ANTIFUNGAL ACTIVITIES OF ETHYL ACETATE FRACTION OF *PSEUDOMONAS FLUORESCENS* FERMENTATION BROTH AGAINST PHYTO-PATHOGENIC FUNGI FROM ROTTEN YAM

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AUGUST, 2018

DECLARATION

I hereby declare that the work in this dissertation entitled **"Antifungal activities of ethyl acetate fraction of *Pseudomonas fluorescens* fermentation broth against phyto-pathogenic fungi from rotten yam."** was performed by me in the Department of Pharmaceutics and Pharmaceutical Microbiology under the supervision of Prof. J.O. Ehinmidu and Dr. B.A. Tytler. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any institution.

Umar DANLADI RASHIDA

.....	.....	.....
Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled "ANTIFUNGAL ACTIVITIES OF ETHYL ACETATE FRACTION OF *PSEUDOMONAS FLUORESCENS* FERMENTATION BROTH AGAINST PHYTO-PATHOGENIC FUNGI FROM ROTTEN YAM*"* by Rashida Danladi UMAR meets the regulations governing the award of the degree of Master of Science in Pharmaceutical Microbiology of the Ahmadu Bello University, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to Allah (SWT) for giving me life and knowledge and to the Danladeez family.

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ABSTRACT

Yam tubers are prone to infestation by microorganism at all stages of growth and in storage. This rot reduces the quality of yam and affects food security. *Pseudomonas fluorescens* possess varieties of promising antifungal properties through production of secondary metabolites which makes it a biocontrol agent against many phyto-pathogens. In this study, the antifungal activities of metabolite produced by *Pseudomonas fluorescens* isolated in Zaria was evaluated. Rhizosphere soil was collected from different farms (sugarcane, rice and soybeans) in Zaria, Nigeria and *Pseudomonas fluorescens* was isolated and identified in accordance with standard microbiological procedure using cultural, microscopical and biochemical characteristics. The four isolate obtained were grown in three (3) fermentation media made from waste such as yam peel, banana peel and sugarcane shaft as carbon sources and groundnut cake as nitrogen source. Crude extract from these fermentations were tested for antifungal activities against *Aspergillus niger, Penicillium citrinum*, and *Rhizopus stolonifer* isolated from rotten yam using agar diffusion assay. Only four isolates labelled sugarcane A_1, sugarcane A_2, Rice B_1 and Rice B_2 were identified as Pseudomonas fluorescens and only fermentation broth extracts of Sugarcane A_2, Rice B_1 and Rice B_2 had remarkable antifungal activities against the fungi with diameter zones of inhibition ranging from 14.00 – 39.00mm in two (2) of the fermentation media. The crude extracts from *P. fluorescens* fermentation broth were extracted in this study and were observed to inhibit the mycelial growth of the test fungi with zone of inhibition ranging from 19.50 - 33.50mm. The *in vitro* & *in vivo* MICs showed significant antifungal activities against test fungi spores. *Rhizopus stolonifer* was observed to be the most susceptible test organism *in vitro* & *in vivo* (0.15 – 5.00mg/ml). This study showed that the *Pseudomonas fluorescens*

isolated in Zaria produced potential antifungal metabolite which can arrest the growth of phyto-pathogenic fungi associated with yam tubers.

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ACRONYMS AND ABBREVIATIONS

In-vitro	Taking place outside living organism
In-vivo	Within the living
DAPG	Di-acetylphloroglucinol
SDA	Sabouraud dextrose agar
DHHA	2,3-dihydro-3-hydroxyanthranillic acid
ADIC	2-amino-2-deoxyisochorismic acid
$CaCO_3$	Calcium Carbonate
$MgSO_4$	Magnesium sulphate
K_2HPO_4	di-potassium phosphate
ANOVA	Analysis of variance
M.I.C.	Minimum Inhibitory Concentration
S.D	Standard Deviation
µg	Micro gram
mm	Millimeter
mg	Milligram
ml	Milliliter
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
HCN	Hydrogen cyanide
PRN	Pyrrolnitrin
PLT	Pyoluteorin
g	grams
Kcal	Kilocalories
PHZ	Phenazines
PCA	Phenazine-1-carboxylic acid

PCN	Phenazine-1-carboxamide
PYO	Pyocyanin
IU	International Unit
RDA	Recommended Dietary Allowance

CHAPTER ONE

INTRODUCTION

1.1 Background

Yam belongs to the genus Dioscorea (Family Dioscoreaceae) and is the second most important tropical root crop in West Africa after cassava (Adisa *et al*., 2015). The genus Dioscorea consists of about 600 species (Degras, 1993). The most cultivated species are the *Dioscorea rotundata* (white yam), *Dioscorea alata* (water yam), *Dioscorea dumetorum* (bitter yam), *Dioscorea esculentum* (loir), *Dioscorea cayenensis* (yellow or guinea yam) and *Dioscorea bulbifera* (aerial yam). White or guinea yam (D. rotunduata poir) is the most popular species in Africa (Lawal *et al*., 2014; Princewill-Ogbonna and Ibeji, 2015).

Yam plays significant roles in the social-cultural and economic wellbeing of thousands of people in Nigeria especially among the people of South Eastern Nigeria (Okigbo *et al*., 2013). Its cultivation is very profitable despite high costs of production and price fluctuations in the markets (IITA, 2013; Izekor and Olumese, 2010).

The quality of yam tubers is affected by rots, which makes them unappealing to consumers (Ogbo and Agu, 2014b; Agu *et al*., 2015). Most rots of yam tubers are caused by pathogenic fungi such as *Aspergillus flavus, Aspergillus niger* (Tiegh), *Fusarium oxysporum, Fusarium solani, Botryodiplodia theobromae, Penicilllium chrysogenum, Rhizoctonia spp, Penicillium oxalicum, Trichoderma viride* and *Rhizopus nodosus* (Okigbo and Ikediugwu, 2000; Okigbo *et al*., 2014; Frank and Kingsley, 2014b; Agu *et al*., 2014). Several methods have been adopted for controlling losses due to post harvest disease of yam; these include the use of chemicals, biological method of control (Okigbo, 2004) and curing by using natural plant extracts (Amusa *et al*., (2003).

Pseudomonas is one of the most ubiquitous bacterial genus and Migula designated and described the species associated with the genus in 1985 (Migula, 1985). Pseudomonads are

well adapted to colonizing roots and the rhizosphere, and they suppress disease by a wide variety of mechanisms (Pieterse *et al*. 2014; Weller 2007) but the most important are the production of antimicrobial metabolites. These characteristics makes Pseudomonas species good candidates for use as seed inoculant and root dips for biological control of soil borne pathogens.

Pseudomonas fluorescens is a gram negative, rod-shaped, and non-pathogenic bacterium that is known to inhibit primarily the soil, plants and water (Peix *et al.,* 2009). It derives its name from its ability to produce fluorescent pigments under iron-limiting conditions (Baysse *et al*., 2003). *P. fluorescens* are well characterized for their ability to produce antimicrobial compounds (Haas and De' fago, 2005). These metabolites range from simple 2, 4-diacetyl phloroglucinol, phenazine-1-carboxylic acid and pyrrolnitrin [3-chloro-4-(2-nitro-3'-chlorophenyl)-pyrrole], as well as the complex macrocyclic lactone 2, 3-de-epoxy-2,3-didehydra-rhizoxin. 2,4-diacetylphloroglucinol (DAPG), is a major factor in control of a range of plant pathogens (Haas and De' fago, 2005) while Pyrrolnitrin is active against *Rhizoctonia* spp, *Fusarium* spp, and other plant pathogen fungi and it has been used as a lead structure in development of a new phenyl pyrrole agricultural fungicide (Ligon *et al*., 2000).

Wastes are leftovers from production and consumption. It includes plant materials, agricultural, household, industrial and municipal wastes and residues (Okonkwo *et al*., 2006). The agricultural and agro-industrial activities generate a large amount of lignocellulosic by products such as bagasse, straw, stem, stalk, cobs, fruit peel and husk, among others (Saranraj and Anbu, 2017). These wastes contain simple and complex sugars that are metabolized by microorganism through secretion of extracellular products (Saheed *et al*., 2013).

1.2 Statement of Problem

Large quantity of yam losses has been reported and these losses have been due to microbial rot diseases. Yam plants are prone to infection by fungi, bacteria, and viruses at all stages of growth and also during storage of tubers (Okigbo, 2002; Okigbo, 2005; Morse *et al*., 2000) Difficulties in yam preservation has compelled majority of the yam farmers are to either consume or sell all their yam products at low prices before the new harvesting season. Consequently, before new harvesting period, they are bound to suffer food security crises. Chemical agents have been used to control this pre harvest and post-harvest losses in the past decades but these chemical agents are not only expensive for farmers, but they are not eco-friendly and micro-organisms are becoming resistant to them (Okigbo, 2004; Okigbo and Ikediugwu, 2000). This has lead scientist to divert their attention to the use of potential beneficial microbes. Biological agents as biocontrol is gaining ground and has proved to be safer, cheaper, effective and eco-friendly.

As a result of high consumption and industrial processing of the edible parts of fruit, fruit wastes (such as banana, grapes, dragon fruit, orange, strawberry, lemon, watermelon, citrus, Pomegranate, pineapple residues, sugarcane bagasse and other fruit) has become one of the main sources of municipal solid wastes which has become an increasingly tough environmental issue (Saranraj and Anbu, 2017). Insufficient and improper disposal of these solid waste result in scenic blights, increase in rodents and insect vectors of disease, and serious hazards to public health.

1.3 Justification of Study

Nigeria is the largest producer of yam in the world (FAOSTAT, 2017) and yam production is regarded as a source of food security and employer of labour in many areas where it is cultivated. It generates employment for more than 50% of people in places of production either

through direct involvement in cultivation, marketing, processing, or indirectly through related services in production (i.e. labour both on the field of production and markets or transportation).

Yam tubers are nutritionally rich and a major source of dietary fiber, carbohydrates, vitamin C, and essential minerals (Charles *et al*., 2005; Polycarp *et al*., 2012).

