Biological Detoxification of Gossypol in Cottonseed Meal

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***Abstract*- India is the second-largest cotton producer globally, contributing to about a quarter of the global cotton production. While the cotton bolls support India's giant cotton textile industry, the seed offers high value in livestock feed and oil production. Cottonseed meal (CSM), a by-product of the cotton oil industry, is an attractive source of feed enriched with high protein and vitamin E content. But its consumption is severely hindered by the presence of a polyphenolic compound known as "Gossypol". Our research elaborates on the biological routes of reducing the gossypol content to permissible limits and making the CSM a lucrative functional food ingredient, ready for the utilization of animal/human consumption.**

***Index Terms*-** Cottonseed meal, Gossypol, Detoxification, enzymes, Protein source.

I. INTRODUCTION

In the present global scenario, malnutrition is a serious concern among the major countries of the Asian and African subcontinent. This has buzzed the alarm to find a sustainable solution to overcome the protein requirement among developing countries [1]. Cotton (Gossypium spp.) is a significant cash crop belonging to the Malvaceae family. It is considered one of the primitive plants cultivated by humans at least 4000 years ago [2]. The global production of cottonseed is estimated to be 44.84 million metric tons (MMT). The cottonseed meal (CSM), a co-product of a cotton oil refinery, is projected to produce 10 MMT annually. This can serve the protein requirement of 590 million people every year [1,3]. However, the consumption of CSM is hindered by the presence of the polyphenolic compound known as gossypol. Gossypol is produced by the pigment gland and is highly concentrated in seeds, with the concentration varying from 0.002-6.64%. The basic structure of gossypol comprises six phenolic hydroxyl groups and two aldehyde groups, making it a highly reactive compound [4]. Gossypol occurs in two vital forms, free and bound. Cottonseed may contain up to 14,000 mg/kg of total gossypol and 7,000 mg/kg of free gossypol, depending upon the species and weather conditions. Previously, several attempts were made on de-gossypolization using the physical, chemical, and biological methods. The physical and chemical methods impose certain constraints as to their efficiency and cost economic point of view. However, biological treatment has emerged as the most attractive and sustainable solution for reducing the gossypol content and improving the nutritional properties.

A. Health implication of gossypol and its processing

Even though few reports highlight the medicinal properties of gossypol, a plethora of literature suggests that intake of gossypol has shown a deleterious effect on growth development and reproductive health of small ruminant and non-ruminant animals. The US FDA has set a limit of 450 ppm for free gossypol in human food products and 500 ppm for free gossypol in feed materials [1]. Thus, it is vital to reduce gossypol content below the prescribed limit for the safe utilization of CSM-based protein. Generally, the gossypol occurs in the unbound free form, and the free gossypol binds to the ε-amino group of lysine, making it unavailable for intestinal absorption. Further, it also leads to health implications such as impaired body weight, respiratory distress, anemia, weight gain, and reduced sperm motility/ concentration [3, 5–7]. To mitigate the gossypol toxicity, several physical methods such as gland flotation, liquid cyclone, supercritical CO2 extraction, and more recently, gamma and electron radiation methods have been employed. However, these methods tend to be expensive. Alternatively, chemical methods such as solvent extraction using green solvents have been attempted. These methods resulted in the reduction of gossypol content but are affected by parameters such as moisture content in seeds [1]. Despite the gossypol level reduction by physical and chemical methods, the safety levels suggested by US-FDA are not achieved. Nevertheless, it is imperative to reduce gossypol cost-effectively to develop a circular bio-economy. Microbial cell factories offer a sustainable advantage over the former methods as they can degrade gossypol and also improve the nutrient content of CSM.

B. Previous work

In the context of the biological route of de-gossypolization, Zhang et.al.[9] performed SSF for 48 hr using disinfected CSM as substrate. Among the microorganisms screened, C. tropicalis ZD-3, S. cerevisiae ZD-5 and A. Niger ZD-8 displayed an excellent detoxifying capacity with the percentage reduction of 94.57%, 88.51%, and 85.16%, respectively. Also, the crude protein content was improved by 10.76% and 22.24% with C. tropicalis ZD-3 and A. Niger ZD-8, respectively highlighting the significance of microbial fermentation.

