Screening of environmental microflora for identification of potential phorbol ester biodegradator and analysis of the degradation product

A Major Project Report submitted in partial fulfillment for the award of the degree

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IN

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Submitted by

RITU SAXENA

(Roll No: 2K13/IBT/06)

Under the supervision of

Dr. JAI GOPAL SHARMA

ASSOCIATE PROFESSOR

Prof. Dr. D. KUMAR

HEAD OF DEPARTMENT

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY

(*FORMERLY DELHI COLLEGE OF ENGINEERING***)**

Delhi-110042

CERTIFICATE

This is to certify that the M.Tech. dissertation entitled **"Screening of environmental microflora for identification of potential phorbol ester biodegradator and analysis of degradation product**" to be submitted by **RITU SAXENA (2K13/IBT/06)** in fulfillment of the requirement for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering), is an authentic record of her work carried out under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for the award of any other degree or diploma elsewhere.

Date:

Dr. D. Kumar
Head of Department
Department of Biotechnology
Delhi Technological University

Dr. Jai Gopal Sharma
Associate Professor
Department of Biotechnology
Delhi Technological University
Delhi- 110042

DECLARATION

I, **RITU SAXENA**, hereby declare that the work entitled "**Screening of environmental microflora for identification of potential phorbol ester biodegradator and analysis of degradation product**" has been carried out by me under the supervision and guidance of **Dr. Jai Gopal Sharma**, Associate Professor in Delhi Technological University, Delhi.

This major project is part of fulfillment for the degree of M.Tech. in Industrial Biotechnology. This is the original work and has not been submitted for any other degree in any other university.

RITU SAXENA

2K13/IBT/06

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DATE: **RITU SAXENA**

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LIST OF ABBREVIATIONS

P E	Phorbol Esters
CP	Crude Protein
Psi	Pound per square inch
g/l	Gram per litre
v/v	Volume by Volume
^{o}C	Degree Celsius
N.A.	Nutrient Agar
FAO	Food and Agriculture Organization
Rpm	Revolutions per minute
Hr	hour
w/v	weight by volume
HPLC	High Pressure Liquid Chromatography
GRAS	Generally recognized as safe
PMA	Phorbol-12-myristate-13-acetate
LB	Luria-Bertani
RP	Reverse phase

1. ABSTRACT

Increased global industrialization and urbanization has led the world to threats of scarce energy resources and thus need for the search of sustainable eco-friendly alternates have emerged in last two decades. Biofuels are partly considered a solution to these issues such as sustainable development, energy security and reduction of greenhouse gas emissions. *Jatropha curcas* has been recognized as a new energy crop for the countries to grow their own renewable energy source with many promising benefits. Large amount of seed cake is generated as by-product during biodiesel production from Jatropha seeds. Seed cake from biodiesel industries contain toxic factors that may be an environmental concern. Further presence of toxic phorbol esters restrict its utilization to be used as a feed in livestock. Safe disposal or meaningful utilization of this major by-product necessitates the degradation of these phorbol esters. *Jatropha* seed cake is the potential and preferred choice for protein and other nutrients for livestock, provided these could be made free of toxic and anti nutritional factors. So, toxic factor degradation in seed cake could serve the dual purpose. The protein content of seed cake came out to be 33%; ash content 4.5%; fat content 7.15% and moisture content 5%.

Biodetoxification is a better approach over physical and chemical treatments with several advantages such as reduction of phorbol esters to non-toxic levels, higher protein content in the residue and environmental friendly with safety and energy concerns. The present study describes the complete degradation of phorbol esters by isolated, screened, most potential phorbol ester biodegradator which has been identified and characterized to be from *Pseudomonas* genera. Degradation was achieved in 12 hr by the treatment of seed cake with cell free fermented broth.

2. INTRODUCTION

With the growing urbanization and ever increasing industrialization, transport industry has become the life line of world economy. Overgrowing population and the depletion of non-renewable energy sources is the biggest problem being faced today. Around 100 years ago, the chief source of energy shifted from solar to the fossil fuel i.e. hydrocarbons. Technology has ultimately led to a greater use of hydrocarbon fuels, making civilization vulnerable to decreased supply of energy. The energy sources that are used in the engines are limited and depleting gradually. This situation urgently leads to search for an alternative fuel for diesel engines. This further necessitates the search for alternative of oil as source of energy. Biodiesel is an alternative fuel or substitute for diesel engine.

The esters of vegetable oils or animal fats are known as Biodiesel. Biodiesel is a promising alternate fuel and has gained significant attention due to the predicted shortage of conventional fuels and environmental concern. The use of biofuels such as biodiesel produced from *Jatropha* oil by trans esterification process represents one of the most promising options over the use of conventional fossil fuels. *Jatropha curcas* is a multipurpose tree which has very high content of oil in its seeds which is a potential source for producing biodiesel.

Jatropha curcas is commonly known as physic nut or purging nut that belongs to Euphorbiaceae family. It grows quickly and can also survive in poor rock textured soil. It reaches to a height of 3-8 m and can be grown on wastelands or barren and marginal agricultural lands where no irrigation facility is available. Further it is resistant to drought and diseases. It does not compete with conventional food crops for land and water and allows production of food crops in the same vicinity (Ameen *et al*, 2011). Thus, it could be an ideal as well as a potential choice to utilize large land resources that are presently unutilized. *Jatropha* has been considered for having a potential advantage over other crops as a new source of protein. The advantage of this non-edible plant is its wide adaptability under various agro-climatic conditions. This crop does not compete for fertile soil, but its growth can be enhanced with fertilization. These characteristics make *Jatropha curcas* regarded as an opportunity for producing oil and preserving food security interests at the same time.

Figure 1: *Jatropha* tree at Delhi Technological University

J. curcas is a genus of approximately 175 succulent plants, shrubs and trees in the Euphorbiaceae family. It has a good adaptation capacity to the large variety of soil conditions. Cultivation on dry, stony and very shallow soils is very frequent. It can be established on marginal land, meadows, contour ridges, hilly slopes and canyons (Wakandigara *et al*, 2013)

Family: Euphorbiaceae

Genus: *Jatropha*

Species: *Jatropha curcas Linnaeus*

The *Jatropha* seeds are of great commercial interest in recent years for its fuel value. Due to its high oil content i.e. 40-60 %, it is used as an alternative source to produce Biodiesel. The residual seed cake is the byproduct of low value which is left after oil extraction and has high protein content. The cake after oil extraction contains 26-29% protein content (Rakshit *et al*, 2010) which is suitable as a protein source for either food or non food products. Hence, it has a potential to complement and substitute soyabean meal as a protein source in livestock diets.

However, the seed cake obtained after pressing them to produce fuel; also contains several toxic and anti-nutritional compounds that can cause serious health problems after long time of contact.

Table 1: Seed Cake Composition (Inekwe U.V. *et al* ,2012)

Moisture content	3.01 ± 0.14
Ash content	3.56 ± 1.39
Lipid	14.55 ± 0.63
Crude Protein	24.83 ± 4.79
Crude Fibre	13.45 ± 0.77
Carbohydrate	38.36 ± 7.43

Biodiesel production from seeds of Jatropha generate large amount of residual deoiled seed cake with an average rate of 500g cake per kg of seed used. Although the oil is an excellent biodiesel feedstock, but potential utilization or safe disposal of huge amounts of seed cake by-product needs to be addressed. The average chemical composition of deoiled seed cake is protein 60%, fat 0.6%, ash 9%, fibre 4% and carbohydrates 26%. The deoiled *J. curcas* seed cake cannot be used as cattle feed, unlike other oilseeds mainly due to the presence of toxic phorbol esters in it. Phorbol esters have been identified as main toxicants in Jatropha seed cake which could not be destroyed even by heating at 160 ºC for 30 min (Chetna *et al*, 2011).

So, the idea of detoxification of pressed seed cake of Jatropha after the extraction of its oil for the biodiesel production will allow its safe disposal avoiding the environmental concern and simultaneously will meet the need for proteins for livestock sector as it is rich in protein content and thus do not conflict with human food security.

The main toxic compounds present are phorbol esters along with anti-nutritional factors present such as trypsin inhibitors, phytic acid, lectins, saponins (Donlaporn & Worapot, 2011). Thus, utilization *J. curcas* seed cake as protein sources for food purposes still needs toxic substance removal. Main toxic compound, phorbol esters are referred to as tigiliane diterpenes in which two hydroxyl groups are esterified to fatty acids and are known for their tumor promoting activity. Phorbol esters are natural plant derived organic compound which is a member of the tigiliane family of diterpene. It is the main toxic component of the press seed cake of Jatropha.

Symptoms of phorbol ester toxicity include dehydration, skin irritation, sunken eyes, loss of appetite and may cause death too. Thus, toxicity is the parameter that limits the utilization of protein rich press cake for animal nutrition and decreases the commercial value of *J curcas.* Following methods can be employed to detoxify the seed cake:

- **Heat and Chemical Treatments**: It can be achieved by treatment with either methanol or treatment with Sodium hydroxide and Sodium hypochlorite or with sodium hydroxide followed by washing with distilled water (Argheore *et al*, 2003).
- **Biodetoxification:** It involves the use of microorganisms in the fermentation of *Jatropha curcas* kernel cake to detoxify toxic substance and antinutritional factors. Fungi *Aspergillus niger, Rhizopus oligosporus, Rhizopus nigricans, Trichoderma longibrachitum* (Belewu & Sam, 2010) have been used for the same. The white-rot

fungi *Bjerkandera adusta, Ganoderma resinaceum* and *Phlebia rufa* have also been used to decrease phorbol esters concentration of *Jatropha curcas* (Candida *et al*, 2011).

Biodetoxification is a better approach because of several advantages as it is more economical, environmentally safe, effectively decrease level of toxic compound (Candida *et al*, 2011) and further environmental friendly with safety and energy concerns (Chin-Feng Chang *et al*, 2014)

Detoxified seed cake has the potential to be exploited as a novel source of functional protein for food applications. The percentage of essential amino acids and mineral contents can be compared to those of other seed and press cakes used as a fodder. It has been reported that except for amino acid-lysine, all other essential amino acids are present in higher concentrations than those of the guidelines of FAO, United Nations (Hipal Gaudani *et al*, 2009) reference pattern suggested for pre-school children. Also it is rich in nitrogen and phosphorus and can be used as manure.

Thus, the possible contribution by the biodetoxification of Jatropa press seed cake may lead to the utilization of agriculture and agroindustry (biodiesel industry) wastes and enabling its nutritional and productive use for animal feed (Candida *et al*, 2011). Therefore, the present study was undertaken with the following broad objectives:

- Biochemical profiling of *Jatropha curcas* seed cake.
- Isolation and screening of environmental microbial flora for potential phorbol ester bio-degradation.
- Selection of potential strains with hydrolytic / esterolytic activity.
- Biochemical identification of selected potential strains.
- Extraction of phorbol esters from pressed seed cake.
- Fermentation strategies for bio-degradation of phorbol ester by selected strain.
- Downstream processing of fermented product for isolation of residual phorbol ester.
- Analysis of degradation product for estimation of degradation efficacy of selected strain and the process thereof, and for the estimation of residual phorbol ester content.

3. REVIEW OF LITERATURE

Biodiesel is now reported as an alternative fuel for diesel engines. In general, the esters of vegetable oils and animal fats are collectively known as biodiesel. It is a domestic, renewable fuel for diesel engine derived from natural oil like Jatropha oil. Biodiesel has an energy content of about 12% less than petroleum-based diesel fuel. Biodiesel refers to a vegetable oil or animal fat based diesel fuel that consist of long-chain alkyl esters(methyl, ethyl or propyl). It is typically made by chemically reacting lipids as vegetable oil, soyabean oil or animal fat with an alcohol, producing fatty acid esters. This process by which biodiesel is produced is Transesterification. Transesterification process involves a reaction in which an ester reacts with an alcohol to form another ester and another alcohol. The catalyst used in this reaction is potassium or sodium hydroxide (KOH or NaOH). Three moles of methanol reacts with one mole triglyceride which produces mixture of fatty esters and glycerine as by-product. Biodiesel is safe and can be used in diesel engines with few or no modifications. The major source of biodiesel is soyabean oil, but other oils include rapeseed, canola, palm, cottonseed, sunflower, and peanut. Jatropha seeds are the primary source from which the oil is extracted. The seeds of Jatropha contains 50-60% oil (Kazi *et al*, 2010)

3.1 *Jatropha curcas*

Jatropha curcas Linnaeus, is a multipurpose plant that contains high amount of oil in its seeds which can be converted into biodiesel. *J. curcas* is presumably the most highly advanced oilseed crop at present in the world. From jatropha seeds, jatropha oil can be extracted which have similar properties as diesel but some properties such as solidifying point, flash point and ignition point are very high in jatropha oil. With the help of chemical reactions, Jatropha oil can be converted into biodiesel. Jatropha oil can also be used directly by blending. The residual seed cake or press cake is a low value byproduct which is left after oil extraction and has a high protein content.

Jatropha curcas is a potential crop having high protein content in the residual seed cake but contains toxins and antinutrients in it. For effective utilization of kernel meal, the removal of antinutrients and toxic principles is necessary. Antinutrients such as trypsin inhibitors and lectin (curcin) can be deactivated by heat treatment, and the adverse effects of phytic acid can be degraded by addition of phytase enzyme while the main toxic compounds: Phorbol esters- are stable to heat to a large extent and are the major factor of concern.

3.2 Phorbol Esters:

The term ‘phorbol esters’ is used to describe a naturally occurring group of compounds mainly distributed in plant species of the Euphorbiaceae family. Phorbol esters are esters of phorbol, a tetracyclic diterpenoid with a tigliane skeletal structure. Terpenoids are classified in accordance to the number of carbon atoms they carry: monoterpenoids have 10 carbons, diterpenoids have 20, etc.

Phorbol itself was isolated as a non-toxic crystalline solid from the plant called *Croton tiglium L.*, but its ester, TPA (12-O-tetradecanoylphorbol-13-acetate), present in croton oil, is the most toxic and most studied phorbol ester, which is known to be a co-carcinogen. However, phorbol esters from *J. curcas* are derivatives of 12-deoxy-16-hydroxyphorbol (Wakandigara *et al*, 2013)

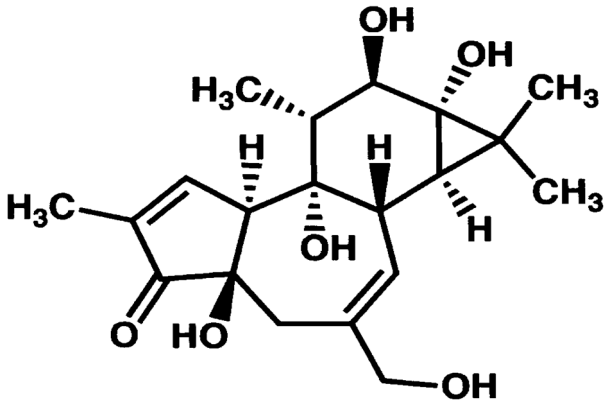


Figure 2: Phorbol

Six phorbol esters have been characterized from *J. curcas* seed and designated as Jatropha factors C1, C2, C3, epimers C4 & C5 and C6, with the molecular formula C_{44} $H_{54}O_8$. All of them are intra-molecular diesters of the same diterpenoid.

