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DEGRADATION OF PHORBOL ESTERS IN *JATROPHA* SEED CAKE BY *PSEUDOMONAS AERUGINOSA* DS1

SHILPI AHLUWALIA[1], JAI GOPAL SHARMA[1*] AND PUSHPENDRA SINGH[2]

[1]Department of Biotechnology, Delhi Technological University, Delhi- 110042, India.
[2]Department of Mechanical Engineering, Delhi Technological University, Delhi- 110042, India.

ABSTRACT

Jatropha curcas is a perennial plant which is widely used as biofuel and is recognized for its medicinal properties. Different parts of *Jatropha* plant are used for the treatment of human and veterinary diseases. Biodiesel production from *Jatropha* seed oil results in large amount of residual seed cake which is rich in lignocellulosic compounds, minerals and proteins but presence of certain toxic compounds limits the use of this nutrient rich cake. Phorbol esters are the major toxic compounds found in *Jatropha curcas* seed cake that make it unsuitable for food and feed applications. Effective utilization of this seed cake necessitates the need of complete removal of its phorbol esters. In this study, we investigated the application of submerged fermentation (SMF) for the detoxification of phorbol esters. SMF was carried out with *Jatropha* seed cake at 37 ˚C, pH 7, 100 rpm for 9, 12 and 15 h using *Pseudomonas aeruginosa* DS1. Complete degradation of phorbol esters resulted when the seed cake was fermented for 15 h at 37 ˚C, pH 7, 100 rpm indicating the potential of this process to completely detoxify the *Jatropha* seed cake in a very short time.

KEYWORDS: Detoxification, *Jatropha curcas*, Phorbol Esters, Submerged Fermentation

JAI GOPAL SHARMA*

Department of Biotechnology, Delhi Technological University, Delhi- 110042, India.

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INTRODUCTION

Jatropha curcas the wonder plant is drought resistant which has the potential to grow in marginal/poor soils. It has achieved tremendous interest because of its high oil content[1] which can be used for the production of biodiesel. Biodiesel can be considered as a substitute for diesel fuel as it gives a similar performance without modifications in the engine. At the same time, biodiesel production from *Jatropha* seeds leads to the production of large amounts of residual seed cake whose disposal or utilization should be guarded[2] which may be helpful in reducing the cost of *Jatropha* biodiesel industry. The *Jatropha* deoiled seed cake is rich in nutrients, but the presence of some anti-nutrients/toxins restricts the use of seed cake as animal feed. Phorbol esters have been identified as the major toxicants in seed cake as they have carcinogenic and mutagenic properties.[3] Many cases of *Jatropha* poisoning have been reported[4-6], but most of them go undocumented as symptoms are not specific and more of like bacterial or protozoal gastroenteritis which includes mainly abdominal pain, vomiting and diarrhoea. Various methods have been reported in the literature[7] to detoxify *Jatropha* seed cake which includes physical, chemical and radiation treatments but these methods require a consortium of treatments which makes the process expensive and time-consuming. Biological detoxification despite of the fact an environmentally and inexpensive process[8] is still at developing stage, although few researchers have reported the use of fungi, and bacteria for the detoxification of *Jatropha* seed cake by solid state fermentation process. The use of fungi for detoxification presented inadequate results as the metabolites produced by fungus are not effective in detoxifying complex phorbol esters.[9] Anjali and Haresh[10] succeeded in total removal of phorbol esters by solid state fermentation (SSF) of *Jatropha* seed cake with white rot fungi, but it took a long time of 20 days. Bacterial cultures may reduce the detoxification time which can make the process rapid and economical. 97% reduction in phorbol ester content was observed in 9 days when SSF of seed cake was done with *Streptomyces fimicarius.*[11] In another study, phorbol- ester content was reduced to an undetectable level when the seed cake was fermented (SSF) with *Pseudomonas aeruginosa* PseA strain within nine days at 30 ˚C, pH 7 and 65% relative humidity.[12] There was 93% reduction in the phorbol ester content when SSF was carried out by *Bacillus smithii* in 3 days.[13] Detoxification of phorbol esters by submerged fermentation process could be a good option than SSF as it requires less time with the process well suited for organisms requiring higher moisture levels for growth. This work aimed at detoxification of *Jatropha* seed cake by SMF using *Pseudomonas* sp. *Pseudomonas* has been well investigated as a lipase and protease producer[14] which may help in the degradation of toxic phorbol esters. SMF using *Pseudomonas* has never been reported in the literature for the detoxification of *Jatropha* seed cake.

