DETOXIFICATION OF RESIDUAL TOXINS IN JATROPHA PRESS CAKE FOR ITS APPLICATION IN ANIMAL FEED DEVELOPMENT

Thesis submitted to Delhi Technological University
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

by

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Detoxification of residual toxins in *Jatropha* press cake for its application in animal feed development**" submitted to Delhi Technological University, Delhi, for the award of Doctor of Philosophy is based on the original research work carried out by me under the supervision of **Prof. Jaigopal Sharma** (Department of Biotechnology) and **Dr. Pushpendra Singh** (Department of Mechanical Engineering), Delhi Technological University, Delhi, India. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I, Shilpi, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of **Prof. Jaigopal Sharma** (Department of Biotechnology) and **Dr. Pushpendra Singh** (Department of Mechanical Engineering), Delhi Technological University, Delhi, for a period of August 2013 to March 2018 at the Department of Biotechnology, Delhi Technological University, Delhi. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their work has been cited in the text and the body of thesis. I further certify that I have not willfully lifted up some other's work, paragraph, text, data, results etc. reported in the journal, books, reports, dissertations, thesis etc., or available at websites and included them in Ph.D. thesis and cited as own my work.

Date
Place

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ACKNOWLEDGEMENT

This thesis has been kept on track and been seen through to completion with support and encouragement of numerous people including my well wishers, my friends and colleagues. I consider myself blessed with a friendly working atmosphere, supportive friends. Though, words would not be enough to express my gratitude to all these people, still I would like to thank everyone for helping in enormous ways to lead me to the conclusive part of this journey.

First of all, I would like to express my extreme sincere gratitude to my supervisor Prof. Jaigopal Sharma and Dr. Pushpendra Singh, for their guidance, suggestions and encouragement. They supported me throughout my thesis with patience and knowledge whilst allowing me the room to work in my own way. The present work is a testimony to their alacrity, inspiration and ardent personal interest, taken by them during the course of this thesis work in its present form.

I would like to express my kind regards to Prof. Yogesh Singh, Vice Chancellor, Delhi Technological University, for providing me necessary facilitiies at DTU. I am thankful to other faculty members of Biotechnology Department and SRC members of my Ph.D. course for their comments, valuable suggestions and guidance during the entire tenure of my Ph.D. Special thanks to CASRAE, DTU for providing me *Jatropha* seed cake for my research work.

I would like to thank Prof. Dr. Rina Chakrabati (Deptt. of Zoology, DU) for her excellent help in fish data analysis. I am highly grateful to Dr. RajKumar Bidlan, PDF, for taking active interest in my work, for his valuable suggestions and helping me in every way for the fulfillment of the research work. Special appreciation to the office staff Mr. Chhail Bihari, Mr. Jitendra Singh and other technical staff for their support.

I am indebted to my friends Mr. Satish Kumar and Dr. Shikha Gupta for providing a stimulating, fun filled and healthy environment in Lab. I would like to extend my sincere thanks to both of them for supporting me at every moment. I would also like

to thank Dr Mohit Tyagi, Deepak Kumar, Ravi and Avanish for helping and supporting me in doing my Ph.D work.

Since a number of research papers and textbooks have been consulted for research work, I would like to thank authors of those research papers and books.

On a personal note, I express my thanks and love to my husband Prof. Ravinderjit Singh Walia for his continuous encouragement, cooperation and support, and my kids Ishwin and Avitaj, who have played the biggest role of stress reliever. I would like to express my special thanks and gratitude to my in-laws S. Rajinder Singh Walia and Mrs. Tarlochan Kaur for their support and encouragement.

Parents are indispensable and pillar strength for the over-all development of the child. A child, especially daughter is incomplete without her family even after marriage too. I would like to give whole hearted credit to my parents S. Swaranjit Singh and Mrs. Kiran Ahluwalia for the completion of the doctorate thesis. Their blessings like catalyst speed up the whole process of thesis formation. I am indebted to my brother Mr. Manmeet Singh and sister in-law Mrs Sumeet Ahluwalia for their love, respect and emotional attachment. I also want to thank Renu for taking care of my children in my absence.

I want to thank Almighty God for giving me blessings, strength and knowledge.

In the end, I would like to apologize to all those people whose names are not mentioned here but they have helped me in completion of Ph.D. thesis in one or another way.

Shilpi

ABSTRACT

Jatropha curcas is an oil-seed plant with good adaptability to grow in barren lands with scanty rainfall. *Jatropha* oil had been well exploited for the production of biodiesel by trans-esterification process. The compressed press cake, after the oil extraction, is a rich source of protein but presence of certain toxic and anti-nutritional factors in the seed cake restricts its use for edible purposes as its ingestion may lead to various health problems. Thus, it becomes imperative to detoxify *Jatropha* press cake for its use as feed supplement. Phorbol esters have been identified as the main toxin present in the *Jatropha* press cake. Various physicochemical have been described for the detoxification of *Jatropha* seed cake and oil which includes use of organic solvents, UV-irradiation etc which were able to reduce phorbol esters completely from *Jatropha* press cake. But these treatments are expensive; also, handling or the disposal of the toxins raises an environmental and health concern. However, for environmental awareness, the biological method would be more advantageous than the others. *Pseudomonas aeroginosa* DS1 was isolated in laboratory and research was done to determine the effects of submerged fermentation (SMF) using Pseudomonas on the phorbol esters (PEs) degradation rate.

For the proper understanding of the process, different parameters affecting the detoxification of *Jatropha* seed cake by submerged fermentation process were studied. Out of several factors, eleven crucial factors were identified and analysed for their inter-relationship using interpretive structural modelling (ISM). Temperature (level IV) was found to be the most crucial factor affecting all other factors for successful implementation of the detoxification process. PE, pH, RPM, esterase, and inoculum (level III) were identified as next crucial set of factors followed by tannins (level II). MICMAC (Matriced' Impacts Croise's Multiplication Appliquée a UN Classement) analysis was done to recognize the factor dependency in detoxification. Out of eleven factors, nine factors were identified as linkage variables; one each as the driver and dependence variable. No autonomous variable was identified.

Taguchi design of experiments L18 orthogonal array was adopted for optimizing the SMF process for detoxification of *Jatropha* seed cake. Process parameters selected for the study were: pH, percentage of seed cake, time, temperature and rpm. The response parameter for detoxification was measured in terms phorbol ester degradation. Analysis of variance (ANOVA) was performed for predicting the optimum process parameters. The percentage contribution of the process parameters with reference to phorbol ester degradation was predicted. The optimum conditions were found to be seed cake (1%), temperature (35 ˚C), pH (7.5) and time (12 h). Fourier Transform Infrared (FTIR) spectroscopy was done to analyze the structural changes occurred in *Jatropha* seed cake during detoxification procedure.

Jatropha seed cake contains good amount of proteins which were extracted from *Jatropha* seed cake by iso-electric precipitation method. For pilot scale experiments, one parameter at- a- time approach was followed and effect of temperature, solubilization pH and precipitation pH on protein content and protein yield of *Jatropha* seed cake was studied. Recovery of protein concentrate was highest when the extraction was carried out at 55°C temperature, solubilization pH 11.5 and precipitation pH 5. The results of these experiments were used to select different factors and their levels to optimize the process using Response Surface Methodology. The factors selected were extraction temperature, time, solubilization pH, precipitation pH and their effect were studied on response variables; dry matter, protein yield and protein content. The optimum values of the variables i.e. temperature, solubilization pH, time and precipitation pH for the extraction of protein from detoxified seed cake were 60°C, 11.0, 4.41 and 0.78 h respectively which yielded maximum dry matter, protein content and protein yield.

The effect of bio-remediated seed cake on seed germination (*Vigna radiata*) and *in vitro* digestibility (*Oreochromis niloticus*, *Cyprinus carpio*) was studied. *Jatropha* seed cake in all forms (Raw, defatted and detoxified) was found to have inhibitory effects on green gram seed germination and overall seedling health. Germination was not affected by the addition of 1 and 5% defatted seed cake while a decrease in germination rate was observed in soil containing 10% defatted seed cake. The detoxified seed cake was found to have positive and eliminatory effect on the toxicity

leading to improvement in both vigor index and pigmentation. Higher percentage of detoxified *Jatropha* seed cake did not affect the root length and very less reduction in the number of secondary roots observed. The vigor index was affected at 10% levels of all the treatments with detoxified seed cake having least effect.

The plant pigments were also affected and found to have reduced in raw and defatted seed cake treatments. Addition of detoxified seed cake concentrations at 1% and 5% level matched with the control in chlorophyll *b* content. *In vitro* digestibility study also showed improvement in digestibility and confirmed the inactivation of toxins in the *Jatropha* protein concentrates. Though, this is a prospective step towards the use of *Jatropha* seed cake as fertilizer and fish feed after detoxification, the avenues are open for various applications.

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ABBREVIATIONS

ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
CCRD	Central composite rotatable design
CI_{CE}	Confidence Interval [confirmation experiments (CE)],
CI_{POP}	Confidence Interval of population
DH	Degree of hydrolysis
DOF	Degrees of freedom
EDTA	Ethylenediaminetetraacetic acid
FAO	*Food and Agriculture Organization*
FTIR	Fourier transform infrared spectroscopy
HPLC	*High-performance liquid chromatography*
ISM	Interpretive structural modeling
JSC	Jatropha Seed Cake
LCMS	*Liquid chromatography–mass spectrometry*
MICMAC	Matriced impacts croises-multiplication applique' and classment
MSD	Mean square deviation
NPK	Nitrogen, phosphorus and potassium
OA	Orthogonal arrays
PE	Phorbol esters
PKC	Protein kinase C
RPM	Revolutions per minute
RSM	Response Surface Modelling
S/N	Signal to noise
SCFE	Supercritical Fluid Extraction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gelelectrophoresis
SMF	Submerged Fermentation
TCD	Thermal conductivity detector
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

There has been substantial increase in energy demand through-out the world due to the strong economic growth especially in developing countries like India. The world's population is expected to shoot from six to nine billion by 2050, with significant change in economic growth, international affairs, biodiversity, human development and health (Nehring, 2009). Nearly 80% of energy requirement is fulfilled by the burning non-renewable fossil fuels and with increase in population it is predicted to increase three times more than current usage (Popp *et al.*, 2014). In India 70% of power generation is from fossil fuels. In the year 2009-10, country imported around 160 million tons of crude oil which accounts for 80% of domestic crude oil consumption. India ranked third after USA and China in oil consumption with 212.7 million tons in 2016. In 2015, India imported 195 million tons of crude oil and 23 million tons of refined petroleum products and also exported 55 million tons refined petroleum products. Excess consumption of fossil fuels may lead to depletion of the non renewable feedstock and also causes a threat to the environment by the emission of green house gases, pollutants including extreme weather conditions, damage to ecosystems along with various health issues like obesity, cancer and many kinds of chronic & lethal respiratory diseases (Fontana *et al.*, 2013). Despite of abundant domestic fossil fuel resources, India's dependence on imported fossil fuels is increasing year by year and it raised upto 38% in 2012 (Figure 1.1) which could be a result of modernization and economic growth. Modernization has made people to move to urban areas where they rely more on fossil fuels than traditional fuels for meeting their energy demands. Over population is another major factor which results in over consumption and unnecessary use and wastage of resources leading to depletion of fossil fuels. Other causes include deforestation, mining of minerals & oils, use of technology for development, contamination of resources and pollution.

Many countries with the help of technology have now developed and started using sources of renewable and inexhaustible energy resources like solar radiation, wind

energy and flowing water & tidal power (water in oceans). These are natural and clean sources of energy and will not pollute the environment. Countries like US and Russia have made significant reductions in CO_2 emissions despite of low economy whereas sizeable increase was observed in countries like in India, Indonesia, Vietnam, and across the Middle East (Figure 1.2). In 2016, India's oil consumption amounted to approximately 212.7 million metric tons of oil equivalent. According to BP Statistical Review of World Energy June 2017, India witnessed the largest increment to CO_2 emissions in the world for a third consecutive year rising by 113.7 million tones or 5% (in percentage terms) in which emissions from coal and oil were mainly responsible.

So to establish a sustainable global development considering the socio-economic scenarios, it becomes necessary to rely more on renewable and green energy sources as alternative to fossil fuels (Bharat and Onkar, 2012). Replacement with these alternative energies with improved energy efficiency mechanisms will attract to utilize more advanced technology which will help to meet the future energy demands.

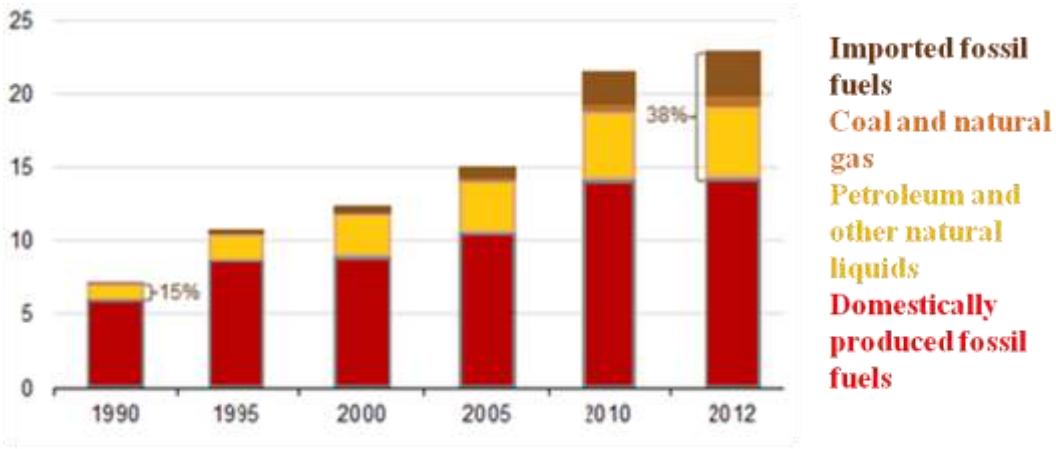


Figure 1.1: India's fossil fuel consumption in Quadrillion British thermal unit.
(https://www.eia.gov/todayinenergy/detail.php?id=17551)

A renewable fuel i.e. biodiesel is considered as one of the best alternate for replacing the diesel fuel. The Indian government announced National Policy on Biofuels in December 2009 in order to meet the energy demands and provide energy security to the country. The policy mainly aimed at development and utilization of various non-food

feed stocks cultivated on unutilized or waste lands, with major focus on research and development on fostering, processing and manufacturing of biofuels. Based on policy, by 2017 blending of 20% biofuels (bio-diesel and bio-ethanol) has also been proposed.

Biodiesel besides reducing the fossil fuel consumption has a photosynthetic origin which does not contribute in raising the CO_2 level in atmosphere. It is biodegradable and significantly reduces the emission of toxic and other gases when used as an automotive fuel. Biofuel production in an area or a village facilitates the supply of its own energy needs and thus providing local energy security, and at the same time generates job opportunities for the people.

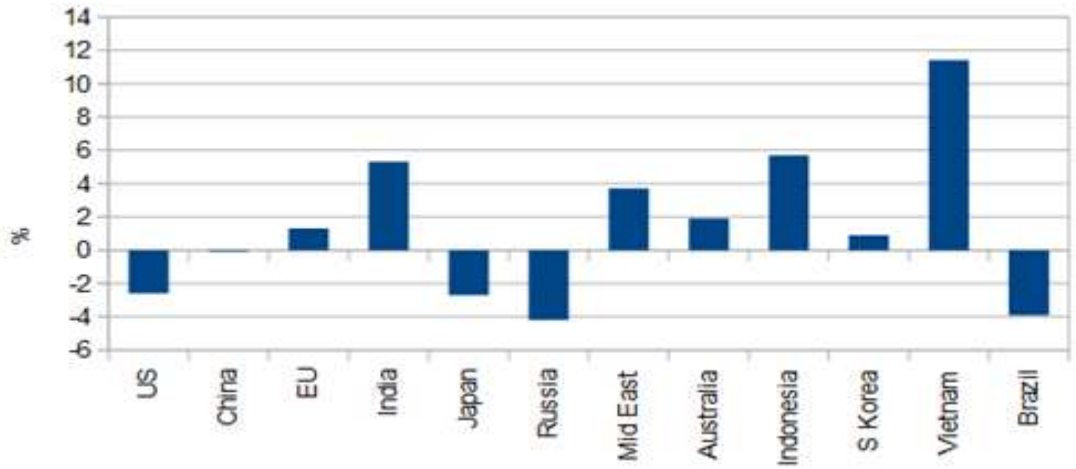


Figure 1.2: Percentage change in CO_2 emissions (2015 v 2014)

[http://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-worldenergy.html]

The use of food crops for the production of biofuels may cause competition between food and fuel (Figure 1.3). In December 2007 report, United Nations Food and Agriculture Organization (UN FAO) calculated a 40% rise in world food prices in one year which included primarily sugarcane, soybeans, corn, palm oil, rapeseed oil and all being major biofuel feedstocks (Tenenbaum, 2008). So, to overcome these problems, the potential of producing biofuel from non-edible crops, e.g. *Jatropha curcas* should be investigated. Indian government focused on utilization of waste or non forest lands and degraded forests for the cultivation of crops and trees containing non-edible oil seeds which can be used for production of bio-diesel. *Jatropha* is a

small tree belonging to *Euphorbiaceae* family which has gained remarkable interest as a raw material for biodiesel industries as the seed of this plant contains a good amount of oil. *Jatropha* had its origin in Central and South America, but lately cultivated in South-East Asia, India and Africa and has around 175 known species (Herrera *et al.*, 2006). *Jatropha* is a fast growing shrub or tree which reaches a height of 3-5 m within three years of its cultivation. It has a smooth grey bark which when cut exudes watery and sticky latex.

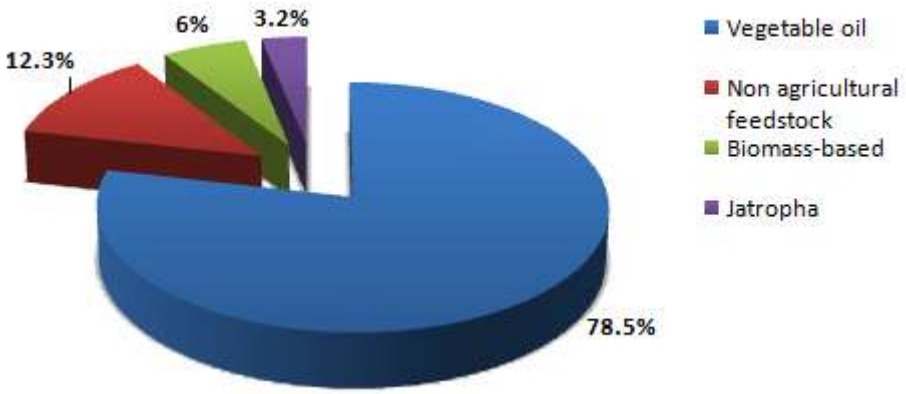


Figure 1.3: Feedstock for the production of Biodiesel

Jatropha curcas has a good adaptability to grow in unfavorable climatic conditions like infertile soil with scanty rainfall and therefore the utilization of wastelands can be made by growing this hardy plant. Almost all parts of this plant, *Jatropha curcas* has been widely used for a variety of purposes (Gubitz *et al.*, 1999; Augustus *et al.*, 2002). The tree itself is being used as a hedge plant providing plant protection. The bark of this plant yields a dark blue dye and is rich in tannin. Leaves of *Jatropha curcas* have been widely used for rearing of silkworm, in production of dyes, medicines, and also have anti-inflammatory properties. Latex of the tree also has medicinal and pesticidal properties and has been used for wound healing. Seeds have been used as insecticide and can also be used as food/fodder when detoxified or non-toxic varieties are used. Fruit hulls of this plant are combustible and can be used for the production of biogas and as green manure. *Jatropha curcas* has gained centre of attention due to its high amount of non-edible oil which has also found applications in

soap and candle industries and its by-product glycerine has been used in many pharmaceutical industries.

The *Jatropha* seed contains 30–35% oil (approx.) which can be used as a substitute for diesel fuel by converting it into high quality biodiesel upon trans-esterification (Makkar and Becker, 2009). The *Jatropha* seed weighs 0.53 -0.86 g (approx.) and because of its high mass per unit volume it can be transported economically without any deterioration. Oil from *Jatropha* seeds is extracted by a mechanical or pneumatic screw press which is then used for biodiesel production. The *Jatropha* oil consists of fatty acids i.e. saturated (14.2% palmitic acid and 7.0% stearic acid) and unsaturated fatty acids (44.7% oleic acid and 32.8% linoleic acid). The seed cake left after oil extraction by screw press contains about 500–600 g/ kg indigestible shells. The shell can be used as fuel and converted to briquettes to be used in cook stoves. Furthermore, the ash remaining after shell combustion can be used for soil enrichment as it has high sodium and potassium content.

It has been estimated that 70- 75 % of the *Jatropha* seed cake is obtained after the extraction of oil from seed (Singh *et al.* 2008). *Jatropha* seed cake can be used as a substitute for chemical fertilizers (Prueksakorn and Gheewala, 2008) and high organic matter of the seed cake makes it appropriate for biogas generation (Staubmann *et al.* 1997; Singh *et al.* 2008). However, it has been reported that biogas production from *Jatropha* seed cake produces 60 % more biogas than cattle dung (Singh *et al.* 2008) with more methane content giving it a higher calorific value. The seed cake has abundant plant nutrients which include lignocellulosic compounds, water, minerals, proteins; also the essential amino acids (except lysine) are higher than the reference protein recommended by Food and Agriculture Organization (FAO). But at the same time it contains many toxic and anti- nutritional compounds such as phorbol esters, phytic acid, trypsin inhibitors, phenolic compounds, lectins (curcin), and saponins in high amounts. Amongst all, phorbol esters have been recognized as the main compound responsible for the toxicity that limits the utilization of *Jatropha* seed cake for animal nutrition. Phorbol esters are tetracyclic diterpenes with tigliane skeletons (Cai-Yan *et al.*, 2010; Devappa, 2012) which are highly toxic, cathartic and skin irritant. Erythema, oedema, necrosis, diarrhea, loss of weight, reduced water intake

are some of the toxicity symptoms found in rodents, rabbits and goats when they were fed with phorbol ester containing feed. Phorbol esters are also known as tumour promoters since they have strong affinity towards Protein kinase C (PKC) which is involved in many cell signaling pathways. Phorbol esters are co-carcinogens which can intensify growth of cancer cells following exposure to subcarcinogenic dose of carcinogen (Goel *et al.*, 2007).

The potential of *Jatropha* as an energy crop can be enhanced by setting up a biorefinery which will increase the overall market value of *Jatropha* seeds. The production of biodiesel from *Jatropha* oil will be the main platform of biorefinery. The by-product, press cake is fractionated into various components e.g. carbohydrates, crude protein, crude fibre, ash, and minerals which can be further processed to produce various profitable products. Among the various components of *Jatropha* seed cake, *Jatropha* protein has a high market value. Proteins play an important role in many biological processes e.g. enzymatic catalysis, serve as transport & storage proteins, and provide mechanical support to the skin and bones. Carboxylic acid and amine groups in amino acids can serve as alternate raw material for the production of N-containing chemicals. Presence of good amino acid content makes proteins to be used as animal feed, food supplements and in many pharmaceutical products. Plant protein isolates being easily available and less expensive are more preferable than animal proteins and are therefore used for fortification, formulation of the food products with required functional properties. Because of certain functional properties like solubility, foaming properties, adhesiveness, emulsifying ability and film-forming properties, proteins are also well suited for technical applications. Comparing with other oilseed proteins like soy protein, rapeseed, or sunflower protein, *Jatropha* proteins have similar properties except the presence of toxic components such as curcin and phorbol esters which makes them unsuitable to be used for feed applications. Several studies have been conducted on *Jatropha* protein extraction which includes steam injection (Devappa and Swamilingaapa, 2008), counter current multistage extraction (Lestari and Mulder, 2010), iso electric precipitation (Hamarneh *et al.* 2010; Makkar *et al.*, 2008), enzyme assisted extraction process (Gofferje *et al.*, 2014) etc. Even though the application of the various extracts of *Jatropha* carries lot of beneficial advantages, yet the toxicity in oil and the compressed cake does not allow the by- products and the oil to be used elsewhere.

Successful utilization of *Jatropha* seed cake can only be achieved by complete removal of all the toxins and anti-nutritional compounds which will help in sustaining and improving the *Jatropha* biofuel production and welfare of the local farmers. Different methods have been employed for the detoxification of *Jatropha* press cake that would allow the use of detoxified meal as a protein-rich dietary supplement in the food or feed diets. Many researchers have made the use of physico-chemical and biological methods to detoxify *Jatropha* seed cake. The physical method for the detoxification mainly employed the use of moist heat but with not much breakthrough (Makkar and Becker, 1997a,b). However, moist heat along when combined with other treatments resulted in partial decrease in phorbol ester contents (Chivandi *et al.*, 2004). The use of chemicals like methanol and ethanol has shown promising results in reducing the toxin contents by 97-100%. Combination of physical and chemical treatments has also been demonstrated with better efficiency of detoxification (Chivandi *et al.*, 2004; Rakshit and Makkar, 2010; Sadubthummarak *et al.*, 2013). Phorbol ester content was reduced from 3850 ppm to 80 ppm by using 90% v/v ethanol-water extraction at room temperature for 2 h followed by treatment with sodium bicarbonate and autoclaving for 20 min (Herrera *et al.*, 2006).

Other methods of detoxification includes hydrothermal processing techniques (Herrera *et al.*, 2006), ionizing radiation, supercritical fluid extraction (SCFE) using CO_2 (Pereira *et al.*, 2013) etc. The aqueous/organic solvent extracts from *Jatropha* seed after detoxification by SCFE method can be effectively used as insecticidal and antimicrobial agent (Pereira *et al.*, 2013). According to Diwani *et al.* (2011), ozone treatment can be the best and less expensive method for removing toxic phorbol esters from *Jatropha* seed cake as it consumes less time and chemicals. The use of γ-radiations have also been made by Gogoi *et al.* (2014) to detoxify the seed cake in which upto 96% reduction in phorbol ester was achieved on exposure of the seed cake to a dose of 125 kGy. Combination of UV irradiation and chemical treatments has also been experimented for the reduction of phorbol esters. Xiao *et al.* (2015) irradiated *Jatropha* oil at wavelengths 220 to 400 nm at 25 °C for 40 min which resulted in 100% reduction of the phorbol esters. Not much research is documented as far as radiation therapy for detoxifying the phorbol esters.

On the contrary, biological detoxification does not involve the use of harsh chemicals or mixes and taking into consideration the safety and energy concerns, the biological methods are more advantageous than the others. Many micro organisms have been employed either using solid state fermentation method or submerged fermentation method for the detoxification of *Jatropha* seed cake. Use of white rot fungi (Barros *et al.,* 2011), *Aspergillus niger, Rhizopus nigricans, Penicillium chrysogenum, Rhizopus oligosporus and Trichoderma longibrachitum* (Belewu and Sam, 2010), *Pseudomonas aeroginosa* (Chetna *et al.,* 2011) and many other micro organisms have been made for detoxifying the seed cake. Submerged fermentation by Bacillus sp. achieved complete detoxification of phorbol esters within a week (Chang *et al.,* 2014).

Detoxifying seed cake with complex and unspecified toxic constituents is still a difficult task for the *Jatropha* biodiesel industry. Detoxification procedures in literature mainly focused on the degradation of the main toxic constituent i.e. phorbol esters. No encouraging advancement has been made from various physical and chemical methods to fully degrade or inactivate phorbol esters from *Jatropha curcas* seed cake. Treatment of seed cake with chemicals such as methanol and ethanol results in complete removal of phorbol esters from the seed cake, however, handling and disposing the toxins extracted raises an environmental and health concern. In addition, employing the use of organic solvent is not economical and may leave a residual effect on the animals consuming the feed and this necessitates the need of more advanced research and techniques. Microorganisms capable of degrading all kinds of the toxins simultaneously in the *Jatropha* press cake would be helpful in solving above-mentioned problems. Effective detoxification of *Jatropha curcas* seed cake would ensure the development of bio-safe animal feed and fertilizer which would help in reducing the cost of biodiesel industry. This objective can be achieved by using an environmental friendly microbial process which should be efficient, fast and should not produce toxins by itself. Detoxification of phorbol esters by submerged fermentation process could be a good option than solid state fermentation as it requires less time and the process well suited for organisms requiring higher moisture levels for growth. Biological degradation of phorbol esters from *Jatropha* seed cake could be a lengthy process which might be considered long for the industry.

So, optimization studies should be carried out to extract maximum phorbol esters faster and more efficiently. Based on this the following objectives were framed for the research work:

OBJECTIVES

To explore the possibility of making potential use of *Jatropha* seed cake, a byproduct of the biodiesel industry, my proposal aims at the following objectives:

1. Analysis of critical factors for phorbol ester degradation in *Jatropha* seed cake using ISM and MICMAC
2. Isolation and screening of esterases producing microbes.
3. Process optimization of detoxification of *Jatropha* press cake toxins using design of experiments.
4. Estimation of protein present in the *Jatropha* press cake for its utilization as animal feed.
5. Comparative study of raw and detoxified seed cake on in-vitro/in-vivo digestibility in animal models.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Jatropha*

The *Jatropha curcas* plant is a native of Mexico and states of Central America and was later grown by Portuguese traders as a hedge plant and got spread to Asia and Africa (Parawira 2010; Trabuccu *et al.*, 2010). *Jatropha curcas* belongs to Division- Magnoliophyta, Class- Mangnoliopsida, Order- Malpighiales, Family- Euphorbiaceae, Sub family- Crotonoideae, Tribe- Jatropheae, Genus- *Jatropha*, and Species- Curcas (Dehgan and Webster, 1979). The genus *Jatropha* has approximately 175 known species. The genus name *Jatropha* is derived from Greek word jatros (doctor), trophe (food), which suggests its medicinal uses, hence the plant has been traditionally used for medicinal purposes (Makkar and Becker, 2009) .The most common vernacular names of *Jatropha* curcas in India are Ratanjyot (hindi), Safedarand, Physic nut (English), Purging nut (English), katamanak (Malayalam), kattamanakku (tamil), Pepalam (telugu), Jepal (gujrati), kananaranda (Sanskrit), Chandrajyot etc (Internet source). *Jatropha* is a large shrub or small tree usually 3-5 m in height with a smooth grey bark (Figure 1), which when cut exudes watery and sticky latex. It grows suitably well on aerated drained soils (Gour, 2006) and is also well adapted to soils having low nutrient content. They are widely distributed in the tropical and subtropical Himalayas, the mountains of Western and Eastern Ghats and plains of South India (Internet source). According to Raju and Ezradanam (2002), *Jatropha curcas* is a perennial plant which grows during the rainy season mainly in July till late October and once the flowering begins, it lasts for 11 days. The growth of this plant varies from state to state under various climatic conditions like in a study done by Bhattacharya *et al.* (2005), in the National Botanic Garden, Lucknow, flowering of *J. curcas* was mainly in the month of July till September. In India, 18 species are found distributed in various parts of the country which are *Jatropha curcas, Jatropha gossypifolia, Jatropha glandulifera, Jatropha heynei, Jatropha integerrima, Jatropha maheshwarii, Jatropha multifida, Jatropha mulendnifera, Jatropha villosa, Jatropha*

nana, Jatropha podagrica, Jatropha hastate, Jatropha tanjovurensis, Jatropha hastate, Jatropha macrofayala, Jatropha acrocurcas, Jatropha diyoka and Jatropha sinera (Figure 2.1). Out of these, *Jatropha curcas* gained importance because of its additional features like adaptability to various habitats, larger fruits and seeds, high oil yield, soil conservation capabilities etc. Paroda and Mal (1989) also reported that *Jatropha* can be cultivated even in poor stony soil, on waste or barren lands where there is lack of irrigation facilities and thus makes an ideal choice for the utilization of waste land resources. Also being a forage crop, it helps in keeping out the cattle and protecting other valuable crops by serving as a hedge.

Figure 2.1: *Jatropha* species grown in India: (A) *Jatropha curcas* (B) *J. multiida* (C) *J. gossypifolia* Red leaf, (D) *J. gossypifolia* Green leaf, (E) *J. integerrima* and (F) *J. podagrica*. (Snehi *et al.*, 2016)

2.2 Composition of *Jatropha*

Jatropha curcas is an economic tree that cultivates well in the sub-tropical and tropical climate, and therefore plays an essential role in controlling soil erosion and land restoration (Inekwe *et al.*, 2012; Estrin, 2009; Achten *et al.*, 2010). Sirisomboon *et al.* (2007) reported that the *Jatropha* fruit hull had high moisture content than nut shell and kernel and the fruit (whole) contained a moisture content of 77.03%. The

moisture content in *Jatropha* seeds varied from 4.75–19.57% (Garnayak *et al.*, 2008). In a study done by Sirisomboon and Kitchaiya (2009) the *Jatropha* kernels has a moisture content of 3.78, 4.01 and 2.82% (on wet basis) at temperatures 40, 60 and 80^0 C respectively. *Jatropha* is considered to be a good source of proteins and lipids indicating good nutritional value. The study of *Jatropha curcas* seeds showed that it contains; moisture 6.62%; protein 18.2%; fat 38.0%; carbohydrates 17.30%; fibre 15.50%; and ash 4.5% (Gubitz *et al.*, 1999). Makkar *et al.*, (1997) distinguished eighteen varieties of *Jatropha* from different sources on the basis of their nutrient and anti-nutritional factors in which a large variation was found in crude protein (19-31%), lipid (43-59%), fibre (3.5-6.1%) and ash (3.4-5%) in *Jatropha* kernels. However, not much difference was observed in gross energy value of kernels which was in the range 28.5-31.2MJ/kg. According to Arab and Salem, (2010), *Jatropha* seeds are also rich in micro nutrients such as Mn (28.37 mg/kg), Fe (0.38 mg/kg) and Zn (47.13 mg/kg) and some macro-elements like K (34.21 mg/kg), Ca (103.13 mg/kg), Mg (109.89 mg/kg), Na (8.44 mg/kg) and P (185.17 mg/kg).

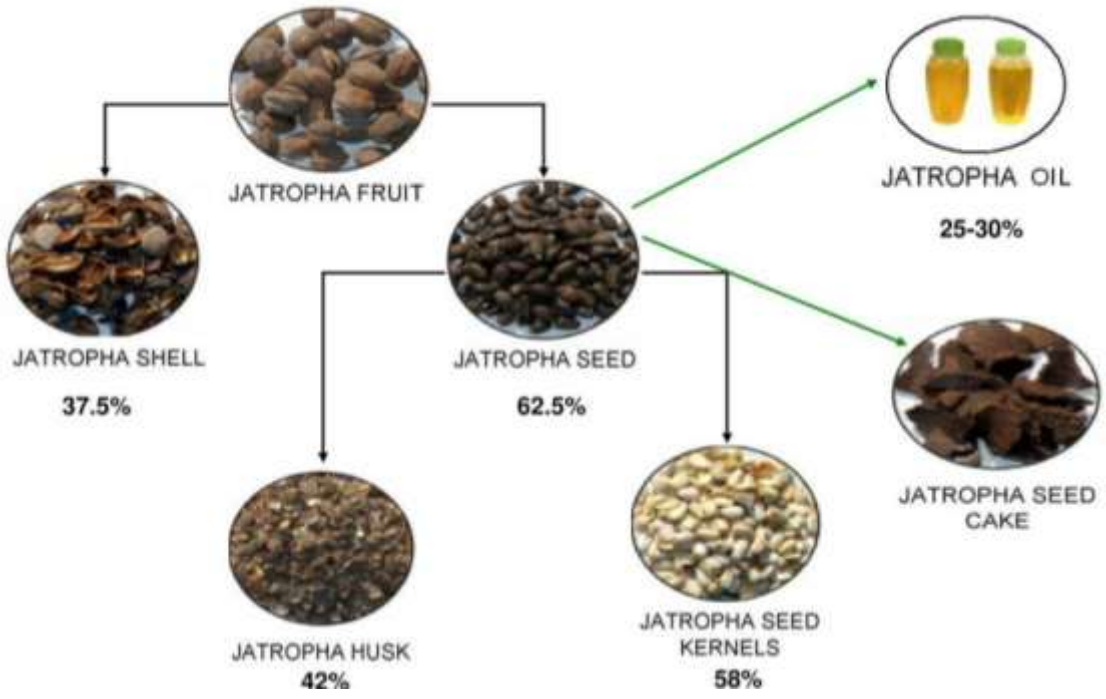


Figure 2.2: **Composition of *Jatropha* (Abreu, 2009)**

The seed oil from *Jatropha curcas* has been used as a reliable raw material for bio-diesel production and for this purpose several tones of seeds are screw pressed which results in the production of de-oiled seedcake. The *Jatropha* oil is usually extracted by crushing *Jatropha* seeds as a whole in a screw press. The oil content of the *Jatropha* seeds ranges between 25-30 % by weight of the seeds and the remaining percentage is of seed cake (Staubmann *et al.,* 1997; Singh *et al.,* 2008). In bio-diesel industry, 3 tons of seed cake renders one ton of biodiesel depending upon the seed quality (Mahajani, 2009). The *Jatropha* seed oil consists of both saturated and unsaturated fatty acids and amongst saturated fatty acids oleic acid (41.5- 48.8%) is present in high amount followed by linoleic acid (34.6-44.4%) and in unsaturated fatty acids, palmitic acid (10.5- 13.0%), and stearic acid (2.3-2.8%) are most commonly found (Herrera *et al.*, 2006).

Jatropha proteins can be obtained from the press cake which contains around 25% w/w proteins. The defatted pressed *Jatropha* seed cake has high protein content and the essential amino acid contents are higher than the FAO amino acid reference except lysine (Herrera *et al.*, 2006). In terms of functional properties and extraction conditions, *Jatropha* protein may be considered as good as to other well-known oilseed proteins such as rapeseed, canola, or sunflower proteins. Proteins from plants are less expensive than animal proteins so they can be effectively used for fortification, and formulation of food products with desirable functional properties. *Jatropha* proteins could be produced as the existing industrial proteins e.g. soy proteins, casein, or wheat gluten.

Jatropha seeds also contain certain antinutritional compounds like curcin, phytates, lectins, saponins and toxic compounds like phorbol esters which restrict the use of *Jatropha* proteins for food applications. Phorbol esters (PEs) are the major barrier which limits the use of *Jatropha* seed cake or proteins for commercial use. During oil extraction from *Jatropha* seeds, a major part (70-75%) of phorbol esters being lipophillic in nature goes with the oil while 25–30% remains bounded with seed meal matrix. The content of phorbol esters varies in the toxic and non-toxic varieties of *Jatropha*, while the highly studied species, *Jatropha curcas*, contains about 1–3 mg/g of phorbol esters in *Jatropha* meal and 3–6 mg/g in *Jatropha* oil (Gogoi *et al.*, 2014).

Anti nutritional factors such as phytates, tannins, saponins are present in 9.4, 0.04 and 2.60 % respectively in *Jatropha* kernel meal (Makkar and Becker, 2009a).

At industrial level, for the extraction of proteins from *Jatropha* with better yields and protein recovery a more efficient method is a required which should extract proteins having good functional properties e.g. water absorption, viscosity, solubility, foaming properties, flavor binding and emulsifying properties which can be used for food as well as technical applications.

2.3 Benefits of *Jatropha*

Owing to multiple uses, *Jatropha* cultivation can be made more profitable if along with *Jatropha* oil, other parts of the tree are made use of (Figure 2.3). There is growing evidence that *Jatropha* tree may provide a range of services which have not been explored properly because of the ignorance of the cultivators about the economics of this plant. It becomes essential to explore unlimited potential of *Jatropha* which would thus help in serving the community and creating employment for the people.

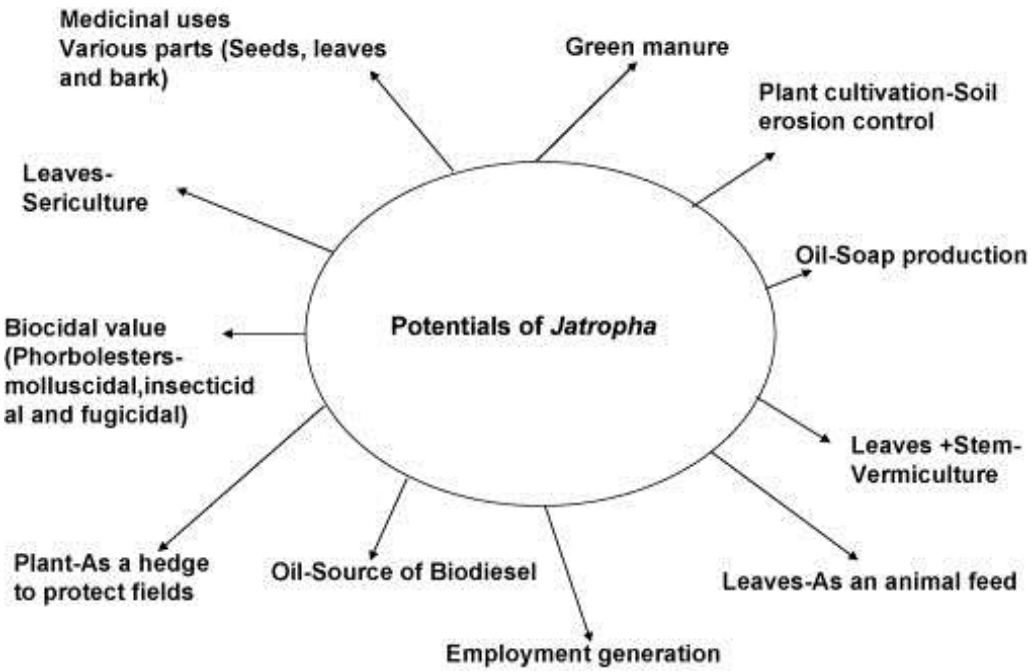


Figure 2.3: Potential uses of *Jatropha* (Kumar and Sharma, 2008)

2.3.1 *Jatropha* Tree

Jatropha curcas grows at a height of 3-4m and therefore commonly grown as live hedge around agricultural fields for keeping out farm animals (Gubitz *et al.*, 1999). The toxicity of this plant hinders the animals from grazing in fields and helps in protecting valuable crops. It can be easily cultivated by growing seeds or branch cutting and not affected by dry weather conditions. *Jatropha* fences also help in preventing wind and soil erosion (Openshaw, 2000) and thus increase the fertility of the agricultural fields. Simple process with low input and maintenance cost of these *Jatropha* hedges adds more features for growing this crop.

2.3.2 Roots and bark

The roots of *Jatropha* help in controlling soil erosion as discussed and also produce yellow colored oil which helps in imparting different shades of brown color to cotton and also has many pharmaceutical applications. The roots of this plant are known to contain an antidote for snake venom (Thomas *et al.*, 2008; Akinpelu *et al.*, 2009) and are also used for treating eczema, scabies, ringworm and gonorrhea (Aiyelaagbe *et al.*, 2007). The bark of this tree contains tannins which can be used in leather industry and for manufacturing blue dyes (Katwal and Soni, 2003).

2.3.3 Leaves, flowers and fruits

Many parts of *Jatropha* plant are used for curing human and veterinary diseases. The leaves of plant have certain compounds which make them effective against malaria (Henning, 1997), rheumatic and muscular pains (Prasad *et al.*, 2012; Heller, 1996). The white latex produced by leaves works as a disinfectant in mouth infections in children and as a hemostatic in cuts and bruises (Esimone *et al.*, 2008). Also, the latex of *Jatropha* has anti-cancerous properties (Oskoueian *et al.*, 2011; Singh *et al.*, 2003) and can be used externally for skin diseases (Prasad *et al.*, 2012) piles and sores among the domestic livestock (Thomas *et al.*, 2008). *Jatropha* flowers attract bees and hence provide an opportunity for production of honey (Openshaw, 2000). *Jatropha* fruit is 2.5-3cm long and ovoid in shape and contains 2-3 seeds per fruit. *Jatropha* fruit consists of 35-40% shell and 60-65 % seed by weight and the seed contains 58% kernel and 42%

husks (Abreu, 2008; Singh *et al.*, 2008). All these components of the *Jatropha* fruit have the potential to be used as sources of bioenergy.

2.3.4 *Jatropha* seed

The weight of the *Jatropha* seed is 0.53 -0.86 g approximately and consists of a kernel which is white in color and covered by a hard, black outer shell. The seed consists of 60–65% crude lipid and 30–34% crude protein. Because of high specific mass of the seeds they can be transported at very low cost without damage. The seeds of *Jatropha curcas* comprise of about 20 to 39% oil which makes them as an important source for bio-fuel production. Also, the oil from its seeds has been found to be useful in cosmetic industry, for the production of candles, soaps and also for medicinal purposes (Gubitz *et al.*, 1999; Akbar *et al.*, 2009). The oil fraction of *Jatropha curcas* consists of both and unsaturated fatty acids.

Table 2.1: Composition of *Jatropha* seed (Marieke *et al*., 2010; Dianika *et al*., 2009)

Components	Seed (%)
Oil	35
Crude Protein	17 - 15
Lignin	20
Fibre	15- 18
Carbohydrate	5- 10
Ash	5

2.3.5 *Jatropha* husks and oilseed shells

The *Jatropha* husk contains 4 % ash, 25% fixed carbon and 72% volatile matter which makes it ideal to be used as potential fuels. Many researchers have successfully used *Jatropha* husk to produce gas, however, due to high ash content in *Jatropha* shell it cannot be easily converted to gas (Cooke, 2009). The shell can be made use by converting it into briquettes and for burning in stoves for cooking purposes. The shell after combustion has a good content of sodium and potassium

which can be used for soil enrichment. The shell contains 34% cellulose 10% hemicellulose and 12% lignin. Along with this, it has a volatile matter, ash and fixed carbon content to be 69%, 15% and 16%, respectively (Singh *et al.*, 2008). A good chemical composition of shell and husk may reduce the need of adding chemical fertilizers in the field, when these are left in field and ploughed.

2.3.6 Antimicrobial activity of *Jatropha* extracts

The *Jatropha* extracts have been shown to exhibit selective activities against the various micro-organisms. This property may be attributed due to the existence of soluble phenolic and polyphenolic compounds in the extracts. Antibiotic activity of *Jatropha* has also been reported against certain micro organisms like *Staphylococcus aureus* (Thomas *et al.*, 2008; Kalimuthu *et al.*, 2010; Ekundayo *et al.*, 2013; Arekemase *et al.*, 2011; Nwankwo *et al.*, 2014; Arun *et al.*, 2012; Leonard *et al.*, 2013; Rachana *et al.*, 2012), *Escherichia coli.* (Thomas *et al.*, 2008; Ekundayo *et al.*, 2013; Arekemase *et al.*, 2011; Nwankwo *et al.*, 2014; Arun *et al.*, 2012; Leonard *et al.*, 2013), *Enterobacter aerogenes* (Beni *et al.*, 2014),*Streptococcus pyogenes* (Nwankwo *et al.*, 2014; Leonard *et al.*, 2013; Thomas, 1989),*Candida albicans* (Arekemase *et al.*, 2011; Leonard *et al.*, 2013; Thomas, 1989), *Salmonella typhimurium, Shigella dysenteriae, Psuedomonas aerugunosa* (Arekemase *et al.*, 2011; Rachana *et al.*, 2012; Egharevba and Kunle, 2013; Rajesh *et al.*, 2012; Deepmala and Gaikwad, 2013), *P. flourescenses, Klebsiella pneumonia* and *K. ozaenae* (Egharevba and Kunle, 2013), *Aspergillus flavus, Neisseria gonorrhea* (Arekemase *et al.*, 2011), *Erwinia carotovora, Xanthomonas* sp. (Deepmala and Gaikwad, 2013), *Aspergillus niger*, *Penicillum fellutanum* (Leonard *et al.*, 2013).

2.4 Toxicity in *Jatropha*

Jatropha plant extracts not only from oil and seed, but from roots, bark, latex and leaf are also toxic to a number of microorganisms and animals. Many cases of accidental consumption of *Jatropha* seeds by children are also well documented (Abdu-Aguye *et al.* 1986; Kulkarni *et al*., 2005, Joubert *et al.*, 1984, Koltin *et al.*, 2006). In a report by Anuj *et al.*, 2016, during 2014 in Agra, while consuming some edible sunflower

seeds, some people because of their ignorance also consumed scattered *Jatropha* fruits & seeds which were then hospitalized. 23 patients were admitted, out of which 20 were minors and 3 adults with a major complaint of abdominal cramps, nausea, vomiting and diarrhea. Children in the age group of 6-12 years were most affected which were then discharged and later recovered successfully because of proper treatment. In another case reported by Singh *et al.* (2010), four children in the age group of 5-8 years were admitted in hospital in emergency department with the symptoms of vomiting and pain in abdomen. On enquiring, children admitted the ingestion of *Jatropha curcas* seeds because of their sweet taste. On giving medication, their symptoms subsided between 5-10 hours and were discharged next day. In both the cases, presence of curcanoleic acid or Jatrophin, curcin, phorbol esters and cyanic acid were held responsible for toxicity. However, the toxic dose is unknown but consuming large quantities of any part of *Jatropha* plant may cause slow damage to kidneys. The seeds and oil of *Jatropha curcas* have found to be toxic because of the presence of some active components like phorbol esters, phytates, lectins, saponins and trypsin inhibitors.

2.4.1 Phorbol esters

Phorbol esters are the major toxic substances found in *Jatropha curcas* which act as a carcinogenesis promoter along with a wide range of biochemical and cellular effects on animals (Ahluwalia *et al.*, 2017). The toxicity of *Jatropha* seed, *Jatropha* oil and *Jatropha* seed cake is predominantly because of this toxin i.e. phorbol esters. Common carp has been found to be extremely sensitive to phorbol esters (Becker and Makkar, 1998). A level as low as 15 ppm in the feed can reduce the growth, feed intake and the production of faecal mucus which makes it mandatory to remove these esters from the feed. It was found to be toxic to mice, rats, sheep, calves, goats, human and chickens and the major organs affected were kidneys, liver, lungs, GIT, spleen and heart (Aregheore *et al.*, 2003; Makkar *et al.*, 2012; Goel *et al.*, 2007; Adam, 1974). The high levels of phorbol esters in the seed cake had been identified as the main agent responsible for toxicity.

2.4.2 Trypsin inhibitors

Trypsin inhibitors are the substances that have the ability to hinder the activity of proteolytic enzymes mainly trypsin in gastrointestinal tract of animals. These enzymes are involved in the breakdown of many proteins in the body and their unavailability interfere the digestion process and hence leads to severe growth depression. Makkar and Becker (1999) reported that trypsin activity can be reduced significantly when *Jatropha* meal (non-toxic) is given 15 min moist heat treatment. However, they also reported high levels of trypsin inhibitors don't have any inhibitory effect on the growth and development of fish. Plant feeds having high level of TIs, a moist heat treatment for 15-30 min is recommended (Norton, 1991).