The phorbol esters are lipophilic, and are present mainly in oil or kernel, and are not affected by heat treatment. The concentration of phorbol esters varies from 1 to 3 mg/g in kernel meal and from 2 to 7 mg/g in oil (Makkar and Becker, 2010)

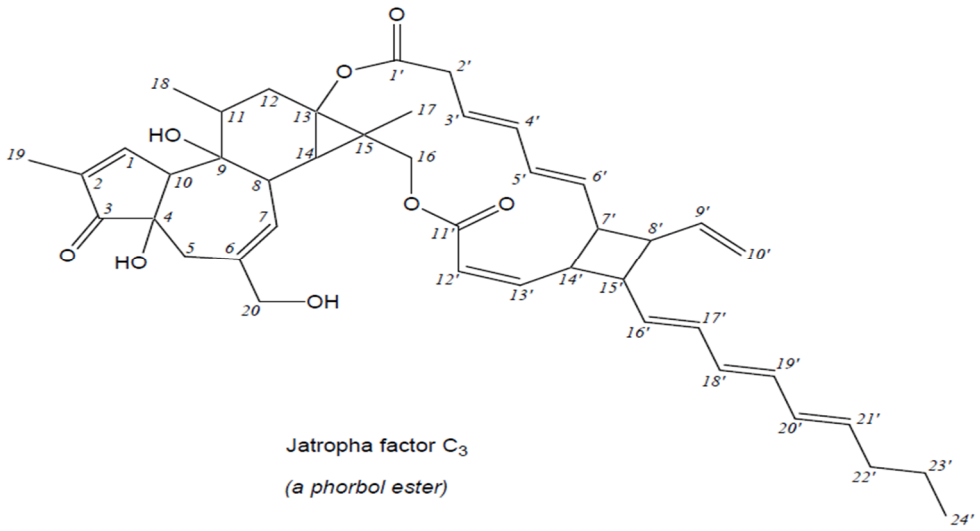


Figure 3: Phorbol esters (Wakandigara *et al*, 2013)

Phorbol esters are 20 carbon tetracyclic diterpenoids made up of four isoprene units. Tigliaine is the fundamental alcohol moiety present in the phorbol esters. Hydroxylation of this basic tigliaine structure at different positions and then ester bonding to various acid moieties results in the formation of large varieties of phorbol ester compounds which are mainly responsible for toxicity in *Jatropha.* Toxicity to goats, rats, snails, pigs, humans, and mice has been reported after the consumption of *Jatropha* seeds or seed cake. In humans and other vertebrates, toxicity of phorbol esters is observed to cause burning pain in the mouth and throat, vomiting, muscle shock, decrease of visual capacity, high pulse rate and other symptoms. All these factors restrict the uses of the seed cake as food (Chetna *et al* , 2011).

Jatropha curcas seed meal with the toxic genotype is considered to have at least six different phorbol esters. The phorbol esters have been reported to mimic the action of diacylglycerol, which is an activator of protein kinase C that in turn regulates the different signal transduction pathways. Any interference with the activity of this protein kinase C can affect number of processes including phospholipid and protein synthesis, enzyme activities, DNA synthesis, phosphorylation of proteins, cell differentiation and gene-expression. Phorbol esters have been also found as co- carcinogens and have purgative and skin-irritant activities. In humans accidental poisoning by *Jatropha curcas* seeds has been reported to cause nausea, vomitings and diarrhoea. Mortality has also been reported in a number of animal species, like chicks, mice and goats.

Even at very low concentrations, the phorbol esters present in the animal feed shows

toxicity. This toxicity limits the use of *Jatropha* constituents as a feed for farm animals and aquaculture species. The term “detection limit of high performance liquid chromatography” refers to a concentration of a substance that is hardly detectable by high performance liquid chromatography (HPLC). Below this HPLC detection limit the substance cannot be detected by HPLC. HPLC is a form of column chromatography to separate, identify and quantify compounds, where a column is utilized that grips the chromatographic packing material, a pump that allows mobile phase to move through the column and a detector that helps in displaying the retention times of the molecules. The detection limit of the HPLC method depends on the experimental conditions for instance to about 10 ppm (Makkar & Becker, 2010)

The most common and only commercially available phorbol ester is phorbol-12-myristate-13-acetate (PMA). In addition to this PMA, six intramolecular diesters that are derived from 12-deoxy-16-hydroxyphorbol have been identified from *J. curcas.* Recently, a dozen of other types of toxins have been purified and identified from *J.curcas* seeds. These toxins are detrimental and deleterious to bacteria, fungi, invertebrates as well as humans. Therefore, the use and safe disposal of the seed cake are the chief challenges to reduce the cost of *Jatropha* biodiesel industry and simultaneously avoiding the environmental concern that it may cause (Xing *et al*, 2013).

PMA is clear colorless (invisible) film or white foam. It is soluble in acetone, dimethyl sulfoxide (DMSO), ethyl acetate, ethanol and methylene chloride, but is practically insoluble in water. To prepare aqueous solutions of PMA, the best practice is to start out with a concentrated solution of the compound in DMSO (20 mM) and dilute a very small aliquot of this solution rapidly with water or buffer. PMA solutions are sensitive to acid and alkaline conditions. PMA when stored in methylene chloride and ethyl acetate or DMSO do not show any detectable autoxidation if stored in diffuse daylight for about 14 days at room temperature.

3.3 Biochemical composition of *Jatropha curcas* seed cake:

The table below describes the content of proteins, carbohydrates, fats, ash, moisture etc. present in seed cake, reported by different researchers.

S.No	PROTEIN CONTENT	FAT CONTENT	ASH CONTENT	MOISTURE CONTENT	CARBOHYDRATE CONTENT	REFERENCE
1	29.4%		5.42%	4.46%		Azza *et al* (2010)
2	23.5%	14.8%	7.8%		42.9%	Donlaporn& Worapot (2011)
3	16.5%	32.24%	3.89%	9.35%		Anna *et al* (2012)
4	44.4%		16%			Ameen *et al* (2011)
5	24.83%	14.55%	3.51%	3.01%	38.36%	Inekwe *et al* (2012)

Table 2: Proximate composition of *Jatropha curcas* seed cake

3.4 Microbial Enzymes as a source of Detoxification:

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the availability of great variety of catalytic activities, high probability of higher yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and fast growth of microorganisms on an inexpensive media. Microbial enzymes have been found to be more stable than their corresponding plant and animal enzymes and their production is more convenient and safer.

Lipases are glycerol ester hydrolases that act on acyl glycerols to liberate fatty acids and glycerol. They can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (M. Veerapagu *et al*, 2013). Lipases or triacylglycerol acyl ester hydrolases are carboxylesterases that catalyze both hydrolysis and synthesis of esters formed from glycerol lipases can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. Lipases can be used as industrial biocatalysts because of their properties like bio-degradability, high

specificity and high catalytic efficiency. *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus,* and *Bacillus alcalophilus* are the most common bacterial lipases. In addition, *Pseudomonas* sp., *Pseudomonas aeruginosa, Burkholderia multivorans, Burkholderia cepacia,* and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers (Ertuguru *et al*, 2007)

Many microorganisms such as bacteria, yeast and fungi are well known to secrete lipase enzyme. Lipase producing microorganisms have been found in various habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc. The oily environment (oil mill effluent) provides a good environment for isolation of lipase producing microorganisms.

Bacterial lipases are mostly extracellular and are more dependent on nutritional and physico-chemical factors, such as temperature, pH, carbon and nitrogen sources, inorganic salts, agitation and dissolved oxygen concentration. Among bacterial lipases, attention has usually been focused on particular classes of enzymes such as the lipases from the genus *Pseudomonas*, which are especially interesting for stream biotechnology (E Mobarak *et al*, 2011). The different species of *Pseudomonas* from diverse environments produce lipases.

The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol. However, nitrogen sources, pH and temperature should also be carefully considered for growth and production optimization (Larbidaouadi *et al*, 2014).

Lipase producers could be isolated from industrial rejection of gas station. One of the sixty isolated strains, exhibiting a higher lipase activity was selected and identified based on their morphological and biochemical characteristics. The effect of incubation time, medium pH, temperature, carbon source and nitrogen source for the lipase production was studied. The lipase production was found to be maximum at pH 8, temperature 40°C and incubation time 48 hours by the lipase producing bacteria *Bacillus licheniformis*. With a selected carbon source, olive oil and glucose were found to be suitable substrates to maximize lipase enzyme production. The optimized concentration of olive oil and glucose was 1% and 1%, respectively. The effect of nitrogen source on lipase production indicated that the yeast

extract was suitable substrates for accelerating lipase production (K. Larbidaouadi *et al* , 2014)

These enzyme producers could also be isolated from oil spilled soil present in vegetable oil processing factories. One of the twenty isolated strain exhibiting a greater zone of clearance than the others, indicated higher lipase activity, it was then selected and identified based on their morphological and physicochemical characteristics. The effect of incubation time, medium pH, temperature, agitation, inoculums concentration, carbon source and nitrogen source for the lipase production was studied. The lipase production was maximum at pH 7, temperature 37ºC and incubation time 48 hours by the lipase producing bacteria *Pseudomonas gessardii*. Increased enzymatic production was obtained when the organisms were cultured in medium supplemented with 1% protease peptone by *Pseudomonas gessardii*. Results demonstrated that *Pseudomonas gessardii* could be an ideal microorganism for extracellular lipase production at industrial level M. Veerapagu *et al*, 2013)

It has been reported that the soil was collected from Himalayan region, for production of thermostable lipase. Tween 20 agar, selective media was used to isolate soil microbe *Bacillus mojavensis*. Different parameters such as substrate selection, pH of the medium, temperature were optimized. Effects of various organic solvents, reducing and oxidizing agents, metal ions and surfactants were analyzed on extracted crude lipase (Sahu *et al*, 2013).

(Kalpana Sagar *et al*, 2013) isolated eighteen bacterial colonies by serial dilution. 100 µl of each dilution was spread on tributyrin agar plates by spread plate technique. The plates were incubated at 37°C for 24-72 hours following which the lipolytic activity was determined (visual observation by the formation of zone of hydrolysis around the bacterial colonies). Out of eighteen isolates, two accessions were found prominent with regard to lipolytic activity. Effect of carbon source, nitrogen source and metals ions were further analysed to determine the maximum lipase activity.

In soil samples collected from Kolli hills, microbes were screened for lipase production and assayed for production of lipase. The production rate was compared with standard *Enterococcus faecium*. Out of the 20 isolates, six isolates were capable of producing lipase more than 3 U/ml in 24 hrs of incubation (M. Prasanna *et al*, 2013).

On isolation of lipase-producing bacteria from wastewater of an oil processing plant, a new strain of *Pseudomonas aeruginosa* was found. Overall 3-times increased lipase production was achieved after improving conditions of production medium. The olive oil and peptones were found to be the most suitable substrate for maximum enzyme production. The enzyme exhibited maximum lipolytic activity at 45°C where it was also found to be stable (Mobarak *et al*, 2011)

3.5 Selection of potential strains with hydrolytic / esterolytic activity:

The potential strains with hydrolytic / esterolytic activity were selected by Tributyrin Clear Zone (TCZ). The bacteria in the nutrient agar plate were isolated and screened for lipolytic activity. Lipolysis was clearly observed by analyzing the changes in the appearance of the substrates such as tributyrin and triolein, that can be emulsified mechanically in various growth media and poured into the petriplates. The bacterial isolates were screened for lipolytic activity on agar plates containing tributyrin (1%, w/v), agar (2%, w/v) in LB media. Lipase enzyme production was indicated by the formation of clear halos around the colonies grown on tributyrin containing agar plates **(**M. Veerapagu *et al*, 2013).

Other method adopted for screening of lipase producing bacterial strains was by spreading 100µl of serially diluted samples on trybutyrin agar plates containing 0.5% (w/v) peptone, 0.3 % (w/v) yeast extract, 1% tributyrin and 2% agar. The plates were incubated at 37°C for 24-72 hours. After incubation period, lipolytic activity was determined by visual observation i.e. the formation of zone of hydrolysis around the bacterial colonies. The criteria for selection was on the basis of utilization of different substrates like tributyrin, olive oil, and anionic detergents tween-20 and tween-80 (Kalpana *et al*, 2013).

M.Prasanna *et al* (2013) isolated lipolytic strains by spreading 0.1ml of diluted soil samples (obtained by serial dilution) on nutrient agar and nutrient agar supplemented with Tween 80. Plates were incubated for 48-72 h at 37°C. Colonies with zone of clearance were screened as potential lipolytic strains. These colonies were then picked aseptically and stored into sterile nutrient slant for future studies.

Well plate assay has been also reported as a method of selecting strains with hydrolytic/esterolytic activity. Tributyrin agar added with 1% (v/v) tributyrin was prepared by emulsifying using homogenizer and autoclaved at 121ºC, 15 psi for 15 min and cooled to 45ºC under laminar air flow and poured into the sterile glass petriplates under aseptic

environment. The agar media was allowed to cool under laminar hood and closed with the lid, kept inverted for 24hrs to obtain the sterile tributyrin agar plates. These sterile agar plates were punched aseptically with sterile cork borer to obtain two wells in the two halves of the plates maintaining a separation distance around. These tributyrin agar plates were loaded with freshly extracted lipase enzyme of 50µl in each well separately and thereafter incubated at 37ºC in the incubator for 48 hrs. The developed clear zones around the wells were measured (in mm) and the data was used for further assessment along with titrimetric assay data to screen the primary isolates for lipase producing bacteria (Bhavani *et al*, 2012).

3.6 Biochemical identification of selected potential strains:

E. Sirisha *et al* (2010) performed citrate, catalase, gelatin liquefaction, nitrate reduction, oxidase, indole, methyl red, voges-proskauer, urease test for biochemical studies.

3.7 Reported approaches so far for degradation of Phorbol esters in seed cake:

Heat treatment in presence of alkali was also effective in reducing phorbol esters. (Martinez-Herrera *et al*, 2006) studied the effect of various treatments, such as hydrothermal processing techniques, solvent extraction, solvent extraction plus treatment with $NaHCO_3$, and ionizing radiation, to inactivate the antinutritional factors in jatropha kernel meal.

Double solvent extraction accompanied with wet extrusion, re-extraction with hexane and moist-heat treatment diminished phorbol ester content by 87.7 %. (Rakshit & Bhagya, 2007) reported that up to 90% of the phorbol esters could be removed by treating the meal with 20 g/L of calcium hydroxide. (Gaur, 2009) developed a process that obtains high yields of Jatropha oil and detoxifies the defatted (oil-free) Jatropha meal. The principle of solid-liquid extraction was utilized to detoxify the meal. Various organic solvents were used for the extraction. Extraction of ground Jatropha seed kernels in a Soxhlet apparatus involving a sequential combination of hexane followed by methanol, proved high efficiency in detoxifying the meal. Phorbol ester content was reduced by 99.6% from 6.05mg/g in untreated meal to about 0.06 mg/g in solvent-treated meal.

Chemical oxidation by ozone gas combined with $NaHCO_3$, 0.2N moist showed its best results at ozonation 2 and 3 mins of ozone dose 50mg/l at 300 mA .Chemical oxidation by ozone or by air stripping for jatropha seed cake meal gave a clean nutrient as animal feed (Diwani *et al*, 2011)

Bacillus species are often subjected to the enzyme production and most of *Bacillus* species are considered as Generally Regarded as Safe (GRAS) by FDA of USA. Moreover, *Bacillus* spp. are often used in the production of food additives and probiotic products. Therefore, *Bacillus* strains have been applied to evaluate the degradation efficiency of phorbol esters in *J. curcas* seed cake. Five *Bacillus* spp. (*B. coagulans licheniformis, B. smithii, B. sonorensis,* and *B. subtilis*) that were isolated from different compost plants and biofertilizers were used form phorbol esters degradation and enzyme production (Chin *et al*, 2011) *Streptomyces fumicarius* is a new strain which is capable of degrading the total toxicity of phorbol esters by 97% and it was found that the detoxified seed cake was non-toxic to plants (Xing *et al*, 2013)

The complete degradation of phorbol esters by *Pseudomonas aeruginosa* PseA strain during the solid state fermentation (SSF) of deoiled Jatropha curcas seed cake has been reported. Phorbol esters were found to get completely degrade in nine days under the optimized SSF conditions i.e. deoiled cake 5g; moistened with 5 ml distilled water; inoculum 1.5 ml of overnight grown *P. aeruginosa*; incubation at temperature 30ºC, pH 7 and RH 65%. SSF of deoiled cake was found to be a potentially viable approach for the complete degradation of the toxic phorbol esters. Under optimized solid state fermentation conditions i.e. deoiled seed cake : water ratio of 1:1 and other mentioned parameters, *P.aeruginosa* PseA completely degraded phorbol esters, the main toxicant of J. curcas seed cake in nine days. The SSF could be a viable a approach for detoxification or removal of toxic factors in seed cakes (Chetna *et al*, 2011).