The scope of developing microbial metabolites for commercial pesticides as an alternative to chemical control agent has gained importance due to increased concerns on environmental pollution, pathogens resistance and high plant protection cost (Reddy *et al*., 2007; Uppal *et al*., 2007)

The agricultural-based industries generate significant quantities of organic wastes including peels from cassava, plantain, banana, oranges and straw from cereals. Rather than allow these wastes to become solid municipal wastes, it is necessary to convert them to useful end products. These wastes could be utilized as cheap raw materials for some industries or as cheap substrates for microbiological processes (Nwabueze and Otowa, 2006). The need to develop alternative media has become imperative as the conventional media are either not readily available or expensive in most developing countries (Saranraj and Anbu, 2017).

1.4 Aim and Objectives

1.4.1 Aim

This study was designed to examine the antifungal activities of ethyl acetate fraction of *Pseudomonas fluorescens* fermentation broth against some phyto-pathogenic test fungi.

1.4.2 Objectives of the Study

1. To isolate & identify *Pseudomonas fluorescens* from rhizosphere of rice, sugar cane and soy beans soil in Zaria, Kaduna state.
2. To Isolate and identify three phyto-pathogenic organisms (*Aspergillus niger, Penicillium citrinum* and *Rhizopus stolonifer)* from rotten yam.
3. To formulate and prepare fermentation media using agricultural waste as different carbon and nitrogen sources for the production of active metabolite from *Pseudomonas fluorescens*.
4. To screen for antifungal activities of the crude extracts using agar well diffusion method.
5. To determine the *in vitro* and *in vivo* antifungal activities of the ethyl acetate fraction from *Pseudomonas fluorescens* fermentation broth on the test fungal spores.

1.5 Hypothesis

1.5.1 Null Hypothesis

The ethyl acetate fraction from *Pseudomonas fluorescens* fermentation broth does not have antifungal activities against phyto-pathogenic fungi spores of *Aspergillus niger, Penicillium citrinum, Rhizopus stonolifer.*

1.5.2 Alternate Hypothesis

The ethyl acetate fraction from *Pseudomonas fluorescens* fermentation broth has antifungal activities against phyto-pathogenic fungi spores of *Aspergillus niger, Penicillim citrinum, Rhizopus stonolifer.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Yam

Scientific classification –
Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Liliopsida*
Order: *Dioscoreales*
Family: *Dioscoreaceae*
Genus: *Dioscorea* L

2.1.1 Description of Yam

Yam is the name given to several plant species in the genus *Dioscorea* that are grown for their edible tubers. These species are not to be confused with the sweet potato, *Ipomoea batatas*, which is often referred to as a yam in the US. Yam plants are herbaceous annual or perennials with climbing or trailing vines. The vines can be smooth or prickly, reaching 10 m (32.8 ft) or more in length depending on the variety. The leaves of the plant are simple and usually oval to heart-shaped with petioles which are the same length, or slightly longer, than the leaf blade itself. Some varieties possess spikes at the bases of the leaves. The plant can produce one singular tuber or several tubers which extend from stolons from a central corm (up to 20) depending on the species. The tubers can be cylindrical, curved or lobed, with brown, grey, black or pink skin and white, orange or purplish flesh. Most yams are annual plants, harvested after one season, but some are perennial with tubers increasing in size each year with the vines dying back at the end of the growing season and re-growing on the return of favourable conditions. The origin of yams is uncertain and genetic information suggests that there may be more than one point of origin.

2.1.2 Species of Yam

There are as many as 600 cultivars of yam throughout the humid tropics, among which there are *Dioscorea rotundata* (white yam); *Dioscorea alata* (soft or water yam); *Dioscorea cayenensis* (yellow yam), *Dioscorea dumetorum* (bitter yam), *Dioscorea nummularia* (wael yam); *Dioscorea esculenta* (Sweet Chinese yam); *Dioscorea trifida* (Africa yam); *Dioscorea bulbifera* (aerial yam); *Dioscorea pentaphila* and *Dioscorea transversa* (Strong yam) (National Advisory Board on Climate Change and Disaster Risk Reduction, 2013; FAO, 2013).

Out of these numerous species, the most important edible species are *D. rotundata* (white yam), *D. alata* (water yam), *D. cayenensis* (yellow yam), *D. dumetorum* (bitter yam), *D. esculenta* (Chinese yam), and *D. bulbifera* (aerial yam) while *D. rotundata* is the most preferred and cultivated, accounting for a large proportion of yam production in West Africa, a region which produces 93 % of the world's yam (FAO, 2013).

i. ***White yam* (*Dioscorea rotundata)* and Yellow yam (*D. cayenensis):*** *Dioscorea rotundata*, the "white yam", and *Dioscorea cayenensis*, the "yellow yam", are native to Africa. They are the most important cultivated yams. In the past they were considered two separate species but most taxonomists now regard them as the same species. White yam's tuber is roughly cylindrical in shape, the skin is smooth and brown and the flesh usually white and firm. Yellow yam is named after its yellow flesh, a color caused by the presence of carotenoids. It looks similar to the white yam in outer appearance; its tuber skin is usually a bit firmer and less extensively grooved. The yellow yam has a longer period of vegetation and a shorter dormancy than white yam.

ii. **Water yam (*Dioscorea alata):*** *Dioscorea alata*, called "water yam", "winged yam" and "purple yam" was first cultivated in Southeast Asia. Although not grown in the same quantities as the African yams, it has the largest distribution world-wide of any cultivated yam, being grown in Asia, the Pacific islands, Africa, and the West Indies (Mignouna *et al*., 2003). Even in Africa, the popularity of water yam is second only to white yam. The tuber shape is generally cylindrical, but can vary. Tuber flesh is white and watery in texture. In the Philippines it is known as "ube" and is

used as an ingredient in many sweet desserts. In Indonesia it is known as "ubi". In Vietnam, it is called *khoai mỡ* and is used mainly as an ingredient for soup. In India, it is known as *ratalu* or *violet yam*. In Hawaii it is known as *uhi*. In Japan it is called *daijo* or *beniimo*.

iii. Aerial yam (*Dioscorea bulbifera):* Also called the "air potato", is found in both Africa and Asia, with slight differences between those found in each place. It is a large vine, 6 meters (20 ft) or more in length. It produces tubers; however, the bulbils which grow at the base of its leaves are the more important food product. They are about the size of potatoes (hence the name "air potato"), weighing from 0.5 to 2 kilograms (1.1 to 4.4 lb). Some varieties can be eaten raw while some require soaking or boiling for detoxification before eating. It is not grown much commercially since the flavor of other yams is preferred by most people.

iv. Bitter yam (*Dioscorea dumetorum)*: *Dioscorea dumetorum*, the bitter yam, is popular as a vegetable in parts of West Africa; one reason being that their cultivation requires less labor than other yams. One marked characteristics of the bitter yam is the bitter flavour of its tubers. The wild forms are very toxic and are sometimes used to poison animals when mixed with bait. It is said that they have also been used for criminal purposes (Kay, 1987)

v. **Chinese yam (*Dioscorea esculenta):*** It is called "Chinese yam" and is native to China. The Chinese yam plant is somewhat smaller than the African, with the vines about 3 meters (10 feet) long. It is tolerant to frost and can be grown in much cooler conditions than other yams. It is now grown in China, Korea, and Japan. It was introduced to Europe in the 19th century when the potato crop there was falling victim to disease, and is still grown in France for the Asian food market. The tubers are harvested after about 6 months of growth. Some are eaten right after harvesting and some are used as ingredients for other dishes, including noodles, and for traditional medicines (Kay, 1987).

2.2 Yam Production in Nigeria

Nigeria is the largest world producer of yam with more than 45,004 million metric tonnes annually (FAOSTAT, 2017). In 1985, Food and Agricultural Organization reported that Nigeria produced 18.3 million tonnes of yam from 1.5 million hectares, representing 73.8 percent of total yam production in Africa. According to 2008 figures, yam production in Nigeria has nearly doubled since 1985, with Nigeria producing 35.017 million metric tonnes with value equivalent of US$5.654 billion while the world's second and third largest producers of yams, Côte d'Ivoire and Ghana, only produced 6.9 and 4.8 million tonnes of yams in 2008 respectively.

In 2014, worldwide production of yams was 68.1 million tonnes where Nigeria farmed yams on 5.4 million hectares (FAOSTAT, 2017). Nigeria exported 72 tonnes of yam to Europe and US on June 29th 2017 as part of moves to diversify its oil-dependent economy and earn much needed foreign exchange.

Table 2.1: World Yam production in 2014

S/No	*Countries*	*Production (millions of tonnes)*
1	*Nigeria*	*45.0*
2	*Ghana*	*7.1*
3	*Ivory coast*	*5.8*
4	*Benin*	*3.2*
5	*Ethiopia*	*1.4*
6	*Togo*	*0.8*
7	*Cameroun*	*0.6*

Source: UN Food & Agriculture Organization (FAOSTAT, 2014)

The major yam producing area in Nigeria include; the middle belt (Benue, Kogi, Ilorin, Niger Nassarawa), Eastern part of Nigeria (Owerri, Onitsha, Porthacourt, Umuahia) and the Western part (Ondo, Oyo, Ibadan).

2.3 Yam Propagation and Harvesting

a) Propagation

Yam crop begins when whole seed tubers or tuber portions are planted into mounds or ridges, at the beginning of the rainy season. The crop yield depends on how and where the seeds are planted, sizes of mounds, interplant spacing, provision of stakes for the resultant plants, yam species, and tuber sizes desired at harvest. Small-scale farmers in West and Central Africa often intercrop yams with cereals and vegetables. Large amounts of material (about 10,000 seed yams) are needed to plant one hectare. If farmers do not buy new seed yams, they must set aside 30% of their harvest for the next year planting (Kabeya *et al*, 2013). In addition, seed yams are bulky and perish quickly (Kabeya *et al*, 2013)

Yam typically grows for six to ten months and is dormant for two to four months, depending on the species. The growth and dormant phases correspond respectively to the wet season and the dry season. For maximum yield the yam requires a humid tropical environment, with an annual rainfall of over 1500 millimetres distributed uniformly throughout the growing season. White, yellow and water yams typically produce a single large tuber per year, generally weighing 5 to 10 kilograms (Calverly, 1998).

b) Harvesting

Yams in West Africa are typically harvested by hand using sticks, spades or diggers (Okpara, 2003). Wood-based tools are preferred to metallic tools as they are less likely to damage the fragile tubers; however, wood tools need frequent replacement. Yam harvesting is labour intensive and physically demanding. For each 2-10kilogram tuber harvested, it involves standing, bending, squatting, and sometimes sitting on the ground, depending on the size of

mound, size of tuber or depth of tuber penetration. Care must be taken to avoid damage to the tuber, because damaged tubers do not store well and spoil rapidly. Some farmers use staking and mixed cropping, a practice that complicates harvesting in some cases.