On similar lines, Mageshwaran et.al.[10] isolated eight fungal strains using gossypol as the sole carbon/energy source and sequenced it using 18s rRNA. It was revealed that most of the strains belonged to the family of Aspergillus and Fusarium spp. When CSM was treated with Fusarium thapsinum, they observed a reduction of 65.2 of free gossypol. The Bacillus subtilis GH38 isolated from the rumen of the fistulated cow have shown a reduction of 8.86% and 49% of free (FG) and total

gossypol (TG), by maintaining the optimum condition of a temperature of 39℃, an inoculum of 107 cells/g, pH of 6.5 and incubation time of 72h [8].

Zhang et.al.[11] investigated the effect of carbohydrate, urea, mineral, and heat treatment on the reduction of free gossypol in the course of solid-state fermentation. The C. tropicalis ZD-3 strain has displayed an exceptional result in reducing FG. The supplementation of sucrose, starch, and minerals maximized the detoxifying percentage. Also, the fermentation resulted in the enhancement of crude protein and amino acid content. Kinetic studies performed under the impact of gossypol concentration on the growth of Candida tropicalis ZAU-1 in the inorganic salt glucose medium showed that the growth of the strain was unaffected even at the concentration of 1000 mg/L, and the degradation was about 94.12%.[12].

Rajarathnam et al. [13] reported that Pleurotus Florida, a white oyster mushroom grown on rice straw supplemented with cottonseed powder, showed a unique ability to degrade the gossypol content in cottonseed powder by using it as a nitrogen source and exhibited incredible growth ability. Furthermore, when the Pleurotus Florida was grown on synthetic media with gossypol as a nitrogen source, about 100 µg of gossypol was reduced with an inoculum load of 10 mg, temperature of 25℃, and incubation time of 10 days. There was no accumulation of gossypol in the budding fungi.

Cheng et.al.[14] has cloned and expressed Helicoverpa armigera CYP9A12 gene encoding cytochrome P-450 enzyme in Pichia pastoris under strong inducible Alcohol oxidase promoter (AOX). The authors adapted response surface methodology to optimize the process parameters and reported 40.91 mg/kg of FG by maintaining enzyme concentration of 2.5 ml, hydrolysis time of 2.5 h, and temperature of 35℃.

Even though many microbial studies have been reported previously to reduce the gossypol content, most organisms such as Candida and Aspergillus belong to the type II category and secrete toxins. The enzyme can be an attractive alternative as it is non-toxic, specific, and degrades with mild heat treatment, but only a few studies have reported on the enzymatic application. Since gossypol is a polyphenol content and is available abundantly in the lignin fraction of the cell wall, several enzymes such as Oxygenase, Phenolic Hydrolases, Manganese peroxidase, Laccase, Cytochrome p450, Hydrogenase, and Methylase are frequently used in the treatment of lignin. Laccase has been shown to oxidize a wide range of phenolic/aromatic compounds [15]. Recently, Wang et.al.[16] showed oxidation of hydroxyl and aldehyde groups associated with gossypol, which decreases the toxicity level significantly. On the other hand, cytochrome P-450 plays a crucial role in the degradation of recalcitrant lignin-associated compounds [17].

The NIT Warangal team is heading in this direction. We aim to screen competent enzymes through the computational tool and improve their stability/specificity via in-silico site-directed mutagenesis. "Our enzyme" will bind with gossypol and convert it into a non-toxic compound, thereby degossypolizing the CSM. This meal can benefit various farmers, livestock, and humans. In addition, the cotton industries will also profit from the process and allow them to be a part of the food industry in the long run. The enzyme will be cloned and expressed in the yeast system. The production will be enhanced using crude glycerol, which is a by-product of the biodiesel industry. Thus, addressing the problems associated with the food and biodiesel industry.