The concentrations of decreased phorbol esters in the seed cake by performing solid state fermentation with three white rot fungi *Bjerkandera adusta, Ganoderma resinaceum, Phlebia rufa* have been evaluated. Erlenmeyer flasks were used for incubation at 28°C for 30 days without agitation. Phorbol esters were analyzed with the help of reverse phase HPLC and was then extracted with a procedure using dichloromethane. Mainly *B.adjusta and P. rufa* were found to significantly reduce the phorbol esters content to non toxic levels i.e. <0.05. These results suggested that the white rot fungi could be a potential approach for biodetoxification of seed cake (Candida *et al*, 2011).

Detoxification of toxic and antinutritional compounds by fermentation with *Bacillus spp.* has been reported. Two generally recognized as safe (GRAS) strains used were *Bacillus*

subtilis and *Bacillus licheniformis* with solid state and submerged fermentations. Result showed *B. licheniformis* with submerged fermentation effectively degraded toxic and antinutritional compounds. Phorbol esters, phytic acid and trypsin inhibitor (due to the presence of enzymes esterase, phytase and protease) were reduced to 62%, 45% and 75% respectively but lectins were not eliminated (Thanyarat and Worapot, 2013).

It was found that autoclaving alone reduces the phorbol ester content by 20% only and addition of sodium hydroxide and sodium hypochlorite followed by heat treatment reduces phorbol ester content by 25%. *P. ostreatus* degraded 99% of the phorbol ester after incubation period of 45-days. This rate of degradation was found to be higher than rates observed when chemical deodorization, deacidification, or bleaching agents were approached to detoxify *J.curcas* oil and seed cake. Antinutritional factor: tannin degradation by *P. ostreatus* increased as a function of the incubation time. The highest observed rate was between 15 and 30 days on the substrate with eucalyptus bark. Phytase activity by *P. ostreatus* caused a 95% decrease of phytic acid in the substrates. The presence of this enzyme has also been observed in *Aspergillus sp , Agaricus sp, Lentinula sp* and *Pleurotus sp.* (Kasuya *et al*, 2013).

The dry matter content of the treated cake was slightly higher than the untreated cake. The crude protein content of the treated diets was higher than the untreated diet due to the addition of microbial protein during fermentation process. Treatment of *Jatropha curcas* cake with *Trichoderma longibrachiatum* was not effective in detoxifying *Jatropha curcas* kernel cake compared to *Aspergillus niger* which was promising in detoxifying *Jatropha curcas* kernel cake (Belewu *et al*, 2010).

An experiment was conducted that consist of three treatments A (Control, without fungi treatment), B (*Aspergillus niger* treated samples) and C (*Mucor mucedo* treated sample). The results showed reduction in the level of the antinutrients after fermentation with the microorganisms. Phytic acid was reduced from 6.67% to 5.92% in *A. niger* treated *J. curcas* seed cake and 6.26% in *M. mucedo* treated sample. Saponin content was found to be reduced from 2.13% to 0.48% in *A. niger* and 0.35% in *M. mucedo* treated samples. Reductions were also reported in the cyanide, tannin and oxalate contents of the fungi treated *J. curcas* seed cakes. This reduction in the toxins of J. curcas seed cake indicated that fermentation might be a good method for the detoxification of toxic seed oils and subsequent uses as a source of feed for livestock (Ameen *et al*, 2011).

A new strain *Streptomyces fumicarius* YUCM 310038 has been found which was capable of degrading the total toxicity by more than 97% in a solid state fermentation carried out for 9 days . The seed cake fermented by YUCM 310038 was non-toxic to plants and was prominently promoting the growth of tobacco plant, which indicates the potential of the strain to transform the toxic seed cake to biologically safer animal feed or organic fertilizer so as to serve the dual purpose to remove the environmental concern as well as to reduce the cost of *Jatropha* biodiesel industry (Xing *et al*, 2013)

3.8 Extraction of phorbol esters from pressed seed cake:

Extraction of phorbol esters have been reported as: 8gm of seed cake was taken in a beaker and 20ml of ethanol was added to extract phorbol esters in an ultrasonic bath. Three complete extractions were carried out. Methanol was evaporated in water bath. For purification of extracted material, 40ml water was added and solution was extracted with ethyl acetate four times. Ethyl acetate was then concentrated on water bath and residue was diluted with methanol (HPLC grade) for further work. Methanol was found to have greater affinity towards phorbol esters and is enough polar solvent to extract phorbol esters present in the cake (Hipal *et al*, 2009).

Other method described for extraction of phorbol esters: 10g of seed cake was taken in a soxhlet extractor for 4 hrs of extraction, methanol was used as solvent. After extraction, methanol was further evaporated by vacuum rotary evaporator until 10ml of solution was obtained (Vittaya *et al*, 2013)

Phorbol esters could also be extracted as in methanol-water (9:1, v/v) mixture in an orbital shaker for 8hr at 180 rpm and 25ºC. The constituents soluble in the methanol-water mixture were extracted in n-hexane by solvent-solvent extraction. Methanol-water layer was concentrated in a rotary evaporator and viscous oily fraction was separated from aqueous solution after concentration and then extracted with diethyl ether. Lower layer was discarded and ether layer was evaporated to obtain crude phorbol ester extract. Phorbol ester fraction was dissolved in THF for its determination by HPLC (Lalit *et al* ,2012).

Xing *et al* (2013) extracted the toxins from seed cake with the help of absolute methanol. 36ml of methanol was added to a flask containing 5g kernel cake and the mixture was incubated in an ultrasound bath for 3×15 min. The extraction process was repeated two

more times and the extract fractions were pooled together at one place. The methanol was removed in a rotary evaporator under vacuum at 60ºC; the extract was then fully dried in an air flow oven at 40ºC. For HPLC analysis, the extract powder was dissolved in methanol at 2mg/ml and then filtered using Whatman filter paper no. 2.

Other method reported for extraction of phorbol esters was: 5g of *J. curcas* crushed seed cake was taken and 20ml of 95% ethanol was added to it and then shaked at 200 rpm for 5 min, then centrifuged at 14,000 *g* for 5 min. The residue was extracted for two additional times with 95% ethanol. The extract fractions were combined and dried under vacuum at 50ºC. The dried extract was dissolved in 1 ml of 95% ethanol and passed through a 0.2µm membrane filter to be used later in HPLC (Chin *et al*, 2011).

3.9 Estimation of Phorbol esters content: HPLC Analysis:

Analysis of Phorbol esters has been done by weighing seed cake, approximately 2 g, followed by extraction with methanol. The phorbol esters content was determined by HPLC (LC-20AT, Shimadzu, Japan) with a photo diode array detector at 280 nm (DAD, SPD-M20A) and a rheodyne injector. The analytical column was C8, controlled at room temperature and the flow rate was 1.3 mL/ min. The mobile phase used was MeOH : H_2O (with 0.1% HCOOH). The four phorbol ester characteristic peaks that appeared between 17 and 21 min were identified and the results were expressed as equivalent to a standard phorbol-12-myristate-13-acetate (Raquel *et al*, 2014).

Experimental studies with RP-18, 5µ, 250 ×4.6 mm analytical column and compound phorbol ester were performed at 280nm using UV detector. Flow rate 1.3 ml/min; column temp. 30ºC; pressure 70-160 bar. Phorbol ester peak was observed between 20-25 min. Standard peak was observed at 29 min. Phorbol esters were found in the range of 0.44-2.15% with an average value of 1.21%. Non-toxic was found to have lowest phorbol ester content as 0.44% (Hipal *et al*, 2009).

Phorbol ester content has been reported to be analysed by HPLC-UV (Shimadzu, LC -10AC, Japan). An aliquot was loaded on HPLC-UV reverse phase C18 column for analysis. Separation was performed at 35ºC, 1 min/min of flow rate and isocratic elution (4:1)(v/v) (acetonitrile : DI water) for mobile phase. The phorbol ester chromatogram had 3 peaks to detect at 280nm and appeared between 8-12min. The results were expressed as equivalent to

a standard, TPA (Phorbol-12-myristate-13-acetate). Result showed phorbol ester content of cake was 2.9259 mg/g and detoxified cake as 0.5482 mg/g (Vittaya *et al*, 2013)

(Lalit *et al*, 2012) determined phorbol ester content by HPLC with C18 column. Temperature 25ºC; Flow rate 1.3 ml/min. Solvents used were 1.75 ml ortho-phosphoric acid (85%) in 1l distilled water (A) and Acetonitrile (B). Phorbol ester peak appeared between 41 and 48 min. Peaks were integrated at 280nm and results were expressed as equivalent of standard phorbol-12-myristate-13-acetate whose peak appeared at 50min. The phorbol ester content of cake was found to be 0.6±0.01 mg/g.

Analysis of phorbol esters has been reported using Waters 515 HPLC system equipped with a 2996 Photodiode Array Detector (Waters, USA) and a Zorbax SB-C18 reverse phase column (5µ, 4.6 × 250mm i.e. Agilent USA). The separation was carried out at 30ºC with a flow rate at 1.0 ml/min starting with 60% water (A) and 40% acetonitrile (B) for 30 min, and decreasing A to 25%, increasing B to 75% for 20 min, then B at 100% for the last 15 min. The detector wavelength was set at 254nm. Standard used was PMA and all chemical agents were obtained from Sigma (U.K.) (Xiang *et al*, 2013).

The phorbol ester concentration could be determined by a method as: an aliquot was loaded on an HPLC–UV reverse phase C18 LiChrophere100, 5µm (250 × 4 mm) (from Merck, Darmstadt, Germany) column. The separation was performed at room temperature 35ºC and the flow rate was 1ml/min using isocratic elution of 1:1 (v/v) deionized water mixed with acetonitrile as mobile phase. The group of peaks were detected at 280nm and appeared at 8-12 min of chromatogram. The results were expressed as equivalent to an external standard, phorbol-12-myristate-13-acetate (Vittaya *et al*, 2012).

Other method described to analyse phorbol esters- HPLC system (Thermo Separation Products, U.S.) that consisted of an AS1000 autosampler, P2000 pump and UV1000 detector. The solvents used were water and acetonitrile: process was started with 60% water and 40% acetonitrile for 15 min, followed by 25% water and 75% acetonitrile for the next 20 min, and finally 100% acetonitrile for the next 20 min. Separation was performed at room temperature (25ºC) with flow rate maintained at 1.3 ml/ min. The detector wavelength was set at 280 nm. The results were expressed as equivalent to PMA (Sigma, U.K.) that was used as an external standard (Chin *et al*, 2011).

4. MATERIALS AND METHODS

Jatropha curcas seed cake was obtained from Biodiesel plant at Delhi Technological University. The seeds were dried, pressed to obtain biodiesel while the residual pressed deoiled seeds called as seed cake was taken. The seed cake so obtained was ground in a grinder and was kept in an air tight zip lock pouch. Ground seed cake powder was kept in refrigerator at 4ºC for further use.

Figure 4: Ground seed cake in zip lock pouch

4.1 Proximate Analysis of *Jatropha curcas* seed cake

It is a method for the quantitative analysis of different macronutrients in feed. Proximate analysis of seed cake:

4.1.1 Ash content – It represents residue remaining after destruction of organic matter . Organic matter of a food stuff is destroyed completely at a very high temperature of 600°C and remaining contents are considered as Ash content. The contents were first charred in the crucible on a burner and was then placed in a temperature controlled furnace heated at 600ºC overnight. After that it was cooled and weighed on an analytical balance (W3).

$$\% \text{ Ash} = \frac{(W1 + W2) - W3}{W1} * 100$$

4.1.2 Moisture Content - It is the amount of water found within a given material at a given time. 10grams of sample was weighed and dried in a Digital oven at 105°C for 1hour and the process was done again and again until it stops losing weight.

$$\% \text{ Moisture content} = \frac{Water\ loss}{original\ weight} * 100$$

4.1.3 Protein content – Protein content was estimated by Lowry method .

4.1.4 Fat content – Fat content was estimated using Soxhlet apparatus.

% Fat content = $\frac{m2 - m1}{E} * 100$

Where m_2 = weight of the vessel containing fat residue after evaporation of solvent in grams

m_1 = weight of the dry empty vessel in grams

E = sample weight in grams

4.2 Isolation and screening of microbial flora for potential Phorbol esters Degradator

4.2.1 Collection of soil samples:

Soil was taken from two sources –

(i) Soil from petrol pump

(ii) Soil near rhizosphere beneath *Jatropha tree.*

Microbial colonies were isolated from two types of soil with the help of Serial dilution.

4.2.2 Isolation:

Nutrient agar was prepared, autoclaved at 121ºC temperature and 15 psi pressure and then cooled to 50ºC. Then it was poured into sterile petriplates and was left to be cooled for solidification. Then, 0.1 ml of dilution was taken from (10^{-4}, 10^{-5}, 10^{-6}) and was spread with the help of L-shaped glass rod on different poured, solidified nutrient agar plates. Plates were then kept in incubator at 37°C for 24 hours.

Media preparation (N.A.) –

Peptones	0.5%
Yeast extract	0.3%
Agar	1.5%
Nacl	0.5%

4.2.3 Screening:

Tween-20 media was prepared, autoclaved at 121ºC temperature and 15 psi pressure and then cooled to 50ºC. Then, Poured plates were prepared. Microbial colonies so obtained from soil were then picked and individual colony was streaked on Tween-20 plates. Plates were then incubated at 37 ºC for 24 hours. Lipase/esterase producers on growth would have produced lipolytic enzymes and acted on tween-20 that resulted in formation of white crystals of calcium salts of fatty acids within the media. Out of 26 streaked microbial colonies, 19 were screened that could be lipolytic enzyme producers.

Media preparation (Tween 20 containing media)-

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

Trybutyrin media was prepared, autoclaved at 121 ºC temperature and 15psi pressure and then cooled to 50 ºC. Then plates were prepared. Microbial colonies from Tween-20 plates were then picked and individual colony was streaked on Tributyrin plates. Plates were then incubated at 37 ºC for 24 hours.

Lipase or esterase activity was determined by the formation of zone of hydrolysis around the bacterial colonies. All 19 colonies that were screened as Tween-20 positive showed clear zone of hydrolysis with tributyrin.

Media preparation (Tributyrin plates) (Jeyaraman *et al,* 2012)

Peptone	0.5% (w/v)
Beef extract	0.3% (w/v)
Trybutyrin	1% (v/v)
Agar	2% (w/v)

4.3 Selection of potential strains with hydrolytic /esterolytic activity:

4.3.1Tributyrin halo zone method

Tributyrin agar containing 1%(v/v) tributyrin was prepared by emulsifying using homogenizer and autoclaved at 121ºC, 15 psi for 15 min and cooled to 45ºC under laminar air flow and then poured into the sterile petriplates under aseptic conditions. Lipase/esterase enzyme production was indicated by the formation of clear halos around the colonies grown on tributyrin containing agar plates**.**

4.3.2 Well-plate Assay or Agar well diffusion assay (Bhavani *et al,* 2012)

Nutrient broth was prepared in tubes to revive the culture.