In forested areas, tubers grow in areas where other tree roots are present. Harvesting the tuber then involves the additional step of freeing them from other roots. This often causes tuber damage. Aerial tubers or bulbils are harvested by manual plucking from the vine. Yields may improve and cost of yam production can be lower if mechanization were to be developed and adopted. However, current crop production practices and species used pose considerable hurdles to successful mechanization of yam production, particularly for small-scale rural farmers. Extensive changes in traditional cultivation practices, such as mixed cropping, may be required. Modification of current tuber harvesting equipment is necessary given yam tuber architecture and its different physical properties (Okpara, 2003).

2.4 Yam Storage

In order to partly ensure food security in the region, yam storage is very important. In other words, yams are stored either for future consumption, higher market prices or for re-plantation (yam seeds) in another season. Due to the difficulties in yam preservation, the majority of the yam farmers are compelled to either consume or sell all their yam products at low prices before the new harvesting season. Consequently, before new harvesting period, they are bound to suffer food security crises.

Certain cultivars of yam store better than others. Easier to store yams are those that are adapted to arid climate, where they tend to stay in dormant low-respiration stage for much longer than yam breeds that are adapted to humid tropical lands where they do not need dormancy. The tubers of many varieties can be stored as long as six months without refrigeration (CGAIR 2006).

2.4.1 Methods of Storage

Various traditional and modern storage techniques are practised in the country, depending on technical know-how, facilities available, climatic factors at the time of storage, risk of exposure to pest attack and the quantity of the farm produce. Although modern techniques are being developed for effective storage of these products in large quantities, most farmers still depend on the traditional methods since these new techniques are not within their reach.

2.4.1.1 Traditional Storage Methods

Various traditional methods used include;

Storing the tubers on wooden platform in cool and well ventilated room to avoid direct sunlight which may cause drying and cracking. The tubers are arranged in irregular rows and covered with dry grass to allow air circulation.

They can also be stored by tying the tubers to a frame of living wooden poles (usually *Ficus* spp since they are not easily attacked by termites) which are fastened together and anchored firmly into the ground. Cross poles are fastened to these upright poles at intervals, usually about 30cm apart. The frames are set up in covered barns.

Yam tuber can be stored by leaving the yams in the soil for temporary storage after the stems have been cut off and by mixing/packing the tubers in ashes, heaping and covering with soil before storing at room temperature. The tubers may be stored in a thatched shed, huts, with a raised platform of about 0.6m-0.9m above the ground. The yams can be suspended singly from branches which shades them. This is practised with small quantities of yams.

Storing yam tubers in layers inside trenches. They are then covered with dry grass followed by a layer of soil. The trenches are normally made in areas where the soil is dry in order to avoid sprouting and decay.

2.4.1.2 Modern Storage Methods

This method usually involves irradiating yam tubers with gamma rays at low dosage of 7.5 - 15 krads with in one months of harvest. This is followed by storage at ambient conditions. This will effectively control factors causing deterioration and keep the yams in good conditions for 8-9 months.

Yam tubers can be stored using chemicals, fungicides and pesticide to prevent and control rotting of stored tubers. Cold room storage at about 15ºC is also being tried in research stations. This has been found to be expensive for traditional farmers.

2.5 Yam Consumption in Nigeria and Around the World

Yams of African species must be cooked to be safely eaten, because various natural substances in raw yams can cause illness if consumed. (Excessive skin contact with uncooked yam fluids can cause the skin to itch. If this occurs, a quick cold bath or application of red palm oil to the affected part of the body will stop the itching). Yams are used differently in different parts of the world. They are consumed after cooking by frying, boiling or roasting. The green parts of some plants can be cooked and consumed as a vegetable. Yams may also be used to produce flour or starch.

Preparing boiled yam involves cutting the tuber into five or so thick slices, then peeling the skin from each cut piece and boiling the whitish starchy flesh. The older the yam, the smaller the chunks must be cut and the more water it will need to boil. Boiled yam is traditionally consumed with palm oil, but is also commonly served with a sauce such as pepper sauce or palaver sauce. In big cities and border towns in West Africa, fried yam and pepper sauce is a popular street food and has a similar position as French fries and ketchup in Western cuisine. Yam balls (styled after meat balls) have also gained some popularity in contemporary West African cuisine.

Among the Akan of Ghana, boiled yam can be mashed with palm oil into *eto* in a similar manner to the plantain dish *matoke*, and is served with eggs. The boiled yam can also be pounded with a traditional mortar and pestle to create a thick, starchy paste known as *iyan* ("pounded yam") or *fufu* which is eaten with traditional sauces such as egusi and palmnut soup. Another method of consumption is to leave the raw yam pieces to dry in the sun. When dry, the pieces turn a dark brown color. These are then milled to create a brown powder known in Nigeria as *elubo*. The powder can be mixed with boiling water to create a thick starchy paste, a kind of pudding known as amala, which is then eaten with local soups and sauces.

2.6 Nutritional Value of Yam

Nutritionally, yam is an essential source of carbohydrates for the consumers around the world. Many yam belt areas in Nigeria continuously proclaimed "yam is food and food is yam" (Maikasuwa and Ala, 2013). Some cultivars of yam tuber have been found to contain protein levels of 3.2-13.9% of dry weight. A yam meal could supply 100% of the energy and protein, 13% of the calcium and 80% of the iron requirement of an adult male (Knoth, 1993).

The tuber is an excellent source of the B-complex group of vitamins. It provides adequate daily requirements of pyridoxine (vitamin B6), thiamin (vitamin B1), riboflavin, folates, pantothenic acid, and niacin. These vitamins mediate various metabolic functions in the body. Yam contains small amounts of vitamin-A, and β-carotene levels both of which are powerful antioxidants Carotenes convert into vitamin-A inside the body.

Table 2.2: Nutritional value of raw yam (*Dioscorea spp*)per 100g

Principle	Nutrient value	Percentage of RDA
Energy	108 Kcal	5%
Carbohydrates	27.88 g	21%
Protein	1.53 g	3%
Total Fat	0.17 g	0.5%
Cholesterol	0 mg	0%
Dietary Fiber	4.1 g	11%
Vitamins		
Folates	23 µg	6%
Niacin	0.552 mg	3.5%
Pantothenic acid	0.314 mg	7%
Pyridoxine	0.293 mg	23%
Riboflavin	0.032 mg	2.5%
Thiamin	0.112 mg	9.5%
Vitamin C	17.1 mg	28.5%
Vitamin A	138 IU	5%
Vitamin E	0.35 mg	2%

Vitamin K	2.3 µg	2%
Electrolytes		
Sodium	9 mg	0.5%
Potassium	816 mg	17%
Minerals		
Calcium	17 mg	2%
Copper	0.178 mg	20%
Iron	0.54 mg	7%
Magnesium	21 mg	5%
Manganese	0.397 mg	17%
Phosphorus	55 mg	8%
Selenium	0.7 µg	0.5%
Zinc	0.24 mg	2%
Phyto-nutrients		
Carotene-Beta	83 µg	-----
Crypto-xanthin	0 µg	-----
Lutein-zeaxanthin	0 µg	-----

(Source: USDA National Nutrient data base)

2.7 Importance of Yam Production

Yams are one of the most highly regarded food products in tropical countries of West Africa where it also has a social and cultural benefit (Abdulsalam *et al*., 2016). It is a highly valued crop which forms, about 10% of the total roots and tuber produced in the world. Yam

production serves as a source of income generation to peasant farmers and the labourers who work on yam farms as well as for those that engage in its sale. Yam serves as source of food security for the country. Peelings and waste from yam are often used for feeding poultry and livestock and can also be used as a source of carbon for the growth of microorganism. According to Lawal *et al*. (2016) yam is considered an herbal medicine in Taiwan. Yam is known to contain a good quantity of bioactive compounds, such as phytochemicals in form of (Alkaloids, Tannis, flavonoids, saponins, glycoside steroids, Anthraquinones etc), polyphenols (flavonoids and phenolic acids), phenols which have been exploited for pharmaceutical products (Mignouna *et al*., 2008). Dioscorin and Diosgenin are the most predominant of the phytochemicals found in yam (Kanu *et al*., 2018)

Yam also plays a significant role in African socio-cultural traditions. There are many traditions associated with yams including ceremonies and festivals, which show their importance in traditional society. A new yam festival is celebrated where people offer yams to gods and ancestors first, before distributing them to the villagers. This is their way of giving thanks to the spirits above them. In Nigeria, the people of south eastern part celebrate. The celebration is followed by various cultural dances with the display of masquerades from different clans or groups. This usually lasts to very late night. During the occasion is also a moment of reuniting old friends and family members.

2.8 Problems Associated with Yam Production in Nigeria

Zaknayiba and Tanko (2013), find that lack of access to farm inputs, high cost of inputs, poor producer prices, high incidences of pests and diseases and inadequate of storage facilities have negatively affected yam production. Similarly, Ike and Inoni (2006); Maikasuwa and Ala (2013) results indicate that the factors of production such as labour, finance and material inputs like fertilizer have influenced on yam production. In the same direction, studies by Etim *et al*.

(2013) suggest that farmers' education, family labour, extension contact and experience of farmers have a positive effect on the farm level technical efficiency and yam output. Similarly, Shehu *et al*. (2010) found out that land, seed yam, family labour, education, and fertilizer are the drivers that influence yam production in Nigeria.

