Chapter 9

Summary & Conclusion
Agro-industrial wastes are the vegetative byproducts or organic waste materials generated as a result of agricultural or industrial operations such as the production and harvest of crops, rearing of animals, slaughter houses, leather processing or litter from household chores. These wastes are generated in very large quantities with high potential to serve as raw materials for production of value added products after processing. However, in most cases the presence of recalcitrant or toxic component limits their utilities. This has led to intense research for development of technologies to convert such agro-wastes into industrially important products, fuel, feed, fertilizer, etc. with simultaneous alternatives to their disposal.

One such agro-industrial waste is deoiled Jatropha seedcake (JSC), which is a by-product obtained upon extraction of oil from Jatropha seeds. In spite of having all the necessary nutrient components and high protein content, it is not suitable as animal feed supplement due to the presence of anti-nutritional factors such as phorbol ester (a polycyclic diterpenoids), lectins, trypsin inhibitors, etc. The disposal of such toxic seed cakes may lead to disruption of biogeochemical cycles of several elements by affecting micro- and macro-fauna at the disposal site, leading to long term unpredictable effects on biosphere. Hence its detoxification is essential for its safe disposal while its value addition will benefit the economics of Jatropha oil based biodiesel production. The detoxification of JSC is currently being attempted by physical or chemical methods however these methods are energy intensive, uneconomical and not at all eco-friendly. Biological detoxification is an alternative eco-friendly approach for converting toxic JSC into safe protein rich animal feed additive/supplements. Such nutrient rich deoiled seedcake also has a prospect of being employed as a substrate for microbial growth and production of commercially relevant enzymes, bioactive compounds, amino acids, vitamins, organic acids, aroma compounds etc. Thus, the use of JSC as a raw material in industrial fermentation would not only serve to significantly reduce the final price of the fermented products but also add value to it, thereby solving its disposal problems. With this background and concept, we decided to develop methods for utilization of JSC as raw material for production of industrially important microbial enzymes and metabolites along with its detoxification.

A bacterial isolate identified as *Pseudomonas aeruginosa* AAU2 (JQ904623) capable of producing lipase was isolated from rotting JSC selected for further studies. Upon optimization of physico-chemical parameters for production of lipase under
submerged fermentation by *P. aeruginosa* AAU2, 11.4-fold enhancement in the lipase production was observed. The *P. aeruginosa* AAU2 yielded maximum lipase 4.92 U/mL in an optimized medium containing 20 g JSC/L, 2.5 g potassium nitrate/L and 0.1% (v/v) Tween 80. The crude lipase from *P. aeruginosa* AAU2 was purified by 40% ammonium sulphate precipitation followed by Sephadex G-100 gel permeation leading to 5.37-fold purification. The optimum pH and temperature for lipase activity were found to be 7.5 and 40°C. The values of *K*<sub>m</sub> and *V*<sub>max</sub> for pNPP hydrolysis reaction catalyzed by partially purified lipase were found to be 0.1 mM and 40 µmoles/mg.min, respectively. This *P. aeruginosa* AAU2 lipase preferred longer carbon chain (C12) fatty acid ester substrates over the shorter ones. The metal ions Ca<sup>2+</sup> and Mg<sup>2+</sup> had stimulatory effect on lipase activity, whereas Cu<sup>2+</sup>, Co<sup>2+</sup> and Hg<sup>2+</sup> strongly inhibited the lipase activity. The *p*CMB, DTT and β-mercaptoethanol significantly reduced the lipase activity suggesting the role of cysteine in catalysis. The AAU2 lipase was resistant to organic solvents, bleach-oxidants, non-ionic and mild commercial laundry detergents. Further, the lipase also exhibited hydrolysis of phorbol 12-myristate 13-acetate (principle *Jatropha* toxin) and efficient trans-esterification activity for synthesis of biodiesel using *Jatropha* oil.

In a separate study, thirty-six proteolytic bacteria were isolated from Jakhao coast which were vigorously screened for organic solvent and salt tolerance. Amongst them *Bacillus tequilensis* P15 (JQ904626) was selected, which was able to produce an extracellular solvent and detergent tolerant protease (116.69 ± 0.48 U/mL). Deoiled JSC was found to be a suitable substrate for protease production under submerged condition. Upon optimization of physico-chemical parameters using one-factor-at-a-time approach, an overall 6.4-fold (860.27 ± 18.48 U/mL) increase in protease production was achieved. The maximum protease yield was obtained using a medium containing 2% (w/v) deoiled JSC as substrate (pH of 8.0) upon 36 h of fermentation at 30°C. The optimum temperature and pH for activity of *B. tequilensis* P15 protease was found to be 50°C and 8.0, respectively. The enzyme exhibited a half-life of 190 min at 50°C, which was enhanced to 270 min in presence of 5 mM Ca<sup>2+</sup>. The P15 protease exhibited significant stability in almost all the solvents tested in the range of log *P*<sub>ow</sub> varying from 8.8 to -0.87. The protease activity was strongly inhibited by PMSF at 5 mM concentration. However, presence of EDTA (5 mM) and *p*CMB (5 mM) enhanced enzyme activity by 20.9% and 13.7%, respectively. The divalent metal ions Ca<sup>2+</sup> and
Zn$^{2+}$ enhanced protease activity by 26.2% and 8.4%, respectively, whereas Cu$^{2+}$ and Fe$^{2+}$ strongly inhibited the enzyme activity. The enzyme was also found to be stable in the presence of surfactants, commercial detergents, oxidizing and bleaching agent ($\text{H}_2\text{O}_2$) at 1% (w/v). This protease was demonstrated to be effective in removing blood stain from fabrics, dehairing of hide and gelatin strip-off from the used photographic films.