MATERIALS AND METHODS

Materials and culture

Deoiled *Jatropha curcas* seed cake was obtained from Biodiesel Plant, DTU Delhi, India and it was ground and stored in ziplock pouches at 4˚C. The culture media were procured from HiMedia Laboratories (Mumbai, India). Standard, i.e., phorbol-12-myristate-13-acetate (Sigma-Aldrich, USA), HPLC grade acetonitrile (Fischer Scientific), HPLC grade methanol (SD fine, India) were used as received from the manufacturer. Other chemicals/solvents were of analytical grade and procured from standard companies. Strain *Pseudomonas aeruginosa* DS1 isolated from soil was used for degradation of phorbol esters.

Preparation of inoculums

Culture was grown overnight in nutrient broth at 37 ˚C and 100 rpm, and 1% of this culture was used to inoculate the media containing (%) starch 2; KH_2PO_4 0.5, KNO_3 1.01, NH_4Cl 0.535, $MgSO_4.7H_2O$ 0.001, $CaCl_2.2H_2O$ 0.01 and $Na_2HPO.12H_2O$ 0.8. The incubation was done in a rotary shaker at 37 ˚C for 24h at 100rpm and was centrifuged at 10000rpm for 20 min at 4˚C for obtaining a cell-free supernatant.

Submerged fermentation of deoiled Jatropha seed cake

Seed cake was autoclaved for 1 h and 5 g of it was added to the cell-free supernatant in different flasks. The flasks were incubated in a rotary shaker at 37 ˚C, pH 7, 100 rpm for 9, 12 and 15 h and phorbol esters were extracted. Control flasks containing 5 g seed cake in minimal medium (KH_2PO_4 0.5%, KNO_3 1.01%, NH_4Cl 0.535%, $MgSO_4.7H_2O$ 0.001%, $CaCl_2.2H_2O$ 0.01% and $Na_2HPO.12H_2O$ 0.8%) were also run alongside under same conditions. All the experiments were done in triplicates.

Extraction of phorbol esters from seed cake

Phorbol esters were extracted with absolute methanol by the method prescribed by Xing *et al.*[11] Briefly, methanol was added to the seed cake in 7:1 ratio and sonicated for 15 min and filtrate was collected. The extraction process was carried out thrice, and the extracted fractions were pooled together. Methanol was removed by rotary evaporator under vacuum at 60˚C; then the extract was fully dried in hot air oven at 40˚C.

Quantification of phorbol esters

Preliminary quantification of phorbol esters was done using thin layer chromatography. The untreated and treated seed cake sample extracts were column chromatographed over silica gel (60-120 mesh) and fractionated by eluting with 5% dichloromethane in acetonitrile. These fractions were analyzed for phorbol esters by TLC before confirmation analysis by HPLC (Agilent, 1260 series, USA) with UV detector (Agilent G1315D MWD). A reverse phase C18 column [5 µm, 4.6 X 250 mm i.d., Thermo Electron Corporation Massachusetts (MA) USA] was used in the method adopted for separation in accordance with Wink *et al.*[15] The solvent system used was water and acetonitrile [Starting with 60% water (A) and 40% acetonitrile (B) for 15 min, decreasing A to 25% and increasing B to 75% for next 20 min, and increasing B to 100% for next 10 min]. Separation was effected at room temperature

(25˚C) with a flow rate of 1.3 ml/min. PMA was used as a standard.

