DETOXIFICATION AND ANTI-NUTRIENT REDUCTION OF *JATROPHA CURCAS* SEED CAKE BY FERMENTATION USING *BACILLUS* SPECIES

BY

AMINAT OLUWADAMILOLA, FAJINGBESI

DEPARTMENT OF MICROBIOLOGY,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

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BY

Aminat Oluwadamilola FAJINGBESI

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DEPARTMENT OF MICROBIOLOGY,

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DECLARATION

I, declare that the work in this dissertation entitled **"Detoxification and Anti-nutrient reduction of *Jatropha curcas* seed cake by fermentation using *Bacillus* Species"** was carried out by me in the Department of Microbiology under the supervision of Prof. C.M.Z. Whong and Prof. J.B. Ameh. The information from the literature was duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for any degree or diploma in any University.

FAJINGBESI, Aminat Oluwadamilola _____ _____

Signature Date

CERTIFICATION

This dissertation entitiled “Detoxification and Anti-nutrient reduction of *Jatropha curcas* seed cake by fermentation using *Bacillus* Species” meets the regulations governing the award of the degree of Masters of Science in the Department of Microbiology, Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Prof. C.M.Z. Whong Chairman, Supervisory Committee	_____ Signature	_____ Date
Prof. J.B. Ameh Member, Supervisory Committee	_____ Signature	_____ Date
Prof. I. O. Abdullahi Head of Department	_____ Signature	_____ Date
Prof. Kabir Bala Dean, School of Postgraduate Studies	_____ Signature	_____ Date

DEDICATION

This dissertation is dedicated to Almighty Allah, my parents Alhaji and Alhaja D.B. Fajingbesi, my siblings and friends.

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All due praises and thanks to Almighty Allah (S.W.T.), for my being and ability to achieve this feat is due to his greatness. Though the struggle was tough and the road was rough, but the journey was well made through the glory of Allah.

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Abbreviations

J. curcas – *Jatropha curcas*

J. curcas L. or JCL – *Jatropha curcas* Linnaeus

LD_{50} – lethal dose 50

GHG Emissions – Greenhouse Gas Emission

FAO – Food and Agriculture Organisation

BamH1 – *Bacillus amyloliquefaciens* H endonuclease

DNA – Deoxyribonucleic Acid

TSAg-medium – Terbium scandium aluminum garnet medium

BAC 1 and BAC 2 – *Bacillus* 1 and 2

0.02N – 0.02 Normal

ABSTRACT

Jatropha curcas seed cake is a by-product generated from the oil extraction of *J. curcas* seed- a biodiesel producing plant's seed. Although, the seed cake contains a high level of protein, it has Phorbol ester and some anti-nutritional factors such as phytic acid, saponin, lectin and trypsin inhibitor making it not to be applied directly in the food or animal feed industries. This study was aimed at detoxifying the toxin and reducing the anti-nutritional factors in *J. curcas* seed cake by fermentation using *Bacillus* species. Three *Bacillus* strains (*Bacillus coagulans, Paenibacillus macerans, Paenibacillus polymyxa)* 1.0×10^8 cells MacFarland's standard per 100ml were used in the study. The seed cake used for the detoxification was extracted both manually and with the use of a machine. This fermentation was carried out on 10g of seed cake in 100ml of distilled water for 5 days with submerged fermentation. Temperature (27^0 C, 30^0 C and 37^0 C), pH (4.5, 6.5, 8.5) and Time (24 h, 48 h, 72 h, 96 h and 120 h) were also varied. After fermentation the toxin and anti-nutritional factor level was determined. Results showed that *Paenibacillus macerans* was able to degrade the toxin and reduce the anti-nutritional factors in the seed cake more than the other two. After fermentation phorbol ester A and B, phytic acid, saponin, lectin and trypsin inhibitor were reduced by 76.4 %, 99.3 %, 56.3 %, 43.6 %, 58.8 % and 64.9 % respectively. The reduction may be due to the activities of esterase, phytase and protease enzymes. *Jatropha curcas* seed cake was detoxified by bacterial fermentation using the three *Bacillus* strains and the rich protein fermented seed cake could be potentially used as animal feed.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Jatropha curcas is a species of flowering plant in the Euphorbiaceae family. It is native to the American tropics, especially Mexico and Central America (Janick and Robert, 2008). It is cultivated in tropical and subtropical regions around the world, becoming naturalized in some areas. The specific epithet, *"curcas"*, was first used by the Portuguese doctor Garcia de Orta more than 400 years ago with uncertain origin. Common names includes Barbados Nut, Purging Nut, Physic Nut and JCL (*J. curcas* Linnaeus), whereas "Lapalapa" (Yoruba) "Binidazugu" (Hausa) and "Owulo idu" (Ibo) in Nigeria. It is a multipurpose tree because of industrial and medicinal uses.

J. curcas is a poisonous, semi-evergreen shrub or small tree, reaching a height of 6 m (Janick and Robert, 2008). It is resistant to a high degree of aridity, allowing it to be grown in deserts. The seeds contain an average of 34.4% oil (Achten *et al.,* 2008) with a range between 27-40% (Achten *et al.,* 2007). Besides the economic potential of processing the oil to produce high-quality biodiesel fuel usable in a standard diesel engine, the seeds also contain the highly poisonous toxalbumin curcin.

Bacillus is a genus of Gram-positive, rod-shaped (bacillus) bacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes (oxygen reliant), or facultative anaerobes (having the ability to be aerobic or anaerobic). They test positive for the enzyme catalase when there has been oxygen used or present (Turnbull, 1996). Ubiquitous in nature, *Bacillus* includes both free-living (non-parasitic) and parasitic pathogenic species.

Under stressful environmental conditions, the bacteria can produce oval endospores and thus remain in a dormant state for very long period of time. (Madigan and Martinko, 2005). The main habitat of endospore-forming *Bacillus* organisms is the soil. *B. subtilis* strains secrete enzymes, such as amylase, protease, pullulanase, chitinase, xylanase, lipase, and esterase are produced commercially and their production represents about 60% of the commercially produced industrial enzymes (Morikawa, 2006).

Esterases and lipases catalyze the hydrolysis of ester bonds and are widely distributed in animals, plants and microorganisms. In organic media, they catalyze reactions such as esterification, intesterification and transesterification (Kawamoto *et al.,* 1987). Esterases differ from lipases mainly on the basis of substrate specificity and interfacial activation (Long, 1971). Esterases are found in plants, animals and microbes, but the majority of industrially produced esterase are derived from microbial sources. This is because they can be engineered for production of esterase with desirable properties for industrial need. The microbial sources include bacteria, fungi, yeasts and actinomycetes (Torres *et al.,* 2005). The applications of esterases are found in various fields, including inorganic synthesis process.

Paenibacillus is a genus of facultative anaerobic, endospore-forming bacteria, originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 (Ash *et al*., 1993). Bacteria belonging to this genus have been detected in a variety of environments such as soil, water, rhizosphere, vegetable matter, forage and insect larvae, as well as clinical samples (Lal and Tabacchioni, 2009: McSpadden-Gardener, 2004: Montes *et al*., 2004: Ouyang *et al*., 2008).

1.2 Statement of Problem

The seeds of *J. curcas* contain oil, which can be used as a renewable biodiesel source and applications in the manufacture of soaps and cosmetics (Makkar *et al.,* 1998). *J. curcas* seed cake is a by-product generated from the oil extraction of *J. curcas* seeds in a biodiesel processing plant. It has high protein content of approximately 50 -60% (Haas and Mittelbach, 2000) and could be used in animal feeds and also as protein hydrolysate. However, it contains the phorbol esters, which are toxic compounds, and the anti-nutritional factors such as trypsin inhibitors, phytic acids, lectins and saponins.

Phorbol esters are the most potent tumor promoters known. They exhibit a remarkable ability to amplify the effect of a carcinogen but are themselves not carcinogenic (Wender *et al.,* 1998). The seeds from *J. curcas* had been reported to be orally toxic to humans, rodents and ruminants of which phorbol esters had been identified as the main toxic agent (Becker and Makkar, 1998). Pure phorbol esters can kill when administered in microgram quantities (Heller, 1996). Ingestion of phorbol esters (LD_{50} for mice: 27mg/kg body mass) can cause lung and kidney damage, resulting in fatality (Li *et al.,* 2010).

Detoxification of toxin is necessary for *J. curcas* seed meal utilization, after which the detoxified seed cake may be used as animal feed and its protein hydrolysate (fermented liquid) as plant growth promoter. Biological detoxification of *J. curcas* seed cake has not been widely studied. However, toxins in cotton seed were successfully detoxified by microbial fermentation (Zhang *et al.,* 2006).

Despite its intrinsic advantages, *J. curcas* seed like soybean seed has the problem of antinutritional factors. In addition to thermos-labile lectins and trypsin inhibitors, *J. curcas*

contains toxic lipo-soluble but thermo-stable phorbol esters (Heller, 1996: Makkar and Becker, 1997). Phorbol esters have to be removed or lowered to levels that do not elicit a toxic response from animals in order for the *J. curcas* seed meal to be used as an ingredient in livestock feeds. Makkah and Becker (1997) reported that phorbol esters were highly soluble in ethanol, giving some possibility of detoxification of the meal.

1.3 Justification

J. curcas seed cake is well adapted to grow in marginal areas with low (480mm) rainfall and poor soils. In such areas, it grows without competing for space with food crops (Gaydou *et al.,* 1982: Heller, 1996). *J.* curcas seed meal (10-20g Kg^{-1} residual oil) has a crude protein content ranging from 580-640g Kg-^{1} of which 90% is true protein (Makkar *et al.,* 1997: Makkar and Becker, 1997). The plant's ability to thrive in marginal areas and its high crude protein makes it an attractive complement and or substitute to soybean meal as a protein source in livestock feeds. The use of *J. curcas* will reduce the competition between man and livestock for soybean that is currently prevailing since soybean is used in both livestock and human feeds. Phorbol esters are the major impediment to the wide commercial use of Jatropha meal as feedstock. During extraction of oil from Jatropha seed, 70-75% of Phorbol esters associate with the oil and 25-30% remain strongly bound to the matrix of seed meal (Wink *et al.,* 1997). The Phorbol esters have been found to be responsible for skin-irritant effects and tumor promotion (Wink *et al.,* 1997). *J. curcas* seed cake is mainly used as manure and can be made more useful when detoxified and hence its use in animal feeds. Thus, this research was set to achieve this following aim and objectives.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to detoxify and reduce the anti-nutritional factors in *J. curcas* seed cake by fermentation using *Bacillus species*.

1.4.2 Specific Objectives

The specific objectives of this study were to:

1. Isolate and identify *Bacillus* species from the soil and kilishi.
2. Determine the proximate composition and phytochemical factors of non-fermented and fermented *J. curcas* seed cake.
3. Determine the reduction of phorbol esters and anti–nutritional factors in *J. curcas* seed cake by fermentation using *Bacillus species*.
4. Determine the optimal environmental condition for the detoxification of the Jatropha curcas seed cake.

1.5 Hypothesis Testing

H_0 - *Bacillus coagulans, Paenibacillus polymyxa* and *Paenibacillus macerans* have no effect on the detoxification and anti-nutritional factors reduction in *J. curcas* seed cake

H_a - *B. coagulans, P. polymyxa* and *P. macerans* have effect on the detoxification and anti-nutritional factors reduction in *J. curcas* seed cake

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and Spread

Jatropha is believed to have been spread by Portuguese seafarers from its centre of origin in Central America and Mexico via Cape Verde and Guinea Bissau to other countries in Africa and Asia. It is now widespread throughout the tropics and sub-tropics. Until recently, *Jatropha* had economic importance in Cape Verde. Since the first half of the nineteenth century, due to its ability to grow on poor soils with low rainfall, it was exploited for oilseed production. Cape Verde exported about 35 000 tonnes of *Jatropha* seeds per year to Lisbon. Along with Madagascar, Benin and Guinea, it also exported *Jatropha* seeds to Marseille where oil was extracted for soap production. However, this trade declined in the 1950s with the development of cheaper synthetic detergents and, by the 1970s, the trade in *Jatropha* oil had disappeared (Wiesenhutter, 2003; Henning, 2004a). In the past, *Jatropha* oil was used for lighting lamps (Gubitz *et al.,* 1999). Today, rural communities continue to use it for its medicinal value and for local soap production. India and many countries in Africa use the *Jatropha* plant as a living hedge to keep out grazing livestock. *Jatropha* is planted in Madagascar and Uganda to provide physical support for vanilla plants. *Jatropha*'s potential as a petroleum fuel substitute has long been recognized. It was used during the Second World War as a diesel substitute in Madagascar, Benin and Cape Verde, while its glycerine by-product was valuable for the manufacture of nitro-glycerine.

2.2 Nomenclature and Taxonomy

J. curcas L. was first described by Swedish botanist Carl Linnaeus in 1753. It is one of many species of the genus *Jatropha*, a member of the large and diverse *Euphorbiaceae* family. Many of the *Euphorbias* are known for their production of phytotoxins and milky white sap. The common name "spurge" refers to the purgative properties of many of these *Euphorbias*. There are 170 known species of *Jatropha*, mostly native to the New World, although 66 species have been identified as originating in the Old World (Heller, 1996). A number of *Jatropha* species are well known and widely cultivated throughout the tropics as ornamental plants. The literature identifies three varieties: Nicaraguan (with larger but fewer fruits), Mexican (distinguished by its less-toxic or non-toxic seed) and Cape Verde. The Cape Verde variety is the one commonly found throughout Africa and Asia.

J. curcas L. has many vernacular names including: physic nut or purging nut (English), *pinhão manso* or *mundubi-assu* (Brazil), *pourghère* (French), *purgeernoot* (Dutch), *Purgiernuss* (German), *purgeira* (Portuguese), *fagiola d'India* (Italian), *galamaluca* (Mozambique), *habel meluk* (Arab), *safed arand* (Hindi), *sabudam* (Thai), *bagani* (Ivory Coast), *butuje* (Nigeria), *makaen* (Tanzania), *piñoncillo* (Mexico), *tempate* (Costa Rica) and *piñon* (Guatemala) (Brittaine and Lutaladio, 2010).

2.3 Description

Jatropha is a succulent perennial shrub or small tree and can attain heights of more than 5 metres depending on the growing conditions. Seedlings generally form a central taproot, four lateral roots and many secondary roots. The leaves, arranged alternately on the stem, are shallowly lobed and vary from 6-15 cm in length and width. The leaf size and shape can

differ from one variety to another. As with other members of this family, the vascular tissues of the stems and branches contain white latex.

The branches and stems are hollow and the soft wood is of little value. *Jatropha* is monoecious, meaning it carries separate male and female flowers on the same plant. There are fewer female than male flowers and these are carried on the apex of the inflorescence, with the more numerous males borne lower down. The ratio of male to female flowers averages 29:1 but this is highly variable and may range from 25-93 male flowers to 1-5 female flowers produced on each inflorescence (Raju and Ezradanum, 2002). It also has been reported that the male-to-female flower ratio declines as the plant ages (Achten *et al.*, 2008), suggesting that fruiting capacity may increase with age.

The unisexual flowers of *Jatropha* depend on pollination by insects, including bees, flies, ants and thrips. One inflorescence will normally produce 10 or more fruits. Fruit set generally results from cross-pollination with other individual plants, because the male flowers shed pollen before female flowers on the same plant are receptive. In the absence of pollen arriving from other trees, *Jatropha* has the ability to self-pollinate, a mechanism that facilitates colonization of new habitats (Raju and Ezradanum, 2002).

The fruits are ellipsoidal, green and fleshy, turning yellow and then brown as they age. Fruits are mature and ready to harvest around 90 days after flowering. Flowering and, therefore, fruiting are continuous, meaning that mature and immature fruits are borne together. Each fruit contains two or three black seeds, around 2 cm x 1 cm in size. On average, the seeds contain 35% of non-edible oil (Brittaine and Lutaladio, 2010).

Jatropha grows readily and rapidly from seeds, which germinate in around 10 days, or from stem cuttings. The plant may reach one (1m) in height and flower within five months under good conditions (Heller, 1996). The growth is sympodial, with terminal flower inflorescences and lateral branching, eventually reaching a height of 3-5m under good conditions. It generally takes four to five years to reach maturity (Henning, 2008). Vegetative growth occurs during the rainy season. During the dry season, there is little growth and the plant will drop its leaves. Flowering is triggered by rainfall and seed will be produced following the end of the rainy season. Seeds are produced in the first or second year of growth. *Jatropha* trees are believed to have a lifespan of 30-50 years or more (Brittaine and Lutaladio, 2010).

2.4 Uses of *Jatropha*

With the present interest in the energy-producing potential of *Jatropha*, it is useful to look at the attributes, both positive and negative of the plant, and to compare the relative energy values of its different parts. It is interesting to note that the energy content of the remaining parts of the fruit after oil extraction exceeds the energy content of the oil.

2.4.1 The *Jatropha* tree erosion control and improved water infiltration

Jatropha has proven effective in reducing the erosion of soil by rainwater. The taproot anchors the plant in the ground while the profusion of lateral and adventitious roots near the surface binds the soil and keeps it from being washed out by heavy rains (Henning, 2004a). *Jatropha* also improves rainwater infiltration when planted in lines to form contour bunds. Furthermore, *Jatropha* hedges reduce wind erosion by lessening wind velocity and binding the soil with their surface roots (Henning, 2004a). Unfortunately, these anti-erosion effects are limited by dry season leaf drop. This means there is less protection at a time when wind

erosion is highest and there is no leaf canopy to protect the soil when the first heavy rains fall. Growth of drought-resistant ground cover plants such as agave may enhance the beneficial effects of Jatropha. It appears that *Jatropha* has little negative allelopathic effect on other plants (Weisenhutter, 2003).

2.4.2 Livestock barrier and land demarcation

In many tropical and subtropical countries, *Jatropha* cuttings are planted as a hedge to protect gardens and fields from wandering animals. Livestock will not eat the mature leaves and even goats will die of starvation if there is only *Jatropha* to browse (Henning, 2004a). For the same reason, *Jatropha* is often planted to mark homestead boundaries. Hedges planted very close together (5 cm) form a barrier that is impenetrable even by chickens.

2.4.3 Fuelwood

The wood of *Jatropha* is soft and hollow and, contrary to some reports in the literature, is not good fuelwood. *Jatropha* groves on the islands of Cape Verde have been used as a fuelwood source, mainly due to the lack of other suitable species (Brittaine and Lutaladio, 2010).

2.4.4 Support for vanilla

Jatropha is grown as a support and shade tree in smallholder vanilla farms in Madagascar and Uganda. The tree stems are pruned while the canopy is left to provide shade. As a result, vanilla plantations report low *Jatropha* seed yields of around 200 kg per ha (Henning, 2004a).

2.4.5 Green manure

Jatropha trees grown from seed develop taproots. Thus, they are able to extract minerals that have leached down through the soil profile and return them to the surface through leaf fall, fruit debris and other organic remains. In this way, *Jatropha* acts as a nutrient pump which helps rehabilitate degraded land (Brittaine and Lutaladio, 2010).

2.4.6 Plant extracts

Jatropha plant extracts have many uses in traditional societies (Heller, 1996). The dried latex resembles shellac and is used as a marking ink. The leaves and bark are used for dyeing cloth. *Jatropha* has medicinal qualities, including a blood coagulating agent and antimicrobial properties that are widely used in traditional medicine and for veterinary use. All parts of the plant are used. Some of these uses that are briefly described below are to some extent anecdotal.

2.4.7 Stem

The latex has a widespread reputation for healing wounds and stopping bleeding, and for curing various skin problems. It is used against pain and the stings of bees and wasps. The fresh stems are used as chewing sticks to strengthen gums and treat gum disease (Brittaine and Lutaladio, 2010).

2.4.8 Bark and roots

The bark has a purgative effect similar to that of the seeds. In the Philippines, fishermen use the bark to prepare a fish poison. The dried and pulverized root bark is made into poultices and taken internally to expel worms and to treat jaundice. A decoction of the roots is used to treat diarrhoea and gonorrhea (Brittaine and Lutaladio, 2010).

2.4.9 Leaves

The leaves also have a purgative effect. Applied to wounds and in decoction, they are used against malaria and to treat hypertension. The leaf sap is used externally to treat haemorrhoids. A hot water extract of the leaves is taken orally to accelerate secretion of milk in women after childbirth. A decoction is used against cough and as an antiseptic following childbirth (Brittaine and Lutaladio, 2010).

2.4.10 Fruits and seeds

The oil-rich seeds and seed oil are used as a purgative and to expel internal parasites. The oil is applied internally and externally to induce abortion, and externally to treat rheumatic conditions and a variety of skin infections. The oil is also an ingredient in hair conditioners. Besides the current interest in the use of the seed oil for biofuel, it is also used for making soap on a small scale and for illumination. In China, the oil is boiled with iron oxide and used to produce furniture varnish. Extracts of the seed oil have been found effective against a number of crop pests and snail vectors of schistosomiasis (Brittaine and Lutaladio, 2010).

2.5 Toxicity and Invasiveness

2.5.1 Toxicity

Although the seeds are considered the most toxic part of the plant, all parts of the *Jatropha* plant contain toxins such as phorbol esters, curcins and trypsin inhibitors (Jongschaap *et al.*, 2007). Varieties commonly found growing in Africa and Asia has seeds that are toxic to humans and animals, whereas some varieties found in Mexico and Central America are known to be non-toxic.

The poisonous and anti-nutrient properties of the seeds are exploited in traditional medicine for de-worming and as a purgative. Just one to three seeds can produce toxic symptoms in humans, mainly those associated with gastro-intestinal irritation. There is acute abdominal pain and a burning sensation in the throat shortly after ingestion of the seeds, followed by nausea, vomiting and diarrhoea. Children are more susceptible. There is no known antidote, but case studies indicate that following accidental ingestion, often by children, full recovery could be achieved.

Toxicity is chiefly due to the presence of phorbol esters, as inferred from the fact that non-toxic Mexican *Jatropha* varieties are deficient in these compounds. Phorbol esters are known to be co-carcinogenic, meaning that they are tumour promoting in the presence of other carcinogenic substances. It has been reported that phorbol esters decompose rapidly – within six days – as they are sensitive to light, elevated temperatures and atmospheric oxygen (Jongschaap *et al.*, 2007), but there is no supporting data for this. The decomposition of phorbol ester and other toxic compounds in the field needs further evaluation before insecticide or molluscicide oil extracts can be widely used, or before the widespread application of seed cake as fertilizer, particularly on edible crops, given that there is no information as to whether such compounds are taken up by plants. Curcin has been described as a major toxic component of *Jatropha*, similar to ricin, which is a well-known poison. Yet, experiments on mice suggest that curcin is, in fact, innocuous and has a median lethal dose (LD_{50}) some 1000 times that of ricin. Curcin is commonly found in edible plants (King *et al.*, 2009).