Crude glycerol was identified as a prospective solution as part of a defined medium to produce the enzyme as it provides a reliable carbon source to the organism to synthesize the enzyme. Crude glycerol is a major by-product in the biodiesel industries and accounts for 10% (w/v) of the total production of biodiesel. Crude glycerol is often dumped into the environment because of its low value. Crude glycerol doesn't have any significant usage (until it's purified) in other industries and thus is considered as waste, and so by utilizing crude glycerol we will be recycling a waste product reducing environmental impact.

Our team screened around 250 enzymes from the families of lignin peroxidases [18], manganese peroxidases [18], laccases [19], cytochrome P450[20], monooxygenases [20], salicylaldehyde dehydrogenases [21], and phenol hydrolases [22] via an online server called CB-Dock which is a free online server that enables its users to identify the binding sites of a protein [8]. After an initial shortlisting based on the server results, docking was carried out in AutoDock v4.2. This is a major step in the screening process as the analysis of the protein-ligand interactions grants an insight into the stability of the resulting compound. After deep scrutiny, we decided to test out *Melanocarpus albomyces* laccase as it gave a relatively good binding energy of -10.49 kJ/mol on AutoDock v4.2. This was also supported by the fact that laccases can catalyze the intramolecular annulation of gossypol's aldehyde and hydroxyl groups on the o-semiquinone radical and generate the released OH radical.[19].

II. Materials and Methods

A. For cloning strategy

Pichia host strain (GS 115), Electrocompetent *E. coli* DH5α for transformation, 3M sodium acetate, 100% ethanol, 80% ethanol, Low salt LB (Luria Bertani) medium, Low Salt LB plates containing Geneticin, YPDS plates containing Geneticin.

B. Trans-esterification of waste cooking oil

As a lipid supply, 20g of waste cooking oil (refined sunflower oil) is mixed with six volumes of methanol and 0.5% sodium hydroxide (catalyst). For 180 minutes, the reaction was kept at 65°C. After that, the reaction mixture was left undisturbed in a separating funnel for 180 minutes to separate crude glycerol and biodiesel. The crude glycerol was utilized to make the pre-inoculum for the BMMY medium. The crude glycerol will also be utilized for medium optimization.

C. Double Digestion reaction for eluting the codon-optimized Laccase gene

To elute the codon-optimized Laccase gene (COL) with *Eco*RI-*Not*I sites, a double digestion reaction was used. The plasmid encoding codon-optimized laccase was gel eluted and cloned into the pPIC9k vector utilizing restriction digestion with the *Eco*RI-*Not*I enzyme. Template - (gel eluted COL-6µl), buffer D

- (1µl), *EcoR*I - (0.2µl) *Not*I - (0.2µl) D/W - (2.6µl) is one PCR reaction setup. The reaction was kept at 37 °C for 3 hours. The process was stopped by heating it to 65°C for 20 minutes and

Experimental Overview

Table 1: The standard steps for cloning and expressing the gene of interest in pPIC9k.

Step	Action
1	The pPIC9k vector was propagated by transforming it into *a recA, endA1 E. coli* DH5α strain.
2	Double digestion was used to isolate the codon-optimized Laccase gene from the plasmid.
3	Developing a codon-optimized the Laccase gene cloning technique in frame with the α-factor secretion signal in the pPIC9k vectors.
4	Selection of transformants on Low Salt LB Plates with 50 µg/ml ampicillin after transformation of the ligated plasmid into *E. coli* cells.
5	Restriction digestion was used to confirm the clone in 10-20 transformants.
6	The recombinant plasmid was purified and linearized before being transformed into *Pichia pastoris* (GS 115).
7	*Pichia* strain (GS 115) transformation and plating onto YPDS plates having GeneticinTM present in the proper concentration.
8	Transformants resistant to GeneticinTM were selected
9	Checking the expression of the gene and determining laccase activity.
10	Laccase treatment of cottonseed meal and determination of gossypol content reduction.