Yam production requires high labour. The crop has low yield per hectare compared to crops such as cassava (manioc) or sweet potato. It is not an efficient food staple given the relatively large amount of planting material that is required and its long growing season. Yam's labour requirement exceeds that of other comparable crops. Yam is also difficult to preserve and store over extended periods of time. Post-harvest food losses are one of the important sources of food insecurity in Africa.

2.8.1 Diseases and Common Pests Affecting Yam

Yam microbial diseases can be classified into two:

- The field diseases and
- The storage diseases.

2.8.1.1 Yam Field diseases

The field diseases are those diseases that cause economic damage to yam in the field from the seedling stage to the point of harvest (Osunde, 2008).

i. Yam Anthracnose

Yam anthracnose is a fungal infection that can impact yam in any geographical area. This is caused mostly by the fungus *Colletotrichum gloeosporioides* (Amusa *et al*.,2003). *G. cingulata* is the perfect state of *C. gloeosporioides*, the form that is usually found causing field anthracnose disease.

On susceptible yam cultivars, symptoms appear as small, dark brown spots or black lesions on leaves which may be surrounded by a chlorotic halo; leaf necrosis; dieback of stem; withered

leaves and scorched appearance. *D. Alata* appears to be more susceptible than other yam species.

ii. Yam Mosaic Virus Disease

This disease is caused by an aphid-transmitted potyvirus that infects several species of Dioscorea, particularly *D. alata*, *D. cayenensis*, *D. rotundata* and *D. Trifida* ss (Amusa *et al*., 2003). Yam mosaic virus (YMV) is considered to cause the most severe losses in yams with symptoms on yam which include; mosaic, green vein banding, shoe string and stunting. These symptoms vary depending on host species and environmental conditions. Other yam viral diseases in West Africa include; yam mild mosaic virus (YMMV), *D. Dumetorum* virus, *D. Alata* bacilliform virus (DaBV), cucumber mosaic virus (CMV), D. Mottle virus (DMoV), and *D. Sansibarensis* virus (DsBV) (Seal & Muller, 2007).

iii. Yam Beetles

The yam beetle *(Heteroligus meles*) is a major pest of yam in West Africa. It lays its egg in moist ground during the early part of the dry season. This means that the eggs are usually laid in swampy areas near rivers and streams and not necessarily in the yam field. Its adult beetles that migrate by flying to yam plots being visually attracted by the foliage of the yam plants.

Yam beetles rarely kill the yam plant but they do considerable damage to the tubers that can be harvested from it. Their feeding activity leaves holes in the body of the tuber. These holes not only make the tuber unsightly and unmarketable, but they may also predispose the tuber to rotting during spoilage.

iv Yam Nematodes

Yam nematodes (*Scutellonema bradys*) and the root knot nematode (*Meloidogyne species*) are the most serious nematodes attacking yam (IITA, 2013; Amusa *et al*., 2003). The yam nematode inhabits the layer just beneath the skin of the tuber. The activity of the yam nematodes results in extensive browning of the sub surface layer of the yam tuber and the

resultant destruction of the meristematic zone. As such, tubers that have been infected with yam nematode may have difficulty sprouting if used as planting material.

The root knot nematode attacks growing yam tubers producing a characteristic warty appearance on the surface of the tubers. Yam nematodes reproduce and build up large population in stored tubers causing severe damage and facilitating fungal and bacterial attacks that cause anthracnose disease, dry rot, soft rot and wet rot.

2.8.1.2 Yam storage diseases

In Storage diseases, during storage, tubers are subject to losses of up to 50% of the fresh matter. Here, the losses due to microbial attack play a predominant role. The fungal pathogens penetrate through wounds in the tubers and infect the inner tuber tissue. Such wounds are caused by insects, nematodes and poor handling before, during and after harvest (Amusa *et al*., 2003).

Morse *et al*. (2000) reported that most of the yam rot induced by fungi in specialized barns near Idah, Kogi State, Nigeria were predisposed by insect attack by mainly storage beetles (*Coleoptera*), mealy bug (*Planococcus citri*) and sclae insect (*Aspidiella hartii*) during storage.

i. **Yam insects**

This is caused by insects such as Mealybug (*Planococcus dioscoreae*) and scale insect (*Aspidiella hartii*) and they attack stored yams. As they feed, these insects reduce the food reserves in the tubers. This both reduces the value of the yams as food, and also makes the yams too weak to sprout vigorously when used as planting setts. Other insect pests such as the yam Moth (*Blastobasis*) and Ginger Weevil (*Elytroteinus*) also damage yams in storage. They lay eggs on stored yams and when these eggs hatch, the larvae bore into the yam and eat the flesh. As the larvae feed, they leave piles of brown droppings which can be seen on the outside of the yam. The larvae mature in the rotten yam.

ii. Tuber rots

Tuber rots caused by mostly fungi are particularly problematic for yams during storage but may also occur in the field. It can be classified into three;

a) Dry rots: Dry is considered as the most devastating of all the storage disease of yam. Dry rots are caused by *Rossellinia* and *Sphaerostilbe.* All organisms which cause rots in the stored tuber are also responsible for rotting of setts after planting.

b) Soft rot: Infected tissues become soft ramified by the fungal mycelium. The causal fungi quickly ramified the tissue which turn brown and become soft and at times wet due to a rapid collapse of the cell walls. Fungi associated with this type of rot are Soft rots are caused by *Penicillium* species, *Fusarium* species *and Botrydiplodia* species. *Rhizopus* spp, *Mucor circinelloides*, *S.rolsii*, and *Rhizoctonia solani* and *Armillariella mellea* (Ikotun, 1983, 1989; Green *et al.*, 1995; Amusa and Baiyewu, 1999).

c) Wet rot: Wet rot characterized by the oozing of whitish fluid out of the tissue when pressed can also be increminated. this symptom is usually associated with a bacterium, *Erwinia carotovora* pv (Amusa and Baiyewu, 1999).

2.9 Management and Control of Pests and Diseases of Yams

A number of treatments and techniques have been developed to reduce these physiological activities and also to protect the tuber from postharvest diseases. These include treatment with chemicals, plant extracts, palm wine and gamma irradiation; storage techniques used include cold storage, improved underground storage and improved yam barns (Osunde, 2008).

Management strategies adopted and advocated for combating the field disease includes;

i. the use of crop rotation, fallowing,
ii. planting of healthy material: The most effective method of controlling the disease is to plant yam varieties that are resistant to anthracnose such as TDA 291 or TDA 297
iii. The destruction of infected crop cultivars.

iv. Control through careful selection of tubers for planting. Cut seed should be dipped in Bordeaux mixture or with a systemic fungicide

Management of the storage diseases include;

v. the use of tecto (thiabendazole),

vi. locally made dry gins or wood ash before storage has been found to protect yam tubers against fungal infection in storage.

vii. Treatments of the yam tubers with insecticide dust (Actellic 2% Dust; ai=pirimiphos-methyl) significantly reduced fungal infections and also ameliorated physical damages acquired during harvest resulting in significantly fewer fungal lesions (Morse *et al.,* 2000).

viii. Mealy bugs can potentially be controlled by natural enemies; chemical pesticides may decrease populations of natural enemies leading to mealybug outbreaks; horticultural oils or soapy solutions can be used to treat heavy infestations

2.10 *Pseudomonas as biocontrol agents*

The genus Pseudomonas is one of the most diverse gram negative bacterial genera and has been isolated from different environments such as water, soils, animals, plants and clinical samples. A total of 128 species is officially recognized for this genus (Peix *et al*., 2009). The members of the genus are the most studied bacterial biocontrol agents with potential to produce diverse range of bioactive secondary metabolites. Some isolates have been used in commercial products in the form of bio-fungicide and bio-fertilizers (Fravel, 2005). Pseudomonas spp have activity against a wide range of plant pathogens and they do so by both direct and indirect mechanisms. Antibiosis involving antibiotics and fungal cell wall degrading enzymes are direct mechanisms of biocontrol. On the other

hand, competition for space and nutrient resources in the rhizosphere and induction of disease resistance in plants are indirect mechanisms of a plant growth promotion.

Antibiotics such as DAPG, pyrrolnitrin, pyoluteorin, phenazines and lipopeptides (LPs) are commonly produced by some of the most effective biocontrol pseudomonads both in culture (Raaijmakers *et al*. 2002; Raaijmakers *et al*. 2009; Raaijmakers *et al*. 2010; Haas and Defago 2005; D'aes *et al*. 2010; Wang *et al.* 2014) and in the rhizosphere (Kwak *et al*. 2012; Mavrodi *et al*. 2012). Extracelluar diffusibles pigments such as pyoverdine, siderophores are responsible for the fluorescence of these organisms.

2,4-diacetylphloroglucinol, oomycin A, phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocanin, anthranilate, pyrrolnitrin, pyoluteorin, hydrogen cyanide, ammonia, viscosinamide and gluconic acid are the mostly reported antimicrobial metabolites of *Pseudomonas* spp (Jayaprakashvel & Mathivanan, 2011). Bacterial isolates that produce these secondary metabolites have biocontrol efficacy against wide range of phyto-pathogens. The list of some antimicrobial metabolites produced by the genera is shown in the Table 3.