For toxic varieties of *Jatropha*, all the products, including the oil, biodiesel and the seed cake, are toxic. Laboratory-scale detoxification of the seed cake to render it usable as a

livestock feed is possible, but it is not straightforward and is unlikely to be economically feasible on a small scale. Mexico has varieties of *J. curcas* that are not poisonous and, in fact, Gubitz *et al.*, (1999) reported that wildlife and livestock feed on the seeds, and that traditional Mexican dishes use the boiled and roasted seeds. Using these varieties in future breeding programmes is the most likely route to non-toxic *Jatropha* products.

2.5.2 Invasiveness

The fact that *Jatropha* can grow and colonize areas that are inhospitable to other plants makes it a potentially invasive species. While some field observers have stated that the plant is not invasive (Henning, 2004a), Australia's Northern Territory and Western Australia have declared it a noxious weed and have biological control programmes in place for its close relative *Jatropha gossypiifolia*. South Africa bans commercial production of *J. curcas* due to these environmental concerns. Brazil, Fiji, Honduras, India, Jamaica, Panama, Puerto Rico and El Salvador classify it as a weed.

2.6 *Jatropha* Cultivation

This aspect brings together available information on the factors required for successful cultivation of *Jatropha* for oil production. It describes climate and soil requirements to guide site selection, followed by information on best crop establishment and management practices.

2.6.1 Climate

Jatropha grows in tropical and sub-tropical regions, with cultivation limits at 30ºN and 35ºS. It also grows in lower altitudes of 0-500m above sea level. *Jatropha* is not sensitive to day length (flowering is independent of latitude) and may flower at any time of the year

(Heller, 1996). It is a succulent shrub that sheds its leaves during the dry season, with deep roots that make it well suited to semi-arid conditions. While *Jatropha* can survive with as little as 250-300 mm of annual rainfall, at least 600 mm are needed to flower and set fruit. The optimum rainfall for seed production is considered between 1000mm and 1500mm (FACT, 2007), which correspond to sub-humid ecologies. While *Jatropha* has been observed growing with 3000mm of rainfall (Achten, 2008), higher precipitation is likely to cause fungal attack and restrict root growth in all but the most free-draining soils. *J. curcas* is not found in the more humid parts of its area of origin, Central America and Mexico. Rainfall induces flowering and, in areas of unimodal rainfall, flowering is continuous throughout most of the year. Optimum temperatures are between 20˚C and 28˚C. Very high temperatures can suppress yields (Gour, 2006). *Jatropha* has been observed to be frost-intolerant. The plant is well adapted to conditions of high light intensity (Jongschaap *et al.*, 2007) and is unsuited to growth under shade.

2.6.2 Soils

The best soils for *Jatropha* are aerated sands and loams of at least 45cm depth (Gour, 2006). Heavy clay soils are less suitable and should be avoided particularly where drainage is impaired, as *Jatropha* is intolerant of waterlogged conditions. Ability to grow in alkaline soils has been widely reported, but the soil pH should be within 6.0-8.5 (FACT, 2007). *Jatropha i*s known for its ability to survive in very poor dry soils in conditions considered marginal for agriculture, and can even root into rock crevices. However, ability to grow in these environments does not translate to high productivity. Being a perennial plant in seasonally dry climates, soil health management under *Jatropha* production would benefit from conservation agriculture practices. This would result in minimum soil disturbance, an

organic mulch cover on the soil surface and legume cover crops as intercrops (Brittaine and Lutaladio, 2010).

2.6.3 Plant nutrition

Jatropha is often described as having low nutrient requirement because it is adapted to growing in poor soils. However, growing a productive crop requires correct fertilization and adequate rainfall or irrigation. Equally, high levels of fertilizer and excessive irrigation can induce high total biomass production at the expense of seed yield. Unfortunately, there is insufficient data on response to fertilizer under different growth conditions for it to be possible to make specific recommendations for optimal crop nutrition (Brittaine and Lutaladio, 2010).

On wasteland in India, Ghosh *et al.* (2007) found that 3.0 tonnes per ha of *Jatropha* seed cake (also known as "press" cake), containing 3.2% N, 1.2% P2O5 and 1.4% K_2O, increased yields significantly when applied to young plants – by +120% and +93% at two different planting densities. A trial at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India showed increasing yield with fertilization to an optimum level (T3), but that over-application suppressed yields.

The optimum levels of inorganic fertilizers have been seen to vary with the age of the tree (Achten, 2008). Site-specific fertilizer trials need to be established for trees of different ages and over a number of seasons. An analysis of the nutrient value of harvested fruit indicates the application rate of nutrients required to maintain soil fertility levels, assuming all other biomass is retained in the field. From the nutrient composition calculated by

Jongschaap *et al.* (2007), the fruit equivalent of 1.0 tonne of dry seed per ha removes 14.3–34.3 kg of N, 0.7–7.0 kg of P, and 14.3–31.6 kg of K per ha.

Mycorrhyzal soil fungi are generally known to improve a plant's ability to absorb mineral nutrients and water from the soil, and to increase drought and disease resistance. The Energy and Resources Institute (TERI) in India has developed mycorrhyzal inoculations for *Jatropha* that improve germination and give earlier fruiting and higher yields. Jongschaap *et al.* (2007) found increased uptake of Phorbol ester and micronutrients. In Brazil, studies on mycorrhyzal inoculation of *Jatropha* are also showing promise in improving uptake of P and K (Parsons, 2008).

2.6.4 Water requirements

There is little quantitative data available on the water needs, water productivity and water-use efficiency of *Jatropha.* It is believed that optimal rainfall is between 1000 and 1500mm (FACT, 2007). On-station trials by ICRISAT confirm this range. *Jatropha* shows a flowering response to rainfall. After short (one month) periods of drought, rain will induce flowering. Thus, the cycle of flowering can be manipulated with irrigation (FACT, 2007). However vegetative growth can be excessive at the expense of seed production if too much water is applied, for example with continuous drip irrigation.

2.6.5 Pests and diseases

It is popularly reported that pests and diseases do not pose a significant threat to *Jatropha,* due to the insecticidal and toxic characteristics of all parts of the plant. Observations of free-standing older trees would appear to confirm this, but incidence of pests and diseases is widely reported under plantation monoculture, and may be of economic significance.

Observed diseases, such as collar rot, leaf spots, root rot and damping-off, may be controlled with a combination of cultural techniques (for example, avoiding waterlogged conditions) and fungicides.

The shield-backed or scutellera bug, regarded as a key pest of plantation stands of *Jatropha* in Nicaragua (*Pachycoris klugii*) and India (*Scutellera nobilis*), causes flower fall, fruit abortion and seed malformation. Other serious pests include the larvae of the moth *Pempelia morosalis* which damages the flowers and young fruits, the bark-eating borer *Indarbela quadrinotata*, the blister miner *Stomphastis thraustica*, the semi-looper *Achaea janata*, and the flower beetle *Oxycetonia versicolor*. Termites may damage young plants. Carefully and judiciously adding an insecticide to the planting pit may be advisable if problems are endemic (Achten, 2008).

Some biological pest control measures are known. For example, in Nicaragua, *Pseudotelenomus pachycoris* have been found to be effective egg parasitoids of *Pachycoris klugii* and in India, the dipteran parasitoid of *Pempelia* also offers promise. Attention to increasing resistance to pests and diseases will be needed in *Jatropha* improvement programs. *Jatropha multifida* is a known host of African cassava mosaic virus as well as a possible source of transmission of the cassava super-elongation disease (*Sphaceloma manihoticola*) (Achten, 2008). This indicates that, as a related species, *J. curcas* probably should not be grown in association with this crop.

2.6.6 Seed yields

Since systematic recording of yields started only relatively recently, it is important to note that there is little data available for seed yields from mature stands of *Jatropha*. Earlier

reported yields used largely inconsistent data, and claims of high yields were probably due to extrapolation of measurements taken from single, high-yielding elderly trees (Jongschaap *et al.,* 2007). Individual tree yields are reported to range from 0.2-2.0 kg of seed annually (Francis *et al.*, 2005).

On an area basis, Openshaw (2000) reports seed yields between 0.4-12 tonnes per ha, and Heller (1996) reports yields between 0.1 and 8.0 tonnes per ha. Mostly, these yield figures are accompanied by little or no information on genetic provenance, age, propagation method, pruning, rainfall, tree spacing, and soil type or soil fertility. Heller (1996) and Tewari (2007) suggest that production in semi-arid areas may be around 2.0–3.0 tonnes per ha, though it appears likely that lower average yields are being realized in these sub-optimal conditions.

Potential yields for *Jatropha* in semi-arid conditions in Andhra Pradesh, India, are forecast at 1.0 tonne per ha (Wani *et al.*, 2008). Furthermore, during a 17-year period, *Jatropha* growers in Nashik, India, averaged yields of less than 1.25 tonnes per ha. On the other hand, with good soil, higher rainfall and optimal management practices, there are reported yields of 5.0 (Achten, 2008), and 6.0–7.0 tonnes per ha (FACT, 2007). Jongschaap *et al.*, (2007) calculated a theoretical potential seed yield of 7.8 tonnes per ha under optimal conditions. *Jatropha* shows a high variability in yield among individual trees, which is a characteristic of the trees in cultivation being essentially composed of wild varieties. Clearly, the greatest prospect for yield improvement lies with improving the germplasm.

The economic life of a *Jatropha* plantation reportedly ranges from 30-50 years, but there is no evidence to substantiate this. However, individual trees are known to live well in excess of 50 years.

2.6.7 Seed harvest, processing and uses of *Jatropha* oil

The oil content of *Jatropha* seed can range from 18.4–42.3% (Heller, 1996) but generally lies in the range of 30–35%. The oil is almost all stored in the seed kernel, which has oil content of around 50–55% (Jongschaap *et al.*, 2007). This compares well to groundnut kernel (42%), rape seed (37%), soybean seed (14%) and sunflower seed (32%).The seed kernel contains predominantly crude fat (oil) and protein, while the seed coat contains mainly fibre. The fruit shell tells more about the fruit pericarp, while the seed consists of the inner kernel and the outer husk or seed coat.

Seeds are ready for harvesting around 90 days after flowering when the fruits have changed from green to yellow-brown. In wetter climates, fruiting is continuous throughout the year, while the harvest may be confined to two months in semi-arid regions. Even then, the fruits do not ripen together, requiring weekly picking and making the harvest labour intensive and difficult to mechanize.

The yellow and brown fruits are harvested by either beating the branches with sticks to knock them to the ground or by hand picking. The fruits are dried and the seeds removed from the fruit shells by hand, by crushing with a wooden board or by using a mechanical decorticator. Work rates for harvesting are given by Henning (2008) as 24 kg per workday while India's National Oilseeds and Vegetable Oils Development Board (NOVOD) gives a rate of 50 kg of seed per workday (NOVOD, 2007). The seeds are shade-dried for sowing but dried in the sun for oil production to reduce moisture content to around 6–10%. If kept dry and ventilated, the seeds may be stored for up to 12 months without loss of germination or oil content, although there may be losses to pests in storage.

2.7 *Jatropha* Oil

2.7.1 Properties of *Jatropha* oil

Oil quality and consistency are important for producing biodiesel. The physical and chemical content of *Jatropha* oil can be extremely variable. Oil characteristics appear to be influenced by both environmental and genetic interaction, as are seed size, weight and oil content. The maturity of the fruits also can affect the fatty acid composition of the oil, and processing and storage further affect oil quality (Achten *et al.*, 2008).

Oil quality is also important when producing *Jatropha* oil for direct use as fuel. More investigation is necessary to determine what oil quality can be attained reasonably in representative rural conditions. In general, it is necessary to ensure low contamination of the oil, low acid value, high oxidation stability and low contents of phosphorus, ash and water.

Crude *Jatropha* oil is relatively viscous, more so than rapeseed. It is characteristically low in free fatty acids, which improves its storability, though its high unsaturated oleic and linoleic acids make it prone to oxidation in storage. The presence of unsaturated fatty acids (high iodine value) allows it to remain fluid at lower temperatures. *Jatropha* oil also has a high cetane (ignition quality) rating. The low sulphur content indicates less harmful sulphur dioxide ($S0_2$) exhaust emissions when the oil is used as a fuel. These characteristics make the oil highly suitable for producing biodiesel (Brittaine and Lutaladio, 2010).

2.7.2 Uses of *Jatropha* oil

2.7.2.1 Jatropha oil as a biodiesel

The production of *Jatropha* biodiesel is a chemical process whereby the oil molecules (triglycerides) are cut to pieces and connected to methanol molecules to form the *Jatropha* methyl ester. An alkali – normally sodium hydroxide (caustic soda) – is needed to catalyze the reaction. Glycerine (glycerol) is formed as a side product. Methanol is normally used as the alcohol for reasons of cost and technical efficiencies. Sodium hydroxide is dissolved in methanol to form sodium methoxide, which is then mixed with *Jatropha* oil. The glycerine separates out and is drained off. The raw biodiesel is then washed with water to remove any remaining methanol and impurities.

Biodiesel may be used as partial blends (e.g. 5% biodiesel or B5) with mineral diesel or as complete replacements (B100) for mineral diesel. In general, B100 fuels require engine modification due to the different characteristics of biodiesel and mineral diesel. Van-Gerpen *et al.* (2007) specifically noted that solvent action may block the fuel system with dislodged residues, damage the hoses and seals in the fuel system, or cause cold filter plugging, poorer performance due to the lower heating value of biodiesel, some dilution of the engine lubricating oil, and deposit build-up on injectors and in combustion chambers.

It is generally accepted by engine manufacturers that blends of up to 5% biodiesel should cause no engine compatibility problems. Higher blends than this may void manufacturers' warranties. *Jatropha* biodiesel has proven to conform to the required European and USA quality standards. For every 1L of biodiesel, 79ml of glycerine are produced, which is equivalent to around 10% by weight. The raw glycerine contains methanol, the sodium hydroxide catalyst and other contaminants, and must be purified to create a saleable

product. Traditional low-volume/ high-value uses for glycerine are in the cosmetic, pharmaceutical and confectionary industries, but new applications are being sought as production shifts to high volume/low value. Glycerine is used in the production of fuel, plastics and antifreeze (Brittaine and Lutaladio, 2010).

The production of biodiesel requires expertise, equipment and the handling of large quantities of dangerous chemicals (methanol is toxic and sodium hydroxide is highly corrosive). It is not a technology suited to resource-poor communities in developing countries (Brittaine and Lutaladio, 2010).

2.7.2.2 Pure Jatropha oil

Jatropha oil may be used directly in some diesel engines, without converting it into biodiesel. The main problem is that *Jatropha* oil has higher viscosity than mineral diesel, although this is less of a problem when used in the higher temperature environment of tropical countries (Brittaine and Lutaladio, 2010).

2.7.2.3 Cooking fuel

There are clear advantages to using plant oil instead of traditional biomass for cooking. These include the health benefits from reduced smoke inhalation, and environmental benefits from avoiding the loss of forest cover and lower harmful GHG emissions, particularly carbon monoxide and nitrogen oxides. The high viscosity of *Jatropha* oil compared to kerosene presents a problem that necessitates a specially designed stove. There are two basic designs – one uses pressure to atomize the oil and the other uses a wick (Brittaine and Lutaladio, 2010).

2.7.2.4 Lighting fuel

The problem of *Jatropha* oil's high viscosity also applies to lamp design. A lamp with a floating wick offers one solution to the oil's poor capillary action. This allows the wick to be kept as short as possible, with the flame just above the oil. It requires periodic cleaning of the wick to remove carbon deposits. Ordinary kerosene lamps may be modified to lower the wick, but the oil level has to be maintained at a constant level and the wick again needs frequent cleaning. There is anecdotal evidence that using a *Jatropha* oil lamp deters mosquitoes (Brittaine and Lutaladio, 2010).

2.7.2.5 Soap making

Jatropha soap is made from a mixture of sodium hydroxide (caustic soda) solution and *Jatropha* oil. This simple technology has turned soap making into a viable small-scale rural enterprise appropriate to many rural areas of developing countries. *Jatropha* soap is valued as a medicinal soap for treating skin ailments. *Jatropha* soap production can be highly profitable, with 4.7 kg of soap produced from 13L of *Jatropha* oil in only five hours (Henning, 2004b). On the other hand, Wiesenhutter (2003) finds that locally produced *Jatropha* soap has limited commercial potential, as the quality is poor in comparison to imported soaps.

2.7.2.6 Other uses for the oil

Jatropha oil has molluscicidal properties against the vector snails of the *Schistosoma* parasite that causes bilharzia. The emulsified oil has been found to be an effective insecticide against weevil pests and houseflies, and an oil extract has been found to control cotton bollworm and sorghum stem borers (Gubitz *et al.*, 1999). Achten *et al.*, (2008) describe the use of oil extracts as an insecticide, molluscicide, fungicide and nematicide.

These potential uses have yet to be commercialized. As previously mentioned, the oil is widely used as a purgative in traditional medicine. It also is used to treat various skin diseases and rheumatism (Heller, 1996).

2.8 Properties and Uses of the Seed Cake

Once the oil is extracted, about 50% of the original seed weight remains as seed cake residue, mainly in the form of protein and carbohydrates. The amount of oil left in the seed cake depends on the extraction process. There are trade-offs for the seed cake. It may be used as fertilizer, fuel or, if it is detoxified or if non-toxic varieties are used, it can be used as animal fodder. However, it is significant that not returning the seed cake to the plantation as fertilizer reduces the utility of *Jatropha* in improving degraded land.

2.8.1 Livestock feed

Jatropha seed cake is high in protein – 58.1% by weight compared to soy meal's 48 percent – and would be a valuable livestock protein feed supplement if it were not for its toxicity. Currently, removal of toxins is not commercially viable. Using non-toxic varieties from Mexico could make greater use of this potentially valuable by-product, but even these varieties may need treatment to avoid sub-clinical problems that could arise with long-term feeding of *Jatropha* seed cake to livestock (Makkar and Becker, 1997).



Plate 1: *J. curcas* Seed Cake from NARICT, Zaria

2.8.2 Organic fertilizer

Jatropha seed cake makes an excellent organic fertilizer with high nitrogen content similar to, or better than, chicken manure. As organic manure, the seed cake can make a valuable contribution to micronutrient requirements (Brittaine and Lutaladio, 2010).

2.8.3 Fuel

The seed cake has a high energy content of 25 MJ kg^{-1}. Experiments have shown that some 60% more biogas was produced from *Jatropha* seed cake in anaerobic digesters than from cattle dung, and that it had a higher calorific value (Abreu, 2008). The residue from the biogas digester can be used further as a fertilizer. Where cow dung is used for household fuel, as in India, the seed cake can be combined with cow dung and cellulosic crop residues, such as seed husks, to make fuel briquettes.

2.9 Using the Fruit Shells and Seed Husks

Biogas has been produced from fruit shells. In addition, trials showed that seed husks could be used as a feedstock for a gasification plant (Achten *et al.*, 2008). *Jatropha* fruit shells and seed husks can be used for direct combustion. The shells make up around 35–40% of the whole fruit by weight and have a calorific value approaching that of fuelwood; therefore, the shells could be useful by-products of *Jatropha* oil production. The calorific values of *Prosopis juliflora* (a fuelwood species of semi-arid areas) and *Jatropha* fruit shells are similar. However, four times the volume of fruit shells is required to equal the heating value of fuelwood, due to their lower bulk density. Seed husks have a higher heating value and greater bulk density, which makes them more valuable than the fruit shells as a combustible fuel. However, the technology required to separate the seed husk from the kernel is more suited to large processing plants than small rural industry. The fruit

shells can be dried and ground to a powder and formed into fuel briquettes. A trial found that 1 kg of briquettes took around 35 minutes for complete combustion, giving temperatures in the range of 525ºC–780ºC (Singh *et al.,* 2008). The ash left after combustion of *Jatropha* shell briquettes is high in potassium, which may be applied to crops or kitchen gardens. The fruit shells and seed husks also can be left around *Jatropha* trees as mulch and for crop nutrition. For *Jatropha* grown on degraded land, this has clear advantages because nutrient re-cycling – through returning the seed cake to the plantation – is unlikely to happen, due to the effort required and the higher utility to be gained from applying the seed cake to high-value crops.

Seeds of *J. curcas* plant are rich in oil and protein. Significant quantities of macrominerals (Na, K, Mg, Ca, P) and microminerals (Mn, Fe, Zn) have also been found in the seeds (Abou-Arab and Abu-Salem, 2010). The seeds contain about 300 – 350g/kg oil, which can be used as a fuel directly or in its transesterified form, as a substitute for diesel. The protein quality of the meal obtained from shelled *Jatropha* seeds is high. Levels of essential amino acids (except lysine) are higher in *Jatropha* seed cake than in the FAO reference protein for a growing child of 3 – 5 years (Makkar *et al.*, 2008).

J. curcas meals contain high true protein, high energy and low fibre. The nutritional value of *Jatropha* meal compares favourably with those from conventional seed meals, such as soyabean (Makkar *et al.,* 1998). Research done by Chivandi *et al.,* (2005) indicated higher crude protein, ash, calcium and phosphorus in *Jatropha* seed meal than in soyabean meal.
J. curcas L. is probably the most highly promoted oilseed crop at present in the world. It is famous for being a potential source of raw material for biodiesel. The popularity of *J. curcas* stems from the widespread general knowledge that it is a non-edible, oil-yielding

tree, well adapted to marginal areas with poor soil and low rainfall, where it grows without competing with annual food crops (Soerawidjaja, 2010).

There are many other uses of *J. curcas L*. Traditionally, the plant has been used as a hedge and living fence, not edible by livestock. Growing *Jatropha* reclaims eroded and waste land. The seed oil can be used as a fuel for lighting and is a raw material for making high quality soap. Residue from seed pressing is good organic fertilizer (Yan, 2008). All parts of *Jatropha* (seeds, leaves and bark) have been used in traditional medicine and for veterinary purposes for a long time (Kumar and Sharma, 2008). This is typical of most members of the Euphorbiaceae family, whose medicinal properties may be due to stress factors such as drought and extreme temperatures that induce synthesis of survival and defense chemicals (Mwine and Van Damme, 2011).