2.4.3 Lectin

Lectin is a glycoprotein mostly found in legume seeds and is highly specific for sugar moieties. They are also called phytohaemagglutinins as they agglutinate red blood cells. The mechanism of action involves the reversible binding of lectins with carbohydrate complexes of glycoconjugates there in cell membranes (Francis *et al.*, 2001). Interaction of lectins with the intestinal epithelium reduces the viability of epithelial cells along with many biological effects such as morphological damage to the villi, interrupting the small intestine metabolism, reduction of insulin levels in blood, breakdown of stored glycogen and fat, difficulty in absorption of iron and fats from diet, etc. (Makkar *et al.*, 2007). However, heat treatment at 100°C for 10 min can easily remove lectin from the seeds. In a study by Aregheore *et al.* (1998), moist heating at 100^{o}C for 10 min reduced lectin content in *Jatropha* seed meal from 102 to 1.17 (haematogglutination units).

2.4.4 Phyates

Phyates are the storage form of phosphorous which is widely found in nuts, edible seeds, grains, beans or legumes. When the phytic acid in the seeds gets bound to mineral it gets converted into phytate. This is one of the anti-nutrients found in high concentration in *Jatropha* meal. Phytate bound phosphorus being indigestible constituent is inadequately utilized by the body and gets excreted in faeces. Also,

phytate bound phosphorus acts as anti-nutritional factor in the diet as it reduces the bioavailability of minerals, mainly Ca, Mn, Zn, Fe as it forms an insoluble phytate-metal complex (Hallberg *et al.*, 1987). Phytates also form an indigestible protein-phytate complex which reduces the protein digestibility and also affects the digestibility of starch by inhibiting the activity of amylase (Francis *et al.*, 2001).

2.4.5. Saponins

Saponins are glycosides of steroids and triterpenes which is widely found in plants. It consists of polycyclic aglycones attached to one or more sugar side chains by glycosidic linkage. Saponins are responsible for giving a bitter taste, formation of foams in aqueous solution and haemolysis of red blood cells (Francis *et al.*, 2001). They also decrease the protein digestibility by forming indigestible saponin-protein complexes and also inhibit the activity of digestive enzymes such as trypsin and chymotrypsin (Sapna *et al.*, 2009). Animal feeds containing saponin have shown adverse effects on the growth, feed intake, histology of fish (Krogdahl *et al.*, 1995; Kaushik *et al.*, 1995; Bureau *et al.*, 1998).

2.4.6 Tannins

Tannins are the astringent, water soluble polyphenolic compounds widely found in food crops and legumes and are categorized into hydrolysable and non-hydrolyzable or condensed tannins (Francis *et al.*, 2001). Tannins interfere in the digestion process by binding to the enzymes or with the feed components such as proteins and minerals. However, the major effect of tannins is due to the decreased efficiency in converting the absorbed nutrients to new substances. Absorption of Vitamin B_{12} is also affected by the presence of tannins. Tannins have been associated for a number of metabolic changes in the experimental animals like decrease in growth rate, net metabolizable energy, feed intake, feed efficiency and protein digestibility (Chung *et al.*, 1998). Because of astringency and bitter taste, tannins are not considered good candidates for food/feed supplements. However, tannins from food processing wastes or edible sources can prove to be more useful in the control of metabolic diseases on animal models (Panzella and Napolitano, 2017). As the tannins are present in the

outer layer of the seed, they can be easily removed by dehulling seeds, by autoclaving or giving alkali treatment to the seeds. However, the *Jatropha curcas* seed cake may serve as very nutritious protein supplement in animal feed but the presence of toxins significantly restricts its use as feed.

2.5 Phorbol esters in *Jatropha*

The toxicity of *Jatropha* is caused by several components and mainly by phorbol esters. *Jatropha* species known and widely studied for toxicity are *Jatropha curcas, Jatropha macarantha, Jatropha elliptica, Jatropha podagrica, Jatropha glauca, Jatropha gossypifolea, Jatropha aceroides, Jatropha tanoresisi, Jatropha integerrima, Jatropha glandulifera, and Jatropha multifida* (Devappa *et al.*, 2010). Some of these species are known to contain a mixture of other toxins along with phorbol esters. Non-toxic varieties of *Jatropha* i.e. *Jatropha curcas* and *Jatropha platyphylla* are being cultivated in Mexico and have been used as food for human while toxic varieties having high phorbol ester content are also cultivated in Mexico, Central America and other parts of the world (Bueso *et al.*, 2016). The phorbol ester content in *Jatropha* varies from region to region and country to country. In a study done by Ahmed and Salimon, (2009), there were significant variations in the phorbol ester content of *Jatropha curcas* seed of three different provenances Malaysia, Indonesia and India. Phorbol esters content was lowest in Malaysian variety (0.23%) while the Indonesian and Indian variety consisted of 1.58 % and 0.58 % respectively. Major anti-nutrients present in toxic and non toxic varieties of *Jatropha* seed meal are presented in Table 2.2.

Table 2.2: Major anti-nutrients in toxic and non toxic *Jatropha* seed meal (Makkar, 2007)

Component	Non Toxic	Toxic
Phorbol esters (mg/g kernel)	2.79	ND to 0.11
Total phenols (tannic acid equivalent %)	0.36	0.22
Tannins (tannic acid equivalent %)	0.04	0.02
Phytates (dry matter %)	9.40	8.90
Saponins (diosgenin equivalent %)	2.60	3.40
Trypsin inhibitor (mg trypsin inhibited per g sample)	21.3	26.5
Lectins (1/mg of meal that produced haemagglutination per ml of assay medium)	102	51

Phorbol esters are tetracyclic diterpenes with a tigliane skeletal structure (Cai-Yan *et al.*, 2010; Devappa, 2012) and are highly toxic, cathartic and skin irritant. Phorbol esters have been known as tumour promoters since they bind and activate protein kinase C that play an important role in cell signaling pathways. Although they are involved in tumour promoting processs, they are not themselves mutagenic or carcinogenic in nature.

Other than *Jatropha curcas,* a number of plants have been reported to contain the toxic phorbols like *Sapium indicum, Sapium japonicum, Euphorbia frankiana* (Beutler *et al.*, 1989). Haas *et al.* (2002) characterized six phorbol esters (*Jatropha* factors C_1–C_6) from *Jatropha curcas* seed oil where all complexes have the same diterpene moiety, namely, 12-deoxy-16-hydroxyphorbol (Figure 2.4). Phorbol esters are widely distributed in different parts of the *Jatropha* plant but they are mainly concentrated in the seed kernel (Ahmed and Salimon, 2009) and during extraction of oil from seeds, being lipophilic in nature 70-75% of phorbol esters go with the oil, and 25-30% remain in the seed meal (Gogoi *et al.*, 2014). *Jatropha curcas* seeds have shown toxicity towards humans and animals due to the presence of phorbol esters. Researchers therefore advised handlers to use precautions like wearing glasses and gloves for protection while handling *Jatropha curcas* and its products (Devappa *et al.*, 2013).

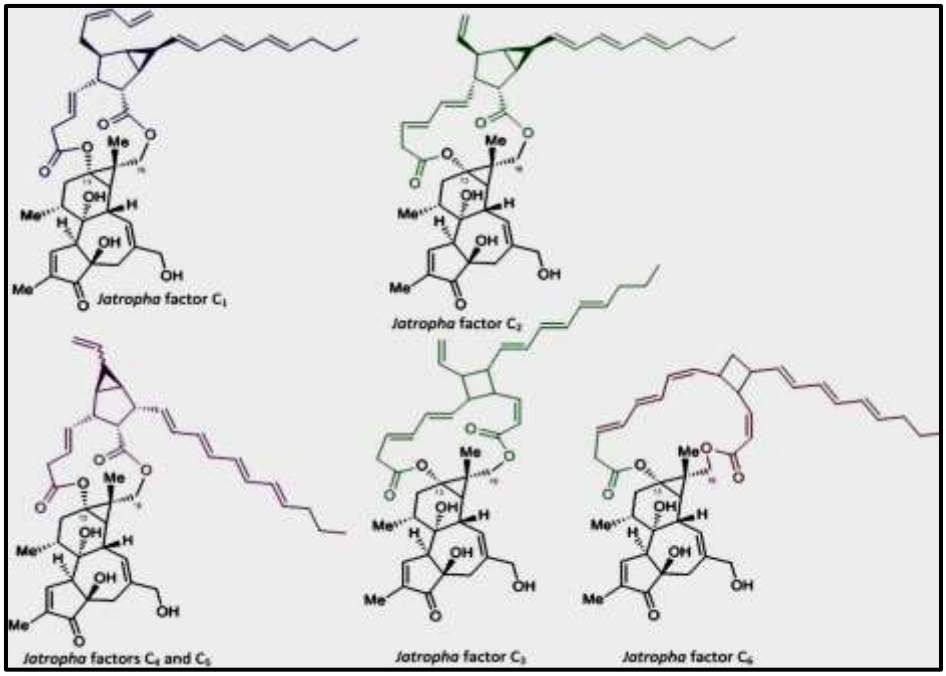


Figure 2.4: Structure of phorbol esters (C_1–C_6) in *Jatropha curcas* oil (Haas *et al.*, 2002)

In humans, phorbol esters intoxication may lead to mouth pain, vomiting, muscle shock, high pulse or even death (Wink *et al.*, 1997). Erythema, oedema, necrosis, diarrhea, loss of weight, reduced water intake are some of the toxicity symptoms found in rodents, rabbits and goats when they were fed with phorbol ester containing feed. Becker and Makkar (1998) carried out the toxicological study of phorbol esters in fish and observed lower metabolic rate, rejection of feed and increase in fecal mucus production as adverse effects of phorbol esters. In a study by Liu *et al.* (1997), phorbol esters extract i.e. 4β-Phorbol-13-decanoate showed molluscicidal activity against two different species of snails, *Oncomelania hupensis* and *Biomphalaria glabrata* and killed both species at a very low concentration of 0.001% (10 ppm). The snail bioassay has been considered the most sensitive of all with 100% mortality rate at 1 µg of phorbol esters.

Phorbol esters were intragastrically given to mice, and the LD_{50} for mice was 27.34 mg/kg body mass. The results of histopathological studies of dead mice revealed that lowest dose (21.26 mg/ kg body mass) did not affect the organs of the mice, doses >or 32.40 mg/kg body mass induced changes like haemorrhage in the lungs, glomerular sclerosis, and atrophy in the kidneys and highest dose of 36.00 mg/kg body mass showed significant changes like abruption of cardiac muscle fibers and anachromasis of cortical neurons (Hirota *et al.*, 2017).

2.6 Conventional uses of *Jatropha* seed cake

India is one of the world's largest oilseed producers producing over 25 MT/annum and exporting over 4.3 MT of oil cake with an assessment of around US$ 800 million annually (www.seaofindia.com)

Seed cake is the residue or by-product obtained after the extraction of oil from the seeds. The oil extraction can be done by two processes i.e. by extrusion process (applying pressure) or by using solvent (multiple extractions). Solvent extraction method removes more oil as compared to extrusion, and the residual material left after any of the process is known as cake. Oil cakes can be categorized into two i.e. edible and non-edible. Seed cakes from coconut, groundnut, cottonseed, mustard oil etc are edible and can be used for animal feeds. Non edible oil cakes such as *Jatropha*

(*Jatropha curcas*), neem (*Azadirachta indica*), karanja (*Pongamia pinnata*), castor (*Ricinus communis*), and mahua (*Madhuca indica*) are rich sources of micro as well as macronutrients but at the same time also contain some toxins and anti-nutritional factors and therefore can be used as plant nutrients/manure only.

The oil cakes usually vary in their composition because of various factors like different growing conditions, variety and quality of seeds, extraction method, storage parameters etc likewise *Jatropha* seed cake's composition also varies because of the same reasons stated above (Table 2.3). *Jatropha* seed cake contains a good amount of proteins and carbohydrates. Though *Jatropha* seed cake is rich in proteins, it is toxic and unpalatable due to the presence of phorbol esters and some anti-nutritional factors (discussed in section 2.4). Because of this reason the *Jatropha* seed cake cannot be directly used as feed for poultry and livestock. Discarding these toxic seed cakes will cause serious environmental problems and also increase the cost of biodiesel production and this call for the effective utilization of *Jatropha* seed cake in other possible ways in an environment friendly manner.

The transformation of *Jatropha* seed cake into valuable by-products may involve a number of processes which could be physical, chemical or biological. Research has been carried out globally on the value addition of the seed cake which is discussed in the following section.

Table 2.3: **Various constituents of *Jatropha* seed cake (Raphael and Kamusoko, 2017)**

Constituent	Quantity (%)
Protein	25–40
Carbohydrate	15–20
Fat	9-12
Fibre	15–32
Starch	0.63
Hemicellulose	5.55
Cellulose	20.3
Lignin	19.46
Ash	3–6

Jatropha seed cake can be used as a substitute for chemical fertilizers because of good content of Nitrogen, phosphorus and potassium (NPK) which is much higher (Table 2.4) than the NPK values of other fertilizers such as cow manure, chicken manure and Karanj-oil cake (Achten *et al.,* 2008). Also, the biological decomposition of *Jatropha* seed cake helps in improving soil fertility which enhances crop production.

Table 2.4: Comparison of N, P and K contents in various seed cakes and organic fertilizers (Achten *et al.*, 2008)

Fertilizer	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Jatropha curcas seed cake	4.44	2.09	1.68
Cow manure	0.97	0.69	1.66
Chicken manure	3.04	6.27	2.08
Compost of raw straw	0.81	0.18	0.68
Compost of municipal wastes	1.25	0.25	0.65
Karanj-oil cake	4.00	1.00	1.00
Neem oil cake	5.00	1.00	1.50

Chaturvedi *et al.* (2009) observed increase in growth and yield of tuberose (a commercial flower) when *Jatropha* press cake was used as an organic fertilizer. In a study done by Penjit *et al.*, 2012, treatment of Chinese kale with the low rate chemical fertilizer and high rate of *Jatropha* seed cake resulted in best marketable yield which was comparable to that obtained with full rate of chemical fertilizer. Chemical fertilizer combined with any grade of *Jatropha* seed cake (low, medium, and high) gave maximum tuber and tomato yield. Wheat yield also improved significantly when inorganic fertiliser was substituted with *Jatropha* press cake (Ghosh *et al.*, 2012)

Jatropha seed cake has a good amount of cellulose, hemicelluloses and lignin (Table 2.3) which makes it a suitable raw material for the production of bioethanol. Several studies have been conducted in which *Jatropha* seed cake has been used as a substrate for bioethanol production. Mishra *et al.* (2011) reported production of 80% ethanol when acid-hydrolysed JPC was fermented with *Sacharromyces cereviciae.* In Brazil, Dos Santos *et al.* (2014) reported in their study that 88.5L of ethanol can be produced by fermenting JPC hydrolysate (obtained after extraction of 1000 kg of oil) with *S. cereviciae.*

Use of *Jatropha* seed cake for biogas production has also been reported in literature (Grimsby *et al.*, 2013; Visser *et al.*, 2007; Staubman *et al.*, 1997). Biogas is a renewable fuel that is being used for supplying heat, electricity, steam and production of methanol. It is mainly composed of 50–60% methane, 38–48% inert CO_2, and 2% trace components. The cake has fairly good amounts of organic matter, proteins and carbohydrates for biogas production by anaerobic bacteria. Anaerobic fermentation of *Jatropha* press cake has been well documented and biogas production from JPC is equivalent to other raw materials like non-edible cakes and cow dung. It has been reported by various researchers that JPC can generate 60% more biogas compared to cattle/cow dung (Singh *et al.*, 2008; Raheman and Mondol, 2012).

Jatropha seed cake has also been used as a raw material for charcoal briquettes. Charcoal briquetting has been known as a recycling technology for treating large tons of *Jatropha* wastes. After the extraction of oil from the seed cake, JSC still contains small amount of oil which helps in increasing the fuel properties of JSC when used as briquettes. Many studies have been conducted to ascertain the use of *Jatropha* press cake briquettes as an energy source (Caezar *et al.*, 2015; Pambudi *et al.*, 2010; Raphael *et al.*, 2010). The briquettes should have less moisture content, ash content and production cost along with that, a good calorific value, combustion performance and compressive strength. The physical and chemical examination of the *Jatropha* seed cake suggested that JPC can be used as briquettes as it gave similar and in some reports even better results when compared to woody pellets or charcoal briquettes.

JPC contains a good amount of proteins which can be used as animal food but the presence of toxic and anti-nutritional components such as phorbol esters, phytates, trypsin inhibitors and lectins makes it inedible. The essential amino acids levels of JPC (except lysine) are also higher as compared to FAO reference protein. Extraction of protein from *Jatropha* seed cake can be done by various methods. Autoclaving seed cake at 121 °C for 15 min before protein extraction at room temperature doesnot affects the molecular weight distributions of the extracted protein (Marasabessy *et al.*, 2011). Many studies have revealed a protein yield ranged between 53-82% after the extraction and recovery. The chemical methods include using NaOH for protein extraction, protein precipitation using ammonium sulphate and a method based on the principle of isoelectric precipitation. Isoelectric precipitation method has been a

method of choice for extraction of proteins from *Jatropha* seed cake. Makkar *et al.* (2008) recovered around 53% of the total proteins from JPC using this method. JPC protein has many potential benefits but due to the presence of toxic components, they are limited to technical applications such as a raw material for production of adhesives, coatings and chemicals (Devappa and Swamylingappa, 2008). In a study by Gofferje *et al.* (2015), it was reported that the cast films made from JPC protein had the desired barrier and mechanical properties which can be used as coating material for food packaging. In another study, formaldehyde-free wood adhesives were prepared from JPC proteins which gave an alternate to formaldehyde-based resins (Zhang *et al.*, 2011). Many industrially important enzymes such as lipase and protease can be produced by solid-state fermentation of JPC (Mahanta *et al.*, 2008). For extracting proteins from *Jatropha* seed cake, a more competent method is required for obtaining high protein yield with high protein content and with good functional properties such as emulsifying properties, foaming properties, solubility, film-forming properties. These protein properties play an essential role in technical applications and are influenced by the extraction and preparation process as the extraction conditions may cause disruption of S-S bridges, denaturation, proteolysis, cross-linking reaction etc. Utilization of *Jatropha* protein in animal feed can only be made after the detoxification of all the toxins from the seed cake. The chemical composition of *Jatropha* seed cake and defatted seed cake is shown in Table 2.5.

Table 2.5: Chemical composition of *Jatropha* seed cake and defatted seed cake (g/ kg dry matter) (Makkar and Becker, 1998)

Component	Seed cake	Defatted seed cake
Dry matter (g kg-1)	937	938
Crude protein (N x 6.25)	244	259
Fat	60	Not detected
Ash	167	178

2.7 Detoxification methods

A lot of research has been done on the extraction of oil from *Jatropha* seeds. Conventional method like solvent extraction is the most extensively used technique for the extraction of oil as it results in higher oil yields along with large amount of

press cake which is rich in proteins, fiber, carbohydrates and some minor components; but for its utilization as feed the press cake should be detoxified. Several methods have been tried for detoxifying defatted cake and kernel meal (Table 2.6) that include physical, chemical and biological methods.

2.7.1 Physical and chemical treatments

The physical methods for the detoxification of *Jatropha* involve the heat treatment that employs the use of moist heat. Research has been done using moist heat alone for the reduction in phorbol esters content with not much breakthrough (Makkar and Becker, 1997a,b). Moist heat along with other treatments resulted in partialdecrease in phorbol ester contents (Chivandi *et al.*, 2004). Combination of physical and chemical treatmentshas been demonstrated with better efficiency of detoxification (Chivandi *et al.*, 2004; Rakshit and Makkar, 2010; Sadubthummarak *et al.*, 2013). The polar nature of phorbol esters has made the use of organic solvents such as ethanol or methanol for detoxification of *Jatropha* cake (Makkar *et al.*, 1997; Chivandi *et al.*, 2006). Treatment of *Jatropha* seed cake with methanol as organic solvent gave better resultsbut the process is time consuming as it requires number of extractions and also toxicity of methanol makes the process non ecofriendly. In a study done by Rakshit *et al.* (2010), 99% reduction in phorbol esters content in oil was achieved using 8 litres of methanol per kg of oil, with a extraction time of 60 min. Pighinelli *et al.* (2012) reported that methanol treatment of *Jatropha* seed cake resulted in 100% reduction of phorbol esters as compared to the treatment with ethanol and sodium bicarbonate which resulted in 64% decrease.Vittaya and Rayakorn (2013) have observed in their study that treatment of *Jatropha* meal with 90% methanol and 85% ethanol has resulted in complete reduction in the phorbol esters. The high extraction yields of phorbol esters according to Severa *et al.* (2013) are due to hydrogen bonding interactions between methanol and hydroxyl groups of phorbol esters. The nontoxic nature of ethanol as compared to methanol has an added advantage if the detoxified meal has to be used as animal feed. Although using a combination of solvent systems is effective in reducing phorbol ester contents but they are less suitable for large scale level as this may complicate the extraction process and make the solvent recovery difficult. Also, the various stages involved in the processing of the biomass up to the detoxification levels increases the expenditure and make the process highly expensive.

These processes may also involve the use of combination of some chemicals that result in presence of other undesirable residues. Combinations of treatments are used by different researchers for the removal of phorbol esters from *Jatropha* (Table 2.6). (Makkar *et al.*, 2012; Chivandi *et al.*, 2004; Guedes *et al.*, 2014; Gaur, 2009)

Some of the other methods of detoxification includes hydrothermal processing techniques (Martinez *et al.*, 2006), ionizing radiation, supercritical fluid extraction (SCFE) (Pereira *et al.*, 2013) etc. Detoxification by SCFE using CO_2 would allow the use of aqueous/organic solvent extracts from *Jatropha* seed as an effective insecticidal and antimicrobial agent (Pereira *et al.*, 2013). Diwani *et al.* (2011) reported that the ozone treatment can be the best and less expensive method for removing toxic phorbol esters from *Jatropha* seed cake compared to other methods as the treatment removes 75.26% phorbol esters and consumes less time and chemicals.

Table 2.6: Various physico0chemical treatments for removal of the toxins in *Jatropha* press cake

S.No.	Process/Treatment	Effects on *Jatropha* esters and other anti- nutritional factors	Reference
Physico- chemical treatments			
1.	Methanol/ethanol (50/50 during 8h)	Phorbol esters reduced by 97.30% in cake	Guedes *et al.*, 2014
2	$NaHCO_3$ or NaOH (3%)	Phorbol esters reduced by 55% in meal	Elangovan *et al.*, 2013
3	90% methanol and 85% ethanol	Phorbol esters reduced 100%	Vittaya and Rayakorn ,2013
4	Plasma generation by helium at high voltage and input power of 50 Win water and methanol	Complete degradation of Phorbol esters in methanol was achieved.	Kongmany *et al.*, 2013
5	Heat treatment + bentonite +zinc oxide + $NaHCO_3$ (4%)	Phorbol ester content reduced to 0.05-0.04 mg/g	Sadubthummarak *et al.*, 2013
6	Methanol	Phorbol ester reduced by 100%	Pighinelli *et al.*, 2012
7	Ethanol (unknown dose) followed by $NaHCO_3$ treatment	Phorbol esters reduced by 97.9%	Makkar *et al.*, 2012
8	$NaHCO_3$0.075%+ ozone treatment for (2,3,6,9 and 12) minutes at dose 50 mg/L and 300m A.	phorbol esters reduced 75.26%	Diwani *et al.*, 2011
9	Alkaline methanol + heat treatment	Phorbol ester reduction and trypsin inhibitor inactivation	Rakshit *et al.*, 2010

S.No.	Process/Treatment	Effects on *Jatropha* esters and other anti- nutritional factors	Reference
10	Solid-liquid extraction with a sequential combination of hexane and methanol	Phorbol esters reduced by 99.6%	Gaur, 2009
11	CaOH (2%)	Phorbol esters reduced by 90% in meal	Rakshit and Bhagya, 2007
12	Ethanol (95%) at 35°C+ heating with pressurized steam at 90°C for 30 minutes + sun-drying and further autoclaving at 121°C during 30 mins	Phorbol esters and antinutrients were still active	Chivandi *et al.*, 2006
13	Petroleum ether extraction	Phorbol esters reduced by 67.7% in kernels	Chivandi *et al.*, 2004
14	Double solvent extraction followed by moist heating	Phorbol esters reduced by 70.8% in kernels	Chivandi *et al.*, 2004
15	Double solvent extraction + wet extrusion, re-extraction with hexane and moist heat treatment	Phorbol esters reduced by 87.7% in kernels	Chivandi *et al.*, 2004
16	Ethanol (80%) or methanol (92%)	Saponins and phorbol esters reduced by 95% after four extractions	Makkar *et al.*, 1997
17	Moist heat treatment at 121°C for 20 mins	No effect on phorbol esters but trypsin inhibitors completely inactivated	Makkar *et al.*, 1997
Radiation treatments			
1	Gamma irradiation of cake (30kGy) at the rate of 2.5kGy/hr	Phorbol ester reduction by 75.8%	Gogoi *et al.*, 2014
2	UV irradiated (220 to 400 nm) *Jatropha* oil at 25 °C for 40 min	100% reduction of phorbol esters	Xiao *et al.*, 2015

2.7.2 Use of Radiations

Years of observations on various species for many generations led to conclude that the irradiated foods are safe and non- toxic to consume (Chen *et al.*, 2012). Irradiation has the advantages of very high performance and less secondary pollution, and therefore can be considered as an additional processing method for removing both heat-stable and heat-labile anti-nutrients (Chen *et al.*, 2012).Irradiation techniques have been employed for the degradation and transformation of anti- nutritional compounds and several suspicious carcinogens (Mir *et al.*, 2013; de Urzedo *et al.*, 2007).γ-radiations have been tried by Gogoi *et al.*(2014) to detoxify the seed cake and 75% reduction was achieved within 12 h of treatment. Combination of UV irradiation

and chemical treatments has also been experimented for the reduction of phorbol esters. Xiao *et al.* (2015) irradiated *Jatropha* oil at 25 °C for 40 min and the wavelengths ranged from 220 to 400 nm which resulted in 100% reduction of the phorbol esters. Not much research is documented as far as radiation therapy for detoxifying the phorbol esters.

2.7.3 Biological Detoxification

On the contrary, bio-detoxification does not involve application of chemicals or mixes and taking into consideration the safety and energy concerns, biological methods are more beneficial than other methods. But at the same time bio- detoxification may be inconvenient and time consuming. Bio-detoxification of *Jatropha* seed has been done using white rot fungi (de Barros *et al.*, 2011), *Aspergillus niger, Trichoderma longibrachitum, Penicillium chrysogenum, Rhizopus oligosporus, Rhizopus nigricans* (Belewu and Sam, 2010) and many other microorganisms have been used to inactivate the toxins and anti-nutritional factors in *Jatropha* kernel meal (Table 2.7).

Many research groups across the globe are involved in parallel research to develop biotechnological strategies to overcome the challenges of phorbol esters toxicity. After detoxification, *Jatropha* proteins can be extracted as they are present in high amounts and can be used as potential components for animal feed, while without detoxification *Jatropha* proteins are limited to technical applications. In a study conducted by Malviya *et al.* (2011), the protein from the seed extract of *Jatropha curcas* was isolated by column chromatography which had a solubility of about 90% above pH 9 and suggested it can be used as a good protein source in food applications, in improving dietary supplement products and protein energy product. *Jatropha* proteins were also extracted from the *Jatropha* seeds using the principle of isoelectric precipitation (Hamarneh *et al.*, 2010; Kumar *et al.*, 2012). Sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE), elemental analysis and Fourier transform infrared spectroscopy (FTIR) were used to analyze the obtained proteins. Liliana *et al.* (2013) extracted *Jatropha* protein by alkaline extraction followed by isoelectric precipitation method by which they were able to obtain a protein isolate with 93.21% of proteins.

Table 2.7: Various biological treatments for removal the of toxins in *Jatropha* press cake

S.No	Process/treatment	Effects on phorbol esters and other anti- nutritional factors	Reference
1.	Fermentation in broth cultures by *Trichodermaharzianum,T. harzianum, Paecilomycessinensi, Cladosporiumcladosporioides, Fusariumchlamydosporum,F. chlamydosporum* and *F. chlamydosporum*	Phorbol esters removed by 88.9%–99.7% after 30 days of incubation.	Azhar *et al.*, 2014
2.	Solid state fermentation of *Jatropha* seed cake with white rot fungi for 20days	Phorbol esters totally removed	Anjali and Haresh *et al.*, 2014
3.	Submerged fermentation by bacillus strains	100% phorbol esters degradation achieved in 7 days.	Chang *et al.*, 2014
2	Solid state fermentation of seed cake with *Streptomyces filmicarius* (YUCM 310038) in 9 days	Phorbol esters reduced by 97% in seed cake.	Xing-Hong *et al.*, 2013
3	Solid state fermentation of seed cake with *Cunninghamellaechinulata*CJS-90 in 12 days.	Degradation of phorbol esters to the extent of 75-100% Phytate degradation in the fermented JSC was in the range of 65-96%. Reduction of saponin was in the range of 55-99%	Somashekar**, 2013**
4	Solid state fermentation of *Jatropha* kernel cake with *Absidiaspinosa, Mucorrouxii*	Tannin, Saponin and phytate contents decreased	Sanusi *et al.*, 2013
5	*Jatropha curcas* fermented by consortium of *Aspergillusniger* and *Neurosporasitophila* (3 g inoculum and 3 hours of fermentation time)	Phorbol esters reduced by 79.69%	**Tuti, 2012**
6	Solid state fermentation of seed cake with white-rot fungi *Bjerkanderaadusta* or *Phlebiarufa*	Phorbol esters reduced by 91-97 % in seed cake	De Barros *et al.*, 2011
7	Solid state fermentation of seed cake with *Pseudomonas aeruginosa* PseA strain within 9 days under 30°C, pH =7 and 65% relative humidity	Phorbol esters reduced to undetectable level	Chetna *et al.*, 2011
8.	Solid state fermentation of *Jatropha curcas kernel* cake with *Aspergillusniger, Penicillium sp., Trichodermaharzanium and Trichodermalongibrachiatum in 10 days.*	Tannin and phytate contents were reduced	Moshood *et al.*, 2011
9.	SSF of *Jatropha* seed cake with zxy-12 strain	92.78% phorbol esters removed	Xiaoyu *et al.*, 2016
10.	SmF of *Jatropha* deoiled seed cake with *Candida parapsilosis* for 3 days	Complete elimination of phorbol esters	Balakrishna *et al.*, 2017

CHAPTER 3

EXPERIMENTAL DESIGN AND ANALYSIS

A properly planning and execution of experiments is must for obtaining unambiguous and precise conclusions from the experimental study. Design of experiments is considered to be a very useful strategy for accomplishing these tasks. In general, it helps in establishing inferences from results obtained which are not accurate but are subjected to variation. It also specifies the most appropriate method for the collection of experimental data. Along with this, it develops techniques which help in proper understanding and analysis of results. Some advantages of design of experiments are: (Peterson, 1985; Adler *et al.*, 1975)

- Identification of important decision variables which influence product/process performance.
- Significant reduction in the number of trials.
- Determination of optimum value of the parameters.
- Determination of "experimental error" can be made
- Helps in getting the inference of the effect of parameters on the properties of the process.

British scientist in 1920 Sir R. A. Fisher was originated the design of experiments technique as a method to maximize the knowledge gained from experimental data. Since then, several innovations have been introduced to extract maximum possible usage from the concept such as full factorial (Montgomery, 2001; Cochran and Cox, 1962) fractional factorial designs (Peterson, 1985), response surface methodology (Ross, 1996; Roy, 1990; Myers *et al.* (1989); Box and Wilson, 1951), and Taguchi method (Taguchi, 1986) etc.

In the present work, three different techniques of design of experiments were used which are Response Surface Methodology (RSM), Taguchi's method and Interpretive structural modeling (ISM).

3.1 Taguchi Experimental Design

In the traditional one-variable-at-a-time approach, only one variable at a time is evaluated keeping remaining variables constant during a test run. This type of experimentation reveals the effect of the chosen variable on the response under certain set of conditions. The major disadvantage of this approach is that it does not show what would happen if the other variables are also changing simultaneously. This method does not allow studying the effect of the interaction between the variables on the response characteristic. The interaction effect comes when one factor collapses and similar effect on response at different levels of other variable is studied (Montgomery, 2001). On the other hand, full-factorial designs require experimental data for all the possible combinations of the factors involved in the study; which demands a very large number of experimental runs. Therefore, in the case of experiments involving relatively more number of factors, only a small fraction of combinations of factors are selected that produces most of the information to reduce experimental effort. This approach is called fractional-factorial design of experiment. The analysis of results in this approach is complex due to non-availability of generally accepted guidelines (Jiang and Komanduri, 1997). The Taguchi method provides a solution to this problem.

3.1.1 Taguchi's Philosophy

Taguchi's **Philosophy** is based on providing quality engineering and focused mainly on the applying engineering techniques than relying on conventional statistical techniques. It consists of two concepts i.e. upstream and shop-floor quality engineering. An upstream method involves the use experiments at small level which reduce the variability, inconsistency and cost of the process, and robust designs derived from upstream experiments are used for large-scale production and business purposes. The shop-floor techniques are cost effective, real time methods for examining, maintaining and sustaining quality in production. Taguchi's philosophy involves simple fundamental concepts which are: (Ross, 1996; Roy, 1990):

- Quality should be devised for a product and not just examine it.
- Best quality can be achieved by reducing variations from the target. The design of the process should not be affected by the environmental factors.

- The cost of quality should be evaluated as a function of deviation from the standard and the losses should be measured system-wide.

Taguchi has proposed an alternative "off-line" strategy to inspect quality of a product on production line. According to him, quality cannot be enhanced by simply inspecting, screening and salvaging the process. Taguchi has recommended a process which involves three stages: *system design*, *parameter design* and *tolerance design* (Ross, 1996; Roy, 1990). In this work, Taguchi design approach is used to study the effect of different factors on phorbol ester degradation in Jatropha seed cake.

3.1.2 Experimental Design Strategy

Taguchi has suggested orthogonal arrays (OA) for designing experiments. For designing an experiment, the most suitable OA is selected and assigned parameters and interactions of interest. By making the use of linear graphs and triangular tables proposed by Taguchi assignment of parameter can be simplified.

The experimental results are analyzed to obtain effect of individual factor or in combination on process, to estimate the response(s) under the optimum condition and to obtain the optimum conditions for a process (Ross, 1996). Information of the contribution of individual parameter helps in deciding the requisite control on a production process. The analysis of variance (ANOVA) is generally applied to the experimental results which help in determining the percentage contribution of each factor against a defined level of confidence. The ANOVA table helps in determining significant factors and their control in the analysis (Ross, 1996).

Taguchi suggested (Roy, 1990) two different ways for analyzing the experimental results. In the first approach, results of a single experiment or the average of the replicates are calculated by using main effect and ANOVA analysis. In second approach, signal-to-noise (S/N) ratio is used for multiple runs in the analysis. The loss function is highly dependent on S/N ratio, higher the S/N ratio, the loss associated will be less.

Taguchi recommends (Ross, 1996) the use of outer OA to force the noise variation into the experiment i.e. the noise is intentionally introduced into the experiment. Generally,

processes are subjected to many noise factors that in combination strongly influence the variation of the response. For extremely 'noisy' systems, it is not generally necessary to identify controllable parameters and analyze them using an appropriate S/N ratio (Roy, 1990). In the present work, both raw data and S/N data analysis have been performed. The effects of different parameters on the response (phorbol ester degradation) have been investigated through main effects based on raw data and the optimum condition for the quality characteristic was found through S/N data analysis.

3.1.3 Loss Function and S/N Ratio

Loss is measured in terms of monetary units and is related to quantifiable product characteristics. Taguchi defines quality loss via his 'loss-function'. He unites the financial loss with the functional specification through a quadratic relationship that comes from Taylor series expansion (Roy, 1990).

$$L(y)=k(y-m)^2$$

where, L = loss in monetary unit

m = value at which the characteristic should be set

y = actual value of a characteristic

k = constant, depends on the magnitude of the characteristic and the monetary unit involved.

The traditional and the Taguchi's loss function concept have been illustrated in Figure 3.1(a) and Figure 3.1(b). The following two observations can be made from Figure 3.1 (a, b) (Roy, 1990).

- The more the value of product's characteristic from the target, greater is the loss and the when quality characteristic meets its target value, loss becomes zero.
- The loss is a continuous function and not a sudden step as in the case of traditional approach (Figure 3.1b).

This consequence of the continuous loss function illustrates the point that merely producing a product within the prescribed limits does not necessarily mean that product is of desired quality.

In a mass production process the average loss per unit is expressed as:

$$L(y)=\left\{k(y_1-m)^2+k(y_2-m)^2+.....+k(y_n-m)^2\right\} \quad (3.1)$$

Where, y_1, y_2, ……y_n = values of characteristics for units 1,2,…..n respectively

n = number of units in a given sample

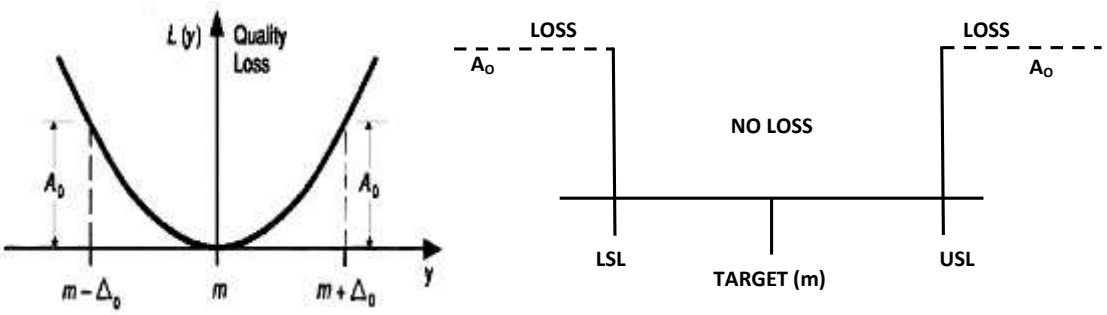
k = constant depending upon the magnitude of characteristic and the monitory unit involved

m = Target value at which characteristic should be set.

Equation (3.1) can be written as: $L(y)=k(MSD)$

Where, MSD is the mean square deviation, which represents the average of squares of all deviations from the target value.

Higher the S/N ratio, optimum value of quality will be obtained with minimum variation. Depending upon the type of response, the following three types of S/N ratio are employed in practice (Byrne and Taguchi,1987).



(a) Taguchi Loss Function (b) Traditional

Figure 3.1: The Taguchi loss-function and the traditional quality philosophy approach (Ross, 1996)

1. Larger the better:

$$\left(S/N\right)_{HB}=-10\log(MSD_{HB}) \quad (3.2)$$

where, $MSD_{HB}=\frac{1}{R}\sum_{j=1}^{R}(1/y_j^2)$

2. Lower the better:

$$\left(S/N\right)_{LB} = -10 \log\left(MSD_{LB}\right) \tag{3.3}$$

where, $MSD_{LB} = \frac{1}{R}\sum_{j=1}^{R}(y_j^2)$

3. Nominal the best:

$$\left(S/N\right)_{NB} = -\log\left(MSD_{NB}\right) \tag{3.4}$$

where, $MSD_{NB} = \frac{1}{R}\sum_{j=1}^{R}(y_j - y_o)^2$

R = Number of repetitions

It is hereby mentioned, the standard definition of MSD has been used for the "nominal the best" type of characteristic. For "smaller the better" type, the target value is zero and for "larger the better" type, the inverse of each large value becomes a small value and again the target value is zero. Therefore, for all the three expressions the smallest magnitude of MSD is being sought.

3.1.4 Taguchi procedure for experimental design and analysis

Figure 3.2 illustrates the stepwise procedure for Taguchi experimental design and analysis. It is described in the following paragraphs.

3.1.4.1 Selection of OA

For the selection of an appropriate OA, the following points should be considered:

- Selection and evaluation of the process parameters and/or their interactions.
- Selecting number of levels for selected parameters.

The determination of parameters to be investigated affects process performance characteristics or responses of interest (Ross, 1996). Various methods have been suggested by Taguchi which help in identifying the significant parameters to be included in the experiments. These include (Ross, 1996):

a. Brainstorming

b. Flow charting

c. Cause-effect diagrams

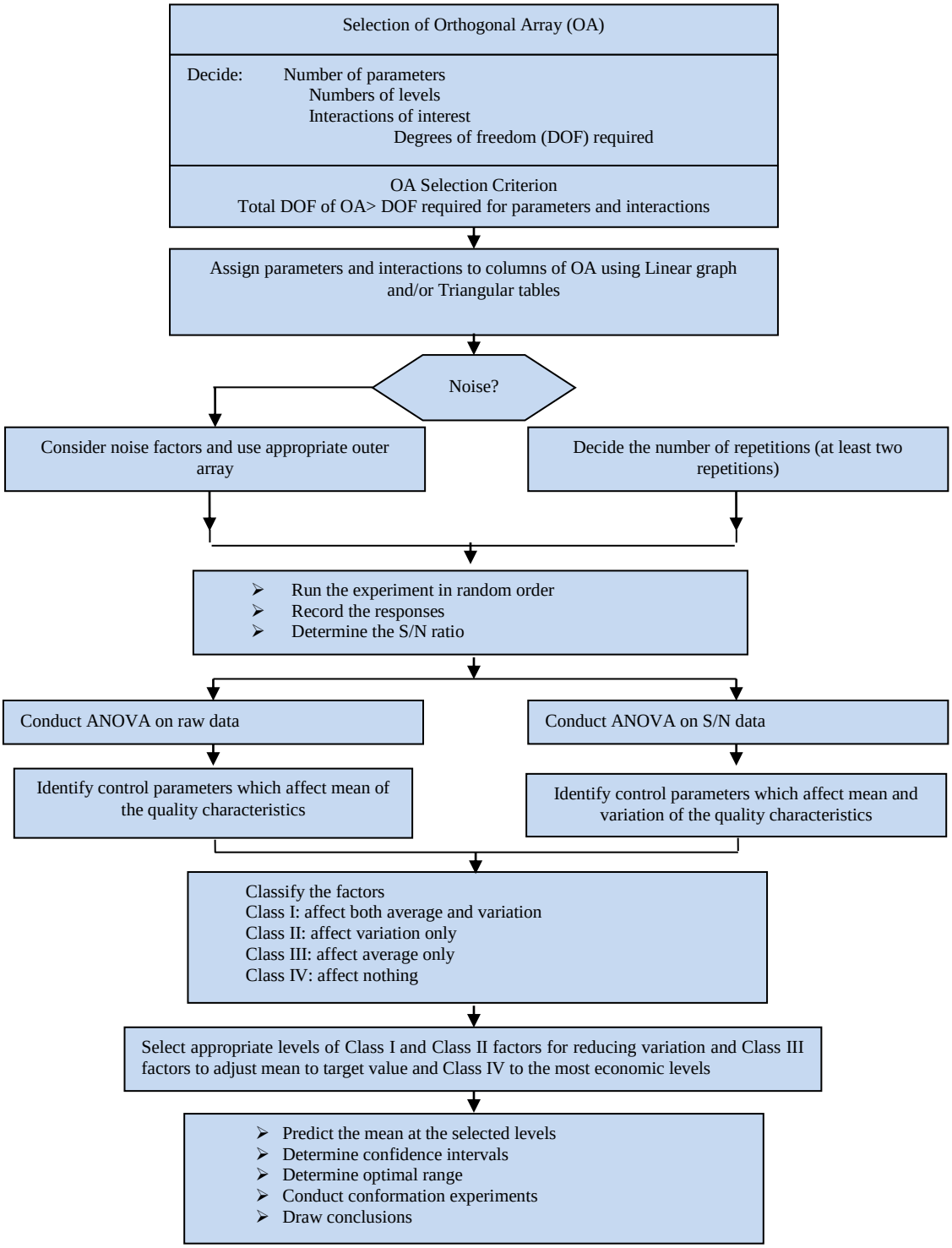


Figure 3.2: Taguchi Experimental Design and Analysis Flow Diagram (Kumar, 1994)

The degree of freedom (DOF) of an experiment is a direct function of total number of experimental runs. DOF of a parameter increases with increase in number of levels because DOF of a parameter is calculated as the number of levels minus one. Thus, the total number of trials in an experiment increases with number of levels and total degrees of freedom.

Thus, two levels for each parameter are recommended to reduce the number of trials in the experiment (Ross, 1996). If curved or higher order polynomial relationship between the parameters under study and the response is expected, at least three levels for each parameter should be considered (Barker, 1990). The standard two-level and three-level arrays (Taguchi and Wu, 1979) are:

a) L_4, L_8, L_{12}, L_{16}, L_{32} (Two-level arrays)

b) L_9, L_{18}, L_{27} (Three-level arrays)

Number in the subscript indicates the number of runs in the array. The formula for calculating degrees of freedom (DOF) in an OA is:

$$f_{L_N} = N - 1$$

where f_{L_N} = total degrees of freedom of an OA

L_N =OA designation

N = number of trials

When a particular OA is selected for an experiment, the following inequality must be satisfied (Ross, 1996):

$f_{L_N} \geq$ Total DOF required for each parameter and their interactions.

Based on the number of levels and total DOF required for the experiment, a suitable OA is selected.

3.1.4.2 Assignment of parameters and interactions to OA

An orthogonal array has several columns to which various parameters and their interactions are assigned. Linear graphs and Triangular tables are two tools, which are useful for deciding the possible interactions between the parameters and their

assignment in the columns of 'OA'. Each 'OA' has its particular liner graphs and interaction tables (Mitra, 2001).

3.1.4.3 Selection of outer array

In Taguchi, factors (parameters) are categorized into two main groups:

- Controllable factors
- Noise factors

Controllable factors can be easily controlled while noise factors are the nuisance variables that are complex and expensive and hence are not possible to control (Byrne and Taguchi, 1987). The noise factors are responsible for the performance variation of a process. Taguchi recommends the use of an outer array for noise factors and inner array for the controllable factors. Use of an outer array forces the noise variation into the experiment. However, experiments against the trial condition of the inner array may be repeated and in this case the noise variation in unforced in the experiment (Byrne and Taguchi, 1987; Taguchi, 1986). The outer array, if used will have the same assignment considerations.

3.1.4.4 Experimentation and data collection

The experiment is performed against each of the trial conditions of the inner array. Every experiment in a trial condition is repeated (if outer array is not used) or according to the outer array (if used). Randomization should be carried for to reduce bias in the experiment.

3.1.4.5 Data analysis

A number of methods have been suggested by Taguchi for analyzing the data: observation method, ranking method, column effect method, ANOVA, S/N ANOVA, plot of average responses, interaction graphs, etc. (Ross, 1996). In the present investigation, following methods are used.

1. Plot of AVERAGE RESPONSE CURVES
2. ANOVA FOR RAW DATA
3. ANOVA FOR S/N data

Plot of average responses is the pictorial representation indicating the trend and effect of a parameter on the response. The S/N ratio is the response of the experiment and measures the variation within a trial in the presence of noise. ANOVA is conducted on S/N ratio and it identifies the significant parameters.

3.1.4.6 Parameter design strategy

Parameter classification and selection of optimal levels

ANOVA (raw data) and S/N ratio classify factors which control the average response and variation in the response respectively. The control factors are classified into four groups:

Group I : Parameters, which affect both average and variation

Group II : Parameters, which affect variation only

Group III : Parameters, which affect average only

Group IV : Parameters, which affect nothing

The parameter design strategy is to select suitable levels of group I and II parameters to decrease variation and group III parameters to adjust the average values to the target value. The group IV parameters may be set at the most economical levels.

Prediction of mean

The next step after determining the optimum conditions is estimation of mean response (μ) at optimum condition which is evaluated from significant parameters only identified by ANOVA. If A and B are significant parameters and second level of both A and B (i.e. A_2B_2) is the optimum value, then optimal value of the response can be estimated (Ross, 1996) as:

$$\mu = \overline{T} + (\overline{A}_2 - \overline{T}) + (\overline{B}_2 - \overline{T})$$
$$= \overline{A}_2 + \overline{B}_2 - \overline{T}$$

$\overline{T}$ = Overall mean of response

$\overline{A}_2, \overline{B}_2$ = Average values of response (at second level) of A and B respectively

Determination of confidence intervals

The mean estimate (μ) is a point estimate calculated from average of results of the experiment. It is a statistical requirement that the value of a parameter should be predicted with a range for a given level of confidence and this range is known as confidence interval (CI). Taguchi suggests two types of confidence intervals for estimated mean of optimal treatment conditions.

1. CI_{CE} – Confidence Interval [confirmation experiments (CE)], used in conformational experiment to validate predictions and is used for a small group only made under specific conditions.

2. CI_{POP} – Confidence Interval of population; is for whole population which covers all parts unlike CI_{CE} made under the specific conditions.

The CI_{CE} and CI_{POP} is calculated by using following equations (Roy, 1990):

$$CI_{CE} = \sqrt{F_{\alpha}(1, f_e) V_e \left[\frac{1}{n_{eff}} + \frac{1}{R}\right]} \tag{3.5}$$

$$CI_{POP} = \sqrt{\frac{F_{\alpha}(1, f_e) V_e}{n_{eff}}} \tag{3.6}$$

where,

$F_{\alpha}(1, f_e)$ = F-ratio at confidence level of (1-α) against DOF 1 and error DOF f_e

f_e = error DOF

N = Total number of results

R = Sample size for confirmation experiments

V_e = Error variance,

$$n_{eff} = \frac{N}{1 + [\text{DOF associated in the estimate of mean responce}]}$$

Conformational experiment

The conformational experiment is an important and most crucial step as it verifies conclusions of the experiments performed. Based on significant parameters, the

optimum conditions are set and a defined number of tests are run. The average values of the conformational experiments are matched with the predicted values and should lie within 95% confidence interval, CI_{CE}. However, these may or may not be within 95% confidence interval, CI_{POP}.

3.2 Response Surface Methodology

Response surface methodology (RSM) was originally developed by Box and Wilson in 1951 for exploring the potential of statistical design in industrial experiments. This approach helps in developing empirical models which can be used both for process improvement and determination of optimal conditions (Myers, 1990). In experimental situations where independent factors are presented in quantitative form, the response can be presented as follows.

$$Y = \varphi(x_1, x_2, ..., x_k) \pm e_r \tag{3.7}$$

Here, the response is between Y and x_1, $x_2 \ldots x_k$ of k quantitative factors. The symbol φ represents response function or surface and residual error e_r measures experimental errors (Cochran and Cox, 1962).

This methodology has been applied in the present work for developing the mathematical models for estimating the protein yield and protein content from Jatropha seed cake. For development of regression equation for response parameters, the second order equation can be assumed as:

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i<j=2}^{2} b_{ij} x_i x_j \pm e_r \tag{3.8}$$

The assumed response (Y) contains linear, squared and interaction terms of the variables. Various techniques are available to find the value of regression coefficients. Box and Hunter (1957) have proposed central composite rotatable design (CCRD) which fits second order equation accurately.

3.2.1 Central Composite Rotatable Design (Second Order)

In rotatable design, standard error at all points is same with distance ρ from the centre of the region i.e. (Box and Wilson, 1951)

$$x_1^2 + x_2^2 + ... + x_k^2 = \rho^2 = \text{constant} \quad (3.9)$$

CCRD is further divided into three parts which are:

- 2^k design, where k is number of parameters and 2 is the number of selected levels.
- Star points located at co-ordinate axes to form a central composite design with a star arm of size α.
- Some more points added at the centre to give equal precision for response Y with a circle of radius 1.

