectively.

Microorganisms

The test microorganisms used in this study viz., *Candida tropicalis* MTCC 1406 and *Saccharomyces*

*Author for Correspondence
E-mail: mageshbioiari@gmail.com

Cerevisiae MTCC 6933 were obtained from Microbiology Lab, ICAR-Central Institute for Research on Cotton Technology, Mumbai. The cultures were grown in malt extract broth at 30° C under shaking conditions for 48 h and maintained in malt agar slants at 4° C.

Inoculum preparation

The cultures mentioned above were grown in malt extract (1X) (Himedia, India, Mumbai) liquid medium (pH 5.5) for 48 h at 30° C under shaking conditions (150 rpm). After growth, the biomass was separated by centrifugation at 10,000 rpm for 5 minutes, washed in sterile water for two times to remove the media residues. The pellet (biomass) was suspended in sterile water of the same quantity of broth culture and kept at 4° C until use. The inoculum of mixed fungal culture for SSF was prepared by mixing equal proportion of individual culture at the time of inoculation. The cell population maintained in inoculum was 10⁶ colony forming units/ml.

Screening of organic acids for chemical disinfection of CSC

The organic acids such as formic, propionic, citric and lactic acids (Fisher scientific, India, New Delhi) were added in five g of basal substrate taken in 100 ml of Erlenmeyer flask at the rate of 0.1, 0.2, 0.5 and 1.0 percentages respectively. The moisture content in the flask was maintained at 70% using sterile distilled water and kept for one hour at room temperature. The samples were drawn and tested for microbial count viz., total bacteria, fungi and actinomycetes by standard serial dilution technique using nutrient agar, martin's rose bengal agar and kennights and munaiers agar respectively. A control was maintained in each treatment in which organic acids were not added in the substrate.

Optimization of SSF in CD CSC

Five g of basal substrate was taken in 100 ml of Erlenmeyer flask, added with 0.5% lactic acid (v/w) along with required moisture content, mixed well and kept for one hour. The pH of initial substrate after lactic acid addition was 5.5. The SSF was performed with mixed fungal culture, *C. tropicalis* with *S. cerevisiae*. The effect of various parameters such as moisture content, inoculum level, incubation temperature and period were tested for gossypol detoxification in CD CSC. Triplicate flasks were set for each experimental variation. A control was maintained in which culture was not added.

Effect of initial moisture content

To understand the effect of initial moisture content of the substrate, various initial moisture contents (40%, 50%, 60%, 70% and 80%) were adjusted using sterile distilled water and fermentation was carried out. The inoculum level was 15% and fermentation was carried out for 48 h at 30°C. Since liquid inoculum was used, size of the inoculum was taken into account for fixing the initial moisture content. The optimum initial moisture content was fixed for subsequent experiments.

Effect of level of inoculum

The various inoculum levels (1%, 3%, 5%, 10%, 15%, 20% and 30%) were tested under optimum moisture content. The fermentation was carried out for 48 h at 30 °C. The optimum inoculum level was fixed for subsequent experiments.

Effect of incubation temperature

To study the effect of incubation temperature on gossypol reduction in CSC, the fermentation was carried out at various incubation temperatures (25, 30, 35 and 37°C) under optimum moisture content and inoculum level for 48 h. The optimum incubation temperature was fixed for subsequent experiments.

Effect of incubation period

Various incubation periods (24, 48, 72 and 96 h) were tested and the fermentation was carried out with other parameters kept at their optimum levels.

Sample processing

After fermentation, the flask containing fermented substrates was dried in an oven at 60°C for 24 h and weight loss was determined. Subsequently the samples were powdered for related analyses.

Related index assay

The moisture content was measured by drying the samples using hot air oven at 105 °C for 5h¹⁵ and the CP content assay was done by Kjeldahl's method¹⁵ using automatic nitrogen analyzer (model Kel plus, M/s Pelican Equipments, India). FG and total gossypol level was determined by the official method of the American Oil Chemist Society¹⁶. The difference between total gossypol and FG gave BG. The CF was determined by automatic fibre estimation system (model Fibra plus, M/s Pelican Equipments, India) based on Weende method¹⁷. The percent reduction in FG and BG were calculated using the formula, Gossypol reduction (%) = Gossypol content

in untreated (control) – Gossypol content in treated (sample)/ Gossypol content in untreated) \times 100. The increase and decrease of CP and CF contents were calculated from difference between the control and sample.