J. curcas seed oil has insecticidal properties, especially against *Callosobruchus maculatus* – a seed beetle in cowpeas (Adebowale and Adedire, 2006), *Cnaphalocrocis medinalis* 116 – rice leaf folder and *Helicoverpa armigera* – cotton boll worm (Dowlathabad *et al.*, 2010). Alcohol extract from *Jatropha* leaf was found to have antibiotic effect on *Escherichia coli* and *Staphylococcus aureus* (Ye *et al.*, 2009). The toxicity of *J. curcas* to mollusks has been investigated because of its relevance in schistosomiasis control. Extracts from *J. curcas L.* were found to be toxic against snails (Goel *et al.*, 2007). Ethanolic extracts of JCL seed cake also showed antifungal activities against the following important fungal phytopathogens: *Fusarium oxysporum, Pythium aphanidermatum, Lasiodiplodia theobromae, Curvularia lunata, Fusarium semitectum, Colletotrichum capsici and Colletotrichum gloeosporioides* (Saetae and Suntornsuck, 2010).

2.10 Phorbol Esters

The term ‘phorbol esters’ is used today 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 (Haas and Mittelbach, 2000).

Phorbol itself was isolated as a non-toxic crystalline solid from the plant, *Croton tiglium L.* (Crombie *et al.,* 1968), but its ester, TPA (12-*O*-tetradecanoylphorbol-13-acetate), present in croton oil, is the most toxic and most studied phorbol ester, known to be a co carcinogen (Devappa *et al.,* 2010; Olivier *et al.,* 1992). However, phorbol esters from *J. curcas* are derivatives of 12-deoxy-16-hydroxyphorbol (Haas *et al.,* 2002). Phorbol esters with the same diterpenoid moiety have also been extracted from the latex of *Euphorbia cooperi* (Gschwendt and Hecker, 1970).

Adolf *et al.* (1984) were the first to identify phorbol esters in *J. curcas L*. Although they concluded that the irritant principles of the plant represented 12-deoxy- 16-hydroxyphorbol-13-acylates with highly unsaturated acid moieties, they could not elucidate their exact chemical structures. Hirota *et al.,* (1988) managed to identify the macrocyclic, dicarboxylic acid, diester structure of one of the phorbol esters and showed that it is a tumor promoter with weaker activity than TPA. Later, Haas *et al.,* (2002) isolated and determined the structures of all phorbol esters found in *J. curcas*.

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

C6 was found to be the least stable. It has been assumed that the diester group biosynthetically arises from two originally separated monoacids (Haas *et al.,* 2002).

The content of phorbol esters in *J. curcas* can vary according to the region in which the plant grows. Ahmed and Salimon (2009) have shown a variation in the phorbol ester content of the *J. curcas* seed oil from Malaysia (0.23%), Indonesia (1.58%) and India (0.58%). Saetae and Suntornsuk (2010) demonstrated a variation in phorbol ester content in *J. curcas* plants from different provinces of Thailand. However, Makkar *et al.* (1998) had previously found similar levels of phorbol esters in plants from Cape Verde (0.27%), Nicaragua (0.22%) and Nigeria (0.23%).

Although the phorbol esters are distributed in different parts of the *J. curcas* plant (Makkar and Becker, 2009), they are concentrated in the seed kernel and their levels in seed oil are higher than those in the seed cake (Hipal *et al.,* 2009).

2.10.1 Phorbol ester toxicity

Phorbol esters are the most potent tumor promoters known. They exhibit a remarkable ability to amplify the effect of a carcinogen but are themselves not carcinogenic (Wender *et al.,* 1998). The seeds from *J. curcas* have been reported to be toxic to humans, rodents and livestock, and phorbol esters have been identified as the main toxic agent (Becker and Makkar, 1998).

Symptoms of intoxication in humans include burning and pain in mouth and throat, vomiting, delirium, muscle shock, decrease in visual capacity and high pulse. In Nigeria and Burkina Faso, seeds and seed oil are added to Strophanthus arrow poison. In Gabon, seeds are grated with palm oil to kill rats. The Shamba of Usambara region of Tanzania

used *Jatropha* seeds as an ordeal poison (Wink *et al.*, 1997). Toxicity to sheep, goats, calves, chicks and fish by consumption of *Jatropha* seed or seed meal has been reported. The seed has also the highest known molluscicidal activity (Demissie and Lele, 2010). Common symptoms of topical application to rabbits, mice and rats were erythema, oedema, necrosis, diarrhea, scaling and thickening of skins. Ingestion of phorbol esters causes lung and kidney damage, resulting in fatality (Li *et al.*, 2010).

The toxicity of phorbol esters is mainly due to their action on biological cell membranes. The phorbol esters are amphiphilic molecules and have a tendency to bind to phospholipid membrane receptors. The receptors are usually the primary targets of the phorbol esters. The most investigated activity of the phorbol esters is their binding and activation of protein kinase C (PKC), an enzyme that plays a critical role in signal transduction pathways and also regulates cell growth and differentiation.

It has been proposed that the phorbol esters convert protein kinase C into a constitutive active form that is irreversibly inserted into the membrane. During normal signal transduction, the enzyme is activated by diacylglycerol, which is then rapidly hydrolyzed. Upon activation, PKC enzymes are translocated to the plasma membrane to conduct various other signal transduction pathways. The phorbol esters act as analogues for diacylglycerol and are stronger PKC activators that are hardly metabolized by the cell. They hyperactivate PKC and trigger cell proliferation, thus amplifying the efficacy of carcinogens. (Goel *et al.,* 2007)

Phorbol esters also affect insulin binding by stimulating PKC-induced phosphorylation of insulin receptors. It is believed that protein kinase C regulates insulin (Jacobs *et al.*, 1983).

Studies on effects of phorbol myristate acetate (PMA), also known as Tetradecanolyphorbol acetate (TPA), on mouse skin showed that its metabolite, phorbolol myristate acetate (PHMA), was nearly as effective a tumor promoter as PMA (Segal *et al.*, 1975). Here, the carbonyl group in PMA is converted to a hydroxyl group to give PHMA. Other esters found to bind specifically to PKC are phorbol-12,13-didecanoate, phorbol-12,13-dibutyrate, phorbol-12,13-dibenzoate, phorbol-12,13-diacetate, phorbol-12,13,20-triacetate, phorbol-13-acetate (Dunphy *et al.*, 1980) and phorbol-12-tetradecanoate (Kikkawa *et al.*, 1983). However, phorbol-13,20-diacetate and 4-*O*-tetradecanoylphorbol-13-acetate were ineffective and are therefore considered to be non-promoters (Yuspa *et al.*, 1976). Interestingly, while phorbol-13-acetate was reported as effective, the closely related 12-deoxyphorbol-13-acetate exhibited inhibitory or antitumor properties (Carrol and Little, 2000).

It has been observed that shorter chain derivatives of phorbol are irritant but not promoting, these being two biological effects that could be considered independent of one another (Szallasi *et al.*, 1993). The presence of a C20 hydroxyl group has been seen to be important for the irritant and tumor promoting activities of phorbol esters. Introduction of an acetyl group in the 20-position gives rise to lower irritancy (Wu *et al.*, 2009).

Therefore, it may be postulated that the spatial position of this function, as determined by their particular carbon skeleton and relative to specifically binding cellular structures, may play a delicate role in the hydrophilic interaction of the esters with membranes and receptors (Hecker, 1987). One way to weaken or abolish tumor-promoting efficacy of a phorbol ester without impairing its mitogenic and irritant activities is to introduce double

bonds into the long chain fatty acid residue at position 12 of the phorbol moiety (Sorg *et al.,* 1982).

Although many biological activities of phorbol esters are mediated through their direct interaction and activation of protein kinase C, there are some phorbol esters that are active without being tumor promoters, such as ostodin and prostratin. It has also been reported that 12-*O*-acetylphorbol-13-decanoate potently inhibits the cytopathic effects of human immunodeficiency virus (HIV) type 1 without appreciable activation of protein kinase C (Abdel-Hafez *et al.*, 2002). Phorbol, the parent diterpenoid of phorbol esters, contains five hydroxyl groups with different reactivities towards acylation.

2.10.2 Detoxification

The *J. curcas* seed cake is a by-product of oil extraction from the seed. Although the seed meal has been found to be protein-rich, its utilization as a stock feed is hampered by the presence of toxic phorbol esters and the anti-nutritional factors that include lectin, saponin, phytic acid, and trypsin inhibitor. Detoxification of the seed meal is therefore of paramount importance. Several biological, chemical and physical detoxification methods have been suggested (Brittaine and Lutaladio, 2010).

Roasting of seeds inactivates trypsin inhibitor and reduces lectin activity. However, saponins, phytic acid and phorbol esters are not affected by roasting. It is not possible to destroy phorbol esters by heat treatment because they are heat stable and can withstand roasting temperature as high as 160^{o}C for 30 minutes (Aregheore *et al.,* 2003). Although rumen microbes can degrade lectins, phytic acid and trypsin inhibitor, they cannot degrade

phorbol esters. This is why ruminants, like cattle, are as prone to phorbol ester toxicity as monogastric animals (Makkar and Becker, 2010).

Complete degradation of phorbol esters by stripping or deodorization of *J. curcas* oil at 260^{o}C, with 3 mbar pressure and 1% steam injection has been reported by Makkar *et al*. (2009). The procedure involves steam injection through the seed oil, in a closed oven, at 260^{o}C and low pressure. Vapours escaping from the oil are condensed in a water cooler that is connected to a vacuum pump. Although Aregheore *et al.* (2003) had suggested that the use of 4% NaOH, followed by washing with water, could effectively reduce phorbol esters, studies by Sumiati *et al.,* (2010) showed that addition of 4% NaOH, then heating in an autoclave at 121^{o}C for 30 minutes, did not produce satisfactory results. Solvent extraction is among the most successful means of detoxifying *J. curcas* seed cake. Chivandi *et al.* (2004) observed that a hexane/ethanol solvent system was useful in reducing phorbol ester content in the seed meal. Sequential extraction involving non-polar solvent followed by polar solvent has been recommended by Gaur *et al.,* (2011). Approximately 80% of phorbol esters present in *Jatropha* oil were extracted using methanol (Devappa *et al.*, 2010).

Nevertheless, Saetae and Suntornsuck (2010) claimed that 90% ethanol was just as effective as 99.5% methanol for complete phorbol ester extraction from *J. curcas* seed cake. They preferred ethanol since it is safer for humans and other animals than methanol. Ethanol was also found to be efficient in removing lectin and reducing saponin, phytic acid and trypsin inhibitor in the seed cake (Saetae and Suntornsuck, 2011). Research done by Martínez-Herrera *et al*. (2006) indicated that extraction with ethanol, followed by treatment

with 0.07% $NaHCO_3$ decreased phorbol ester content by 98% in *Jatropha* seeds, including a considerable decrease in lectin activity.

Devappa *et al*. (2010) have demonstrated that *Jatropha* phorbol esters are biodegradable in the environment. The degraded products are innocuous. Degradation rates of phorbol esters in the soil are influenced by temperature and moisture levels. The occurrence of storage fungi in *J. curcas* seeds points to the use of these microorganisms in detoxifying the seed meal. Different species of *Aspergillus, Penicillium, Mucor* and *Rhizopus* were isolated from stored *Jatropha* seeds (Jayaraman *et al.,* 2011). Solid-state fermentation of *J. curcas* seed cake, using various microorganisms has been tried by a number of researchers. Here, the method constitutes a fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in the absence of free flowing liquid. The same technique had been employed to eliminate or reduce anti-nutritional factors in other plants, such as saponin from whole soybean tempe by *Rhizopus oligosporus*, caffeine and tannins from coffee husk by *Aspergillus* sp. and gossypol from cotton seed by *Candida tropicalis* (Joshi *et al.*, 2011).

Belewu and Akande (2010) studied the effect of *Aspergillus niger* and *Penicillium* sp. treated *J. curcas* kernel cake, as stock feed for West African dwarf goat. They concluded that fungi-treated *J. curcas* seed meal can partially replace soybean cake for growing goat by as much as 50%. Other work by Belewu *et al*. (2010a) showed that innoculation of *J. curcas* kernel cake with *Aspergillus niger* and incubation at room temperature for 7 days resulted in the detoxification of the seed meal. In yet another study by Belewu *et al*. (2010b), it was concluded that growing goats can be fed a diet consisting of 50% soybean cake plus 50% *Rhizopus oligosporus* treated *J. curcas* kernel cake.

Sumiati *et al.* (2009) used *Rhizopus oryzae* to detoxify *J. curcas* seed meal by solid-state fermentation for 3 days. The procedure eliminated phorbol esters, reduced trypsin inhibitor by 68% and fat content by 93%. Fermentation using *Rhizopus oligosporus* was also described as an effective method of detoxifying *J. curcas* seed meal and increasing its nutritive value for poultry because there was reduction of phytic acid, an increase in protein utilization efficiency and retention of calcium and phosphorus in broilers (Sumiati *et al.,* 2010). Again, Rosa *et al*. (2010) have demonstrated that *Aspergillus niger* is not only a detoxifier but a nutritional enrichment additive. Joshi *et al.,* (2011) have claimed that phorbol esters can be completely degraded in nine (9) days by *Pseudomonas aeruginosa* under optimized solid-state fermentation of deoiled *J. curcas* seed cake. It is interesting to note that Tjakradidjaja *et al*. (2009) obtained poor results on trials with *Aspergillus niger, Rhizopus oligosporus, Rhizopus oryzae, Trichoderma viridae and Trichoderma reesei* in the development of mice feed. This led them to conclude that the use of fermented *J. curcas* seed meal with various moulds at 5% in ration is not effective as protein source for male mice.

2.11 *Bacillus*

Bacillus is a genus of Gram-positive, rod-shaped (bacillus), bacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes (oxygen reliant), or facultative anaerobes (having the ability to be aerobic or anaerobic). They will test positive for the enzyme catalase when there has been oxygen used or present (Turnbull, 1996). Ubiquitous in nature, *Bacillus* includes both free-living (non-parasitic) and parasitic pathogenic species. Under stressful environmental conditions, the bacteria can produce oval endospores that are not true spores but which the bacteria can reduce themselves to and

remain in a dormant state for very long periods. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera of Firmicutes (Madigan and Martinko, 2005).

Many species of Bacillus can produce copious amounts of enzymes, which are made use of in different industries. Some *Bacillus* species can form intracellular inclusions of polyhydroxyalkanoates under certain adverse environmental conditions, as in a lack of elements such as phosphorus, nitrogen, or oxygen combined with an excessive supply of carbon sources. *Bacillus amyloliquefaciens* is the source of a natural antibiotic protein barnase (a ribonuclease), alpha amylase used in starch hydrolysis, the protease subtilisin used with detergents, and the BamH1 restriction enzyme employed for molecular biology purposes.

Bacillus species are known to be able to synthesize esterase enzyme which helps in the break-down of phorbol ester. Esterases represent a diverse group of hydrolases catalyzing the cleavage and the formation of ester bonds. They have been extensively exploited in the synthesis of flavour esters, in the resolution of racemic mixtures, and in the degradation of natural material as well as industrial pollutants (Panda and Gowrishankar, 2005).

2.11.1 *Bacillus coagulans*

Bacillus coagulans is a lactic acid-forming bacterial species within the genus *Bacillus*. The organism was first isolated and described as *Bacillus coagulans* in 1915 by B.W. Hammer at the Iowa Agricultural Experiment Station as a cause of an outbreak of coagulation in evaporated milk packed by an Iowa condensary (Hammer, 1915).

The species Lactobacillus sporogenes was originally isolated and described in 1933 by Horowitz-Wlassowa and Nowotelnow and subsequently reclassified as *Bacillus sporogenes*. More recently, it has been shown that *B. sporogenes* shares the same characters of B. coagulans, and therefore it has been moved into *B. coagulans* group (De-Vecchi and Drago, 2006). Accordingly to the 8th edition of Bergey's Manual of Determinative Bacteriology, spore-bearing rods capable of (i) producing lactic acid, (ii) either facultative or aerobic respiration and, (iii) decomposing hydrogen peroxide to water and oxygen (i.e. catalase positive), are to be classified within the genus *Bacillus.* The phenotypic heterogeneity of the species makes a satisfactory description of the species for practical use rather difficult (De Clerck *et al*., 2004). This diversity has been confirmed by genotypic assays on several strains from different sources. For example, a considerable variability within *B. coagulans* species has been shown both by 16S rDNA sequence comparison and total DNA-DNA relatedness analysis, thus permitting the definition of some common genomic traits of this species (De Clerck *et al*., 2004). Even if some commercial products are still labelled as "*L. sporogenes*", it is well known that *L. sporogenes* is to be renamed as *B. coagulans*.

However, *B. coagulans* differs from the other bacteria of the genus Bacillus for position of endospore in the cellular body (terminal in *B. coagulans*, centrally or subterminally located in other bacilli), lack of cytochrome C oxidase and inability to reduce nitrate to nitrite. In the vegetative form, *B. coagulans* cells appear as Gram-positive mobile rods, occurring singly or, rarely, in short chains of variable lengths. They optimally grow at a temperature range of 35-50°C and at pH values between 5.5 and 6.5. Metabolically, they are facultative anaerobes and produce acids but no gas from fermentation of maltose, mannitol, raffinose,

sucrose and trehalose. These characteristics favour growth of *B. coagulans* in acid foods and it has often been reported to spoil milk products, vegetables or fruits due to the production of high amounts of lactic acid (Anderson, 1984; Cosentino *et al*., 1997; Ramon-Blanco *et al*., 1999; DeClerck *et al*., 2004). By contrast, production of lactic acid and of other products such as thermostable enzymes may be exploited at the industrial level (Payot *et al*., 1999; Batra *et al.*, 2002; Yoon *et al*., 2002).

Bacillus coagulans has been added by the European Food Safety Authority (EFSA) to their Qualified Presumption of Safety list and has been approved for veterinary purposes as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration's Center for Veterinary Medicine, as well as by the European Union, and is listed by Association of American Feed Control Officials (AAFCO) for use as a direct-fed microbe in livestock production. It is often used in veterinary applications, especially as a probiotic in pigs, cattle, poultry, and shrimp. Many references to the use of this bacterium in humans are documented, especially in improving the vaginal microbiota (Sanders, *et al*., 2003), improving abdominal pain and bloating in irritable bowel syndrome patients, (Hun, 2009) and increasing immune response to viral challenges (Baron, 2009). There is evidence from animal research that suggests that *Bacillus coagulans* is effective in both treating as well as preventing recurrence of *Clostridium difficile* associated diarrhea (Fitzpatrick, 2013). One strain of this bacterium has also been assessed for safety as a food ingredient (Endres *et al*., 2009). Spores are activated in the acidic environment of the stomach and begin germinating and proliferating in the intestine. Spore-forming *B. coagulans* strains are used in some countries as probiotics for patients on antibiotics.

2.12 *Paenibacillus*

Paenibacillus is a genus of facultative anaerobic, endospore-forming bacteria, originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 (Ash *et al*., 1993). Bacteria belonging to this genus have been detected in a variety of environments such as: soil, water, rhizosphere, vegetable matter, forage and insect larvae, as well as clinical samples (Lal and Tabacchioni, 2009: McSpadden, 2004: Montes *et al*., 2004: Ouyang *et al*., 2008). The Latin word *paene* means almost; therefore, *Paenibacilli* literally translates to *almost Bacilli*. The genus includes *P. larvae*, which is known to cause American foulbrood in honeybees, the *P. polymyxa*, which is capable of fixing nitrogen and therefore is used in agriculture and horticulture, the *Paenibacillus* sp. JDR-2 which is known to be a rich source of chemical agents for biotechnology applications, and pattern forming strains such as *P. vortex* and *P. dendritiformis* discovered in the early 90s (Ben-Jacob and Cohen, 1997: Ben-Jacob *et al*., 1998: Ben-Jacob *et al*., 1992: Ben-Jacob *et al*., 1995), which are known to develop complex colonies with intricate architectures (Ben-Jacob, 2003: Ben-Jacob *et al*., 2000a: Ben-Jacob *et al*., 2000b: Ben-Jacob and Levine, 2005: Ingham and Ben-Jacob, 2008).

There has been a rapidly growing interest in *Paenibacillus* spp. since many were shown to be important (Choi *et al*., 2004: Konishi and Maruhashi, 2003: Nielsen and Sorensen, 1997) for agriculture and horticulture (e.g. *P. polymyxa*), industrial (e.g. *P. amylolyticus*), and medical applications (e.g. *P. peoriate*). These bacteria produce various extracellular enzymes such as polysaccharide-degrading enzymes and proteases, which can catalyze a wide variety of synthetic reactions in fields ranging from cosmetics to biofuel production. Various *Paenibacillus spp.* also produce antimicrobial substances that affect a wide

spectrum of microorganisms (Girardin *et al*., 2002: Piuri *et al*., 1998: Von der Weid *et al*., 2003) such as fungi, soil bacteria, plant pathogenic bacteria and even important anaerobic pathogens such as *Clostridium botulinum*.

More specifically, several *Paenibacillus* species serve as efficient plant growth promoting rhizobacteria (PGPR). PGPR competitively colonize plant roots and can simultaneously act as biofertilizers and as antagonists (biopesticides) of recognized root pathogens, such as bacteria, fungi and nematodes (Bloemberg and Lugtenberg, 2001). They enhance plant growth by several direct and indirect mechanisms. Direct mechanisms include phosphate solubilization, nitrogen fixation, degradation of environmental pollutants and hormone production. Indirect mechanisms include controlling phytopathogens by competing for resources such as iron, amino acids and sugars, as well as by producing antibiotics or lytic enzymes. Despite the increasing interest in *Paenibacillus spp.*, genomic information of these bacteria is lacking. More extensive genome sequencing could provide fundamental insights into pathways involved in complex social behavior of bacteria, and can discover a rich source of genes with biotechnological potential (Kloepper *et al*., 1980: Ryu *et al*., 2003).