Table 2.3: Antibiotics produced by biocontrol *Pseudomonas* spp and their target plant pathogens

S/No	*Compounds Produced*[1]	*Producer organisms*[2]	*Target plant pathogens*[3]	*References*
1	*2,4-DAPG*	P. aeruginosa	Ac, Cg, Fo, Ggt	*Chung* et al. *(2008)*
		P. brassicacearum	Pc, Pu, Rs	*Haas and Keel (2003), Raajimakers* et al. *(2002), Ryu* et al. *(2000)*
		P. chlororaphis	St, Tb	
		P. fluorescens	Pst	*Weller* et al. *(2012)*
		P. protegens		*Ramette* et al. *(2011)*
2	*Phenazines (PCA, PCN, PYO)*	P. aeruginosa	Bs, Cgr, Co, Fo	*Haas and Keel (2003), Sharmuugaiah* et al. *(2010)*
		P. chlororaphis	Ggt, Lk, Mp, Fo	*Kapsalis* et al. *(2008), Morohoshi* et al. *(2013)*

		P. fluorescens	Psp, Pu	*Ligon* et al. *(2002)*
		P. oryzihabitans	Phythium *spp*	*Powell* et al. *(2000)*
		Pseudomonas spp	Rs, Sh, Ti	*Raajimakers* et al. *(2002)*
				Mazzola et al. *(1992)*
		P. aureofaciens	Fo	
3	*Pyrrolnitrin (PRN)*	P. fluorescens	Ggt, Alternaria spp	*Upadhyay and Srivastava (2011), Haas and Keel (2003)*
			Bc, Fsa, Pe, Ptr, Rs,	
		Pseudomonas *spp*	Ss, Tb, Vd	*Ligon* et al. *(2000)*, *Raajimaker* et al. *(2002)*, *Athukorala* et al. *(2010)*
4	*Pyoluteorin (PLT)*	P. flourescens	Pu, Rs, Tb	*Haas and Keel (2003)*
		Pseudomonas *spp*		*Raajimaker* et al *(2002)*
5	*Hydrogen cyanide (HCN)*	P. corrugata	Bc, Tb	*Gross and Loper (2009)*
		P. aeruginosa	Mj	*Siddiqui* et al. *(2003)*
		P. fluorescens		*Guo* et al. *(2007)*
		Pseudomonas *spp*		*Haas and Keel (2003)*
		P. brassicacearum		*Zhou* et al. *(2012)*
6	*Aerugine*	P. fluorescens	Co, Pu, Pc	*Lee* et al. *(2003)*
7	*Iturins (Cyclic lipopeptides)*	P. asplenii	Gc, Pd, Pi, Pim	*Hultberg* et al. *(2010)*
		P. fluorescens	Pu, Rp, Rs	*Raajimakers* et al. *(2006)*
8	*Oomycin A*	P. fluorescens	Pu, Phythium *spp*	*Ligon* et al. *(2000)*
9	*Rhamnolipids*	P. aeruginosa	Phythium *spp*	*Ligon* et al. *(2000)*
		P. chlororaphis		

10	*Trigliceropeptides*	P. chlororaphis P. putida	Aa, Fusarium *spp*, Hs, Pf	*Chetverikov and Loginov (2005)*

Adapted from Someya *et al* (2013)

[1]Compounds produced

[2]species that are responsible for the production of each antibiotics

[3]plant pathogen controlled by the antibiotics. Abbreviations: *Ac, Aphanomyces cochlioides, Cg Collectotrichum gleosporioides, Fo Fusarium oxysporum, Ggt Gaeumannomyces graminis var tritici, Pc Phytophthora capsici, Pu Phythium ultimum, Rs Rhizoctonia solani, St Septonia tritici, Tb Thielabiopsis basicola, Pst Pseudomonas syringae pv tomato, Bs Bipolaris sorokiniana, Cgr Collectotrichum graminicol, Co Collectotrichum orbiculae, Lk Leptosphaeria korrae, Mp Magnaporthe poae, Psp Phythium splendens, Sh Sclerotinia homoecarpa, Ti Typhyla incarnata, Bc Botrytis cinerea, Fsa Fusarium sambucinum, Pe Penicillium expansum, Ptr Pyrenophora triticirepentis, Ss Sclerotinia sclerotiorum, Vd Verticillium dahliae, Mj Meloidogyne javanica, Gc Geotrichum citriaurantii, Pd Penicilium digitatum, Pi Phytophthora infestans, Pim Phythium intermedium, Rp Rhodotorula pilimanae, Aa Alternaria alternata, Hs Helminthosporium sativum, Pf Penicillium funiculosum*

Although detailed mechanisms of action for most of these antibiotics are not known, the mechanism of biocontrol activity have been demonstrated for some. One of the better known antibiotics produced by biocontrol Pseudomonas isolates, DAPG, causes growth inhibition and excessive branching of hypha in fungi by disrupting the filamentous actin cytoskeleton (Islam and Fukushi, 2010). Hydrogen cyanide is generated due to oxidation of glycine that produces HCN and CO2 by a membrane-bound flavoenzyme (HCN synthase). HCN is a potent inhibitor of cytochrome C oxidase and other mettalloenzymes (Blumer and Haas, 2000). Cyclic lipopetide (CLP) causes immobilization and subsequent cell lysis of the entire zoosphere of *Phytophthora infestans* (de Bruijin *et al*., 2007). Pyrrolnitirn inhibits growth synthesis of protein, RNA, DNA and uptake of metabolites of pathogens (Pang *et al*., 2009)

2.11 *Pseudomonas fluorescens*

Pseudomonas fluorescens encompasses a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environments. *Pseudomonas fluorescens* is a gram negative, rod-shaped, and non-pathogenic bacterium that is motile by means of multiple polar flagella.

It is an obligate aerobe, except for some strains that can utilize NO_3 as an electron acceptor in place of O_2.

Pseudomonas fluorescens is a widely known biocontrol agent against many soil borne plant pathogens because they are well adapted in soil. Some strains of the *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many fungal diseases (Hoffland *et al*. 1996, Wei *et al*. 1996). *P. fluorescens* are well characterized for their ability to produce many antimicrobial compounds (Haas and De' fago, 2005).

2.11.1 Bioactive Metabolites of *Pseudomonas fluorescens*

Some bioactive metabolites produced by *Pseudomonas fluorescens* and their synthesis are discussed as follows;

2.11.2 Pyrrolnitrin (PRN) and Pyoluteorin (PLT)

Pyrrolnitrin and pyoluteorin are tryptophan-derived metabolites produced mainly by gram negative bacteria as *Pseudomonas* spp, *Burkholderia* spp., and *Serratia* spp. Pyrrolnitrin are monochlorinated heteroaromatic pyrrole rings while pyoluteorins possess dichlorinated rings (Pang *et al*., 2015; Schmidt *et al*., 2009). No mode of action has been published for pyoleutorin although pyrrolnitrin has been reported to inhibit respiratory electron transport and synthesis of proteins. They also combine with cell membrane phospholipids thus affecting transport (Haas and De fago 2005; Nose and Arima 1969; Tripathi and Gottlieb, 1969).

Arima *et al.* (1964) described it for the first time as antifungal substance produced by *Burkholderia pyrrocina.* However, its production has also been reported from *P. fluorescens, Pseudomonas chlororaphis, Pseudomonas aureofaciens, Burkholderia cepacia,* and *Myxococcus fulvus.*

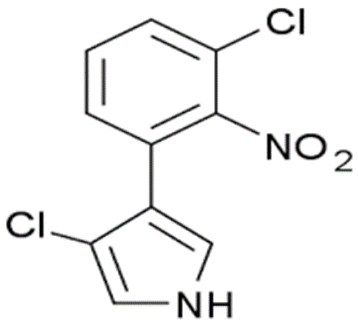


Figure 1: Chemical Structure of Pyrrolnitin

(https://pubchem.ncbi.nlm.nih.gov/compound/Pyrrolnitrin)

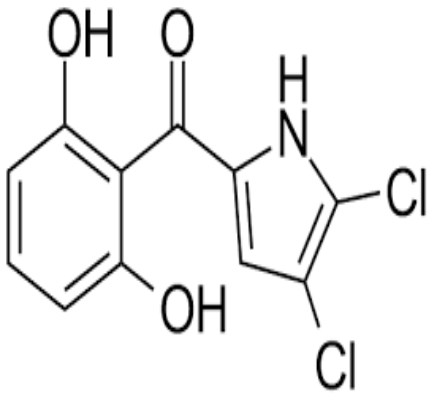


Figure 2: Chemical Structure of Pyoluteorin

(https://pubchem.ncbi.nlm.nih.gov/compound/Pyoluteorin)

2.11.2.1 Biosynthesis of Pyrrolnitrin

Synthesis of PRN occurs from aromatic amino acid, tryptophan, in a multistep process mediated by various enzymes encoded by prnA, prnB, prn C, and prnD genes of PRN operon (Hamill *et al.*, 1970). Product of prnA gene catalyzes the chlorination of l-tryptophan to 7- chlorotryptophan

followed by its conversion to aminopyrrolnitrin (APRN) via, monodechloroamino pyrrolnitrin (MDA) byproduct of prnB and prnC genes while as product of prnD gene catalyzes the final step of oxidation of APRN to PRN. PRN operon is regulated by a global regulator gene, gac A (gac is an acronym for global activator of antibiotic and cyanide synthesis) (Duffy and Defago 2000; Haas and Keel, 2003). Howell and Stipanovic (1979) reported pyrrolnitrin as an effective metabolite produced by the soil-inhabiting *P. fluorescens* responsible for imparting protection to cotton seedlings against infection by *Rhizoctonia solani*. Tazawa *et al.* (2000) reported that pyrrolnitrin produced by *P. fluorescens* is associated with decline in takeoff disease of wheat.