Tributyrin agar containing 1% (v/v) tributyrin was emulsified using homogenizer and autoclaved at 121ºC, 15 psi for 15 min and then cooled to 45ºC under laminar air flow. Then, media was poured in to the sterile petriplates aseptically. These sterile agar plates

were punched aseptically with sterile cork borer to obtain a 8.5mm diameter wells. These tributyrin agar plates were loaded with freshly extracted crude lipase enzyme of 100 µl in each well separately for each extracted crude enzyme and incubated at 37ºC in the incubator for 48 hrs. The developed clear zones around the wells were measured (in mm) and the data was used for further studies to screen most potential phorbol esters degradator.

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

⇩

Flasks were incubated in a rotary shaker at 200rpm for 22 hrs

⇩

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 30mins

⇩

Supernatant decanted into other falcon tubes

⇩

Wells were made in trybutyrin plates using cork borer

⇩

100µl of supernatant was added into plate wells

⇩

Plates were incubated in an incubator for 16hrs, 24hrs

4.4 Biochemical identification of selected potential strains:

4.4.1 Gram Staining

Gram staining is a differential staining method to separate bacterial species into two groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell-wall. Basically, it detects the presence of peptidoglycan, which is present as a thick

layer in Gram-positive bacteria and a thin layer in gram-negative. A Gram-positive bacteria results in a purple/blue color while a Gram-negative results in a pink/red color.

The gram stain is a very important preliminary step in the initial characterization and classification of bacteria. It is also a key procedure in the identification of bacteria based on staining characteristics, that enables the bacteria to be examined using a light microscope. After getting stained, the morphology and arrangement of the bacteria can be observed.

The gram staining was carried out as follows:

smears were prepared using an inoculation loop by putting a drop of cultured broth on an sterilized glass slide

⇩

smear was then heat fixed on a burner flame

⇩

flooded with Crystal Violet solution for 1min

rinsed with distilled water

⇩

smear then flooded with Gram's Iodine solution ,allowed to remain for 1min and again rinsed with distilled water

⇩

slides were then treated with Gram's decolouriser (95% ethanol)

⇩

washed with distilled water

⇩

safranin was applied for about 1 min as counterstain followed by washing with distilled water

Slides were then viewed under microscope.

4.4.2 Catalase Test:

Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water. In order to survive, organisms rely on a defense mechanism that allows them to repair or escape the oxidative damage of hydrogen peroxide (H_2O_2). Some bacteria produce the enzyme catalase which facilitates cellular detoxification by neutralizing the toxic and bactericidal effects of hydrogen peroxide.

Test cultures were grown freshly on Nutrient agar, onto which was added few drops of 3% (v/v) hydrogen peroxide. Culture tubes were observed for the liberation of nascent oxygen

in the form of bubbles. The production of bubbles indicates the production of enzyme catalase showing positive test.

4.4.3 Methyl Red and Voges-Proskauer (MR-VP) Test:

These two tests were performed using MR-VP broth. Test cultures were individually inoculated into the broth medium and incubated for 24 hr at 37ºC. The culture broth was divided into two parts; one part was used for MR reaction and the other for VP reaction.

4.4.3.1 **Methyl Red Test:**

Methyl Red (MR) test determines the microbial performance to carry out mixed acids (acetic, lactic and succinic) fermentation when supplied with glucose. These large amount of acids produced result in a prominent decrease in the pH of the medium which is below 4.4. If pH goes below 4.4 with the fermentation process, culture medium turns red on addition of methyl red that indicates positive test and vice-versa.

Methyl Red indicator was prepared by dissolving 0.1g of methyl red in 300ml of ethanol 95% ethanol and later 500ml with distilled water. The MR reagent in 5-6 drops was added to one part of the culture broth. Development of pink color indicates positive reaction.

4.4.3.2 Voges-Proskauer Test:

The Voges-Proskauer test determines the ability of some microorganisms to produce a neutral reacting end product, such as acetyl methyl carbinol (acetoin), from organic acids like pyruvic acid resulting from the metabolism of glucose.

The reagent used in this test is Barritt's reagent, consisting of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol serves as a catalyst to bring out a red complex. As a result, a pink complex is formed, imparting a rose color to the medium. Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent indicates the presence of acetyl methyl carbinol and represents a positive test. The absence of rose color shows negative result.

VP test reagent consists of 2 solutions:

Solution A		**Solution B**	
Alpha-napthol	5.0 g	Potassium Hydroxide	40 g
Absolute alcohol	100 ml	Creatine	0.5 g
		Distilledwater	100ml

To second part of culture broth, 0.6 ml of solution A and 0.2 ml of solution B were added, mixed well and tubes were kept unplugged so as to facilitate aerobic environment. Formation of eosin pink color indicates positive reaction.

4.4.4 Urease test:

Many of the urinary tract infecting microorganisms have an enzyme called urease that has the ablity to split urea in the presence of water to release ammonia and carbon dioxide. Ammonia formed then combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, further turning the indicator phenol red from its original orange yellow color to bright pink.

Individual test cultures were inoculated into the prepared slants by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated for 24-48 h at 37°C. Positive urease reaction (i.e. alkaline) is indicated by a deep cerise (cherry red/ bright red) color of the whole medium.

Media (g/l)

Beef extract	2.0
Peptone	15.0
Yeast extract	2.0
Dextrose	1.0
Mannitol	10.0
Phenol red	0.05
Agar	15.0

Final pH of the medium: 7.2 ± 0.2. Prior to use, 25 mL of 40% membrane filtered urea solution was added to the molten sterile medium and mixed well. 1 inch butt slants were prepared using sterile glass tubes.

4.4.5 Citrate Utilization Test:

The citrate test detects the ability of an organism to use citrate as the main source of carbon and energy. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced as a result ultimately. The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive.

Freshly prepared slants of Simmon's citrate agar were inoculated with individual test organisms and incubated for 24-48h at 37°C. The formation of deep blue colour in the incubated slants indicate positive reaction for citrate utilization.

Simmon's citrate medium (g/l):

$MgSO_4.7H20$	0.2
Ammonium dihydrogen phosphate	1
Dipotassium hydrogen phosphate	1
Sodium citrate	2.0
Sodium chloride	5
Bromothymol blue	0.08
Agar	1.5

4.4.6 Nitrate Reduction Test:

Individual test cultures were inoculated into the prepared nitrate broth and incubated for 24 h at 37°C. The culture broth was tested for nitrate reduction using the following reagent, which consisted of two solutions:

Solution 1:

Sulphanilic acid	8g
5N Acetic acid	1 l

Solution 2:

α-napthol	5g
5N Acetic acid	1 l

To 5 mL of the 24 h-old culture broth, was added 2 drops each of solution (1) and (2). Development of orange/brick red colour is indicator of nitrate reduction to nitrite.

Nitrate Broth (g/l):

Beef extract	3.0
Peptone	5.0
KNO_3	1.0

Bacterial Morphology:

Bacteria are very small unicellular microorganisms ubiquitious in nature. They have cell walls composed of peptidoglycan and reproduce by binary fission. They vary in their morphological features.

The most common morphologies are:

i. **Cocci:** Spherical bacteria; may occur in pairs (diplococci) , in groups of four (tetracocci) , in grape-like clusters (staphylococci), in chain (streptococci) or in cubical arrangements of eight or more (sarcinae).

ii. **Bacilli:** Rod-shaped bacteria ; generally occurs as singles but occasionally found in pairs (diplococcic) or in chains (streptobacilli).

iii. **Spirilla:** spiral-shaped bacteria.

Some bacteria have other shapes such as :

- **Coccobacilli:** Elongatedspherical or ovoid form
- **Filamentous:** Bacilli that occurs in long chains or threads.
- **Fusiform:** Bacilli

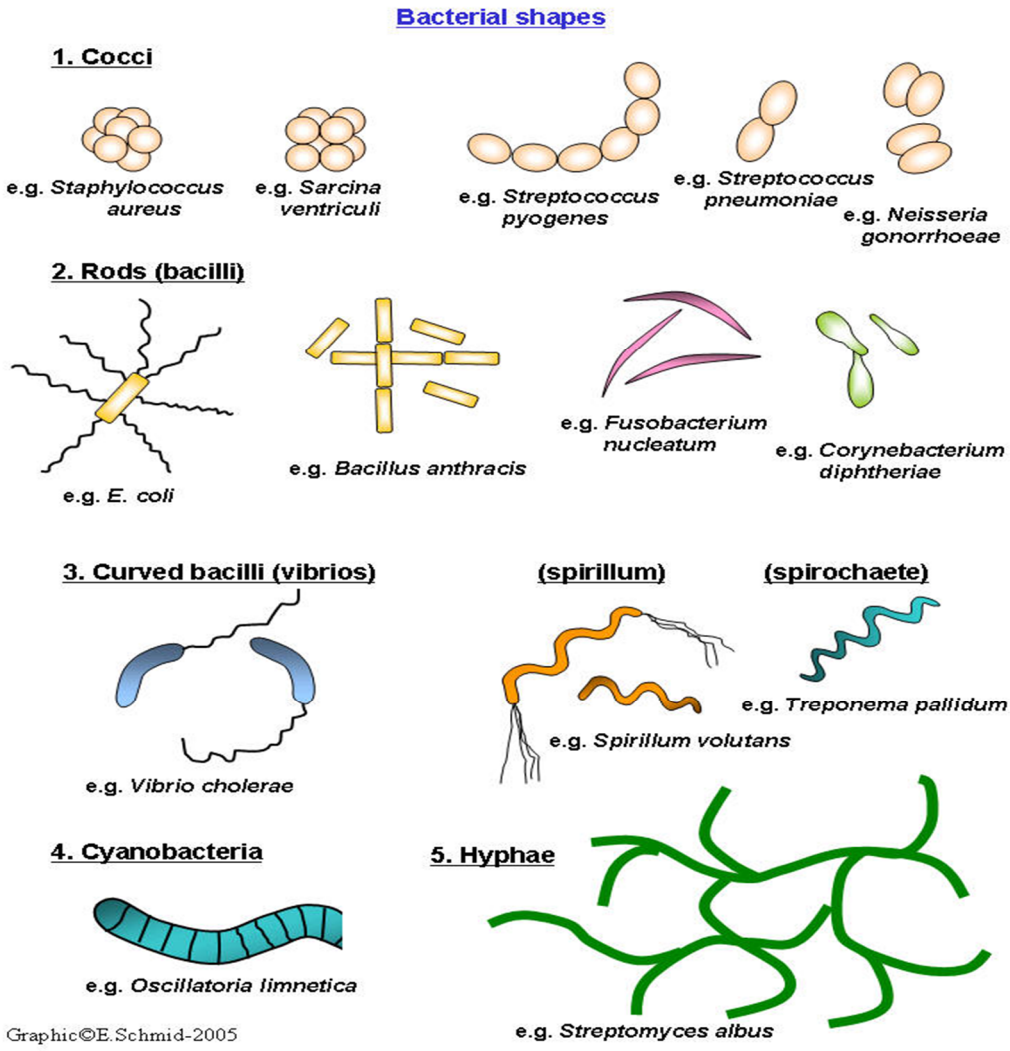


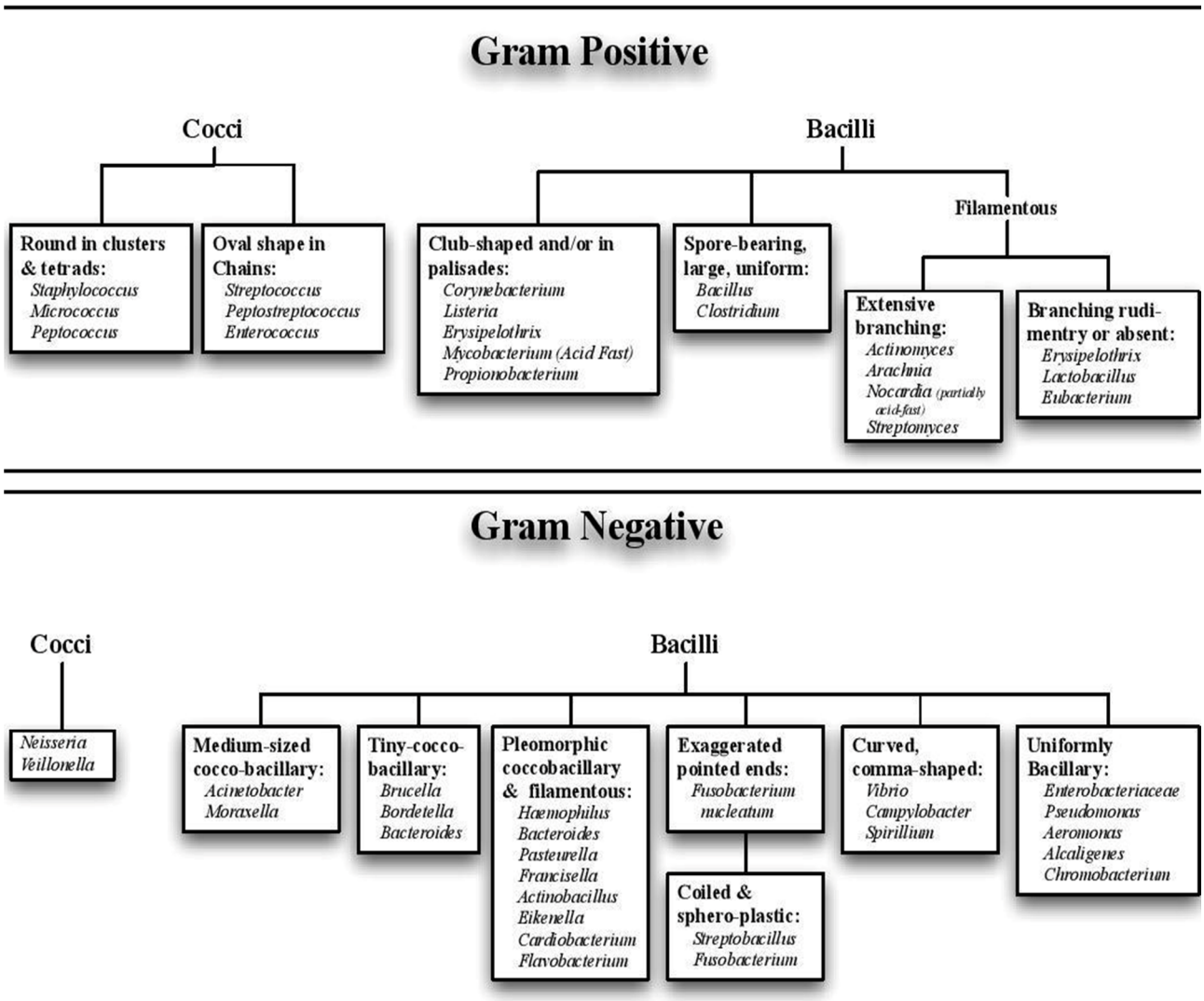
Figure 5: Bacterial morphology

Bergey's Manual of Determinative Bacteriology:

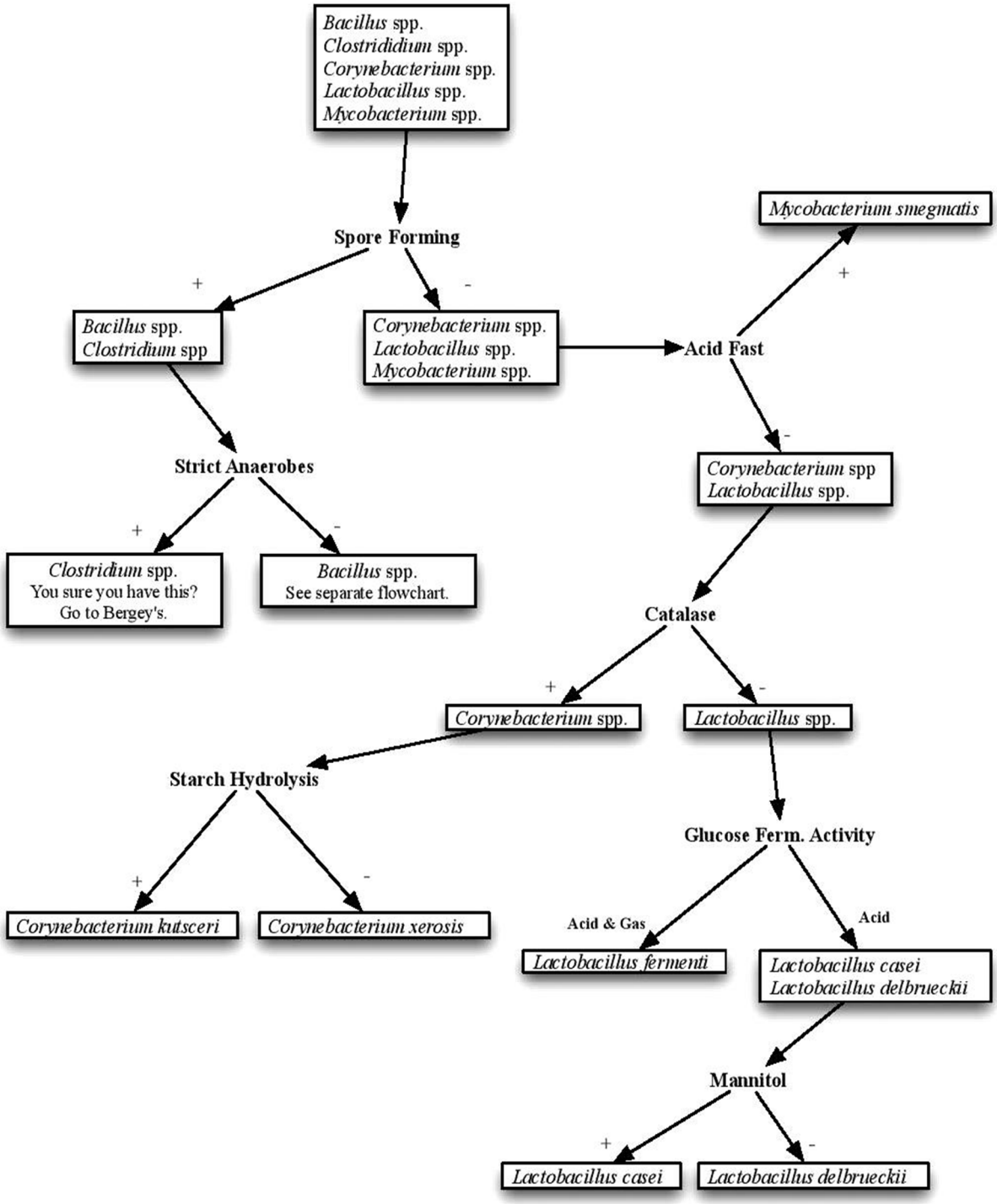
Differentiation via Gram stains and cell morphology.

Gram Stain & Morphological Flowchart

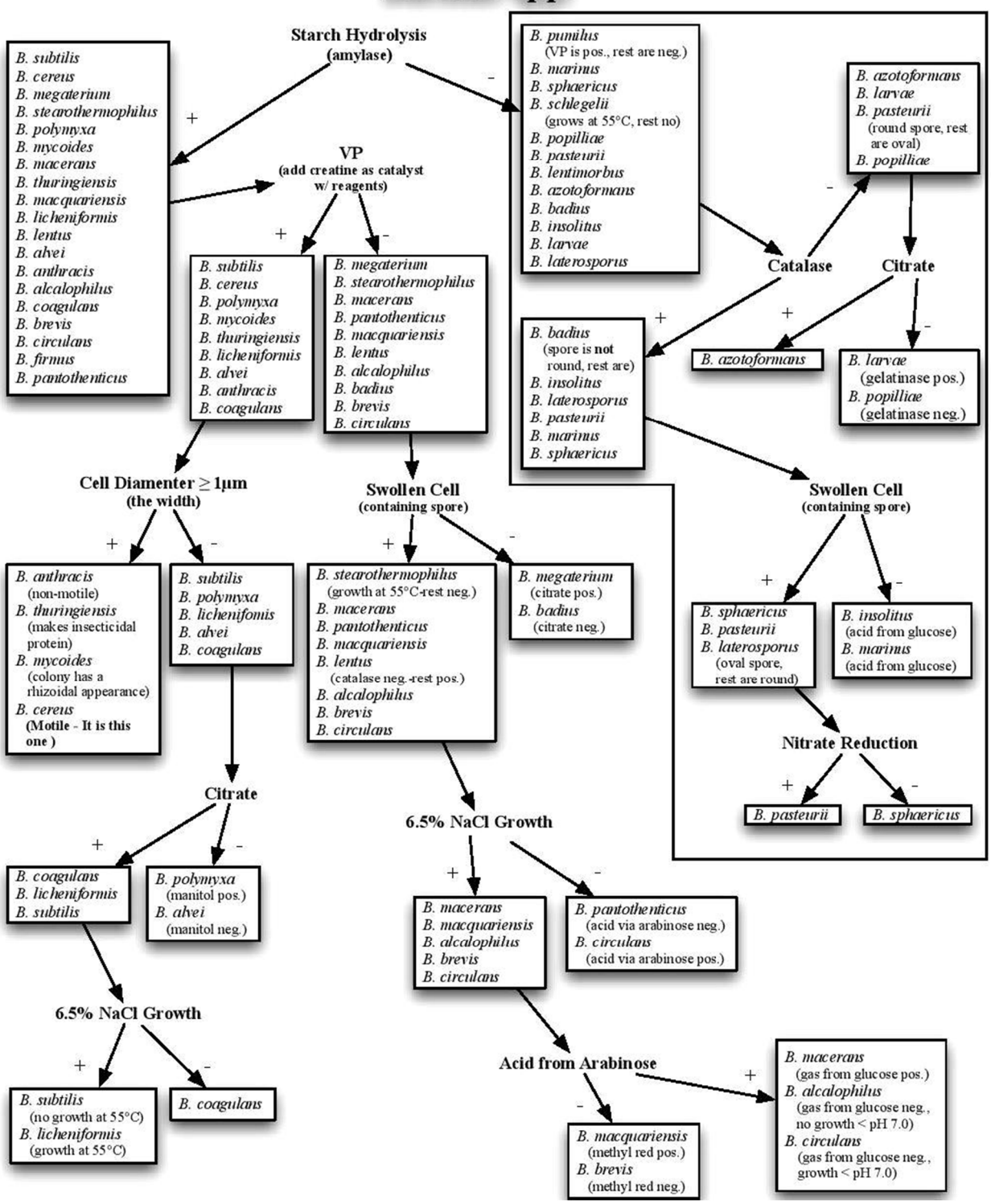
Some Examples



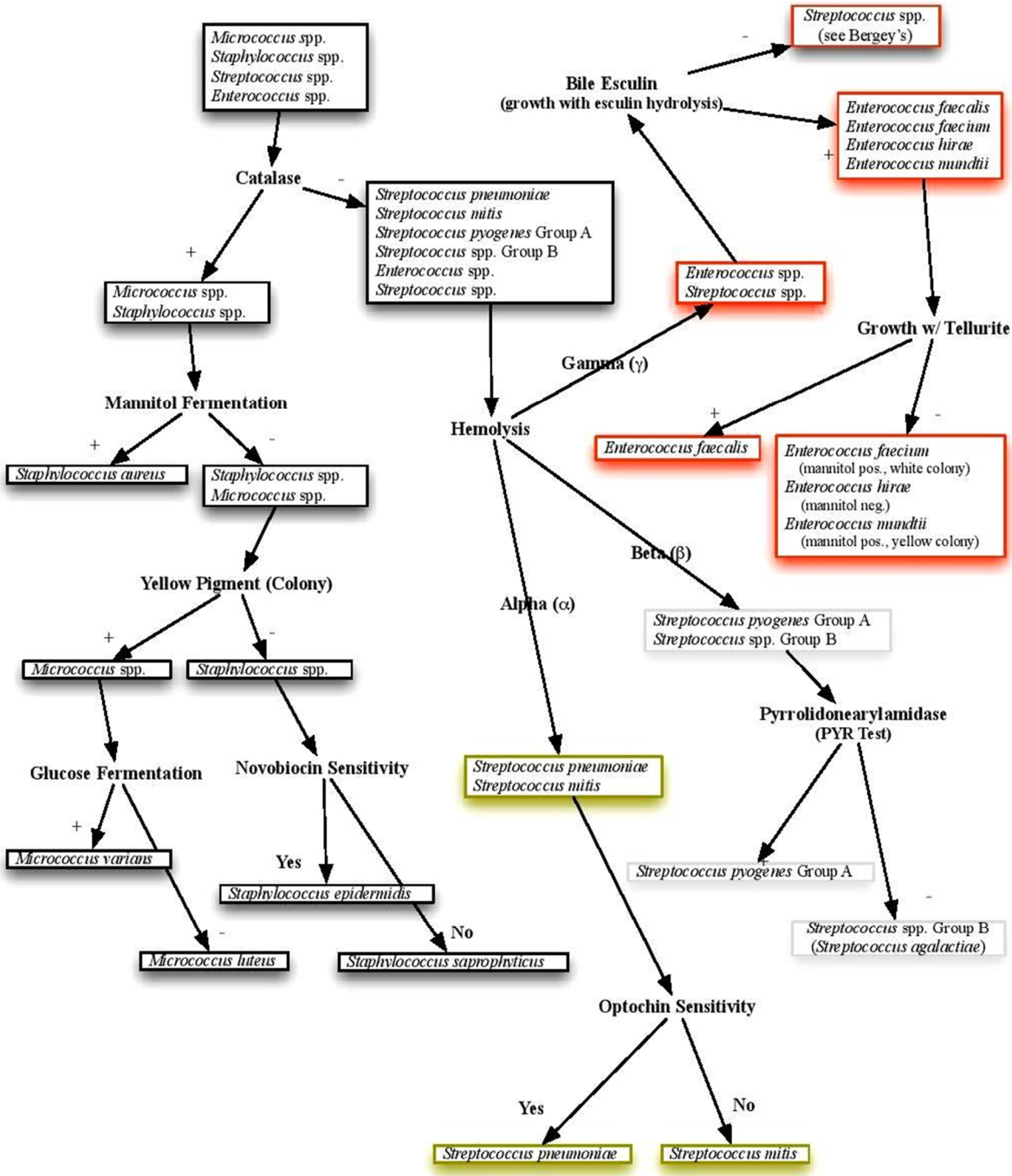
Gram Positive Rods ID Flowchart



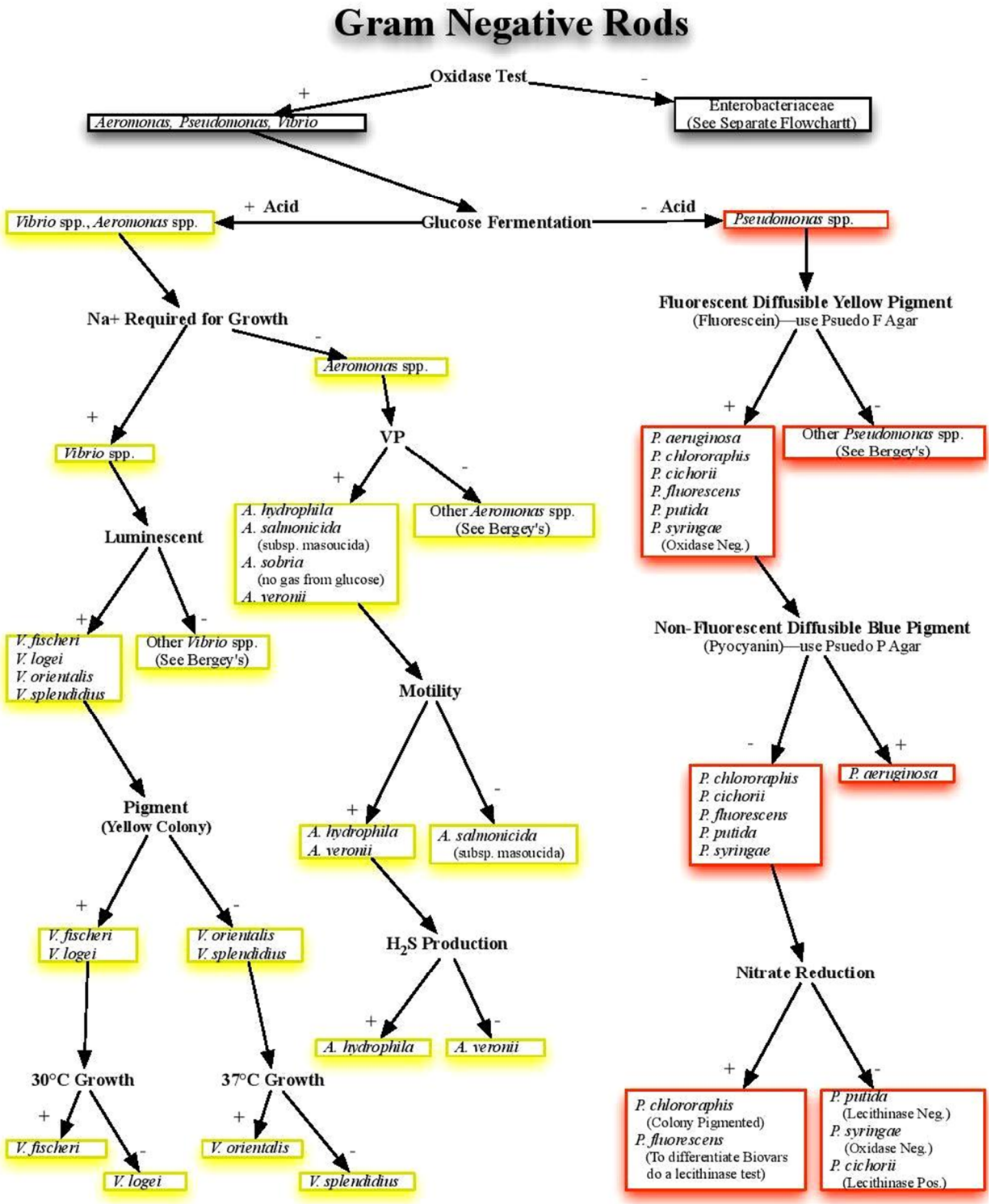
Bacillus spp. ID Flowchart



Gram Positive Cocci ID Flowchart



Gram Negative Rods ID Flowchart



4.5 Extraction of phorbol esters from pressed seed cake: (Xiang *et al*, 2013)

Phorbol esters, the toxins were extracted with absolute Methanol.

5g of seedcake was weighed and was mixed to a flask

36ml of methanol

⇩

Incubation in an ultrasound bath for 15 min

⇩

Extract fraction was separated into other flask

⇩

Extraction process was repeated for another two times

and the extract fractions were pooled together

⇩

Methanol was removed in a Rotary Evaporator under vacuum at 60ºC

⇩

extract was then fully dried in an air flow oven at 40ºC

For HPLC analysis, the extract powder was dissolved in methanol at 2mg/ml and then filtered using Whatman filter paper no. 2.

Figure 6: Rotary Evaporator

4.6 Fermentation strategy for degradation of PE by selected strain:

Media preparation:

Starch	2.0% (w/v)
KH_2PO_4	0.5% (w/v)
NH_4NO_3	0.8% (w/v)

$MgSO_4.7H_2O$ 0.001% (w/v)

$CaCl_2.2H_2O$ 0.01% (w/v)

$Na_2HPO.12H_2O$ 0.8 % (w/v)

100 ml media was taken in a 250ml Erlenmeyer flask.

Inoculum: 1.0%

Culture was revived in a nutrient broth overnight at 37ºC under shaking conditions and this overnight culture was then used to inoculate the media so prepared. Flasks were then incubated in a rotary shaker at 37ºC for 24 hrs at 100 rpm. After the completion of incubation time, culture media was transferred into falcon tubes, centrifuged at 4000rpm for 20 min, then the supernatant was decanted into another sterile flask aseptically. Seed-cake was autoclaved and 5g of it was then added to each of the flask.