2.12.1 *Paenibacillus macerans*

Paenibacillus macerans is a diazotroph bacterium found in soil and plants capable of nitrogen fixation and fermentation. This bacterium was originally discovered in 1905 by an Austrian biologist named Schardinger and thought to be a bacillus. *Paenibacillus macerans* is a part of the *Paenibacillaceae* family, which are facultative anaerobes. It is Gram-variable, being Gram-positive or Gram-negative rods. It does not have a capsule and has

peritrichous flagella for movement. It does form ellipsoidal, terminal, or subterminal spores, which may last in the soil for many years. *P. macerans* can be grown in the lab on a nutrient agar with a slightly acidic pH around 5. Optimal growth temperature is 30°C. It is also able to grow in 5% NaCl making it a halotolerant bacteria. *P. macerans* has been shown to have some of the broadest metabolic capabilities of any of the *Paenibacillus* genus. It is able to ferment hexoses, deoxyhexoses, pentoses, cellulose, hemicellulose and glyerol under anaerobic conditions (Gupta *et al*., 2009).

The high fermentation rates of glycerol make this an important organism in the study of fuel and chemical production. *P. macerans* also produces a significant amount of histamines which may cause allergies in some individuals if ingested (Rodriruez-Jerez *et al*., 1994). This bacterium is a facultative anaerobe capable of nitrogen fixation. In the absence of oxygen it is able to convert nitrogen gas to ammonia which is more easily used by plants. *P. macerans* is usually found in soil and plant materials but has also been identified in blood cultures of infants with neonatal infection (Noskin *et al*., 2001).

2.12.2 *Paenibacillus polymyxa*

Paenibacillus polymyxa is a Gram-positive bacterium capable of fixing nitrogen. The species may also be known as *Bacillus polymyxa*. It is found in soil, plant roots, and marine sediments (Lal and Tabacchioni, 2009). Strains of this species were also isolated from cod intestines by students at the University of Tromsø in February 2009. *P. polymyxa* can be grown in the laboratory on TSAg-medium. *P. polymyxa* is used as a soil inoculant in agriculture and horticulture. Biofilms of *P. polymyxa* growing on plant roots have been shown to produce exo-polysaccharides, which protect the plants from pathogens. The

interactions between this bacterial species and plant roots also cause the root hairs to undergo physical changes (Yegorenkova *et al*., 2013). Some strains of *P. polymyxa* produce polymyxin antibiotic compounds (Shaheen *et al*., 2011). Surfactant complexes isolated from *P. polymyxa* have been shown to be effective in disrupting biofilms of *Bacillus subtilis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus bovis* (Quin *et al*., 2012).

2.13 Optimization

Fermentation medium and fermentation condition play an important role because they affect the formation, concentration and yield of a particular fermentation end product thereby affecting the overall process (Schmidt, 2005). In bioprocess industry, it is often important to conduct optimization experiments because new and mutant strains are continuously been introduced. In fermentation process, optimizing different combination and sequence of process condition and medium component are required to determine the growth condition that produces the biomass with the physiological state best suited for product formation (Stanbury *et al.*, 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Sample

Bacterial strains, *Bacillus coagulans* and *Paenibacillus polymyxa and Paenibacillus macerans* were used in this study. *P. macerans* and *P. polymyxa*- were obtained from dump site (soil) *and B. coagulans* from kilishi (food) using a minimal medium that contains *Jatropha curcas* seed cake as the only source of carbon.

3.2 Isolation of *Bacillus* Species

These strains were isolated from soil samples using the surface spread method on a minimal medium containing the *J. curcas* seed cake. The minimal medium was be prepared according to the modified method of Lateef *et al.,* (2010) as follows; $NaNO_3$ 2g/L, NaCl 2g/L, KH_2PO_4 2g/L, $MgSO_4$ 0.05g/L, $FeSO_4.7H_2O$ 0.1g/L, $CaCO_3$ 0.1g/L, *Jatropha curcas* seed cake 2g/L, Agar Powder 15g/L.

The medium was sterilized in the autoclave at 121^{o}C for 15 minutes, and supplemented with 0.05g/l of sterile nystatin to inhibit the growth of fungi. The *J. curcas* seed cake that was used served as the sole source of carbon, nitrogen, sulfur and energy. The plates were incubated at 37^{o}C for up to 3 days. Distinct colonies, observed using morphological features, were selected, isolated, and purified on minimal medium and nutrient agar medium.

3.3 Morphological Identification

Gram staining and characterization was done using the conventional biochemical technique according to the flow chart of Bergey's manual and also with the Microgen kit for *Bacillus* identification. The strains, after being characterized, were stored on Nutrient agar slants at 4^{o}C till further use.

3.3.1 Gram staining

A clean sterile slide was obtained, after which a drop of distilled water using a sterile wire loop was placed on the slide. A sterile wire loop was used to pick a distinct colony from the already purified minimal medium onto the slide and homonized to prepare a smear. The smear was heat-fixed and covered with crystal violet stain for 45 seconds and rapidly washed off with clean water. The smear was then covered with Gram's iodine, washed off with clean water, rapidly decolorized for few seconds with acetone and washed off immediately with clean water. The smear was then covered with safranin for 2 minutes and then washed off with clean water. The back of the slide was wiped clean and placed on a draining rack to air dry and microscopically examined with the oil immersion objective (100x).

3.3.2 Endospore staining

Endospore staining was done according to Schaeffer-Fulton method (Schaeffer and Fulton, 1933). A smear of the isolates was prepared, air-dried and heat fixed on a glass slide and covered with a blotting paper. The blotting paper was saturated with malachite green stain solution and steamed for about 5 minutes, keeping the paper moist by adding more of the dye. The slide was washed in Tap water and counterstained with safranin for 30 seconds,

washed off and blotted dry. The slides were examined under the oil immersion lens for the presence of endospores. Endospores are bright green and the vegetative cells are brownish red to pink.

3.4 Biochemical Characterization

3.4.1 Microgen Kit for *Bacillus* ID

The Microgen Bacillus-ID identification system consists of 2 microwell strips (labelled BAC 1 and BAC 2), each containing 12 dehydrated substrates for the performance of either carbohydrate fermentation tests or other biochemical based tests. The last well in the second strip is a carbohydrate fermentation control well for use as a reference well in the interpretation of these tests. The selection of the substrates included in the test panel has been determined using computer based analysis of all available substrates for the identification or differentiation of *Bacillus* species (Lapage *et al*., 1973).

Identification of isolates is achieved by recording the results visualised by a colour change after 24 and 48 hours incubation at 30°C and the addition of appropriate reagents (Indole, Nitrate and VP tests) after 48 hours. These results are then analysed using the Microgen Identification System Software (MID-60) for the identification of *Bacillus* species.

3.5 Raw Material and Proximate Composition

J. curcas seed cake was collected from the National Research Institute for Chemical Technology (NARICT) Zaria. It was analysed for moisture, crude protein, crude fat, ash and crude fibre using the AOAC (Association of Official Analytical Communities) methods (AOAC, 1995).

3.5.1 Moisture content

The aluminum dish used was brushed and oven dried at 105^{o}C for one hour and placed in a desiccator to cool before weighing. Forty (40g) of the sample was weighed into the weighed dish and placed in the oven at 105^{o}C overnight. The dishes were removed with covers placed on top and placed in the desiccator and weighed as quickly as possible to prevent moisture absorption.

$$\% \text{ dry matter} = \frac{(\text{weight of dish+ dried sample}) - \text{weight of dish}}{\text{Weight of sample before drying}} \times 100$$

% moisture content = 100 - % dry matter

3.5.2 Ash content

Crucibles were cleaned and placed into a muffle furnace at 600^{o}C for one hour and then transferred to the desiccator from the furnace to cool at room temperature. The crucibles, after cooling, were weighed as quickly as possible to avoid moisture absorption. Exactly 2.0g of the sample was weighed into porcelain crucible and placed in a muffle furnace at 600^{o}C overnight, then transferred into the desiccator to cool at room temperature and weighed immediately to prevent moisture absorption.

$$\% \text{ Ash} = \frac{\text{weight of sample after incinerating} - \text{weight of crucible}}{\text{Weight of sample}} \times 100$$

3.5.3 Crude protein

Half (0.5g) of the sample were weighed each into a digestion flask, to which 30cm^{3} of concentrated sulphuric acid and two (2) kjeltabs were added and heated gently on a digestion apparatus by means of an electric element. At this stage of digestion, the reaction mixture turned black due to the dehydrating action of the sulphuric acid and formation of free carbon. As the reaction of free carbon oxidizes, a clear yellow solution remains. After

heating strongly for 2 hours, the sulphuric acid were seen by its condensation in the lower neck of the flask, which was allowed to cool after digestion and small quantity of distilled water was added to dissolve the crystals that were formed, then made to mark with distilled water in a 100ml volumetric flask.

Ten (10ml) of 2.0% boric acid solution and 2 drops of indicator were mixed in a 100ml conical flask while 10ml of the diluted sample was pipetted into the Markham distiller with 10ml of 40% sodium hydroxide. The mixed boric acid and indicator in the flask was placed at the bottom of the condenser to collect the ammonia, while all other possible channels of escape of the ammonia closed. When about 50ml of the distillate was collected in the now green boric acid and indicator solution, the distillate was then titrated with 0.02N sulphuric acid, which gave a grey-mauve end point.

$$\%N_2 = \frac{\text{(sample titre-blank titre) x Normailty of the acid x 14.02 x10}}{\text{Weight of sample (g) x aliquot}} \times 100$$

% Crude protein = $\%N_2$ x 6.25 (there is an average of 16% N_2 protein therefore 100/16 = 6.25)

3.5.4 Crude fibre

Two (2g) of sample was weighed into a beaker, then 200ml of warm sulphuric acid 0.128N solution was added and boiled for 30 minutes with the use of Lavconco heating apparatus. The boiled sample was filtered and washed with hot water. The residue was collected back into the beaker and 200cm^3 of warm sodium hydroxide solution was added and boiled for another 30 minutes. The boiled sample were then filtered and washed with hot water while the residue washed with acetone and collected into the crucible for drying at 105^oC overnight.

The dried residue was weighed together with the crucible as quickly as possible after it was cooled in the desiccator for 30 minutes to avoid moisture absorption. The crucible and the content were incinerated to ash at 600^{o}C for 3 hours, allowed to cool in the desiccator for 30 minutes and weighed as quickly as possible to prevent moisture absorption.

$$\% \text{ crude fibre} = \frac{\text{weight of ash (g)}}{\text{Weight of sample}} \times 100$$

3.5.5 Digesstible carbohydrate

The digestible carbohydrates was determined by subtracting the calculated percentages for each nutrient from 100. That is:

Carbohydrate by difference = 100 – (moisture + ash + crude fat + crude protein)

3.6 Analysis for Toxin and Anti –Nutritional Factors

3.6.1 Phorbol ester

Phorbol esters were determined according to the method of Saetae and Suntornsuk (2010). The dry seed cake was extracted with 95% ethanol. The phorbol ester concentration was determined by High performance liquid chromatography (HPLC), using phorbol 12–myristate 13–acetate (Sigma, Steinheim, Germany) as a standard.

3.6.2 Phytic acid

Phytic acids were determined according to the method of Reddy *et al* (1982). Four (4.0g) of sample was soaked in 100ml of 2% hydrochloric acid for 5 hours and filtered. Twenty-five (25ml) of the filtrate was decanted into a conical flask and 5ml of 0.3% ammonium thiocyanate solution was added. The mixture was titrated with a standard solution of iron

(III) chloride until a brownish yellow colour persists for 5 minutes. 1ml of 0.025M $FeCl_2$ will give 6.601mg phytate.

3.6.3 Lectin

Lectins were determined according to the modified method of Gordon and Marqaurdt (1974). The dry seed cake was extracted with normal saline. A serial dilution of sample with normal saline (1:1) in a micro titration plate was prepared. Sheep red blood cells were added into the diluted sample. The lectin concentration was displayed as of a haemagglutination unit which is the minimum amount of the sample required to show the agglutination.

3.6.4 Trypsin inhibitor

Trypsin inhibitor was determined by spectrophotometric method and N-α-benzol-L-arginine-*p*-nitroanilide (BAPNA) was used as a standard (Onwuka, 2005). One gram (1g) of the sample was dispensed in 50ml of 0.5M NaOH solution, stirred for 30 minutes and centrifuged at 3,600rpm and then filtered. Exactly 2ml of standard trypsin was added to 4ml of aliquot in a test tube and was allowed to stand for 5 minutes. The aliquot was then measured in a spectrophotometer at 410nm. The blank analysis was also carried out simultaneously. One trypsin inhibitor unit is equal to an increase of 0.01 absorbance unit from the blank.

3.6.5 Saponin

Saponins were determined according to Hudson and El-Difrawi (1979). Ten grams (10g) of the sample was added to 100ml of 20% aqueous ethanol in water and agitated with a

magnetic stirrer for 12 hours at 55^0C. The solution was filtered using Whatmann No 1 filter paper and the residue was re-extracted with 300ml of 20% aqueous ethanol. The extracts were combined and reduced to about 40ml under vacuum using a rotary evaporator. The extract and 20ml diethyl ether were transferred into a 250ml separatory funnel and shaken vigorously. The aqueous layer was discarded. The process of purification was continued until a colourless aqueous extract was obtained. The pH of the remaining aqueous solution was adjusted to 4.5 by adding 4g of sodium chloride and the solution then shaken successively with butanol. The butanolic extract was washed twice with 10ml of 5% (w/v) sodium chloride and evaporated to dryness in a fume cupboard, to give the saponin, which was weighed and expressed as a percentage.

3.7 Submerged Fermentation using Different *Bacillus* Strains

Ten grams (10g) of seed cake and 100ml of water were added into 250ml Erlenmeyer flask. The flask was sterilized and then inoculated aseptically with each bacterial strain at 1.0×10^8 cells MacFarland's standard per 100ml. The control contained the 10g of the seed cake and 100ml of water. The cultures and control were incubated at 30^0C on a rotary shaker operated at 150rpm. The flasks were sampled at days 0 and 5 to determine the levels of toxin- phorbol ester and anti-nutritional factors- Phytic acid, Lectin, Trypsin inhibitor and Saponin. Samples were withdrawn at different time intervals for residual toxin and anti-nutritional factors analyses. The percentage (%) reduction of toxin- Phorbol ester and anti-nutritional factors were calculated from differences of samples in day 0 and 5.

3.8 Optimization of Detoxification Conditions

Using the modified method of Kamath *et al*. (2010), Various process parameters influencing detoxification include fermentation time (24 hours, 48 hours, 72 hours, 96 hours, 120 hours), pH (4.5, 6.5, 8.5) and temperature (27^0C, 30^0C, 37^0C). The optimization medium contained the 10g of the seed cake, 100ml of water and the bacterial strains at 1.0 × 10^8 cells MacFarland's standard per 100ml. At constant temperature other parameters like pH and Time were varied.

CHAPTER FOUR

4.0 RESULTS

Three organisms were isolated and further characterized as shown in Tables 10-12 to be *Paenibacillus macerans*, *Paenibacillus polymyxa* and *Bacillus coagulans*. Proximate composition of *J. curcas* seed cake is shown in Table 4.1. The cake contains moisture content (5.45%), crude proteins (23.75%), crude fibre (11.38%), crude fat (9.80%) and digestible carbohydrate (46.45%).

Figure 4.1 shows the phytochemical composition of dried *J. curcas* seed cake in which the extraction of the oil was done both manually and with the use of a machine. The phytochemical composition showed that the manually extracted seed cake has higher phorbol ester, phytic acid, lectin and trypsin inhibitor concentrations than those extracted with the use of a machine, while the saponin content is lower in the manually extracted seed cake compared to that which was extracted with the use of a machine.

Table 4.2 shows the phytochemical composition of fermented *J. curcas* seed cake. There was 76.35% reduction of phorbol ester A and also 99.32% for phorbol ester B. Phytic acid reduced by 56.26% while saponin reduction was 43.56%. There was a reduction of 58.75% for lectin and 64.94% for trypsin inhibitor.

Table 4.1: Proximate Composition of Dry *J. Curcas* Seed Cake

Parameters	Seed cake (%)
Moisture	5.45
Ash content	8.20
Crude Protein	23.75
Fat and oil	9.80
Digestible carbohydrate	46.45
Crude fibre	11.38

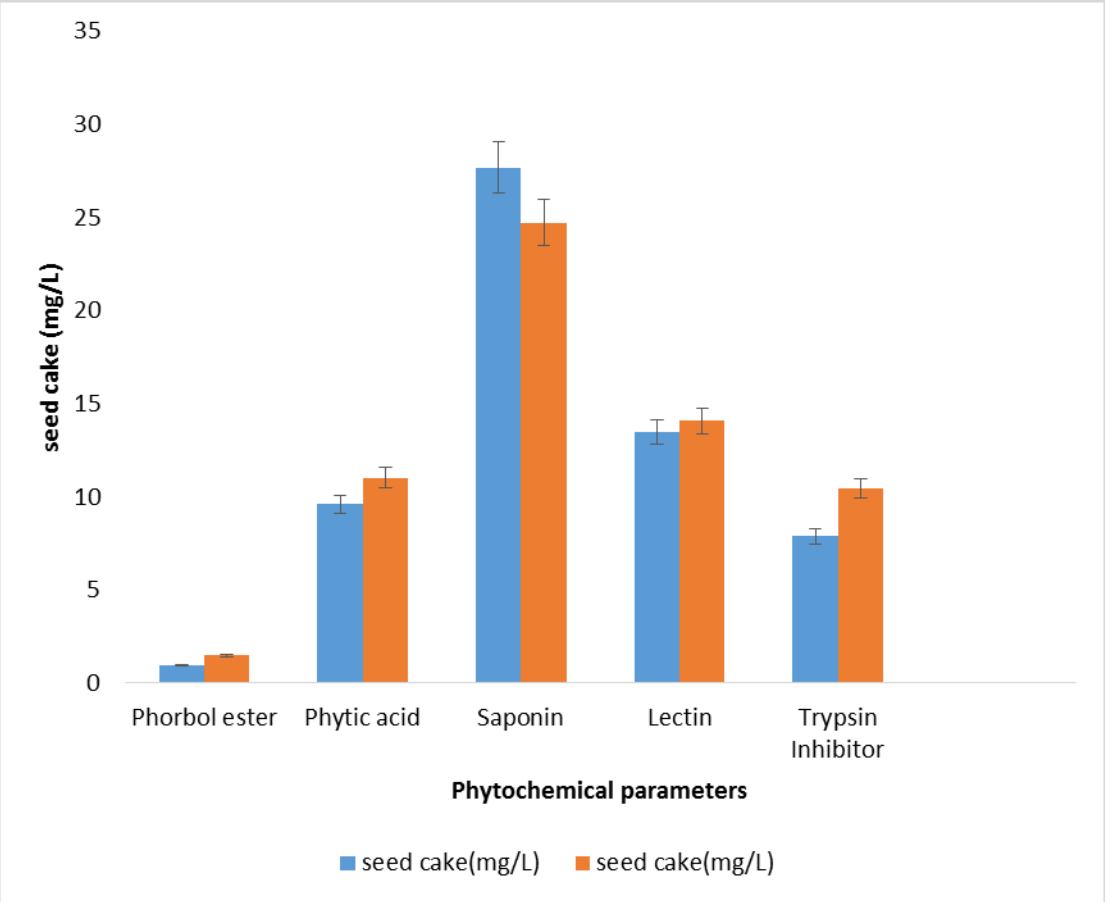


Figure 4.1: Phytochemical Content Of Dry *J. Curcas* Seed Cake Processed Using Different Oil Extraction Methods

Table 4.2: Percentage Reduction of Fermented *J. curcas* Seed Cake

Anti-nutrient	Conc. (mg/l) 0 Day	5 Days	% Reduction
Pborbol ester A	1.48	0.35	76.35
Phorbol ester B	1.48	0.01	99.32
Phytic acid	11.02	4.82	56.26
Saponin	24.70	13.94	43.56
Lectin	14.06	5.80	58.75
Trypsin inhibitor	10.44	3.66	64.94

Keys: Phorbol ester A- Before Optimization, Phorbol ester B- After Optimization

Table 4.3 shows the phorbol ester analysis of the fermented *J. curcas* seed cake. The phorbol ester concentration containing the three isolated bacteria reduced with time when compared with the control. This reduction was more pronounced in fermented seed cake containing *Paenibacillus macerans* (0.35mg/L) than *Bacillus coagulans* (0.81mg/L) and *Paenibacillus polymyxa* (0.58mg/L).

Table 4.4 shows the phytic acid analysis of the fermented *J. curcas* seed cake. The phytic acid concentration of fermented *J. curcas* seed cake containing the isolated bacteria reduced with time when compared with the control. There was a reduction in phytic acid concentration in the three isolate but the reduction was more in fermented seed cake containing *Paenibacillus macerans* (4.82mg/L) than *Bacillus coagulans* (7.83mg/L) and *Paenibacillus polymyxa* (6.02mg/L).

Table 4.5 shows the saponin analysis of the fermented *J. curcas cake.* The saponin concentration of the fermented *J. curcas* seed cake containing the isolated bacteria reduced with time when compared with the control. There was a reduction in saponin concentration in the three isolates but the reduction was more in fermented seed cake containing *Paenibacillus macerans* (13.94mg/L) than in those containing *Bacillus coagulans* (19.84mg/L) and *Paenibacillus polymyxa* (16.98mg/L).

Table 4.3: Effect of Isolates on Reduction of Phorbol Ester during Fermentation of *J. curcas* Seed Cake

	Conc. (mg/L)			
	Control	*B. coagulans*	*P. polymyxa*	*P. macerans*
time(hr)				
0	1.48	1.48	1.48	1.48
24	1.38	0.93	0.77	0.52
48	1.30	0.90	0.68	0.47
72	1.25	0.88	0.66	0.40
96	1.19	0.84	0.61	0.38
120	1.14	0.81	0.58	0.35
% reduction	22.97%	45.27%	60.81%	76.35%

Table 4.4: Effect of Isolates on Reduction of Phytic Acid during Fermentation of *J. curcas* Seed Cake

	Conc. (mg/L)			
	Control	*B. coagulans*	*P. polymyxa*	*P. macerans*
time(hr)				
0	11.02	11.02	11.02	11.02
24	10.05	8.89	7.01	5.78
48	9.85	8.62	6.99	5.49
72	9.62	8.04	6.63	5.00
96	9.43	7.94	6.55	4.91
120	9.21	7.83	6.02	4.82
% reduction	16.42%	28.95%	45.37%	56.26%

Table 4.5: Effect of Isolates on Reduction of Saponin During Fermentation of *J. curcas* Seed Cake

	Conc. (mg/L)			
	Control	*B. coagulans*	*P. polymyxa*	*P. macerans*
time(hr)				
0	24.70	24.70	24.70	24.70
24	23.50	21.60	19.66	15.56
48	22.98	21.42	18.81	15.20
72	22.57	21.03	18.48	14.81
96	22.23	20.55	17.89	14.48
120	21.87	19.84	16.98	13.94
% reduction	11.46%	18.87%	31.26%	43.56%

Table 4.6 shows the lectin levels in the fermented *J. curcas* seed cake. The lectin concentration of the fermented *J. curcas* seed cake containing the isolated bacteria reduced with time when compared with the control. There was a reduction in lectin concentration using the three isolates but the reduction was more in fermented seed cake containing *Paenibacillus macerans* (5.80mg/L) compared to those containing *Bacillus coagulans* (9.52mg/L) and *Paenibacillus polymyxa* (8.21mg/L).