Here, α is radius of the circle. When $k \geq 5$, the experimental size can be reduced by using a half replication of 2^k factorial designs in which the value of α becomes $2^{(k-1)/4}$. Error mean square can be found out by replication of the centre points. Table 3.1 contains various components of CCRD (second order) design for different number of variables.

3.2.2 Estimation of the Coefficients

The second order regression equation representing response surface can be written as (equation 3.8):

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i<j=2}^{2} b_{ij} x_i x_j \pm \varepsilon_r$$

Where Y is estimated response, b's are coefficients and x_i's are independent variables.

Table 3.1: Centre composite 2nd order rotatable design components (Peng, 1967)

Variables (k)	Factorial Points (2^k)	Star Points (2k)	Center Points (n)	Total (N)	Value of (α)
3	8	6	6	20	1.682
4	16	8	7	31	2.000
4	16	8	6	30	2.000
5	16*	10	6	32	2.000
6	32*	12	9	53	2.378
* Half replication					

Least square method is generally used for finding out the regression coefficients (Hines and Montgomery, 1990). In matrix form, the second order response surface may be written as:

$$Y = X\beta + \varepsilon$$

where,

$$Y = \begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ \vdots \\ y_N \end{bmatrix},\ X = \begin{bmatrix} 1 & x_{11} & x_{21}....x_{k1} & x_{11}^2....x_{11}x_{21}.... \\ 1 & x_{12} & x_{22}....x_{k2} & x_{12}^2....x_{12}x_{22}.... \\ \vdots & \vdots & \vdots\ \vdots & \vdots \\ \vdots & \vdots & \vdots\ \vdots & \vdots \\ 1 & x_{1N} & x_{2N}....x_{kN} & x_{1N}^2....x_{1N}x_{2N}.... \end{bmatrix},\ \beta = \begin{bmatrix} b_o \\ b_1 \\ \vdots \\ \vdots \\ b_p \end{bmatrix},\ \varepsilon = \begin{bmatrix} e_1 \\ e_2 \\ \vdots \\ \vdots \\ e_N \end{bmatrix}$$

N= Total number of experiments

P= Total number of coefficients

Y= (N × 1) vector of the observations,

X= (N × P) matrix of the levels of independent variables

β= (P × 1) vector of the regression coefficients

ε= (N × 1) vector of random errors.

The least square estimator is:

$$L = \sum_{q=1}^{N} \varepsilon_q^2 = \varepsilon'\varepsilon = (Y - X\beta)'(Y - X\beta) \tag{3.10}$$

This may be expressed as:

$$L = Y'Y - \beta'X'Y - Y'X\beta + \beta'X'X\beta \tag{3.11}$$

Since β'X'Y is a (1×1) matrix and its transpose will also be a (1×1) matrix. Then

$$(\beta'Y)' = \beta X Y'$$

Hence the equation 4.11 has been written as:

$$L = Y'Y - 2\beta'X'Y + \beta'X'X\beta$$

which on simplification gives the values of different coefficients of regression equation as [Kumar (1994)].

$$X'X\beta = X'Y$$
$$\beta = (X'X)^{-1} X'Y \tag{3.12}$$

3.2.3 Analysis of Variance

In analysis of variance (Peng, 1967; Cochran and Cox, 1962), the total sum of squares may be divided into four parts:

- Contribution of first order terms.
- Contribution of second order terms.
- 'Lack of fit' component, which measures the variation of response from the fitted surface.
- Experimental error which is obtained from centre points.

The formula used for computing the sum of squares for ANOVA is given in the Table 3.2 (Steel and Torrie, 1986; Peng, 1967). Where N, n_o, y_s, and y_o represent the total number of experimental points, total number of observations, s^{th} response value and mean value of response respectively at the centre point of experimental region. The design matrix for four variables is shown in Table 3.3.

3.2.4 Significance testing of the coefficients

For determining the significance of the individual coefficients, one has to set up a null hypothesis, which calculates the difference of estimated coefficients from its mean value using the student's t-test (Hines and Montgomery, 1990; Steel and Torrie, 1986). Where design is completely randomized, it may be shown that the analysis of variance could be used in place of t-test to compare two treatments. This is due to the reason that the one tailed F-test with 1 and n degree of freedom (DOF) corresponds to the two tailed t-test with n DOF i.e. t^2 = F for 1 DOF (Kumar, 1994). Hence for significance testing of individual coefficients F test with 1 and n_o DOF has been used, where n_o is the total number of observations of the centre point.

F-ratio can be calculated as:

$$F(1, n_o) = \frac{\left(b_i^2 / c_{ii} \right)}{S_e^2} \tag{3.13}$$

where, b_i = Regression coefficients

c_{ii} = Element of the error matrix $(X' X)^{-1}$

S_e = Standard deviations of experimental error calculated as:

$$S_e = \frac{1}{n_o - 1} \sum_{s=1}^{n_o} (y_s - \bar{y}_o)^2$$

where $$\bar{y}_o = \frac{1}{n} \sum_{s=1}^{n_o} y_s$$

y_s = s^{th} response value at the centre.

Calculated F_{cal} is compared with tabulated F_{tab} values at a particular confidence level and if $F_{cal} > F_{tab}$ for a coefficient, effect of term is considered significant. The insignificant second order terms have been deleted from the equations and remaining coefficients have been recalculated (Kumar, 1994).

3.2.5 Adequacy of the model

Once the coefficients have been estimated and tested for their significance, the estimated regression equation is derived. This regression equation is then tested for the adequacy of fit as per the following procedure (Kumar, 1994):

i) Find the residual sum of squares as:

$$S_1 = \sum_{q=1}^{N} (y_p - \bar{y}_q)^2 \tag{3.14}$$

Where, y_q's are the observations at experimental points and $\bar{y}_q$ is the mean of all observations. N is total number of observations and k is total number of variables. The DOF for residual sum of squares is

$$f_1 = N - \frac{(k+2)(k+3)}{2}$$

ii) From repeated observations at the centre point, the error sum of squares is found as:

$$S_2 = \sum_{s=1}^{n_o} (y_s - \bar{y}_o)^2 \quad (3.15)$$

where y_s is the s^{th} response values at the centre point. $\bar{y}_o$ is the mean of all the responses at the centre point. n_o is the total number of experimental points at the centre. The DOF for error sum of squares is $f_2 = n_o - 1$.

iii) The inadequacy of fit sum of squares is estimated as:

$$S_3 = S_1 - S_2 \quad (3.16)$$

For which DOF is

$$f_3 = f_1 - f_2 = N - \frac{(k+1)(k+2)}{2} - n_o - 1$$

Table 3.2: Analysis of variance for central composite 2nd order rotatable design (Peng, 1967)

S. No.	Source	Sum of Squares	Degree of freedom
1	First order terms	$\sum_{q=1}^{k} b_i \left(\sum_{q=1}^{N} x_{iq} Y_q \right)$	k
2	Second order terms	$b_o \left(\sum_{q=1}^{N} y_q \right) + \sum_{i=1}^{k} b_{ii} \left(\sum_{q=1}^{N} x_{iq}^2 Yq \right) + \sum_{i<j}^{k} b_{ij} \left(\sum_{q=1}^{N} x_{iq} x_{jq} y_q \right) - \frac{\left(\sum_{q=1}^{N} y_q \right)^2}{N}$	$\frac{k(k-1)}{2}$
3	Lack of fit	Found by subtraction	$N - n_o - \frac{k(k+3)}{2}$
4	Experimental error	$\sum_{s=1}^{n_o} (y_s - \bar{y}_0)^2$	n_o-1
5	Total	$\left(\sum_{q=1}^{N} y_q \right)^2 - \left[\frac{\left(\sum_{q=1}^{N} y_q \right)^2}{N} \right]$	N-1

Table 3.3: Central composite second order rotatable design matrix for 5 variables

No. of Expts	X1	X2	X3	X4
1	1.000	-1.000	1.000	-1.000
2	1.000	1.000	-1.000	1.000
3	2.000	0.000	0.000	0.000
4	-1.000	1.000	-1.000	1.000
5	-1.000	-1.000	1.000	1.000
6	0.000	0.000	0.000	0.000
7	1.000	-1.000	-1.000	-1.000
8	1.000	1.000	-1.000	-1.000
9	-1.000	-1.000	-1.000	-1.000
10	-1.000	1.000	1.000	-1.000
11	0.000	0.000	-2.000	0.000
12	0.000	-2.000	0.000	0.000
13	0.000	0.000	0.000	0.000
14	0.000	0.000	0.000	0.000
15	1.000	1.000	1.000	-1.000
16	1.000	1.000	1.000	1.000
17	0.000	0.000	0.000	0.000
18	-2.000	0.000	0.000	0.000
19	-1.000	-1.000	1.000	-1.000
20	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	-2.000
22	0.000	0.000	0.000	2.000
23	-1.000	1.000	-1.000	-1.000
24	0.000	2.000	0.000	0.000
25	0.000	0.000	0.000	0.000
26	1.000	-1.000	1.000	1.000
27	-1.000	1.000	1.000	1.000
28	-1.000	-1.000	-1.000	1.000
29	1.000	-1.000	-1.000	1.000
30	0.000	0.000	2.000	0.000

iv) Applied F-test to test the adequacy of fit:

$$F = \frac{S_3 / f_3}{S_2 / f_2} \tag{3.17}$$

The estimated regression equation fits the data adequately if $F < F_{0.05}(f_3, f_2)$ at 95% confidence level.

3.3 Conclusion

Design of experiments is a very essential tool for the accomplishment of the experimental study. It stipulates the most suitable method for collection of experimental data and helps in drawing inferences from the results even with variations. Along with this, it helps in developing techniques for proper understanding and analysis of results.

CHAPTER 4

MODELING AND ANALYSIS OF CRITICAL FACTORS FOR PHORBOL ESTER DEGRADATION IN *JATROPHA* SEED CAKE USING ISM AND MICMAC

4.1 Introduction and background

Jatropha is an oilseed plant which has gained significant interest as a raw material for biodiesel industries for the reason that it can grow in unfavourable climatic regions like infertile soils and scanty rainfall (Fersi *et al.,* 2012). The *Jatropha* seed contains approximately 30–35% oil that can produce high-quality biodiesel upon transesterification (Sohpal *et al.,* 2011; Makkar and Becker, 2009). Post oil-extraction, the residual seed cake has a good amount of nutrients like lignocellulosic compounds, water, minerals, proteins, and all the essential amino acids except lysine (Martinez *et al.,* 2006). However, the presence of certain toxic and anti- nutritional compounds makes the seed cake unfit for consumption as food. Phorbol esters, phytic acid, trypsin inhibitors, phenolic compounds, lectins (curcin), and saponins are some of the anti-nutrients found in *Jatropha* seed cake and amongst all phorbol esters have been identified as one of the main toxic compounds (Chivandi *et al.*, 2004; Saetae and Suntornsuk, 2011). Attempts made to detoxify *Jatropha* seed cake using various physical and chemical treatments (Aregheore *et al.*, 2003; Makkar and Becker, 2009; Abou and Abu, 2010; Gogoi *et al.,* 2014) allow the use of detoxified meal as a protein-rich dietary supplement in the food or feed diets. All these methods require a combination of treatments which not only have negative impacts on the environment but also make the process expensive and time-consuming. At the industrial level, a more efficient and environmentally friendly process is a prerequisite which should be able to detoxify *Jatropha* seed cake in less time and cost, and this can be accomplished by using the biological method (Ahluwalia *et al.,* 2017). Removal of phorbol esters by biological treatment is dependent on various factors which include

the type of microbes, incubation period, incubation time, Shaking (rpm), substrates used, and many more (Abdel *et al.,* 2002; Jain *et al.*, 2013; Ahluwalia *et al.*, 2017).

Most researchers focused on different factors for detoxification of *Jatropha* seed cake. This research aimed to comprehensively evaluate and identify various factors through extensive literature review and analyse them by interpretive structural modelling (ISM). To visualise the direct relationship among the considered factors, ISM based model was developed. MICMAC analysis was done to find the driving and dependence power of different enablers. The outcomes of this study helped in presenting a new approach of applying a biological method to ISM in the identification of factors important for detoxification of *Jatropha* seed cake with lesser practical work. The main objectives of this work were:

1. Identification and ranking the factors affecting the detoxification of *Jatropha* seed cake;
2. Evaluation of interactions between identified factors and dependent variables;
3. Understanding technical implications of this research work.

The flow chart of how this work was carried out in solitary view is shown in Figure 4.1.

4.2 Research Methodology

4.2.1 Identification of driving factors

Various physical and chemical ways have been identified for degrading phorbol esters in seed cake. Degradation of phorbol esters from *Jatropha* seed cake is influenced by intrinsic factors (composition of *Jatropha*), environmental factors (temperature, RH, etc.), and methods and conditions of detoxification methods. Based on literature review and discussions with the subject experts, eleven main factors were identified. Then, a questionnaire was designed, and opinions of field expertswere gathered. Also during the study, specialists from Indian Industry and academicians from various reputed institutes were communicated for the opinion on detoxification factors of *Jatropha* seed cake.

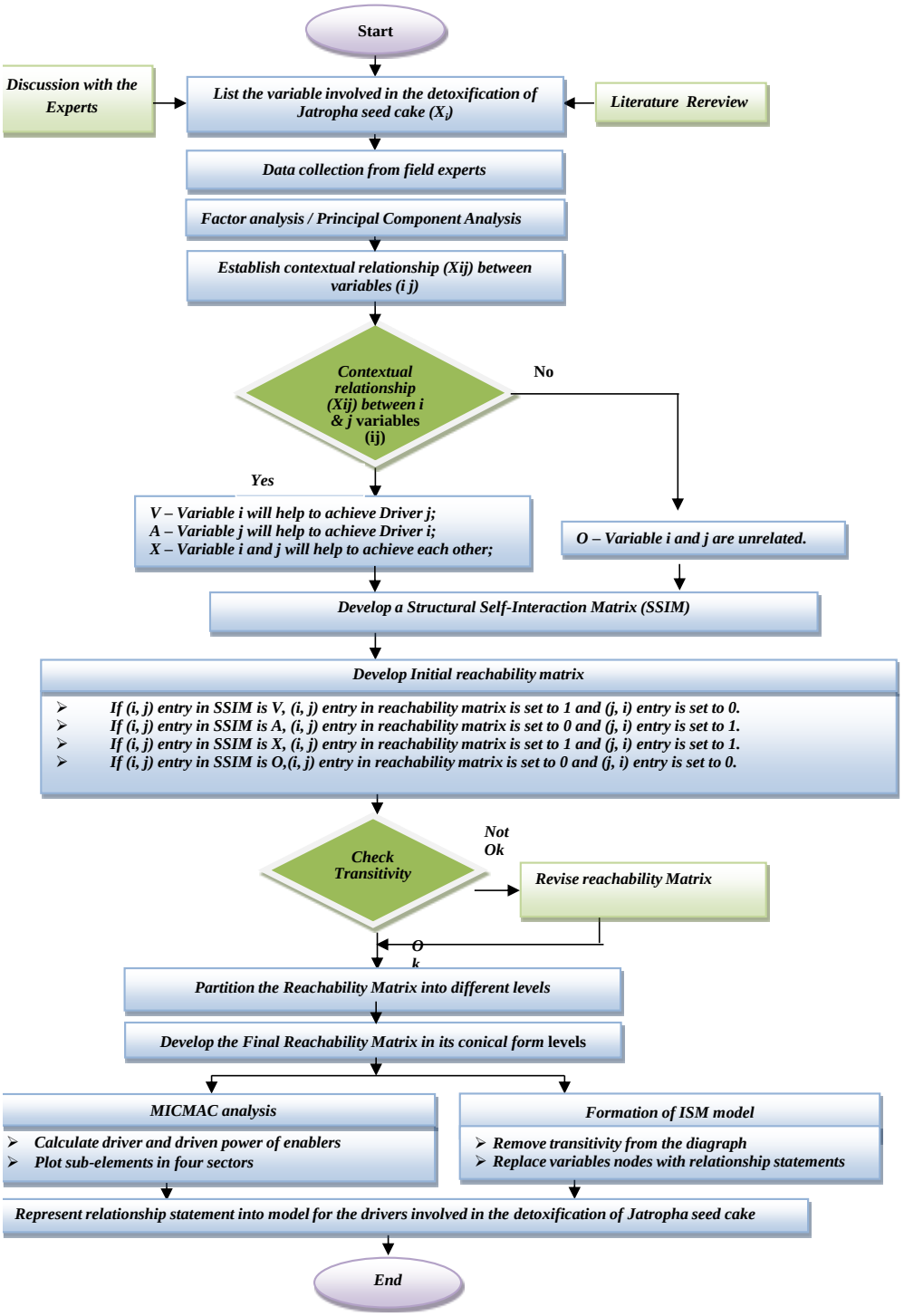


Figure 4.1: Flow chart illustrating ISM research direction

4.2.2 Factor analysis: Factor analysis (FA) is an imperative tool for data reduction and provides the close measures of different variables by determining the common parameters based on account of observed correlations (Fabrigar *et al.*, 1999; Hayton *et*

al., 2004). FA is one of the most popular systematic and theoretical approaches in decisions making for researchers to retain the factors. At the initial level, a questionnaire was structured using a 5-point Likert scale for eleven main factors and was sent to seventy field experts to collect their opinion regarding the importance of factors. Out of seventy, twenty- seven responses were received, which shows the 38.6% response rate. According to Malhotra and Grover (1998), if response rate is thirty percent then it is sufficient to drive the reliability analysis.

For the collected data to be considered reliable, the value of Cronbach's alpha coefficient (α) should lie between 0.7 to 1.0. George and Mallery, (2003); Gliem and Gliem, (2003) provided the following rules of thumb, if the value α is 0.9 – 1.0 means 'Excellent', 0.8 – 0.9 means 'Good', 0 .7 – 0.8 means 'Acceptable', 0.6 – 0.7 means 'Questionable', 0.5 – 0.6 means 'Poor', and < 0.5 means 'Unacceptable'. Cronbach's alpha coefficient is within limits. Hencethe collected data is considered reliable. Then FA is done for the filtration of relevant factors by same software and extracted and listed in Table 4.1.

4.3. ISM Methodology

ISM methodology is an interactive learning process which helps in improving order and direction of complex relationships amongst variables of a system (Grover *et al.,* 2004). Number of variables, different and directly related affecting the detoxification of *Jatropha* seed cake are arranged to form a comprehensive systemic model. Mostly, performance of a process is evaluated considering a few of the controllable variables with the objective of optimising dependent variables. Based on technical and economic requirements, some criteria's may play important roles in the overall performance evaluation of a process. However, accurate and absolute evaluation of performance can be estimated only by considering all the factors and dependent variables simultaneously. Simultaneous consideration of all the factors and dependent variables has not been reported in literature for detoxification of *Jatropha* seed cake so far. There is a need for a simple and systematic procedure for improving order and direction on complex relationships among variables of detoxification methods. Efforts

need to be extended which could help in determining factors that influence the overall performance of detoxification of *Jatropha* seed cake.

The ISM model developed helps in identifying structure of a complex problem which involves a system of field study in a carefully designed pattern employing the use of graphics and words (Nishat *et. al.* 2006). ISM is a powerful qualitative method which can be effectively applied in various fields. Ikno and Junzo (2009) established a DNA-based algorithm to solve decision support problems based on innovative bioscience technologies using ISM. Attri and Grover (2015) applied ISM for improving decision-making ability among executives working in different areas in an organization. Chakraborty *et al.* (2015) applied ISM for the evaluation of sustainability issues in the tea sector of Assam. The ISM can be judiciously hired for improving the system under consideration. The mathematical approach of the ISM method can be found in different reference works (Harary *et. al.*, 1965), while the philosophical theory for application of this approach is presented by Warfield (1973). The main advantage of using ISM methodology is that it converts vague and poorly articulated models of systems into evident and well-defined models (Sage, 1977), at the same time ISM methodology has certain drawbacks also (Kannan *et al.*, 2010). There is possibility of subjective bias by a person who is judging the variables, as the relations among the variables are dependent on that person's knowledge and familiarity with the system understudy. To avoid these difficulties the retrospective review has been done by the authors. Furthermore, in ISM no weight age is given to the variables to confer their relative importance. ISM approach starts with an identification of strategies or factors or any variables, which are related to the problem followed by choosing a contextually appropriate subservient relation (Attri *et al.*, 2013). The flowchart (Figure 4.2) summarizes the work plan of this research work. ISM methodology involves various steps which are as follows (Sharma and Garg, 2010, Tyagi *et al.*, 2015).

1. Identifying and enlisting different factors of detoxification of *Jatropha* seed cake.
2. Creating a contextual relationship between various factors for detoxification of *Jatropha* seed cake.

3. Development of structural self-interaction matrix (SSIM) for detoxification of *Jatropha* seed cake which shows interactions amongst the factors under concern.
4. Developing reachability matrix and checking the transitivity.
5. Development of flow chart without representing transitive links.
6. Translating resultant relationship into ISM by substituting factors with statements.
7. Checking conceptual variation and relevant amendments and developing contextual correlation among different factors.

ISM is used to show the direct relationship between various detoxification factors i.e. Protein, Lipids, Phorbol Esters, pH, RPM (Shaking), Esterase, Temperature, Inoculum, Time, Saponin and Tannins. ISM creates a model to analyse the considered detoxification factors by their driving and dependency behaviour. This model helps to study the different detoxification factors in an appropriate framework with the help of graph. This methodology uses the experience as well knowledge of field experts for constructing the structural framework. Various factors considered for ISM which affects the detoxification of *Jatropha* seed cake are shown in Table 4.1.

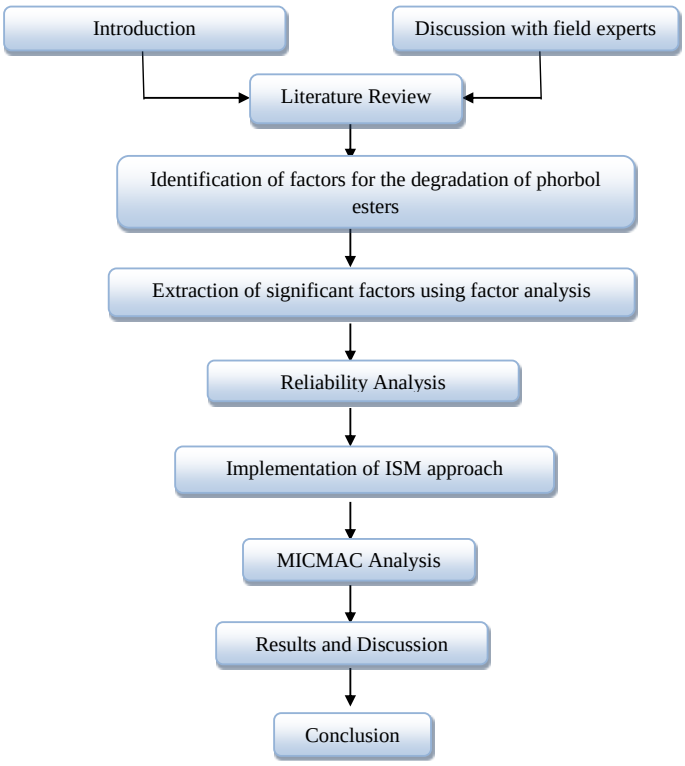


Figure 4.2: Research Flow Chart

4.3.1 Structural Self-Interaction Matrix (SSIM)

Structural Self-Interaction Matrix helps in finding the contextual relationship among different extracted factors using expert's opinion (Table 4.2). The matrix provides the pair-wise relationship of each factor. The symbols (V, A, X and O) are used for determining relationship of factors (i and j).

V –Factor i will assist to enhance factor j

A -Factor j will assist to enhance factor i

X -Factor i and j will assist to enhance each other

O - Factors i and j are isolated

Table 4.1: Factors affecting for detoxification of *Jatropha* seed cake

S.No.	Factors	Relevance	Reference
F1	Protein	Detoxification procedures should not affect the protein quality	Iftikhar, 2008; Belewu and Sam, 2010
F2	Lipids	Act as concentrated energy source in animal feed	Deleers *et al.*, 1981; Jacobson *et al.*, 1975
F3	Phorbol Esters	Phorbol esters are the main toxicant in *Jatropha* seed cake	Sharath *et al.*, 2014; Xing *et al.*, 2013; Chang *et al.*, 2014
F4	pH	Action of enzymes is dependent on pH	Mohankumar *et al.*, 2014; Wardhani *et al.*, 2016
F5	Rpm	Rotation speed affects the heat and mass transfer of the process.	Venogopal *et al.*, 2007
F6	Esterase	One of the important enzyme which helps in hydrolyzing different compounds	Wardhani *et al.*, 2016
F7	Temperature	Temperature affects the rate of microbial growth	Mohankumar *et al.*, 2014
F8	Inoculum	Inoculum level affects the fermentation time and growth of bacterial cells	Mohankumar *et al.*, 2014; Ajeeta and Somashekar, 2015
F9	Time	Fermentation time plays an important role in detoxification	Faisal et al., 2014; Wardhani *et al.*, 2016
F10	Saponin	*Saponins* are the bitter compounds which may reduce the palatability of livestock feeds.	Sharath *et al.*, 2014
F11	Tannins	Tannins are polyphenolic biomolecule which have an astringent taste whose recovery is affected by pH, temperature and time	Sharath *et al.*, 2014

4.3.2 Reachability Matrix

The successive phase in ISM methodology is the preparation of initial reachability matrix. Based on the above pair-wise relationship of the factors in SSIM (Table 4.2), initial reachability matrix (Table 4.3) was built using binary system i.e. 0 and 1. This conversion is done by applying rules which are:

Table 4.2: Structural self-interaction matrix for the factors

S.No.	Factors	11	10	9	8	7	6	5	4	3	2	1
F1	Protein	A	X	A	A	O	O	A	A	O	O	-
F2	Lipids	X	X	X	A	O	A	A	A	A	-	
F3	Phorbol Esters	O	O	X	A	A	A	A	O	-		
F4	pH	V	X	A	V	A	V	A	-			
F5	RPM	O	X	A	X	O	V	-				
F6	Esterase	V	V	X	A	A	-					
F7	Temperature	O	V	O	V	-						
F8	Inoculum	O	V	X	-							
F9	Time	V	X	-								
F10	Saponin	O	-									
F11	Tannins	-										

If (i, j) result in the SSIM is V, then set (i, j) result in reachability matrix to 1 and (j, i) result to 0.

If (i, j) result in SSIM is A, then set (i, j) result in reachability matrix to 0 and (j, i) result to 1.

If (i, j) result in SSIM is X, then set (i, j) result in reachability matrix to 1 and (j, i) result to 1.

If (i, j) result in SSIM is O, then set (i, j) result in reachability matrix to 0 and (j, i) result to 0.

The final reachability matrix (Table 4.4) was constructed with the help of initial reachability matrix by applying transitivity rule, according to which if strategy 'X' is related to 'Y' and 'Y' is related to 'Z', then it is essential that X will also be related to Z. The element/cell which shows the transitivity is marked by star (*).

4.3.3 Level Partition

From final reachability matrix, different levels of sets are partitioned for each factor to find reachability set and antecedent set (Sage, 1977; Warfield, 1974). The reachability set of a definite factor includes itself and the other factor, which may help to accomplish the detoxification of *Jatropha* seed cake. The antecedent set includes the factors themselves and other factors that may contribute to achieve it which results in the intersection of these sets for all considered factors.

Table 4.3: Initial reachability matrix for the variables

Factors	1	2	3	4	5	6	7	8	9	10	11
F1	1	0	0	0	0	0	0	0	0	1	0
F2	0	1	0	0	0	0	0	0	1	1	1
F3	0	1	1	0	0	0	0	0	1	0	0
F4	1	1	0	1	0	1	0	1	0	1	1
F5	1	1	1	1	1	1	0	1	0	1	0
F6	0	1	1	0	0	1	0	0	1	1	1
F7	0	0	1	1	0	1	1	1	0	1	0
F8	1	1	1	0	1	1	0	1	1	1	0
F9	1	1	1	1	1	1	0	1	1	1	1
F10	1	1	0	1	1	0	0	0	1	1	0
F11	1	1	0	0	0	0	0	0	0	0	1

Table 4.4: Final reachability matrix for the variables

Factors	1	2	3	4	5	6	7	8	9	10	11	Driver power
F1	1	1*	0	1*	1*	0	0	0	1*	1	0	**6**
F2	1*	1	1*	1*	1*	1*	0	1*	1	1	1	**10**
F3	1*	1	1	1*	1*	1*	0	1*	1	1*	1*	**10**
F4	1	1	1*	1	1*	1	0	1	1*	1	1	**10**
F5	1	1	1	1	1	1	0	1	1*	1	1*	**10**
F6	1*	1	1	1*	1*	1	0	1*	1	1	1	**10**
F7	1*	1*	1	1	1*	1	1	1	1*	1	1*	**11**
F8	1	1	1	1*	1	1	0	1	1	1	1*	**10**
F9	1	1	1	1	1	1	0	1	1	1	1	**10**
F10	1	1	1*	1	1	1*	0	1*	1	1	1*	**10**
F11	1	1	0	0	0	0	0	0	1*	1*	1	**5**
Dependence power	**11**	**11**	**9**	**10**	**10**	**9**	**1**	**9**	**11**	**11**	**10**	

The reachability set and antecedent sets for each factor were calculated from final reachability matrix and are given in Tables 4.5 and 4.6. The factor acquiring the same reachability set and intersection set obtains level 'I' i.e. the highest position in ISM model (Kannan and Haq, 2007).

Table 4.5: Level partition (Iteration I)

Factors	Reachability Set	Antecedent Set	Intersection	Level
F1	1,2,4,5,9,10	1,2,3,4,5,6,7,8,9,10,11	1,2,4,5,9,10	I
F2	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F3	1,2,3,4,5,6,8,9,10,11	2,3,4,5,6,7,8,9,10		
F4	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10		
F5	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10		
F6	1,2,3,4,5,6,8,9,10,11	2,3,4,5,6,7,8,9,10		
F7	1,2,3,4,5,6,7,8,9,10,11	7		
F8	1,2,3,4,5,6,8,9,10,11	2,3,4,5,6,7,8,9,10		
F9	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F10	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F11	1,2,9,10,11	2,3,4,5,6,7,8,9,10,11		

This procedure is completed on a different level of iteration. The factors acquring level 'I' are deleted in next iterations. The next iteration was then completed with the remaining factors and by repeating in the same manner and continuously performing these iterations until each factor obtains a certain level.

4.3.4 ISM based model

The bus diagram as shown in Figure 4.3 represents the different factors and their corresponding inter-relation. From the above inter-relationships, the selected manipulative steps have been divided into four levels for the factors related to detoxification of *Jatropha* seed cake. Temperature (F7) is the most important factor which drives all the other factors which result in the successful integration of detoxification. Temperature plays a major role in various metabolic processes like protein denaturation, enzyme inhibition, production of metabolites, cell death,etc (Selwal *et al*., 2010).

Table 4.6: Level partition (Final Iteration)

Factors	Reachability Set	Antecedent Set	Intersection	Level
F1	1,2,4,5,9,10	1,2,3,4,5,6,7,8,9,10,11	1,2,4,5,9,10	I
F2	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F3	3,4,5,6,8	3,4,5,6,7,8	3,4,5,6,8	III
F4	3,4,5,6,8	3,4,5,6,7,8	3,4,5,6,8	III
F5	3,4,5,6,8	3,4,5,6,7,8	3,4,5,6,8	III
F6	3,4,5,6,8	3,4,5,6,7,8	3,4,5,6,8	III
F7	7	7	7	IV
F8	3,4,5,6,8	3,4,5,6,7,8	3,4,5,6,8	III
F9	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F10	1,2,3,4,5,6,8,9,10,11	1,2,3,4,5,6,7,8,9,10,11	1,2,3,4,5,6,8,9,10,11	I
F11	11	3,4,5,6,7,8,11	11	II

Detoxification of *Jatropha* seed cake employs the use of microorganisms so the temperature can be considered as a significant parameter since enzyme-catalyzed

reactions are sensitive to little changes in temperature. So temperature (F7) at level 4 builds the base of this ISM model.

Temperature (F7) escorts the five factors at level 3 i.e. Phorbol Esters (F3), pH (F4), RPM (F5), Esterase (F6) and Inoculum (F8). These five factors have strong relation among them. pH is another significant factor for any biological process, as it changes with metabolic activities of an organism and thus affects the production of enzymes. In a research done by Wardhani *et al.*, (2016), phorbol ester degradation increased with an increase in buffer pH from 5 to 7 and decreased when the pH further increased to 9. Hence the highest PE degradation was obtained at pH 7. Inoculum level also has a considerable control on enzyme production; lower inoculum level may result in insufficient biomass, leading to reduced product formation. On the other hand, higher inoculum level may enhance biomass production but leads to the poor product because of depletion of nutrients (Selwal *et al.*, 2010). In a study done by Muhammad *et al.*, 2016, low inoculum level resulted in less xylanase production but when the inoculum level was increased the enzyme production also increased. In another finding, phorbol ester degradation increased with an increase in inoculum level and a further increase in inoculum did not have a significant effect on PE degradation (Wardhani *et al*., 2016).

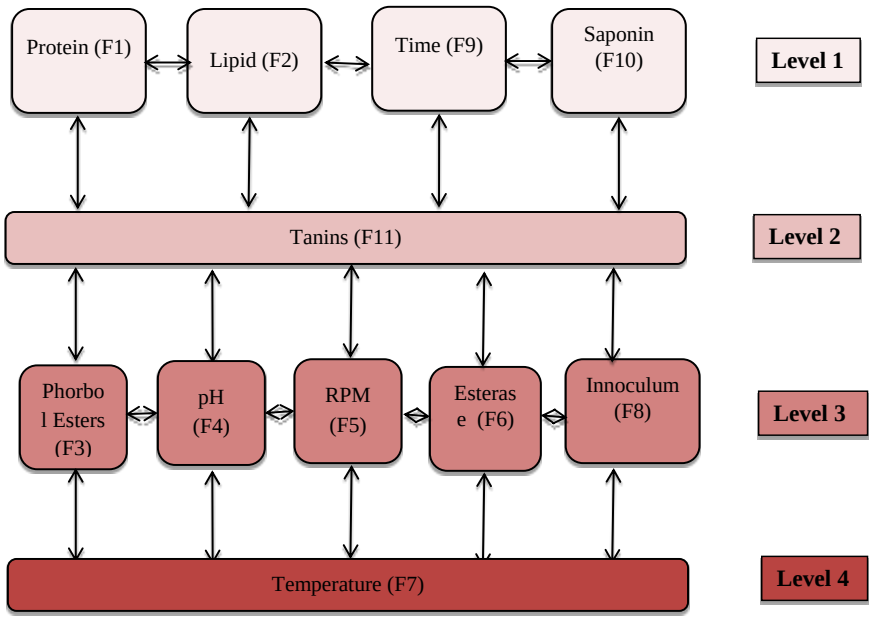


Figure 4.3: Model based on ISM

To optimise the inoculum size, spore suspensions containing 10^3- 10^8 spores/ml were tested for maximum lipase (457.41 U/g) and protease (1553.23 U/g) activities that were obtained with an inoculum containing 1 x10^7 spores/5 g *Jatropha* seed cake (Veerabhadrappa *et al.,* 2014).Therefore it is important to have an optimum inoculum level which could help in attaining maximum degradation of phorbol esters. The agitation speed can influence the extent of mixing in the submerged fermentation systems and may also affect the nutrient availability. Agitation helps in anadequate mixing, improves the mass, heat transfer and dissolved oxygen in the culture medium (Darah *et al*., 2015). At low agitation speed, insufficient oxygen in the medium affects the microbial growth, whereas high agitation speed would sometimes lower the enzyme production (Seth and Chand, 2000).

At level 2 factor, Tannins (F11) ushered by level 4. In a study done by Rakic *et al*. (2004), the thermal treatment caused a significant reduction in the tannin content. Obiang and Ryu (2013) obtained similar results in their study in which extrusion processing at a temperature of 120-140 ˚C led to a reduction in the tannin content. Makkar and Becker (1996) concluded in their research that the tannin recovery decreased with increase in temperature, pH and storage time. They suggested that alkaline pH, higher temperature and presence of oxygen or any other oxidising agent helps in inactivating the tannins.

Protein (F1), Lipid (F2), Time (F9) and Saponin (F10) at level 1 guided by level 2 and are the desired outcomes of the figures. These four factors achieve the topmost position of this hierarchical representation. Many researchers have described the effect of tannins on protein and lipid content (Bartosz *et al*., 2012; Marwan *et al*., 2016). Extraction time also plays a major role in the extraction of tannins from the plant (Baldosano *et al.,* 2015; Villamor, 2009). A significant increase in tannin contents with an increase in time and temperature were observed by Rehman *et al*. (2002). So we can conclude that the optimum values of the factors like Protein (F1), Lipid (F2), Time (F9) and Saponin (F10) can help in getting desired quality of *Jatropha* seed cake.

4.4 MICMAC analysis

'MatricedImpacts croises-multiplication applique' and classment (cross- impact matrix multiplication applied to classification) simply known as MICMAC analysis, is based on properties of matrices multiplication (Dewangan *et al.*, 2015; Khan and Haleem, 2012; Diabat and Govindan, 2011; Kannan *et al*., 2009; Kandasamy *et al*., 2007) .i.e. MN = Max {min (m_{ij}, n_{ij})} where,

M = [m_{ij}] and N = [n_{ij}] are two fuzzy matrices.

ISM Fuzzy MICMAC analysis involves the following steps (Gorane and Kant, 2013; Dubey and Singh, 2015):

I: Development of binary direct relationship matrix (Gorane and Kant, 2013)

II: Development of Fuzzy direct reachability matrix

III: Creating stabilised fuzzy MICMAC matrix

MICMAC analysis is done on the basis of driving and dependence power in final reachability matrix. This analysis then categorised into four segments as shown in Figure 4.4.

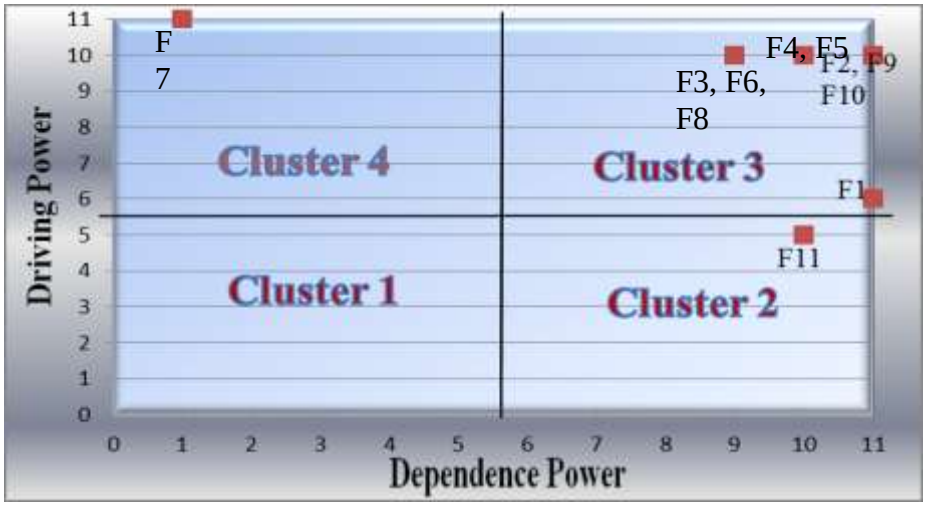


Figure 4.4: MICMAC Analysis Graph

Segment I (Autonomous): This segment has low driving power and dependence power. No factor fell in this segment.

Segment II (Dependence): It has low driving power but high dependence power; only one factor fell in this segment.

Segment III (Linkage): This segment has high driving as well as dependence power. A total of nine factors acquired this segment.

Segment IV (Driving): This segment has high driving power but less dependence power. Only one factor had fallen in this segment.

Cluster 1 (Autonomous Cluster): Factors in this cluster have very low driving and dependence power. They have no relation with others and are not influenced by any other factors. There was no factor in our study that fell into this cluster.

Cluster 2 (Dependence Cluster): Factor that have high dependence power but low driving power is known as dependence cluster. This cluster represents factors which are dependent on other factors. The dependency of the factors falling in this cluster shows that they need other factors for their implementation into the system. Tannins (F11) is the only factor which fell in this category.

Cluster 3 (Linkages Cluster): Factors in this cluster have very high driving and dependence power and have very strong relation among them. Most of the factors in this study fell in this cluster. Any change in one factor will show immediate effect on other factors. A total nine factors were categorised in this cluster i.e. protein (F1), lipids (F2), phorbol esters (F3), pH (F4), RPM (F5), esterase (F6), inoculum (F8), time (F9) and saponins (F10).

Cluster 4 (Independent Cluster): Factors in this cluster have very high driving power but low dependence power. Factors that fall in this cluster are also known as driving cluster. Temperature (F7) is the only factor that acquired this cluster.

4.5. Conclusion

It is clear that different factors in detoxification of *Jatropha* seed cake have a different impact in their implementation. Thus, it was essential to analyse the mutual relationship among these factors to identify the critical issues which push the other factor and help researchers to eliminate them. To achieve the objective, eleven major

factors in detoxification of *Jatropha* seed cake were identified through extensive literature review and discussions with experts and the selected factors were assessed using the structural modelling. This assessment was then used to develop the ISM to understand the mutual relationship among various detoxification factors. "Temperature" has been identified as a most crucial factor which affects all other factors for successful implementation of the process. "Phorbol Esters", "pH", "RPM", "Esterase", "Inoculum" are the factors which were identified at the third level of the ISM model which shows that all these factors are driven by the level- two factor i.e. Tannins and level- one factors namely "Protein", "Lipids", "Time", "Saponins". This ISM can be used for lab scale trials for studying the PE detoxification before scaling up the process.

CHAPTER 5

ISOLATION AND SCREENING OF ESTERASES PRODUCING MICROBES AND PROCESS OPTIMIZATION OF DETOXIFICATION OF *JATROPHA* PRESS CAKE

5.1 Introduction

Jatropha plant belongs to *Euphorbiaceae* family and has achieved significant interest in biodiesel production because of its high oil content. *Jatropha* biodiesel industry is still in developing stage because of high cost, lack of utilization of toxic by-products, and no support from government in supply chain and infrastructure in some of the countries. Mechanical extraction of oil from *Jatropha* seed for biodiesel production results in a considerable amount of solid cake which is rich in proteins, minerals, amino acids etc. (Abhilash *et al.*, 2011). Despite of high nutritional quality, *Jatropha* seed cake is non-edible because of several anti-nutritional factors like tannin, saponin, lectin, protease inhibitors, phytate and most importantly the phorbol esters (Pradhan *et al.*, 2012). Phorbol esters are bioactive diterpene derivatives and have been recognized as major toxic constituent in *Jatropha* plant. Co-carcinogenicity and tumor promotion are some of the biological effects of phorbol esters along with other biochemical and cellular effects (Blumberg, 1980 &1981; Devappa, 2010). Phorbol esters are detrimental to many insects, birds, humans as well as most of the microorganisms including bacteria, fungi (Xing *et al.*, 2013). At the same time *Jatropha curcas* contains an alkaloid in its latex which has anti-cancerous and antimicrobial properties which can be used externally for diseases like rheumatism, skin problems etc. (Usman and Osuji, 2007).

Several methods to remove phorbol esters from *Jatropha* seed cake have been reported that would allow the use of detoxified meal as food/feed supplement. Heat, radiations and different chemical treatments are being used to detoxify *Jatropha curcas* seed cake but all these methods require a combination of treatments which makes them expensive and a time consuming processes. Detoxification using

microorganisms is still at infancy stage, although few researchers have reported detoxification of *Jatropha* seed cake using fungi and bacteria by solid state fermentation process. In a work done by Anjali and Haresh (2014), solid state fermentation (SSF) with white rot fungi for 20 days resulted in total removal of phorbol esters from *Jatropha* seed cake. In a study conducted by Xing *et al.* (2013), there was 97% reduction in phorbol ester content in 9 days when SSF of seed cake was done with *Streptomyces fimicarius*. Chetna *et al.* (2011) reported the decrease in phorbol ester content to an undetectable level when the seed cake was fermented (SSF) with *Pseudomonas aeruginosa* PseA strain within 9 days at 30°C, pH 7 and 65% relative humidity.

Detoxification by SSF has economical advantages but at the same time it is a time consuming process. On the other hand, submerged fermentation is a rapid and best suited process for the microorganisms that require high moisture content for their growth such as bacteria. In a work done by Chang *et al.* (2014), the *Bacillus* strains took 7 days under submerged fermentation for 100% degradation of phorbol esters. Present work aimed at isolation and identification of lipase/esterase producing microorganisms preferably *Pseudomonas* for detoxification of *Jatropha* seed cake by SMF. To best of our knowledge, SMF using *Pseudomonas* has never been investigated in literature for the detoxification of *Jatropha* seed cake. *Pseudomonas* has been well reported as a lipase and protease producer (Mahanta *et al.*, 2008). The investigation includes the morphology and growth characteristics of the microorganism isolated. The fermentation process was optimized using Taguchi technique which helps in studying large number of factors and their effects on responses with a few experimental runs. L18 orthogonal array was used to study the effect of fermentation conditions on the phorbol ester content of *Jatropha* seed cake. Multi-factorial optimization of phorbol ester degradation by SMF using Taguchi method has not been reported until to date.

5.2 Materials

Deoiled *Jatropha curcus* seed cake was obtained from Biodiesel Plant (CASRAE), DTU Delhi, India. The seeds were dried, pressed to obtain oil and the residual

pressed deoiled seed cake was ground and stored at 4°C in zip lock pouches. Culture media were procured from Hi media Laboratories (Mumbai, India). Standard i.e Phorbol-12-myristate 13-acetate (Sigma Aldrich, ≥99 %), HPLC grade acetonitrile (Fischer Scientific), HPLC grade methanol (SD fine) were used as received from manufacturers. Other chemicals/solvents used for study were procured from standard companies and were of analytical grade.

5.3 Methods

5.3.1 Chemical composition of *Jatropha curcas* seed cake

The moisture content of *Jatropha* seed cake samples was determined by hot air oven method to a constant weight at 105°C (Method 930.15, AOAC, 2005). Crude protein (Method 954.01, AOAC, 1990), lipid (Method 945.16, AOAC, 2000) and ash contents (Method 942.05, AOAC, 1990) were determined according to the standard methods of AOAC.

Figure 5.1: Biodiesel centre (a) and *Jatropha* Tree (b) at DTU, Delhi

5.3.2 Isolation and screening of microbial flora for potential phorbol esters degradator

5.3.2.1 Collection of soil samples: Soil for the isolation of micro-organisms was taken from two sources i.e. from petrol pump and near rhizosphere beneath *Jatropha* tree. Microbial colonies were isolated from two types of soil using serial dilution method.

5.3.2.2 Initial Isolation of microorganisms: 10 g of each soil sample was weighed and added to 90 ml sterile distilled water. The samples were stirred and dilutions were made (10^{-1} to 10^{-7} dilution factor). 0.1ml from each dilution was inoculated into nutrient agar petri dishes. The plates were incubated at 37°C for 3 days. Isolated colonies were picked and re-streaked onto a fresh nutrient agar plates. The plates were incubated at 37°C for 2 days.

Nutrient agar composition

Peptone	0.5%
Beef extract	0.3%
Agar -agar	1.5%
NaCl	0.5%

***5.3.2.3 Screening for lipolytic bacteria*:** After successful isolation from soil, the bacterial colonies were sub-cultured on Tween-20 media and their morphology was observed. Tween-20 media was prepared by taking the following:

Tween 20 media

Tween-20	10 v/v
Peptone	10 g/l
Nacl	5 g/l
$CaCl_2$	0.1 g/l
Agar-agar	20 g/l

The contents were autoclaved at 121ºC [15 psi] for 15min and the colonies were streaked on Tween-20 plates and incubated at 37 ºC for 24 h. A total of 26 colonies were streaked and screened for lipolytic activity.

5.3.2.4 Screening of isolates for lipase/esterase activity: Isolates from Tween-20 plates were grown in Tributyrin agar. Colonies from Tween-20 plates were picked and individual colony was streaked on Tributyrin plates which were then incubated at

37 ºC for 24 h. Lipase or esterase activity was determined by the formation of zone of hydrolysis around the bacterial colonies.

Tributyrin Media (Prasanna *et al.* 2012)

Peptone	0.5 % (w/v)
Beef extract	0.3 % (w/v)
Trybutyrin	1.0 % (v/v)
Agar	2.0 % (w/v)

5.3.2.5 Selection of potential strains with hydrolytic/esterolytic activity: Tributyrin agar containing 1% (v/v) Tributyrin was emulsified using homogenizer and autoclaved at 121°C [15min] and cooled to 45°C under laminar air flow. Media was aseptically poured into sterile petri plates. The plates were then punched aseptically with sterile cork borer to obtain 8.5mm dia wells. These Tributyrin agar plates were loaded with freshly extracted 100 µl crude lipase enzyme in each well separately and incubated at 37°C for 48 h. The developed clearance zones around the wells were measured (in mm) and data was used for further studies.

500 µl of revived culture was added to 100ml of Tributyrin broth in 250ml flasks

⇩

Flasks were incubated in a rotary shaker at 200rpm for 24h

⇩

Sodium azide was added to flasks (to have antimicrobial action in the supernatant)

⇩

Culture broth was transferred to centrifuge tubes and centrifuged

at 4000rpm for 30 mins

⇩

Supernatant decanted into other falcon tube

⇩

Wells were made in tributyrin plates using cork borer

⇩

100 µl of supernatant was added into wells

⇩

Plates were incubated for 16h and 24h

5.3.3 Characterization and identification of the isolates

5.3.3.1 Based on morphological characteristics: After isolation of colonies, colony morphology formed a major criteria in the identification of bacteria. The bacterial colonies were characterized in terms of size, shape, colour, elevation, consistency and transparency. The observed morphological characteristics included shape as (circular, filamentous, irregular), elevation (flat, raised, pulvinate), pigmentation (green, yellow, pink, white, transparent), size (small, moderate, large) and texture.

5.3.3.2 Based on Gram Staining: Preliminary identification was done with the help of Gram staining. Isolates showing positive results were subjected to Gram-staining and the isolates were classified based on morphological and Gram-staining results. Gram-positive organisms retained the crystal violet stain, while Gram-negative organisms were decolorized by solvent and attained safranin as counter-stain. In the Gram-stain procedure, smear was heat-fixed and then stained with a basic crystal violet dye. The slides were then treated with Gram's iodine for a minute to fix the stain, washed briefly with 95% ethanol and finally stained with counter stain safranin. The slides were then washed with distilled water and dried in air and observed under microscope.

5.3.3.3 Based on biochemical tests and 16S rRNA sequence: Various biochemical tests were performed for the identification of bacterial isolates such as catalase production, MR-VP test, oxidase test, Urease test, citrate utilization, indole production, nitrate reduction, motility test, hydrogen sulphide production, gelatin & starch hydrolysis and carbohydrates utilization tests (Inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose etc), according to the procedure described by Tokuyasu *et al.* (2012). The identification of strain DS1 was performed at Helix Biogenesis, India. In brief, the 16S rDNA sequencing was conducted using purified amplicons obtained employing universal primers on ABI 3500 Genetic Analyzer.