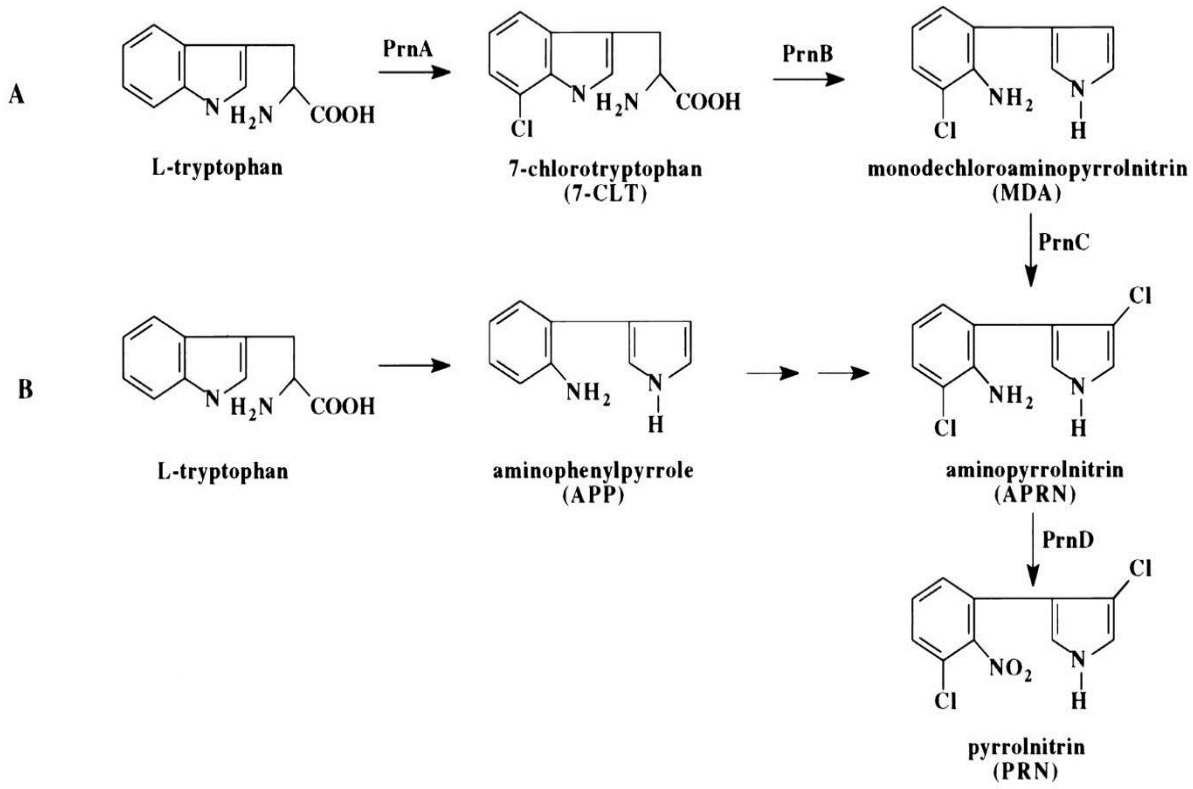


Figure 3: Biosynthetic pathways for PRN as proposed by Van Pee *et al*. (1980) (A) and by Chang *et al*. (1981) (B). Adapted from Sabine Kirner *et al*.

2.11.2.2 Biosynthesis of Pyoleutorin

This occurs from amino acid proline in a series of coupled condensation reactions followed by chlorination and oxidation at different stages of the multistep reaction catalyzed by at least 10 enzymes encoded by 10 genes (plt L, M, R, A, B, C, D, E, F, G) of PLT operon (Kraus and Lopez 1995; Nowak-Thompson *et al.,* 1997). Among them, plt B and plt C encode Type I polyketide synthetase; plt A, plt D, and plt H encode three halogenases; and plt G encodes thioesterase. PLT operon is regulated by plt R gene, which acts as transcriptional activator of the operon. It shows resemblance with Lys R family of transcriptional factors. Production of PLT is mainly associated with the inhibition of oomycete class of fungus including *Phythium ultimum* causing Phythium damping disease (Nowak-Thompson *et al.*, 1999).

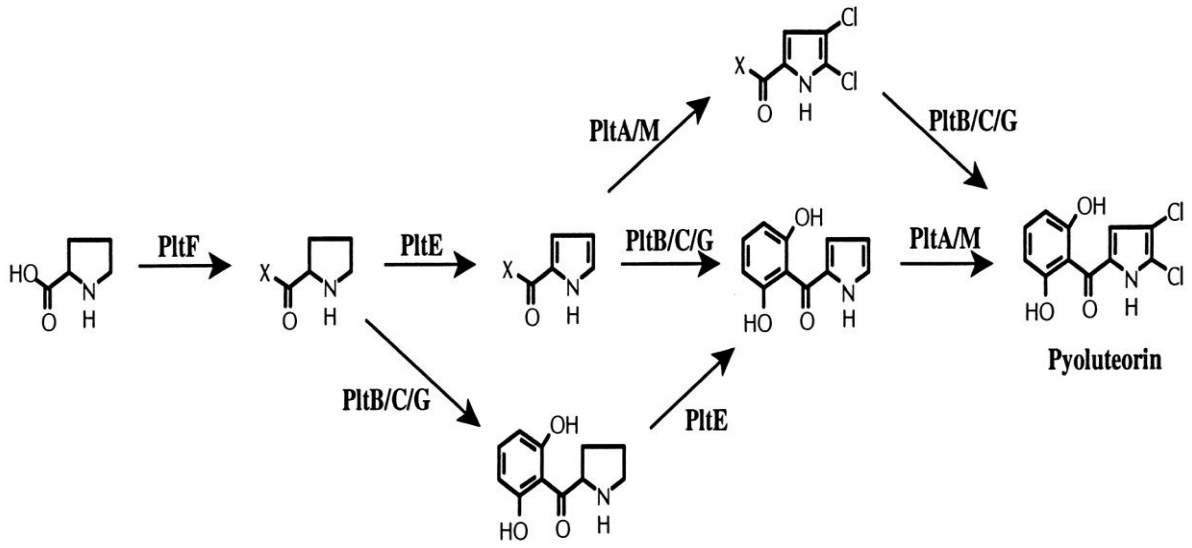
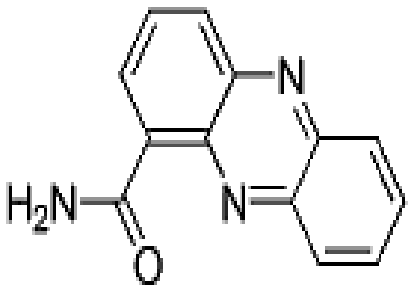


Figure 4: Hypothetical pyoluteorin biosynthetic pathways as described by Brian Nowak-Thompson *et al*. (1999).

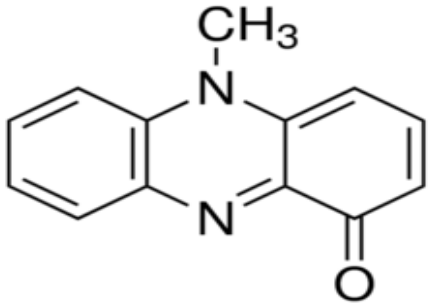
2.11.3 Phenazines (PHZ)

Phenazines are nitrogen containing heterocyclic pigmented compounds, known for their broad spectrum antifungal activity, synthesized by a wide range of bacterial genus including *Pseudomonas, Burkholderia, Brevibacterium, Nocardia, Erwinia, Vibrio, Pelagiobacter* and some actinomycetes like Streptomyces (Kennedy *et al*., 2015; Mavrodi *et al*., 2010; Morohoshi *et al*., 2013). The core structure of phenazines is a pyrazine ring (1,4- diazabenzene) exhibiting two annulated benzenes. Fluorescent Pseudomonad members including strains of *P. fluorescens, P. chlororaphis,* and *P. aeruginosa* are best known PHZ producers. *P. aeruginosa*, a common soil inhabitant and opportunistic human pathogen, is the first PHZ-producing micro-organism reported to produce characteristic blue green pigment pyocyanin (PYO, 5-N-methyl-1-hydroxyphenazine). Besides

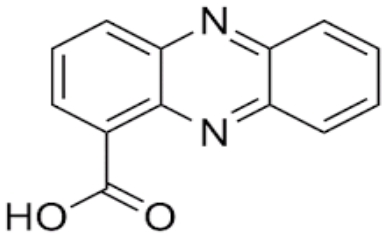
producing PYO, it also produces several other PHZs like 1-hydroxy-phenazine, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), aeruginosin A (5-methyl-7- amino-1-carboxymethylphenazinium betaine), and aeruginosin B (5-methyl-7-amino- 1- carboxy-3-sulfophenazinium betaine) (Holliman 1969; Byng *et al.*, 1979). Phenazines, such as PCA, PCN and PYO are toxic to a wide range of organisms. More than 6,000 phenazines derivatives have been described with one or other bioactivity (Mavrodi *et al*., 2006; Pierson & Pierson, 2010). Several modes of actions of phenazines have been identified, which include the reduction of molecular oxygen to reactive oxygen species (ROS), (Mahajan *et al*., 1999), the facilitation of energy generation (Glasser, *et al*., 2014; Wang *et al*., 2010; Prince-Whelan, 2007), involvement in iron homeostasis via Fe(III) reduction (Hernandez *et al*., 2004; Wang and Newman, 2008), participation as signal molecules via the activation of the Fe-containing transcription factor SoxR (Dietrich *et al*., 2008; Dietrich *et al*., 2006; Gu and Imlay, 2011) DNA p–p interaction and intercalation (Hollstein and vanGemert, 1971) and biofilm morphogenesis (Dietrich *et al*., 2013) through influencing the intracellular redox state. Phenazines are structurally related compounds but PCN and PCA have distinct effects on *Fusarium oxysporum f.sp. radicis-lycopersici*, which illustrate the difficulty of structure based prediction of antifungal activity (Chin-A-Woeng *et al*., 2001). Differences between phenazines in their functional groups also likely change their solubility and capacity for electron shuttling, especially at different pH levels (Cezairliyan et al., 2013; ChinA-Woeng et al., 2003; Pierson and Pierson, 2010).



Phenazine-1-carboxamide (PCA)



Pyocyanin



Phenazine-1-carboxylic acid (PCN)

Figure 5: Structures of some common phenazines produced by fluorescent *Pseudomonas* spp (Pierson and Pierson, 2010)

2.11.3.1 Phenazine Biosynthesis

The phenazines are a class of over 150 bacterial secondary metabolites with redox properties that enable them to act as broad specificity antibiotics as well as virulence and survival factors e.g. in infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* (Okegbe *et al*., 2012; Guttenberger *et al*., 2017). The five enzymes responsible in phenazine synthesis namely phzB, phzD, phzE, phzF and phzG are conserved among all phenazine-producing bacteria. Phenazine synthesis was shown to proceed via the shikimic acid pathway with chrosmic acid as the branch point intermediate and glutamine the source of nitrogen in the tricyclic moiety (Mentel *et al*., 2009; Turner and Messenger, 1986). Their biosynthesis involves the condensation of two chorismate-derived

precursors into the symmetrical phenazine scaffold (Fig. 6)). In this pathway, PhzF was found to act as an isomerase of (2 *S*,3 *S*)-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) (Parsons *et al*., 2004; Blankenfeldt *et al*., 2004) The reaction is believed to require two chemical steps, namely a hydrogen shift from C3 to C1 of the substrate and a subsequent tautomerization of the enol intermediate. Two molecules of the ensuing highly reactive ketone (1 *R*,6 *S*)-6-amino-5-oxo-2-cyclohexene-1-carboxylic acid (AOCHC) then undergo condensation to hexahydro-phenazine-1,6-dicarboxylic acid (HHPDC), followed by oxidations to dihydro-phenazine-1,6-dicarboxylic acid ($PDCH_2$) and/or dihydro-phenazine-1-carboxylic acid ($PCAH_2$) as precursors for strain-specific derivatives (Mentel *et al*., 2009; Blankenfeldt *et al*., 2014).