White rot fungi are well known for their ability to degrade a wide range of xenobiotics, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyl and synthetic dyes due to their enzymatic systems. In addition, the enzymatic complexes produced by white rot fungi also have an enormous potential to be used in the treatment of fibrous feedstuffs in order to improve its nutritive value through sequential degradation of certain refractory cell wall components. The ten white rot fungi, viz., *Ganoderma lucidum*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus florida*, *Pleurotus sapidus*, *Pleurotus sajor-caju*, *Trametes versicolor*, *Trametes hirsuta*, *Trametes zonata* and *Trametes gibbosa* were screened to decompose JSC with simultaneous degradation of phorbol esters under solid state conditions. Upon fermentation, the phorbol esters were extracted with methanol and analyzed by HPLC. All the white rot fungi tested, exhibited phorbol ester degradation but the extent of degradation varied with the fungal culture. The white rot fungi, *G. lucidum* and *T. zonata* were found to completely degrade phorbol esters in JSC. However, *T. versicolor*, *T. gibbosa*, and all the *Pleurotus* sp. were able to significantly reduce the phorbol ester content of JSC by 70%. Further, no significant loss in nutritive content was observed in the fermented JSC, irrespective of the white rot fungi used. Alternatively the JSC after decomposition by white rot fungi may be tested for its potential application as animal feed supplement as it is expected to get enriched with additional growth factors of fungal origin. However, the applicability and potential of such fermented residue as feed supplement needs be assessed effectively through feed trials on animals.

The other utility of fermented JSC would be as soil in-put and the synthesis of phytohormone, indole acetic acid (IAA) by these fungi while fermentation would further add value to the fermented JSC residue. Therefore, all the above mentioned white rot fungal sp. were investigated for the production of IAA in L-tryptophan supplemented JSC as sole nutrient and energy source. Both solid state and submerged fermentation methods were evaluated. Under solid state fermentation there was no IAA
production. However, in submerged condition, *T. versicolor*, *Pl. ostreatus* and *Ph. chryosporium* exhibited IAA production. The maximum IAA production (473.55±3.32 µg/mL) was observed in *Pl. ostreatus* after 18 d of incubation in medium containing 0.1% (w/v) L-tryptophan, 2% (w/v) JSC at pH 7.0 and 37 °C. The IAA produced by *Pl. ostreatus* was extracted and characterized by TLC as well as mass spectrometry. The biological activity of fungal IAA obtained from the culture supernatant of *Pl. ostreatus* was determined using wheat coleoptile bioassay.

Since the development of the concept of biologically compatible nanoparticle, a great deal of effort has been put into the biosynthesis of inorganic materials, especially metal nanoparticles using microorganisms. In recent past, the use of plant parts for nanoparticle synthesis has gained momentum, mainly as it eliminates the elaborate process of maintaining cell cultures. In the same context, silver nanoparticles were successfully synthesized from AgNO₃ through a simple green route using the aqueous extract of JSC as reducing and stabilizing agent. The resulting silver nanoparticles were characterized with the help of UV-visible absorption spectroscopy, transmission electron microscopy (TEM), selected area electron diffraction (SAED) and X-ray diffraction (XRD). The nanoparticles exhibited an absorption peak around 430 nm, a characteristic surface plasmon resonance band of silver nanoparticles. They were found to be mono-dispersed and spherical in shape with average particle size of 10.48 ± 2.74 nm. The crystalline nature of the nanoparticles with face centered cubic geometry was confirmed by the presence of characteristic (111), (200), (220), (311) and (222) diffraction planes of face centered cubic structure with 4.083 Å lattice parameter. Further, the FTIR analysis revealed the involvement of proteins and phenols in reduction and stabilization of nanoparticles. The synthesized silver nanoparticles exhibited significant antibacterial activity, suggesting its prospective applications in antibacterial formulations.