5.3.3.4 Enzyme activity: The culture showing the maximum zone of clearance around the wells was then grown in lipase production medium. The composition of the medium:

Enzyme Production medium (%)

Starch	2
$Na_2HPO.12.H_2O$	0.8

KH_2PO_4	0.5
KNO_3	1.01
NH_4Cl	0.535
$MgSO_4.7H_2O$	0.001
$CaCl_2$	0.01
Olive oil	1

Enzyme was prepared from *Pseudomonas* by growing in enzyme production media followed by incubation in a rotary shaker at 37 ˚C for 24 h at 100 rpm. Enzyme was obtained by removing bacterial cells by centrifuging at 10000 rpm for 10mins.

Lipase Assay: Lipase assay was done by titrimetric method using olive oil as a substrate. Olive oil emulsion was prepared using olive oil (10% v/v) and gum Arabic (5% w/v) in 100mM potassium phosphate buffer pH 7.0. 100 µl of crude enzyme was added to emulsion and incubated for 30 mins at 37°C. The reaction was stopped by addition of acetone: ethanol (1:1) mixture and fatty acids liberated were estimated by titrating with 0.05M NaOH using phenophathelin indicator. One unit of enzyme is defined as the amount of enzyme required to hydrolyse one µmol of fatty acid from triglycerides. Selection of optimum conditions for the production of lipases was done by varying one factor at a time and keeping other factors at fixed entre value. The assay mixture was incubated at temperature range of 25 - 45°C, pH 6.5-8.5 and time 12 - 36 h.

5.4 Submerged fermentation of deoiled *Jatropha* seed cake

5.4.1 Preparation of catalyst

Enzyme was prepared from *Pseudomonas aeruginosa* DS1 isolated in our lab as mentioned in Section 5.3.3.4.

5.4.2 Pilot experiment

Seed cake was autoclaved for 1 h and 5 g of it was added to 100ml crude enzyme broth 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.

5.4.3 Submerged fermentation of deoiled *Jatropha* seed cake

Seed cake was autoclaved for 1 h and was added into the supernatant (prepared above) for different combinations as per L18 orthogonal array. The flasks were incubated in rotary shaker set for each trial for different periods of time and the phorbol esters were extracted. All the experiments were done in triplicates.

5.5 Phorbol esters extraction from seed cake

Extraction of phorbol esters was done with absolute methanol by the method described by Xing *et al.* (2013). Briefly, methanol was added to seed cake in 7:1 ratio and the mix was incubated in an ultrasonic bath for 15 min and filtrate was collected. The extraction process was repeated twice and the methanol extract fractions were pooled. Methanol was recovered by rotary vacuum evaporator at 60°C and the extract was fully dried to constant weight in hot air oven at 40˚C.

5.6 Quantification of phorbol esters

For preliminary quantification of phorbol esters, 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. The fractions were analyzed for phorbol esters by TLC before confirmation analysis by HPLC. The untreated and treated seed cake samples were run on HPLC [Agilent-1260, USA] (Figure 5.2) with UV detector (Agilent G1315D MWD) and a reverse phase C18 column [5 µm, 4.6 X 250 mm i.d. Thermo Electron Corporation Massachusetts (MA) USA]. Method adopted for quantification was in accordance with Wink *et al.* (2000). The solvent system used was: water (A) and acetonitrile (B), programmed A: B as 60:40 (15 min), 25:75 (20 min), and 100: 0 (10 min) with a flow rate of 1.3 ml/min.

All the HPLC experiments were performed at room temperature (25˚C). PMA (*Phorbol* 12-myristate 13-acetate) was used as external standard. The characterization was done by Mass spectrometry analysis (LCMS, Waters-SYNAPT-G2) using ES+ technique (Figure 5.3).

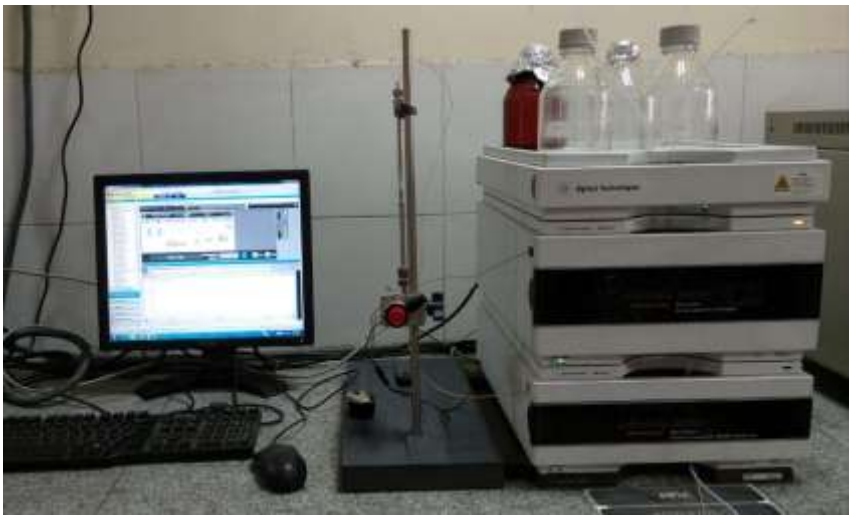


Figure 5.2 HPLC Unit (Agilent-1260, USA)

Figure 5.3: Liquid Chromatography–Mass Spectrometry Unit (LCMS, Waters-SYNAPT-G2)

5.7 Optimization using Taguchi Method

The process parameters which influence phorbol ester degradation in the most significant way were selected for investigating their effect by submerged fermentation process. The process parameters considered were: pH (A), percentage of the seed cake (B), time (C), temperature (D) and frequency of rotation (RPM) (E). The design parameters and their different levels considered for optimization of the study by Taguchi method are shown in Table 5.1. The effect of these variables was identified using ANOVA based on S/N ratio. Taguchi L18 orthogonal array (Table 5.2) was followed for performing experiments at each level and response was measured in the terms of phorbol ester degradation.

Table 5.1: Design parameters and their levels selected for the Taguchi experimentation

Parameter	Parameter Name	Range	Level 1	Level 2	Level 3
A	pH	6.5 and 7.5	6.5	7.5	-
B	Seed cake	1-9 (%)	1	5	9
C	Time	9-12 (h)	9	12	15
D	Temperature	25-45 (˚C)	25	35	45
E	RPM	100-150	100	125	150

5.8 Analysis of the Experimental Data

The results of fermentation runs were analyzed using the software MINITAB® (USA) and Design Expert v.10.0.3.1 (Stat-Ease Inc.,Minneapolis, MN, USA). The quality characteristic or the response can be obtained in three different forms i.e. the lower-the-better, the higher-the-better, and the nominal-the-better. Phorbol ester degradation was taken as higher-the-better type in the present work because a lower or nominal value of phorbol ester degradation may result inadequate detoxification of the seed cake which is not desired if the seed cake is to be used for edible purpose. According to Taguchi, S/N ratio is an important characteristic for the evaluation of the quality, higher the S/N ratio better will be the quality. Therefore, the highest S/N ratio is considered as an optimum level of the process parameters. The S/N ratio for higher-the-better type of response can be calculated (Ross, 1988; Roy, 1990) as:

$$(S/N)_{HB} = -10 \log \left[\frac{1}{R} \sum_{j=1}^{R} \frac{1}{Y_j^2} \right]$$

Where, Y_j, j= 1, 2... R is the observed response values repeated R number of times. The F ratio of the parameters in the design was accounted to be statistically significant.

5.9 Fourier Transform Infrared Spectrophotometry (FTIR)

FTIR analysis was done using a Perkin Elmer spectrum (Version 10.5.3, US) equipped with an IR microscope. All spectra ranged between wave numbers 4000 and 550 cm^{-1}.

Table 5.2: Orthogonal array for Taguchi L18 with response (raw data and S/N ratios)

Input parameters						Response			S/N ratio
Trial No.	**pH**	**Seed cake (%)**	**Time (h)**	**Temperature (˚C)**	**RPM**	**R1**	**R2**	**R3**	
1	6.5	1	9	25	100	49.7	43.4	47.9	33.40
2	6.5	1	12	35	125	78.6	88.4	77.1	38.16
3	6.5	1	15	45	150	89.4	90.8	87.1	38.99
4	6.5	5	9	25	125	32.6	31.3	30.1	29.91
5	6.5	5	12	35	150	67.3	65.5	67.3	36.48
6	6.5	5	15	45	100	74.8	69.1	67.7	36.94
7	6.5	9	9	35	100	51.1	49.9	52.1	34.15
8	6.5	9	12	45	125	47.2	49.9	48.7	33.73
9	6.5	9	15	25	150	32.8	37.1	37.8	31.05
10	7.5	1	9	45	150	81.3	87	89.5	38.66
11	7.5	1	12	25	100	89.4	88.9	86.5	38.91
12	7.5	1	15	35	125	98.2	97.1	97.9	39.80
13	7.5	5	9	35	150	64.5	66.4	62.6	36.18
14	7.5	5	12	45	100	69.2	67.8	68.8	36.73
15	7.5	5	15	25	125	49.6	48.7	48.5	33.79
16	7.5	9	9	45	125	64.7	61.1	60.1	35.83
17	7.5	9	12	25	150	62.4	63.2	61.6	35.90
18	7.5	9	15	35	100	71.8	72.9	75.2	37.30
	Total					1174.6	1178.5	1166.5	645.92
	Mean					65.18			
R1, R2 and R3 represent the phorbol ester degradation values for three replicates of each trial.									

5.10 Results and discussion

5.10.1 Chemical composition of *Jatropha curcas* seed cake

The proximate composition of defatted *Jatropha* seed cake was found out and presented in Table 5.3. The protein content of *Jatropha* seed cake was 18% and it was slightly lower than the values reported by Makkar *et al.* (1997) which ranged from 22% - 27%. This slight variation in protein content could possibly be due to difference in the variety of the seed or the climatic conditions of the country. There was significant difference in the fat content (6.15%) compared to the values reported by Inekwe *et al.* (2012) i.e. 14-19% which implies that our method of oil extraction from the seed cake is more efficient as compared to methods reported in literature. Moisture content of seedcake was 5.2% which is comparable to that reported in literature. The ash content was fairly high (3%) which indicates that seed cake could be a good source of minerals to be used as animal feed. The phorbol ester i.e. the main toxin in the seed cake was 0.3 mg/g which is similar to that reported by Gogoi *et al.* (2014).

Table 5.3 Chemical composition of *Jatropha* seed cake

		Reference
Protein	18%	Method 954.01, AOAC, 1990
Fat	6.15%	Method 945.16, AOAC, 2000
Moisture	5.2%	Method 930.15, AOAC, 2005
Ash content	3%	Method 942.05, AOAC, 1990
Dry matter	948 g/kg	Method 934.0, AOAC, 1990
Phorbol esters	0.3 mg/g	Makkar *et al.*, 2007

5.10.2 Initial isolation of microorganisms

Microbial colonies were isolated from two types of soil i.e. from petrol pump and near rhizosphere beneath *Jatropha* tree by serial dilution technique (Figure 5.4). Many bacterial colonies were observed in the first two days from which single and independent colonies were chosen based on distinct size, shape, elevation, pigmentation, consistency and margin for sub-culturing. A total of 26 bacterial colonies were isolated as pure culture from soil samples and were labelled from A to Z.

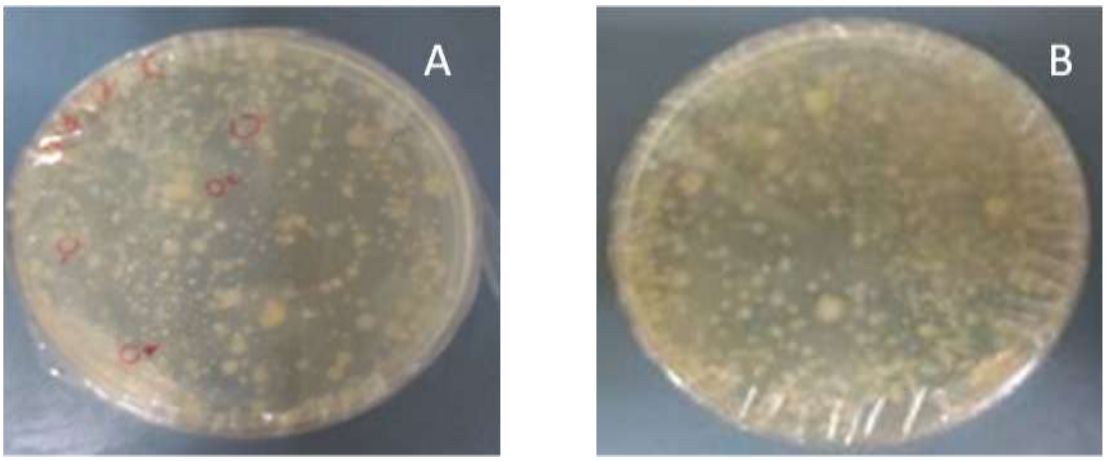


Figure 5.4: A: Mixed population from petroleum contaminated soil sample. B: Mixed population from rhizosphere near *Jatropha curcas*

5.10.3 Screening for lipolytic bacteria

Preliminary screening of the bacterial isolates was done using Tween 20 medium. Lipase/esterase producers synthesize lipolytic enzymes that act on Tween 20 resulting in formation of white insoluble crystals of calcium salts of fatty acids within the media. Out of 26 streaked microbial colonies, 19 were screened on the basis of white precipitation around the colonies which is an indicative of lipase activity. The morphological characteristics and Gram staining was done for these 19 bacterial isolates. Figure 5.5 shows some of the isolates growing on Tween 20 medium.

5.10.4 Screening of isolates for lipase/esterase activity

The extracellular lipase or esterase activity was confirmed by the formation of zone of hydrolysis around the bacterial colonies. The 19 isolates were grown in Tributyrin medium and observed for clear halos around the colonies after 24-48 h of growth. All the Tween 20 positive isolates showed clear zone of hydrolysis with tributyrin. Figure 5.6 shows the zone of hydrolysis around some of the isolates.

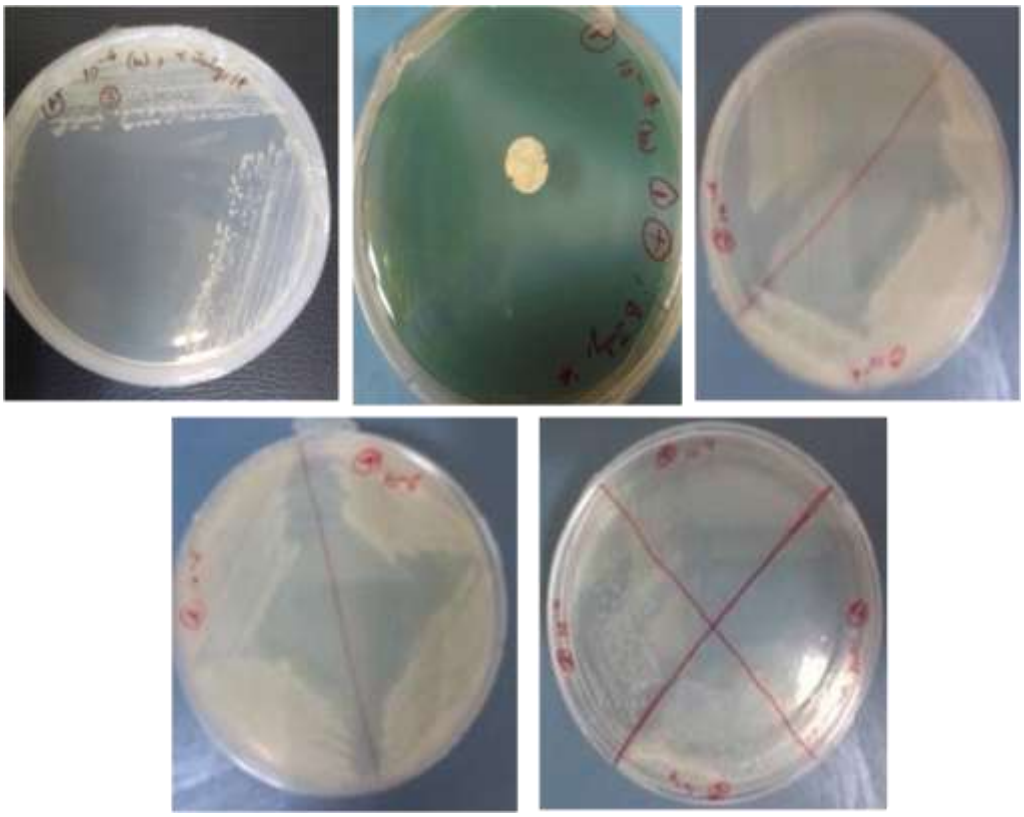


Figure 5.5: Individual isolates growing on Tween medium

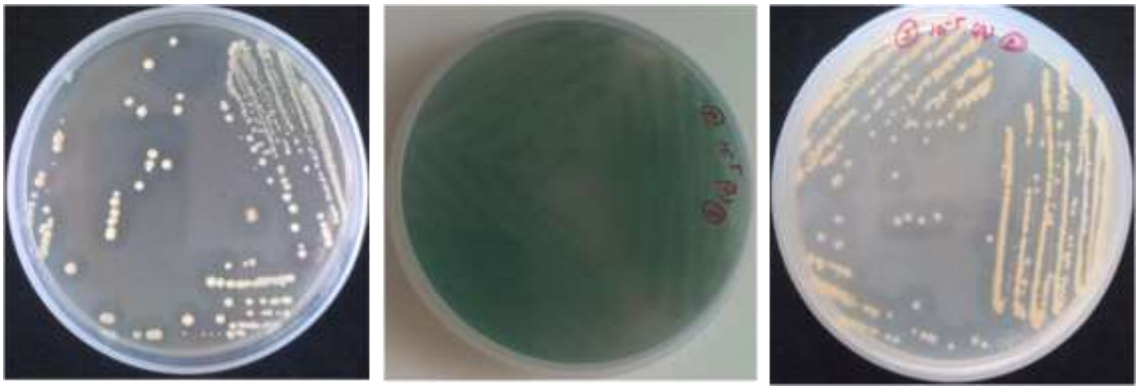


Figure 5.6: Lipolytic activity is shown by the clearance zone of hydrolysis around the bacterial colonies

5.10.5 Selection of potential strains with hydrolytic/esterolytic activity

i) Agar plate assay: Tributyrin agar plate assay was done with the help of tributyrin halo zone method for the selection of most potential strain producing enzyme for phorbol ester degradation. Lipase/esterase enzyme production was indicated by the formation of clear

halos around the colonies grown on tributyrin containing agar plates. Out of 19 screened strains, 5 were found to produce greater halo zones (Figure 5.7)

Figure 5.7: Plate assay showing halo zone formation around the bacterial streaks

ii) Well plate assay: Well plate assay was done for five strains which were screened on the basis of halo zone method to select the most potential strain that could be carried further for the detoxification of seed cake. The strain that showed the maximum clearance zone was selected. Figure 5.8 shows the clear halos around the well of the four best cultures. Clear zones developed around the wells were measured (mm) for a period of seven days (Table 5.4). The bacteria (strain S) showing the largest zone of clearance on well plate assay was finalized as a potential strain for the detoxification experiments.

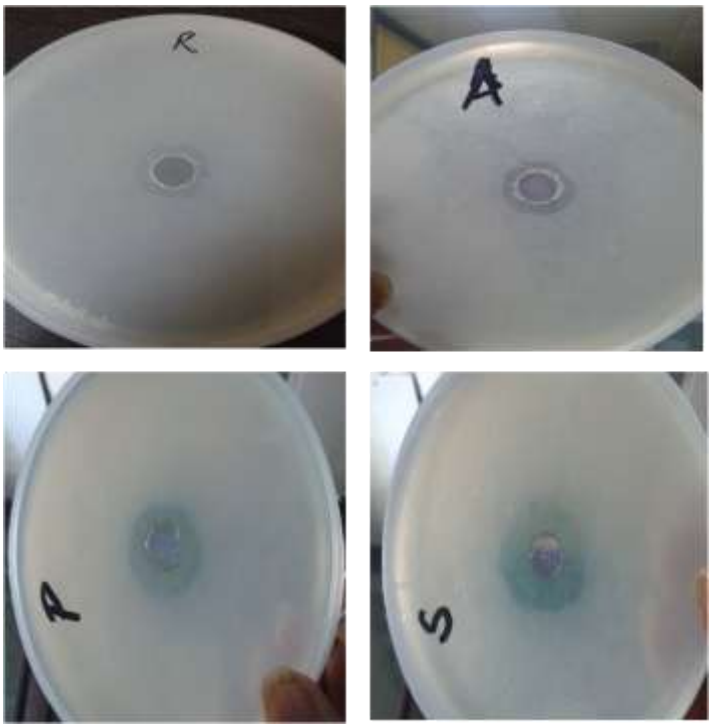


Figure 5.8: Well plate assay of some of the isolates

Table 5.4: Diameters of zone of clearance of the four potential strains

S.No.	Strain Code	2 days (mm)	4 days (mm)	6 days (mm)
1	**P**	08	12	19
2	**S**	10	18	24
3	**A**	05	09	12
4	**R**	03	05	09

5.10.6 Morphological characteristics and gram staining

Bacteria were characterized based on morphology of colonies and Gram staining. Morphological characteristics of 19 pure cultures isolated from different soil samples were studied (Table 5.5). The taxonomical characteristics of some of the strains were identical in morphology and Gram-staining. The isolated colonies were either yellow, pale or whitish in color. Microbial observations showed that both Gram negative and Gram positive bacteria were present in the soil sample but Gram-negative sphere and rods were pre-dominant. Figure 5.9 shows the microscopic view of the isolates.

Table 5.5: Morphological characteristics and gram staining of 19 cultures

Cultures from petroleum contaminated soil				
Culture code	**Gram staining**	**Morphology**	**Arrangement**	**Color**
A	Negative	Coccus	Diplococcus	Pale
G	Negative	Cocci	Diplococcus	Yellow
H	Negative	Mixed rods and Cocci	Singlets	Yellow
I	Negative	Cocci	in groups	Pale
L	Positive	Cocci	diplococcus	White
M	Negative	Cocci	Singlets	White
O	Negative	Cocci	Singlets	Yellow
P	Negative	Rods	Tetrads	Yellow
Q	Negative	Cocci	in groups	Pale
R	Negative	Cocci	Diplococcus and tetrads	Pale
S	Negative	Rods	Doublets	White
Cultures from soil near rhizosphere beneath *Jatropha* tree				
B	Negative	Rods	In tetrads	Brown
C	Negative	Coccus	Singles and in groups	Yellow
D	Negative	Coccus	In tetrads	Yellow
E	Negative	Rods	In long chains	White
F	Negative	Rods	Diplobacilli	Yellow
J	Negative	Coccus	Singlets	Pale
K	Negative	Coccus	Diplococci	White
N	Negative	Rods	Diplobacilli	White

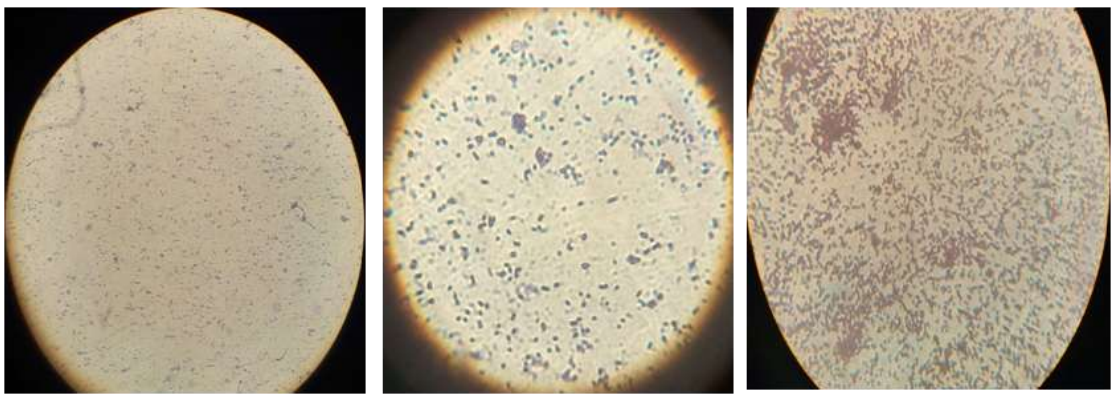


Figure 5.9: Microscopic view of isolates

5.10.7 Biochemical tests and 16S rRNA sequencing

The bacterial strain 'S' showing the largest zone of clearance on well plate assay was identified based on morphological, biochemical characterization according to Bergey's Manual of Determinative Bacteriology- 9 Ed. Biochemical tests were performed of the selected strain 'S'. All the results were noted (Table 5.6) and the strain was identified and designated as *Pseudomonas* strain. The lipolytic activity of *Pseudomonas* strain was further screened on minimal media agar containing 1% olive oil and fluorescent dye Rhodamine. Orange color fluorescent halos around lipase producing colonies were seen when the plates were exposed to UV light at 350nm.

Table 5.6: Results of biochemical tests for identification of selected strain

Test	Results	Test	Results
Gram staining	Gram negative	Starch	+
Catalase	+	Citrate	+
Motility	+	TDA	-
Nitrate	+	Gelatin	-
Lysine	+	Malonate	+
Ornithine	-	Inositol	-
H_2S	-	Sorbitol	-
Glucose	+	Rhamnose	+
Mannitol	-	Sucrose	-
Xylose	+	Lactose	-
ONPG	-	Arabinose	+
Indole	-	Adonitol	-
Urease	+	Raffinose	-
MR	+	Salicin	-
VP	-	Arginine	+
		Pigment	Sap green

Figure 5.10: DNA sequence chromatogram

AAAGGGCAGCCGAACCTAACACTGAATTGCGGAGGAAGGGAAGTTGCTCCTGGA
TTCAGCGGCGGACGGGTGAGTAATGCCTCAGGAATCTGCCTGGTAGTGGGGGAT
AACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGG
ATCTTCGGACCTCCACGCTATCAGATGAGCCTTAGGTCGGATTAGCTAGTTGAGT
GGGGTAAAGGCCTCACCAAGGCGACGATTCCGTAACTGGTCTGAGAGGATGATC
AGTCACACTGGAACATGAGACACGGTTCCAGACTCCTTACGGGAGGCAGCAGTG
GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAAT
ACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGC
AGGCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAA
CTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTG
TAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC
TGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGAT
CTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG
GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTAAT
CCAGAGATGGATTGGTGCCTTCGGGAACNGTGACACAGGTGCTGCATGGCTGTCG
TCAGCTCNTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTC
CTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGGATGACGTCCAAGTCATCATGGCCCTTACGGCCAGGGCTAC
ACACGTGCTACAATAGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGNGCTA
ATCCCATAAAACCGATCGNNGTCCGGATCGCAGTCTCCCAACTNGACTGCGTGAA
GTCGGTNTCGCTAGTAATCGTGANNTCAGTAATGTCACGGTGANNNCGTTCCCGN
GCCTTGTANNNACCA

Figure 5.11 DNA sequence

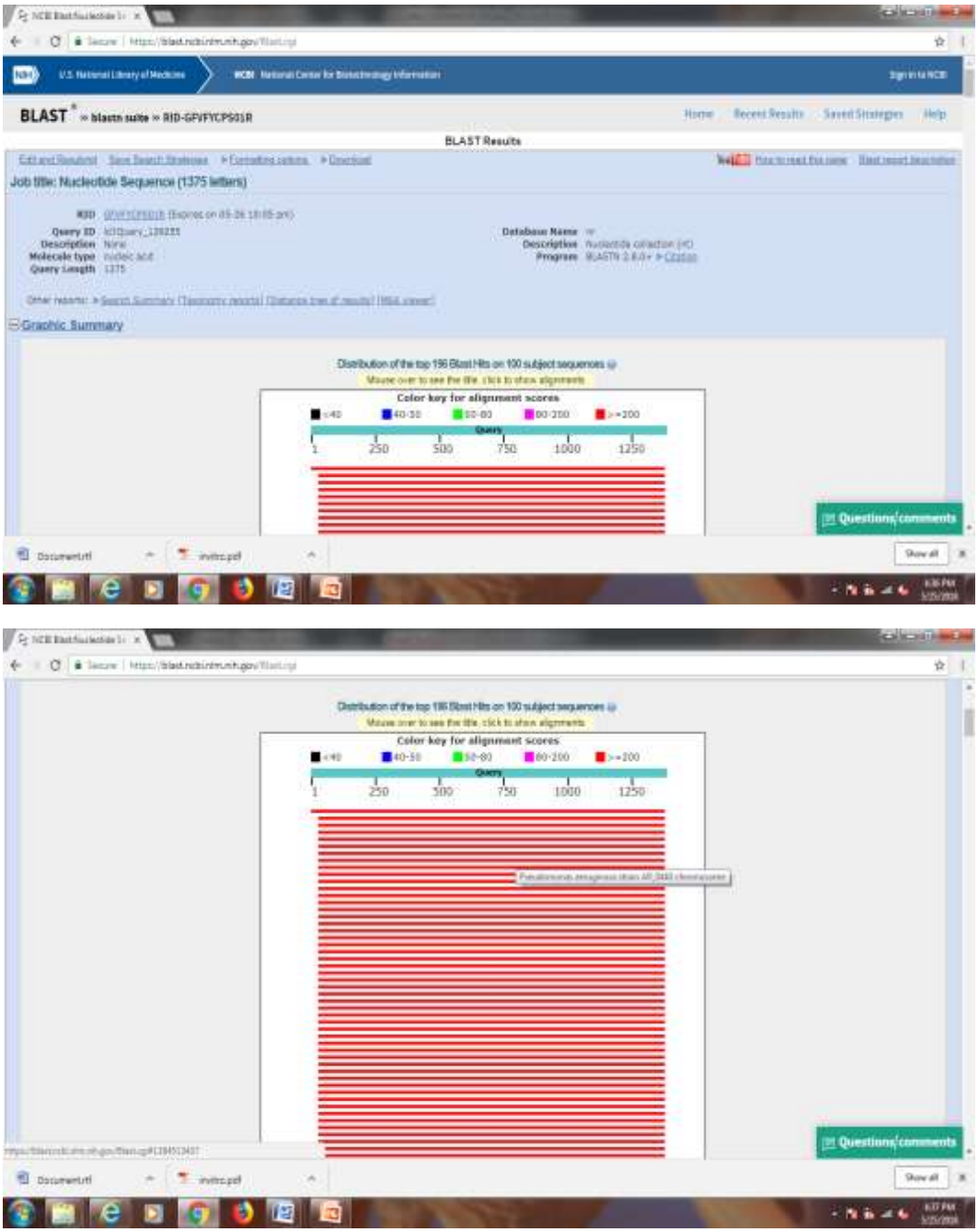


Figure 5.12a: BLAST results for the bacterial culture

The sequence obtained was 1375 base long and is presented in Figure 5.11. The sequence was analysed using the online tool BLAST from NCBI site. The hit results BLAST are shown in Figure 5.12(a,b). Our bacterial culture indicated a good match with DNA database with 98% homology (Figures 5.10-5.13). Hence, it can be inferred that the microbe used in the present study was probably strain of *Pseudomonas aeruginosa*.

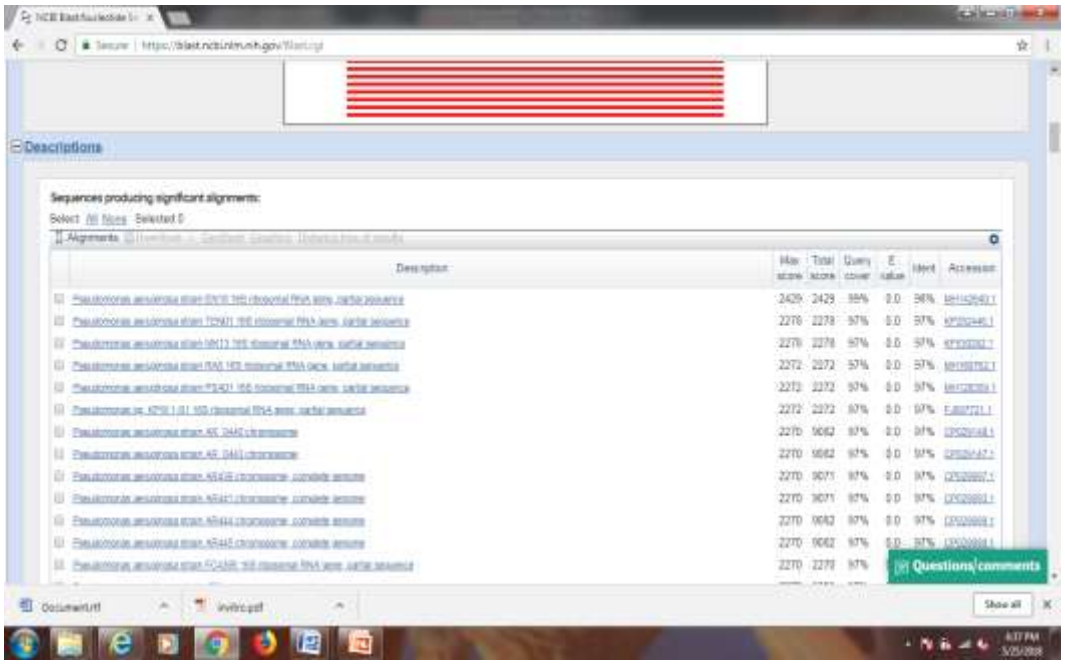


Figure 5.12b: BLAST results for the bacterial culture

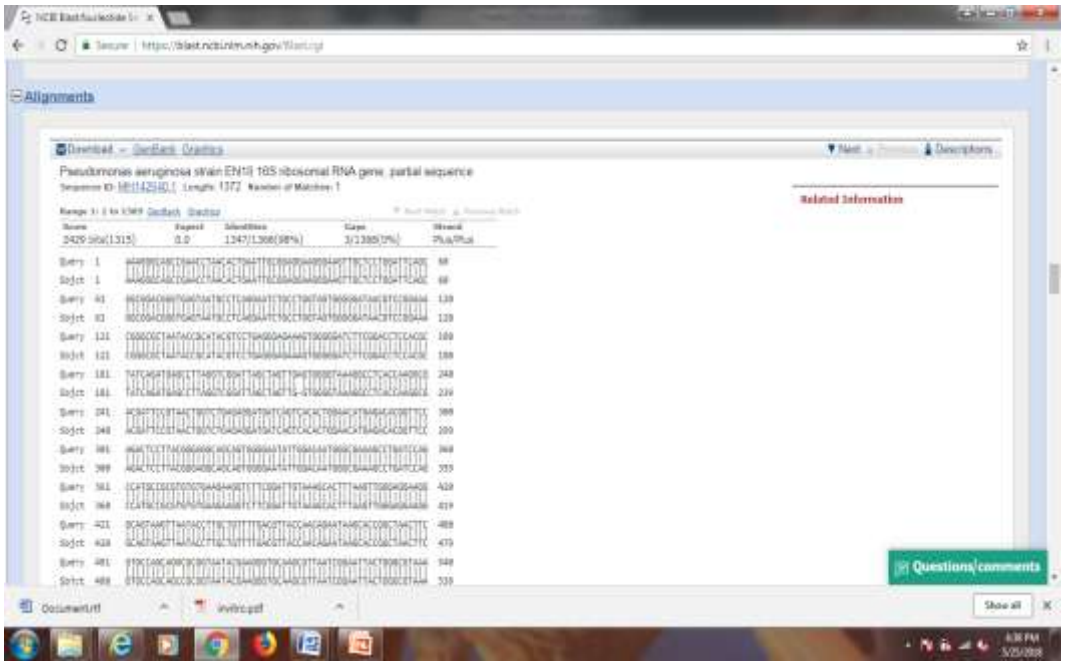


Figure 5.13: Homology analysis

5.10.8 Lipase activity

The selected strain of *Pseudomonas aeruginosa* DS1 was used for lipase production and it showed maximum lipase activity of 23.8 IU/ml after 24 h of incubation. Further

incubation had a negative effect on the activity (Figure 5.14). It has been reported that some fast growing bacteria are able to secrete lipase within 24 h of incubation (Sharma *et al.*, 2002b). Bacteria grow fairly well in a complex medium containing all the necessary nutrients such as carbon sources, nitrogen sources, phosphorous and mineral salts. It was observed that the enzyme activity was optimal at 35°C, at which lipase activity was 22.5 IU/ml. With further increase in temperature lipase activity reduced that could be due to denaturation of enzyme/proteins. Similarly, pH also had a significant effect on lipase activity. Highest lipase activity of 20 IU/ml was achieved at slightly alkaline pH (7.5).

The lipase activity was affected by the acidic and extreme alkaline pH which is in accordance with the results of Tembhurkar *et al*. (2012). The lipase activity was optimum at 35°C, pH 7.5 and 24 h of incubation period. So these conditions were further used for enzyme production in detoxification experiments.

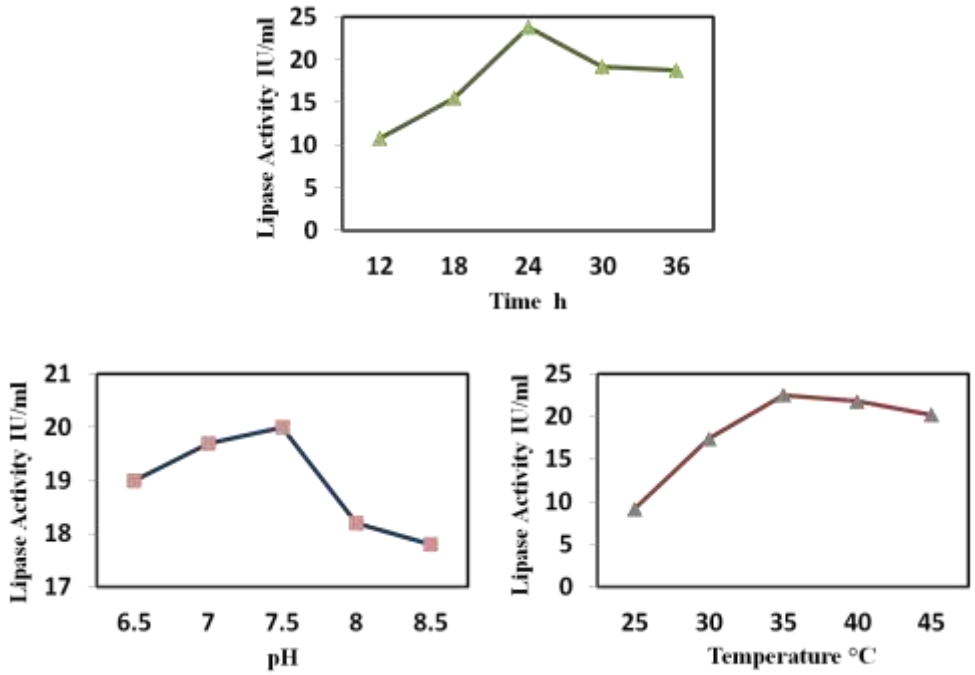


Figure 5.14: Effect of time, pH and temperature on lipase activity

5.10.9 Phorbol esters in seed cake

Jatropha curcas contains six phorbol esters with diterpene moiety i.e. 12-deoxy-16-hydroxyphorbol in *Jatropha curcas* seed oil (Haas *et al.*, 2002). 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 (Martinez-Herrera *et al.*, 2006; Makkar *et al.*, 1997; Haas *et al.*, 2002). In a study by Ahmed and Salimon (2009), three different varieties of tropical *Jatropha curcas* were identified from Malaysia, Indonesia and India with two, five and four phorbol ester peaks respectively. HPLC chromatograms of *Jatropha* seed cake extract showed the presence of a major phorbol esters peak which had two minor peaks at 35.4 and 35.9 min (Figure 5.15a). Total phorbol esters were calculated by summing up the area of PE peaks. We observed two phorbol esters peaks in our *Jatropha* seed cake sample which is similar to the findings of Chetna *et al.* (2011) 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.

Phorbol ester concentration in the raw *Jatropha* seed cake was found to be 0.3 mg/g. Enzyme catalyzed degradation of phorbol esters in 15 h was found to be 99.7%. The conditions were further optimized by Taguchi model. The precursor ion analysis of PMA and phorbol esters at m/z 311 represented the protonated phorbol nucleus ($[C_{20}H_{22}O_3{}^{+}H]^{+}$) which gave the confirmation of the presence of phorbol esters in the raw *Jatropha* seed cake. The results were in accordance with Baldini *et al.* (2014) who also reported protonated phorbol nucleus common to all phorbol ester family.

5.10.10 Pilot Experimentation

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 5.15 (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.

Submerged fermentation technique has been the method of choice for bacterial enzyme production because of their higher moisture requirement (Subramaniyam and Vimala, 2013). In work done by Phengnuam and Suntornsuk (2013), 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 (de Barros *et al.* 2011; Azhar *et al*. 2014). In a work done by Chetna *et al.* (2011), 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.

5.10.11 Optimization of the Process by Taguchi Methodology

The average values of phorbol ester degradation, the raw data and the S/N ratio for each parameter at three levels were calculated (Tables 5.7 and 5.8) and plotted. In Figure 5.16 (a), the variation in phorbol ester degradation with respect to the pH of the production media can be observed. These results show that phorbol ester degradation at pH 6.5 is comparatively lesser but increases when the pH of the medium is 7.5. Most bacterial lipases work better in alkaline as well as acidic conditions (Sztajer *et al.,* 1991; Sunna *et al.,* 2002; Anbu *et al.* 2011); however, *Pseudomonas* strain shows maximum lipase activity at slightly alkaline pH (Gilbert, 1991; Ghosh *et al.,* 1996; Sidhu, 1998b; Tembhurkar *et al.,* 2012; Kathiravan *et al.,* 2012). Higher degradation of phorbol esters at pH 7.5 can be attributed to optimum lipase activity by our strain *Pseudomonas aeruginosa* DS1 which indicates maximum the enzyme activity, the more will be phorbol ester degradation. The higher value of S/N ratio for pH 7.5 indicates that it may be regarded as optimal level of pH for obtaining higher phorbol ester degradation.

Table 5.7: Average values and main effects of phorbol ester degradation (raw data)

Level	pH	Seed cake (%)	Time (h)	Temperature (^{0}C)	RPM
L1	57.95	81.57	56.96	52.31	66.46
L2	72.40	58.43	69.32	72.44	61.66
L3	*	55.53	69.25	70.79	67.42
L2-L1	14.45	-23.13	12.36	20.13	-4.80
L3-L2	*	-2.90	-0.07	-1.65	5.77

L1, L2, L3 represent parameter levels 1, 2 and 3 respectively, L2-L1 is mean effect when corresponding parameter changes from level 1 to level 2. L3-L2 is mean effect when corresponding parameter changes from level 2 to level 3.

Table 5.8: Average values and main effects of phorbol ester degradation (S/N ratio)

Level	pH	Seed cake (%)	Time (h)	Temperature (^{0}C)	RPM
L1	34.76	37.99	34.69	33.83	36.24
L2	37.01	35.01	36.659	37.01	35.20
L3	*	34.66	36.319	36.81	36.21
L2-L1	2.26	-2.98	1.969	3.19	-1.04
L3-L2	*	-0.35	-0.34	-0.20	1.01

L1, L2, L3 represent parameter levels 1, 2 and 3 respectively, L2-L1 is mean effect when corresponding parameter changes from level 1 to level 2. L3-L2 is mean effect when corresponding parameter changes from level 2 to level 3.

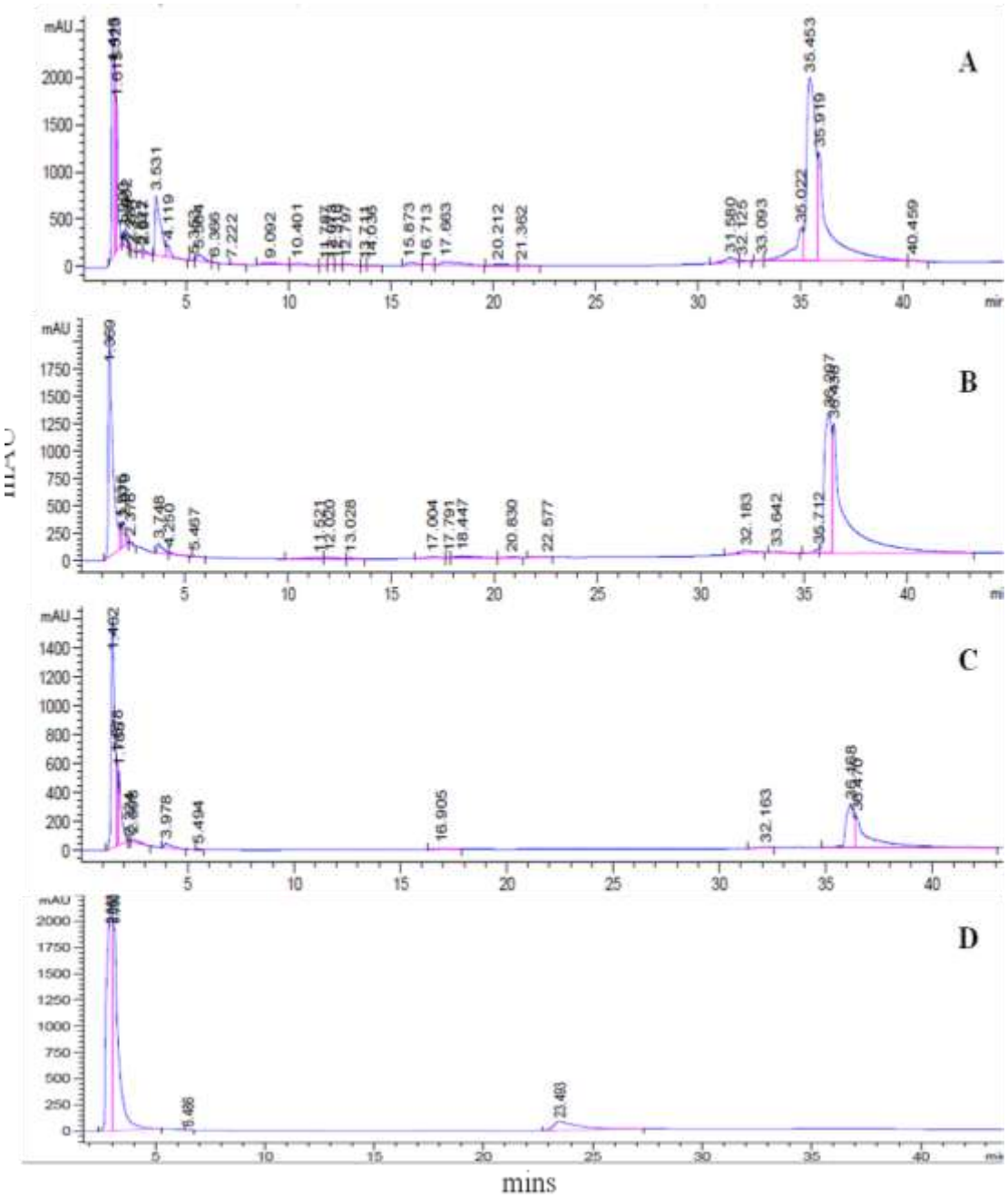


Figure 5.15: Chromatograms of untreated (A-for all sampling hours) and treated (B-9h; C-12h; D-15h) *Jatropha* seed cake samples.

It was observed from Figure 5.16 (b) that the phorbol ester degradation is more when the seed cake is 1 %. However, it is lower when the percentage seed cake is 5 % and lowest for 10 %. A higher value of S/N ratio for the seed cake (%) when fixed at 1 shows that it may be regarded as optimum percentage for obtaining higher phorbol ester degradation. As the enzyme units (inoculum size) are equal for all the

concentrations of the seed cake, enzyme units will be limiting factor for the degradation. Therefore, to enhance the degradation rate of higher concentrations of seed cake, greater volumes of biomass need to be added. Further if higher inoculum is used, the detoxification process may be shortened to certain extent. Also, certain reports have discussed the effect of phorbol ester on the integrity of cellular proteins (Gomperts *et al.,* 2009; Ehsan *et al.,* 2014). This could possibly be another reason that can be attributed to lower degradation of phorbol esters at higher concentrations of seed cake and requires further studies.

From Figure 5.16 (c), it can be noted the degradation of phorbol esters is more when the seed cake is incubated for 12 h and further the rate of degradation slowed down. S/N ratio also suggests higher degradation of phorbol esters at 12 h as compared to other two levels. Although complete detoxification of the seed cake was achieved in 15 h, detoxification at 12 h can be regarded as optimal time since the phorbol ester content in the seed cake was found to be within permissible limit of 0.09 mg/g (Gogoi *et al.,* 2014). Most of the researchers have worked with the biomass for detoxification while our work mainly involved the enzymes in the broth (after centrifugation to remove the cells). In a work done by Azhar *et al.* (2014), phorbol ester concentration decreased with incubation time because of certain enzymes released by the strain that degraded diterpene esters i.e. phorbol esters. According to de Barros *et al.* (2011), lipases and esterases are the enzymes responsible for hydrolyzing these ester bonds. Chetna *et al.* (2011) reported that lipase production by *Pseudomonas* was responsible for the degradation of phorbol esters during solid state fermentation of the *Jatropha* seed cake. So, it can be stated that the incubation time has significant effects on the levels of phorbol esters in the seed cake in the presence of biomass or free enzymes. However, as seen in Figure 5.16 (c), there is slight decline in phorbol ester degradation rate at 15 h; this may be due to the change in pH because of the secondary metabolites produced during fermentation which further affect the enzyme activity and the degradation of phorbol esters.