Statistical analysis

Data of study was analysed in the completely randomized design (CRD) using one way analysis of variance (ANOVA) (WASP.1; ICAR research complex, Goa). For all analysis, the differences were considered to be significant at $P < 0.05$.

Results and Discussion

Microbial fermentation of gossypol detoxification is the best alternative among different gossypol detoxification methods, since fermentation process enhances the nutritional value of CSM¹⁸. Previous work on SSF showed that fungal cultures *C. tropicalis*, *S. cerevisiae*, *A. niger* and *P. florida* greatly reduced the FG content in CSM¹⁹⁻²⁴. The mixed fungal cultures, *C. tropicalis* ZD-3 with *A. niger* ZD-8 and *S. cerevisiae* with *A. niger* had higher FG detoxification rate in CSM^{18,22}. In this study, the mixed fungal culture, *C. tropicalis* with *S. cerevisiae* was used for SSF. In any SSF, the heat treatment is the most common method used for sterilization of substrate. However, the huge infrastructure requirement for steam sterilization imposes SSF uneconomical particularly under scale-up conditions^{25,26}. Hence, in this study SSF was performed with CD CSC. For chemical disinfection, different organic acids such as formic, propionic, citric and lactic acids were screened for its effect on control of initial microflora in basal substrate. The results showed that there were no fungal growth in control (untreated) as well as in organic acids treated CSC. In general, the CSC treated with organic acids (0.5 or 1.0%) had significant reduction in bacterial and actinomycetes population ($P < 0.05$). The lactic acid (0.5 and 1.0 %) treated CSC

had 1.5 log reduction in numbers of bacteria and significant reduction in actinomycetes population (Table 1). Hence, lactic acid @ 0.5 % was chosen for chemical disinfection of CSC for its use in SSF. Organic acids are used as feed additive in animal feed to improve the palatability, nutrient utilization and feed conversion ratio²⁷. Broiler chicken fed diets supplemented with lactic acid (3%) had significant improvement in body weight gains and feed conversion ratio²⁸. In another study, lactic acid was proved to be more effective in reducing numbers of *L. monocytogenes* in fresh cut vegetables when exposed to 1% solutions for ten minutes²⁹. The initial pH of CD basal substrate was 5.5 and this had been kept constant in all optimization experiments. Invariably, previous reports showed that gossypol detoxification in CSM were found to be better at its natural pH (5.5 to 6.0)^{18,26}. The initial moisture content is one of the factors determining the efficiency of SSF. The results showed that the moisture content had positive effect on gossypol detoxification. The optimum moisture content for gossypol detoxification in SSF was found to be 70% in CD CSC fermented with fungal culture, *C. tropicalis* with *S. cerevisiae* ($P < 0.05$) (Figure 1a). The increase in moisture content substantially increase the solubility of nutrients in the substrate thereby effecting the growth of microorganisms however, the excess moisture may lead to reduce the oxygen transfer which limits the microbial growth³⁰⁻³². In a similar study, the optimum moisture content for gossypol detoxification in CSM was 50 to 55 %^{21, 26}. The difference in the results might be due to more absorption of moisture by high fibre content in CSC. The fibre content is higher in undecorticated CSC among various cottonseed extractions³³. Among different inoculum level tested (1, 3, 5, 10, 15, 20 and 30%), the addition of inoculum size of 15 % was found to be optimum in CD CSC fermented with

Table 1 — Effect of organic acids on initial microbial count (colony forming units/g) in cottonseed cake

Concentration (%)	Formic acid		Propionic acid		Citric acid		Lactic acid	
	B	A	B	A	B	A	B	A
0.1	17800 (4.2) ^a	340 (2.5) ^a	18800 (4.2) ^a	420 (2.6) ^a	18800 (4.3) ^a	350 (2.5) ^a	18900 (4.3) ^a	390 (2.6) ^a
0.2	16300 (4.2) ^a	350 (2.5) ^a	18200 (4.2) ^a	460 (2.6) ^a	18200 (4.3) ^a	230 (2.4) ^{ab}	18700 (4.3) ^a	320 (2.5) ^a
0.5	3500 (3.5) ^b	140 (2.1) ^b	18100 (4.2) ^a	450 (2.6) ^a	4360 (3.6) ^b	140 (2.1) ^b	750 (2.8) ^b	130 (2.1) ^b
1.0	980 (2.9) ^c	40 (1.5) ^c	3280 (3.5) ^b	130 (2.1) ^b	2290 (3.4) ^c	80 (1.9) ^c	540 (2.7) ^b	50 (1.7) ^c
Control (untreated)	19100 (4.3) ^a	400 (2.6) ^a	18900 (4.3) ^a	480 (2.6) ^a	18900 (4.3) ^a	350 (2.5) ^a	19200 (4.3) ^a	400 (2.6) ^a