Time: PE Detoxification tests were conducted at different intervals as 3 hr, 6 hr, 9 hr and 12 hr.

Incubation: Incubation was done at 37ºC at 100 rpm so as to treat the seed cake with cell free fermented broth. The cultures were incubated for 3hr, 6hr, 9hr, and 12hr. Control was prepared for each time interval.

For the extraction of residual PE from test and control, incubation process was terminated at different set intervals and seed-cake was separated from the seed-cake media as:

seed-cake media was transferred into falcon tube, centrifuged at 4000rpm for 20 min, supernatant decanted and seed-cake as pellet was separated. Further, phorbol esters were extracted according to (Xiang *et al*, 2013).

4.7 HPLC analysis:

HPLC analysis was done in accordance to Hipal *et al* (2009) in which conditions maintained were as follows:

Reverse phase C-18, 5µ, 250 ×4.6 mm analytical column and compound was studied at 280nm using UV detector.

Flow rate 1.3 ml/min;

column temp 30 ºC;

pressure 70-160 bar.

Mobile phase- acetonitrile and deionized water in ratio of 4:1.

RESULTS AND DISCUSSION:

5.1 Proximate composition of seed cake analysis

5.1.1 Ash percentage = 4.5%

5.1.2 Moisture content percentage of seed cake was found to be 5 %.

5.1.3 Protein content was found to be 33%.

5.1.4 Fat content = 7.15 %

Table 3: Result of proximate composition

	Estimated	Reference (Inekwe U.V. et al ,2012)
Ash content	4.5%	3.56 ± 1.39
Moisture content	5%	3.01 ± 0.14
Protein content	33%	24.83 ± 4.79
Fat content	7.15 %	7.2 ± 2.5

5.2 Isolation and Screening Results:

Microbial colonies were isolated from two types of soil i.e. from petrol pump and near rhizosphere beneath *Jatropha tree* with the help of Serial dilution.

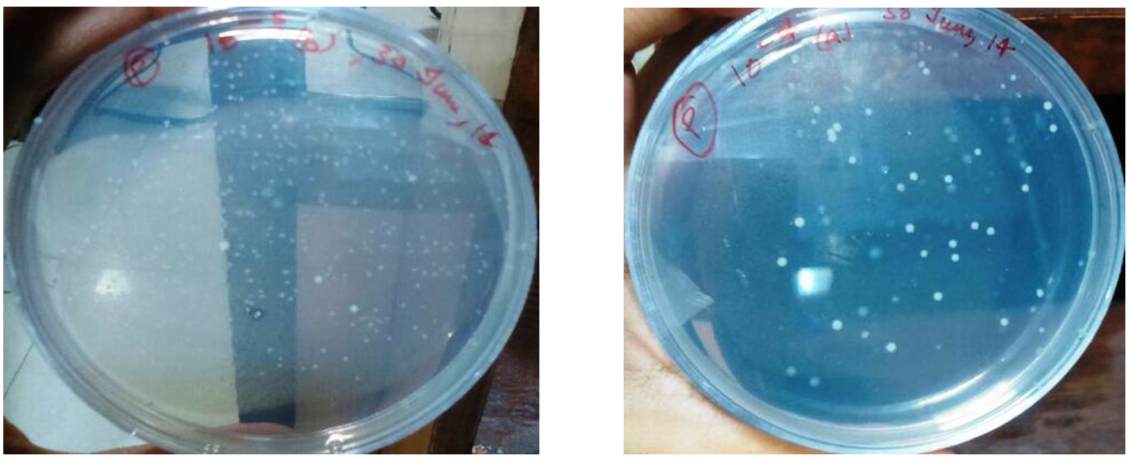
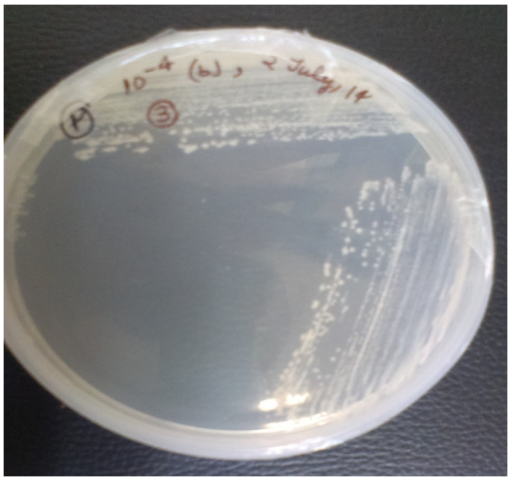


Figure 7: serial dilution plates

(a) Screening using Tween-20 plates:

Preliminary screening was done using tween-20. Lipase/esterase producers on growth would have produced lipolytic enzymes and acted on tween-20 that resulted in the formation of white crystals of calcium salts of fatty acids within the media. Out of 26 streaked microbial colonies, 19 were screened that could be esterolytic enzyme producers.



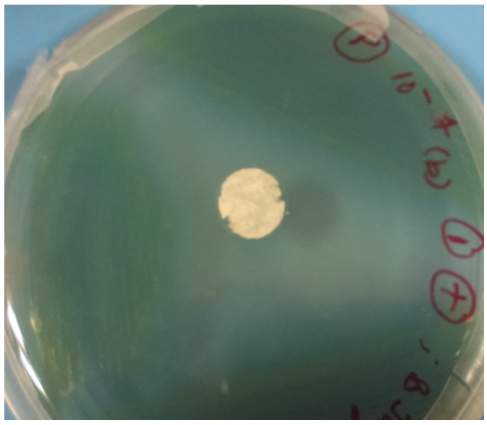
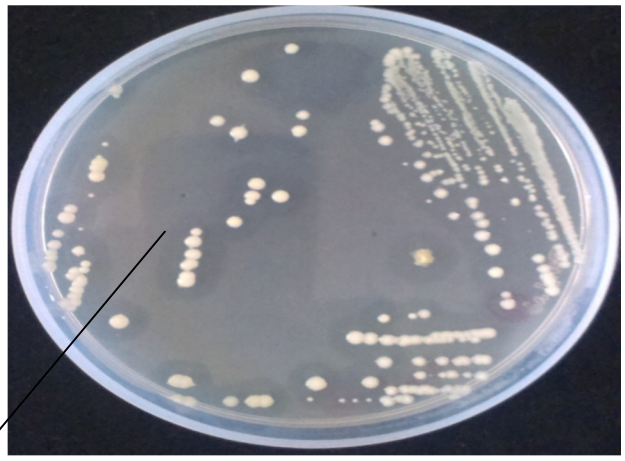
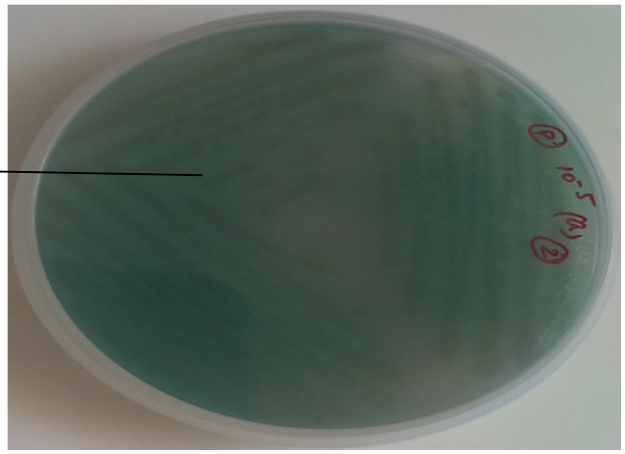


Figure 8: Tween-20 plates with white calcium salts of fatty acids formed in media

Then, lipase or esterase activity was confirmed by the formation of zone of hydrolysis around the bacterial colonies. All 19 colonies that were screened as Tween-20 positive showed clear zone of hydrolysis with tributyrin.

(b) Screening using Tributyrin plates –





Lipolytic/esterolytic activity is shown by the CLEARANCE ZONE OF HYDROLYSIS around the Bacterial colonies . .

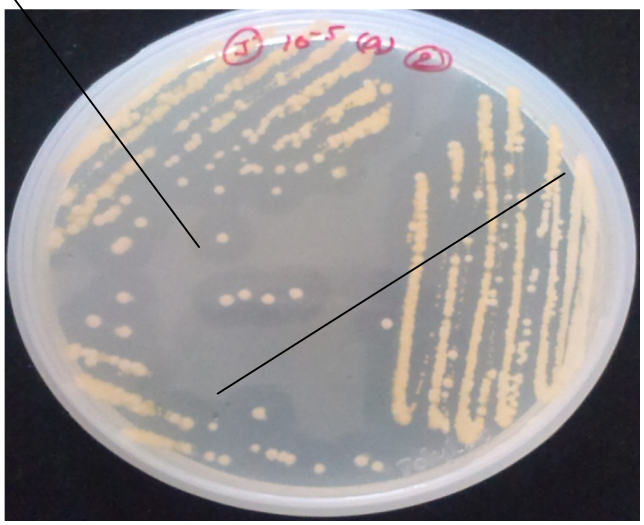


Figure 9: Tributyrin plates depicting clearance zones around bacterial colonies

5.3 Plate Assay for potent PE degradator (Tributrin halo zone method)

Tributyrin agar plate assay was done with the help of Tributyrin halo zone method for the selection of most potential strain producing enzyme for the phorbol esters 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.

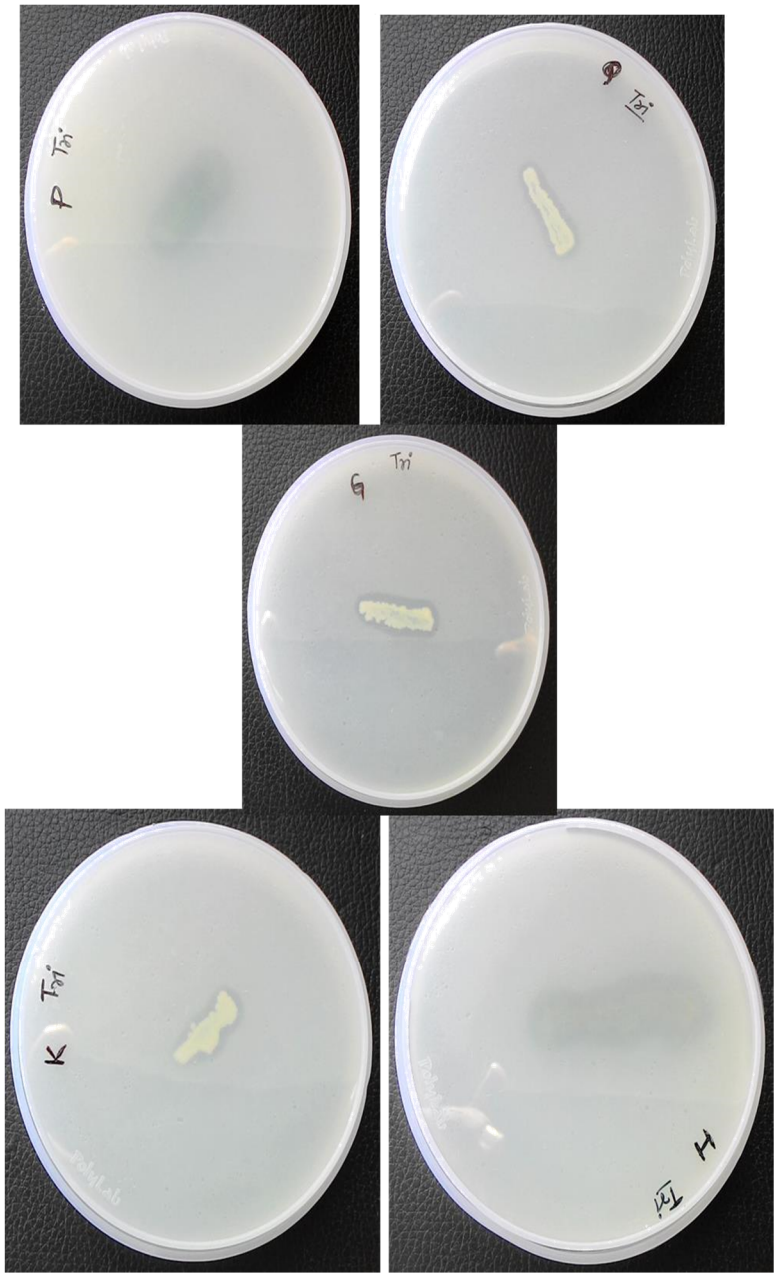


Figure 10: Plates showing halozone formation

5.4 Well Plate Assay:

Five strains screened on the basis of halo zone method, then undergone well plate assay to select the most potential strain that could be carried further for the detoxification of seed cake. The strain that will show maximum clearance zone will be selected.

Figure 11: Plates showing well plate assay

Developed clear zones around the wells were measured (mm) and the data was used for further. Well diameters (in well-plate assay) of the potential lipolytic/esterolytic strains:

Diameter of well => 8.5mm

P => 1.3cm

S => 1.5cm

A => 1.2cm

R => 1.3cm

H => 1.3cm

Out of these five strains, strain S showed maximum clearance zone around the well and was taken further for setting experiments to degrade Phorbol esters present in the seed cake.

5.5 Biochemical identification of selected potential strains:

5.5.1 Gram stain Test

Gram negative rods were found under light microscope.

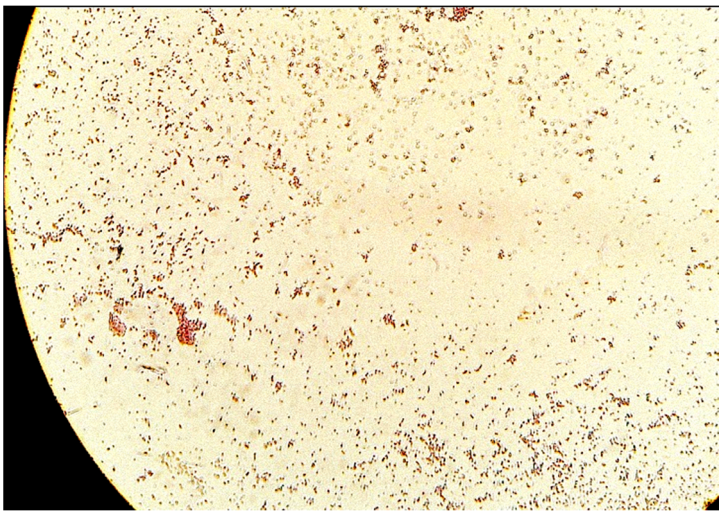


Figure 12: Microscopic view of Gram negative rod shaped bacteria

5.5.2 Methyl Red test

Strain ‘S’ showed negative test . No pink color was developed .

Figure 13: Absence of pink color indicating negative methyl red test

5.5.3Voges–Proskauer Test

Absence of rose color showed negative result for strain ‘S’

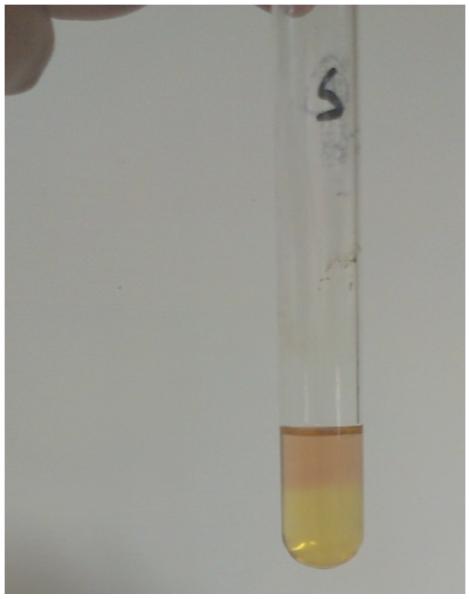


Figure 14: negative voges proskauer test

5.5.4 Catalase Test

Strain ‘S’ was observed with the liberation of nascent oxygen in the form of bubbles. This indicates the production of catalase. Thus it showed positive catalase test.