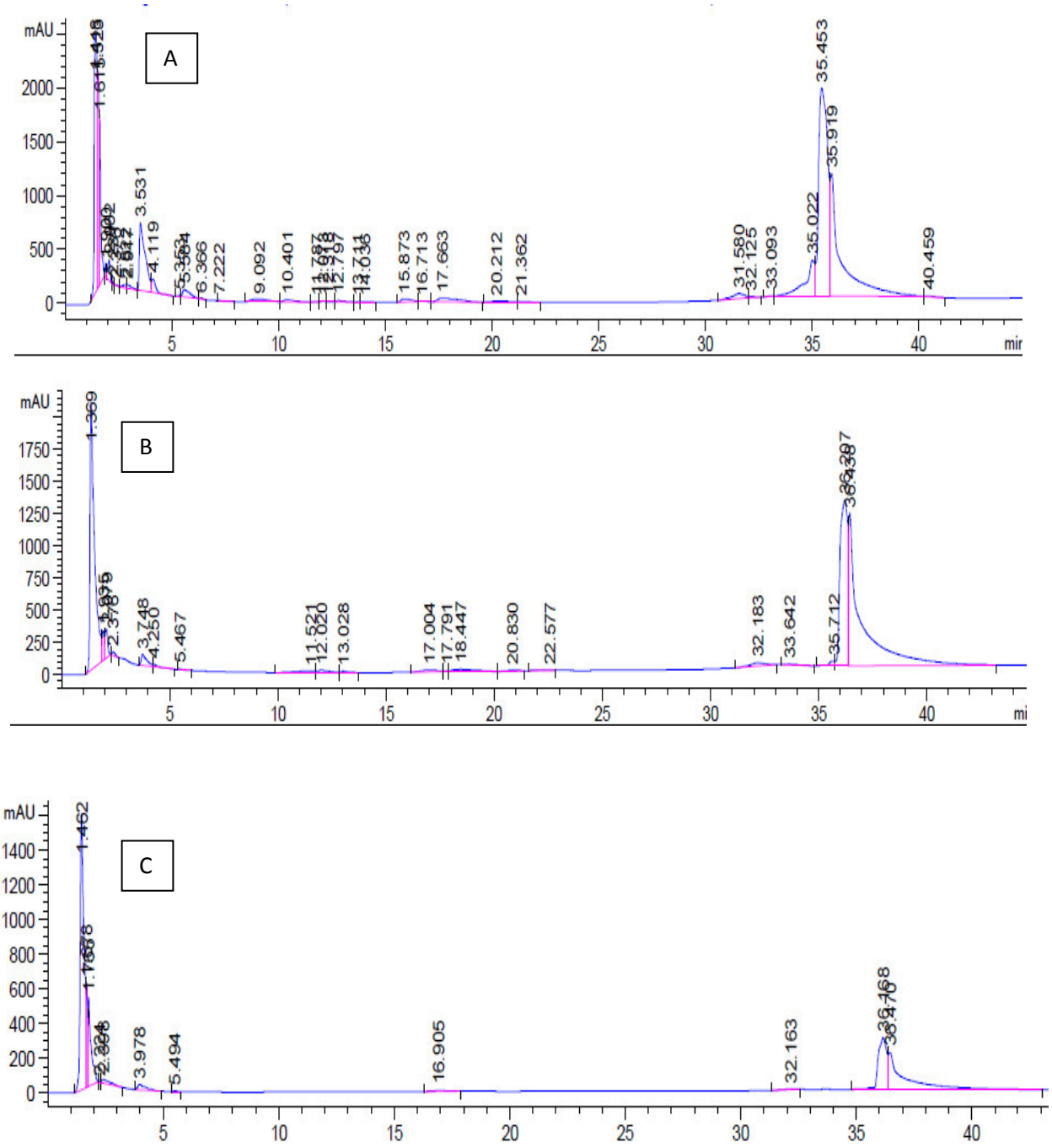
RESULTS

Phorbol esters in seed cake

Phorbol-12-myristate-13-acetate (PMA) is the only standard commercially available for investigating the presence of phorbol esters by HPLC. Methanol extracted phorbol esters from the deoiled seed cake were analyzed by HPLC. The quantification was done by taking PMA as the standard whose peak appeared at 37 min. HPLC chromatograms of *Jatropha* seed cake extract showed the presence of a major phorbol esters peak which consisted of two minor peaks at retention times of 35.4 and 35.9 min (Fig. 1A). We could identify two peaks for phorbol esters in the seed cake extracts. Total phorbol esters were calculated by summing up the area of phorbol ester peaks. The degradation was determined by comparing the area of the peaks for the samples with those of untreated seed cake.

Degradation of phorbol esters during submerged fermentation

Submerged fermentation was carried out using *Pseudomonas aeruginosa* DS1. The flasks containing the medium, seed cake and the inoculum were incubated in a rotary shaker at 37 ˚C, pH 7,100 rpm for 0, 9, 12 and 15 h and phorbol esters were extracted. Figure 1(A-D) shows the chromatograms of untreated and treated *Jatropha* seed cake samples at different time intervals. Phorbol ester concentration decreased gradually with increase in fermentation time. 31.57% reduction in phorbol ester peaks was observed after 9 h of fermentation period, which increased to 55.26% after 12 h. No phorbol ester peak was detected after 15 h of fermentation, and the phorbol ester content in the reference sample (uninoculated) remained same as that of 0 h.