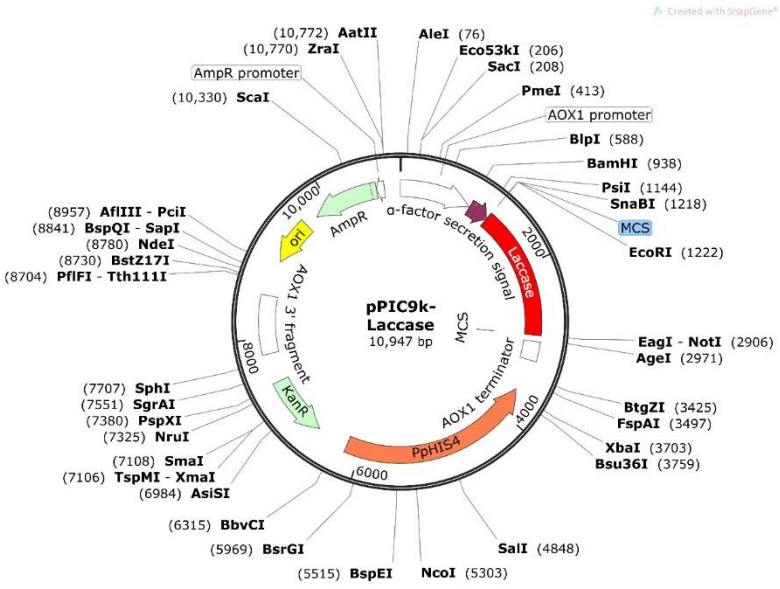

Fig 1: pPIC9k – Laccase Plasmid

then loading it onto an agarose gel with a 0.8% concentration. A gel elution kit (NEB, USA) was used to remove restriction digested reaction products from cut gel pieces.

D. Double digestion of pPIC9k vector

The *Eco*RI-*Not*I enzyme was used for restriction digestion of the pPIC9k empty vector. As previously stated, the same reaction setup was used.

E. Ligation reaction of pPIC9k plasmid with COL double digested and gel eluted product

Sticky-end ligation: A ligase enzyme was used to ligate *Eco*RI-*Not*I restriction digested COL gel eluted product into *Eco*RI-*Not*I restriction digested pPIC9k vector. pPIC9k vector (1.98µl), ligase enzyme (1µl), ligase buffer (1µl), and insert (1µl) D/W-(10.22µl) were used in the procedure. For overnight incubation, the ligation reaction was kept refrigerated at 16°C.

F. Transformation of ligation reaction into the E. Coli, Top10 cells

The two tubes (C and T) of 50µl of *E. Coli* competent cells were taken, and 15µl of the ligation reaction was transformed into 50µl of *E. Coli* proficient cell (T). Both tubes were placed on ice for 20 minutes, and heat shock treatment was given at 42°C for 90 seconds. For 10 minutes, the C and T tubes were placed on ice. Each tube was filled with 900µl of fresh LB broth. Both tubes were maintained at 37°C for 1 hour in a Thermomixer. 200µl was taken from the aforementioned culture and distributed onto LB medium plates with 25 µg/ml of Geneticin. Plates were incubated at 37°C overnight. Colonies were selected from LB plates and inoculated into vials containing 2ml of fresh LB broth.PCR was used to select positive clones' colonies, and plasmids were isolated from the positive clones using a plasmid isolation kit (NEB, USA).

G. Confirmation by double digestion

Plasmids were isolated using a plasmid isolation kit for the selection of positive clones (NEB, USA). *Eco*RI and *Not*I digestions were used to confirm the clones.

H. Linearization of pPIC9k COL construct by using enzyme SacI

Using the enzyme *Sac*I, the pPIC9k COL construct was linearized for integration into the Pichia genome. Plasmid (30µl), buffer D (5µl), *Sac*I (1µl), and D/W (14µl) were part of one reaction setup. The reaction was kept at 37°C for 3 hours in a dry bath. After 20 minutes of heating at 65°C, the reaction was stopped. A small portion of the digest was tested for complete linearization using agarose gel electrophoresis. 10% of volume 3 M sodium acetate and 2.5 volumes of 100% ethanol were added to the linearization reaction and centrifuged to pellet down the DNA.