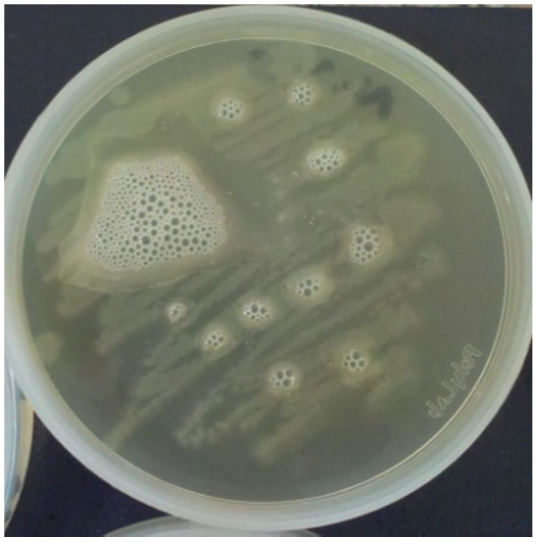


Figure 15: Bubbles showing positive catalase test

5.5.5 Urease Test

Positive urease reaction is indicated by a deep cerise (cherry red/ bright red) colour . Strain ‘S’ showed positive urease test .

Figure 16: Red color indicating positive urease test

5.5.6 Citrate Test

Formation of deep blue colour in the incubated slants indicate positive reaction for citrate utilization. Strain ‘S’ did not show such color and showed negative citrate test .

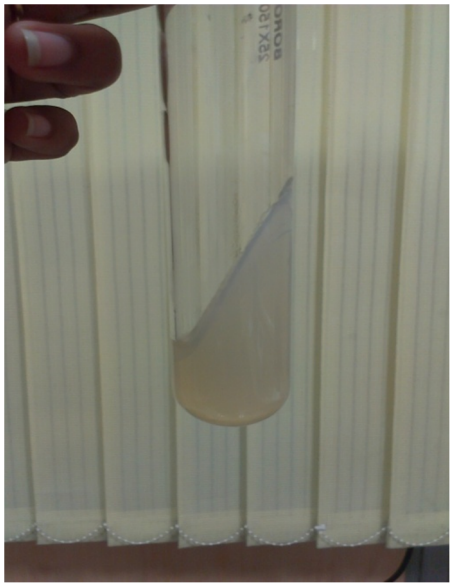


Figure 17: Negative citrate utilization test

5.5.7 Nitrate Reduction Test

Development of orange/brick red colour is indicator of nitrate reduction to nitrite. Strain ‘S’ stayed colorless during nitrate reduction test .

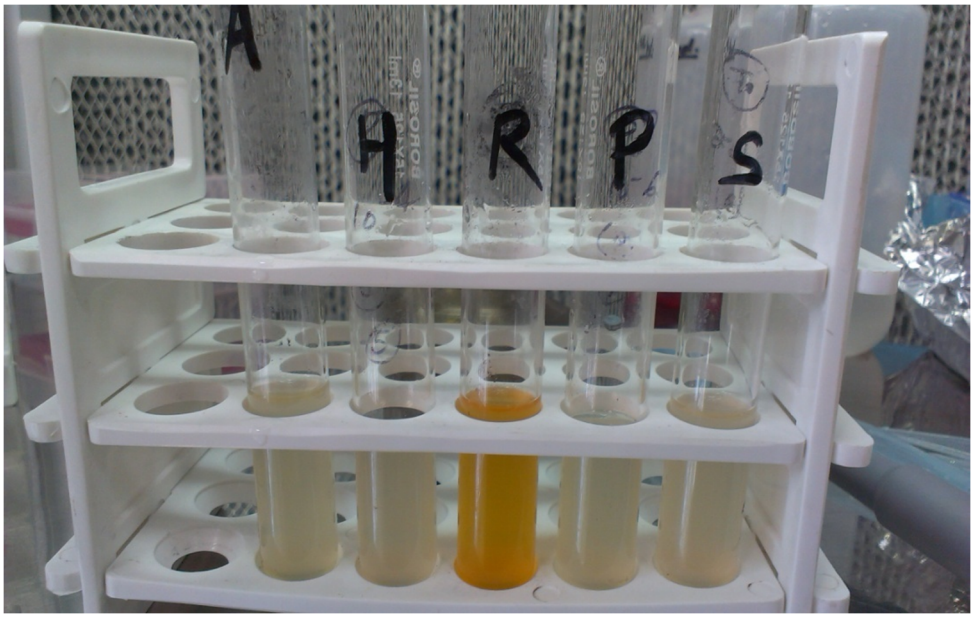


Figure 18: Tube S stayed colorless

TESTS	STRAIN 'S'
Grams stain test	-ve Rods
Methyl Red test	–
Voges- Proskauer test	–
Catalase test	+
Citrate test	–
Urease test	+
Nitrate	–

Table 4: Morphological and Biochemical characterization of strain 'S'

Identification and characterization of strain 'S' was done according to Bergey's Manual of Determinative Bacteriology. Strain 'S' was found to be of ***Pseudomonas*** genera.

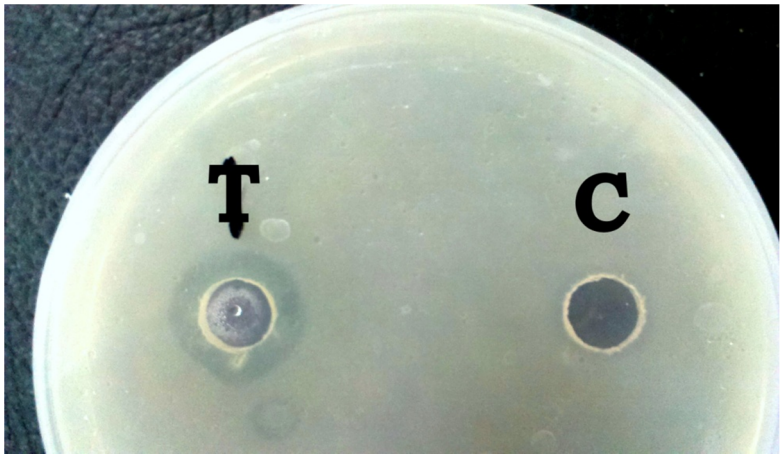


Figure 19: Supernatant from Test and Control; showing esterolytic enzyme activity in Test.

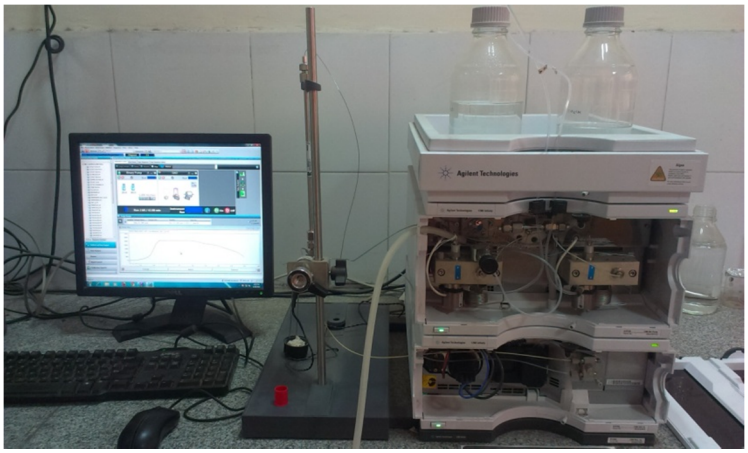
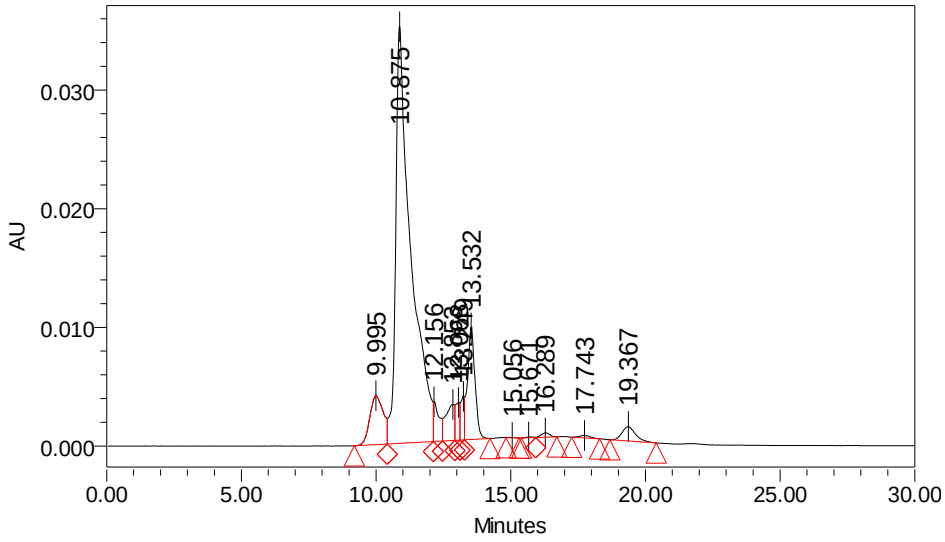


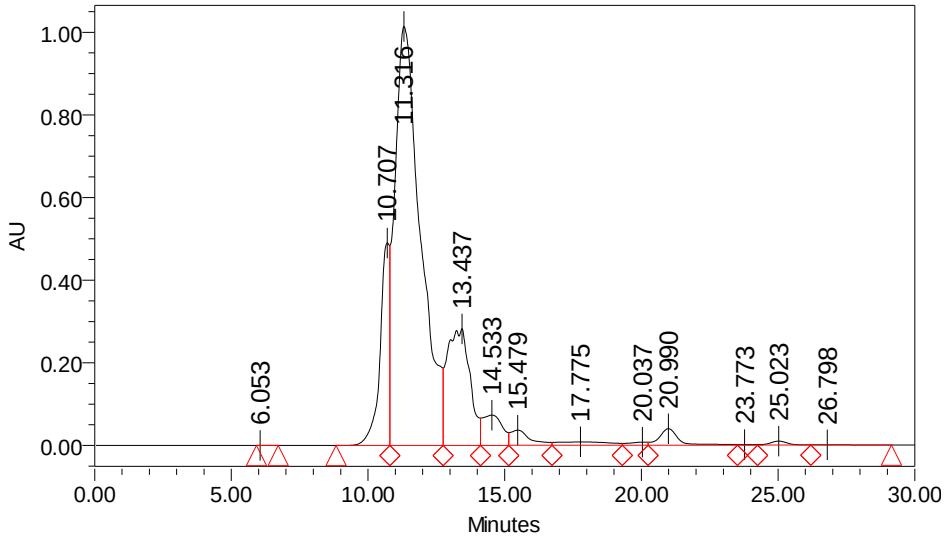
Figure 20: HPLC instrument

1. Standard



	Name	Retention Time	Area	% Area	Height	Int Type	Amount	Units	Peak Type	Peak Codes
1		9.995	142883	7.34	4115	BV			Unknown	
2		10.875	1374079	70.62	35179	VV			Unknown	
3		12.156	48874	2.51	3360	VV			Unknown	I38
4		12.853	71003	3.65	3027	VV			Unknown	
5		13.068	34064	1.75	3176	VV			Unknown	I19
6		13.249	34435	1.77	3675	VV			Unknown	
7		13.532	183028	9.41	9407	VB			Unknown	
8		15.056	333	0.02	25	BB			Unknown	
9		15.671	1147	0.06	60	BV			Unknown	
10		16.289	7988	0.41	366	VB			Unknown	
11		17.743	5673	0.29	196	BB			Unknown	
12		19.367	42097	2.16	1208	BB			Unknown	

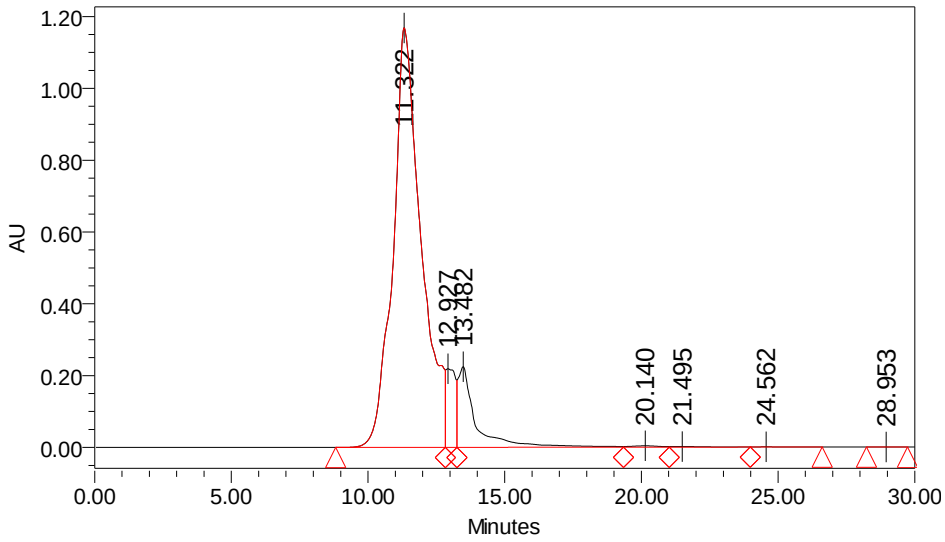
2. C5



	Name	Retention Time	Area	% Area	Height	Int Type	Amount	Units	Peak Type	Peak Codes
1		6.053	637	0.00	23	BB			Unknown	
2		10.707	11655086	11.24	489928	BV			Unknown	
3		11.316	66081784	63.75	1014043	VV			Unknown	
4		13.437	16437199	15.86	282199	VV			Unknown	
5		14.533	3703402	3.57	73091	VV			Unknown	
6		15.479	1849243	1.78	36703	VV			Unknown	

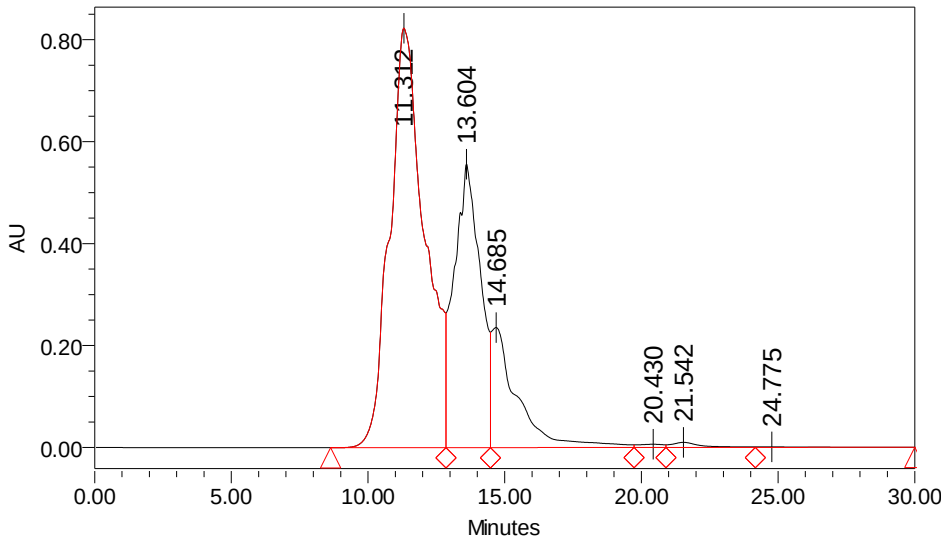
7		17.775	1073867	1.04	8220	VV			Unknown	
8		20.037	350965	0.34	7515	VV			Unknown	
9		20.990	1952546	1.88	39979	VV			Unknown	
10		23.773	62410	0.06	1469	VV			Unknown	
11		25.023	435919	0.42	9670	VV			Unknown	
12		26.798	61080	0.06	743	VB			Unknown	

3. T5



	Name	Retention Time	Area	% Area	Height	Int Type	Amount	Units	Peak Type	Peak Codes
1		11.322	83380407	83.82	1168396	BV			Unknown	
2		12.927	5270032	5.30	218679	VV			Unknown	
3		13.482	10367159	10.42	224504	VV			Unknown	
4		20.140	268614	0.27	3723	VV			Unknown	
5		21.495	155226	0.16	1732	VV			Unknown	
6		24.562	35485	0.04	585	VB			Unknown	
7		28.953	1350	0.00	29	BB			Unknown	

4. U



	Name	Retention Time	Area	% Area	Height	Int Type	Amount	Units	Peak Type	Peak Codes
1		11.312	73289559	57.42	822890	BV			Unknown	
2		13.604	37321698	29.24	556308	VV			Unknown	
3		14.685	15741584	12.33	235592	VV			Unknown	
4		20.430	402041	0.31	6302	VV			Unknown	
5		21.542	749734	0.59	9987	VV			Unknown	
6		24.775	139842	0.11	1158	VB			Unknown	

From above results, it can be observed that Control shows the presence of phorbol esters with the peak observed having retention time around 38 min and the low peak area in comparison with untreated seed cake can be attributed to the subjection of seed cake to autoclave. Peak with retention time around 23 min is due to the presence of PM or PA as impurities due to self degradation of PMA; they will also get degraded due to degradation into their respective components phorbol, acetate, myristate. Test for 3hr, 6hr, 9hr and 12hr shows the decrease in the amount of phorbol esters as a result of esterolytic enzyme. In 12hr almost all phorbol esters degraded.