B: Bacteria; A: Actinomycetes. Treatment values followed by same alphabet do not differ significantly ($P < 0.05$). Figures in parentheses are logarithmic transformed val

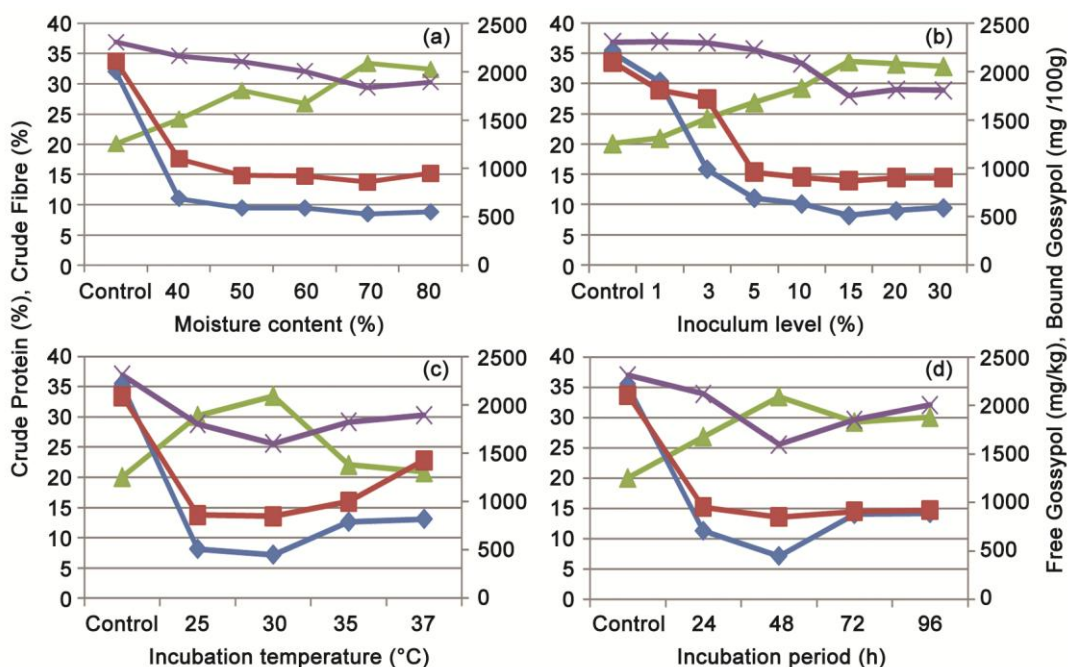


Fig.1 — Effect of fermentation conditions on gossypol detoxification by *C. tropicalis* with *S. cerevisiae* in chemical disinfected (CD) CSC. (a) Moisture content (b) Inoculum level (c) Incubation temperature (d) Incubation period. Crude protein (▲), Crude fibre (×), Free Gossypol (◆), Bound gossypol (■).

C. tropicalis with *S. cerevisiae* ($P < 0.05$) (Figure 1b). The results showed that with increase in inoculum up to certain level, the detoxification rate increases beyond which, not much change occurred. The results are in agreement with previous reports where 5 % inoculums level was found to be optimum for gossypol detoxification in CSM^{21,24,26}. The high protein level in the substrate accelerates the initial growth of microbes. The higher inoculum requirement for gossypol detoxification in CSC might be due to low protein content in CSC than CSM. The optimal temperature for gossypol detoxification by *C. tropicalis* with *S. cerevisiae* in CD CSC was 30°C ($P < 0.05$). The results are presented in Figure 1c. The results are in agreement with the previous reports where the optimum temperature for biodegradation of gossypol reported was 30°C^{20-22,26}. The optimal incubation period for detoxification of gossypol and other nutritive quality improvement was 48 h ($P < 0.05$) (Figure 1d). In a similar study, the optimum incubation period for detoxification of gossypol in CSM reported was 24 to 48 h^{20,21,23,24} and 60 to 72 h²⁶. The fermented CSC incubated for 48 hours had higher CP and low CF contents. Hence, under optimized conditions, the fermented CSC had 450 mg/kg FG, 850 mg/100g BG, 33.5% CP and

25.6 % CF. The US FDA limits the FG content in food products and ingredients to 450 mg/kg. While the protein advisory group of UN (FAO/WHO) has a guideline which limits FG to 600 mg/kg and BG to 1140 mg/100g³⁴. In the present study, fermented CSC meets the gossypol level as set by the standards. The SSF significantly increased CP and decreased CF contents in fermented CSC. In a similar study, the fermented CSM had significantly reduced fibre content^{18,35}. The SSF increased the protein content from 3 to 7% and improved aminoacids profile in CSM²⁰⁻²².