I. Transformation of pPIC9k COL plasmid into Pichia pastoris

Pichia pastoris was inoculated into YPD broth and incubated at 28°C overnight. The cells were centrifuged for 5 minutes at 4°C at 1500 x g, and the pellet was resuspended in sterile MQ. The cells were centrifuged one more and resuspended in sterile 1M sorbitol. 80µl of cells from the above step was mixed with 5 - 10 µg of linearized plasmid DNA and transferred to an electroporation cuvette. The cells were pulsed using yeast-specific conditions, and sorbitol was added right away to stabilize the cells. On YPDS plates with 100µg/ml of geneticin, 10, 25, 50, 100, and 200µl were distributed. For three to five days, the plates were incubated at 28°C.

J. Determination of Mut phenotype

Using a sterilized toothpick, Geneticin-resistant transformants were patched onto the MMH and MDH plates. For two days, plates were incubated at 28°C. Mut+ strains will grow normally on both plates, whereas MutS strains will grow normally on the MDH plate but not at all on the MMH plate.

K. Expression of Laccase

Using a single colony, 30ml of MGY (Minimal Glycerol Medium) was inoculated and incubated overnight at 28°C. At room temperature, cells were extracted by centrifuging at 1,500 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 100ml of BMGY and BMMH medium. To maintain growth, the flask containing BMGY and BMMH medium was incubated overnight at 28°C. After 24 hours, 1ml of 100% methanol was added to keep the induction going. 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube at each of the times listed below. The samples were examined for expression levels and the ideal time to harvest after induction. At room temperature, samples were centrifuged at maximum speed for 5 minutes in a microcentrifuge. The supernatant was separated and kept at minus 80°C in a separate tube. The following periods were used to assess activity and expression levels: 0, 24, 48, 72, and 96 hours (four days).

L. Laccase Activity

The oxidation of the ABTS technique was used to evaluate laccase activity [23]. Laccase oxidizes ABTS, a non-phenolic dye, into a more stable and desired cation radical. The concentration of cation radicals that provide the vivid blue-green color codon-optimization can be linked to enzyme activity and is measured at 420 nm [24]. 0.5 mM ABTS, 0.1 M sodium acetate (pH 4.5), and an appropriate amount of enzyme was included in the assay mixture. The increase in A420 (ε420, 3.6 × 104 M−1·cm−1)., which indicates ABTS oxidation, was measured. The reaction mixture was incubated for 5 minutes and contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer at pH 4.5, and 100 µl of culture supernatant. In a spectrophotometer, absorbance was measured at 420 nm against a suitable blank. The amount of laccase that oxidized 1 µmol of ABTS substrate per minute was defined as one unit. Bradford's dye-binding method was used to quantify protein concentration using BSA as a reference.

M. Defatting of cottonseed meal

100gm of cottonseed is grounded in a blender and sieved with 0.2 mm mesh. About 10gm of the cottonseed meal was mixed with 100 ml of n-hexane and the mixture was placed in a sealed air-tight container and kept in the shaking incubator maintained at 35°C for 60 minutes to extract oil. The supernatant liquid was separated from the solid seeds and the defatted CSM was stored at -20°C and used for further experiments.

N. Biodegradation of gossypol using crude enzyme extract

The culture supernatants consisting of crude laccase enzymes are used to treat the defatted CSM and were evaluated for gossypol biodegradability. The reaction mixtures were prepared in 5 mL vials; 0.5 mL of culture supernatants was added in 2.5 mL of 0.1M phosphate buffer (pH 6.0). Furthermore, 0.1 mL of the enzyme extracts was added in 2.5 ml of the same buffer containing CSM at 1g in sterile Petri plates. The reaction mixture was incubated in a horizontal shaker at 65°C for 1 h. The reaction mixture was dried and used for gossypol estimation. Residual gossypol level in the mixture was analyzed by AOCS Ba 7-58[25].

III. RESULTS AND FINDINGS

A. Cloning and expression of laccase gene in P. pastoris GS115

The 1.6 Kb codon-optimized laccase gene of *Melanocarpus albomyces* was cloned downstream of signal peptide α - mating factor of pPIC9K vector for extracellular protein expression. The resultant recombinant vector was transformed into *P. pastoris* GS115, and the recombinant strain is designated as GS-COL. The recombinant strain is evaluated for the production of laccase in the shake flask.