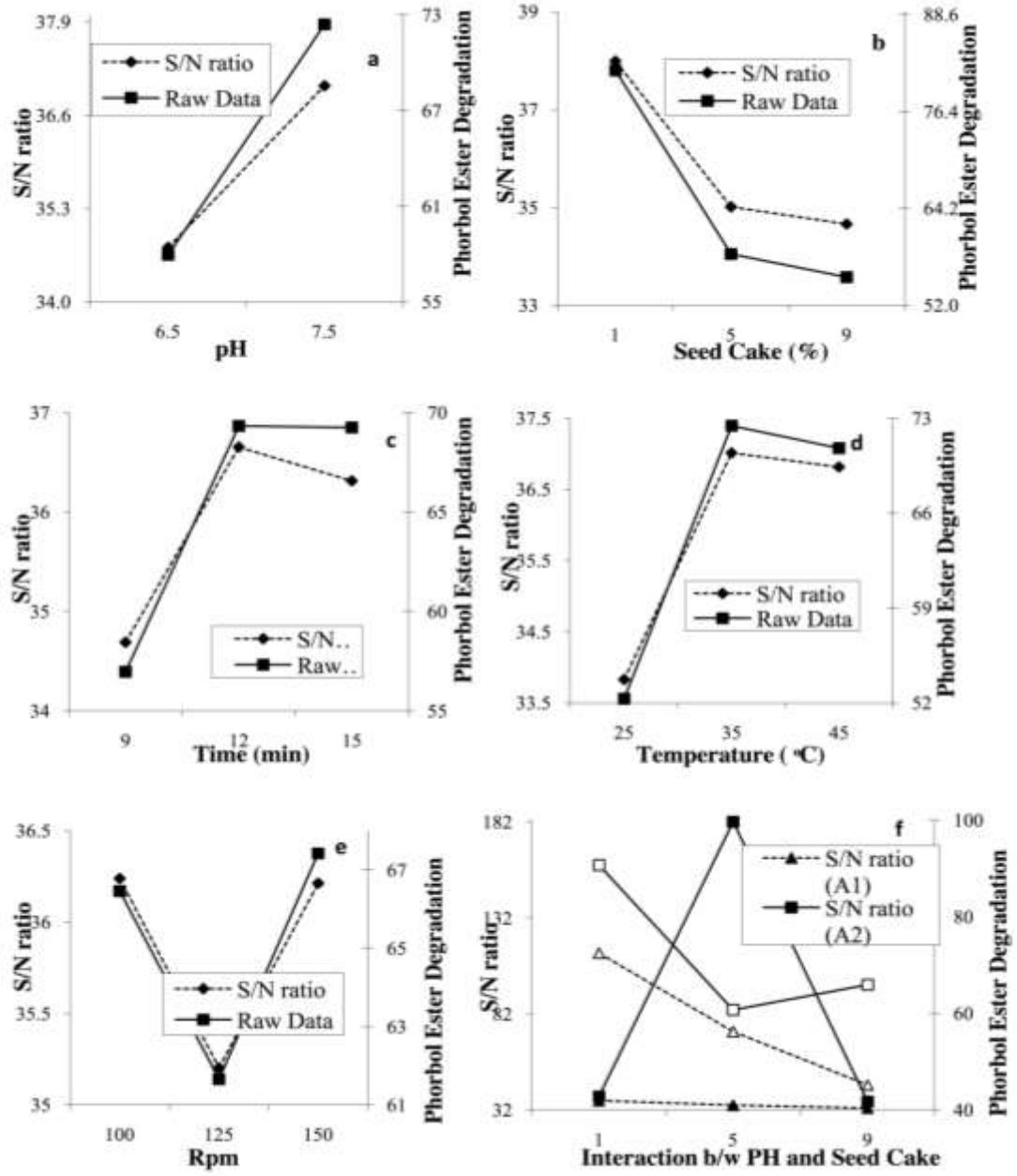


Figure 5.16: Variation of phorbol ester degradation (raw data and S/N ratio) with process parameters: a) pH; b) seed cake (%); c) time (h); d) temperature (˚C); e) rpm and f) interaction between pH and seed cake

Figure 5.16 (d) shows the variation in phorbol ester degradation rate with respect to different temperatures of the process. Results show that the phorbol ester degradation is maximum at 35 ˚C and starts to decline when the temperature is increased to 45 ˚C. However, on decreasing the temperature from 35˚C to 25˚C, the degradation decreases. These results are in agreement with Devappa *et al.* (2010b) who have observed that

increase in temperature to a certain point results in reduction in phorbol ester content in *Jatropha* oil and the rate start decreasing with further increase in temperature. The S/N ratio, however, is maximum at 35˚C, comparable when the temperature is 45˚C and decreases when the temperature is decreased to 25˚C. Figure 5.16 (b) shows the chromatogram of 12h treated 1% seed cake at 35˚C and pH 7.5 in which no phorbol esters peak appeared. Our findings indicate that the process is certainly catalyzed by the enzymes that have temperature optima of 35 ˚C beyond which they slow down due to change in the kinetic behavior and also due to the structural violation (denaturation) of the enzymes at higher temperatures. At low temperatures, most of the enzymes show low activity and our enzyme also behaved in similar manner.

The S/N ratios depict a similar values at all the three levels of RPM indicating that the response is not affected by variations in the speed. From Figure 5.16 (e), it can be observed that the phorbol ester degradation is equal at 100 and 150 rpm. On contrary to expectations, the degradation of phorbol esters decreased when the rpm was increased from 100 to 125 and again increased when the speed was changed from 125 to 150 rpm (need more extensive study to explain this phenomenon). The RPM shows insignificant effect on raw data and S/N ratio. Thus, any of the three levels of RPM can be selected for the study taking into consideration the user requirement and the cost.

As phorbol ester degradation comes under higher-the-better type category, higher values of degradation were required. Each parameter at level, i.e. A-1, B-1, C-2, and D-2 may result in maximum degradation of phorbol esters [Figure 5.16 (a-e)]. However, it can be observed from Figure 5.16, the highest values of mean responses correspond to the highest values of S/N ratios.

ANOVA was performed to study the significance of the input parameters in the degradation of phorbol esters. The ANOVA of raw data and S/N data for phorbol ester degradation are shown in Tables 5.9 and 5.10. From these tables, it was observed that pH, seed cake (%), time and temperature had a significant effect on phorbol ester degradation values while RPM had an insignificant effect. The percentage contribution of seed cake (%) was highest (39.39%), followed by temperature (24.02 %), pH (15.11%) and time (9.47%).

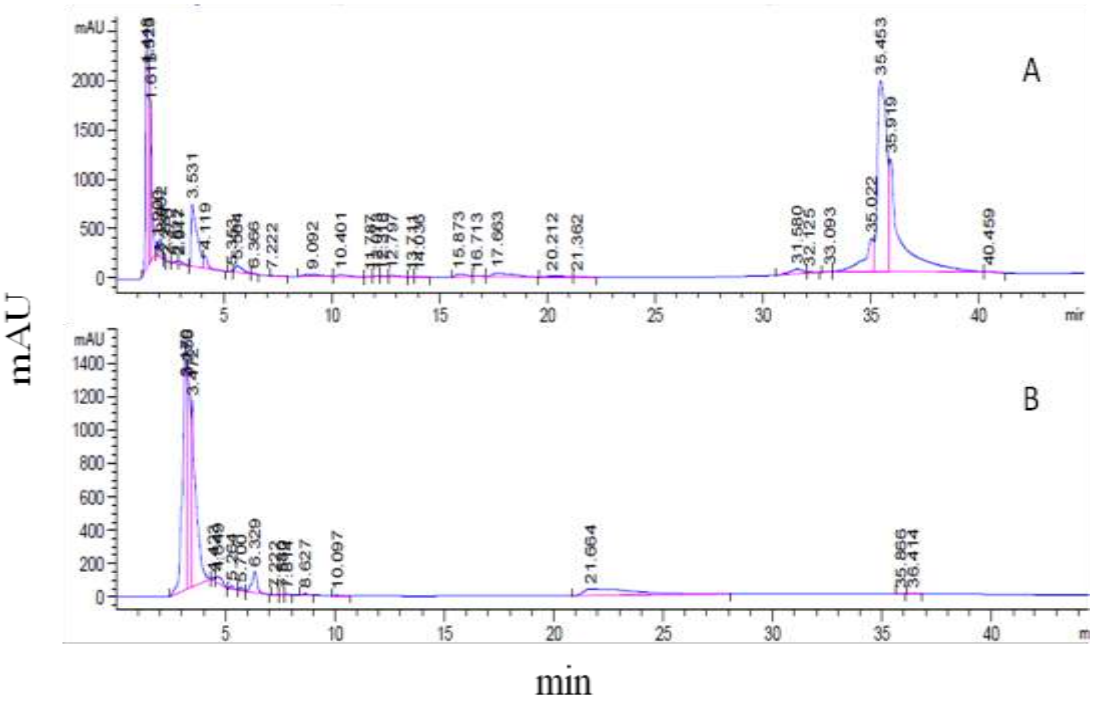


Figure 5.17: HPLC chromatogram of (a) untreated *Jatropha curcas* seed cake (b) treated seed cake at pH = 7.5, Seed cake = 1%, Time= 12 h, Temperature = 35 ˚C, RPM = 100 rpm

Table 5.9: Pooled ANOVA (raw data)

Source	SS	DOF	V	F-ratio	SS'	P (%)
pH	2819.56	1	2819.56	70.45*	2819.56	15.11
Seed cake (%)	7327.77	2	3663.89	91.54*	7327.77	39.39
Time	1822.91	2	911.46	70.45*	1822.91	9.47
Temperature	4498.24	2	2249.12	56.19*	4498.24	24.02
RPM	168.17	2	84.08	2.10	-	-
Error	1761.10	44	40.02	-	1929.27	12.01
Total	18397.75	53	-	-	18397.75	100

*Significant at 95% confidence level.

SS= Sum of squares; DOF= Degree of freedom; V= Variance; SS'= Pure sum of squares; P= Percentage contribution of parameter.

Table 5.10: Pooled ANOVA (S/N data)

Source	SS	DOF	V	F-ratio	SS'	P (%)
pH	22.87	1	22.87	18.30*	22.87	16.80
Seed cake (%)	40.20	2	20.10	16.08*	40.19	29.23
Time	13.21	2	6.60	5.28*	13.21	8.32
Temperature	38.22	2	19.11	15.29*	38.22	27.76
RPM	4.19	2	2.09	1.67	-	-
Error	9.99	8	1.25	-	14.19	17.82
Total	128.68	17	-	-	128.68	100

*Significant at 95% confidence level.

SS= Sum of squares; DOF= Degree of freedom; V= Variance; SS'= Pure sum of squares; P= Percentage contribution of parameter.

5.10.11.1 Estimation of Optimum Levels

The significant parameters at selected levels were used for predicting optimum value of response (phorbol ester degradation) and the mean of the response factor can be calculated as follows (Walia *et al.,* 2006; Goyal *et al.,* 2012):

Significant parameters: A, B, C and D

Optimal values: A_2, B_1, C_2, and D_2

Average value (from Table 5.11) of phorbol ester degradation at

2nd level of **pH** ($\overline{A}_2$) = 72.40

1st level of **Seed cake** ($\overline{B}_1$) = 81.57

2nd level of **Time** ($\overline{C}_2$) = 69.32

2nd level of **Temperature** ($\overline{D}_2$) = 72.44

The overall mean of phorbol ester degradation ($\overline{T}_{ped}$) = 65.18 (from Table 5.2)

The predicted optimum value of phorbol ester degradation (μ_{ped}) has been calculated as:

$$\mu_{SSR} = \overline{A}_2 + \overline{B_1} + \overline{C}_2 + \overline{D}_2 - 3\overline{T}_{ped} = 100.19$$

Confirmation experiments (CI_{CE}) and of population (CI_{POP}) at 95% confidence can be computed by the equation:

$$CI_{CE} = \sqrt{F_{\alpha}(1, f_e) V_e \left[\frac{1}{n_{eff}} + \frac{1}{R}\right]} \quad \text{and} \quad CI_{POP} = \sqrt{\frac{F_{\alpha}(1, f_e) V_e}{n_{eff}}}$$

N = 18 × 3 = 54 (treatment = 18. R= repetition = 3); f_e (error DOF) = (53 - 7) = 46; v_e (error variance) = 40.03 (calculated from Table 5)

$$n_{eff} = \frac{N}{1 + [\text{DOF associated in the estimate of mean responce}]} = 5.4$$

$F_{0.05}$ (1, 46) = 4.05 (Tab F value)

CI_{POP} = ±5.48

CI_{CE} = ±9.17

The 95% confidence intervals (CI_{POP} and CI_{CE}) of the predicted optimum scatter of phorbol ester degradation are:

CI_{POP} : 94.71< μ_{SSR} (µm) <105.67

CI_{CE} : 91.02< μ_{SSR} (µm) <109.36

The optimum values of the process parameters for phorbol ester degradation are as follows:

pH (A, 2nd level) = 7.5

Seed cake (B, 1st level) = 1%

Time (C, 2nd level) = 12 h

Temperature (D, 2nd level) = 35 °C

RPM (E, 1st level) = 100 rpm

Table 5.11: Average values of responses at optimum levels

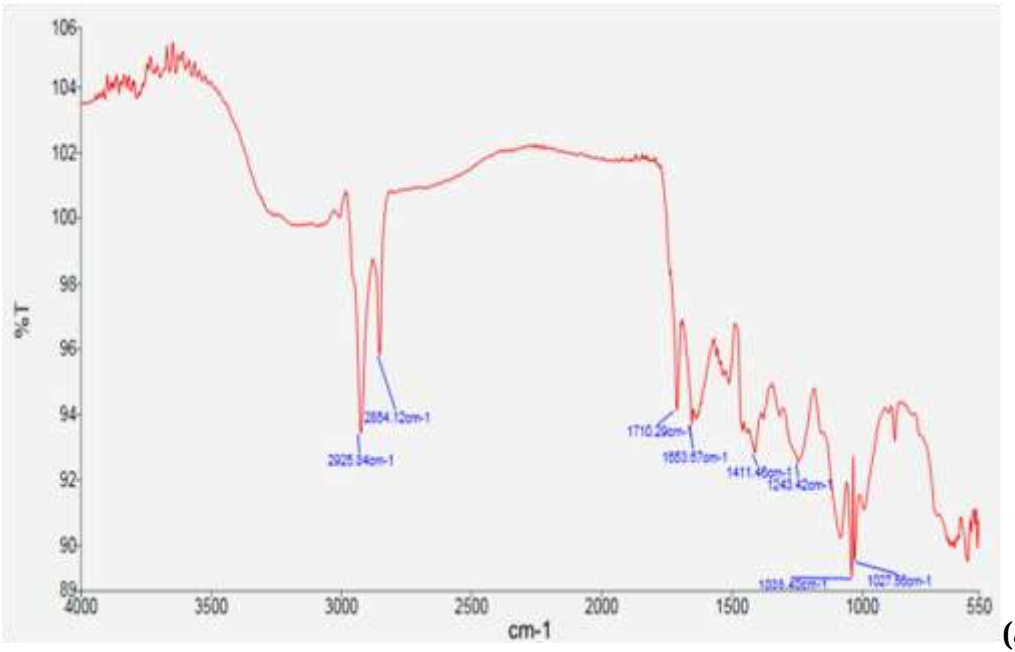
Optimal level	Average value of phorbol ester degradation
A2	72.40
B1	81.57
C2	69.32
D2	72.44
E3	67.42

5.10.11.2 Conformation Experiments

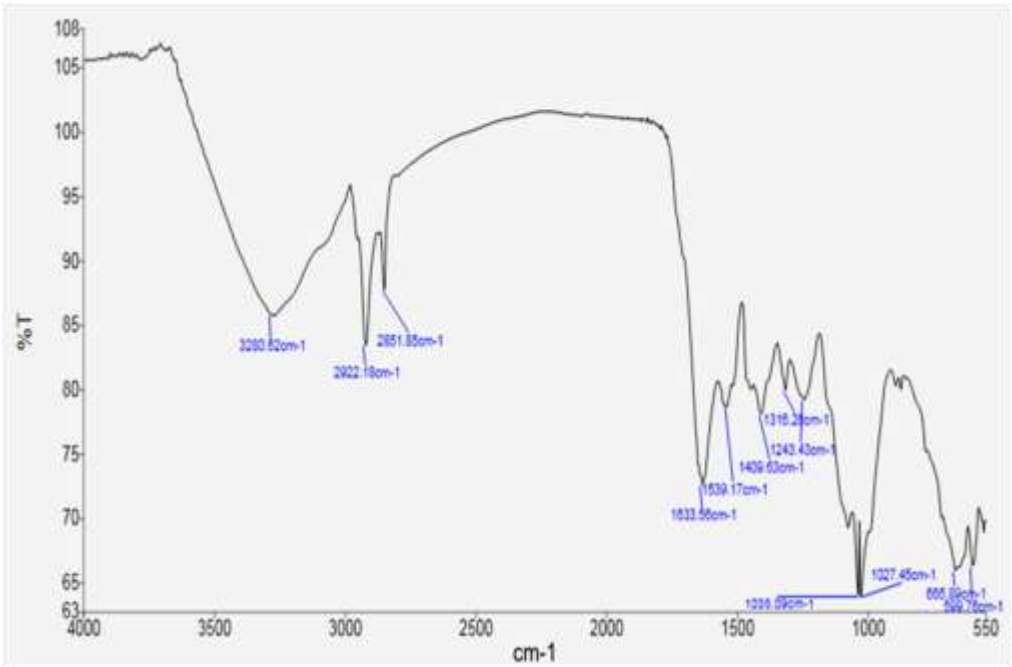
Three confirmational experiments for response characteristic i.e. phorbol ester degradation were performed at optimized levels of process parameters for the validation of results. The pH was set at the second level (A2), seed cake % was set at the first level (B1), time was kept at the second level (C2), temperature was set at second level (D2) and rpm was set at first level (E1). The average phorbol ester degradation was found to be 95.2% (0.08mg/g, residual phorbol ester concentration) which was within the confidence interval of the predicated optima and also within the permissible limit of 0.09mg/g (Gogoi *et al.*, 2014).

5.10.12 FTIR analysis

FTIR spectra of *Jatropha* seed cake and detoxified seed cake are illustrated in Figure 5.18. Peaks were observed in the range 2800-2950 cm^{-1} confirming the presence of strong aliphatic C-H stretch. Strong peaks at 1710 cm^{-1} and 1653 cm^{-1} is evident of carbonyl group C=O and C=C respectively present in the phorbol esters. NO_2 stretching can be observed at 1411 cm^{-1} (Biradar *et al.*, 2014) and peak at 1243 cm^{-1} indicates the bending of CH_2 and CH_3 aliphatic groups. Peaks at 1027 and 1038 cm^{-1} are indicative of C-C bonds of aromatic groups. Comparing the graphs of detoxified seed cake and raw *Jatropha* seed cake, a new peak at 3280 cm^{-1} was observed characteristic of O-H stretching which could be due to presence of water molecules added during submerged fermentation process. Very small differences were observed, where the peaks at 2925, 2854, 1653 and 1411 cm^{-1} were shifted to 2922, 2854, 1633 and 1409 cm^{-1} respectively. Another indicative of phorbol ester degradation could be attributed to peak observed at 1539 cm^{-1} of N-H bending which could be a result of NO_2 reduction that can be seen at peak 1411 cm^{-1}. Peaks present in the region 1000-1250 cm^{-1} represents C-O stretching of esters, alcohols, ethers, phenols (Nisar *et al.*, 2017). Additional peak at 665 cm^{-1} can be seen in detoxified seed cake that indicates C-Cl bond which could possibly be a result of chlorine addition from Ammonium chloride and Calcium chloride during submerged fermentation.



(a)



(b)

Figure 5.18: FTIR graphs of (a) *Jatropha* seed cake and (b) detoxified seed cake

5.11 Conclusion

The morphology and growth characteristics of the isolated culture were studied. The fermentation process for detoxification of *Jatropha* seed cake was optimized using Taguchi technique. L18 orthogonal array was used to study the effect of fermentation

conditions on the phorbol ester content of *Jatropha* seed cake. Analysis of variance (ANOVA) was performed for predicting the optimum process parameters.

The percentage contribution of the process parameters with reference to phorbol ester degradation was predicted. Optimum value of the process parameters i.e. seed cake (1%), temperature (35 ˚C), pH (7.5) and time (12 h) helped in achieving the degradation of phorbol esters within the permissible limits in the Jatropha press cake.

CHAPTER 6

EXTRACTION OF PROTEINS FROM DETOXIFIED *JATROPHA* SEED CAKE AND ITS OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

6.1 Introduction

Jatropha seed cake which is the byproduct of biodiesel industry is rich in proteins, carbohydrates and contain many bioactive compounds, has found many economical and technical applications. The seed cake have high mineral content of nitrogen, phosphorous, potassium so it can be used as an organic fertilizer and for biogas production. Being nutrionally rich, the seed cake can be used as animal feed but presence of toxins like phorbol esters makes it unsuitable for animal consumption and therefore the seed cake needs to be detoxified (Ahluwalia *et al.*, 2017a, b). Despite of this, the use of *Jatropha* protein is advantageous as the protein quality and the essential amino acids content (except lysine) is high in *Jatropha* seed cake as compared to FAO reference protein. The proteins extracted from raw *Jatropha* seed cake were successfully used in polyketone- based wood adhesive formulations (Hamarneh *et al.*, 2010). Also, *Jatropha* proteins have good functional properties which widens up the possibilities of using this protein for various technical applications. Several studies have been conducted on *Jatropha* protein extraction which includes steam injection (Devappa and Swamylingappa, 2008), counter current multistage extraction (Lestari *et al.*, 2010), iso electric precipitation (Hamarneh *et al.*, 2010; Makkar *et al.*, 2008), enzyme assisted extraction process (Gofferje *et al.*, 2014) etc. For this study, the principle of isoelectric precipitation was applied for the extraction of proteins from *Jatropha* seed cake and the effect of factors such as extraction temperature, time, solubilization pH, precipitation pH variations on protein yield was studied and optimized using Response Surface Methodology.

6.2 Materials and Methods

6.2.1 Materials

Jatropha seed cake, after extraction of the oil from whole seeds by screw press was obtained from Biodiesel centre, DTU. All the chemicals used in the work were purchased from Fischer Scientific and were of analytical grade.

6.2.2 Methods

6.2.2.1 Protein extraction

Extraction of protein from *Jatropha* seed cake at different conditions was done according to method of Makkar *et al.* (2008) which followed the principle of isoelectric precipitation. 10g seed cake was suspended in 100 ml water and the pH was adjusted using 1 mol L^{-1} NaOH. The mixture was stirred for 1 h at different temperatures. The pH was checked after every 20 min and adjusted using 1 M NaOH. The contents were then centrifuged at 7000 rpm for 15 min to collect the supernatant. The acidic pH of the supernatant was adjusted using 4M HCL. The mixture was stirred for 10 min and centrifuged at 7000 rpm for 15 min for the separation of protein precipitates. The pellet was air dried and the weight (dry wt.) was noted. Each experiment was done in duplicates.

6.2.2.2 Pilot scale experiments

The alkaline mixture was stirred for 1 h at different temperature levels i.e. 25, 40, 55, 70, 85°C to evaluate the effect of temperature on yield and protein content of the samples. The effect of alkaline and precipitation pH on the protein content and protein yield was also studied. The alkaline pH of the mixture (seedcake + water) varied from 7.5-11.5 and the precipitation pH was varied from 2-6 (Table 1).

Table 6.1: Parameters and their range

	1	**2**	**3**	**4**	**5**
Temperature ($^{\circ}$C)	25	40	55	70	85
Solubilisation pH	7.5	8.5	9.5	10.5	11.5
Extraction pH	2	3	4	5	6

6.2.2.3 Protein analysis

Protein content of the *Jatropha* seed cake and protein precipitates was done by Kjeldahl method (AACC, 2000), which consists of three steps: digestion, distillation and titration (Figure 6.1). The sample is digested in boiling concentrated sulfuric acid in the presence of a catalyst for complete dissolution and oxidation. The nitrogen in the sample is converted to ammonium sulfate. In the distillation step, sodium hydroxide is added into the solution, which converts the ammonium ion to ammonia, which is distilled and received in a boric acid solution. The amount of ammonia released and thus the amount of nitrogen present in the sample is then determined by titration. The results are expressed in % N, % NH_3 or protein (%N x factor). Protein content of the supernatant was determined by Lowry's method (Lowry *et al.*, 1951). Bovine serum albumin was used for plotting standard curve. Dry matter recovery and protein yield was calculated according to the formula:

$$Dry\ matter\ (\%) = \frac{Dry\ matter\ weight\ of\ protein\ precipitate}{Initial\ dry\ weight\ of\ seed\ cake}\ X\ 100$$

$$Protein\ yield\ (\%) = \frac{dry\ mass\ extract\ (g) X\ Protein\ content\ extract\ (\%)}{initial\ weight\ of\ seed\ cake\ (g) X\ protein\ content\ in\ seed\ cake\ (\%)} X100$$

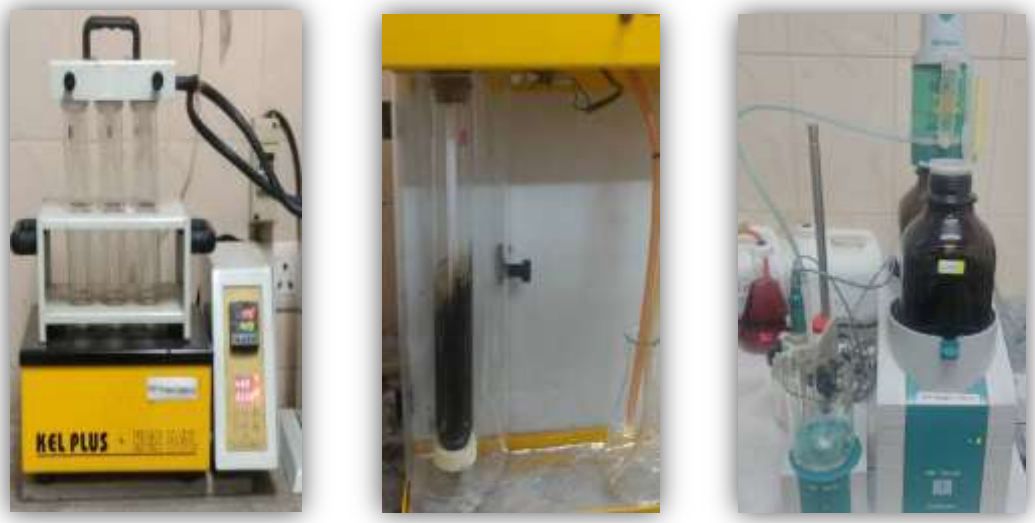


Figure 6.1: Kjeldahl apparatus: Digestion, distillation and titration (Kelplus Model KES06 L)

6.2.2.4 Optimization of protein extraction using RSM

Four different parameters i.e. Temperature, solubilization pH, precipitation pH and time were varied at five levels for studying the combined effect of these independent variables on three response variables i.e. dry matter, protein content and protein yield. Response surface methodology (RSM) was used to study the process and optimization of different parameters for the extraction of proteins from *Jatropha* seed cake. The experimental plan consisted of four-factors at five levels. Different levels of variation and codes of the independent variables of Temperature (A), solubilization pH (B), precipitation pH (C) and time (D) are presented in Table 6.2. As per CCRD, thirty set of experiments were performed with varying levels of parameters as shown in Table 6.3. Analysis for variance was performed to evaluate the effect of the independent variables on the response variables. Design Expert (DX-11) was used for the selecting appropriate model and multiple regression analysis was performed considering a full second order polynomial model.

Table 6.2: Levels and codes of the independent variables

Variables	Codes	Levels (coded and real values)				
		-2	-1	0	1	2
Temperature (°C)	A	30	40	50	60	70
Solubilization pH	B	8	9	10	11	12
Precipitation pH	C	3.5	4	4.5	5	5.5
Time (h)	D	0.5	0.75	1	1.25	1.75

6.3 Results and discussion

6.3.1 Recoveries of protein concentrates

The data in Table 6.4 show that the recoveries of dry matter, protein yield and protein content of protein concentrate (from *Jatropha* raw seed cake). The protein concentrate had protein content in the range from 29.33-34.84%. The protein yield varied from 19.6-66.2% and it was substantially higher when the extraction temperature was high. Many parameters like solvents, temperature, pH, time, agitation speed etc can affect the protein extraction efficiency.

6.3.2 Effect of temperature on protein content and extraction yield of protein concentrates

No significant change in the protein yield was seen at temperatures 25, 40 and 55°C; however a slight increase in protein yield was observed. As the temperature was increased from 55 to 85°C, a significant decrease in protein yield was observed from 45.5 to 17.6% (Figure 6.2a).

Table 6.3: Real and coded values for independent variables Temperature (A), solubilization pH (B), precipitation pH (C) and time (D)

No.of expts	Temperature		Solubilization pH		Precipitation pH		Time	
	Real Value	Coded value (A)	Real Value	Coded value (B)	Real Value	Coded value (C)	Real Value	Coded value (D)
1	40	-1.0	9	-1.0	4.00	-1.0	0.75	-1.0
2	60	1.0	9	-1.0	4.00	-1.0	0.75	-1.0
3	40	-1.0	11	1.0	4.00	-1.0	0.75	-1.0
4	60	1.0	11	1.0	4.00	-1.0	0.75	-1.0
5	40	-1.0	9	-1.0	5.00	1.0	0.75	-1.0
6	60	1.0	9	-1.0	5.00	1.0	0.75	-1.0
7	40	-1.0	11	1.0	5.00	1.0	0.75	-1.0
8	60	1.0	11	1.0	5.00	1.0	0.75	-1.0
9	40	-1.0	9	-1.0	4.00	-1.0	1.25	1.0
10	60	1.0	9	-1.0	4.00	-1.0	1.25	1.0
11	40	-1.0	11	1.0	4.00	-1.0	1.25	1.0
12	60	1.0	11	1.0	4.00	-1.0	1.25	1.0
13	40	-1.0	9	-1.0	5.00	1.0	1.25	1.0
14	60	1.0	9	-1.0	5.00	1.0	1.25	1.0
15	40	-1.0	11	1.0	5.00	1.0	1.25	1.0
16	60	1.0	11	1.0	5.00	1.0	1.25	1.0
17	30	-2.0	10	0.0	4.50	0.0	1.00	0.0
18	70	2.0	10	0.0	4.50	0.0	1.00	0.0
19	50	0.0	8	-2.0	4.50	0.0	1.00	0.0
20	50	0.0	12	2.0	4.50	0.0	1.00	0.0
21	50	0.0	10	0.0	3.50	-2.0	1.00	0.0
22	50	0.0	10	0.0	5.50	2.0	1.00	0.0
23	50	0.0	10	0.0	4.50	0.0	0.50	-2.0
24	50	0.0	10	0.0	4.50	0.0	1.50	2.0
25	50	0.0	10	0.0	4.50	0.0	1.00	0.0
26	50	0.0	10	0.0	4.50	0.0	1.00	0.0
27	50	0.0	10	0.0	4.50	0.0	1.00	0.0
28	50	0.0	10	0.0	4.50	0.0	1.00	0.0
29	50	0.0	10	0.0	4.50	0.0	1.00	0.0
30	50	0.0	10	0.0	4.50	0.0	1.00	0.0

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and hydrogen bonds which increase the protein dissolution rate resulting in high protein content in the protein concentrates (Liliana *et al.*, 2013).

Table 6.4: Dry matter, protein yield and protein content in the protein concentrate (from *Jatropha* raw seed cake)

S.No	Wt. of empty petridish	Wt. of petridish + sample	Wt. of sample	Dry matter content (%)	Protein content (%)	Protein yield (%)
A	12.44	15.07	2.31	23.1	29.33	42.345
B	14.22	15.22	2.28	28	29.98	42.722
C	12.9	16.26	2.36	23.6	30.85	45.504
D	14.12	15.41	1.29	12.9	31.59	25.469
E	14.17	15.06	0.89	8.9	31.72	17.644
F	13.95	14.52	0.57	5.7	29.69	10.577
G	13.97	15.65	1.68	16.8	30.55	32.078
H	13.99	16.32	2.33	23.3	33.51	48.799
I	13.8	16.47	2.67	26.7	34.64	57.806
J	13.96	16.84	2.88	28.8	34.69	62.442
K	13.81	16.14	2.33	23.3	31.12	45.319
L	13.66	16.22	2.56	25.6	31.2	49.920
M	14.09	16.22	2.13	21.3	33.23	44.237
N	13.99	16.27	3.18	31.8	33.35	66.283
O	13.92	16.31	2.39	23.9	33.15	49.518

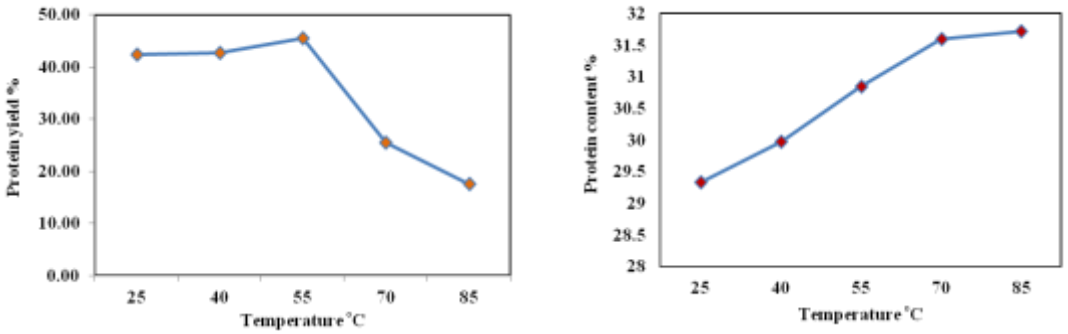


Figure 6.2: Effect of temperature on protein yield (a) and protein content (b) of concentrates.

6.3.3 Effect of solubilization pH on protein content and extraction yield of protein concentrates

It can be observed from the Figure 6.3 a, increasing pH has a positive effect on the protein content of the concentrates. There was gradual increase in the protein content

when the pH was raised from 7.5 to 10.5; however no significant change in protein content was observed when the pH reached 11.5. Highest protein content of 34.69% was observed in samples when they were solubilized at 11.5. According to Beatriz *et al.* (2002), extractions at high pH (>10) results in the degradation of alkali labile amino acids such as lysine which affects its biological value and protein digestibility and may result in decrease in protein content. The graphs in Figure 6.3b clearly shows that the alkaline conditions significantly affect the protein extraction yield and higher yields were obtained at pH 11.5 compared with pH 10.5. In a work done by Oliyaei *et al.* (2017), extraction of proteins from fish was done by iso-electric precipitation method, in which high protein recovery was observed at pH12 than pH10. Different studies have shown an increase in solubility at alkaline pH.

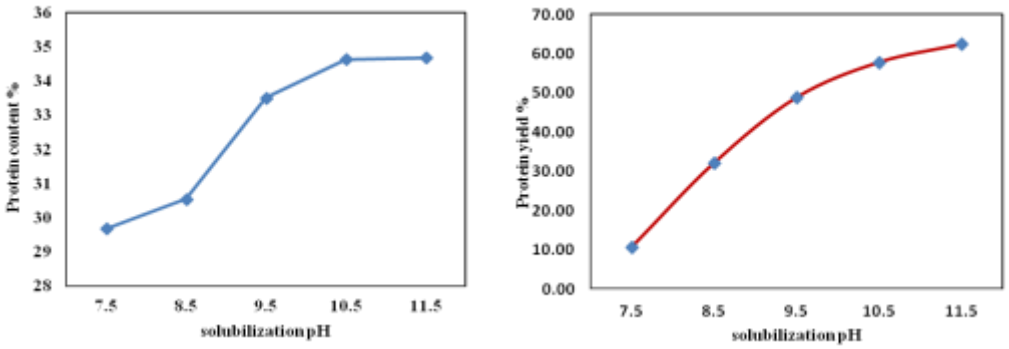


Figure 6.3: Effect of solubilization pH on the protein content (a) and protein yield (b) of concentrates

6.3.4 Effect of precipitation pH on protein content and protein yield of *Jatropha* seed cake

Increase in protein content was observed when precipitation pH was raised from pH 2 to 4 and it was comparable at pH 4 and 5 with 33.23 and 33.35% protein content respectively (Figure 6.4a). A decline in protein content was observed when the extraction was performed at pH6. As seen in Figure 6.4, there was steady increase in protein yield when the precipitation pH was increased from 2 to 5. A protein yield of 66.28% was obtained when the extraction was done at pH5. However further increase in pH resulted in sharp decline in protein yield (Figure 6.4b). This suggests that the isolectric point of *Jatropha* protein is between pH 4.5-5 which results in maximum recovery of the solubilized proteins at this pH. According to Kain *et al.* (2009)

solubility of proteins is minimum at isolectric pH because of balance between positive and negative charges which minimizes the repulsion forces amongst amino acids and thus reduces the solubility of proteins.

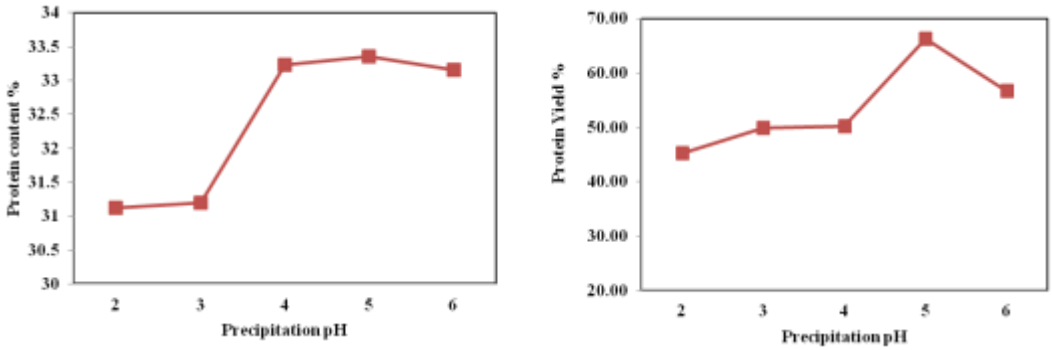


Figure 6.4: Effect of precipitation pH on the protein content (a) and protein yield (b) of concentrates

6.3.5 Protein content in the supernatant

As not all the proteins were extracted from *Jatropha* seed cake by isoelectric precipitation; it is assumed that some of the proteins must have lost in the supernatant. The protein content in the supernatant ranged from 6.88-26.78µg/ml at different levels of temperature, solubilization pH and precipitation pH. Effect of temperature, solubilization pH and precipitation pH on protein content in supernatant can be seen in Figure 6.5. It was observed that higher extraction temperatures (>55°C) resulted in more amount of protein in the supernatant. Recovery of proteins from *Jatropha* seed cake was highest at 55°C, alkaline pH 9.5 and precipitation pH 4. So it can be concluded that desirable temperature for the extraction of proteins should be in the range of 40-55°C.

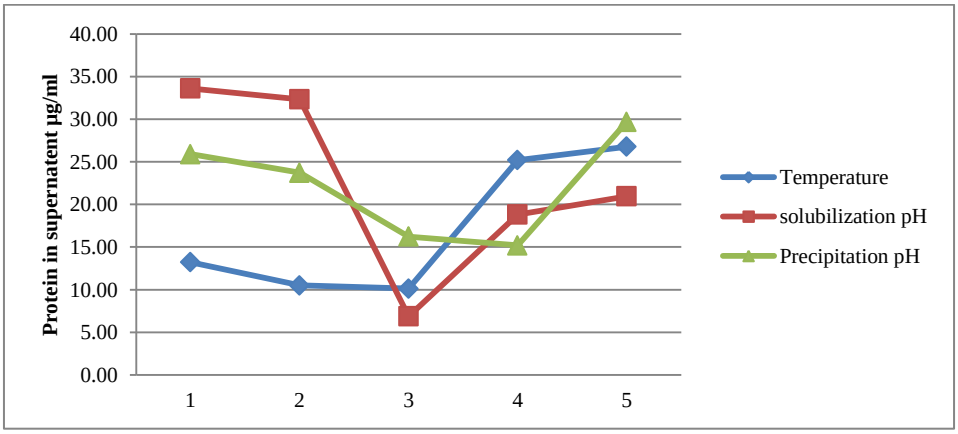


Figure 6.5: Effect of temperature, solubilization pH and precipitation pH on protein content in supernatant

A protein content of 33.61, 6.88 and 18.82 µg/ml was observed in supernatant when the solubilization pH was 7.5, 9.5 and 10.5 respectively. From Figure 6.5, it can be seen that alkaline pH above 9.5 resulted in less losses of proteins in supernatant. So it can be inferred that solubilization pH towards alkalinity results in less losses of protein in supernatant which will help in more recovery of proteins from the *Jatropha* seed cake. The precipitation pH for the extraction of proteins ranged from pH 2-6 and it was observed, 16.22 and 15.20 µg/ml of proteins were recovered in supernatant precipitated at pH3 and pH4 respectively which is very less as compared to other levels of precipitation pH. So precipitation pH in the range 3-4 shall help in getting maximum protein yield.

6.3.6 Optimization of protein extraction from detoxified *Jatropha* seed cake using RSM

Thirty experiments were performed according to CCRD to determine the effect of four parameters i.e. temperature, solubilization pH and precipitation pH and time on dry matter, protein content and protein yield. All the experiments were performed in triplicates and the average values of the responses were calculated given in Table 6.5.

6.3.6.1 Effect of parameters on dry matter

Experimental runs were performed and the results were modeled according to a polynomial quadratic equation to identify the variables that affected significantly or non- significantly. The analysis of variance (ANOVA) was performed and is given in Table 6.6. Determination coefficient (R^2=0.95) illustrates that only 5% of the total variations are not described by the model. Adjusted determination coefficient (adjusted R^2=0.92) is also high, which indicates the model is highly significant. As seen in Table 6.6, the value of lack of fit (0.095) was non-significant which implies accuracy of the model. The response surface equation for dry matter obtained after eliminating the non-significant terms is:

$$\textbf{Dry Matter} = 16.55 + 1.68A + 2.42B + 0.40C + 2.17D - 0.83AC + 1.01AD - 1.39BD + 0.77CD - 0.73A^2 - 0.71B^2 - 0.45D^2$$

The probability value F is less than 0.05 which indicates that the model is significant. The coefficients in the equation indicated that all the parameters significantly affect

the dry matter content. The dry matter content was found to increase with increase in temperature, pH and time. In a work done by Makkar *et al.*, 2008, increase in temperature increased the dry matter and protein recovery in seed cake when the extraction was carried out at pH 10.

Table 6.5: Average values of the response parameters.

S.No.	Temperature °C (A)	Solubilization pH (B)	Precipitation pH (C)	Time h (D)	Dry matter (%)	Protein content (%)	Protein Yield (%)
1	40	9	4	0.75	6.1	31.72	11.7
2	60	9	4	0.75	10.8	30.07	18.4
3	40	11	4	0.75	15	31.11	26
4	60	11	4	0.75	17.5	37.02	36
5	40	9	5	0.75	8.2	30.12	13.7
6	60	9	5	0.75	8	33.58	15
7	40	11	5	0.75	15.6	31.72	27.5
8	60	11	5	0.75	15.2	40.87	34.5
9	40	9	4	1.25	12.5	32.98	23
10	60	9	4	1.25	18.3	33.72	34.2
11	40	11	4	1.25	11.7	33.17	21.6
12	60	11	4	1.25	20.1	42.18	47.1
13	40	9	5	1.25	15.3	31.72	31
14	60	9	5	1.25	17.5	35.18	34.2
15	40	11	5	1.25	16.7	31.94	29.6
16	60	11	5	1.25	23.1	42.75	50.9
17	30	10	4.5	1.00	11.2	34.54	21.5
18	70	10	4.5	1.00	16.7	45.62	42.3
19	50	8	4.5	1.00	9.1	29.47	14.9
20	50	12	4.5	1.00	19	39.56	41.8
21	50	10	3.5	1.00	16	30.61	27.2
22	50	10	5.5	1.00	17.1	33.35	31.7
23	50	10	4.5	0.50	11.8	33.12	21.7
24	50	10	4.5	1.50	18.4	36.31	37.1
25	50	10	4.5	1.00	16.8	34.88	32.6
26	50	10	4.5	1.00	17.5	35.33	34.3
27	50	10	4.5	1.00	16.1	34.01	30.4
28	50	10	4.5	1.00	17.3	35.01	32.6
29	50	10	4.5	1.00	16.4	34.98	32.9
30	50	10	4.5	1.00	15.9	35.97	31.4

In our study, there was significant rise in dry matter content when the extractions were carried out at high temperatures. The residual distribution graph (Figure 6.6) for dry matter shows that the residuals are distributed near the normal line; therefore, the developed mathematical model is appropriate. The response surface plots of dry matter content affected by temperature, solubilization pH, time and precipitation pH are shown in Figure 6.7.

Table 6.6: Pooled ANOVA showing the variables as a linear, quadratic and interaction terms on dry matter of *Jatropha* seed cake

Source	Sum of Squares	Df	Mean Square	F- value	p-value
Model	421.46	11	38.31	34.06	< 0.0001
A-Temp	68.01	1	68.01	60.46	< 0.0001
B-Solubilization pH	140.17	1	140.17	124.62	< 0.0001
C-Precipitation pH	4.00	1	4.00	3.56	0.0755
D-Time	112.67	1	112.67	100.17	< 0.0001
AC	11.22	1	11.22	9.98	0.0054
AD	16.40	1	16.40	14.58	0.0013
BD	30.80	1	30.80	27.39	< 0.0001
CD	9.61	1	9.61	8.54	0.0091
A^2	15.23	1	15.23	13.54	0.0017
B^2	14.21	1	14.21	12.64	0.0023
D^2	5.67	1	5.67	5.04	0.0376
Residual	20.25	18	1.12		
Lack of Fit	18.15	13	1.40	3.34	0.0957^{ns}
Pure Error	2.09	5	0.4187		
Cor Total	441.70	29			
				R^2	0.95
* significant at 95% confidence, ns insignificant				Adj R^2	0.92

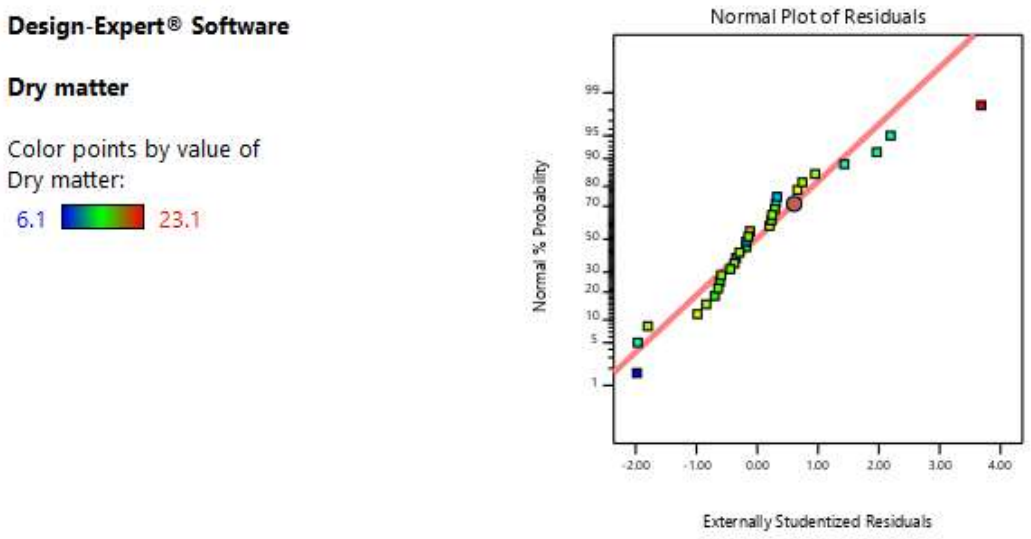


Figure 6.6: Residual distribution of dry matter content

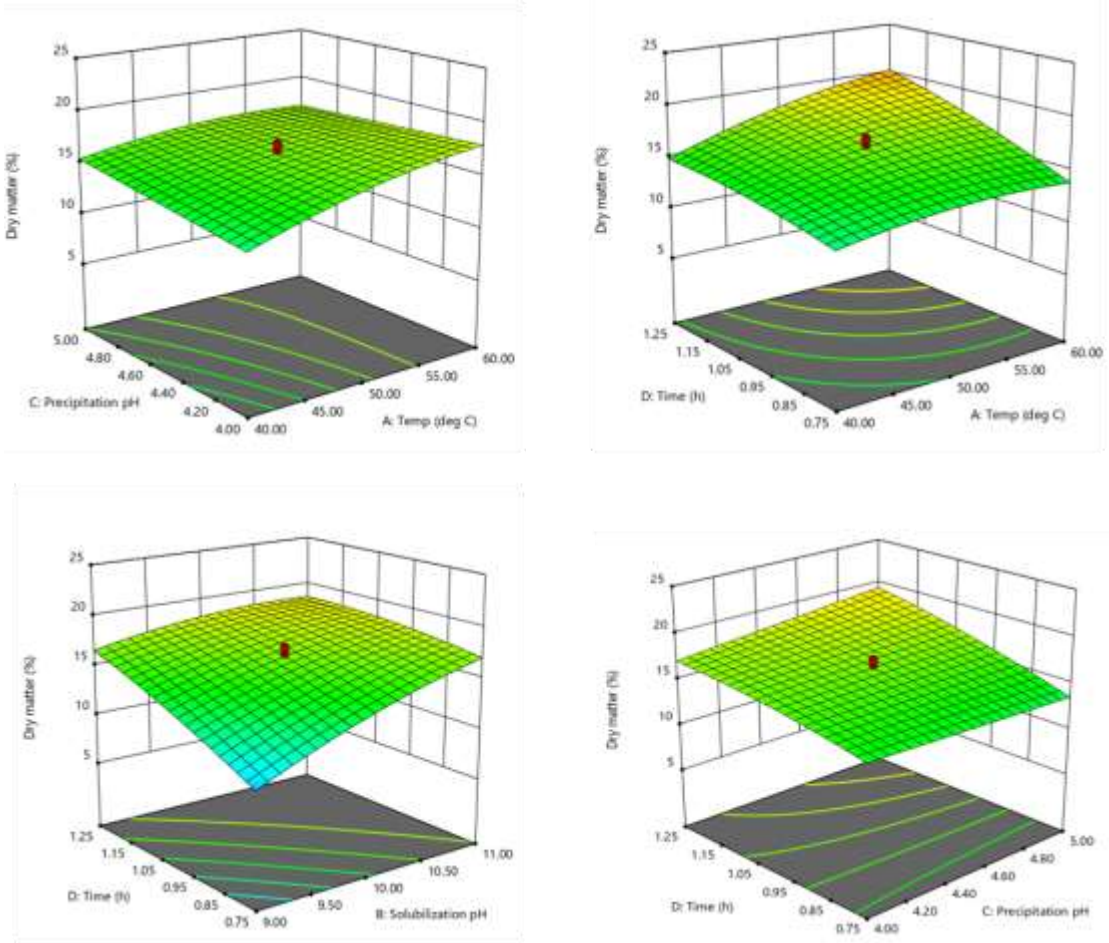


Figure 6.7: Response surface plots of dry matter content affected by temperature, solubilization pH, time and precipitation pH.

6.3.6.2 Effect of parameters on protein content

The protein content of the detoxified seed cake ranged from 30.07 – 45.62 %**.** The second-order polynomial model had a high correlation coefficient (R^2=0.95) and it indicated that the model fitted suitably with the observed data. The analysis of variable (ANOVA) after omitting the non-significant terms is given in Table 6.7. The 3-D and residual plots of protein content as a function of temperature, solubilization pH, precipitation pH and time are shown in Figure 6.8. The residuals fell around straight line implying that the errors are distributed uniformly and the model is adequate. The response surface equation after removing the non-significant terms, for protein content is:

Protein Content = 34.50 + 2.63A + 2.16B + 0.47C + 0.99D + 1.80AB + 0.80AC - 1.17A^2 - 0.85C^2

Table 6.7: ANOVA showing the variables as a linear, quadratic and interaction terms on protein content of *Jatropha* seed cake

Source	**Sum of Squares**	**Df**	**Mean Square**	**F- value**	**p-value**
Model	435.99	8	54.50	52.00	< 0.0001
A-Temp	165.64	1	165.64	158.03	< 0.0001
B-Solubilization pH	112.02	1	112.02	106.87	< 0.0001
C-Precipitation pH	5.41	1	5.41	5.16	0.0338
D-Time	23.62	1	23.62	22.54	0.0001
AB	52.09	1	52.09	49.70	< 0.0001
AC	10.35	1	10.35	9.88	0.0049
A^2	38.86	1	38.86	37.08	< 0.0001
C^2	20.85	1	20.85	19.89	0.0002
Residual	22.01	21	1.05		
Lack of Fit	19.97	16	1.25	3.06	0.1105 ^{ns}
Pure Error	2.04	5	0.4079		
Cor Total	458.00	29			
				R^2	0.95
* significant at 95% confidence, ns insignificant				Adj R^2	0.93

The coefficients of the first-order term in the equation showed that the protein content of the concentrate increased with increase in temperature, solubilization pH, precipitation pH and time. In a study by Oliyaei *et al.* (2017) an increase in protein content was observed with increase in temperature which could possibly be due to the reason that high temperatures break the bonds in protein structure which increases the dissolution rate resulting in high protein content. However, temperature beyond a certain range may cause overheating and result in protein denaturation and addition of impurities which may affect the protein content in the protein concentrate. In our study, temperature upto 70°C was also found to be suitable for extraction of proteins from detoxified *Jatropha* seed cake with good protein content.