Table 4.7 shows the trypsin inhibitor levels in the fermented *J. curcas* seed cake. The trypsin inhibitor concentration of the fermented *J. curcas* seed cake containing the isolated bacteria reduced with time when compared with the control. There was a reduction in trypsin inhibitor concentration using the three isolates but the reduction was more in fermented seed cake containing *Paenibacillus macerans* (3.66mg/L) compared to those containing *Bacillus coagulans* (7.20mg/L) and *Paenibacillus polymyxa* (5.55mg/L).

Table 4.8 shows the mean optimization of phorbol ester with respect to temperature. The mean values were statistically significant for the different temperature varied for each organism used with a calculated P-value of 0.000, ($P<0.05$). At $27^{o}C$ *Bacillus coagulans* and *Paenibacillus polymyxa* had their highest mean of 0.81 and 0.73 respectively, while *Paenibacillus macerans* had a mean value of 0.67.

Table 4.6: Effect of Isolates on Reduction of Lectin During Fermentation of *J. curcas* Seed Cake

	Conc. (mg/L)			
	Control	*B. coagulans*	*P. polymyxa*	*P. macerans*
time(hr)				
0	14.06	14.06	14.06	14.06
24	13.01	11.39	9.33	7.58
48	12.68	11.01	9.00	6.93
72	12.32	10.94	8.78	6.44
96	12.01	9.78	8.53	6.10
120	11.78	9.52	8.21	5.80
% reduction	16.07%	32.29%	41.61%	58.75%

Table 4.7: Effect of Isolates on Reduction of Trypsin Inhibitor During Fermentation of *J. curcas* Seed Cake

	Conc. (mg/L)			
	Control	*B. coagulans*	*P. polymyxa*	*P. macerans*
Time(hr)				
0	10.44	10.44	10.44	10.44
24	9.74	8.78	6.98	5.10
48	9.43	8.36	6.40	4.87
72	9.23	8.01	6.00	4.29
96	8.99	7.97	5.96	3.95
120	8.79	7.20	5.55	3.66
% reduction	15.8%	31.03%	46.84%	64.94%

Table 4.8: Effect of Temperature on Reduction of Phorbol Ester by Isolates During Fermentation of *J. curcas* Seed Cake.

Temp (^{O}C)	***B. coagulans***	***P. polymyxa***	***P. macerans***
	mean±SE	mean±SE	mean±SE
27	0.81±0.05[a]	0.73±0.08[a]	0.67±0.07[a]
30	0.65±0.03[b]	0.45±0.8[b]	0.37±0.08[b]
37	0.49±0.07[c]	0.19±0.8[c]	0.12±0.07[c]
P-value	**0.000***	**0.000***	**0.000***

Mean values followed by different letter within the same column are statistically significant Duncan Multiple Range Test ($p<0.05$)

Table 9 shows the mean optimization of phorbol ester with respect to time. The mean values were statistically insignificant for the different fermentation time varied for phorbol ester reduction. The calculated P-value were 0.486, 0.357 and 0.399 for *Bacillus coagulans*, *Paenibacillus polymyxa* and *Paenibacillus macerans* respectively.

Table 10 shows the mean optimization of phorbol ester with respect to pH. The calculated mean values were statistically insignificant for the different pH values varied for phorbol ester reduction. The calculated P-value were 0.942, 0.908 and 0.940 for *Bacillus coagulans*, *Paenibacillus polymyxa* and *Paenibacillus macerans* respectively.

Fig 4.2 shows the phorbol ester concentrations of the fermented seed cake at 27^0C and pH 4.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 4.5 and temperature of 27^0C starting with a concentration of 0.78 and reduced to 0.58 for *Paenibacillus macerans*, a concentration of 0.84 and reduced to 0.68 for *Paenibacillus polymyxa* and a concentration of 0.89 and reduced to 0.74 with *Bacillus coagulans*.

Table 4.9: Effect of Fermentation Period on Reduction of Phorbol Ester on isolates During Fermentation of *J. curcas* Seed Cake

Time (Hours)	***B. coagulans*** mean±SE	***P. polymyxa*** mean±SE	***P. macerans*** mean±SE
24	0.71±0.13[a]	0.56±0.23[a]	0.49±0.24[a]
48	0.67±0.14[a]	0.51±0.23[a]	0.44±0.24[a]
72	0.64±0.15[a]	0.46±0.23[a]	0.38±0.24[a]
96	0.62±0.15[a]	0.40±0.24[a]	0.34±0.24[a]
120	0.59±0.14[a]	0.35±0.24[a]	0.28±0.23[a]
P-value	**0.486**	**0.357**	**0.399**

Mean values followed by same letter within the column are not statistically significantly ($p>0.05$).

Table 4.10: Effect of pH on Reduction of Phorbol Ester by Isolates During Fermentation of *J. curcas* Seed Cake

pH	***B. coagulans***	***P. polymyxa***	***P. macerans***
	mean±SE	mean±SE	mean±SE
4.5	0.65±0.14[a]	0.47±0.24[a]	0.40±0.24[a]
6.5	0.65±0.14[a]	0.45±0.24[a]	0.39±0.24[a]
8.5	0.64±0.14[a]	0.44±0.24[a]	0.37±0.24[a]
P-value	**0.942**	**0.908**	**0.940**

Mean values followed by same letter within the column are not statistically significantly ($p>0.05$).

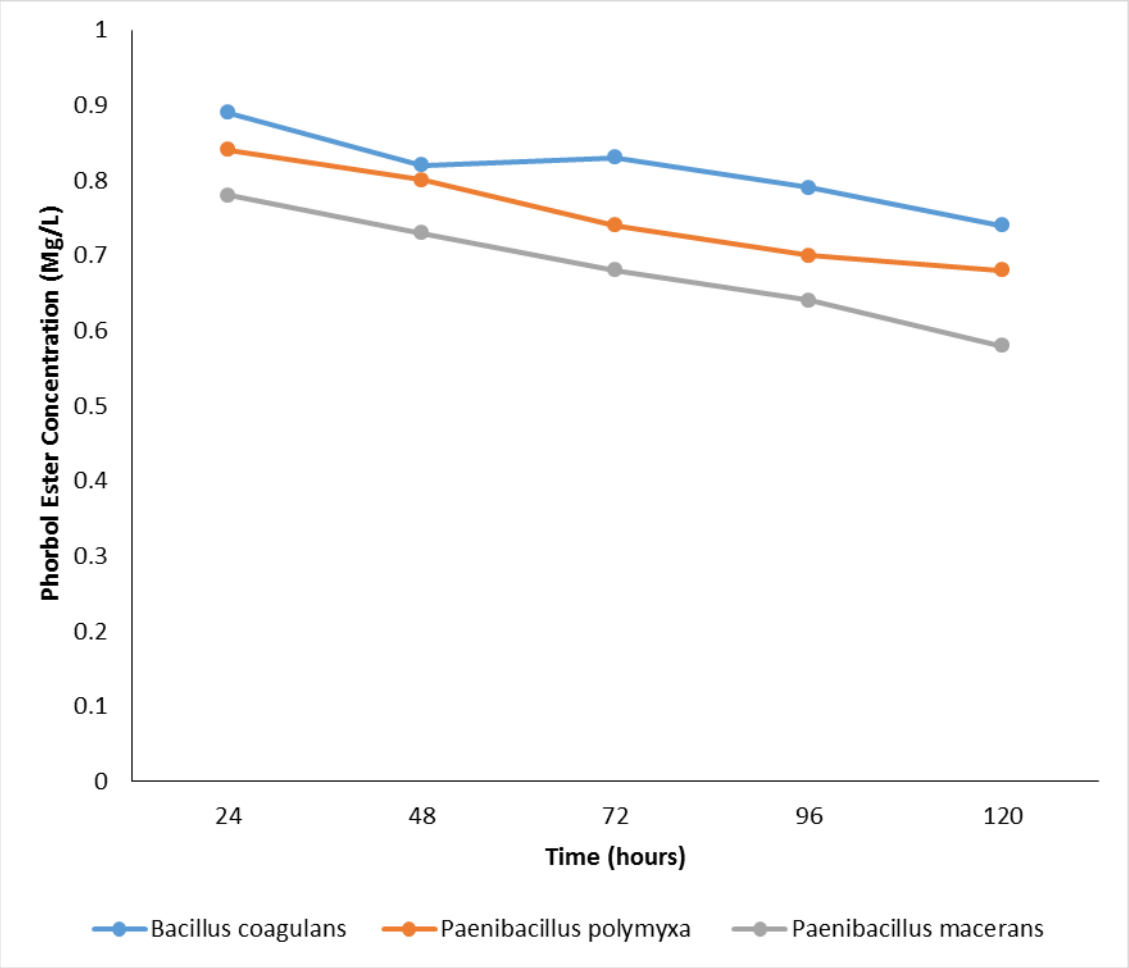


Fig 4.2: Phorbol ester concentration at 27^0C and pH 4.5

Fig 4.3 shows the phorbol ester concentrations of the fermented seed cake at 27^0C and pH 6.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 6.5 and temperature of 27^0C starting with a concentration of 0.76 and reduced to 0.56 for *Paenibacillus macerans*, a concentration of 0.82 and reduced to 0.62 for *Paenibacillus polymyxa* and a concentration of 0.84 and reduced to 0.75 for *Bacillus coagulans*.

Fig 4.4 shows the phorbol ester concentrations of the fermented seed cake at 27^0C and pH 8.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 6.5 and temperature of 27^0C starting with a concentration of 0.75 and reduced to 0.54 for *Paenibacillus macerans*, a concentration of 0.81 and reduced to 0.59 for *Paenibacillus polymyxa* and a concentration of 0.84 and reduced to 0.73 with *Bacillus coagulans.*

Fig 4.5 shows the phorbol ester concentrations of the fermented seed cake at 30^0C and pH 4.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 4.5 and a temperature of 30^0C starting with a concentration of 0.52 and reduced to 0.28 for *Paenibacillus macerans*, a concentration of 0.57 and reduced to 0.35 for *Paenibacillus polymyxa* and a concentration of 0.71 and reduced to 0.66 for *Bacillus coagulans*.

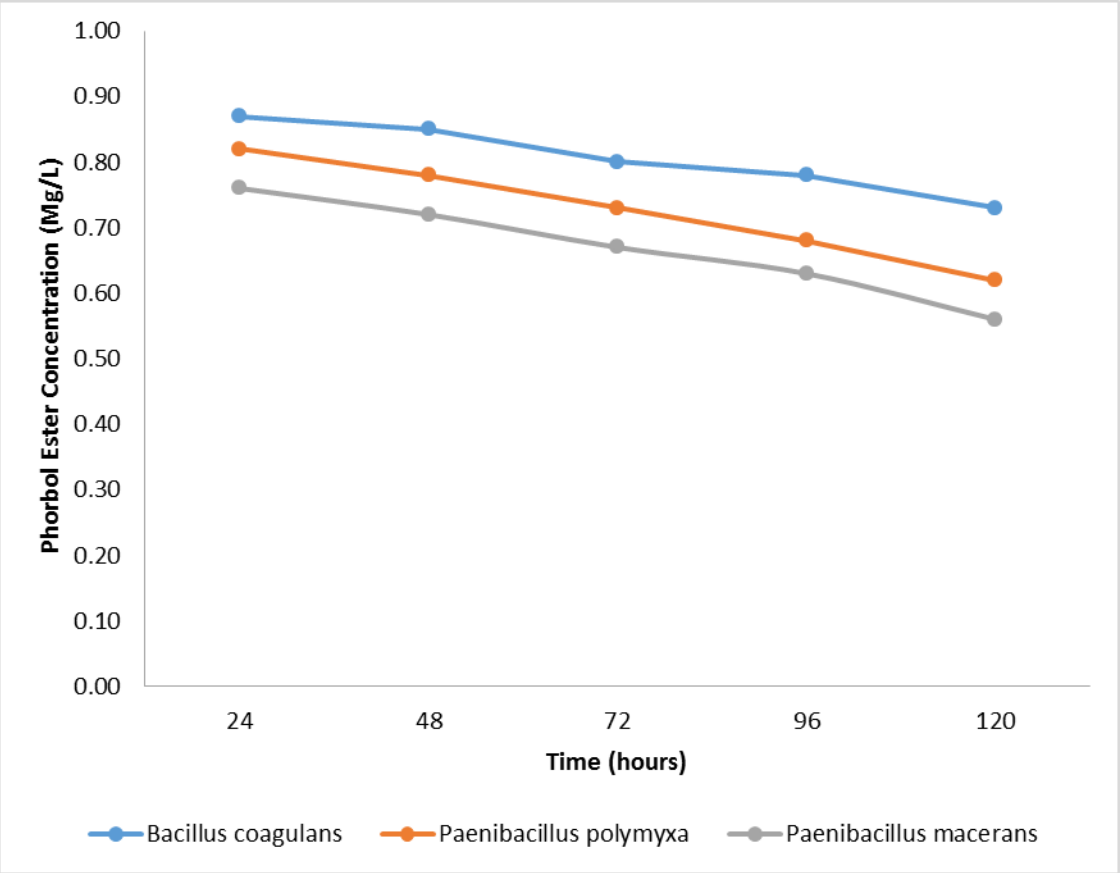


Fig 4.3: Phorbol ester concentration at 27^0C and pH 6.5

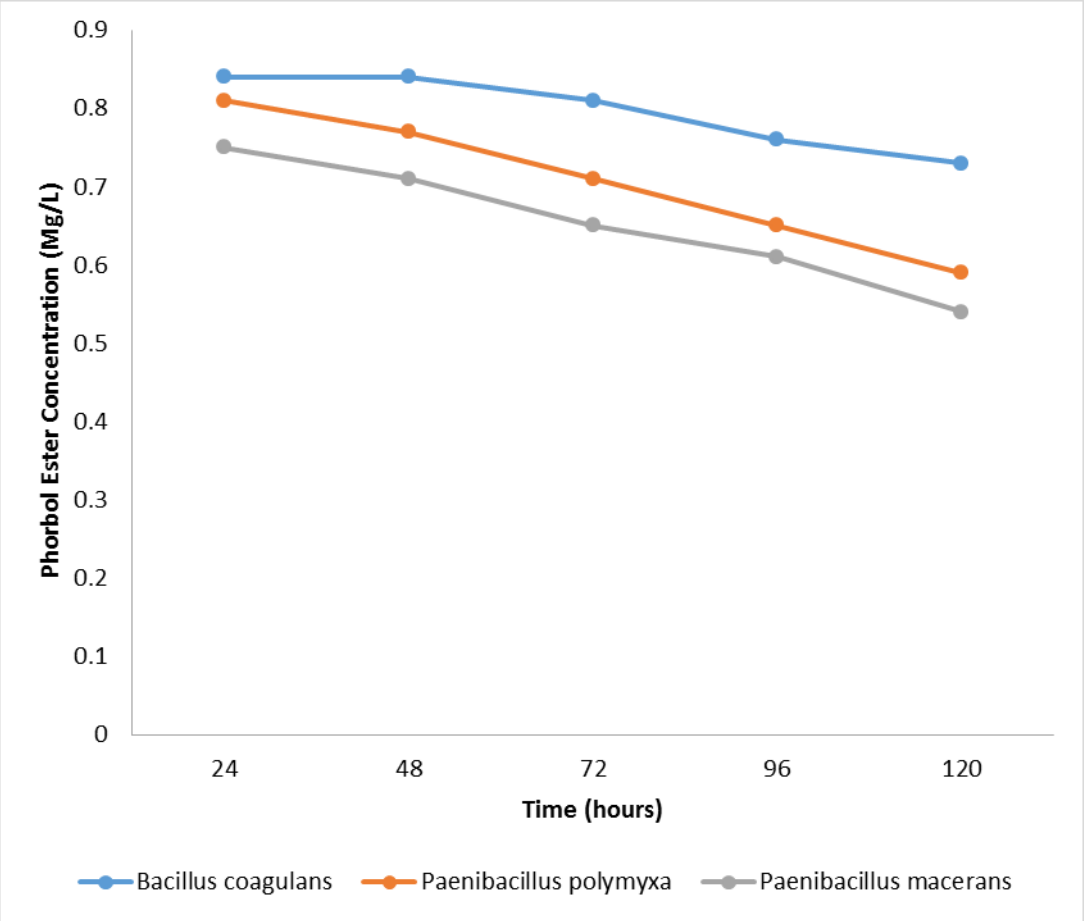


Fig 4.4: Phorbol ester concentration at 27^0C and pH 8.5

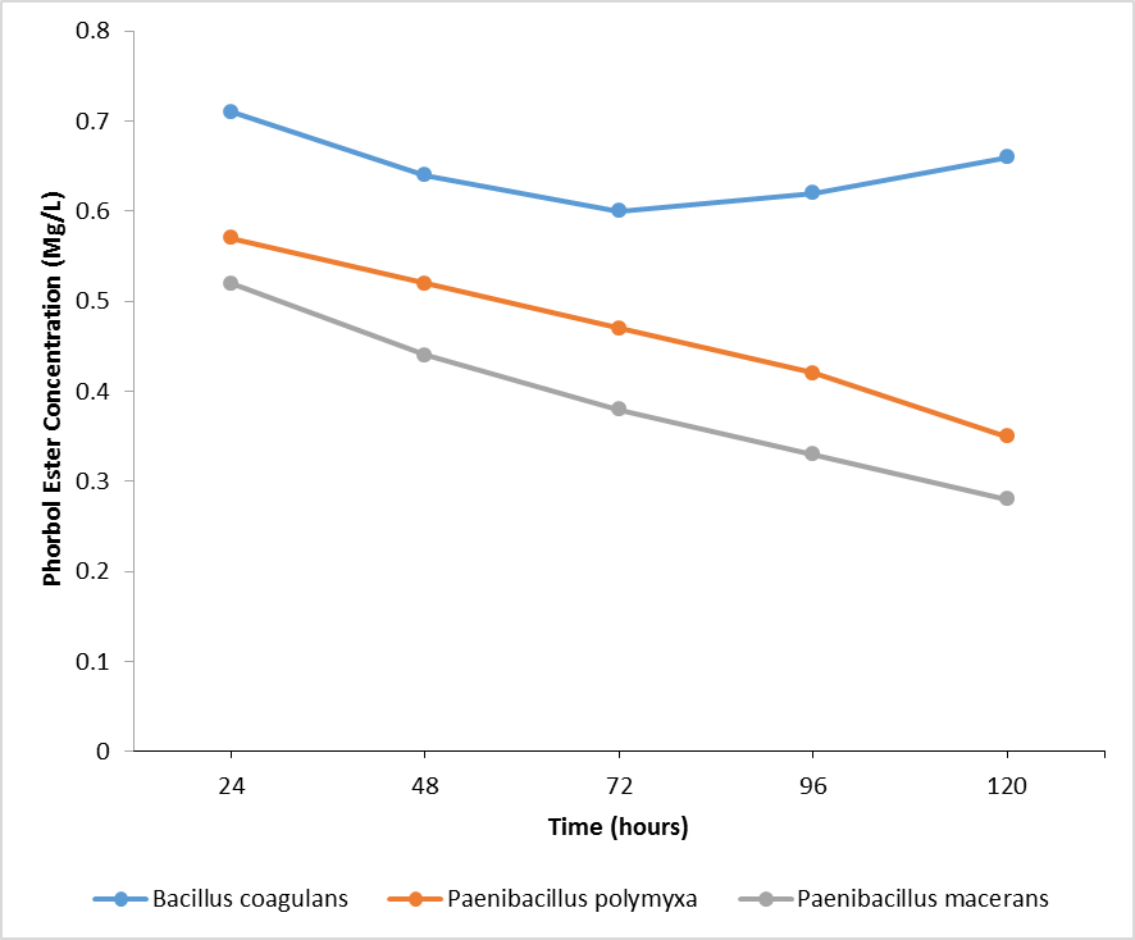


Fig 4.5: Phorbol ester concentration at 30^0C and pH 4.5

Fig 4.6 shows the phorbol ester concentrations of the fermented seed cake at 30^0C and pH 6.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 6.5 and temperature of 30^0C starting with a concentration of 0.49 and reduced to 0.27 for *Paenibacillus macerans*, a concentration of 0.55 and reduced to 0.34 for *Paenibacillus polymyxa* and a concentration of 0.84 and reduced to 0.75 with *Bacillus coagulans*.

Fig 4.7 shows the phorbol ester concentrations of the fermented seed cake at 30^0C and pH 8.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 8.5 and a temperature of 30^0C starting with a concentration of 0.48 and reduced to 0.25 for *Paenibacillus macerans*, a concentration of 0.54 and reduced to 0.32 for *Paenibacillus polymyxa* and a concentration of 0.66 and reduced to 0.60 for *Bacillus coagulans*.

Fig 4.8 shows the phorbol ester concentrations of the fermented seed cake at 37^0C and pH 4.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 4.5 and a temperature of 37^0C starting with a concentration of 0.23 and reduced to 0.04 for *Paenibacillus macerans*, a concentration of 0.30 and reduced to 0.1 for *Paenibacillus polymyxa* and a concentration of 0.61 and reduced to 0.40 for *Bacillus coagulans*.