B. Shake Flask Studies for Laccase Expression

The recombinant strain GS-115 COL was pre-cultured in BMGY medium, where pure glycerol and crude glycerol, in both mediums the recombinant strain showed substantial growth, displaying its ability to consume the impurities associated with crude glycerol. Further, the pre-inoculum was transferred to the BMMH medium and induced with 1% methanol.

The maximum production of laccase was found to be 9.75 U/ml at 48h time interval with a concomitant increase in cell concentration of 8.9 using pure glycerol-based pre-inoculum (Fig 2-A). Similarly using crude glycerol-based pre-inoculum, the maximum laccase and the cell concentration were found to be 6.75 and 7.59, respectively (Fig 2-B). The laccase displayed a growth-associated product.

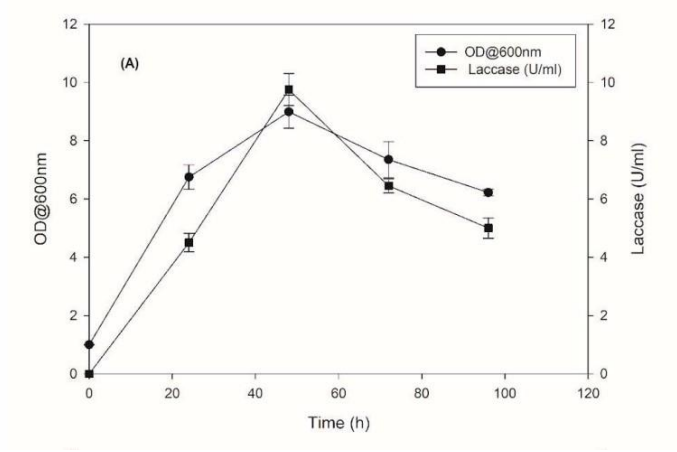

Fig 2-A: Growth and product profile of GS-COL strain grown in pure glycerol-based pre-inoculum and BMMH medium with 1% methanol induction.

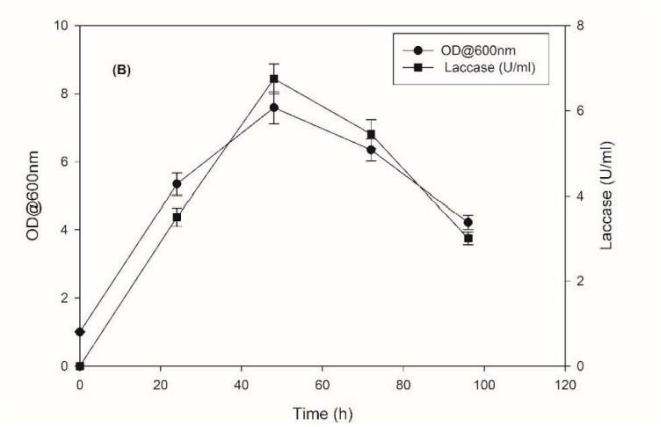

Fig 2-B: Growth and product profile of GS-COL strain grown in Crude glycerol-based pre-inoculum and BMMH medium with 1% methanol induction.

IV. CONCLUSION

In this research, we attempted to find an efficient enzymatic method for the degradation of gossypol. By using in-silico methods, we screened nearly 250 enzymes before finalizing our enzyme to Melanocarpus Albomyces laccase. To develop a cost-effective and environmentally safe process, we utilize crude glycerol as a carbon source for producing gossypol-degrading enzymes. The laccase was produced by growing the strain *Pichia Pastoris* GS115 in the crude glycerol medium. Cottonseed meal was treated with the produced laccase to degrade gossypol. In the future, we plan to scale up this experiment by optimizing the experiment parameters and by carrying out in silico mutations to increase specificity, and by also testing other potential enzymes which can degrade gossypol. By doing this, we could achieve gossypol-free cottonseed meal, which is a good protein source that potentially meets the protein requirements of the rapidly growing population.

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