Many researchers have observed increased protein content at higher pH because of better solubility of proteins at alkaline pH which may attribute to good protein content in the protein concentrate (Rhee *et al.*, 1972; Gisele *et al.*, 2014). An increase in protein content was also observed with time as longer extraction times results in more solubility of proteins with a good amount of protein. According to Kain *et al.* (2009) solubility of proteins is minimum at iso-electric pH because of balance between positive and negative charges which minimizes the repulsion forces amongst amino acids and thus reduces the solubility of proteins. Rhee *et al.*, (1972) observed a decrease in protein content when the pH was increased beyond the iso-electric point. The positive coefficient of the interaction terms (AB and AC) indicated an increase in protein content with addition of these variables.

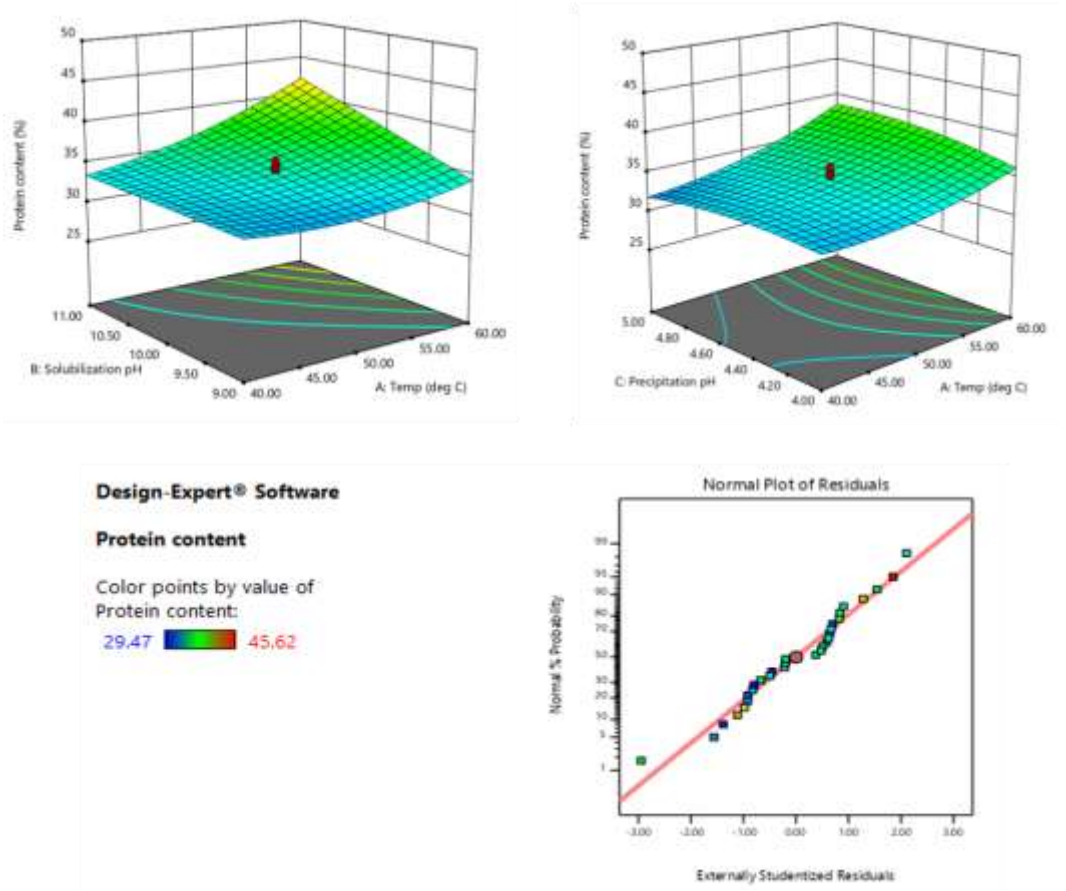


Figure 6.8: Response surface plots of protein content affected by temperature, solubilization pH, time and precipitation pH and residual distribution plot

6.3.6.3 Effect of parameters on protein yield

Analysis of variance (ANOVA) was performed to evaluate the effect of four factors on the protein yield. As seen in Table 6.8, the value of lack of fit (0.091) was non-significant which implies accuracy of the model. The R^2 and the adjusted R^2 value of the model was 0.96 and 0.94 respectively so it can be conferred that the obtained model is accurate and can be predicted for mentioned response. The independent variables having $p<0.05$ indicates the significant model terms.

Table 6.8: ANOVA showing the variables as a linear, quadratic and interaction terms on protein yield of *Jatropha* seed cake

Source	Sum of Squares	Df	Mean Square	F- value	p-value
Model	2598.74	12	216.56	44.53	< 0.0001
A-Temp	680.53	1	680.53	139.94	< 0.0001
B-Solubilization pH	885.73	1	885.73	182.14	< 0.0001
C-Precipitation pH	31.28	1	31.28	6.43	0.0213
D-Time	596.01	1	596.01	122.56	< 0.0001
AB	107.12	1	107.12	22.03	0.0002
AC	26.52	1	26.52	5.45	0.0320
AD	81.90	1	81.90	16.84	0.0007
BD	92.16	1	92.16	18.95	0.0004
CD	28.09	1	28.09	5.78	0.0279
B^2	39.25	1	39.25	8.07	0.0113
C^2	23.13	1	23.13	4.76	0.0435
D^2	23.77	1	23.77	4.89	0.0410
Residual	82.67	17	4.86		
Lack of Fit	73.74	12	6.14	3.44	0.0910 ^{ns}
Pure Error	8.93	5	1.79		
Cor Total	2681.41	29			
				R^2	0.96
* significant at 95% confidence, ns insignificant				Adj R^2	0.94

The response surface equation for protein yield after removing the non-significant terms in coded values is:

Protein Yield = 31.97 + 5.32A + 6.08B + 1.14C + 4.98D + 2.59AB – 1.29AC + 2.26AD - 2.40BD + 1.33CD – 1.18B^2 – 0.90C^2 – 0.92D^2

It can be observed from the response equation that all the four independent variables i.e. Temperature (A), solubilization pH (B), precipitation pH (C) and time (D) were showing significant effect on the protein yield. The interaction of temperature and solubilization pH had a positive effect on protein yield while all other interaction

terms were negative. Figure 6.9 shows the residual distribution plot for protein yield and it was observed that the residual points were distributed around the normal line which implies the developed model is appropriate for predicting and investigating the effect of parameters.

As seen in Figure 6.10, increase in protein yield was observed with increase in temperature. In a work done by Selling *et al.* (2013), highest protein yield (35%) was obtained when pennycress seeds were extracted with water at 77°C. The rate of extraction varies linearly with temperature, and increase in temperature causes molecules to move fast, thereby increasing the mass transfer rate between solid and liquid resulting in good solubility and increased extraction rate. But further increase in temperature resulted in decrease in protein yield. In a work done by Kain *et al.* (2009), extraction of proteins from peanut at high temperatures (60°C) resulted in decrease in protein yield. According to Chenyan *et al.*, (2011) extractions at higher temperatures results in a decline in protein yield which could be due to thermal degradation or denaturation of proteins. In our study, temperature upto 60°C resulted in a good protein content and protein yield but as the temperature was further raised to 70°C, protein yield declined but the protein content was still higher. pH also plays a significant role on the extraction of proteins and protein yield. As the solubilization pH was increased an increase in protein yield was observed which could be due to increased extractability of proteins. In a study, extraction of proteins from fish was done by iso-electric precipitation method and high protein recovery was observed at pH12 than pH10 (Oliyaei *et al.* 2017). In another study, protein yield increase was attributed to less protein denaturation at high pH (Saha *et al.,* 2016). Similar results were obtained in various studies where a high protein yield was obtained at high alkaline pH (Martin *et al.* 2010; Srivastava and Roy, 2011). Different studies have also shown an increase in solubility at precipitation pH. The graphs of precipitation pH indicated a good protein yield at pH5. This suggests that the isolectric point of *Jatropha* protein is between pH 4.5-5 which results in maximum recovery of the solubilized proteins at this pH. According to Kain *et al.* (2009) solubility of proteins is

minimum at isolectric pH because of balance between positive and negative charges which minimizes the repulsion forces amongst amino acids and thus reduces the solubility of proteins. Each protein has its own iso-electric pH depending upon its amino acid composition. Likewise, the method of extraction also affects the iso-electric pH of a particular protein. According to Yu *et al.* (2007), maximum amount of peanut protein was extracted from supernatant when the extraction was carried out at pH4. While in another work reported by Wu *et al.* (2009), pH 4.5 was found to be the iso-electric pH for precipitation of peanut protein. The protein yield also increased with increase in extraction time which is similar to the findings of Qiaoyun *et al.*, 2017 who also observed a sharp increase in protein yield when the extractions were carried out for longer periods.

The interaction terms of solubilization pH and precipitation pH (BC) and precipitation pH and time (CD) were also found to be positive and significantly affecting the protein yield. It has been cited in many reports that both solubilization pH and precipitation pH influence the solubility properties of proteins in a solution and hence the protein yield. Since the interaction terms of solubilization pH and time was not significant, these factors are not dependant on each other in the chosen range.

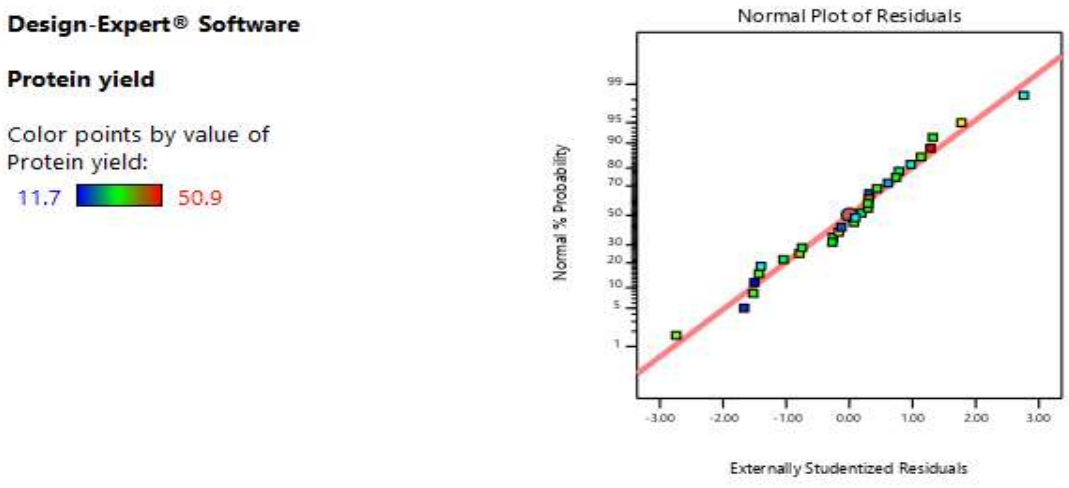


Figure 6.9: Residual distribution of protein yield

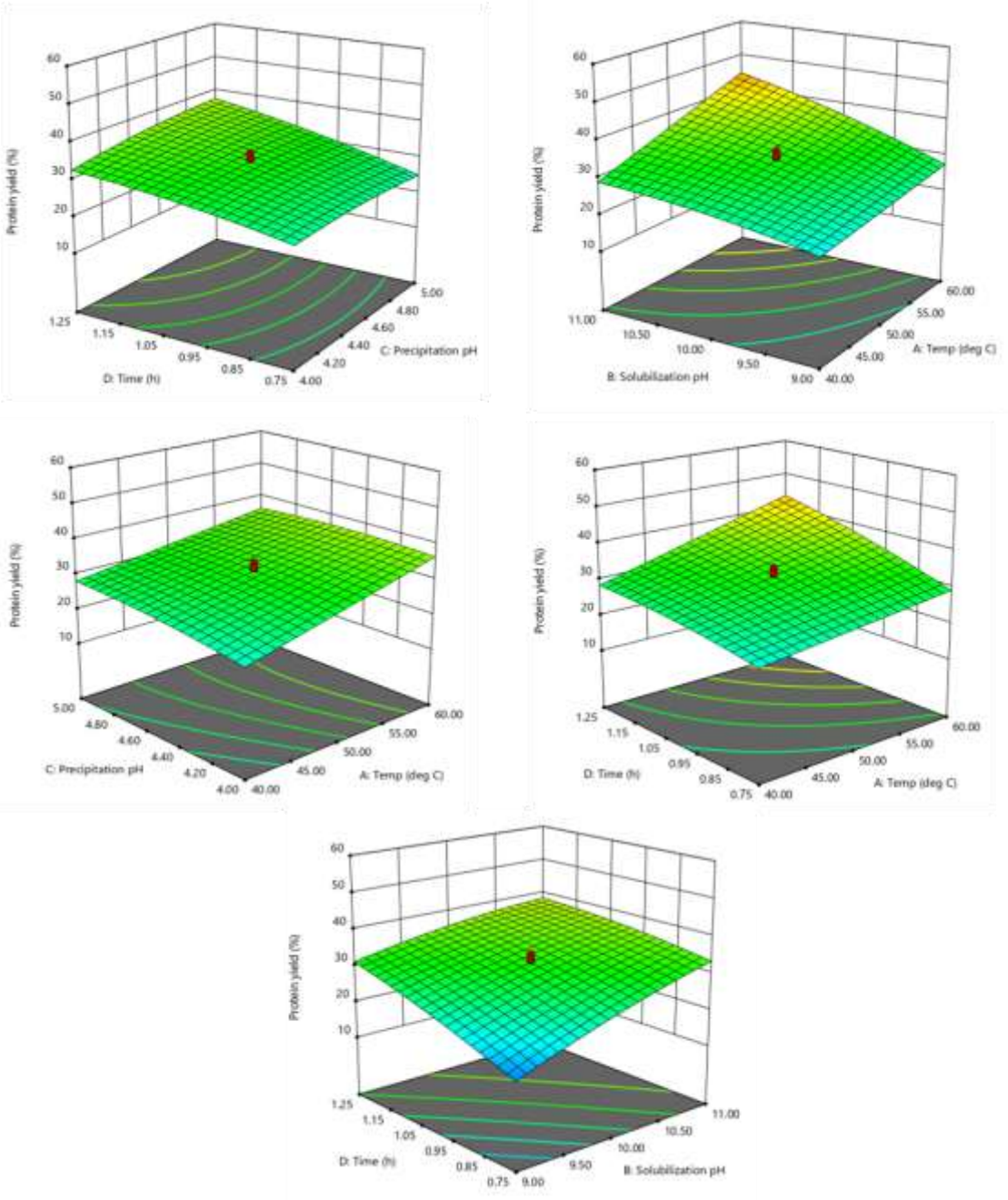


Figure 6.10: Response surface plots of protein yield affected by temperature, solubilization pH, time and precipitation pH.

6.3.6.4 Optimization

The values for dry matter, protein content and protein yield as proposed by the final quadratic model are shown in Figure 6.11. The optimum values as obtained by differentiation of the quadratic model, for achieving dry matter, protein content and protein yield were Temperature = 60°C, solubilization pH = 11, time = 0.78h and

precipitation pH = 4.41. The predicted optimal dry matter, protein content and protein yield corresponding to these values were 17.4 %, 41.12 % and 39.86 % respectively. Additional experiments were performed in triplicates using these optimized levels to verify the accuracy of the model for predicting the three responses i.e. dry matter, protein content and protein yield. The average of triplicate experiments yielded a dry matter, protein content and protein yield of 17.1 %, 41.98 % and 38.66 % respectively (Table 6.9). On comparing, a good compliance between the experimental and predicted values was observed which confirms the reliability of model as well as existence of optimal point.

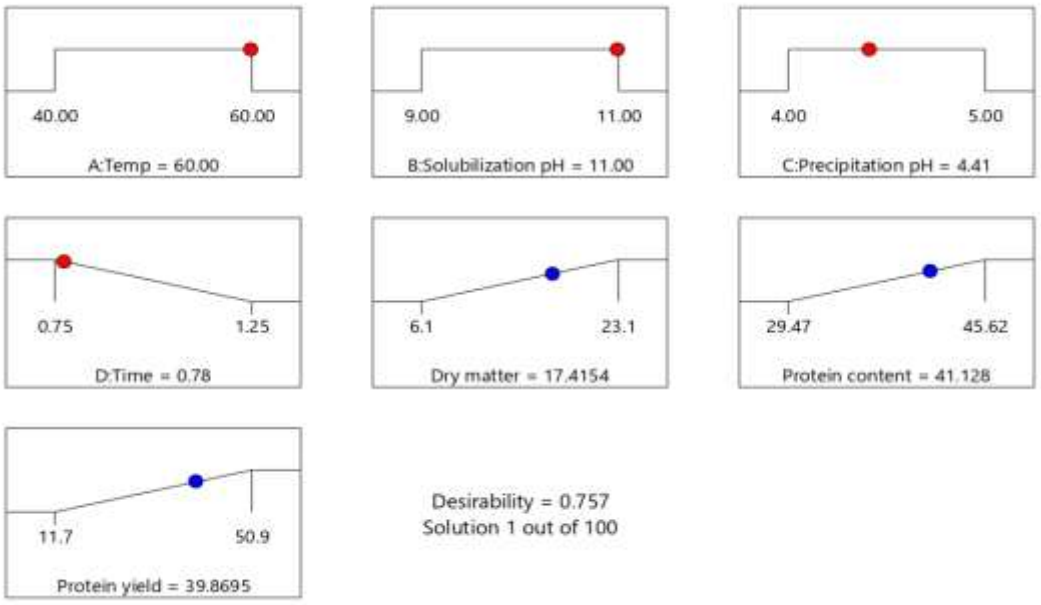


Figure 6.11: Desirability ramp of optimization for protein extraction from detoxified seed cake

6.4 Conclusion

A highest protein yield of 66.28% was obtained from *Jatropha* seed cake by iso-electric precipitation method. Temperature and pH had a significant effect on the protein content and protein yield. Recovery of proteins was highest when the extraction process was carried out at 55°C. Solubilization pH towards alkalinity resulted in less protein losses in supernatant which should help in more recovery of proteins from the *Jatropha* seed cake.

Table 6.9: Predicted values vs experimental values for optimum dry matter, protein content and protein yield

Variable	Optimized values	Dry Matter (%)		Protein Content (%)		Protein Yield (%)	
		Pred value	Exper. value	Pred value	Exper. value	Pred value	Exper. value
Temperature	60°C	17.4	17.1	41.12	41.98	39.86	38.66
Solubilization pH	11.0						
Precipitation pH	4.41						
Time	0.78h						

The extraction of protein from detoxified *Jatropha* seed cake was optimized using RSM. The optimum values of the variables i.e. temperature, solubilization pH, precipitation pH and time for the extraction of protein from detoxified seed cake were 60°C, 11.0, 4.41 and 0.78 h respectively which yielded maximum dry matter, protein content and protein yield.

CHAPTER 7

TESTING THE EFFECT OF BIO-REMEDIATED SEED CAKE ON SEED GERMINATION AND *IN VITRO* DIGESTIBILITY FOR USE AS ANIMAL FEED SUPPLEMENT

7.1 Introduction

Jatropha seeds also contain certain antinutritional compounds like curcin, phytates, lectins, saponins and toxic compounds like phorbol esters which restrict the use of *Jatropha* for food/feed applications. The toxicity of *Jatropha* seed, *Jatropha* oil and *Jatropha* seed cake is predominantly because of this toxin i.e. phorbol esters. Phorbol esters are widely distributed in different parts of the *Jatropha* plant but they are mainly concentrated in the seed kernel (Ahmed and Salimon, 2009). Anti nutritional factors such as phytates, tannins, saponins are present in 9.4, 0.04 and 2.60 % respectively in *Jatropha* kernel meal (Makkar and Becker, 2009a). So the handling and disposal of *Jatropha* seed cake should be guarded or it should be detoxified before disposing for environmental and health concern.

For the formulation of animal feeds, digestibility data is very crucial and important factor. Protein in animal feeds is considered as the main and the most expensive ingredient. Therefore, it becomes very important to identify low-cost ingredients rich in proteins which would help in cutting down the production costs of feeds. In recent years, search for alternate protein sources for aquafeeds is high on demand (Tacon and Metian, 2015; Marie-Helene *et al*., 2017). Some of these ingredients are from plant origin, such as soybean meal but their use is limited because of the presence of certain anti-nutritional factors such as trypsin inhibitors and digestive proteases which affect organism digestive process (Silvia *et al*., 2016). This demands the addition of other protein ingredients which contains all essential amino acids (Cordoba and Garcia, 2002). Bone meal is a by-product of slaughterhouses and can also be used as protein

supplement but the quality of the meal is a major concern as it depends upon the production process and the raw materials used (Tacon and Akiyama, 1997). In aquaculture, feeding trials are generally done for measuring the effect of feeds on the growth performance but these are expensive and time taking procedures and may get affected by environmental factors (Wasielesky *et al*., 2003). In vitro digestibility methods using digestive enzymes of the specific species have been developed which are faster and comparatively less expensive than in vivo methods**.** But in vivo digestibility data is also needed to validate the data obtained by in vitro studies; both In vivo and in vitro assays should correlate to access the protein quality (Ezquerra *et al*., 1997).

Very few studies have been conducted to evaluate the effects of detoxified *Jatropha* protein concentrate on in vitro digestibility of fishes, especially for *Oreochromis niloticus*, *Labeo rohita*. With this aim, the present study was planned to evaluate the effect of detoxified *Jatropha* protein concentrate on *in vitro* digestibility. Along with this, the effect of addition of raw *Jatropha* seed cake, defatted seed cake and detoxified seed cake on germination and development of green gram (*Vigna radiata*) was also studied.

7.2 Materials and Methods

7.2.1 Materials

a) Seeds and soil: Green gram seeds used for the study were purchased from the local market. Seeds of uniform size and weight were selected for study. Soil was collected from Delhi Technological University ground and was sieved before use. Whatman No.1 filter papers were used in the present study. *Jatropha* seed cake (raw and defatted) was received from centre for Advance Studies and Research in Automotive Engineering, DTU. A part of this was detoxified by subjecting it to microbial remediation of phorbol esters for 12h as described by Shilpi *et al.*, (2017).

b) Fish species: *Oreochromis niloticus*, tilapia (length: 19.12 ±1.54 cm, weight: 124.12±23.10 g) and *Labio rohita*, (length: 23.6±2.4 cm, weight: 106.4±22 g) were used for in vitro digestibility study for detoxified *Jatropha* seed cake. The fishes were obtained from Jahangirpuri, Delhi and were brought to the wet laboratory facility carefully; Fishes were maintained in the plastic tanks separately for 7 days for

acclimation. Water temperature, pH and dissolved oxygen level were maintained at 25-30°C, 7.8-8.5 and ≥ 6 mg/l respectively. Diet containing 40% protein was fed to fish twice daily. For the complete evacuation of the digestive tract, fishes were starved for 72 h before sampling.

7.2.2 Sterilization

Soil was autoclaved at 121°C for 40 min at 1.05 Kg/cm^2 (115 lb psi) every alternate day for a week ensuring proper sterilization. Distilled water and glass wares were sterilized by autoclaving at 121°C/20 min at 115 lb psi. Petri dishes, plastic cups, forceps and needles were cleaned with 90% ethanol before use. Green gram seeds were surface sterilized using teepol and were washed thoroughly under tap water. The seeds were then treated with an antioxidant i.e. Citric acid solution (1%) in a flask, placed on rotary shaker (100 rpm for 10min) followed by washing with distilled water. The seeds were re-sterilized with freshly prepared $HgCl_2$ solution (0.1%) under constant shaking (100 rpm/ RT) for 5 min and washed with sterile distilled water. Distilled water, cups, tissue paper, lamp, etc. except seeds, were exposed to UV light in a laminar air flow cabinet for 30 min prior to use. Laminar air flow surface was cleaned with 70% ethanol before starting with the seeds germination experiments.

7.2.3 Germination Test in Soil

Different concentrations of raw, defatted and detoxified *Jatropha* seed cake were mixed thoroughly with sterilized soil according to Table 7.1 and filled in plastics cups (8.0 cm diameter). Controls were maintained separately. The sides and bottom of plastic cups were pricked facilitating the air and extra water removal. 10 seeds per cup equidistant and at 1 cm depth were sown with help of flame- sterilized forceps. After sowing, soil was moistened by adding 10 ml of sterile distilled water with the help of pipette. 10 replicates of each concentration were made and kept in air conditioned room (28±2°C) under natural light. During the incubation period, sterile distilled water (2 ml) was added daily in the evening in each cup to maintain the water content. The experiments were terminated after five days and the growth parameters of seedling such as morphogenesis, rhizogenesis and leaves surface area etc. were measured and calculated.

Table 7.1: Different combinations of soil and *Jatropha* seed cake

	1% (Ratio of seed cake to soil)	**5% (Ratio of seed cake to soil)**	**10% (Ratio of seed cake to soil)**
Raw *Jatropha* seed cake	1:99	5:95	10:90
Defatted seed cake	1:99	5:95	10:90
Detoxified seed cake	1:99	5:95	10:90
Control	100:0	100:0	100:0

7.2.4 Analytical

After five days, seedlings were counted, and their root and shoot lengths were measured. Seedling vigor index was calculated according to Bidlan *et al.* (2004) as

$$V.I = \frac{(Mean\ Root\ Length + Mean\ Shoot\ Length)\ X\ Germination\ \%}{10}$$

7.2.4.1 Growth of seedling

Observations were recorded after 5 days of sowing of seeds. The following observations were made: a) number of seedlings b) Percentage seed germination c) Average shoot length (cm) d) Average root length (cm) e) Average number of leaf per shoot f) Average number of roots per shoot. The *in vivo* raised shoots were excised immediately and maintained at 4°C until further analysis for photosynthetic pigments.

7.2.4.2 Photosynthetic pigments

Chlorophyll was estimated by using method described by Holden (1960) with slight modifications. Fresh leaf samples of 0.5g were weighed and homogenized using mortar-pestle with 80% acetone until all the color was extracted from the tissue. The extract was then centrifuged at 1200 rpm for 10min and supernatant was collected. The residue was re-extracted with 10ml of 80% acetone and centrifuged and the supernatants were pooled together. The test tubes were covered with black sheet so as to protect chlorophyll from photolytic degradation. The Bio-spectrophotometer (Eppendorf) was set at wavelengths 663 nm, 645 nm, and 480 nm for chlorophyll *a*, chlorophyll *b*, and carotenoid respectively. The optical density was measured and the content of chlorophyll *a*, chlorophyll *b*, and carotenoid (in $mg.g^{-1}$) was calculated

using standard formula. For total chlorophyll, the values of chlorophyll a and chlorophyll b were added.

$$Chlorophyll\ a\ (mg\ g^{-1}) = \frac{12.3\ X\ A_{663} - \ 0.86\ X\ A_{645}}{a\ X\ 1000\ X\ W}\ X\ V$$

$$Chlorophyll\ b\ (mg\ g^{-1}) = \frac{19.3\ X\ A_{645} - \ 3.6\ X\ A_{663}}{a\ X\ 1000\ X\ W}\ X\ V$$

$$Total\ Cholorophyll\ (mg\ g^{-1}) = Chlorophyll\ a + Chlorophyll\ b$$

$$Carotenoid\ (mg\ g^{-1}) = \frac{4\ X\ OD\ X\ total\ volume\ of\ acetone}{Weight\ of\ fresh\ leaf}$$

where,

OD = Optical Density

V = Final volume of chlorophyll extract in 80% acetone

W = Dry weight of plant material

a = Path length (usually 1 cm)

7.2.5 Sampling and crude enzyme extract preparation

Fish were anesthetized using MS 222 (Sigma, USA) and was dissected to collect the digestive tract and associated glands of each fish which were then cleaned properly. The digestive tissue of each species was pooled (5 *Rohita* and 5 for *Tilapia*) and the pooled samples of both fishes were collected in petri plates separately. Tissues were then weighed and homogenized using chilled distilled water (1:3, w/v). The homogenate was filtered through cheese cloth pre-treated with EDTA (ethylenediaminetetraacetic acid, 0.5%). The filtrate was then centrifuged at 10,000 x *g* for 30 min at 4°C and supernatant was collected. This supernatant is called as crude enzyme extract. The crude extract of both the fishes were stored separately at -20 °C for further use.

7.2.6 Elemental analysis

Five best samples (from previous chapter) of dried protein concentrates were selected for this study and were analyzed for carbon, hydrogen, oxygen, nitrogen and sulphur contents in CHNSO analyzer (Vario micro tube, Germany) [Figure 7.1].

Figure 7.1 CHNS Analyzer (Elementar Germany, Vario Micro Cube)

Combustion of organic compounds at high temperature in the presence of oxygen releases gaseous products like CO_2, H_2O, SO_2, N_2 and NO_x. The elemental composition of the samples is measured by calculating the weights of the combustion products. The combustion products (CO_2, SO_2, H_2O and N_2) are separated from other components in a chromatographic column. N_2 passes with He carrier gas and CO_2, H_2O and SO_2 gets trapped in adsorption column. Heating the gases in the column by Temperature Programmed Desorption (TPD) allows separation of the gases resulting in gas chromatograms of three different peaks eluting in the order of N_2, CO_2, and H_2O. The gases eluting from TPD column are quantitatively analyzed using Thermal conductivity detector (TCD). IR detector in the system allows to measure sulphur content even in trace quantities. Finally, the concentration of the elements is calculated from detector signals and weight of the sample.

7.2.7 Estimation of protein in crude enzyme extract

The protein (soluble) in the enzyme extract was estimated by Bradford (1976) method. Absorbance was measured at 595 nm using UV-visible Spectrophotometer (Shimadzu, Japan). BSA (1 mg/ ml) was used as standard for calculating protein content. Briefly, crude enzyme extract (5 µl) was taken in eppendorf and volume was made upto 100 µl by adding distilled water. 1 ml of Bradford reagent was added and vortexed.

The amount of protein in crude enzyme extract was calculated as:

Protein in µg /µl = (absorbance – intercept)/ slope.

As the aliquot taken was 5 µl so the obtained value was divided by 5.

7.2.8 Total protease activity

Estimation of total protease activity of the crude extract was done using azocasein as substrate (Garcia-Carreno, 1993). 10 µl of crude extract was incubated at 25°C for 10 min with 500 µl of 1% azocasein in presence of Tris-HCl 50 mM (pH 7.5). Reaction was terminated by adding 500 µl of 20% TCA (trichloroacetic acid). The assay mixture was then centrifuged at 10,000 x *g* for 10 min and the absorbance was read at 366 nm. Total protease activity was expressed as:

Activity Units = Abs_{366} / min/ mg protein in the reaction mixture

where, Abs366 = Assay Abs. - Blank Abs.

7.2.9 *In vitro* digestibility: pH-Stat method

Degree of hydrolysis (DH) of protein sources (crude enzyme extract) was evaluated using pH-Stat titration method (Garcia-Carreno *et al.,* 1997, Kumar *et al.,* 2007). Substrate suspension (8 mg/ml) for each plant ingredient was prepared and pH was adjusted to 8 using 0.1 N NaOH. The crude enzyme was diluted appropriately to obtain an activity of 0.250 U mg/protein. The pH of enzyme sample was adjusted at 8.0 using 0.1N NaOH before the assay.

The *in vitro* digestibility assay was performed in the pH-Stat Autotitrator interfaced to desktop computer with a preinstalled Stat-titration programme (Tiamo Version 2.2). The following parameters were set: mode - Stat titration, control pH at 8.0; dynamics, 0.2; maximum rate - 0.1 ml /min ; minimum rate - 10.0 µl /min ; dosing rate - 0.1 ml /min ; stop time - 3600 s and report output.

10 g of the substrate suspension at pH 8.0 was taken in a vessel and addition of 200 µl of crude enzyme extract was done to start the reaction at 25°C. The reaction mixture's pH (pH8) was maintained by adding 0.1N NaOH. All the experiments were

performed in triplicates. Volume of NaOH used for keeping a constant pH (8.0) was recorded and used in calculating the degree of hydrolysis for plant ingredient using the formula (Adler-Nissen, 1986):

$$Degree\ of\ hydrolysis(\%) = \frac{BXN_BX\ 1.4\ X\ S\%\ X\ 100}{8\ X\ 100}$$

where, B = ml of 0.1 N NaOH consumed to maintain the reaction mixture at pH 8.0

N_B = normality of the titrant

S% = protein content in the reaction mixture expressed as %.

7.2.10 Amino acid analysis

The amino acid content of the five protein concentrates was estimated by Amino Acid Analyzer (Hitachi, Model L-8900) [Figure 7.2]. The protein sample is made to pass through a cation-exchange column where it is separated into amino acid components which are detected at a wavelength of 570 nm. Quantification was done by comparing sample chromatogram peaks with the standards. Proline and hydroxyproline (imino acids), have no absorbance maxima within the visible light spectrum, and are therefore detected at 440 nm. In the deamination column, 20 μL of sample was injected with a flow rate of 0.35 mL min^{-1} and the column temperature was 30-70 °C. In reaction column, reaction temperature was 135 °C with a Ninhydrin flow rate of 0.30 mL min^{-1}.

a) Procedure for sample preparation (except for amino acids cysteine, methionine & tryptophan): 30 mg of sample was taken in hydrolysis tube along with 10ml 6N HCl and the reaction mixture was exposed to nitrogen for 3–4 min for removing oxygen and creating inert conditions. The tube was tightly closed ensuring no leakage and was kept in hot air oven at 110 °C for 22 h. After hydrolysis, tube was brought to room temperature and transferred the contents in 25 ml volumetric flask and the volume was made up with distilled water. The sample was then filtered through Whatman filter paper (110 mm). 10 ml sample was taken in round bottom flask to recover HCl either by Vacuum evaporator or Nitrogen evaporator followed by drying in hot water bath (60 – 70 °C) to remove HCl. The dried sample was washed

thrice with 5ml distilled water to remove traces of acid. The sample was diluted to a volume of 8.4ml with 0.02N HCl and filtered through syringe filter (Whatman 0.20 µm PES Filter). The sample (1.5ml) was put in glass vial of Hitachi L- 8900 AAA auto sampler.

Figure 7.2: Amino acid analyzer (Hitachi, Model L-8900)

b) Sample prepration for cysteine and methionine: 1-2 mg sample was taken in hydrolysis tube and 2mL performic acid was added and left for 4 to 24 h at 0 °C. 48% HBr (0.3 ml) was added and the sample was dried under reduced pressure (in Nitrogen evaporator). 6N HCl was added and the reaction mixture was exposed to the nitrogen 3 -4 min. The tube was closed tightly ensuring no air leakage from the tube and kept in oven at 110 °C for 22 h followed by drying in water bath for removing HCl. The sample was then washed thricewith 5ml distilled water to remove acid. Addition of 1 mL of 0.2N NaOH was done and the sample was left for 1 h. The pH was adjusted with 0.05N- 0.1N HCl and the sample was filtered through syringe filter Whatman 0.20 µm PES Filter. The sample (1.5 mL) was put in glass vial of Hitachi L- 8900 AAA auto sampler rack.

c) Sample preparation for recovery of tryptophan (Trp): Weighed the sample (3 mg/ml or less) and added 4N Methanesulfonic acid and 0.2 % 3-(2-Aminoethyl) indole in a digestion tube. The reaction mixture was exposed to the nitrogen 3 - 4 min and kept in hot air oven for hydrolysis at 110°C for 22 h. After decomposition, added 4N NaOH to adjust the pH to 2. Adjusted the volume by 0.02N HCl as a sample (0.5 mg protein/mL) and filtered through syringe filter Whatman 0.20 µm PES Filter. Put the sample (1.5 mL) in glass vial of Hitachi L- 8900 AAA auto sampler rack.

7.3 Results and discussion

7.3.1 Effect of addition of raw seed cake on seeds of green gram

The effect of seed cake at different levels (1, 5, and 10%) on the germination and growth of green gram was observed (Table 7.2). A maximum of 96.6% seeds germinated with an average of 20.95 cm length of seedling on addition of 1% raw seed cake to the soil. However, in control (without seed cake) 100% seeds germinated with an average length of 29.98 cm.

Table 7.2: Effect of seed cake (raw, detoxified and defatted) at different levels on the germination and growth of green gram

Sample	% Germination	Shoot length	Root Length	Secondary roots	No. of leaves	Leaf area
Control	100	22.01	7.97	12.2	2	1.36
Raw 1%	96.6	16.56	4.39	8.27	1.86	0.737
5%	100	5.17	0.623	0.4	1.8	0.486
10%	50	1.06	0.18	0	0	0
Control	100	22.01	7.97	12.2	2	1.36
DT- 1%	100	18.43	2.39	6.93	2	1.05
5%	100	14.67	0.94	7.4	2	0.726
10%	93.3	7.33	0.437	5.9	1.6	0.33
Control	100	22.01	7.97	12.2	2	1.36
DF- 1%	100	17.11	1.08	7.5	2	0.792
5%	100	14.39	0.85	7.3	1.93	0.633
10%	93.3	4.125	0.376	4.06	1.53	0.31

Interestingly, higher germination rate was observed in 5% as compared to 1% of seed cake while at 10% a much lower percentage of seed germination and survival was observed. Inhibition in germination of tobacco plant on the application of *Jatropha* kernel cake was also observed by Xing *et al.* (2013). In another study by Larissa *et al.* (2014), *Jatropha curcas* oil significantly affected the germination of lettuce with increasing concentrations of the *Jatropha* oil indicating the phytotoxicity of the oil. Mitodepressive effects on the cell cycle were observed on analyzing meristematic cells along with chromosomal and nuclear alterations indicating the cytogenotoxicity of the oil (Andrade *et al.*, 2010). As the percentage of raw *Jatropha* seed cake was increased, a significant decrease in root and shoot length was observed (Figure 7.3). No secondary roots and leaves were observed in samples containing 10% raw *Jatropha* seed cake. According to Xing *et al.* (2013), there was no significant improvement in the tobacco seedling growth on the application of the kernel cake to the soil, but the root growth decreased by 48% indicating the phytotoxicity of the kernel cake on root growth. Phorbol esters are mainly responsible for the toxicity of *Jatropha* plant. After extraction of oil from *Jatropha* meal, most of the phorbol esters (70-75%) being lipophilic show affinity towards oil and 25-30% is left in the seed cake. Phorbol ester toxicity is known to cause various physiological and morphological changes in the plant. Reduction in mitotic index results in decreased root growth which can be seen in the percentage of dividing cells (Andrade *et al.* 2010).

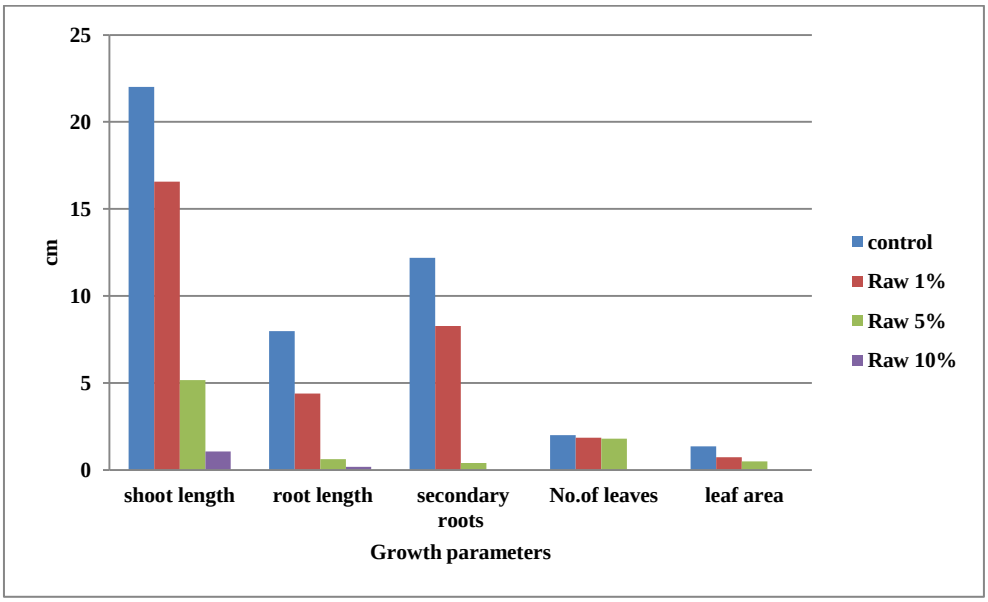


Figure 7.3: Effect of different concentrations of raw seed cake on growth parameters of green gram

Phorbol esters are responsible for the activation of many *iso*-forms of protein kinase - C (PKC) which is a cell protein that regulates many cellular processes in the cell cycle and results in interaction between DNA and toxic compounds which leads to reduction in mitotic index (Zhang *et al.,* 1995; King *et al.,* 2009). Overall an inhibitory growth pattern was observed with increase in % age of raw *Jatropha* seed cake as shown in Figure 7.3.

7.3.2 Effect of addition of defatted seedcake on seeds of green gram

The effect of defatted seedcake at different levels (1, 5, 10%) on the germination and growth of green gram was observed. Germination was not affected by the addition of 1 and 5% defatted seed cake while a decrease in germination rate (93.3%) was observed in soil containing 10% defatted seed cake. Addition of higher percentage of defatted *Jatropha* seed cake showed an inhibitory effect on the growth parameters of the green gram. There was reduction in root, shoot length and secondary roots (Figure 7.4). Not much difference was observed in growth parameters in samples containing 1% and 5% defatted seed cake but in samples containing 10% seed cake difference was clearly visible. A better root & shoot length and leaf area was observed as compared to samples containing raw *Jatropha* seed cake as defatted seed cake contained less amount of oil and hence the phorbol esters. In a study done by Penjit *et al.* (2012), treatment of Chinese kale with the low rate chemical fertilizer and high rate of *Jatropha* deoiled seed cake resulted in best marketable yield which was comparable to that obtained with full rate of chemical fertilizer. Chemical fertilizer combined with any grade of *Jatropha* seed cake (low, medium, and high) gave the maximum tomato and tuber yield.

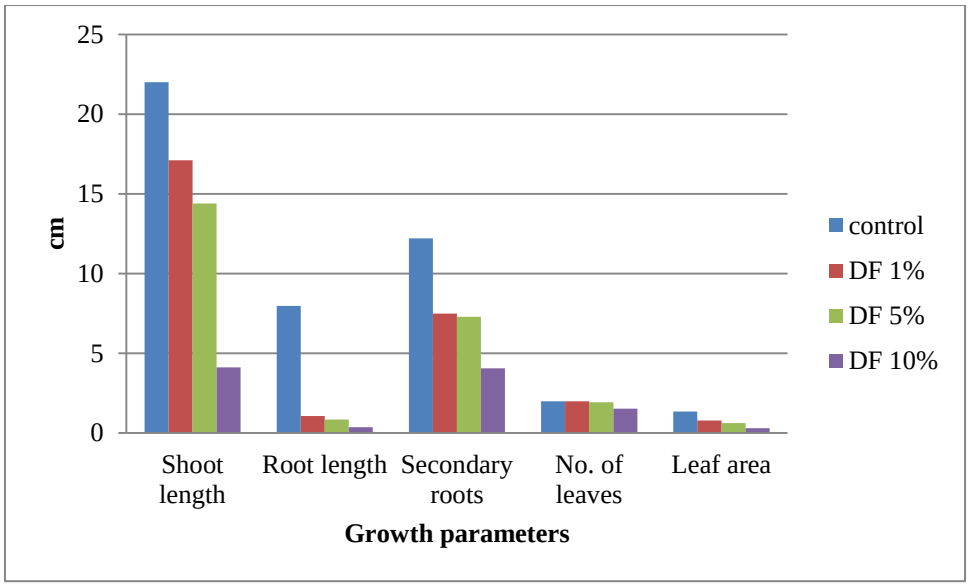


Figure 7.4: Effect of different concentrations of defatted seed cake on growth parameters of green gram

7.3.3 Effect of addition of detoxified seedcake on seeds of green gram

Detoxified seedcake at three different levels (1, 5, 10%) was added and its effect was studied on the germination and growth of green gram. No effect on seed germination was observed in samples containing 1 and 5% detoxified seed cake while 10% had a decreased germination rate (93.3%). Addition of higher percentage of detoxified *Jatropha* seed cake affected the root length but interestingly there was very less reduction in the number of secondary roots and leaves (Figure 7.5). There was improvement in the number of leaves and leaf area compared to other two seed cakes. In a study by Xing *et al.* (2013) application of the kernel cake fermented by *S. fimicarius* YUCM 310038 significantly improved the leaf length and tobacco seedling growth. This implies that the *Jatropha* seed cake can be used as organic fertilizer after it has been detoxified by various micro-organisms which have the potential to degrade phorbol esters. Chaturvedi *et al.* (2009) observed increase in growth and yield of tuberose (a commercial flower) when *Jatropha* press cake was used as an organic fertilizer. Wheat yield also improved significantly when inorganic fertilizer was substituted with *Jatropha* press cake (Ghosh *et al.*, 2012).

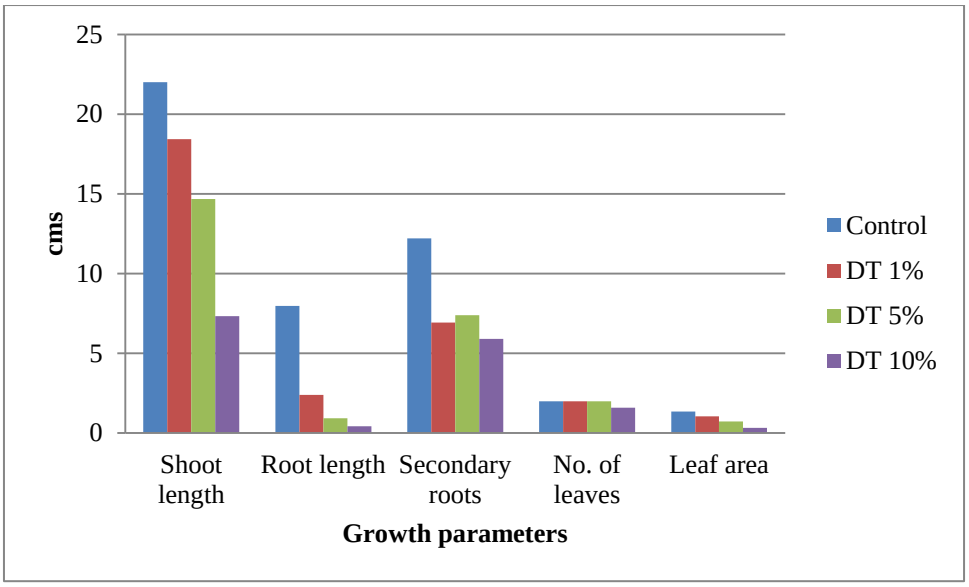
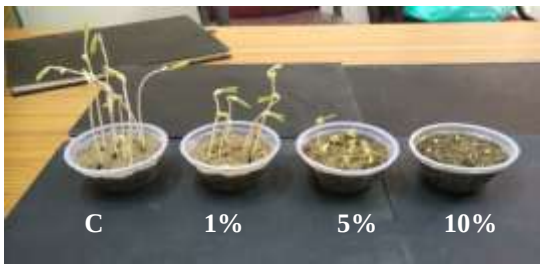


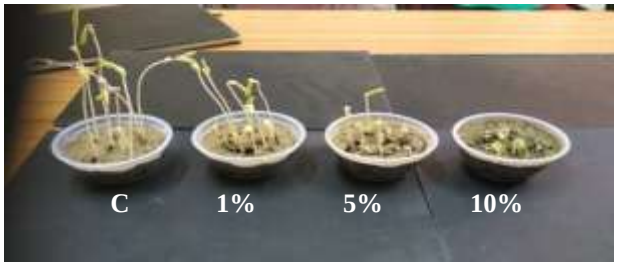
Figure 7.5: Effect of different concentrations of detoxified seed cake on growth parameters of green gram

The relative growth and germination patterns for different treatments of green gram are depicted in Figures 7.6-7.8. The apparent growth indicates the toxic effects on the germination with increasing concentrations in all the treatments. The highest concentration studied i.e. 10% show very little growth even at the end of 5 days of plantation.



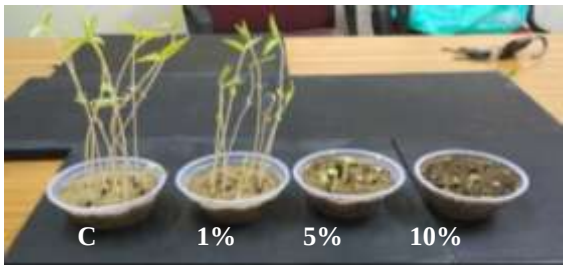
Raw seed cake

Detoxified seed cake

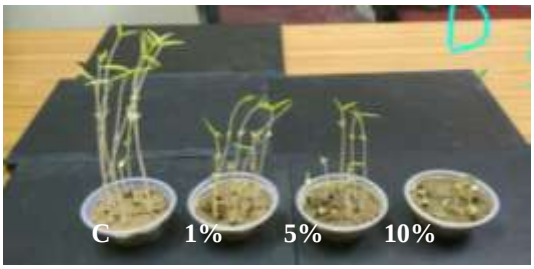


Defatted seed cake

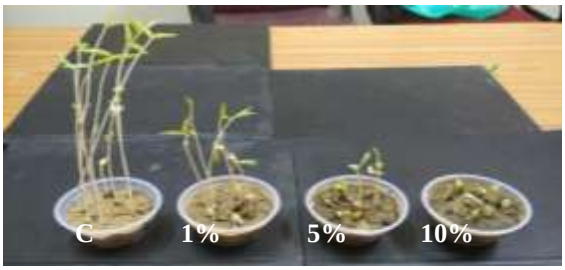
Figure 7.6: Seed germination observed after three days at different concentrations of raw, detoxified and defatted *Jatropha* seed cake



Raw seed cake



Detoxified seed cake



Defatted seed cake

Figure 7.7: Seed germination observed after five days at different concentrations of raw, detoxified and defatted *Jatropha* seed cake. C: Control.