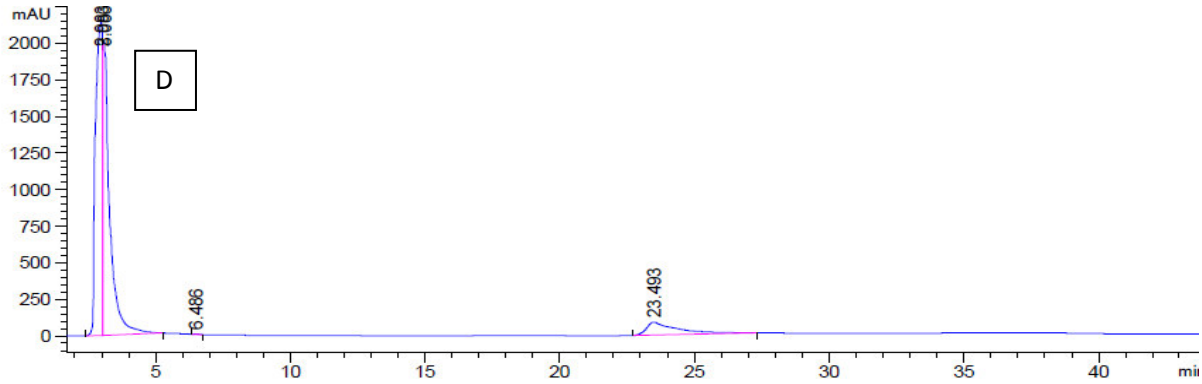


Figure 1

Chromatograms of untreated (A-for all sampling hours) and treated (B-9h; C-12h; D-15h) *Jatropha* seed cake samples.

DISCUSSION

Jatropha curcas contains six phorbol esters with diterpene moiety i.e. 12-deoxy-16-hydroxyphorbol in *Jatropha curcas* seed oil.[16] The quantity and the type of phorbol esters in *Jatropha* plant vary from country to country and is influenced by the species, soil fertility and the climatic conditions of the area where they grow.[16-18] In work done by Ahmed and Salimon [19], three different varieties of tropical *Jatropha curcas* were identified from Malaysia, Indonesia and India with two, five and four phorbol ester peaks respectively. We observed two phorbol esters peaks in our *Jatropha* seed cake sample which is similar to the findings of Chetna *et al.* [12] in which two peaks of phorbol esters were identified in the *Jatropha* seed cake, out of which the first peak with a retention time of 23.1 min was collected and quantified. Submerged fermentation technique has been the method of choice for bacterial enzyme production because of their higher moisture requirement. [20] In work done by Phengnuam and Suntornsuk[21] submerged fermentation resulted in more phorbol ester degradation in *Jatropha* seed cake as compared to solid state fermentation. While most of the research has been done with the biomass for detoxification, our work involved the use of enzymes in the broth after centrifugation. Degradation of phorbol esters was more when the inoculated seed cake was incubated for 15 h. Decrease in phorbol ester concentration with incubation time is attributed to certain enzymes like lipases and esterases released by the strain that hydrolyzes these diterpene esters.[22,23] In work done by Chetna *et al.*[12] lipase production by *Pseudomonas* was responsible for the possible degradation of phorbol ester during solid state fermentation of the *Jatropha* seed cake. So it can be inferred that the incubation time has a significant effect on the levels of phorbol esters of seed cake in the presence of biomass or free enzymes and our results clearly demonstrate that *Pseudomonas aeruginosa* DS1 could degrade phorbol esters in *Jatropha* seed cake to an undetectable level.

CONCLUSION

Submerged fermentation (SMF) was carried out to detoxify *Jatropha* seed cake by using *Pseudomonas aeruginosa* DS1. Complete degradation of phorbol esters was achieved when 5 g seed cake was inoculated at 37 ˚C, pH 7,100 rpm for 15 h which is very short treatment time compared to other methods of detoxification.The detoxification conditions may further be optimized to make the submerged fermentation process more suitable for commercial applications to produce animal feed supplements from *Jatropha curcas* seed cakes.

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CONFLICT OF INTEREST

Conflict of Interest declared none.

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Reviewers of this article

Dr. Gaganpreet Kaur Saini

Doctorate in Biotechnology, FSQA Coordinator,Kiskadee Ventures Limited,130 Dynamic Drive,Scarborough Canada,M1V 5C9

Prof. Y. Prapurna Chandra Rao

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