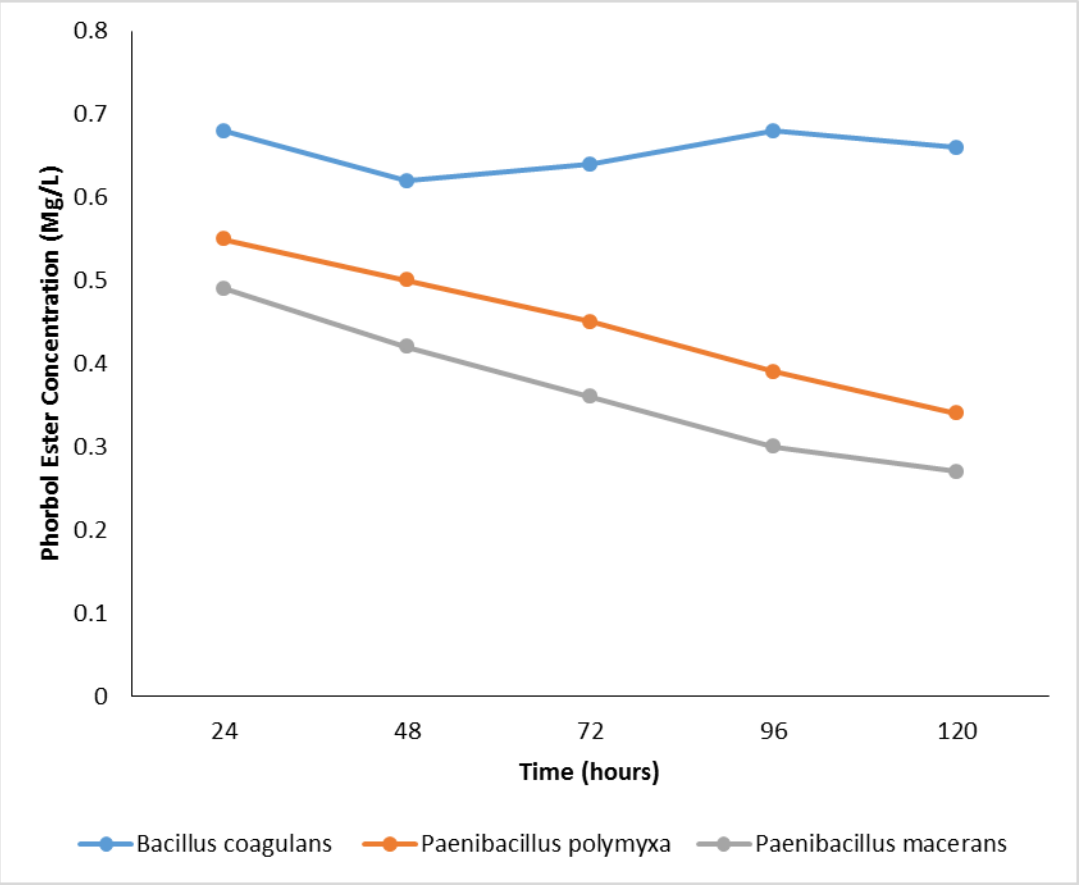


Fig 4.6: Phorbol ester concentration at 30^0C and pH 6.5

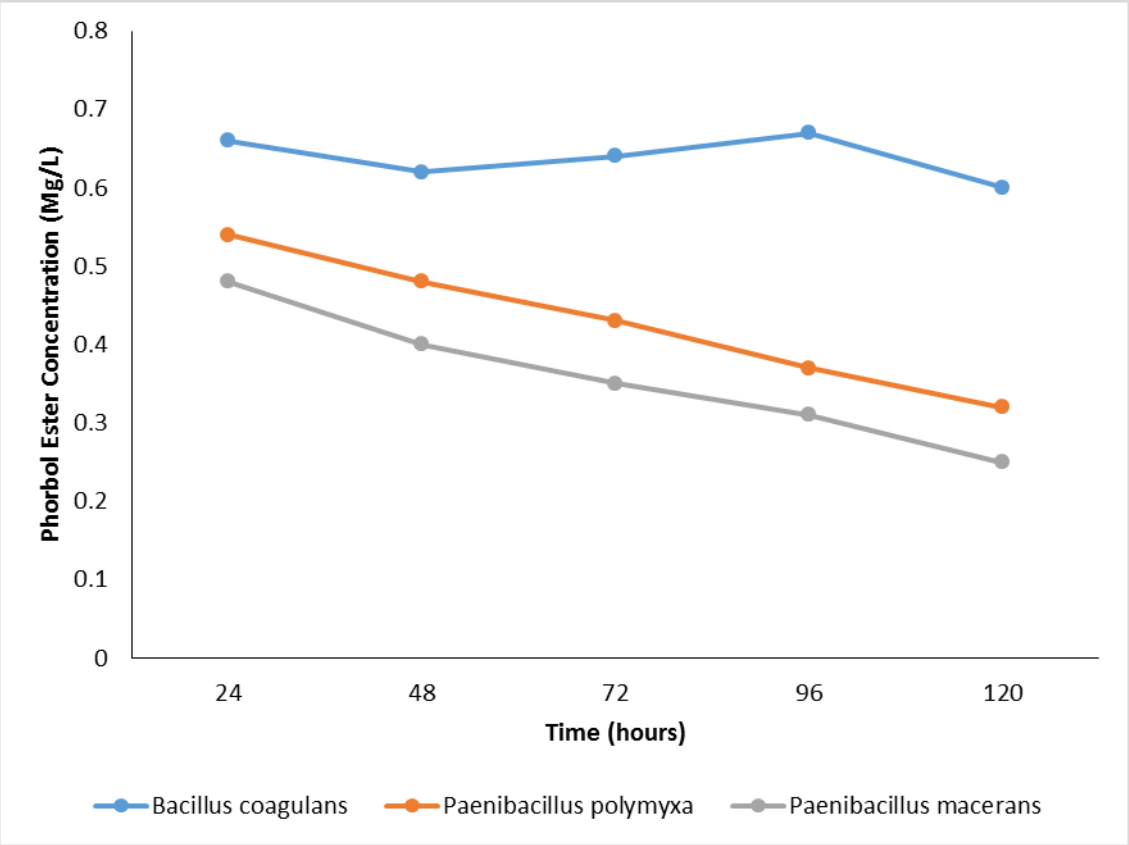


Fig 4.7: Phorbol ester concentration at 30^0C and pH 8.5

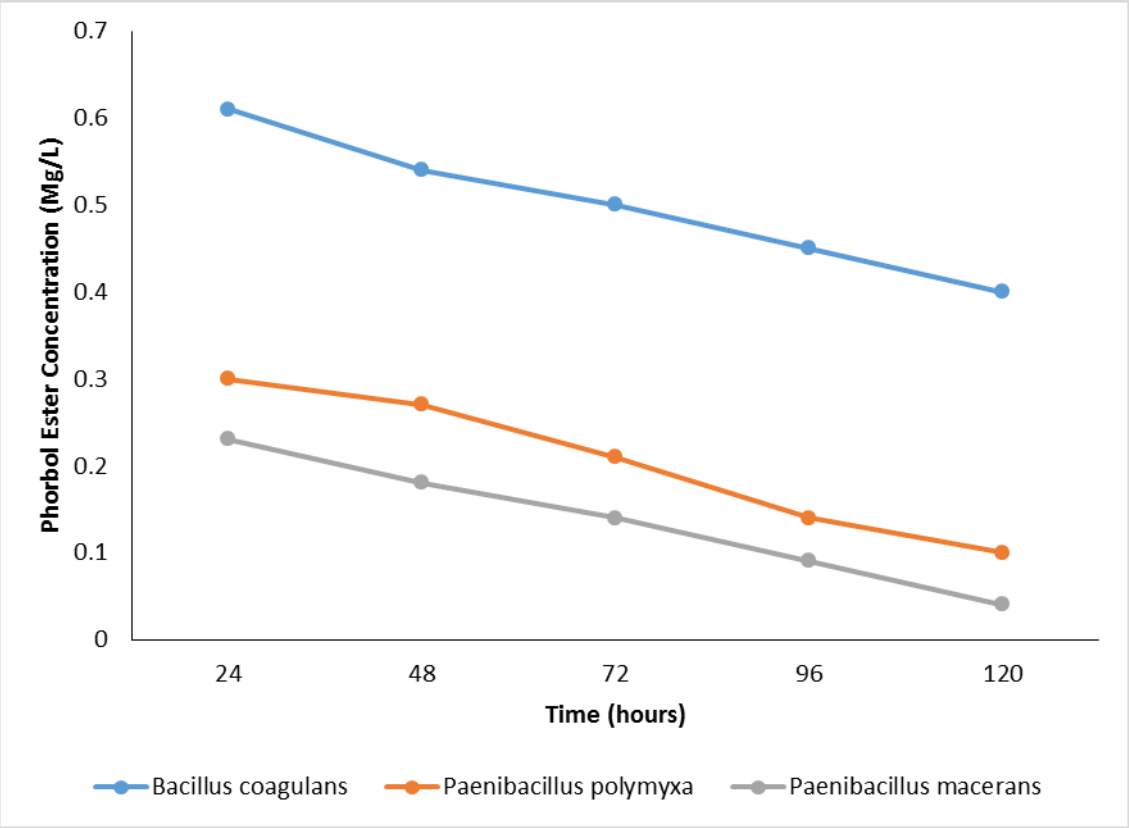


Fig 4.8: Phorbol ester concentration at 37^0C and pH 4.5

Fig 4.9 shows the phorbol ester concentrations of the fermented seed cake at 37^0C and pH 6.5. *Paenibacillus macerans* reduces phorbol ester faster with time than the *Bacillus coagulans* at pH 6.5 and a temperature of 37^0C starting with a concentration of 0.21 and reduced to 0.03 for *Paenibacillus macerans,* a concentration of 0.29 and reduced to 0.08 for *Paenibacillus polymyxa* and a concentration of 0.57 and reduced to 0.41 with *Bacillus coagulans*.

Fig 4.10 shows the phorbol ester concentrations of the fermented seed cake at 37^0C and pH 8.5. *Paenibacillus macerans* reduces phorbol ester faster with time when compared to *Paenibacillus polymyxa* and *Bacillus coagulans* at pH 8.5 and a temperature of 37^0C starting with a concentration of 0.20 and reduced to 0.01 for *Paenibacillus macerans,*a concentration of 0.28 and reduced to 0.07 for *Paenibacillus polymyxa* and a concentration of 0.55 and reduced to 0.42 for *Bacillus coagulans*.

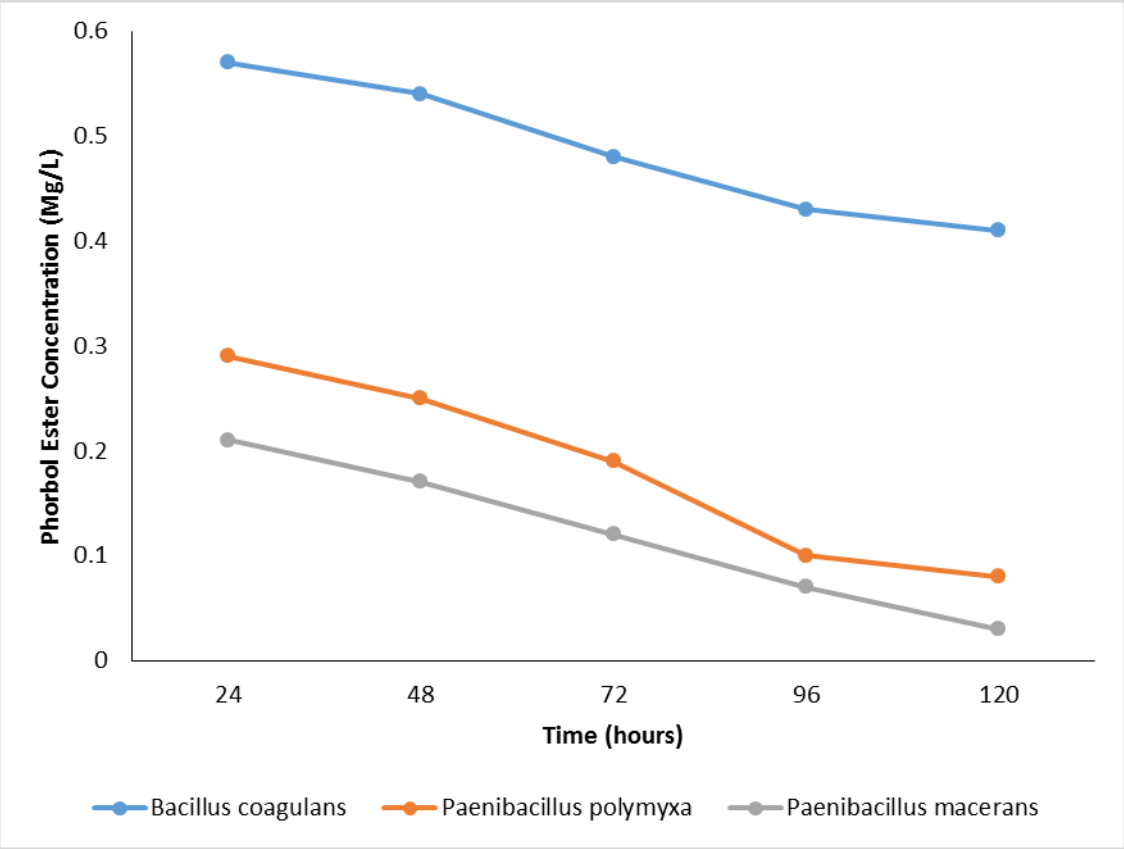


Fig 4.9: Phorbol ester concentration at 37^0C and pH 6.5

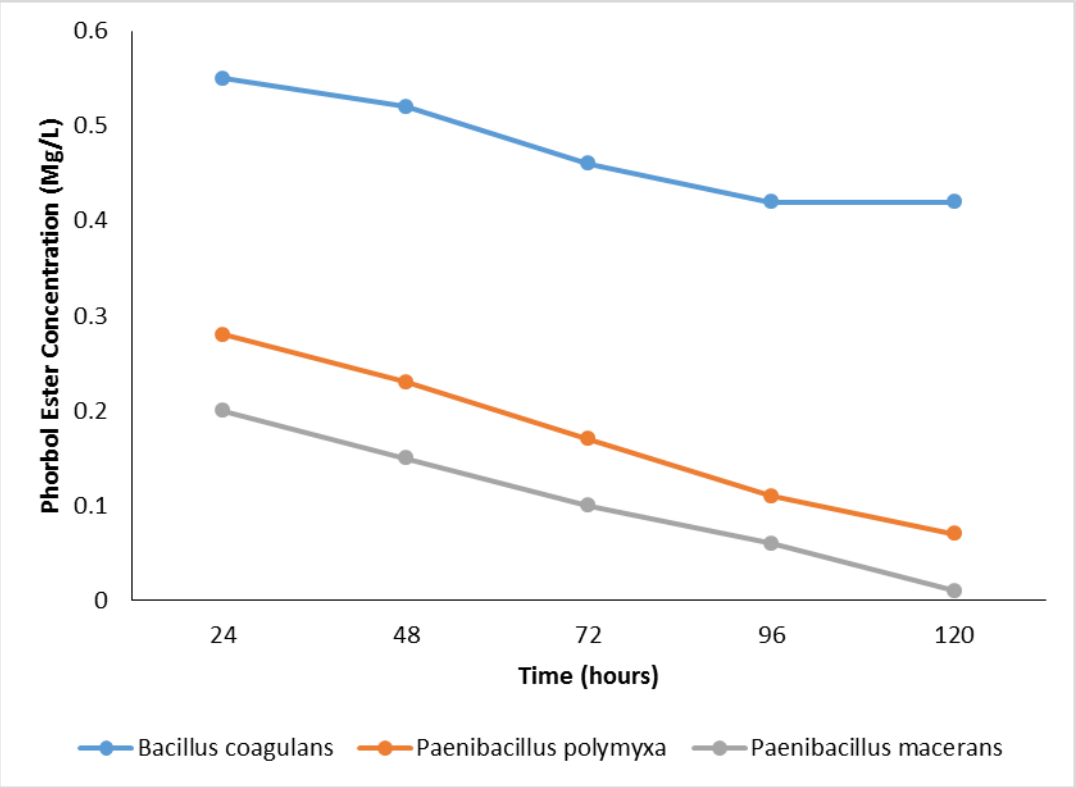


Fig 4.10: Phorbol ester concentration at 37^0C and pH 8.5

CHAPTER FIVE

5.0 DISCUSSION

5.1 Proximate Analysis

The proximate composition in Table 4.1 revealed that *J. curcas* seed cake has a moisture content of 5.45% which indicates that the seed cake used for this study had little or no moisture content. Crude protein (23.75%), crude fibre (11.38%), fat and oil (9.8%), ash content (8.2%) and digestible carbohydrate (46.45%). This study is in agreement with the study of Makkar *et al.,* (1997) and Makkar and Becker (1997), who reported *J. curcas* seed cake to have a crude protein content ranging from 580-640g Kg^{-1} of which 90% is true protein. It is also in agreement with the study of Runmi *et al.,* (2014), who reported to have obtained moisture content (5.87%), Ash content (6.23%), Fat content (12.4%), Protein content (41.14%) and residue oil (12%).

5.2 Phytochemical Analysis

Phorbol ester, phytic acid, lectin and trypsin inhibitor were found to be higher in the *J. curcas* seed cake in which the extraction was carried out manually to be 1.48, 11.02, 14.0 and 10.44mg/l respectively when compared to the seed cake in which the biodiesel was extracted with a machine whose values were 0.94, 9.58, 13.48 and 7.87mg/l respectively. This could be due to the fact that with the use of machine there would be complete extraction and so most of the anti-nutrients and toxin would have been crushed out

alongside with the biodiesel thereby making the concentration lower than in the manually extracted biodiesel.

Post fermentation as shown on Table 4.2, the concentration of phorbol ester, Phytic acid, Saponin, Lectin and Trypsin inhibitor were reduced by 76.35, 56.26, 43.56, 58.75, and 64.94% which is in agreement with the work of Phengnuam and Suntornsuk (2013), whose Phorbol ester, phytate and trypsin inhibitor reduced after fermentation by 62, 42 and 75%, respectively.

Jatropha seeds are mechanically pressed in order to obtain oil, and generating a large amount of oil cake (41–57 percent on dry matter basis) as a by-product (Makkar *et al*., 1997). Jatropha oil cake is rich in nitrogen, phosphorus and potassium (Kumar and Sharma, 2008), thus it is thought to be an excellent candidate for use as a fertilizer for plant nutrient source. However, toxic Jatropha phorbol esters restrict its utilization as fertilizer.

5.3 Effect of the Isolates on Phorbol Esters Detoxification

Fermented *J. curcas* seed cake revealed that the Phorbol ester in the seed cake reduced as the fermentation time increases. Fermented *J. curcas* seed cake solution containing the *Paenibacillus macerans* ferments *J. curcas* seed cake more with a percentage of 76.35% when compared to the *J. curcas* seed cake solution containing *Paenibacillus polymyxa* (60.81%) and *Bacillus coagulans* (45.27%) as well as the control (22.97%). This may be because *Paenibacillus macerans* capable of synthesizing comparably higher esterase concentrations to break down the phorbol ester. This is in agreement with the work of Chin-Feng *et al.* (2014) who was able to detoxify the seed cake by 76.5, 77.1 and 78.4% using *B. smithii, B. sonorensis* and *B. licheniformis* respectively.

Degradation of Jatropha phorbol esters has been usually conducted by various chemical and physical methods. It has been reported that high heat and pressure treatments (260^0C, 3 mbar) with moisture degraded Jatropha phorbol esters in oil completely (Makkar *et al*., 2009). Combination of alkali and autoclave treatments (121^oC, 30 min) decreased Jatropha phorbol esters up to 89 percent in seed meal (Rakshit *et al*., 2008). An autoclave treatment of Jatropha seed meal followed by four repeated washes with methanol degraded 95% of phorbol esters (Aregheore *et al*., 2003). Although these chemical and physical treatments decreased Jatropha phorbol esters efficiently, detoxification method of Jatropha oil cake by incubation with a bacteria for five days is milder and more cost-effective than other methods.

5.4 Effect of the Isolates on Phytic Acid Reduction

Fermented *J. curcas* seed cake revealed that the phytic acid content of the fermented seed cake also reduces with time. Phytate which is the common storage form of phosphorus in plant seeds and cereal grains (*Reddy et al*., 1982) is considered to be an anti-nutritional factor for humans and animals because of its high chelating ability with cations and complex formation with the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients (Wodzinski and Ullah, 1996: Martinez *et al.,* 1996).

The fermented *J. curcas* seed cake solution containing the *Paenibacillus macerans* ferments *J. curcas* seed cake more by reducing the Phytic acid level up to 53.26% when compared to *P. polymyxa* (45.37%) and *B. coagulans* (28.95%) as well as the control (16.42%). This may be because *Paenibacillus macerans* can readily synthesize phytases for catalysis of phytate. This is in agreement with the work of Saetae and Suntornsuk, (2011)

whose phytic acid reduced after detoxification. It is also in agreement with the work of Phengnuam and Suntomsuk, (2013) whose phytate after detoxification reduced to about 42%. Phytate is the major storage form of phosphorus in seeds and is found in diets of many animals and humans. Phosphorous is one of the major feed ingredients and is supplied to animals in required amounts through raw material and added phosphates. 50−80% of phosphorous is bound in phytates, which cannot be broken down by endogenous enzymes in poultry (Wodzinski and Ullah, 1996).

5.5 Effect of the Isolates on Saponin Reduction

There was a reduction of the saponin content more in *Paenibacillus macerans* than in *P. polymyxa* and *B. coagulans* as well as the control. The saponin contents were reduced by 43.56, 31.26, 18.87 and 11.46% for *Paenibacillus macerans, P. polymyxa, B. coagulans* and the control respectively. This is in partial agreement with the work of Saetae and Suntornsuk, (2011) whose saponin content reduced by 80% after fermentation. Saponins may serve both as anti-feedants and plant anti-microbial agents, but some plant saponins may enhance nutrient absorbtion and aid in animal digestion. However, saponins are often bitter to taste, and so can reduce plant palatability (Foerster, 2006).

5.6 Effect of the Isolates on Lectin Reduction

There was a reduction in the lectin concentration of the *J. curcas* seed cake which was more in *P. macerans* than in *P. polymyxa* and *Bacillus coagulans* as well as the control. The lectin contents were reduced by 58.75, 41.61, 32.29 and 16.07% for *Paenibacillus macerans, P. polymyxa, B. coagulans* and the control respectively. This reduction could be as a result of *P. macerans* being able to utilize lectin more as a source of nutrient than the

others. This is in disagreement with the work of Saetae and Suntornsuk, (2011) whose results show that the lectin contents were not detected in the detoxified *J. curcas* seed cake, whereas they were observed in high levels in *J. curcas* seed cake.