Conclusion

The use of CSC in non-ruminants feed is limited due to the presence of gossypol and high fibre content. A SSF process was optimized in which, the fermented CSC had significantly lower level of FG, BG and CF contents and higher CP compared to untreated. This study revealed that chemical disinfection of CSC with 0.5 % lactic acid would be a suitable alternative for heat sterilization of CSC thus saves energy and cost of SSF process for gossypol detoxification and nutritive quality improvement. The FG and BG levels in fermented CSC meets the international standard requirements which show its potential to be used as

safer non-ruminants feed. Further, the potential of this simple, economical and ecofriendly process for its industrial adoption may be explored.

Acknowledgement

Grateful to National Agricultural Science Fund (NASF), ICAR, New Delhi for financial support and thanks to Director, ICAR-CIRCOT for his moral support and guidance.

References

- Gadella I C N, Fonseca N B S, Oloris S C S, Melo M M & Blanco B S, Gossypol toxicity from cottonseed products, *The Sci World J*, DOI.10.1155/2014/231635.
- Kenar J A, Reaction chemistry of gossypol and its derivatives, *J Am Oil Chem Soc*, **83** (2006) 269-302.
- Fernandez S R, Aoyagi S, Han Y, Parsons C M & Baker D H, Limiting order of amino acids in corn and soybean meal for growth of the chick, *Poult Sci*, **73** (1994) 1887-1896.
- Berardi LC & Goldblatt L A, Gossypol, in Toxic constituents of plant food stuffs, **2nd edn**, edited by I E Liener, *Academic Press*, New York, (1980) 183 – 237.
- Francis G, Makkar H P S & Becker K, Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish, *Aquaculture*, **119** (2001) 197 – 227.
- Robinson P H, Getachew G, De Peters E J & Calhoun M C, Influence of variety and storage for up to 22 days on nutrient composition and gossypol level of Pima cottonseed (*Gossypium* spp), *Anim Feed Sci Technol*, **91** (2001) 149-156.
- Nagalakshmi D, Rama Rao S, Panda A & Sastry V, Cottonseed meal in poultry diets, *J Poult Sci*, **44** (2007) 119-134.
- Yang X, Guo J-L & Sun J-Y, Biodegradation of free-gossypol by a new fungus isolated from cotton planted soil, *Afr J Microbiol Res*, **5** (2011) 3066-3072.
- Shi A H, Zhang Y, Qu P, Yan J G & Xiao H J, Screening and breeding of highly-effected degrading cotton-phenol strains and study on detoxification technology and conditions, *Acta Microbiol Sin*, **38** (1998) 318-320.
- Wu X Y & Chen J X, The utilization of microbes to break down FG in cottonseed meal, *Sci Agric Sin*, **22**(1989) 82-86.
- Kalaiarasi K & Parvatham R, Optimization of process parameters for α - amylase production under solid state fermentation by *Aspergillus awamori* MTCC 9997, *J Sci Ind Res*, **74** (2015) 286 – 289.
- Anusree M, Nampoothiri K M & Pandey A, Sugarcane baggase as inert support for L-lysine production in solid state fermentation, *Indian J Biotechnol*, **14** (2015) 193-199.
- Deschamps F, Giuliano C, Asther M, Huet M C & Rooussos S, Cellulase production by *Trichoderma harzianum* in static and mixed solid state fermentation reactions under non-aseptic conditions, *Biotechnol Bioeng*, **27** (1985) 1385-1388.
- Shaikh A, Kathe A A & Mageshwaran V, Reduction of gossypol and increase in crude protein level of cottonseed cake using mixed culture fermentation, *KKU Res J*, **19** (supplement issue) (2014) 67-73.
- AOAC, *Official Methods of analysis*, **16th edn**, Association of Official Analytical Chemists, Washington, DC, (1999).
- AOCS, Free and total gossypol methods, *Official and tentative methods of the AOCS*, **4th edn**, American oil chemists society, Chicago, (1989).