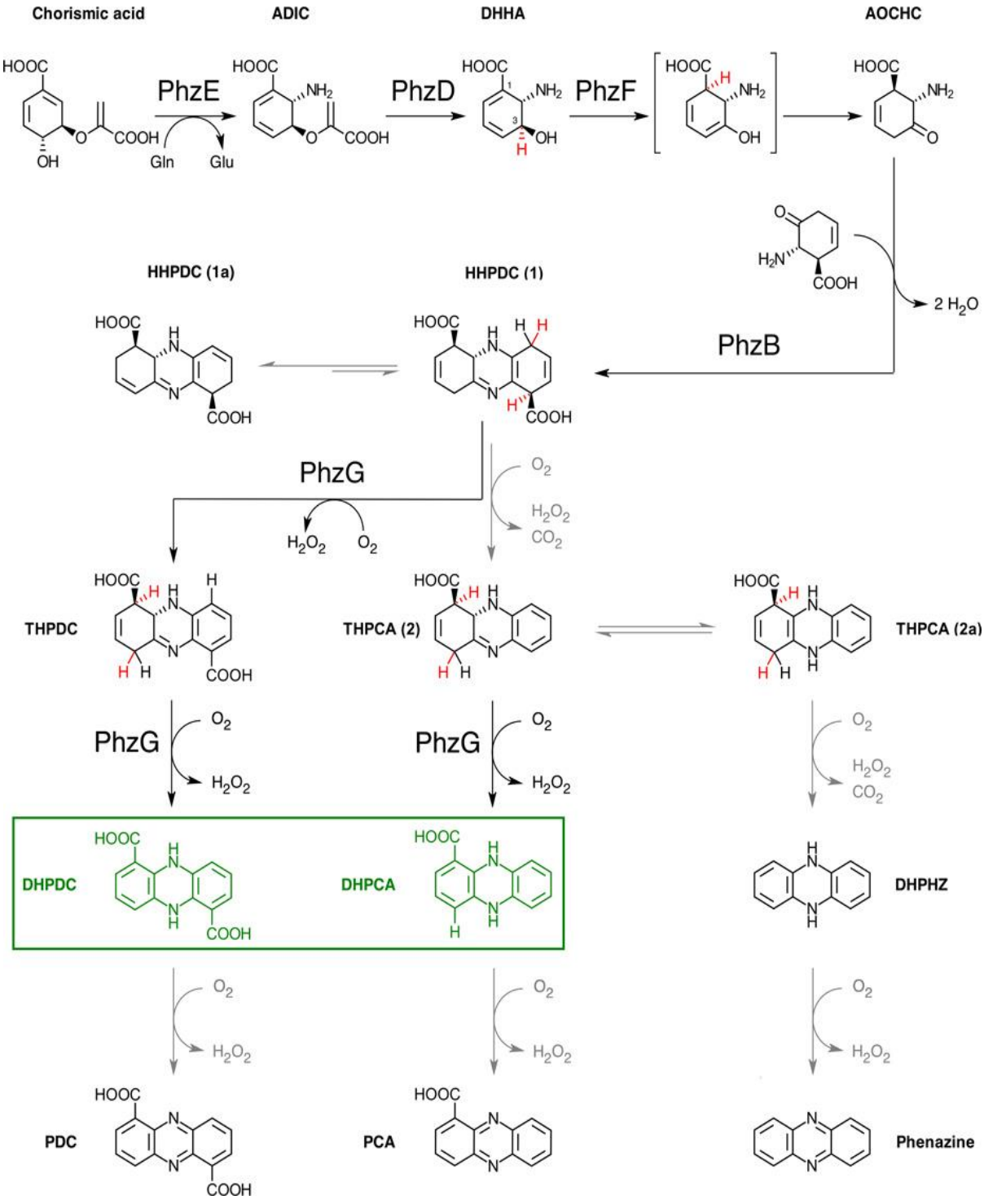


Figure 6: Current understanding of core phenazine biosynthesis (Blankenfeldt and Parson, 2014)

2.11.4 2, 4-Diacetylphloroglucinol

Among the phloroglucinols, 2,4-diacetylphloroglucinol (2,4-DAPG) is well known. DAPG, is a benzenetriol in which two of the ring hydrogen are replaced by acetyl group. 2,4-Diacetylphloroglucinol (DAPG) is a polyketide antibiotic that has antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (Bangera and Thomashow, 1999; de Souza *et al*., 2003a, b; Haas and Keel, 2003; Isnansetyo, *et al*., 2003; Kamei and Isnansetyo, 2003; Keel *et al*.,1992). It has been known as an excellent bio-control agent to suppress the take-all, a serious soil borne disease of wheat all over the world (Weller *et al*., 2002; Kwak *et al*., 2012).

The mechanism of action of 2, 4-DAPG against phytopathogens is not fully elucidated, but it was reported to damage membranes and zoospores of *Pythium* spp. (de Souza *et al.*, 2003a). (Troppens *et al* 2013) demonstrated that DAPG produced by *Pseudomonas fluorescens* alters mitochondrial morphology with loss of the membrane potential and increase of cytotoxic Ca2+ in *Neurospora crassa*. Besides the direct activity against pathogens (Keel *et al.*, 1992; Ramette *et al.*, 2001), the antibiotic also protects plants indirectly by inducing resistance in *Arabidopsis thaliana* against *Peronospora parasitica* (Iavicoli *et al.*, 2003) and *Pseudomonas syringae* pv. *tomato* (Weller *et al.*, 2004). 2, 4-DAPG also affects root physiology directly by blocking amino acid uptake (Phillips *et al.*, 2004). 2, 4-DAPG is an auto-inducer of its own synthesis and can function as a molecular signal (Schnider-Keel *et al.*, 2000), influencing antibiotic expression in neighboring strains (Maurhofer *et al.*, 2004). However, its production is affected by a large number of abiotic factors, such as Fe3+, Zn2+ Cu2+, Mo2+, and glucose.

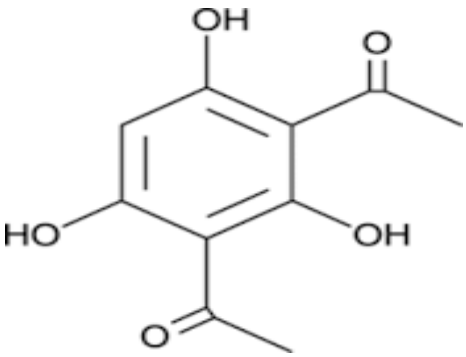


Figure 7: Chemical Structure of 2,4-diacetylphloroglucinol

(https://pubchem.ncbi.nlm.nih.gov/compound/2_4-Diacetylphloroglucinol)

2.11.4.1 Biosynthesis of DAPG

DAPG is synthesized by condensation of three acetyl-CoenzymeA molecules with one molecule of malonyl-CoenzymeA to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate PHL utilizing a CHS-type enzyme (Shanahan *et al*., 1992a).

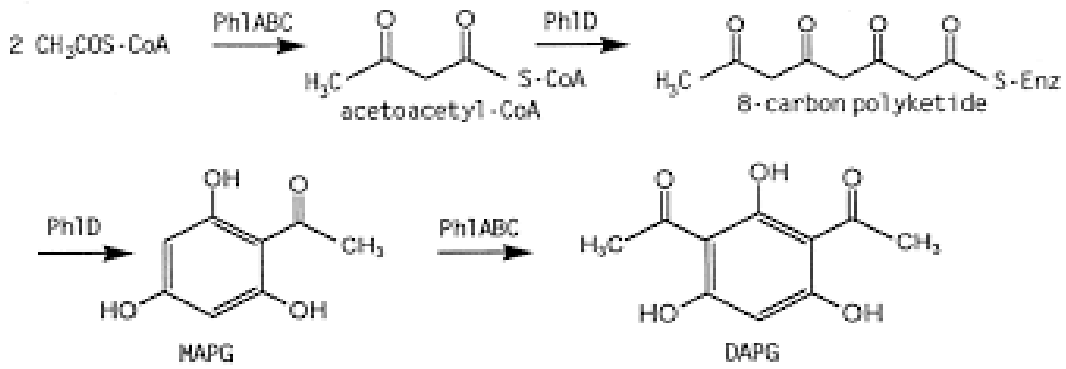


Figure 8: Proposed roles of PhlA, PhlB, PhlC, and PhlD in the biosynthesis of MAPG and 2,4-DAPG (Bangera and Thomashow, 1999**)**

The biosynthetic locus includes the five-gene operon phlACBDE (Bangera and Thomashow,1999). Particularly noteworthy is phlD, which is required for the synthesis of phloroglucinol, a precursor of monoacetylphloroglucinol (MAPG) and 2,4-DAPG (Achkar *et al*., 2005). PhlD has remarkable homology with members of the chalcone synthase/stilbene synthase (CHS/STS) family of plant enzymes (Bangera and Thomashow,1999; Ramette *et al*., 2001).

2.12 Use of agro-waste as part of media formulation for growth of microorganisms

Large amount of wastes is generated every year from the industrial processing of agricultural raw materials. Most of these wastes are used as animal feed or burnt as means for elimination. However, such wastes usually have a composition rich in sugars, minerals and proteins, and therefore, they should not be considered "wastes" but raw materials for other industrial processes.