5.7 Effect of the Isolates on Trypsin Inhibitor Reduction

There was a reduction in the trypsin inhibitor concentration of the *J. curcas* seed cake which was more in *P. macerans* than in *P. polymyxa* and *Bacillus coagulans* as well as the control. The trypsin inhibitor contents were reduced by 64.94, 46.84, 31.03 and 15.80% for *Paenibacillus macerans, P. polymyxa, B. coagulans* and the control respectively. This reduction could be as a result of *P. macerans* being able to utilize trypsin more as a source of nutrient than the others. This is in agreement with the work of Phengnuam and Suntomsuk, (2013) whose trypsin inhibitor levels were reduced by 75% after detoxification. It is also in agreement with the work of Saetae and Suntornsuk, (2011) whose trypsin inhibitor level found in the detoxified seed cake were much lower than those found in *J. curcas* seed cake.

5.8 Effect of pH, Temperature and Time on Detoxification of Phorbol Esters by Isolates

The mean values obtained from Tables 4.8 showed statistical difference in each of the organism. *Bacillus coagulans* had mean values of 0.81, 0.65 and 0.49 at 27, 30 and 37^0C respectively. *Paenibacillus polymyxa* had mean values of 0.73, 0.45 and 0.19 at 27, 30 and 37^0C respectively. *Paenibacillus macerans* had mean values of 0.67, 0.37 and 0.12 at 27, 30 and 37^0C respectively. The lowest mean value of 0.12 observed for *Paenibacillus macerans* indicates that the organism had the best detoxification and anti-nutrient potential

at a temperature of 37^0C. There were no statistical difference in the mean optimization of phorbol ester reduction with respect to time and pH.

Conversely, *Bacillus coagulans* displayed the least detoxification and reduction in the anti-nutrients potential. This is in contrast to the work of Mohamed and Ashraf (2014) whose results after fermenting *J. curcas* seed cake with a *Bacillus* (*Bacillus pumilus*) indicated that fermented *J. curcas* seed cake can replace up to 50% dietary fish meal. Varying temperature, pH and time of fermentation showed that *Paenibacillus macerans* at 37^oC, pH 8.5 and within the duration of 5 days will detoxify *J. curcas* seed cake in a submerged fermentation medium provided pH and temperature remains constant. This reduction is related to the activities of esterase, phytase and protease enzymes. *Jatropha curcas* seed cake could be detoxified by bacterial fermentation and the high protein fermented seed cake could be potentially applied to animal feed.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Three Bacterial strains used for this study were isolated and identified to be *Paenibacillus macerans, Paenibacillus polymyxa* and *Bacillus coagulans.*

The proximate compositions of Jatropha curcas seed cake were moisture of 5.45%, Ash of 8.20%, crude protein of 23.75%, crude fat of 9.80%, crude fibre of 11.38% and digestible carbohydrate of 46.45%. Phytochemical factors of non-fermented *J. curcas* seed cake include phorbol ester- 1.48mg/L, phytic acid- 11.02mg/L, saponin- 24.70mg/L, lectin- 14.06mg/L, trypsin inhibitor- 10.44mg/L while that of fermented *J. curcas* seed cake were phorbol ester- 0.35mg/L, phytic acid- 4.82mg/L, saponin- 13.94mg/L, lectin- 5.80mg/L, trypsin inhibitor- 3.66mg/L.

The percentage reduction of phorbol ester and Anti- nutritional factors when *Bacillus coagulans* was used is 45.27% for phorbol ester, 28.95% for phytic acid, 32.29% for lectin, 18.87% for saponin and 31.03% for trypsin inhibitor, when *Paenibacillus polymyxa* was used is 60.81% for phorbol ester, 45.37% for phytic acid, 41.61% for lectin, 31.26% for saponin and 46.84% for trypsin inhibitor, and when *Paenibacillus macerans* was used is 76.35% for phorbol ester, 56.26% for phytic acid, 58.75% for lectin, 43.56% for saponin

and 64.94% for trypsin inhibitor. They all did better than the control which was 22.97% for phorbol ester, 16.42% for phytic acid, 16.07% for lectin, 11.46% for saponin and 15.80% for trypsin inhibitor.

The optimal temperature for the detoxification of *J. curcas* seed cake was achieved at around 37^0C for the three Bacterial strains (*Paenibacillus macerans, Paenibacillus polymyxa* and *Bacillus coagulans*) used for this study.

6.2 Conclusion

J. curcas seed cake, a by-product of *J. curcas* with both high toxin and anti-nutritional factor levels, were detoxified during the course of this study by three Bacterial species, *Paenibacillus macerans, Paenibacillus polymyxa* and *Bacillus coagulans*. In this study, *Paenibacillus macerans* was discovered to be the best organism with a detoxification and anti-nutritent potential in the *J. curcas* seed cake meal followed by *Paenibacillus polymyxa* and *Bacillus coagulans* respectively.

Temperature is a factor which influences the rate of reduction of the phorbol ester in *J. curcas* seed cake during fermentation.

The reduction of phorbol esters, phytate and trypsin inhibitor may be due to the presence of esterase, phytase and protease activities, respectively. *J. curcas* seed cake could be detoxified by bacterial fermentation using *Bacillus* strains and the rich-protein fermented seed cake could be potential for use in animal feed.

6.2 Recommendations

1. More work should be done on the complete detoxification of the phorbol ester in the *J. curcas* seed cake using *Paenibacillus macerans* and *Paenibacillus polymyxa* by submerged and solid state fermentation so that if complete removal or detoxification can be achieved, it can be used as feed supplements for animals and even humans.

2. Identification of the gene coding for the detoxification of the ester will enhance the understanding of the mechanism involved.

3. The use of genetically modified bacteria can also be employed in the detoxification process (fermentation). The use of mutant strains and also a consortium of *Bacillus* species can also be investigated.

REFERENCES

Abdel-Hafez, A.A.M., Nakamura, N. and Hattori, M. (2002). Biotransformation of phorbol by human intestinal bacteria *Chemical and Pharmaceutical Bulletin*, **50**(2): 160 – 164.

Abou-Arab, A.A. and Abu-Salem, F.M. (2010). Nutritional quality of *J. curcas* seeds and effect of some physical and chemical treatments on their anti-nutritional factors. *African Journal of Food Science*, **4**(3): 93 – 103.

Abreu, F. (2008). Alternative By-products from *Jatropha*. In: International Consultation on Pro-poor *Jatropha* Development. 10–11 April 2008, Rome. *IFAD.*

Achten, W.M.J., Mathijs, E., Verchot, L., Singh, V.P., Aerts, R. and Muys, B. (2007). *Jatropha* biodiesel fueling sustainability? Biofuels, Bioproducts and Biorefining. *The Jatropha Archives*, **1**(4): 283-291.

Achten, W.M.J., Verchot, L., Franken, Y.J., Mathijs, E., Singh, V.P., Aerts, R. and Muys, B. (2008). *Jatropha* bio-diesel production and use (a literature review). *Biomass and Bioenergy the Jatropha Archives*, **32**(12): 1063-1084.

Adebowale, K.O. and Adedire, C.O. (2006). Chemical composition and insecticidal properties of underutilized *J. curcas* seed oil. *African Journal of Biotechnology*, **5**(10): 901 – 906.

Adler-Nissen, J. (1986). Enzymatic Hydrolysis of Food Proteins. *Elsevier Applied Science Publishers*. New York, pp 110-169.

Adolf, W., Opferkuch, H.J. and Hecker, E. (1984). Irritant phorbol derivatives from four *Jatropha* species. *Phytochemistry*, **23**: 129 – 132.

Ahmed, W.A. and Salimon, J. (2009). Phorbol ester as toxic constituents of tropical *J. curcas* seed oil. *European Journal of Scientific Research*, **31**(3): 429 – 436.

Anderson, RE. (1984). Growth and corresponding elevation of tomato juice pH by Bacillus coagulans. *Journal of Food Science*, **49**: 647-649.

Aregheore, E.M., Becker, K. and Makkar, H.P., (2003). Detoxification of a toxic variety of *J. curcas* using heat and chemical treatments, and preliminary nutritional evaluation with rats. *South Pacific Journal of Natural and Applied Sciences*, **21**: 51–56.

Ash, C., Priest, F.G. and Collins, M.D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus. Antonie Van Leeuwenhoek*, **64**: 253-260.

Association of Official and Analytical Chemists (AOAC). (1995). Official method of analysis. 16th edition. *The association of official analytical chemists. Arlington, Virginia,* pp. 1-6

Baron, M. (2009). A patented strain of *Bacillus coagulans* increased immune response to viral challenge. *Postgraduate Medicine,* **121**(2): 114–118.

Batra, N., Singh, J., Banerjee, U.C., Patnaik, P.R. and Sobti R.C. (2002). Production and characterization of a thermostable beta-galactosidase from *Bacillus coagulans* RCS3. *Biotechnology and Applied Biochemistry,* **36**: 1-6.

Becker, K. and Makkar, H.P.S. (1998). Effects of phorbol esters in Carp (*Cyprinus carpio L*). *Veterinary and Human Toxicology,* **40**(2): 82-86.

Belewu, M.A. and Akande, B.A. (2010). Biological upgrading of the nutritional quality of *J. curcas* kernel cake: effect on performance characteristics of goat. *International Research Journal of Biotechnology,* **1**(2): 19 – 22.

Belewu, M.A., Belewu, K.Y. and Ogunsola, F.O. (2010a). Nutritive value of dietary fungi treated *J. curcas* kernel cake: Voluntary intake, growth and digestibility coefficient of goat. *Agriculture and Biology Journal of North America,* **1(2):** 135 – 138.

Belewu, M.A., Eniolorunda, O.O. and Ilori, G.I. (2010b). Response of goat to fungi (*Rhizopus oligosporus, Rhizopus nigrican*) treated *J. curcas* kernel cake. *Archives of Applied Science Research,* **2**(4): 255 – 261.

Ben-Jacob, E. (2003). Bacterial self-organization: co-enhancement of complexification and adaptability in a dynamic environment. *Philosophical Transactions of the Royal Society of London A.,* **361**: 1283-1312.

Ben-Jacob, E. and Cohen, I. (1997). Cooperative formation of bacterial patterns. In Bacteria as Multicellular Organisms Edited by Shapiro JA, *Dworkin M. New York: Oxford University Press,* pp. 394-416.

Ben-Jacob, E. and Levine, H. (2005). Self-engineering capabilities of bacteria. *Journal of the Royal Society Interface,* **3:** 197-214.

Ben-Jacob, E., Cohen, I. and Gutnick, D.L. (1998). Cooperative organization of bacterial colonies: from genotype to morphotype. *Annual Review of Microbiology,* **52:** 779-806.

Ben-Jacob, E., Cohen, I. and Levine, H. (2000b). Cooperative self-organization of microorganisms. *Advanced Physiology,* **49**: 395-554.

Ben-Jacob, E., Cohen, I., Golding, I., Gutnick, D.L., Tcherpakov, M., Helbing, D. and Ron, I.G. (2000a). Bacterial cooperative organization under antibiotic stress. *Physica A.,* **282**: 247-282.

Ben-Jacob, E., Shmueli, H., Shochet, O. and Tenenbaum, A. (1992). Adaptive self-organization during growth of bacterial colonies. *Physica A.*, **187**: 378-424.

Ben-Jacob, E., Shochet, O., Tenenbaum, A. and Avidan, O. (1995). Evolution of complexity during growth of bacterial colonies. *In NATO Advanced Research Workshop; Santa Fe, USA*, pp. 619-633.

Bird, R. and Hopkins, R. H. (1954). The action of some alpha-amylases on amylase. *Biochemistry. Journal*, **56:** 86–99.

Bloemberg, G.V. and Lugtenberg, B.J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current Opinion Plant Biology*, **4**: 343-350.

Brittaine, R. and Lutaladio, N. (2010). Jatropha: A smallholder Bioenergy crop, the potential for Pro-Poor development. *Integrated crop management*, **8**: 14-53

Carroll, G.L. and Little, D.R. (2000). Inter- and intramolecular reductive coupling reactions: An approach to the phorbol skeleton. *Organic Letters,* **2**(18): 2873 – 2876.

Cassey, A. and Walsh, G. (2004). Identification and characterization of a phytase of potential commercial interest. *Journal of Biotechnology,* **110**: 313-322.

Chin-Feng, C., Jen-Hsien, W., Kao-Yung, L., Li-Yun, Liu and Shang-Shyng, Y. (2014). Phorbol esters degradation and enzyme production by *Bacillus* using *Jatropha* seed cake as substrate. *International Journal of Environmental Pollution and Remediation,* **2:** 30-36.

Chivandi, E., Kachigunda, B. and Fushai, F. (2005). A comparison of the nutrient and antinutrient composition of industrially processed Zimbabwean *J. curcas* and Glycine max meals. *Pakistan Journal of Biological Sciences*. **8**(1): 49 – 53.

Chivandi, E., Mtimuni, J.P., Read, J.S. and Makuza, S.M. (2004). Effect of processing method on phorbol esters concentration, total phenolics, trypsin inhibitor activity and the proximate composition of the Zimbabwean *J. curcas* provenance: A potential livestock feed. *Pakistan Journal of Biological Sciences*, **7**(6): 1001 – 1005.

Choi, K.K., Park, C.W., Kim, S.Y., Lyoo, W.S., Lee, S.H. and Lee, J.W. (2004). Polyvinyl alcohol degradation by *Microbacterium barkeri* KCCM 10507 and Paeniblacillus amylolyticus KCCM 10508 in dyeing wastewater. *Journal Microbiology Biotechnology,* **14**: 1009-1013.

Cosentino, S., Mulargia, A.E., Pisano, B., Tuveri, P. and Palmas, F. (1997). Incidence and biochemical characteristics of Bacillus flora in Sardinian dairy products. *International Journal of Food Microbiology*, **38**: 235-238.

Crombie, L., Games, M.L. and Pointer, D.J. (1968). Chemistry and structure of phorbol, the diterpene parent of co-carcinogens of Croton oil. *Journal Chemical Society*, pp 1347 – 1362.

De Clerck, E., Rodriguez-Diaz, M., Forsyth, G., Lebbe, L., Logan, N.A. and De Vos, P. (2004). Polyphasic characterization of Bacillus coagulans strains, illustrating heterogeneity within this species, and emended description of the species. *Systematic Applied Microbiology*, **27**: 50-60.

De-Vecchi, E. and Drago, L. (2006). L. sporofenes or B. coagulans: Misidentification or Mislabelling? *International Journal of Probiotics and Prebiotics*, **1(1)**: 2-10.

Demissie, A.G. and Lele, S.S. (2010). Bioassay-assisted identification of phorbol ester from *J. curcas* (Linn.) tissue culture. *International Journal of Pharmacy and Biosciences*, **1(3):** 1 – 7.

Dowlathabad, M.R., Sreeyapureddy, A., Adhikari, A., Bezawada, K. and Nayakanti, D. (2010). Pharmaceutical investigation and biopesticidal activity of *J. curcas* L. seed oil on digestive enzymic profiles of *Cnaphalocrocis medinalis* (Rice leaf folder) and *Helicoverpa armigera* (Cotton boll worm). *International Research Journal of Pharmacy*, **1**(1): 194 – 200.

Dunphy, W.G., Delclos, K.B. and Bumberg, P.M. (1980). Characterization of specific binding of [3H] phorbol 12, 13-dibutyrate and [3H] phorbol 12-myristate 13-acetate to mouse brain. *Cancer Research*, **40**: 3635 – 3641.

Endres, J. R., Clewell, A., Jade, K. A., Farber, T., Hauswirth, J. and Schauss, A. G. (2009). Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. *Food and Chemical Toxicology*, **47(6):** 1231–1238.

Errington, J. (2003). Regulation of endospore formation in Bacillus subtilis. *Nat Rev Microbiology*, **1**: 117–126.

Essamri, M., Deyris, V. and Comeau, L. (1998). Optimization of lipase production by *Rhizopus oryzae* and study on the stability of lipase in organic solvents. *Journal of Biotechnology*, **60**: 97-103.

FACT (2007). Position Paper on *J. curcas* L. State of the art, small and large scale project development. *Fuels from Agriculture in Communal Technology* (available at http://www.fact-fuels.org).

Fitzpatrick, L.R. (2013). Probiotics for the treatment of Clostridium difficile associated disease. *World Journal of Gastro-intestinal Pathophysiology*, **4**(3): 47–52.

Foerster, H. (2006). Metacyc pathway: saponin biosynthesis. *Annals of the New York Academy of Sciences,* **138(2):** 14-18

Francis, G., Edinger, R. and Becker, K. (2005). A concept for simultaneous wasteland reclamation, fuel production, and socio-economic development in degraded areas in India: need, potential and perspectives of *Jatropha* plantations. *Natural Resources Forum,* **29:** 12–24.

Gaur, S., Viswanathan, B., Seemamahannop, R., Kapila, S. and Book, N.L. (2011). Development and evaluation of an efficient process for the detoxification of *Jatropha* oil and meal. *Nature Precedings*; doi 10.1038/npre.2011.6118.1.

Gaydou, A.M., Menet, L., Ravelojaona, G. and Geneste, P. (1982). Vegetable energy sources in Madagascar: ethyl alcohol and oil seeds. *Oleagineux,* **37**: 135-141

Ghosh, A., Patolia, J.S., Chaudhary, D.R., Chikara, J., Rao, S.N., Kumar, D., Boricha, G.N. & Zala, A. (2007). Response of *J. curcas* under different spacing to *Jatropha* de-oiled cake. *The Netherlands: FACT Foundation*, pp 5-6.

Girardin, H., Albagnac, C., Dargaignaratz, C., Nguyen-The, C. and Carlin, F. (2002). Antimicrobial activity of foodborne *Paenibacillus* and *Bacillus spp*. against *Clostridium botulinum. Journal of Food Prot*, **65**: 806-813.

Goel, G., Makkar, H.P.S., Francis, G. and Becker, K. (2007). Phorbor ester: structure, biological activity, and toxicity in animals. *International journal of toxicology*, **26:** 279-288.

Gordon, J.A. and Marquardt, M.D. (1974). Factors affecting hemagglutination by concanavalin A and agglutinin. *Biochimica et Biobpysica Acta.,* **322**: 136-144.

Gour, V. K. (2006). Production practices including post-harvest management of *J. curcas*. In: Singh, B., Swaminathan, R., Ponraj, V. (eds). *Proceedings of the biodiesel conference toward energy independence – focus of Jatropha, Hyderabad, India,* June 9–10. New Delhi, Rashtrapati Bhawan, pp 223–251.

Graumann, P. (editor) (2012). *Bacillus: Cellular and Molecular Biology* (2nd ed). *Caister Academic Press.* ISBN 978-1-904455-97-4. [1].

Gschwendt, M. and Hecker, E. (1970). Tumor-promoting compounds from Euphorbia cooperi: Di- and triesters of 16-hydroxy-12-deoxy-phorbol. *Tetrahedron Letters,* **8**: 567 -570.

Gubitz, G.M., Mittelbach, M. & Trabi, M. (1999). Exploitation of the tropical oil seed plant *J. curcas* L. *Bioresource Technology*, **67:** 73–82.

Gupta, A., Murarka, A., Campbell, P. and Gonzalez, R. (2009). Anaerobic Fermentation of Glycerol in *Paenibacillus macerans*: Metabolic Pathways and Environmental Determinants. *Applied and Environmental Microbiology*, **75**(18): 5871–5883.

Haas, W., Sterk, H. and Mittelbach, M. (2002). Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *J. curcas*. *Journal of Natural Products*, **65**(10): 1434 – 1440.

Hammer, B. W. (1915). Bacteriological studies on the coagulation of evaporated milk. *Iowa Agricultural Experiment. Strain. Resource Bulletin*, **19**: 119-131

Hass, W. and Mittelbach, M. (2000). Detoxification experiments with the seed oil from *J. curcas L*. *Industrial Crops and Products*, **12**(2): 111-118.

Hecker, E. (1987). Three stage carcinogenesis in mouse skin – Recent results and present status of advanced model system of chemical carcinogenesis. *Toxicologic Pathology*, **15**(2): 245 – 258.

Heller, J. (1996) Physic nut. *J. curcas L*. Promoting the conservation and use of underutilized and neglected crops 1. Institute of Plant Genetics and Crop Plant Research, Gatersleben / International Plant Genetic Resources Institute: Rome, Italy.

Henning, R.K. (2004a). The *Jatropha* System – an integrated approach of rural development (available at www.*Jatropha*.de).

Henning, R.K. (2004b). *J. curcas* L. in Africa. *Case Study. Weissenberg, Germany, Bagani.*

Henning, R.K. (2008). Identification, selection and multiplication of high yielding *J. curcas* L plants and economic key points for viable *Jatropha* oil production costs. Paper presented to: *International Consultation on Pro-poor Jatropha Development.* 10–11 April 2008, Rome, IFAD (available at http://www.ifad.org/events/*Jatropha*/).

Hipal, G., Mayank, G., Nikhil, G., Soham, T., Prasad, P., Girish, G., Vamsi, K.K., Reddy, M.P., Sethiya, B.D. and Rathod, M.R. (2009). Isolation and characterization of phorbol esters (toxin) from the *J. curcas* L. *International Journal of Microbiology Research*, **1**(1): 1 – 7.

Hirota, M., Suttajit, M., Suguri, H., Endo, Y., Shudo, K., Wongchai, V., Hecker, E. and Fujiki, H. (1988). A new tumor promoter from the seed oil of *J. curcas* L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. *Cancer Research*, **48**: 5800 – 5804.

Hobman, J. L., Penn, C. W. and Pallen, M. J. (2007). Laboratory strains of Escherichia coli: model citizens or deceitful delinquents growing old disgracefully? *Molecular Microbiology*, **64**: 881–885.

Hudson and El-Difrawi, (1979). The method for sapogenins determination in seeds of Lupine spp. and Jatropha plant. *Food Chemistry*, **3:** 181-186.

Hun, L. (2009). Bacillus coagulans significantly improved abdominal pain and bloating in patients with IBS. *Postgraduate Medicine* ,**121** (2): 119–124.

Ingham, C.J. and Ben-Jacob, E. (2008). Swarming and complex pattern formation in *Paenibacillus vortex* studied by imaging and tracking cells. *BMC Microbiology,* **8** pg 36.

International Seed Testing Association (ISTA) (1999). *International Rule for Seed Testing Association. Bassersdorf. Switzerland.*

Ishida, H., Hata, Y., Kawato, A., Abe Y. and Kashiwagi, Y. (2004). Isolation of a novel promoter for efficient protein production in *Aspergillus orygae*. *Bioscience, Biotechnology, and Biochemistry,* **68**: 1849-1857.