Recently attention has been diverted towards the agro-industrial waste materials for heavy metal removal from aqueous effluents. The basic components of these waste biomass include hemicellulose, lignin, lipids, proteins, simple sugars, hydrocarbons, starch containing variety of functional groups viz. acetamido, alcoholic, carbonyl, phenolic, amido, amino, sulphhydryl etc, that facilitates metal complexation and aids in sequestration of heavy metals. Therefore, we investigated JSC for its use as biosorbent for Cr(VI) removal from wastewater. The acid pre-treated biomass exhibited 1.9-fold
higher biosorption efficiency for Cr(VI). The Cr(VI) biosorption efficiency was found to increase with decrease in pH of aqueous medium. The adsorption capacity of biosorbent for Cr(VI) increased with increasing concentration of Cr(VI). The biosorption of Cr(VI) by acid treated JSC followed a pseudo-second order kinetics. The results of equilibrium studies showed that the biosorption process fitted the Langmuir isotherm model with a maximum adsorption capacity of 22.727 mg of Cr(VI)/g of biosorbent at 30°C. The activation energy for Cr(VI) biosorption was found to be 27.114 kJ/mol, suggesting it to be predominantly a physical process. The important thermodynamic parameters of adsorption (ΔG, ΔH and ΔS) were determined which indicated that the Cr(VI) sorption by JSC is a spontaneous and endothermic process.

Another hard-to-degrade agro-industrial residue is keratin which is the main constituent of low commercial value agrowastes of animal origin and includes feathers, nails, hair, horns, hoofs, etc., and generated in large amount from poultry and cattle slaughter houses. The increased health consciousness has directed to greater consumption of white meat (bird meat) for table purpose in place of red meat (animal meat) due to presence of less saturated fat and cholesterol. This increased requirement for bird meat has resulted in generation of huge amount of feather waste which is currently disposed off either by dumping or incineration both of which are environmentally unacceptable methods. Alternately, it is possible to decompose such wastes employing a biotechnological approach, for instance, as low-cost substrates for the production of enzymes (especially keratinase) and other value-added microbial products such as nutrient-rich animal feed or as agricultural inputs. On the other hand, inadequate effectiveness of chemical control and increasing restrictions on the use of fungicide and pesticides have given increasing impetus to biological control as an alternative tool for integrated disease management in agriculture. Consequently, hydrolysis of bird feathers employing a keratinolytic bacterial strain which also produces antifungal metabolites, could offer a dual environmental advantage: solving disposal problem of bird feathers and production of biocontrol agents. From this perspective, thirteen proteolytic bacteria with potential to degrade feather meal were isolated from poultry soil. On the basis of ability to grow and efficiently degrade whole feathers, bacterial isolate designated as *Bacillus amyloliquefaciens* 6B (JQ904625) was selected for further studies. An overall 2.58-fold (610.13 U/mL) increase in enzyme production could be achieved upon optimization of process parameters for keratinase
production. The maximum enzyme production was obtained using BHM containing 0.5% (w/v) feather meal as substrate and pH 8.0 upon 12 h of fermentation at 37 °C in shake flask (150 rpm) condition. Additionally, the keratinolytic protease exhibited significant stability in organic solvents tested in the range of log $P_{ow}$ varying from 3.0 to 4.5 with maximum stability in isooctane. The keratinolytic protease was concentrated by 40-80% ammonium sulphate precipitation with 38.26% recovery and 24.2-folds purification. The optimum temperature and pH for the keratinolytic protease was found to be 50 °C and 8.0, respectively. The half-life of the enzyme at 50 and 60 °C was found to be 44 and 19 min, respectively. The values of $K_m$ and $V_{max}$ for casein hydrolysis reaction catalyzed by crude enzyme concentrate were found to be 1.14 mg/mL and 1.43 mg/mL.min, respectively. The enzyme activity was strongly inhibited by PMSF at 5 mM concentration suggesting it to be serine hydrolase. The EDTA (5 mM) resulted in increase in enzyme activity by 6.27%. The divalent metal ions Mn$^{2+}$, Zn$^{2+}$ and Ca$^{2+}$ had stimulatory effect on protease activity, whereas Cu$^{2+}$ and Fe$^{2+}$ strongly inhibited the enzyme activity. The enzyme was also found to be stable in the presence of bleach oxidant, non-ionic and anionic surfactants at 1% (w/v), suggesting its potential application as detergent additive. Furthermore, the $B. amylo liquefaciens$ exhibited antagonistic activity against plant-pathogenic fungi. Consequently, the feather hydrolysates resulting from the microbial conversion of feather keratin offer a prospective utility as a biocontrol agent as well as nitrogen-rich organic agricultural input.

To conclude, in the present work, we have demonstrated prospective applications of JSC as raw material for production of microbial enzymes such as lipase and protease and phyto-hormone (indole acetic acid). The degradation of phorbol esters in JSC was demonstrated by microbial lipase as well as during solid state fermentation by white rot fungi. The application of aqueous extract from JSC in biosynthesis of silver nanoparticles was also demonstrated and would provide a value to waste seed cake if a commercial scale technology is developed. The ability of acid pretreated JSC for sorption of Cr(VI) suggests the development of efficient biosorbents from waste JSC, which have vast applications in environment sector. The degradation of bird feather degradation using a soil bacterial isolate with simultaneous production of solvent and detergent tolerant keratinolytic protease was demonstrated, which offers a cost-effective technology for industrial protease production, while adding value to poultry feather
waste. Through present work we have demonstrated the value added resourceful management of two important toxic and/or recalcitrant agrowastes, viz., Jatropha seedcake and bird feather waste while addressing the environmental concern for their safe disposal.