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.691	BV	0.0996	209.62411	32.33964	4.0038
2	2.019	VB	0.0759	7.24987	1.36632	0.1385
3	2.549	BB	0.2654	134.80873	6.35840	2.5748
4	3.200	BV	0.1033	11.96904	1.56335	0.2286
5	3.366	VB	0.1029	9.55538	1.25363	0.1825
6	4.047	VB	0.1196	12.78485	1.70333	0.2442
7	4.333	BB	0.0963	6.21700	1.00356	0.1187
8	5.073	VB	0.1208	20.67245	2.49062	0.3948
9	5.699	BV	0.1352	85.70481	9.50450	1.6370
10	5.879	VB	0.1344	39.51454	4.33250	0.7547
11	7.676	BB	0.2142	32.15160	2.28374	0.6141

Table 5: Standard- PMA

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.405	BB	0.5841	304.24060	6.24839	0.0954
2	2.052	BV	0.1332	2.92082e4	2679.10889	9.1617
3	2.118	VV	0.2990	6.04394e4	2680.95093	18.9580
4	3.276	VB	0.0922	161.76709	26.88833	0.0507
5	3.530	VB	0.1406	77.92155	8.69137	0.0244
6	3.955	BB	0.1112	611.72675	79.98375	0.1919
7	4.429	BB	0.0915	239.21608	41.32730	0.0750
8	4.757	BV	0.1565	876.17395	79.54966	0.2748
9	5.072	VB	0.1391	2751.17554	317.52225	0.8630
10	5.484	BB	0.1878	155.24573	14.17260	0.0487
11	5.821	BV	0.1316	325.38269	37.36731	0.1021
12	5.967	VB	0.1279	216.04521	24.73594	0.0678
13	6.536	BV	0.1511	155.90022	16.07503	0.0489
14	6.774	VB	0.1519	92.54050	9.48126	0.0290
15	7.344	BB	0.1865	376.25409	29.95807	0.1180
16	8.071	BV	0.2702	1051.00476	53.26932	0.3297
17	8.412	VB	0.2716	353.66574	18.29193	0.1109
18	9.192	BB	0.2234	33.75985	2.22042	0.0106
19	9.869	BB	0.3552	64.92682	2.40345	0.0204
20	11.877	BB	0.3204	100.51727	4.40280	0.0315
21	13.511	BB	0.4015	129.55475	4.53201	0.0406
22	15.401	BB	0.4244	1438.19519	48.43310	0.4511
23	20.794	BV	0.2537	4382.74023	226.52908	1.3747
24	21.308	VB	1.0192	1.78995e5	2453.46582	56.1453
25	34.308	BB	0.6260	701.86444	13.60115	0.2202
26	37.001	BBA	0.8291	3.55646e4	586.79218	11.1555
Totals :				3.18807e5	9466.00232	

Table 6: Untreated seed-cake

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.019	BV	0.1285	2.68902e4	2691.96875	10.3748
2	2.132	VV	0.3646	7.30434e4	2691.46851	28.1817
3	3.179	VB	0.1220	745.02252	83.56947	0.2874
4	3.377	BB	0.0630	89.21720	23.15129	0.0344
5	3.959	BB	0.0970	522.73553	81.35871	0.2017
6	4.438	BB	0.0979	368.22885	59.73011	0.1421
7	4.771	BV	0.1462	752.21069	74.24472	0.2902
8	5.076	VB	0.1120	2131.90015	296.24564	0.8225
9	5.365	BV	0.0918	60.68470	10.13939	0.0234
10	5.481	VB	0.1438	99.82471	9.56000	0.0385
11	5.783	BB	0.0936	86.69151	14.96523	0.0334
12	6.107	BB	0.1525	324.87814	33.10575	0.1253
13	6.561	BV	0.1430	123.28854	13.69926	0.0476
14	6.805	VB	0.1242	51.49969	6.66016	0.0199
15	7.122	BB	0.1529	51.12844	5.47587	0.0197
16	7.386	BV	0.1513	201.72272	20.05930	0.0778
17	7.599	VV	0.1750	317.36859	25.88593	0.1224
18	7.878	VV	0.1815	414.17239	33.21493	0.1598
19	8.118	VV	0.2176	911.60767	60.57335	0.3517
20	8.450	VB	0.2402	260.43103	15.64445	0.1005
21	9.964	BB	0.1592	13.02414	1.25352	5.025e-3
22	10.482	BB	0.2392	40.36226	2.46275	0.0156
23	11.260	BB	0.2385	31.77261	2.17380	0.0123
24	13.601	BB	0.3403	203.86353	8.79834	0.0787
25	15.424	BB	0.3812	1786.82288	70.62739	0.6894
26	18.544	BB	0.3353	30.23150	1.41491	0.0117
27	21.606	BB	1.0683	1.18664e5	1530.90100	45.7830
28	38.078	BBA	1.2471	3.09715e4	330.54950	11.9494
Totals :				2.59188e5	8198.90204	

Table 7: Control 5

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
3	2.146	VB	0.0881	6.44654	1.01442	32.1448
Totals :				20.05467	3.73651	

Table 8: Test 5

6. CONCLUSION AND FUTURE PERSPECTIVES

Proximate composition of ash content, moisture content and protein has been done and it indicates that *J. curcas* seed cake is rich in protein content. For the degradation of phorbol ester, any microbial flora requires extracellular esterolytic activity for the hydrolysis of ester bond. Therefore, in this dissertation we tried to identify potential lipase producer that is why environmental samples were collected from the soil oil spills. For detoxification, enzymes from bacterial sources have been utilized. The selected bacterial strain has been characterized and identified to be from *Pseudomonas* genera. *Pseudomonas* are well known for their esterolytic activity. The extracellular enzyme from this *Pseudomonas* strain has been utilized for the degradation of phorbol esters present in the seed cake. It has been found that selected strain *Pseudomonas* completely degraded phorbol esters in 10 hours. This is due to the action of esterolytic enzymes on the phorbol esters that break the compound into phorbol, myristic acid and acetic acid. Since these compounds are non-toxic, this fulfills our aim of detoxifying the seed-cake. The seed cake now is safer for disposal thus eliminating the environmental concern and further its high protein content allows it to be used in animal feeds. So, this approach could be viable for breaking down the toxic phorbol esters or seed-cake detoxification.

In future, the detoxified seed-cake could be checked for its in-vitro digestibility and further it could be checked as a feed for rats, fish or poultry.

7. REFERENCES

A. Wakandigara, L. R. M. Nhamo and J. Kugara; 2013; Chemistry of Phorbol Ester Toxicity in Jatropha curcas Seed–a Review; International Journal of Biochemistry Research & Review; 146-161

Ameen, O. M., Belewu, M. A.Onifade, O. Adetutu; 2011 ;Chemical Composition Of Biologically Treated Jatropha Curcas Kernel Cake; Vol.2 pp.757-759

Anna L.M.T. Pinghelli, Roseli A. Ferrari, Maria C.N.A. Machado, Kil J Park; 2012; Study of *Jatropha curcas* L. cake detoxification; CIGR-Ageng International Conference of Agricultural Engineering

Azza A. Abou-Arab and Ferial M. Abu-Salem; 2010; Nutritional quality of *Jatropha curcas* seeds and effect of some physical and chemical treatments on their anti-nutritional factors; African Journal of Food Science Vol. 4(3) pp. 93-103

Belewu, M.A., Belewu, K.Y and Ogunsola; 2010; Nutritive value of dietary fungi treated Jatropha curcas kernel cake: Voluntary intake, growth and digestibility coefficient of goat; Agriculture And Biology Journal Of North America

Bhavani M, G V Chowdary, David M, Archana G; 2012; Screening, Isolation and Biochemical Characterization of Novel Lipase Producing Bacteria from Soil Samples; International Journal of Biological Engineering 2012, 2(2) pp. 18-22

Candida R. M. de Barros, Luis M. M. Ferreira, Fernando M. Nunes, Rui M. F. Bezerra, Albino A. Dias, Cristina V. Guedes, John W. Cone, Guilhermina S. M. Marques and Miguel A. M. Rodrigues; 2011; The potential of white-rot fungi to degrade phorbol esters of *Jatropha curcas* L. seed cake

Chetna Joshi, Priyanka Mathur, S.K. Khare; 2011; Degradation of phorbol esters by *Pseudomonas aeruginosa* PseA during solid-state fermentation of deoiled *Jatropha curcas* seed cake; Bioresource Technology 102, pp. 4815–4819

Chin-Feng Chang, Jen-Hsien Weng, Kao-Yung Lin, Li-Yun Liu, Shang-Shyng Yang; 2011; Phorbol Esters Degradation and Enzyme Production by Bacillus using Jatropha Seed Cake as Substrate; International Journal of Environmental pollution and remediation.

Danielle Branta Lopes, Laira Priscila Fraga, Luciana Francisco Fleuri, Gabriela Alves Macedo; 2011; Lipase and esterase-to what extent can this classification be applied accurately?; pp. 608-613

Donlaporn Saetae and Worapot Suntornsuk; 2011; Toxic Compound, Anti-Nutritional Factors and Functional Properties of Protein Isolated from Detoxified Jatropha curcas Seed Cake ; International Journal of Molecular Sciences

E. M. Aregheore, K. Becker and H.P.S. Makkar; 2003; Detoxification of a toxic variety of Jatropha curcas using heat and chemical treatments and preliminary nutritional evaluation with rats; pp. 50-56

E Mobarak-Qamsari, R Kasra-Kermanshahi, and Z Moosavi-nejad; 2011; Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110; Iran J Microbiol.; pp. 92–98

E. Sirisha, N. Rajasekar and M. Lakshmi Narasu; 2010; Isolation and Optimization of Lipase Producing Bacteria from Oil Contaminated Soils; Advances in Biological Research 4 (5); pp. 249-252

Gaur S.; 2009; Development and evaluation of an effective process for the recovery of oil and detoxification of meal from *Jatropha curcas*; Master Thesis: Missouri University of Science and Technology, USA.

Garode Am, Sonune Na; 2014; Screening and Identification Of Lipase producing Bacteria from Domestic Wastewater; International Journal Of Pharma and Bio Sciences 5(2) pp. 265 – 268

Inekwe U.V., Odey M.O., Gauje B, Dakare A.M., N Keonya O, Bala S., Mohammed G.; 2012; Comparative Proximate Composition Of Partially Deoiled *Jatropha curcas* seed from India, Kaduna, Edu; pp.151- 154

K. Larbidaouadi, Z. Benattouche and B. Abbouni; Screening selection identification production and optimization of bacterial lipase isolated from industrial rejection of gas station; Journal of Chemical and Pharmaceutical Research, 2014, 6(6); pp. 455-459

Kalpana Sagar, Yasir Bashir, Mayur M Phukan, B. K. Konwar; October 2013; Isolation Of Lipolytic Bacteria From Waste Contaminated Soil: A Study With Regard To Process Optimization For Lipase; International Journal Of Scientific & Technology Research Volume 2, Issue 10

Kazi Mostafijur Rehman, Mohammad Mahsud, Md. Roknuzzannam and Assadullah Al Galib; 2010; Biodiesel from Jatropha oil as an alternative fuel for diesel engine; International Journal of Mechanical and Mechatronics Engineering IJMME-IJENS, Vol 10 No.3

M. Prasanna Rajeshkumar, V.S. Mahendran and V. Balakrishnan; 2013; Isolation and identification of lipase producing organisms from diverse soil samples of Kolli hills; International Journal of current Microbiology and Applied sciences Volume 2 Number 5, pp. 205-210

M.Veerapagu, Dr .A. Sankara Narayanan, K.Ponmurugan, K.R.Jeya; 2013; Screening Selection Identification Production And Optimization Of Bacterial Lipase From Oil Spilled Soil; Asian Journal of Pharmaceutical and Clinical Research; Vol 6, Suppl 3

Maria Catarina Megumi Kasuya, José Maria Rodrigues da Luz, Lisa Presley da Silva Pereira, Juliana Soares da Silva, Hilário Cuquetto Montavani and Marcelo Teixeira Rodrigues; 2013; Bio-Detoxification of Jatropha Seed Cake and Its Use in Animal Feed

Rakshit, K.D. & Bhagya, S.**;** 2007; Effect of processing methods on the removal of toxic and anti-nutritional constituents of *Jatropha* meal: A potential protein source. Journal of Food Science and Technology, 3, pp. 88–95.

Rakshit K. Devappa, Harinder P. S. Makkar And Klaus Becker; 2010; Nutritional, Biochemical, and Pharmaceutical Potential of Proteins and Peptides from Jatropha: Review ; J. Agric. Food Chem.

S. Ertugrul, G. Donmez, S. Taka; 2007; "Isolation of lipase producing *Bacillus sp.* from olive mill wastewater and improving its enzyme activity", Journal of Hazardous Materials, vol.149, pp.720– 724

Sahu Rahul, Awasthi G., Kumar L., Singh Kamraj; Isolation And Characterization Of Bacteria From Soil Collected From Himalayan Region For The Production Of Lipase By Solid State Fermentation Using Tween-20; Oct. Jour. Env. Res. Vol. 1(1), pp. 23-29

Thanyarat Phengnuam and Worapot Suntornsuk; 2013; Detoxification and anti-nutrients reduction of Jatropha curcas seed cake by Bacillus fermentation .

Harinder P.S. Makkar, Vikas Kumar and Klaus Becker; 2012; Biofuel co-product as livestock feed; Chapter 21-Use of detoxified jatropha kernel meal and protein isolate in diets of farm animals; pp. 351-374

Verma N, Thakur S and Bhatt A.K; December, 2012; Microbial Lipases: Industrial Applications and Properties; International Research Journal of Biological Science, Vol. 1(8), pp. 88-92.

Xing-Hong Wang, Lingcheng Ou, Liang-Liang Fu, Shui Zheng, Ji-Dong Lou, Josè Gomes-Laranjo, Jiao Li, Change Zhang; 2013; Detoxification of Jatropha curcas kernel cake by a novel Streptomyces fimicarius strain; Journal of hazardous materials 260, pp. 238-246