Jacobs, S., Sahyoun, N.E., Saltiel, A.R. and Cuatrecasas, P. (1983). Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. *Biochemistry*, **80**: 6211 – 6213.

Janick, J. and Robert, E.P. (2008). *The Encyclopedia of Fruit & Nuts*. CABI, pp 371–372.

Jayaraman, P., NesaPriya, S., Parameshwari, S., Priya, S.S., Jawahar, N. and Babu, H.S. (2011). Occurrence of storage fungi in *Jatropha* (*J. curcas* L.) seeds *African Journal of Microbiology Research*, **5**(5): 475 – 480.

Jongschaap, R.E.E., Corré, W.J., Bindraban, P.S. and Brandenburg, W.A. (2007). Claims and Facts on *J. curcas* L. *Wageningen, Plant Research International*.

Joshi, C., Mathur, P. and Khare, S.K. (2011). Degradation of phorbol esters by *Pseudomonas aeruginosa* PseA during solid-state fermentation of deoiled *J. curcas* seed cake. *Bioresource Technology,* **102:** 4815 – 4819.

Juhász, A.C.P., Pimenta, S., Soares, B.O., Batista Morais de Lourdes, D. and Rabello de Oliveira, H. (2009). Floral biology and artificial polinization in physic nut in the north of Minas Gerais state, Brazil [Biologia floral e polinização artificial de pinhão-manso no norte de Minas Gerais] *Pesquisa Agropecuaria Brasileira*, (in Portuguese), **44**(9): 1073–1077

Kamath, P., Subrahmanyam VM., Rao, J.V. and Vasanth Raj, P. (2010). Optimization of cultural conditions for protease production by a fungal species. *Indian journal of pharmaceutical sciences.* **72**:161-166.

Kawamoto, T., Sonomoto, K. and Tanaka, A. (1987). Esterification in organic solvents: selection of hydrolases and effects of reaction conditions. *Biocatalysis,* **1**:137-145.

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y. (1983). Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *The Journal of Biological Chemistry,* **258**(19): 11442 – 11445.

King, A.J., He, W., Cuevas, J. A., Freudenberger, M., Ramiaramanana, D. and Graham, I. A. (2009). Potential of *J. curcas* as a source of renewable oil and animal feed. *Journal of Experimental Botany*. **5:** 12-15

Kloepper, J.W., Leong, J., Teintze, M. and Schroth, M.N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature,* **286:** 885-886.

Konishi, J. and Maruhashi, K. (2003). 2-(2'-Hydroxyphenyl) benzene sulfinate desulfinase from the thermophilic desulfurizing bacterium *Paenibacillus* sp. strain A11-2: purification and characterization. *Applied Microbiology Biotechnol*ogy, **62**: 356-361.

Kumar, A. and Sharma, S. (2008). An evaluation of multipurpose oil seed crop for industrial uses (*J. curcas* L.): A review. *Industrial Crops and Products,* **28**(1): 1 – 10.

Lal, S. and Tabacchioni, S. (2009). Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. *Indian Journal of Microbiology,* **49**(1): 2–10.

Lateef, A., Oloke, J.K., Gueguim Kana, E.B., Sobowale, B.O., Ajao, S.O. and Bello B.Y. (2010). Keratinolytic activities of a new feather-degrading isolate of *Bacillus cereus* LAU 08 isolated from Nigerian soil. *International Biodeterioration and Biodegradation,* **64**(2):162-165.

Lapage, S.P., Bascombe, S., Willcox, W.R. and Curtis, M.A. (1973). Identification of Bacteria by Computer: General Aspects and Perspectives. *Journal of General Microbiology.* **77**: 273 -290.

Li, C.Y., Devappa, R.K., Liu, J.X., Lv, J.M., Makkar, H.P.S. and Becker, K. (2010). Toxicity of *J. curcas* phorbol esters in mice. *Food and Chemical Toxicology,* **48**: 620-625.

Long, C. (1971). *Biochemists handbook. Redwood, London,* pp 273-274.

Lui, K. and Markakis, P. (1989). Trypsin inhibition Assay as related to limited hydrolysis of inhibitors. *Analytical Biochemistry*. **178(1):**159-165.

Madigan, M. and Martinko, J. (2005). *Brock Biology of Microorganisms* (11th ed).

Makkar, H.P., Becker, K., Sporer, F. and Wink, M., (1997). Studies on nutritive potential and toxic constituents of different provenances of *J. curcas. Journal of Agriculture and Food Chemistry*, **45**: 3152–3157.

Makkar, H.P., Maes, J., De Greyt, W. and Becker, K., (2009). Removal and degradation of phorbol esters during pre-treatment and trans-esterification of *J. curcas* oil. *Journal American Oil Chemist Society*, **86**: 173–181.

Makkar, H.P.S. and Becker, K. (1997). Potential of *J. curcas* seed meal as a protein supplement to livestock feed, constraints to it utilization and possible strategies to overcome constraints. *In: Biofuels and industrial products from J. curcas, "Jatropha 97", Managua Nicaragua.*

Makkar, H.P.S. and Becker, K. (2009). *J. curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *Eur. Journal of Lipid Science Technology*, **111**: 773 – 787.

Makkar, H.P.S. and Becker, K. (2010). Are *J. curcas* phorbol esters degraded by rumen microbes? *Journal of the Science of Food and Agriculture*, **90**: 1562 – 1565.

Makkar, H.P.S., Aderibigbe, A.O. and Becker, K. (1998). Comparative evaluation of non-toxic and toxic varieties of *J. curcas* for chemical composition, digestibility, protein degradability and toxic factors. *Food Chemistry*, **62**(2): 207 – 215.

Makkar, H.P.S., Francis, G. and Becker, K. (2008). Protein concentrate from *J. curcas* screw-pressed seed cake and toxic and antinutritional factors in protein concentrate. *Journal of the Science of Food and Agriculture*, **88**: 1542 – 1548.

Martinez, C., Ros, G., Periago, M.J., Lopez, G., Ortuno, J. and Rincon, F. (1996). Phytic acid in human nutrition. *Food sciences and technology international*, **2**: 201-209.

Martínez-Herrera, J., Siddhuraju, P., Francis, G., Dávila-Ortíz, G. and Becker, K. (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *J. curcas* L. from Mexico. *Food Chemistry*, **96**: 80 - 89.

Matsumiya, Y., Sumiyoshi, S., Matsukura, T. and Kubo, M. (2007). Effect of Epidermal Cell of Soybean Protein-degraded Products and Structural Determination of the Root Hair Promoting Peptide. *Applied Microbiology and Biotechnology*, **77:** 37-43.

McSpadden-Gardener, B.B. (2004). Ecology of *Bacillus* and *Paenibacillus* spp. in Agricultural Systems. *Phytopathology*, **94**: 1252-1258.

Mohamed, S.H. and Ashraf, M.A-S.G. (2014). Fish nutrition laboratory, National Institute of Oceanography and Fisheries (NIOF), Cario, Egypt. *World Aquaculture Society Aquaculture America 2014-Meeting Abstract,* pg. 20

Montes, M.J., Mercade, E., Bozal, N. and Guinea, J. (2004). *Paenibacillus antarcticus* sp. nov., a novel psychrotolerant organism from the Antarctic environment. *International Journal of System Evolutionary Microbiology*, **54**: 1521-1526.

Morikawa, M. (2006). Beneficial Biofilm Formation by Industrial Bacteria *Bacillus subtilis* and Related Species. *Journal of Bioscience and Bioengineering*, **101**(1): 1-8.

Mwine, J.T. and Van-Damme, P. (2011). Why do Euphorbiaceae tick as medicinal plants? A review of Euphorbiaceae family and its medicinal features. *Journal of Medicinal Plants Research,* **5**(5): 652 – 662.

Nielsen, P. and Sorensen, J. (1997). Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. *Fems Microbiology Ecology* 1997, **22:** 183-192.

Noskin, G.A., Suriano, T., Collins, S., Sesler, S. and Peterson, L.R. (2001). *Paenibacillus macerans* pseudobacteremia resulting from contaminated blood culture bottles in a neonatal intensive care unit. *American Journal of Infection Control,* **29**(2): 126–129.

NOVOD (2007). *Jatropha* – An Alternate source for Biodiesel. *New Delhi: National Oil Seeds and Vegetable Oils Development Board* (available at http://www.novodboard.com).

Olivier, G.W.J., Rowan, M.G., Branch, S.K., Mahon, M.F. and Molloy, K.C. (1992). Two esters of Synadenol, a new lathyrane diterpenoid, from the latex of Synadenium compactum (Euphorbiaceae): A crystal structure analysis. *Journal of the Chemical Society*, **1**: 1831 – 1835.

Onwuka, G.I. (2005). Food Analysis and Instrumentation. *Theory and Practice*. Naphtali Prints, Lagos, Nigeria, pp 133-137.

Openshaw, K. (2000). A review of *J. curcas*: an oil plant of unfulfilled promise. *Biomass and Bioenergy,* **19**: 1–15.

Ouyang, J., Pei, Z., Lutwick, L., Dalal, S., Yang, L., Cassai, N., Sandhu, K., Hanna, B., Wieczorek, R.L., Bluth, M. and Pincus, M.R. (2008). Case report: Paenibacillus

thiaminolyticus: a new cause of human infection, inducing bacteremia in a patient on hemodialysis. *Annual Clinical and Laboratory Science*, **38**: 393-400. .

Panda, T. and Gowrishankar, B.S. (2005). Production and applications of esterases. *Applied Microbiology and Biotechnology,* **67**: 160-169.

Parsons, A.T. (2008). Financing *Jatropha* Development. In: International Consultation on Pro-poor *Jatropha* Development. 10–11 April 2008, Rome, *IFAD.*

Payot, T., Chemaly, Z. and Fick, M. (1999). Lactic acid production by Bacillus coagulans-kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme and Microbial Technology,* **24**: 191-199.

Phengnuam, T. and Suntomsuk, W. (2013). Detoxification and anti-nutrients reduction of *J. curcas* seed cake by *Bacillus* fermentation. *Journal of biosciences and bioengineering*, **115**: 168-172.

Piggot, P. J. and Hilbert, D. W. (2004). Sporulation of Bacillus subtilis. *Current Opinion in Microbiology,* **7:** 579–586.

Piuri, M., Sanchez-Rivas, C. and Ruzal, S.M. (1998). A novel antimicrobial activity of a *Paenibacillus polymyxa* strain isolated from regional fermented sausages. *Letter of Applied Microbiology*, **27**: 9-13.

Quinn, G.A., Maloy, A.P., McClean, S., Carney, B. and Slater, J.W. (2012). Lipopeptide biosurfactants from *Paenibacillus polymyxa* inhibit single and mixed species biofilms. *Biofouling* **28** (**10**): 1151–1166.

Raju, A.J.S. and Ezradanum, V. (2002). Current Science, **83**: pg 11.

Rakshit, K.D., Darukeshwara, J., Rathina Raj, K., Narasimhamurthy, K., Saibaba, P. and Bhagya, S., (2008). Toxicity studies of detoxified Jatropha meal (*J. curcas*) in rats. *Food Chemical Toxicology,* **46**: 3621–3625.

Ramon-Blanco, C., Sanz-Gomez, J.J., Lopez-Diaz, T.M., Otero, A. and Garcia-Lopez, M.L. (1999). Numbers and species of Bacillus during the manufacture and ripening of Castellano cheese. *Milchwissenschaft - Milk Science International,* **54**: 385-388.

Reddy, N.R., Sathe, S.K. and Salunkhe, D.K. (1982). Phytates in legumes and cereals. *Advanced Food Resources,* **28**: 1-92.

Rodriguez-Jerez, J.J., Giaccone, V., Colavita, G. and Parisi, E. (1994). "Bacillus macerans—a new potent histamine producing micro-organism isolated from Italian cheese". *Food Microbiology,* **11**(5): 409–415.

Rosa, T.S., Castro, A.M., Torres, A.G. and Freire, D.M.G. (2010). Analysis of nutritional composition and detoxification of *J. curcas* cake after solid-state fermentation. *The 32nd Symposium on Biotechnology for Fuels and Chemicals, April 19 – 22, 2010.*

Runumi, G., Utpal, K.N. and Ajay, K.T. (2014). Reduction of phorbol ester content in *Jatropha* cake using high energy gamma radiation. *Journal of Radiation Research and Applied Sciences,* **7**(3): 305-309.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Pare, P.W. and Kloepper, J.W. (2003). Bacterial volatiles promote growth in Arabidopsis. *Proc Natl Acad Science USA,* **100**: 4927-4932.

Saetae, D. and Suntornsuk, W. (2010). Antifungal Activities of Ethanolic Extract from *J. curcas* Seed cake. *Journal of Microbiology and Biotechnology,* **20**: 319-324.

Saetae, D. and Suntornsuk, W. (2011). Toxic compound, anti-nutritional factors and functional properties of protein isolated from detoxified *J. curcas* seed cake. *International Journal of Molecular Sciences,* **12(1)**: 66 - 77.

Sanders, M. E., Morelli, L. and Tompkins, T. A. (2003). Sporeformers as Human Probiotics: *Bacillus, Sporolactobacillus,* and *Brevibacillus. Comprehensive Reviews in Food Science and Food Safety,* **2**(3): pg 101.

Schaeffer, A.B. and Fulton, M. (1933). A simplified method of staining Endospores. *Science* **77** pg 194

Schmidt, F.R. (2005). Optimization and scale up of industrial fermentation processes. *Applied Microbiology and Biotechnology,* **68:**425-435.

Segal, A., Van-Duuren, B.L. and Maté, U. (1975). The identification of phorbolol myristate acetate as a new metabolite of phorbol myristate acetate in mouse skin. *Cancer Research,* **35**: 2154 – 2159.

Shaheen, M., Li, J., Ross, A.C., Vederas, J.C. and Jensen, S.E. (2011). *Paenibacillus polymyxa* PKB1 produces variants of polymyxin B-type antibiotics. *Chemistry and biology,* **18**(12): 1640–1648.

Singh, R. N., Vyas, D. K., Srivastava, N. S. L. and Narra, M. (2008). SPRERI experience on holistic approach to utilize all parts of *J. curcas* fruit for energy. *Renewable Energy,* **33**(8): 1868-1873.

Soerawidjaja T.H. (2010). Potential of *J. curcas* L. as a non-edible feedstock of biodiesel fuel. *7th Biomass-Asia Workshop; 29 Nov. – 1 Dec., 2010; Jakarta, Indonesia.*

Sorg, B., Fürstenberger, G., Berry, D.L., Hecker, E. and Marks, F. (1982). Preparation of retinoic acid esters of phorbol derivatives. *Journal of Lipid Research*, **23**: 443 – 447.

Stannbury, P.F., Whitakar, A. and Hall, S.J. (1997). Principles of fermentation technology. *Elsevier*, London UK.

Sumiati, Y., Sudarman, A., Nurhikmawati, I., and Nurbaeti, (2010). Detoxification of *J. curcas* meal as poultry feed. *The 2nd International Symposium on Food Security, Agricultural Development & Environmental Conservation in Southeast and East Asia; Bogor, Indonesia*, pp. 61 – 66.

Sumiati, Y., Yusriani, Y., Astuti, D.A. and Suharti, S. (2009). Feeding fermented *J. curcas* L. meal supplemented with cellulose and phytase to kampong chicken. *The 1st International Seminar on Animal Industry 2009; Bogor, Indonesia.* pp. 191 -197.

Szallasi, Z., Krsmanovic, L. and Blumberg, P.M. (1993). Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate induced tumor promotion in CD-1 mouse skin. *Cancer Research,* **53**: 2507 – 2512.

Tewari, D. N. (2007). *Jatropha and biodiesel*. 1st ed. New Delhi: Ocean Books Ltd.

Thilborg, S.T., Christensen, S.B., Comett, C., Olsen, C.E. and Lemmich, E. (1994). Molluscicidal Saponin from a Zimbabwean Strain of *Phytolacca dodecandra. Phytochemistry*, **36**: 753-759.

Tjakradidjaja, A.S., Suryahadi, and Mahajati, R. (2009). Effectivity of *J. curcas* seed meal fermented with various moulds as protein source for male mice (*Mus musculus*). *The 1st International Seminar on Animal Industry 2009; Bogor, Indonesia*, pp. 157 – 164.

Torres, S., Baigori, M.D. and Castro, G.R. (2005). Effect of hydroxylic solvents on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86. *Process Biochemistry,* **40**: 2333-2338.

Turnbull, P.C.B. (1996). *Bacillus.* In: *Barron's Medical Microbiology* (Baron S *et al.*, eds.) (4th ed.). *University of Texas Medical Branch*.

Vaintraub, I.A. and Lapteva, N.A. (1995). A modified Method for the indirect quantitative analysis of phytate in foodstuffs. *Analysis Biochemistry*, **225**(2): 206-212.

Van-Gerpen, J. H., Peterson, C. L. and Goering, C. E. (2007). Biodiesel: An Alternative Fuel for Compression Ignition Engines. In: 2007 *Agricultural Equipment Technology Conference, 11-14 February 2007, Kentucky. Michigan: The American Society of Agricultural and Biological Engineers.*

Von der Weid, I., Alviano, D.S., Santos, A.L., Soares, R.M., Alviano, C.S. and Seldin, L. (2003). Antimicrobial activity of *Paenibacillus peoriae* strain NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi. *Journal of Applied Microbiology,* **95**: 1143-1151.

Wani, S., Sreedevi, T. K. and Marimuthu, S. (2008). Pro-poor biodiesel initiative for rehabilitating degraded drylands. In: International Consultation on Pro-poor *Jatropha* Development. 10–11 April 2008, Rome, *IFAD* (available at http://www.ifad.org/events/*Jatropha*/).

Wender, P.A., Martin-Cantalejo, Y., Carpenter, A.J., Chiu, A., DeBrabander, J., Harran, P.G., Jimenez, J.M., Koehler, M.F.T., Lippa, B., Morrison, J.A., Müller, S.G., Müller, S.N., Park, C.M. Shiozaki, M., Siedenbiedel, C., Skalitzky, D.J., Tanaka, M. and Irie, K. (1998). The chemistry-medicine continuum: Synthetic computer, spectroscopic and biological studies on new chemotherapeutic leads. *Pure and Applied Chemistry*, **70**(3): 539 – 546.

Wiesenhütter, J. (2003). Use of the Physic Nut (*J. curcas* L.) to combat desertification and reduce poverty. *CCD Project Document. Bonn,* GTZ.

Wink, M., Koschmieder, C., Sauerwein, M. and Sporer, F. (1997). Phorbol esters of J. curcas – Biological activities and potential applications. In Gübitz, G.M., Mittelbach, M. and Trabi, M. *Biofuel and Industrial Products from Jatropha curcas;* Graz, Austria, pp. 160 – 166.

Wodzinski, R.J. and Ullah, A.H.J. (1996). *Advanced Applied Microbiology,* **42**:263−302.

Wu, Q.C., Tang, Y.P., Ding, A.W., You, F.Q., Zhang, L. and Duan, J.A. (2009). 13 CNMR data of three important diterpenes isolated from Euphorbia species. *Molecules*, **14:** 4454 – 4475.

Yan`, H. (2008). Is *J. curcas* a solution to sustainable energy supply? *Jatropha International Congress; December 17 – 18, 2008; Singapore.*

Ye, M., Li, C., Francis, G. and Makkar, H.P.S. (2009). Current situation and prospects of *J. curcas* as a multipurpose tree in China. *Agroforest System*, **76:** 487 – 497.

Yegorenkova, I.V., Tregubova, K.V. and Ignatov, V.V. (2013). *Paenibacillus polymyxa* Rhizobacteria and Their Synthesized Exoglycans in Interaction with Wheat Roots: Colonization and Root Hair Deformation. *Current Microbiology*, **66** (5): 481–486.

Yoon, H.G., Lee, K.H., Kim, H.Y., Kim, H.K., Shin, D.H., Hong, B.S. and Cho, H.Y. (2002). Gene cloning and biochemical analysis of thermostable chitosanase (Tch-2) from Bacillus coagulans Ck 108. *Bioscience Biotechnology and Biochemistry*, **66**: 986-995.

Yudkin, M. D. and Clarkson, J. (2005). Differential gene expression in genetically identical sister cells: the initiation of sporulation in *Bacillus subtilis*. *Molecular Microbiology*, **56**: 578–589.

Yuspa, S.H., Lichti, U., Ben, T., Patterson, E., Hennings, H., Slaga-663, T.J., Colburn, N. and Kelsey, W. (1976). Phorbol esters stimulate DNA synthesis and ornithine decarboxylase activity in mouse epidermal cell cultures. *Nature,* **262:** 402 – 404.

Zhang, W.J., Zhao, S.H., Sun, J.Y. and Yang, X. (2006). Development of a microbial fermentation process for detoxification of gossypol in cottonseed meal. *Animal Feed Science and Technology*, **135**: 176-186.

APPENDICES

Appendix I: Gram Staining Characteristics of the Isolates

Isolates	Gram reaction	Morphology	Arrangement
A	positive(+)	rod	singly
B	positive(+)	rod	singly
C	positive(+)	rod	singly

Appendix II: Endospore Staining Characteristics of the Isolates

Isolates	presence of endospore	location of the spore
A	present	sub terminal
B	present	sub terminal
C	present	sub terminal

Appendix III: Biochemical test characteristics of the isolates

Organisms	ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	V P	NIT	Tentative identity of the isolates
A	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	-	+	+	*B coagulans*
B	+	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	-	-	+	-	-	+	+	*P. polymyxa*
C	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	+	+	*P. macerans*

Key: A- *Bacillus coagulans* B- *Paenibacillus polymyxa* C-*Paenibacillus macerans*
+ Positive - negative

ARA- Arabinose
CEL- Cellobiose
INO- Inositol
MAN- Mannitol
MNS- Mannose
RAF- Raffinose
RHA- Rhamnose
SAL- Salicin
SOR- D-Sorbitol
SUC- Sucrose
TRE- Trehalose
XYL- Xylose
ADO- Adonitol
GAL- Galactose
MDM- Methyl-D-Mannoside
MDG- Methyl-D-Glucosides
INU- Inulin
MLZ- Melezitose
IND- Indole
ONPG- ONPG Hydrolysis
ARG- Arginine Dihydrolase
CIT- Citrate utilization
V P- Voges Proskauer
NIT- Nitrate