Raw

Detoxified

Defatted

Figure 7.8: Comparative lengths of seedlings under treatment with different concentrations of raw, detoxified and defatted seed cake

7.3.4 Effect of different treatments on vigor index (V.I) of green gram

The effect of different seed cakes (raw, defatted and detoxified) on vigor index of green gram is shown in Table 7.3. The highest value of vigor index was obtained from detoxified seed cake (1%) and raw seed cake (1%) which recorded 208.2 and 202.3 respectively. Vigor index was significantly affected by the different concentrations of the raw *Jatropha* seed cake in soil. Soil with 1% raw seed cake had the higher *V.I.* than 10%. Not much difference was observed in vigor indexes at 1 and 5% levels of defatted and detoxified seed cake, however difference increased when the level was raised to 10%. This reveals the negative impact of higher concentrations of the seed cake on the overall health of the seedlings. The 10% level of detoxified seed cake, though had a lower impact on the plant health compared to the other two treatments. This indicates that the detoxified seed cakes can be used as fertilizer with further treatments after strategic analyses. We can say that the detoxification reduces the inhibitory effects of *Jatropha* seed cake.

Table 7.3: Effect of different treatments on vigor index of green gram

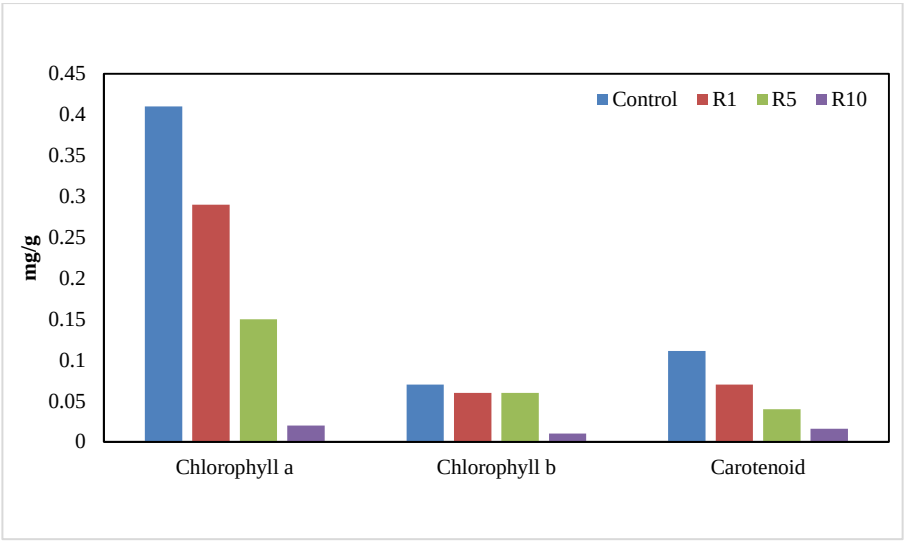
	Raw	**Defatted**	**Detoxified**
1%	202.3	181.9	208.2
5%	57.9	152.4	156.1
10%	6.2	41.9	72.4
Control	299.8	299.8	299.8

7.3.5 Effect of different concentrations of seed cake on photosynthetic pigments in green gram

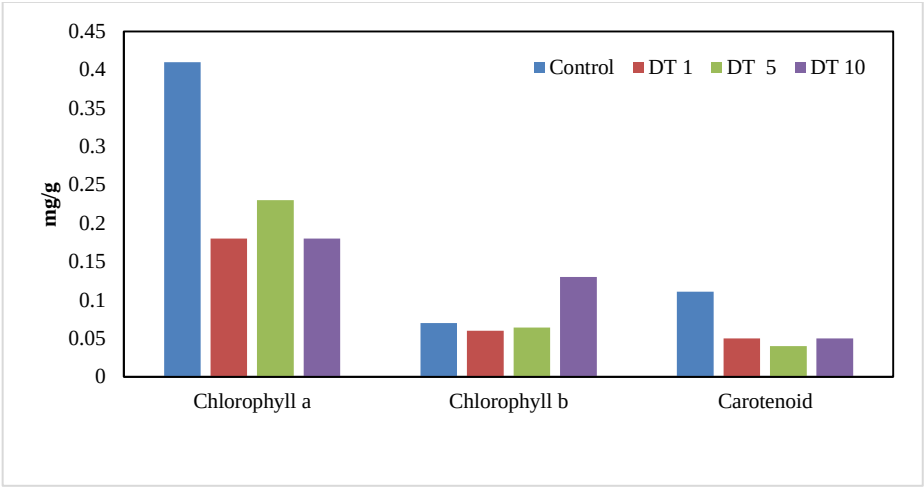
In the present investigation, 6 days old seedling was evaluated for the analysis of various photosynthetic pigments, chlorophyll a, chlorophyll b and carotenoid. Chlorophyll is the most valuable primary metabolite which captures sunlight and is responsible for photosynthesis. The effects of *Jatropha* seed cake on chlorophyll a, chlorophyll b is shown in Figure 7.9. It was observed that increase in concentration of raw *Jatropha* seed cake affected the chlorophyll a content

(Figure 7.9a) which implies that raw *Jatropha* seed cake inhibits the production of chlorophyll in green gram. There were no significant changes observed in chlorophyll b content at lower levels, it drastically decreased at higher concentration of 10% raw *Jatropha* seed cake. Addition of detoxified seed cake concentrations at 1% and 5% level matched with the control in chlorophyll b content (Figure 7.9b). At all levels of detoxified seed cake addition, carotenoid and chlorophyll a content decreased by more than 50%. It was also observed that chlorophyll b content at 10% detoxified seed cake was comparatively higher (almost double) than the control. In the presence of defatted seed cake at all concentrations, there was significant reduction in chlorophyll b and carotenoid content while at 1%, chlorophyll a and at 5% chlorophyll b were observed to be slightly higher and at 10% level, chlorophyll a was comparatively more. However, chlorophyll and carotenoid contents were better in all the controls (Figure 7.9c).

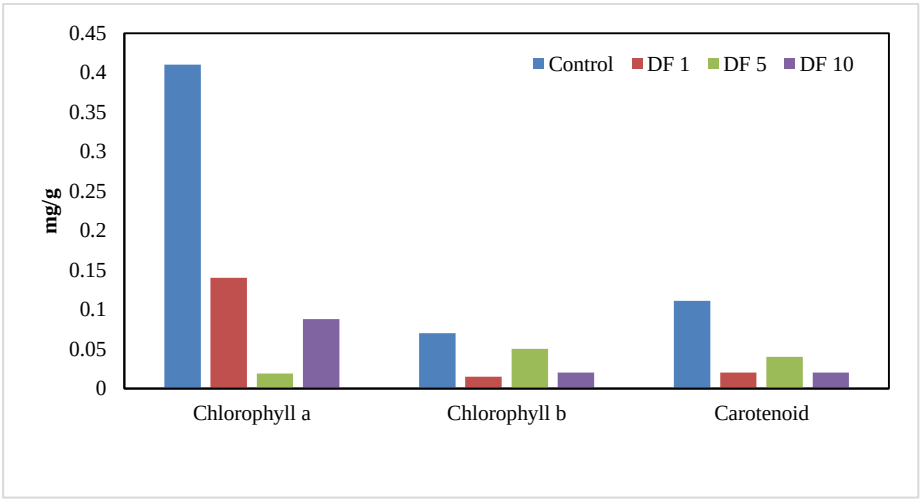
The presence of *Jatropha* seed cake shows significant changes at all concentrations under study. Exceptional enhancement in chlorophyll b content at 10% level of detoxified seed cake was observed indication metabolic interference under normal conditions in the absence of factors that are being supplied by detoxified seed cake for its bio synthesis. It was also observed that at 5% level of defatted seed cake, chlorophyll a content drastically reduced compared to raw and detoxified seed cake while not much difference was observed at 1% level in case of detoxified and defatted seed cakes, but it was almost two-third as compared to 1% raw seed cake level.



(a)



(b)



(c)

Figure 7.9: Effect of different treatments on pigments of green gram

7.3.6 Elemental analysis

Five best samples of dried protein concentrates on the basis of highest protein content and protein yield were selected for this study and were analyzed for carbon, hydrogen, oxygen, nitrogen and sulphur contents in CHNSO analyzer (Vario micro tube, Germany). The results are given in Table 7.4.

Table 7.4: CHNS analysis of protein concentrates

	Samples				
	1	**2**	**3**	**4**	**5**
N (%)	6.33	5.81	5.90	6.75	7.30
C (%)	53.79	55.94	54.94	55.43	53.75
H (%)	7.21	8.41	8.32	8.43	8.05
S (%)	0.29	0.19	0.39	0.50	0.67
P (%)	39.56	36.31	36.87	42.18	45.62
N area	13664	16999	15528	20345	16993
C area	71450	102715	90143	106530	78773
H Area	31311	51201	45077	53763	38781
S Area	23970	23639	29308	35801	36130
C/N Ratio	8.49	9.62	9.31	8.21	7.36
C/H Ratio	7.46	6.64	6.59	6.57	6.68

CHNS analysis was carried out in CHNS Analyzer (Elementar Germany, Vario Micro Cube)

7.3.7 Protease activity

The protease activity of both fishes used for *in vitro* digestibility study was estimated. After adjusting the total protease activity to 0.250 U/mg, the final protease activities were found to be 0.259±0.002 U/mg protein and 0.261 U/mg protein for Talipia and Rohita respectively.

7.3.8 Degree of hydrolysis

Estimation of degree of hydrolysis (DH) by pH Stat method helps in determining the suitability of different plant proteins for preparation of diets for carps such as *Oreochromis niloticus* and *Labeo rohita*. The degree of hydrolysis of five different samples of detoxified *Jatropha* seed cake ranged from 13.84-18.85 % and 6.21-7.70 % in *Oreochromis niloticus* and *Labeo rohita* respectively (Table 7.5). While in control sample, degree of hydrolysis was 2.65±0.44% in *Oreochromis niloticus* and 3.22±1.02% in *Labeo rohita* which is much lower than the other samples of detoxified seed cake. In Talipia, low (13.84 ±0.31%) and high (18.85±0.47%) were observed for Sample No. 3 and 2 respectively.

Table 7.5: Degree of hydrolysis of detoxified *Jatropha* protein concentrates

Sample No.	Protein (%)	Degree of hydrolysis	
		Oreochromis niloticus	*Labeo rohita*
1	39.56	15.41±0.65	6.21±0.57
2	36.31	18.85±0.47	6.91±0.31
3	36.87	13.84±0.31	6.31±0.73
4	42.18	17.63±0.94	7.70±1.41
5	45.62	18.19±1.73	6.58±0.47

The degree of hydrolysis was high in all the detoxified *Jatropha* press cake samples. The DH in *Oreochromis niloticus* was higher for proteins (sample 2) extracted at 50°C, solubilization pH 10, precipitation pH 4.5 and extraction time of 1.5 h followed by sample 3 where the extraction conditions were 60°C, solubilization pH 11, precipitation pH 4.0 and extraction time 1.25 h. While in *Labeo rohita*, similar values was obtained for all the protein samples with very little variation, amongst all, Sample 4 had the highest DH i.e. 7.70%. Protein's solubility and buffering capacity,

availability of amino acids to alkali proteases, cleavage of peptide bonds by proteases are few factors that may affect the degree of hydrolysis of protein concentrates (Sharma *et al*., 2016). Many other factors may also be responsible for the species specific hydrolysis of the protein concentrates. According to Shamna *et al*. (2015), a high protease activity was observed in *Labeo rohita* fed with fermented *Jatropha* protein concentrate than non-fermented because of the certain toxins such as phorbol esters, phytates or trypsin inhibitors. Limited information is available on literature on *in vitro* digestibility of the *Jatropha* in freshwater fishes. Many researchers has reported the replacement of fish meal by low cost ingredients and were successful in replacing 50% of fish meal by *Jatropha* meal without affecting the fish's performance (Hassaan *et al*. 2016; Latif *et al*. 2015). This *in vitro* digestibility study showed a significant improvement in digestibility and thus confirmed the inactivation of toxins in the *Jatropha* protein concentrates which indicates that the detoxified *Jatropha* seed cake can be used as one of the ingredients in the fish feed formulations.

7.3.9 Amino acid profile of protein concentrates from detoxified *Jatropha* seed cake

Amino acid composition of protein concentrates is an indicative of their nutritional value. The concentrations of amino acids in all the samples varied considerably (Table 7.6-7.10). Sample 5 was found to have higher content of essential amino acids than sample 1 indicating extraction of protein at high temperature 60°C didn't affect the amino acid concentration. The levels of amino acids in sample 5 was slightly greater than sample 4 which suggested that extraction of proteins at pH 5 (precipitation pH) increased the concentration of amino acids.

Table 7.6: Amino acid content of Sample 1 (temp 50°C, solubilization pH 12, precipitation pH 4.5, time 1h)

S.No.	Amino acids	Concentration (g/100g)	±SE
Essential			
1	Histidine (His)	1.211	0.19
2	Isoleucine (Ile)	2.327	0.42
3	Leucine (Leu)	3.916	0.07
4	Lysine (Lys)	1.408	0.17
5	Methionine (Met)	0.818	0.01
6	Phenylalanine (Phe)	2.514	0.65
7	Threonine (Thr)	1.770	0.89
8	Tryptophan (Trp)	0.313	0.00
9	Valine (Val)	2.781	0.41
Non-Essential			
1	Alanine (Ala)	2.489	0.27
2	Arginine (Arg)	5.749	0.87
3	Aspartate (Asp)	4.615	0.42
4	Cysteine (Cys)	0.592	0.24
5	Glutamic acid (Glu)	8.864	0.32
6	Glycine (Gly)	2.569	0.45
7	Proline (Pro)	1.332	0.18
8	Serine (Ser)	2.163	1.00
9	Tyrosine (Tyr)	1.486	0.13
10	Phosphoserine (p- Ser)	0.170	0.08
11	Phospho ethanol amine (PEA)	0.0124	0.02
12	α Amino-n- adipic acid (a-AAA)	0.0248	0.00
13	α Amino-n- butaric acid (a-ABA)	0.0509	0.02
14	Cystathionine (Cysthi)	0.0963	0.00
15	β Alanine (b-Ala)	0.0493	0.03
16	β Amino isobutyric acid (b-AiBA)	0.0334	0.00
17	ϒ Amino butyric acid (g-AiBA)	0.0130	0.00
18	Ethanol amine ($EOHNH_2$)	0.0119	0.00
19	Ornithine (Orn)	0.0439	0.00
20	1 Methyl histidine (1 Mehis)	0.0847	0.00

(Amino acid content was determined in Amino Acid Analyzer, Hitachi, L-8900, Japan)

Table 7.7: Amino acid content of Sample 2 (temp 50°C, solubilization pH 10, precipitation pH 4.5, time 1.5h)

S.No.	Amino acids	Concentration (g/100g)	±SE
Essential			
1	Histidine (His)	1.099	0.46
2	Isoleucine (Ile)	1.706	0.71
3	Leucine (Leu)	3.590	2.17
4	Lysine (Lys)	1.370	0.72
5	Methionine (Met)	0.642	0.80
6	Phenylalanine (Phe)	2.219	1.35
7	Threonine (Thr)	1.202	0.00
8	Tryptophan (Trp)	0.297	0.00
9	Valine (Val)	2.239	2.83
Non-Essential			
1	Alanine (Ala)	2.200	0.98
2	Arginine (Arg)	5.380	0.69
3	Cysteine (Cys)	0.847	0.106
4	Glutamic acid (Glu)	8.371	0.00
5	Glycine (Gly)	2.322	0.41
6	Proline (Pro)	1.064	0.46
7	Serine (Ser)	1.558	0.00
8	Tyrosine (Tyr)	1.370	0.37
9	Phosphoserine (p- Ser)	0.120	0.15
10	Phospho ethanol amine (PEA)	0.037	0.00
11	α Amino-n- butaric acid (a-ABA)	0.0463	0.01
12	Cystathionine (Cysthi)	0.8470	0.10
13	β Alanine (b-Ala)	0.0571	0.01
14	β Amino isobutyric acid (b-AiBA)	0.1061	0.00
15	ϒ Amino butyric acid (g- AiBA)	0.0117	0.01
16	Ethanol amine (EOHNH2)	0.0127	0.00
17	Ornithine (Orn)	0.0319	0.04
18	1 Methyl histidine (1 Mehis)	0.0855	0.00
19	Hydroxyproline (Hypro)	0.0363	0.00

(Amino acid content was determined in Amino Acid Analyzer, Hitachi, L-8900, Japan)

Table 7.8: Amino acid content of Sample 3 (temp 60°C, solubilization pH 11, precipitation pH 5.0, time 0.75h)

S.No.	Amino acids	Concentration (mg/g)	±SE
Essential			
1	Histidine (His)	1.193	0.09
2	Isoleucine (Ile)	2.282	1.19
3	Leucine (Leu)	3.945	0.178
4	Lysine (Lys)	1.438	0.04
5	Methionine (Met)	0.691	0.57
6	Phenylalanine (Phe)	1.442	0.10
7	Tryptophan (Trp)	0.463	0.00
8	Valine (Val)	2.442	1.43
Non-Essential			
1	Alanine (Ala)	2.380	0.04
2	Arginine (Arg)	6.296	0.67
3	Cysteine (Cys)	0.944	0.22
4	Glutamic acid (Glu)	4.946	0.00
5	Glutamine (Gln)	3.995	0.00
6	Glycine (Gly)	2.465	0.33
7	Proline (Pro)	1.118	1.61
8	Serine (Ser)	2.767	0.00
9	Tyrosine (Tyr)	1.627	1.82
10	Phosphoserine (p- Ser)	0.0838	0.00
11	Phospho ethanol amine (PEA)	0.0426	0.00
12	α Amino-n- butaric acid (a-ABA)	0.0707	0.16
13	Cystathionine (Cysthi)	0.1325	0.53
14	β Alanine (b-Ala)	0.0601	0.05
15	β Amino isobutyric acid (b-AiBA)	0.111	0.00
16	ϒ Amino butyric acid (g- AiBA)	0.009	0.04
17	Ethanol amine (EOHNH2)	0.0417	0.00
18	Ornithine (Orn)	0.033	0.03
19	1 Methyl histidine (1 Mehis)	0.0655	0.00

(Amino acid content was determined in Amino Acid Analyzer, Hitachi, L-8900, Japan)

Table 7.9: Amino acid content of Sample 4 (temp 60°C, solubilization pH 11, precipitation pH 4.0, time 1.25h)

S.No.	Amino acids	Concentration (g/100g)	±SE
Essential			
1	Histidine (His)	1.383	0.69
2	Isoleucine (Ile)	2.579	2.19
3	Leucine (Leu)	4.444	2.28
4	Lysine (Lys)	1.732	0.75
5	Methionine (Met)	0.736	1.24
6	Phenylalanine (Phe)	2.829	1.26
7	Threonine (Thr)	2.178	0.00
8	Tryptophan (Trp)	0.510	0.00
9	Valine (Val)	2.87	2.45
Non-Essential			
1	Alanine (Ala)	2.724	1.35
2	Arginine (Arg)	6.662	1.71
3	Cysteine (Cys)	0.791	0.59
4	Glutamic acid (Glu)	9.951	0.00
5	Glycine (Gly)	2.841	1.31
6	Proline (Pro)	1.460	0.40
7	Serine (Ser)	3.027	2.88
8	Tyrosine (Tyr)	1.910	0.60
9	α Amino-n- butaric acid (a-ABA)	0.070	0.12
10	Cystathionine (Cysthi)	0.121	0.20
11	β Alanine (b-Ala)	0.038	0.11
12	β Amino isobutyric acid (b-AiBA)	0.035	0.00
13	ϒ Amino butyric acid (g- AiBA)	0.020	0.00
14	Ethanol amine (EOHNH2)	0.056	0.00
15	Ornithine (Orn)	0.036	0.02
16	1 Methyl histidine (1 Mehis)	0.043	0.00
17	Hydroxyoroline (Hypro)	0.042	0.00

(Amino acid content was determined in Amino Acid Analyzer, Hitachi, L-8900, Japan)

Table 7.10: Amino acid content of Sample 5 (temp 60°C, solubilization pH 11, precipitation pH 5.0, time 1.25h)

S.No.	Amino acids	Concentration (g/100g)	±SE
Essential			
1	Histidine (His)	1.450	0.70
2	Isoleucine (Ile)	2.633	2.21
3	Leucine (Leu)	4.636	2.19
4	Lysine (Lys)	1.828	0.81
5	Methionine (Met)	0.845	0.12
6	Phenylalanine (Phe)	2.917	0.88
7	Threonine (Thr)	2.102	2.60
8	Tryptophan (Trp)	0.573	0.00
9	Valine (Val)	2.964	2.75
Non-Essential			
1	Alanine (Ala)	2.896	1.33
2	Arginine (Arg)	7.187	2.07
3	Aspartate (Asp)	5.719	0.00
4	Cysteine (Cys)	0.677	1.32
5	Glutamic acid (Glu)	10.92	3.95
6	Glycine (Gly)	2.993	1.21
7	Proline (Pro)	1.468	0.10
8	Serine (Ser)	3.005	0.66
9	Tyrosine (Tyr)	1.993	0.88
10	Phosphoserine (p- Ser)	0.246	0.00
11	Phospho ethanol amine (PEA)	0.009	0.00
12	α Amino-n- butaric acid (a-ABA)	0.076	0.12
13	Cystathionine (Cysthi)	0.677	1.32
14	β Alanine (b-Ala)	0.126	0.00
15	β Amino isobutyric acid (b-AiBA)	0.058	0.09
16	ϒ Amino butyric acid (g- AiBA)	0.014	0.00
17	Ethanol amine (EOHNH2)	0.057	0.00
18	Ornithine (Orn)	0.032	0.04
19	1 Methyl histidine (1 Mehis)	0.055	0.00

(Amino acid content was determined in Amino Acid Analyzer, Hitachi, L-8900, Japan)

7.4 Conclusion

Jatropha seed cake in all forms was found to have inhibitory effects on green gram seed germination and overall seedling health. The plant pigments were also affected and found to have reduced in raw and defatted seed cake treatments. The detoxified seed cake was found to have positive and eliminatory effect on the toxicity leading to improvement in both vigor index and pigmentation. *In vitro* digestibility also study showed the improvement in digestibility and confirmed the inactivation of toxins in the *Jatropha* protein concentrates. Though, this is a prospective step towards the use of *Jatropha* seed cake as fertilizer and fish feed after detoxification, the avenues are open for various applications.

CHAPTER 8

SUMMARY AND FUTURE PERSPECTIVES

Detoxifying *Jatropha* press cake with multifaceted toxic constituents is still a great challenge to the *Jatropha* industry. Several chemical and physical methods have failed to make head way to fully degrade phorbol esters from *Jatropha curcas* seed cake to convert it to animal feed. However, methanol and ethanol extractions have the potential to completely remove (not degrade) the phorbol esters from the kernel cake. But, handling or the disposal of the toxins raises an environmental and health concern. In addition, the use of organic solvent is expensive and could have a residual effect on the animals and human beings consuming the feed. However, among three detoxification techniques, for environmental awareness with safety and energy concerns, the biological method would be more advantageous than the others.

Design of experiments is the most appropriate method for analyzing experimental data and drawing inferences from the results. Eleven major factors were identified in detoxification of *Jatropha* seed cake and analysed for their inter-relationship using interpretive structural modelling (ISM). Temperature was found to be the most crucial factor followed by PE, pH, RPM, esterase, and inoculum level for successful implementation of the detoxification process. MICMAC analysis was done to recognize the factor dependency in detoxification. Out of eleven factors, nine factors were identified as linkage variables; one each as the driver and dependence variable. No autonomous variable was identified.

L18 orthogonal array was adopted for optimizing the SMF process for detoxification of *Jatropha* seed cake by Taguchi method. Analysis of variance (ANOVA) was performed for predicting the optimum process parameters. The percentage contribution of the process parameters with reference to phorbol ester degradation was predicted. Optimum value of the process parameters i.e. seed cake (1%), temperature (35 ˚C), pH (7.5) and time (12 h) helped in achieving the degradation of phorbol esters within the permissible limits in the *Jatropha* press cake.

Proteins were extracted from *Jatropha* seed cake by iso-electric precipitation method. Effect of temperature, solubilization pH and precipitation pH on protein content and protein yield of *Jatropha* seed cake was studied. Recovery of protein concentrate was highest when the extraction was carried out at 55°C temperature, solubilization pH 11.5 and precipitation pH 5. The extraction of protein from detoxified *Jatropha* seed cake was optimized using RSM. The optimum values of the variables i.e. temperature, solubilization pH, time and precipitation pH for the extraction of protein from detoxified seed cake were 60°C, 11.0, 4.41 and 0.78 h respectively which yielded maximum dry matter, protein content and protein yield.

Jatropha seed cake in all forms was found to have inhibitory effects on green gram seed germination and overall seedling health. The plant pigments were also affected and found to have reduced in raw and defatted seed cake treatments. The detoxified seed cake was found to have positive and eliminatory effect on the toxicity leading to improvement in both vigor index and pigmentation. *In vitro* digestibility also study showed the improvement in digestibility and confirmed the inactivation of toxins in the *Jatropha* protein concentrates. Though, this is a prospective step towards the use of *Jatropha* seed cake as fertilizer and fish feed after detoxification, the avenues are open for various applications.

Even though *Jatropha* is known to harbor many anti nutritional factors including phorbol esters, its utility for bio-diesel production is remarkable. The by-product of bio-diesel industry is the defatted seedcake that still carries many inhibitory compounds including phorbol esters. The healthy utilization of this by product can be achieved by detoxifying it biologically. We have demonstrated effective means to bio-remediate phorbol esters from *Jatropha* seed cake retaining high percentage of proteins that can be further enhanced and used for supplementing animal feed. Various models have been proposed that can further be scaled up. Instrumentally, phorbol esters have been found to get eliminated by bio-remediation. Before using the detoxified seed cake as a supplement in feed or agricultural purposes in the form of fertilizer, the overall toxic effects of the bio-remediated product need to be satisfied. We tried to test the comparative effect of raw and detoxified seed cake on few food crops and found that the detoxification through microbial enzymes had enhanced the

growth and development of these crop seeds. Further studies are required to learn the toxicity of detoxified product on other life forms and environment. This can be achieved initially by tests on microbes followed by lower life forms such as fishes, mice etc and later with higher animals.

In the present era, it is known that time is money. Therefore studies can be improvised to further reduce the time for detoxification. The present report describes the work by single bacteria *Pseudomonas aeroginosa* DS1. To maintain bacteria in axenic form is economically not easy. Moreover if more number of synergistic bacteria are employed they can themselves maintain the integrity of the consortium. Models can be designed using certain defined consortia for effective bioremediation of *Jatropha* seed cake in open. The mechanism of degradation can also be worked out to know the intermediary metabolites and their effect on the population of consortium. This will help to engineer certain genes that can further shorten the detoxification process and eliminate any toxic intermediates formed.

Environmentally and economically biotechnology has proven to be useful. Instead of dumping toxic pollutants in the environment, biotechnological approach is always recommended to reduce and transform these pollutants into simpler and non-toxic metabolites. Though this is one of the reports describing the use of microbial enzymes for detoxifying *Jatropha* toxins, the future holds astonishing promises of improving the strategies and making commercial profits apart from keeping the environment clean and green. This is just a small step towards nutritional and commercial use of wastes the avenues are not yet closed.

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SUPPLEMENTARY

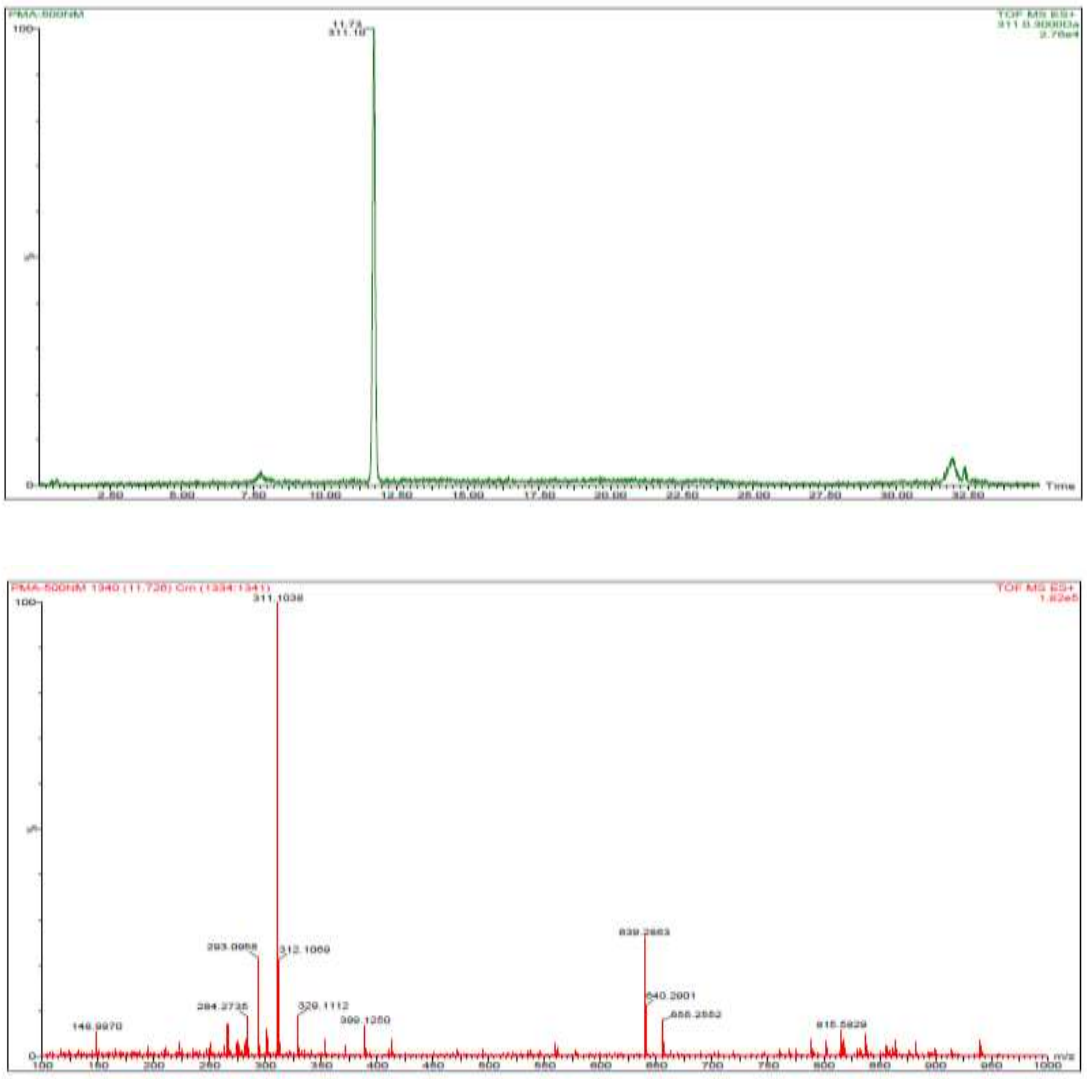


Figure 1: LC-MS Chromatogram and ESI precursor-ion (m/z 311)-scan chromatogram of PMA

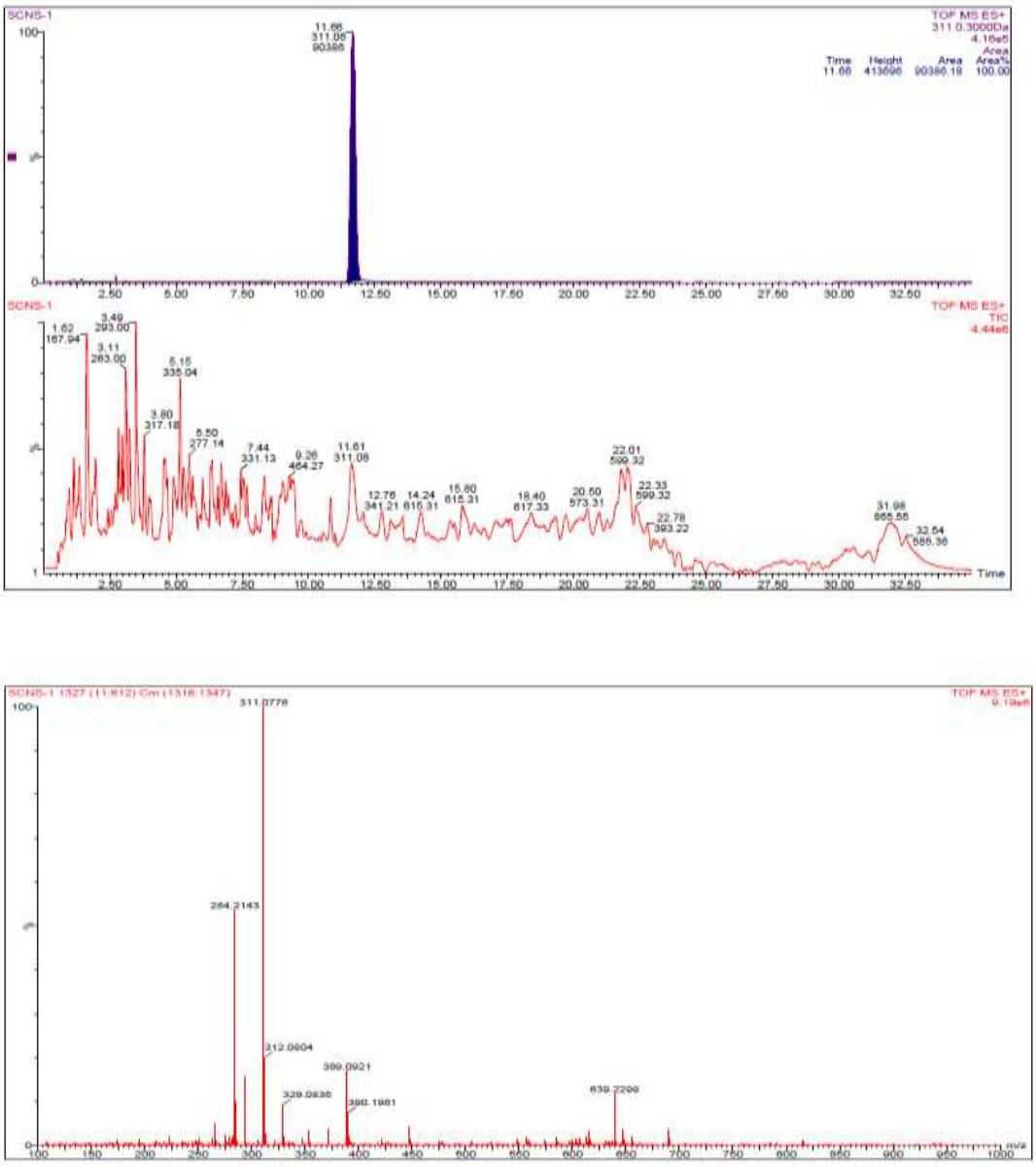


Figure 2: LC-MS Chromatogram and ESI precursor-ion (m/z 311)-scan chromatogram of untreated *Jatropha*seed cake sample

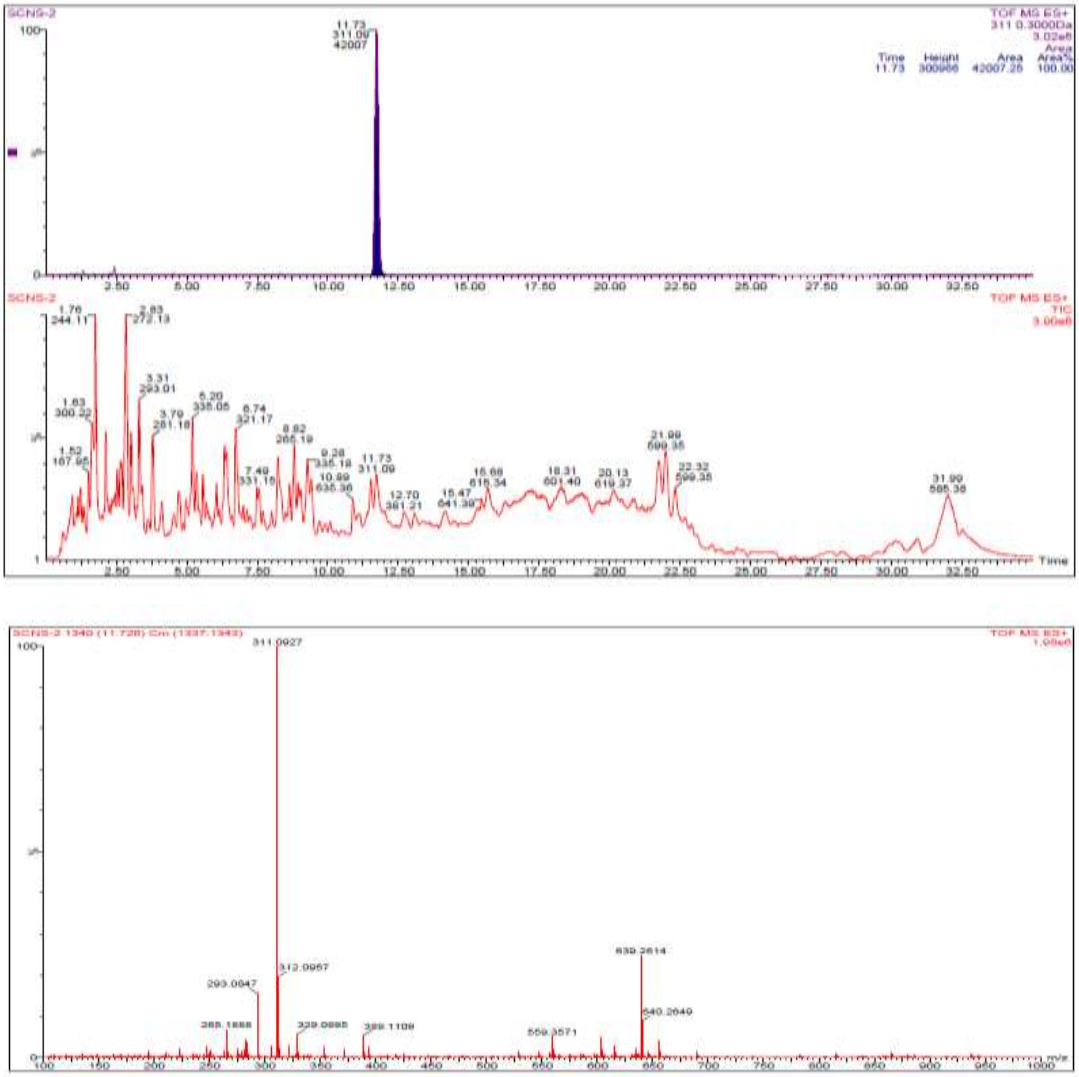


Figure 3: LC-MS Chromatogram and ESI precursor-ion (m/z 311)-scan chromatogram of treated *Jatropha*seed cake sample

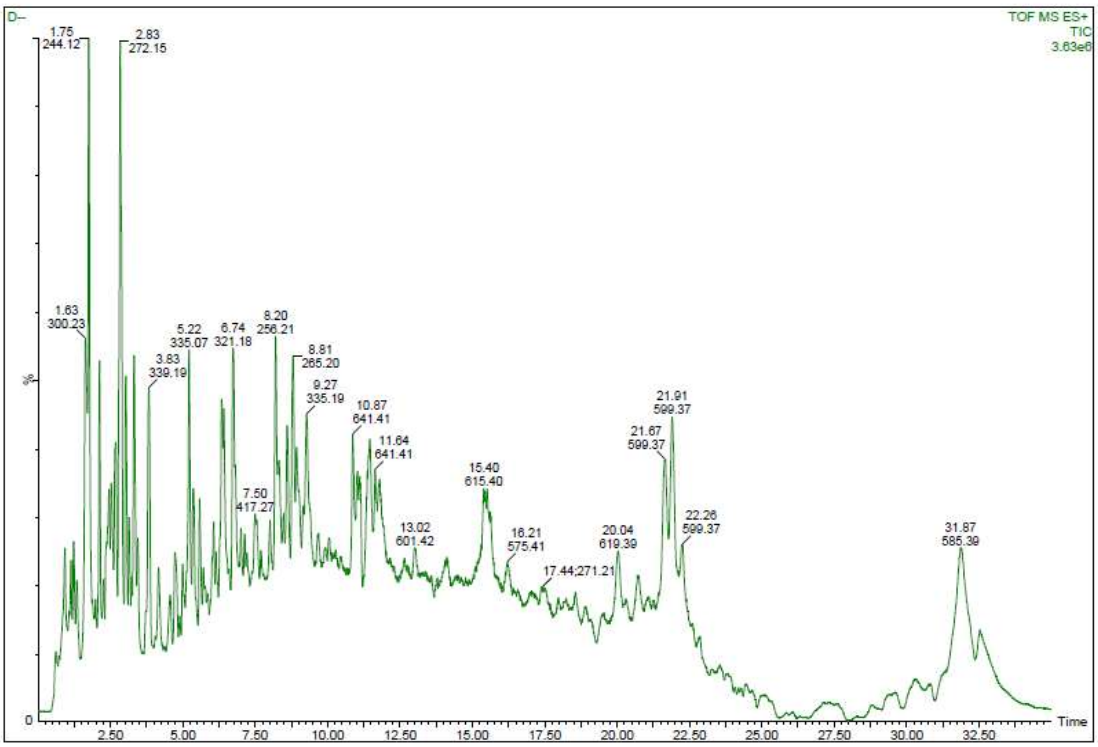
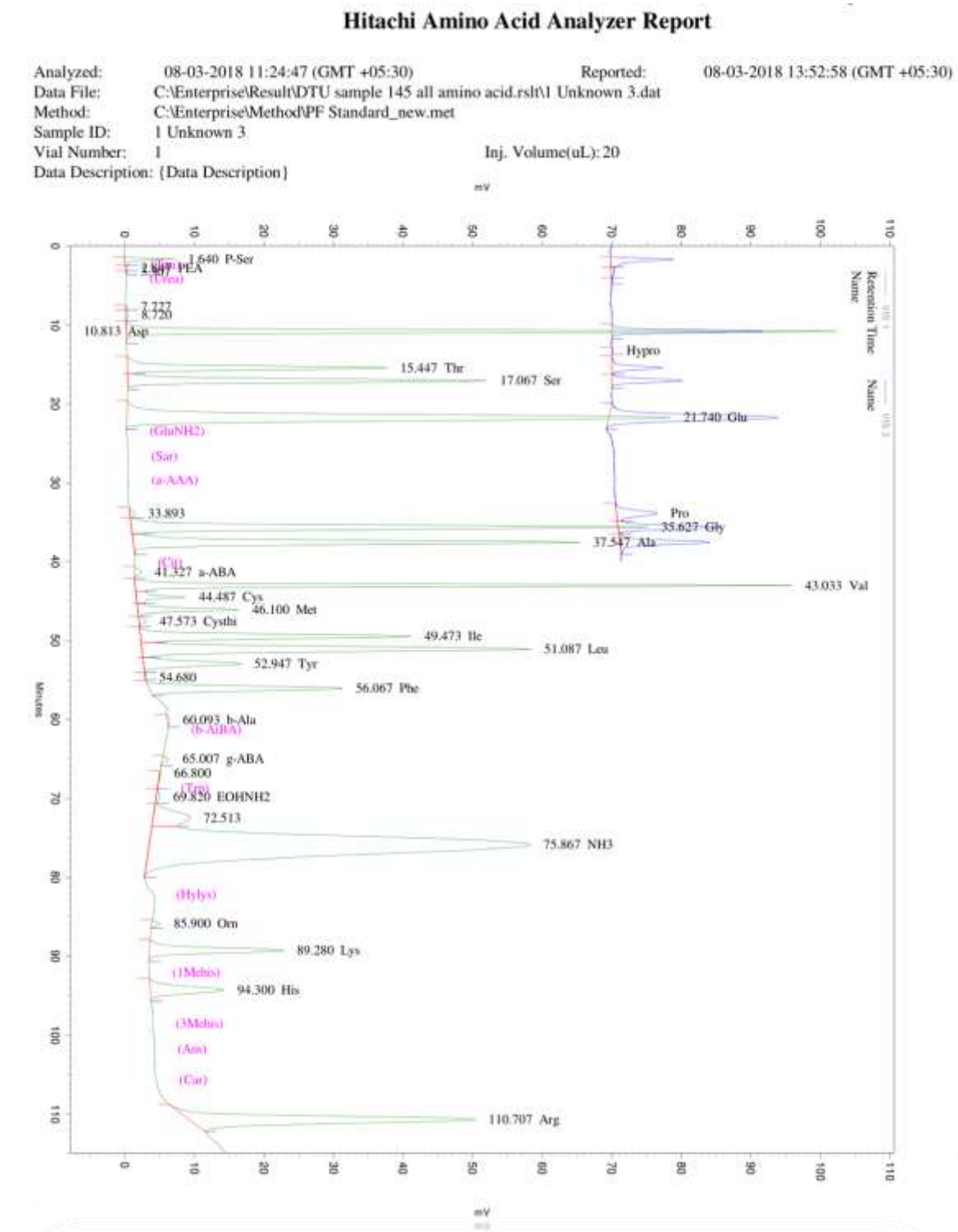


Figure 4: Chromatogram of detoxified *Jatropha*seed cake

Sample 1 (all amino acids)



Hitachi Amino Acid Analyzer Report of Sample 1 (all amino acids)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
1	1.640	P-Ser	28851	506279	0.408	75.527
2	2.847	PEA	1210	40346	0.050	7.082
6	10.813	Asp	408941	11259628	8.897	1184.125
7	15.447	Thr	149353	5232500	3.971	472.954
8	17.067	Ser	206568	7268111	5.477	575.595
9	21.740	Glu	312630	17118798	15.377	2261.957
11	35.627	Gly	297012	11143949	8.856	664.844
12	37.547	Ala	256681	10056317	7.181	639.755
13	41.327	a-ABA	4050	170312	0.120	12.417
14	43.033	Val	377683	8717344	6.077	711.592
15	44.487	Cys	28379	952670	0.653	156.834
16	46.100	Met	58160	1911256	1.393	207.807
17	47.573	Cysthi	4192	177574	0.110	24.515
18	49.473	Ile	155656	6397444	4.509	591.583
19	51.087	Leu	223698	10137515	7.605	997.714
20	52.947	Tyr	56107	2560293	2.105	381.491
22	56.067	Phe	111029	4630816	3.769	622.618
23	60.093	b-Ala	1257	71468	0.085	7.551
24	65.007	g-ABA	3912	132430	0.092	9.456
26	69.820	EOHNH2	2064	143567	0.133	8.113
28	75.867	NH3	219044	34638311	13.419	228.527
29	85.900	Orn	4820	140479	0.084	11.040
30	89.280	Lys	77113	3595388	2.481	362.686
31	94.300	His	42578	2625163	1.953	303.098
32	110.707	Arg	164546	10243649	8.519	1483.932
Totals			3195534	149871607	103.322	
VIS 2 Results						
5	13.240	Hypro	823	24812	0.082	
9	33.860	Pro	23429	1241445	2.985	343.585
Totals			24252	1266257	3.067	

Sample 1 (Trytophan)

Hitachi Amino Acid Analyzer Report

Analyzed: 09-03-2018 12:39:14 (GMT +05:30) Reported: 09-03-2018 15:07:26 (GMT +05:30)
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Vial Number: 1 Inj. Volume(uL): 20
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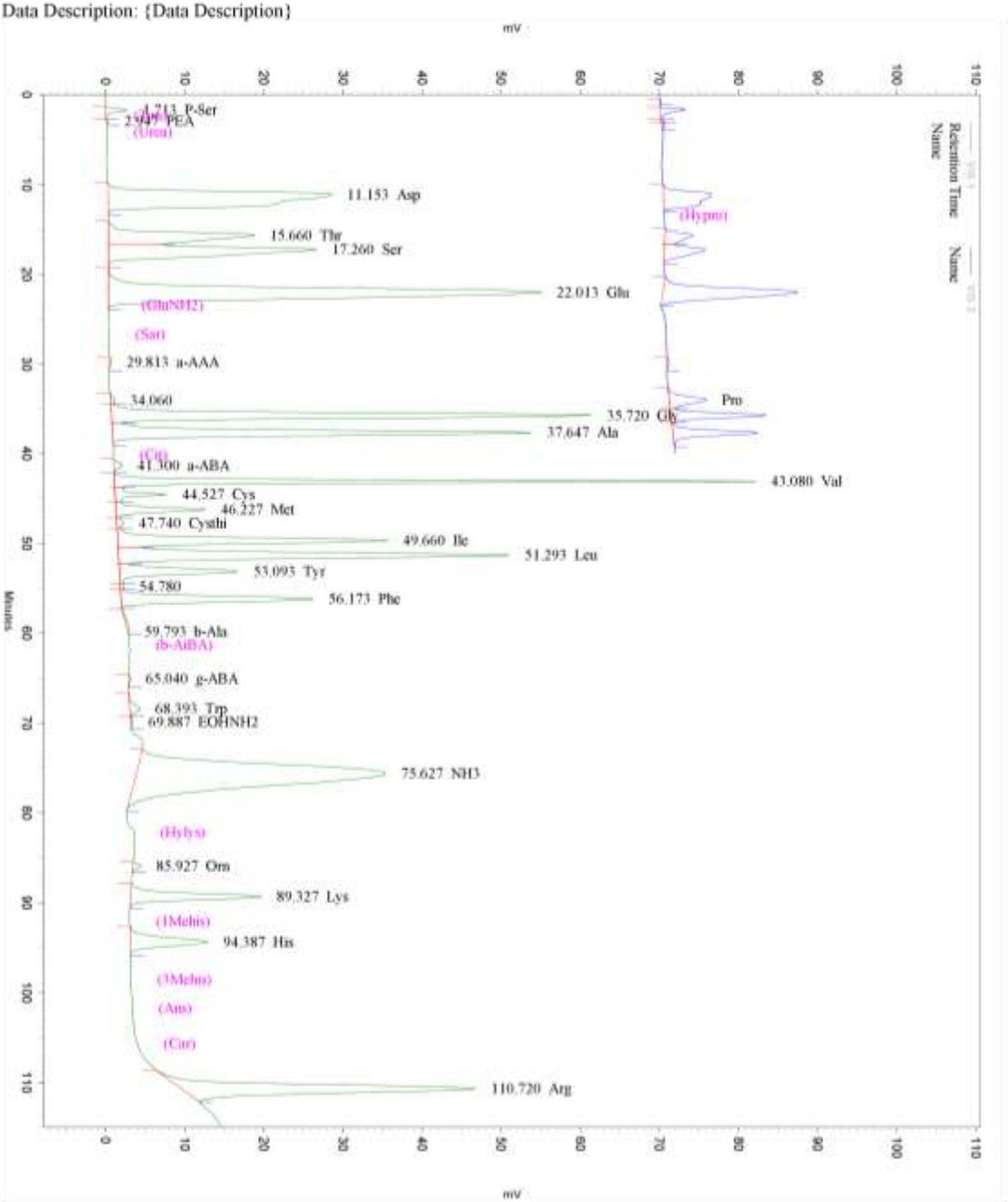