- Tecator, Determination of crude fibre in some feed and food samples by using the fibertec system and weende method, Application no 01, Tecator AB box 70, S-26301, Hoganaes, Sweden, (1978).
- Atia A I & Rahim G A A, Detoxification treatments of free gossypol in cottonseed meal by microbial treatment of mixed cultures and biochemical evaluation on rabbits, *J Rad Res Appl Sci*, **2** (2009), 397-415.
- Rajarithnam S, Sashirekha M N & Bano Z, Biodegradation of gossypol by the white oyster mushroom, *Pleurotus florida*, during culturing on rice straw growth substrate supplemented with cotton seed powder, *World J Microbiol Biotechnol* **17** (2001) 221-227.
- Zhang W, Xu Z, Sun J & Yang X, Effect of selected fungi on the reduction of gossypol levels and nutritional value during solid substrate fermentation of cottonseed meal, *J Zhejiang Univ SCIENCE B*, **7**(2006 a) 690-695.
- Zhang W, Xu Z, Zhao S-H, Jiang J-F, Wang Y-B & Yan X-H, Optimization of process parameters for reduction of gossypol levels in cottonseed meal by *Candida tropicalis* ZD-3 during solid state fermentation, *Toxicon*, **48**(2006 b) 221-226.
- Zhang W-J, Xu Z- R, Sun J-Y & Yang X, A study on the reduction of gossypol levels by mixed culture solid state fermentation of cottonseed meal, *Asian-Aust J Anim Sci*, **19** (2006 c) 1314 -1321.
- Zhang W-J, Xu Z-R, Zhao S-H, Sun J-Y & Yang X, Development of a microbial fermentation process for detoxification of gossypol in cottonseed meal, *Animal Feed Sci Technol*, **135**(2007) 176 – 186.
- Khalaf M A & Meleigy S A, Reduction of free gossypol in cottonseed meal by microbial treatment, *Int J Agril Biol*, **10** (2008) 185-190.
- Wang X, Tang J-W, Yao X-H, Wu Y-F, Sun H & Xu Y-X, Effect of *Bacillus cereus* Br on bacterial community and gossypol content during fermentation in cottonseed meal, *Afr J Microbiol Res*, **6** (2012)6537-6544.
- Weng X-Y & Sun J-Y, Biodegradation of free gossypol by a new strain of *Candida tropicalis* under solid state fermentation: Effects of fermentation parameters, *Process Biochem*, **41**(2006) 1663 – 1668.
- Dibner J J & Buttin P, Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism, *J Appl Poultry Res*, **11** (2002) 453–463.
- Adil S, Banday T, Bhat G A, Mir M S & Rehman M, Effect of dietary supplementation of organic acids on performance, intestinal histomorphology, and serum biochemistry of broiler chicken, *Vet Med Intl*, DOI. 10.4061/2010/479485. 2010.
- Zhang S & Farber J M, The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. *Food Microbiol*, **13**(1996) 311-321.
- Zadrazil F & Brunnert H, Investigation of physical parameters important for the SSF of straw by white rot fungi, *Eur J Appl Microbiol Biotechnol*, **11** (1981) 183-188.
- Ohno A, Ano T & Shod M, Production of antifungal antibiotic, itruin in solid state fermentation by

- Bacillus subtilis* NB 22 using wheat bran as substrate, *Biotechnol Lett*, **14** (1992) 817-822.
- 32 Murthy M V R, Mohan E V S & Sadhukhan A K, Cyclosporin A production by *Tolypocladium inflatum* using solid state fermentation, *Process Biochem*, **34**(1999) 269-280.
- 33 Mageshwaran V, Shaikh A, Kambli N D, Kathe A A & Patil P G, Gossypol detoxification in various cottonseed extractions by fungal cultures during solid state fermentation, *Cotton Res J*, **5**(2014)132-136.
- 34 Rhee K C, Detoxification and deallergenation of oilseed meals, Proceedings of the World Conference on Oilseed Technology and Utilization (Ed Applewhite, T H) Budapest, Hungary, (1992).
- 35 Tang J W, Sun H, Yao X H, Wu Y F, Wang X, & Feng J, Effects of replacement of soybean meal by fermented cottonseed meal on growth performance, serum biochemical parameters and immune function of yellow-feathered broilers, *Asian-Aust J Anim Sci* **25** (2012) 393-400.