Hitachi Amino Acid Analyzer Report of Sample 1 (Tryptophan)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
1	1.713	P-Ser	10793	275334	0.222	41.074
2	2.947	PEA	1449	21506	0.027	3.775
3	11.153	Asp	113181	11054297	8.734	1162.531
4	15.660	Thr	73947	4729314	3.589	427.472
5	17.260	Ser	105388	6617865	4.987	524.099
6	22.013	Glu	219046	16235896	14.584	2145.296
7	29.813	a-AAA	841	38550	0.039	6.328
9	35.720	Gly	242812	9739013	7.740	581.026
10	37.647	Ala	212009	8790423	6.277	559.222
11	41.300	a-ABA	4347	185244	0.131	13.505
12	43.080	Val	324441	7581831	5.285	618.901
13	44.527	Cys	26209	785957	0.538	129.389
14	46.227	Met	45360	1673422	1.219	181.948
15	47.740	Cysthi	3477	177186	0.110	24.461
16	49.660	Ile	136606	5987472	4.220	553.672
17	51.293	Leu	197795	9112684	6.836	896.852
18	53.093	Tyr	59967	2798365	2.301	416.965
20	56.173	Phe	97275	4296517	3.497	577.671
21	59.793	b-Ala	835	128246	0.152	13.550
22	65.040	g-ABA	1111	44930	0.031	3.208
23	68.393	Trp	5101	295023	0.390	79.652
24	69.887	EOHNH2	1115	52087	0.048	2.943
25	75.627	NH3	126243	20302240	7.865	133.944
26	85.927	Orn	4118	124246	0.074	9.764
27	89.327	Lys	66168	3146082	2.171	317.362
28	94.387	His	39202	2456776	1.828	283.657
29	110.720	Arg	148771	9288475	7.724	1345.562
Totals			2267607	125938981	90.620	
VIS 2 Results						
		Hypro			0.000 BDL	0.000
10	33.993	Pro	18739	1059931	2.549	293.348
Totals			18739	1059931	2.549	

Sample 2 (all amino acids)

Hitachi Amino Acid Analyzer Report

Analyzed: 10-03-2018 13:58:21 (GMT +05:30) Reported: 10-03-2018 16:26:36 (GMT +05:30)
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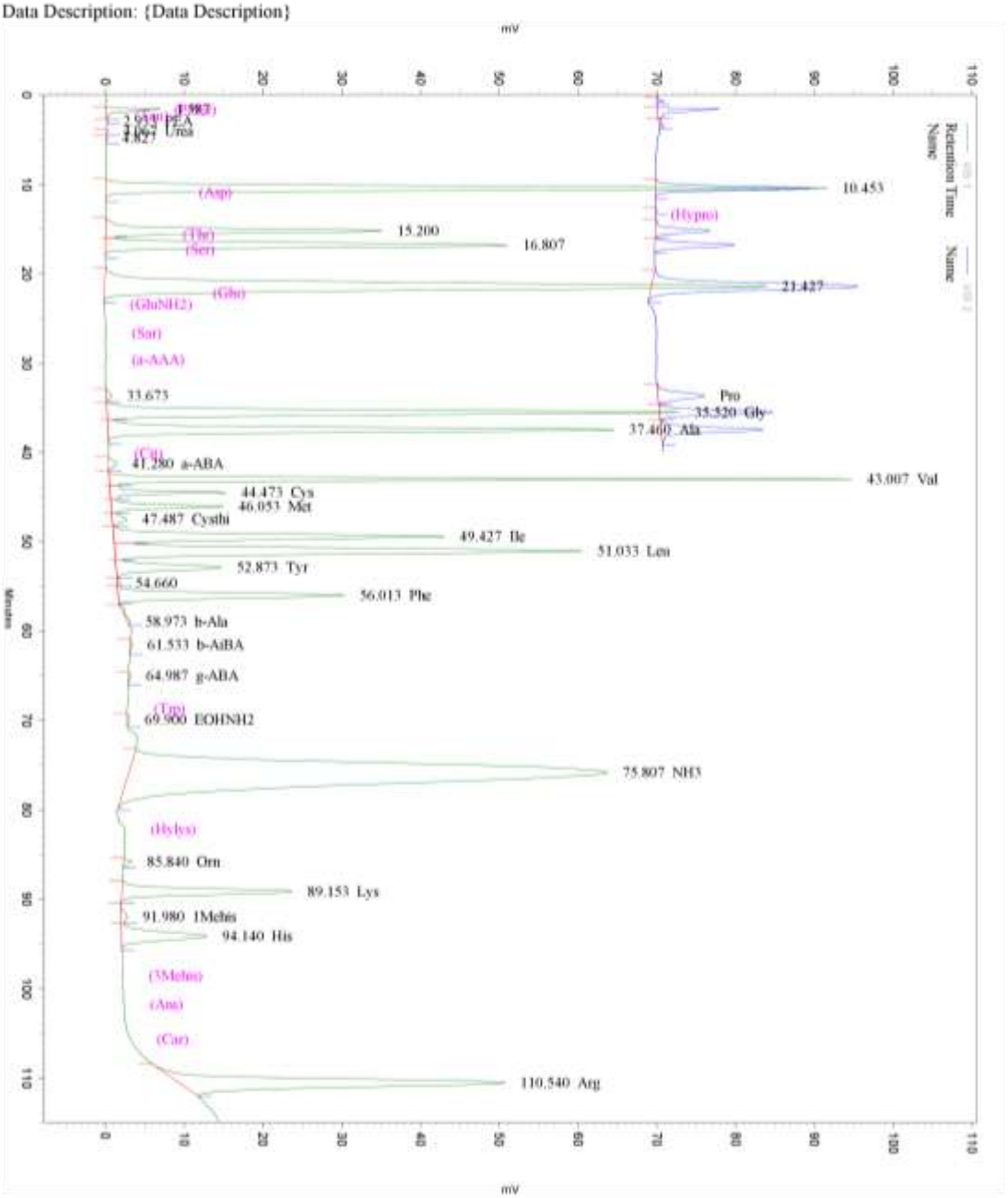

Hitachi Amino Acid Analyzer Report of Sample 2 (all amino acids)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
2	2.933	PEA	454	7952	0.010	1.396
3	4.067	Urea	485	9665	0.331	19.870
10	35.520	Gly	290704	10906383	8.668	650.670
11	37.460	Ala	257058	9945658	7.102	632.715
12	41.280	a-ABA	4097	176046	0.124	12.835
13	43.007	Val	377153	8506332	5.930	694.367
14	44.473	Cys	58773	1433809	0.982	236.041
15	46.053	Met	56785	1830424	1.334	199.019
16	47.487	Cysthi	7324	371329	0.231	51.264
17	49.427	Ile	168075	6924593	4.881	640.330
18	51.033	Leu	237231	10647740	7.987	1047.929
19	52.873	Tyr	53476	2463495	2.026	367.068
21	56.013	Phe	114815	4819598	3.923	648.000
22	58.973	b-Ala	1271	151834	0.180	16.043
23	61.533	b-AiBA	1219	72230	0.097	9.957
24	64.987	g-ABA	1161	52380	0.036	3.740
25	69.900	EOHNH2	1194	57648	0.053	3.258
26	75.807	NH3	243024	37351431	14.470	246.426
27	85.840	Orn	4340	129137	0.077	10.149
28	89.153	Lys	86508	3935880	2.716	397.034
29	91.980	1Mehis	3208	208285	0.139	23.541
30	94.140	His	43355	2732257	2.033	315.463
31	110.540	Arg	165590	10356000	8.612	1500.208
Totals			2177300	113090106	71.940	
VIS 2 Results						
		Hypro			0.000 BDL	0.000
9	33.680	Pro	23626	1225099	2.946	339.061
Totals			23626	1225099	2.946	

Sample 2 (Trytophan)

Hitachi Amino Acid Analyzer Report

Analyzed: 11-03-2018 11:19:08 (GMT +05:30) Reported: 11-03-2018 13:47:29 (GMT +05:30)
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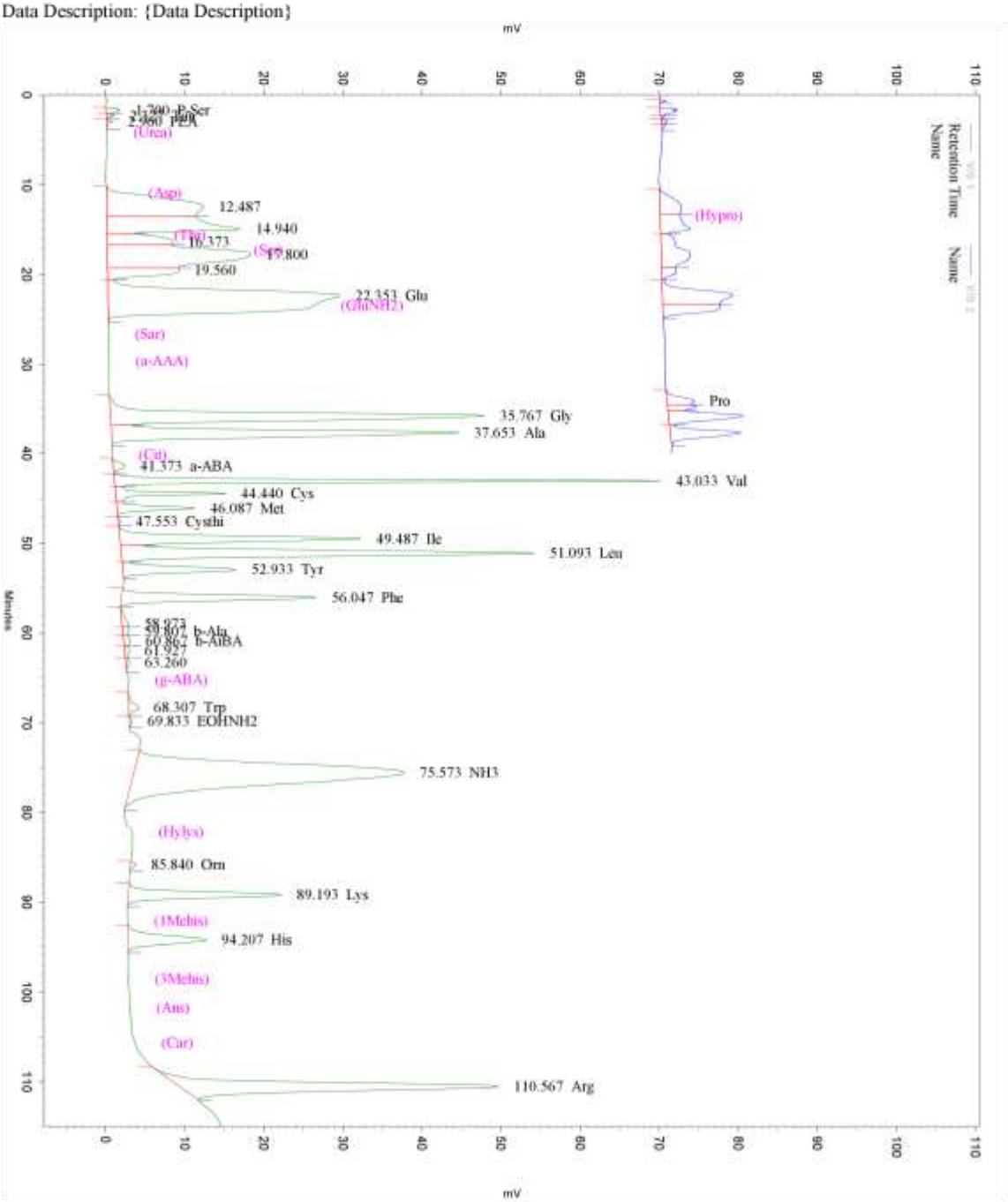

Hitachi Amino Acid Analyzer Report of Sample 2 (tryptophan)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
1	1.700	P-Ser	7074	194245	0.157	28.978
2	2.327	Tau	4022	69796	0.067	8.401
3	2.960	PEA	3073	59175	0.074	10.387
9	22.353	Glu	117464	17438581	15.664	2304.210
10	35.767	Gly	189456	10524321	8.364	627.877
11	37.653	Ala	175774	9091967	6.492	578.406
12	41.373	a-ABA	6052	304293	0.215	22.185
13	43.033	Val	276384	6597765	4.599	538.572
14	44.440	Cys	55754	1398460	0.958	230.222
15	46.087	Met	39485	1338012	0.975	145.480
16	47.553	Cysthi	1196	38018	0.024	5.249
17	49.487	Ile	122064	5289079	3.728	489.091
18	51.093	Leu	209083	9435971	7.078	928.669
19	52.933	Tyr	57200	2600204	2.138	387.438
20	56.047	Phe	98732	4267217	3.473	573.732
22	59.807	b-Ala	3235	146084	0.173	15.435
23	60.867	b-AiBA	3456	211981	0.283	29.223
26	68.307	Trp	5104	303905	0.402	82.050
27	69.833	EOHNH2	1516	66867	0.062	3.779
28	75.573	NH3	136800	21191084	8.210	139.808
29	85.840	Orn	2837	83795	0.050	6.585
30	89.193	Lys	77442	3540619	2.443	357.162
31	94.207	His	39305	2511979	1.869	290.030
32	110.567	Arg	161128	10091793	8.392	1461.934
Totals			1793636	106795211	75.890	
VIS 2 Results						
		Hypro			0.000 BDL	0.000
12	34.140	Pro	13862	726319	1.746	201.017
Totals			13862	726319	1.746	

Sample 3 (all amino acids)

Hitachi Amino Acid Analyzer Report

Analyzed: 10-03-2018 16:28:30 (GMT +05:30) Reported: 10-03-2018 18:56:45 (GMT +05:30)
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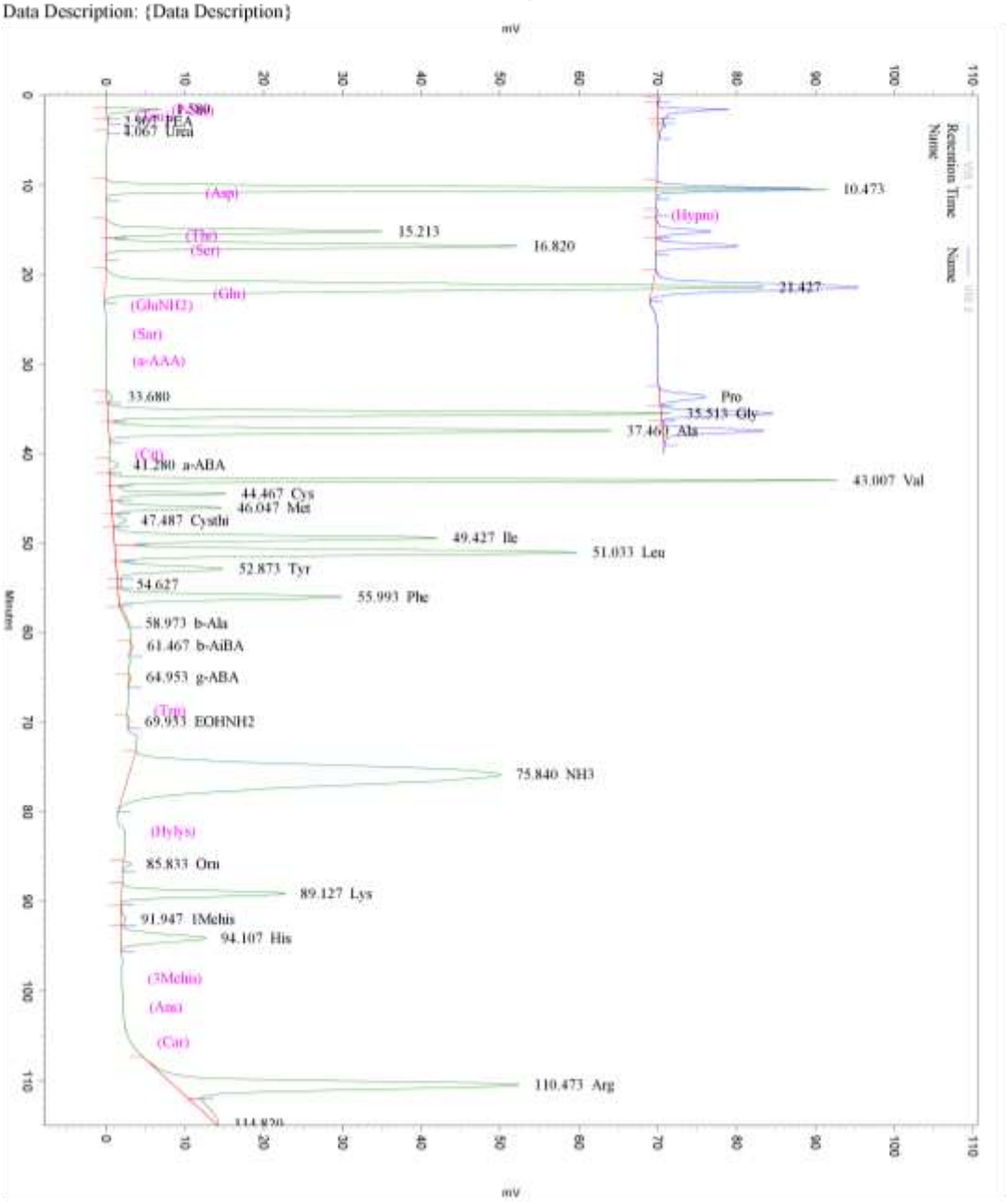

Hitachi Amino Acid Analyzer Report of Sample 3 (all amino acids)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
2	2.907	PEA	510	10136	0.013	1.779
3	4.067	Urea	380	4962	0.170	10.201
9	35.513	Gly	285897	10635460	8.452	634.507
10	37.460	Ala	255145	9774952	6.980	621.855
11	41.280	a-ABA	4510	195791	0.138	14.274
12	43.007	Val	369433	8262753	5.760	674.484
13	44.467	Cys	58498	1460787	1.001	240.483
14	46.047	Met	55867	1795017	1.308	195.169
15	47.487	Cysthi	6605	350453	0.218	48.382
16	49.427	Ile	164243	6775410	4.775	626.535
17	51.033	Leu	234521	10506459	7.881	1034.025
18	52.873	Tyr	54328	2530818	2.081	377.099
20	55.993	Phe	113500	4760344	3.874	640.033
21	58.973	b-Ala	1265	135865	0.161	14.355
22	61.467	b-AiBA	1071	66322	0.089	9.143
23	64.953	g-ABA	1171	53317	0.037	3.807
24	69.933	EOHNH2	1102	51496	0.048	2.910
25	75.840	NH3	188673	29088829	11.269	191.914
26	85.833	Orn	4064	120888	0.072	9.500
27	89.127	Lys	83031	3731286	2.575	376.395
28	91.947	1Mehis	2399	151197	0.101	17.089
29	94.107	His	42992	2717053	2.021	313.708
30	110.473	Arg	175228	11465119	9.534	1660.879
Totals			2104433	104644714	68.558	
VIS 2 Results						
		Hypro			0.000 BDL	0.000
10	33.680	Pro	23570	1206256	2.900	333.846
Totals			23570	1206256	2.900	

Sample 3 (Trytophan)

Hitachi Amino Acid Analyzer Report

Analyzed: 11-03-2018 13:49:30 (GMT +05:30) Reported: 11-03-2018 16:17:45 (GMT +05:30)
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Sample ID: 2 Unknown 3
Vial Number: 2 Inj. Volume(uL): 20
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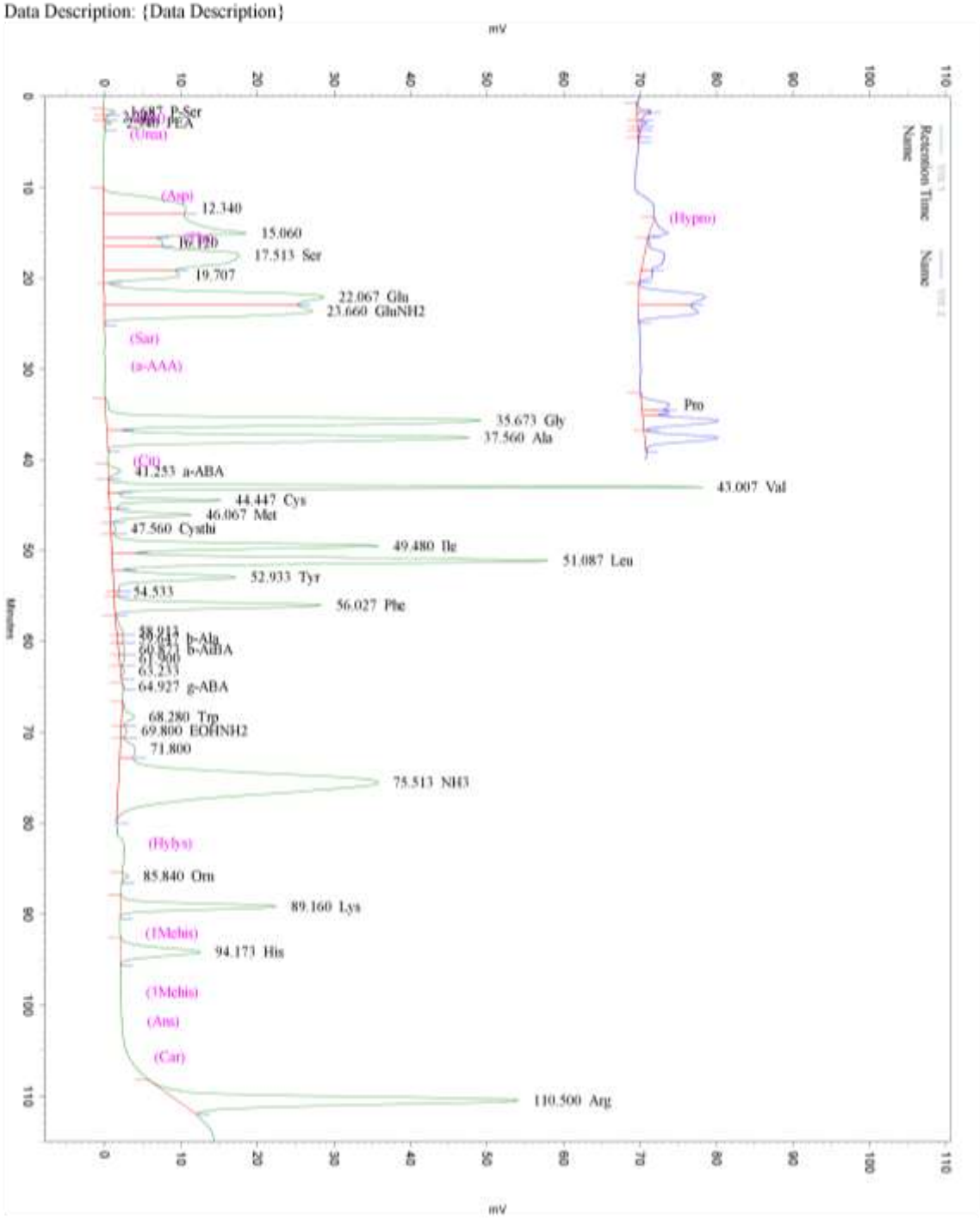


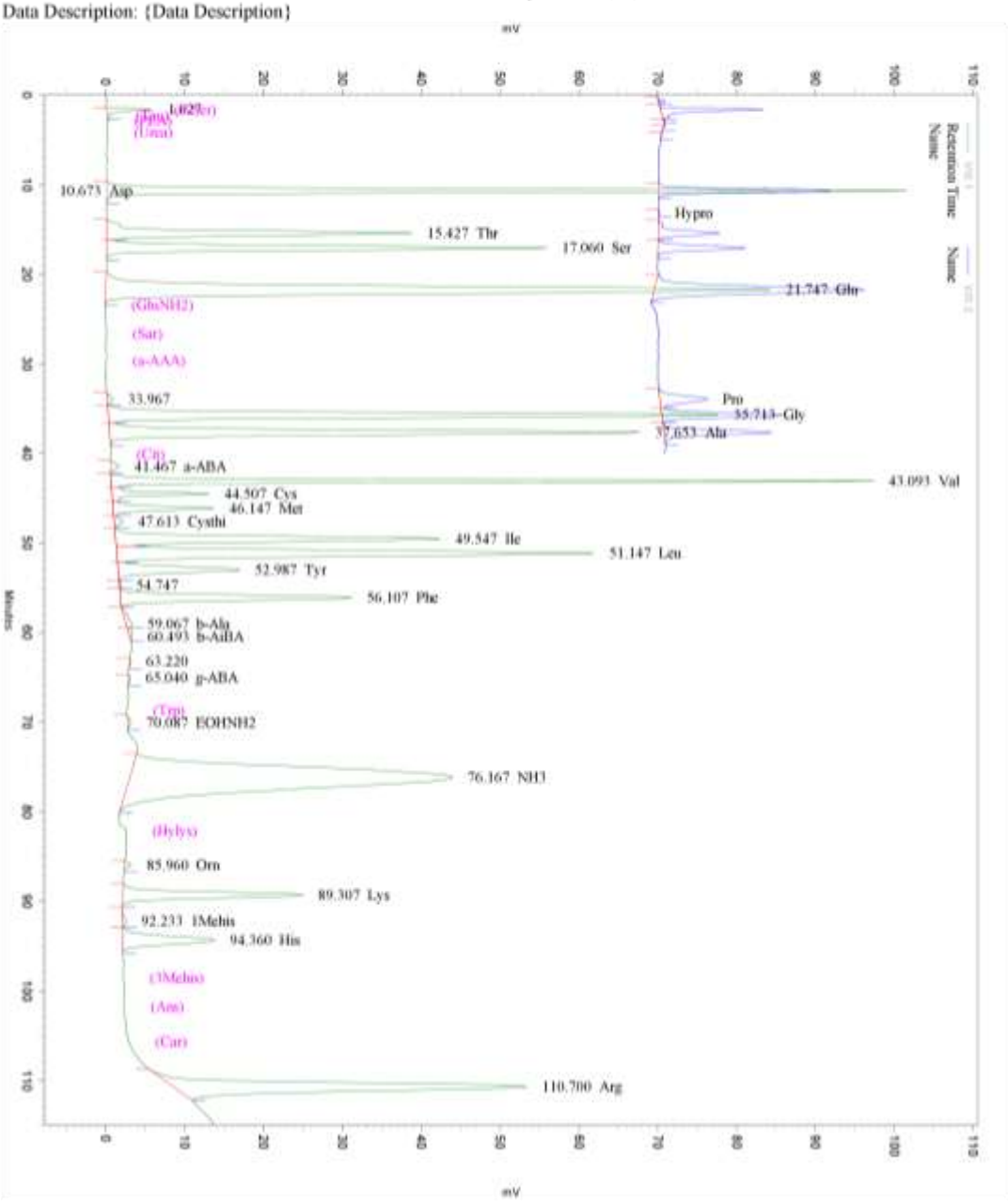
Hitachi Amino Acid Analyzer Report of Sample 3 (Tryptophan)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
1	1.687	P-Ser	5668	146725	0.118	21.889
3	2.940	PEA	3680	63443	0.079	11.136
7	17.513	Ser	70754	9114712	6.868	721.836
9	22.067	Glu	114900	9802499	8.805	1295.233
10	23.660	GluNH2	108595	8655974	6.598	963.977
11	35.673	Gly	196034	10929422	8.686	652.045
12	37.560	Ala	189191	9740695	6.956	619.676
13	41.253	a-ABA	6320	310474	0.220	22.635
14	43.007	Val	310434	7349510	5.123	599.936
15	44.447	Cys	58182	1532575	1.050	252.301
16	46.067	Met	42333	1522472	1.109	165.536
17	47.560	Cysthi	2878	150364	0.093	20.759
18	49.480	Ile	139682	6101844	4.301	564.249
19	51.087	Leu	227932	10412237	7.811	1024.752
20	52.933	Tyr	64223	3166322	2.604	471.791
22	56.027	Phe	108257	4719532	3.841	634.546
24	59.647	b-Ala	3344	161223	0.191	17.035
25	60.873	b-AiBA	3178	211287	0.283	29.128
28	64.927	g-ABA	783	19587	0.014	1.399
29	68.280	Trp	6568	448087	0.592	120.977
30	69.800	EOHNH2	2973	193307	0.179	10.924
32	75.513	NH3	135902	21798040	8.445	143.813
33	85.840	Orn	3314	101091	0.060	7.945
34	89.160	Lys	81392	3708562	2.559	374.103
35	94.173	His	41840	2683101	1.996	309.788
36	110.500	Arg	178358	11223727	9.334	1625.910
Totals			2106715	124266812		87.913
VIS 2 Results						
		Hypro			0.000 BDL	0.000
12	33.947	Pro	14619	904641	2.175	250.370
Totals			14619	904641	2.175	

Sample 4 (all amino acids)

Hitachi Amino Acid Analyzer Report

Analyzed: 08-03-2018 16:24:55 (GMT +05:30) Reported: 08-03-2018 18:53:08 (GMT +05:30)
Data File: C:\Enterprise\Result\DTU sample 145 all amino acid.rslt\3 Unknown 3.dat
Method: C:\Enterprise\Method\PF Standard_new.met
Sample ID: 3 Unknown 3
Vial Number: 3 Inj. Volume(uL): 20
Data Description: {Data Description}



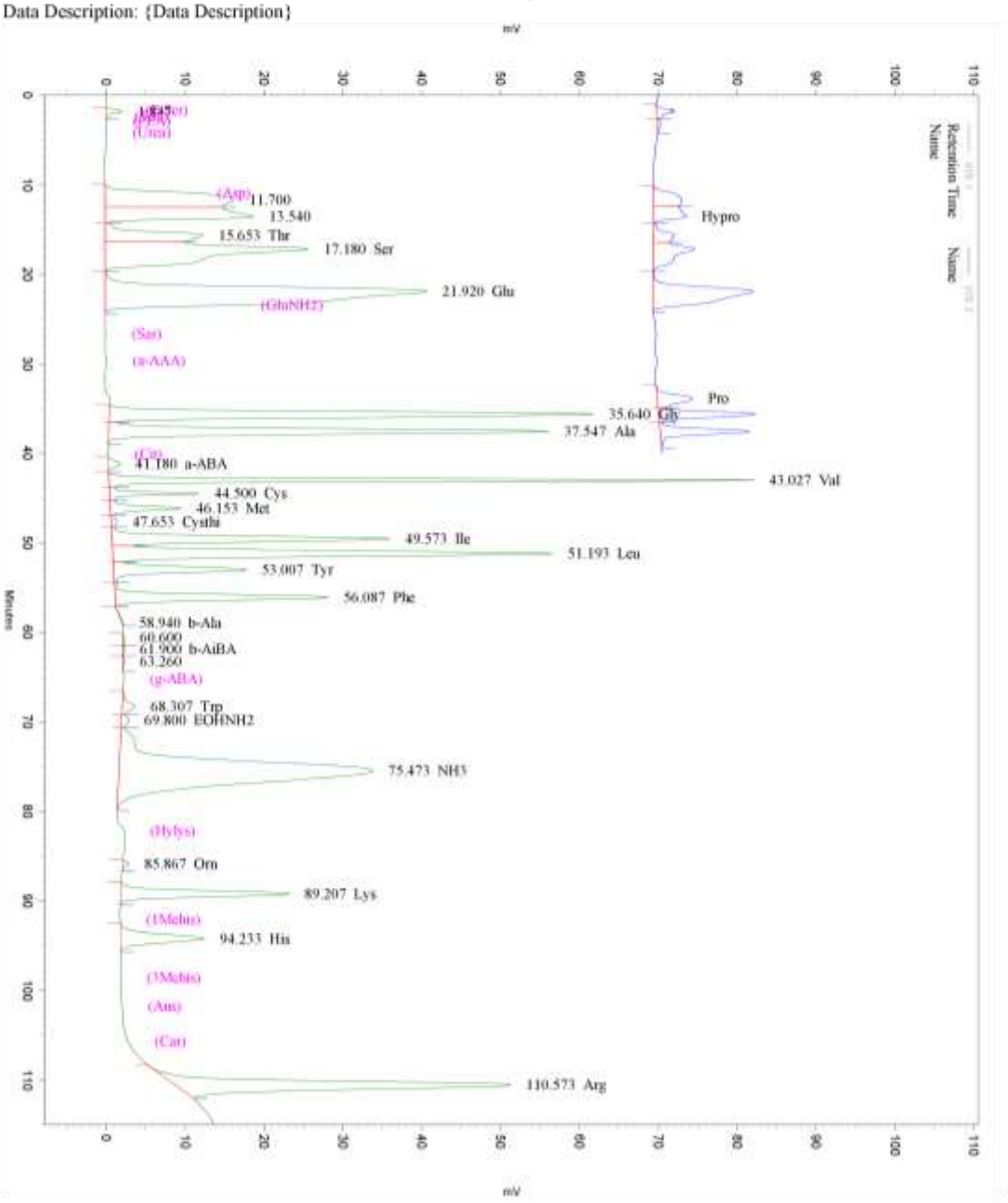
Hitachi Amino Acid Analyzer Report of Sample 4 (all amino acids)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
2	10.673	Asp	405749	11952348	9.444	1256.975
3	15.427	Thr	154729	5747473	4.362	519.501
4	17.060	Ser	223183	8163468	6.151	646.503
5	21.747	Glu	337328	18820906	16.906	2486.861
7	35.713	Gly	309461	11505961	9.144	686.441
8	37.653	Ala	268993	10467910	7.475	665.940
9	41.467	a-ABA	4437	191855	0.136	13.987
10	43.093	Val	386819	8722205	6.080	711.989
11	44.507	Cys	49355	1214145	0.832	199.879
12	46.147	Met	51162	1683681	1.227	183.064
13	47.613	Cysthi	4202	208033	0.129	28.720
14	49.547	Ile	164581	6782621	4.780	627.201
15	51.147	Leu	241914	10843627	8.134	1067.208
16	52.987	Tyr	61704	2810378	2.311	418.755
18	56.107	Phe	117262	4914162	3.999	660.714
19	59.067	b-Ala	2793	262699	0.312	27.757
20	60.493	b-AiBA	1188	108162	0.145	14.911
22	65.040	g-ABA	924	45733	0.032	3.266
23	70.087	EOHNH2	1892	97121	0.090	5.488
24	76.167	NH3	163674	26176498	10.141	172.700
25	85.960	Orn	3364	100034	0.059	7.861
26	89.307	Lys	91261	4160913	2.871	419.734
27	92.233	1Mehis	1723	107510	0.072	12.151
28	94.360	His	46679	2896192	2.155	334.391
29	110.700	Arg	179080	11218347	9.329	1625.131
Totals			3273457	149201982	106.315	
VIS 2 results						
7	13.187	Hypro	729	19304	0.064	8.328
11	33.967	Pro	24172	1271956	3.058	352.029
Totals			24901	1291260	3.122	

Sample 4 (Trytophan)

Hitachi Amino Acid Analyzer Report

Analyzed: 09-03-2018 15:09:15 (GMT +05:30) Reported: 09-03-2018 17:37:29 (GMT +05:30)
Data File: C:\Enterprise\Result\DTU sample 145 Tryptophan.rslt\2 Unknown 3.dat
Method: C:\Enterprise\Method\PF Standard_new.met
Sample ID: 2 Unknown 3
Vial Number: 2 Inj. Volume(uL): 20
Data Description: {Data Description}



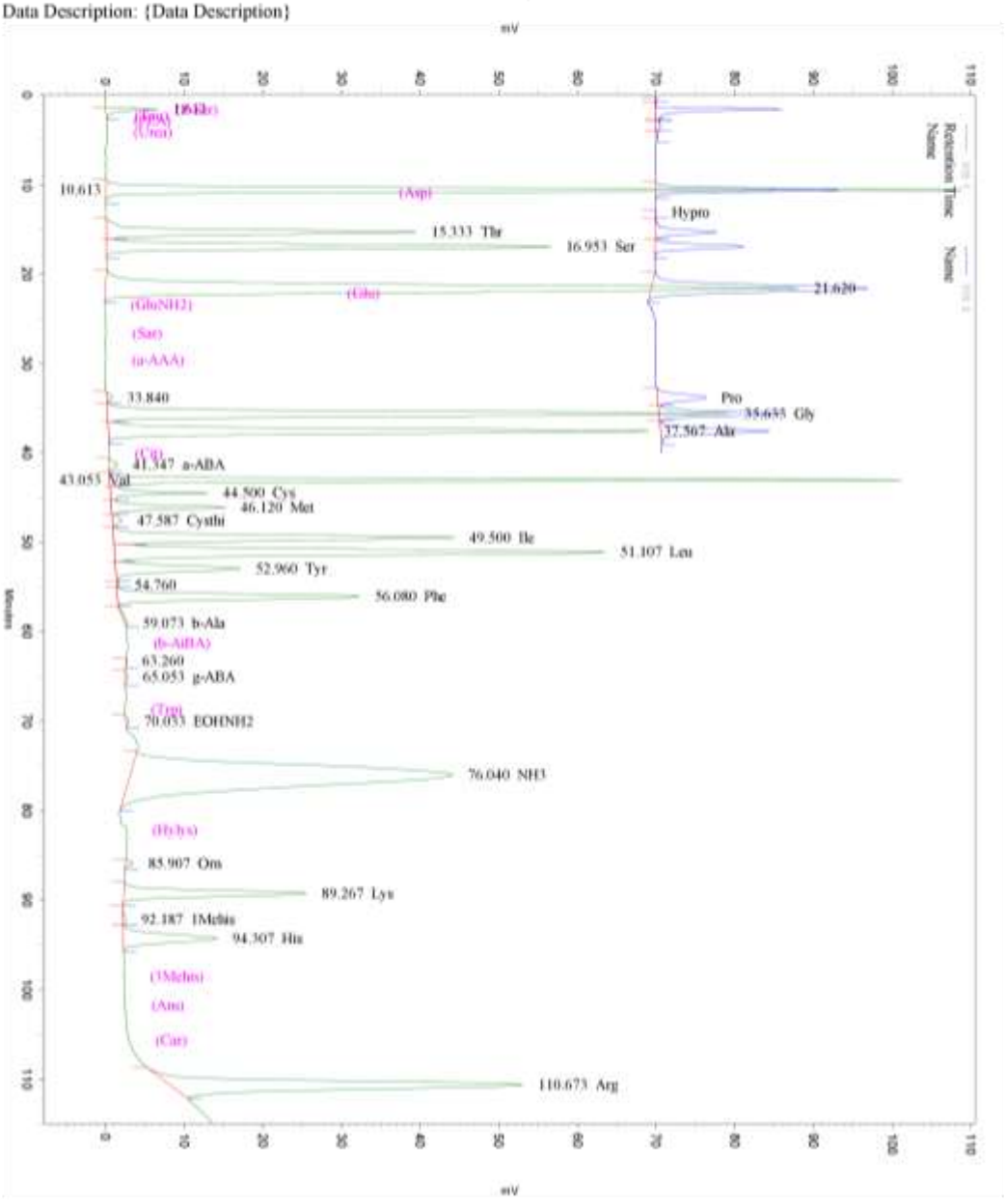
Hitachi Amino Acid Analyzer Report of Sample 4 (Tryptophan)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
4	15.653	Thr	49740	3462045	2.627	312.926
5	17.180	Ser	103262	9925092	7.479	786.014
6	21.920	Glu	163305	17861569	16.044	2360.101
7	35.640	Gly	245701	10773139	8.562	642.721
8	37.547	Ala	223319	9654340	6.894	614.182
9	41.180	a-ABA	5957	271197	0.192	19.772
10	43.027	Val	327304	7624904	5.315	622.417
11	44.500	Cys	45366	1055082	0.723	173.693
12	46.153	Met	35862	1333926	0.972	145.035
13	47.653	Cysthi	3191	172769	0.107	23.852
14	49.573	Ile	141074	6051241	4.265	559.569
15	51.193	Leu	223141	10125820	7.596	996.563
16	53.007	Tyr	67433	3138033	2.580	467.576
17	56.087	Phe	108258	4770154	3.882	641.352
18	58.940	b-Ala	1059	60685	0.072	6.412
20	61.900	b-AiBA	1215	60120	0.080	8.288
22	68.307	Trp	6625	447890	0.592	120.924
23	69.800	EOHNH2	3836	249226	0.231	14.084
24	75.473	NH3	128786	21588623	8.364	142.431
25	85.867	Orn	3313	100268	0.060	7.880
26	89.207	Lys	85397	3895812	2.688	392.992
27	94.233	His	42495	2699046	2.008	311.629
28	110.573	Arg	170049	10627125	8.837	1539.484
Totals			2185688	125948106	90.170	
VIS 2 results						
4	13.527	Hypro	16937	1306779	4.300	563.783
8	33.880	Pro	18220	1216789	2.926	336.761
Totals			35157	2523568	7.226	

Sample 5 (all amino acids)

Hitachi Amino Acid Analyzer Report

Analyzed: 08-03-2018 13:54:50 (GMT +05:30) Reported: 08-03-2018 16:23:02 (GMT +05:30)
Data File: C:\Enterprise\Result\DTU sample 145 all amino acid.rslt\2 Unknown 3.dat
Method: C:\Enterprise\Method\PF Standard_new.met
Sample ID: 2 Unknown 3
Vial Number: 2 Inj. Volume(uL): 20
Data Description: {Data Description}



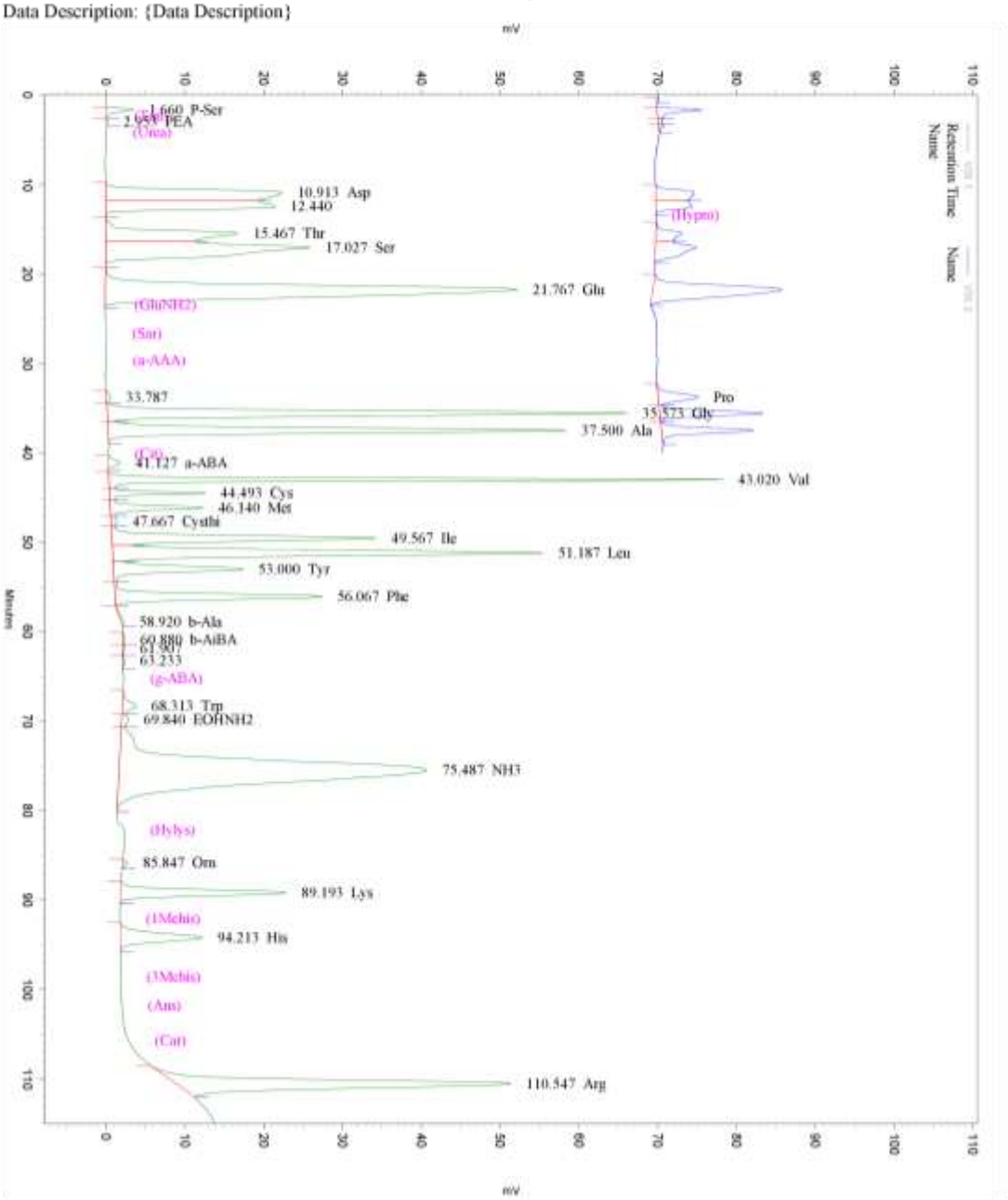
Hitachi Amino Acid Analyzer Report of Sample 5 (all amino acids)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
3	15.333	Thr	157597	5715415	4.338	516.603
4	16.953	Ser	226077	8204520	6.182	649.754
7	35.633	Gly	316239	11815062	9.390	704.882
8	37.567	Ala	274807	10661369	7.613	678.247
9	41.347	a-ABA	4404	189975	0.134	13.850
10	43.053	Val	402429	9046946	6.307	738.497
11	44.500	Cys	49545	1224675	0.839	201.613
12	46.120	Met	57947	1877126	1.368	204.097
13	47.587	Cysthi	4827	244862	0.152	33.804
14	49.500	Ile	173218	7177867	5.059	663.750
15	51.107	Leu	249549	11264706	8.450	1108.650
16	52.960	Tyr	63413	2944857	2.422	438.792
18	56.080	Phe	123048	5215427	4.245	701.220
19	59.073	b-Ala	1264	111074	0.132	11.736
21	65.053	g-ABA	1350	67506	0.047	4.820
22	70.033	EOHNH2	1683	82106	0.076	4.640
23	76.040	NH3	163861	25745146	9.974	169.854
24	85.907	Orn	3920	116905	0.069	9.187
25	89.267	Lys	92968	4250120	2.933	428.733
26	92.187	1Mehis	1394	90419	0.060	10.219
27	94.307	His	47745	2985131	2.221	344.660
28	110.673	Arg	179328	11187961	9.304	1620.729
Totals			2596613	120219175	81.313	
VIS 2 results						
7	13.127	Hypro	783	22801	0.075	9.837
11	33.840	Pro	24833	1286464	3.093	356.044
Totals			25616	1309265	3.168	

Sample 5 (Trytophan)

Hitachi Amino Acid Analyzer Report

Analyzed: 09-03-2018 17:39:19 (GMT +05:30) Reported: 09-03-2018 20:07:37 (GMT +05:30)
Data File: C:\Enterprise\Result\DTU sample 145 Tryptophan.rslt\3 Unknown 3.dat
Method: C:\Enterprise\Method\PF Standard_new.met
Sample ID: 3 Unknown 3
Vial Number: 3 Inj. Volume(uL): 20
Data Description: {Data Description}



Hitachi Amino Acid Analyzer Report of Sample 5 (Tryptophan)

Pk #	RT	Name	Height	Area	ESTD Conc/nmol	Conc/ng
1	1.660	P-Ser	14034	362501	0.292	54.078
2	2.953	PEA	717	11905	0.015	2.090
3	10.913	Asp	89511	6338231	5.008	666.564
5	15.467	Thr	67222	4486442	3.405	405.519
6	17.027	Ser	103892	8526958	6.425	675.289
7	21.767	Glu	209331	17507615	15.726	2313.332
9	35.573	Gly	263995	10579520	8.408	631.170
10	37.500	Ala	232631	9546904	6.817	607.348
11	41.127	a-ABA	5980	267941	0.189	19.534
12	43.020	Val	311845	7241649	5.048	591.132
13	44.493	Cys	48673	1080774	0.740	177.923
14	46.140	Met	47069	1734883	1.264	188.631
15	47.667	Cysthi	3057	165595	0.103	22.861
16	49.567	Ile	134104	5734510	4.042	530.281
17	51.187	Leu	218333	9864871	7.400	970.881
18	53.000	Tyr	65821	3071125	2.525	457.607
19	56.067	Phe	105128	4624818	3.764	621.812
20	58.920	b-Ala	1423	85110	0.101	8.993
21	60.880	b-AiBA	1411	78591	0.105	10.834
24	68.313	Trp	7009	466532	0.617	125.957
25	69.840	EOHNH2	3343	223469	0.207	12.628
26	75.487	NH3	156276	26135248	10.125	172.428
27	85.847	Orn	2619	77491	0.046	6.090
28	89.193	Lys	83890	3805927	2.626	383.925
29	94.213	His	41425	2627103	1.954	303.322
30	110.547	Arg	169751	10594496	8.810	1534.757
Totals			2388490	135240209	95.764	
VIS 2 results						
		Hypro				0.000 BDL
10	33.787	Pro	20480	1157400	2.783	320.324
Totals			20480	1157400	2.783	

RESEARCH PUBLICATIONS

1. **Shilpi Ahluwalia,** Jai Gopal Sharma and Pushpendra Singh. Modeling and Analysis of critical factors for phorbol ester degradation in *Jatropha* seed cake using ISM and MICMAC. International Journal of Environment and Waste Management, 2018, 21(4): 287-305.

2. **Shilpi Ahluwalia**, Rajkumar Bidlan, Tanya Saxena, Shreya Dhamija, Satish Kumar, Pushpendra Singh, Jaigopal Sharma. Effect of raw, defatted and detoxified *Jatropha*seed cake on germination of green gram (*Vigna radiata*). International Journal of Research and Applied Science Engineering Technology 2018; 6(1): 1033-1041.

3. **Shilpi Ahluwalia**, Rajkumar Bidlan, Jai Gopal Sharma, Pushpendra Singh. Review on phorbol ester degradation of *Jatropha* seed cake for its use as animal feed. International Journal of Pharmacy and Pharmaceutical Sciences 2017; 9(1):7-13.

4. **Shilpi Ahluwalia,** Jai Gopal Sharma and Pushpendra Singh. Degradation of phorbol esters in *Jatropha* seed cake by *Pseudomona*s *aeruginosa* DS1. International Journal of Pharmacy and Bio Sciences 2017; 8(2): 542-546.

5. **Shilpi Ahluwalia**, Jai Gopal Sharma and Pushpendra Singh, Optimizing the degradation of by-product (*Jatropha* seed cake) from biodiesel industry for recovery and utilization of useful compounds using Taguchi method, International Journal of Environment and Waste Management. (Under Review)

6. **Shilpi Ahluwalia**, Rajkumar Bidlan, Pushpendra Singh and Jai Gopal Sharma, Extraction of proteins and amino acid analysis from detoxified *Jatropha* seed cake: Optimization using Response Surface Methodology, International Journal of Environment Science and Technology. (Communicated)

7. **Shilpi Ahluwalia**, Jai Gopal Sharma and Pushpendra Singh. Effect of process parameters on extraction of proteins from *Jatropha* seed cake- a byproduct of

biodiesel industry. International Conference on Future Learning Aspects of Mechanical Engineering- 2018 (Abstract accepted).

8. **Shilpi Ahluwalia** and Jai Gopal Sharma. Potential uses of *Jatropha* seed cake-byproduct of biodiesel industry, Proceedings of International Conference of Smart Technology for Mechanical Engineering-2013, 1-6.

9. **Shilpi Ahluwalia**, Jai Gopal Sharma and Pushpendra Singh. Effect of pH and temperature on the extraction of *Jatropha curcas* proteins, International Conference on New Frontiers of Engineering, Science & Technology (NFEST-2018), Delhi Technological University (*Abstract)*