Wastes derived from agricultural activities include materials such as straw, stem, stalk, leaves, husk, shell, peel, lint, seed/stones, pulp or stubble from fruits, legumes or cereals (rice, wheat, corn, sorghum, barley), bagasses generated from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many others. The presence of carbon sources, nutrients and moisture in these wastes provides conditions suitable for the development of microorganisms. Additionally, the agro-industrial wastes may be used in these processes as solid support, carbon, nitrogen and/or mineral sources, which would allow obtaining more economical fermentation processes avoiding the use of expensive chemical components in the media formulation.

Media are used for selective and differential cultivation of microorganisms (Seddon and Boriello, 1989; Taylor and Holland, 1989; Pelczar *et al*., 1993). Culture media used in the laboratory for the cultivation of microorganism supply the nutrients required for their growth and maintenance. Microorganisms can obtain energy directly from sunlight (autotrophs) while carbon can be made available in organic forms such as carbohydrates or inorganic forms such as carbon dioxide (Madigan *et al*., 2000).

Plant materials have been used to recover both fungi and bacteria from different sample sources such as Groundnut, Sorghum extracts, local food stuff waste, cassava whey, three – leaf yam, African oil bean, maize, beans and pigeon pea (Famurewa and David, 2008).

Different media for the growth and isolation of organisms have been reported from different substrates. Nkene (2015) used cassava peel, maize corn powder, banana peel and malt powder as carbon sources while soybeans and groundnut cake were used as nitrogen sources for the production of bioactive metabolites by *Pseudomonas fluorescens* against *Erwinia carotovora* isolates from rotten yam. The use of legume seeds as alternative nutrient media for bacteria and fungi has been reported (Tharmila *et al*., 2011; Arulanantham *et al*., 2012; Ravimannan *et al*., 2014).

Laleye (1990) reported a modification of potato medium supplemented with cow dung, soy milk and other growth factors. These media supported the growth of the test organisms luxuriantly when compared with growth on conventional Nutrient (Oxoid) and Potato Dextrose Agar (Oxoid). Also Adesemoye and Adedire (2005) studied the feasibility of developing alternative media to Potato Dextrose Agar (PDA) using local cereal species as the basal media.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Autoclave (Portable 230V and 1850W Adelphi MFG CO. Ltd, England), incubator (National Appliance Co. Ltd, Oregon, USA: model 1630, 240V and 2340W), hot air oven (Baird and Tatlock (London) Ltd, Chad Well Health Essex, England), refrigerator (Haier Thermocool: Model No. HRF-688-FF/A), microscope (Wild Heerbrugg M11, made in Switzerland), bunsen-burner, UV-Visible spectrophotometer (Eppendorf Biophotometer 8,5mm, Lichtstrahihohe), UV iluminator (Vilberb Lourmat TFX-35-M serial no NºV02 8104), centrifuge (Eppendorf 5417R), micropipette, microwave oven (HINARI Life Style 800watts model MX310TCSL), Electronic weighing balance (QT 600), Vortexing machine (Touch plate Super Mixer, CAT No 1291 Lab-line Instrument Inc USA) were the equipment used for this study

3.1.2 Glass wares

Glass wares such as Petri-dishes, beakers, bijou bottles, universal bottles, glass slides, test tubes, measuring cylinder, separating funnel, Eppendorf tube, magnetic stirrer (W and T Avery Ltd., England) were used for this study.

3.1.3 Reagents and Chemicals

All reagents and chemicals that were used in this study include; Methyl red, Phenol red, Kovac's reagent, calcium carbonate, magnesium sulfate, α-napthylamine, sulphanilic acid, zinc dust, buffer, potassium hydroxide, sodium hydroxide, ethanol, ethyl acetate, dipotassium hydrogen phosphate, glycerol, peptone mixture from BDH Chemical Ltd., England.

3.1.4 Culture media

The media that were used in this study include; Nutrient agar, King's B Agar, Cetrimide agar, SDA (Sabourand Dextrose Agar) from Oxoid Ltd, England.

3.2 Methods

3.2.1 Isolation of *Pseudomonas fluorescens*

The isolation of *Pseudomonas fluorescens* was carried out on Cetrimide agar. Briefly, one gram (1g) of soil sample from rice farm, sugar cane farm and soybeans farm was suspended in 9ml of sterile distilled water. 0.1ml of sample was spread on plates containing the medium and incubated at 37°C for 24hours.

3.3 Identification of *Pseudomonas fluorescens*

Standard microbiological tests were conducted for rapid identification of *Pseudomonas* colonies on the plates. Two parameters were used for the identification and confirmation of *P. fluorescens*, namely; cultural characteristics and biochemical test.

3.3.1 Microscopic characteristics

Gram staining of the isolate was carried out as described by Chessbrough (2006). A smear of the isolate was made on a slide. The smear was heat fixed and stained with the primary stain crystal violet and allowed to stand for 60 seconds. The stain was rinsed gently with water. The culture was fixed with Lugol's iodine solution for c60seconds, decolourized with ethanol and then counterstained with safranin. The stained slide was examined under an oil immersion lens and the isolate was classified as gram positive or gram negative.

3.3.2 Cultural characteristics

The colony morphology and whether there was production of pigment was observed by growing the isolate on cetrimide agar.

3.3.3 Growth at 4°C and 42°C

The ability of the isolates to grow at this temperature was determined by growing them in Cetrimide agar medium at respective temperatures.

3.3.4 Biochemical tests

Biochemical tests such as oxidase, catalase, gelatin hydrolysis, and nitrate reduction test (Reynold, 2004) was carried out to confirm the organism.

3.3.4.1 Oxidase Test

The oxidase test for the suspected organism was carried out as described by Chessbrough (2006). Bacterial inoculum was smeared on a moist filter paper and a drop of 1% tetramethyl-p-phenylenediaminedihydrochloride was added to the inoculum. Immediate colour change to purple gave a positive result.

3.3.4.2 Catalase Test

Two (2) Millilitres of the hydrogen peroxide solution was poured into a test tube and a sterile glass rod (not a nichrome wire loop) was used to remove several colonies of the test organism and immerse in the hydrogen peroxide solution (Chessbrough, 2006). Formation of bubbles indicated a positive reaction, while the ones without any bubbles showed negative reaction.

3.3.4.3 Indole Test

The indole test was carried out as described by Chessbrough (2006). Bacterial inoculum was inoculated in 5ml of peptone water in Bijou bottle and it was incubated at 30°c for 24hours after which some drops of Kovac's reagent was added. Formation of red ring indicated indole positive.

3.3.4.4 Gelatin Hydrolysis Test

This test was performed by stabbing the inoculum of the isolate into a tube containing Nutrient broth with gelatin used as the solidifying agent. Liquefied gelatin showed a positive result, while solid gelatin or non-liquefied showed negative result.

3.3.4.5 Nitrate Reduction Test

This test was conducted to determine the ability of the isolates to reduce nitrate to nitrite or further to free nitrogen gas. Peptone Nitrate broth with Durham tube was prepared in a screw-cap tube. The medium was inoculated with a loop-full of test culture and the inoculated broth was incubated for 24 hours at about 37° C. After incubation, 0.5 ml of reagent A which is Sulphanilic acid was added and then reagent B α- napthylamine was added and the test tube was observed for development of red colour.

3.3.4.6 Sugar Fermentation Test

An inoculum from a 24hours culture of the bacteria was transferred aseptically to a tube containing 5ml of nutrient broth tube containing phenol red indicator and different sugars; glucose, lactose, galactose, fructose, mannitol, arabinose, maltose and sorbitol. The inoculated tube was incubated at 37°C for 24 hours. A positive test result for the sugar fermentation showed a colour change from red to yellow, indicating a pH change to acidic.

3.3.4.7 Arginine Di-hydrolase Test

An inoculum from a 24hours culture was transferred aseptically to a sterile tube of arginine di-hydrolase broth. The inoculated tube was incubated at 37° C for 24 hours and the preliminary results was determined. The microbe first uses the glucose present to cause the pH to drop. This was indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme arginine di-hydrolase is activated. The culture was incubated an additional 24 hours at 37°C to allow the microbe to now use the arginine. The final results were obtained by observing the tube at 48 hours. Change back to purple from yellow indicated a positive test for

arginine di-hydrolase. Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicated a negative result.

3.4 Isolation and Identification of Phyto-pathogenic fungi

The three (3) test organisms (*A. niger, P. citrinum*, *Rhizopus stolonifer)* were isolated on Sabourand Dextrose Agar (SDA) medium from rotten yam using the method described by Okigbo (2005). The rotten yam tubers were first surfaced sterilized by swapping with cotton wool soaked in 70% ethanol. A sterilized knife was used to cut open the yam and portion of the rotten part was crushed & mashed into small pieces and teased out in 10ml of sterile distilled water in a bottle and this was shaken to distribute the pathogen from the yam evenly. A loopful of the inocula was collected from the suspension of the test sample and cultured on the SDA medium.

The medium was prepared according to manufacturer's instruction. A measured quantity of the powdered medium was dispensed into 1L of distilled water in a conical flask and mixed thoroughly. This was sterilized by autoclaving at 121°C for 15minutes. The sterile medium was allowed to cool and it was poured into sterile petri dishes and allowed to cool and gel. The inoculated plates with the test samples were then incubated at room temperature for 7 days and on establishment of growth, the plates were examined for distinct mycelia and from such mycelia, the test organisms were collected and sub-cultured on SDA slants.

The mycelial growth was identified as the test fungi based on morphology (such as, colour, texture, presence of visible spores on the culture plate and microscopy. Microscopic examination was done by observing their asexual or sexual reproductive structures (conidia, spores and hyphae) using lacto phenol dye to stain the isolates and examine under microscope.

A drop of 70% alcohol was dropped on a microscope slide and the fungal inoculum was dropped on the alcohol. A drop of the lactophenol was added before the alcohol dried out. A cover slip was placed in a way to avoid air bubbles and this preparation was viewed under a microscope using an oil immersion lens.

3.5 Screening for in vitro antifungal activity of crude extract of *Pseudomonas fluorescens* fermentation broth

3.5.1 Preparation of formulated fermentation media

The compositions of the formulated fermentation media for *P. fluorescens* used in the study is shown in table 3.1

Table 3.1: Composition of the fermentation media for growth of *Pseudomonas fluorescens*

	Amount(g/L)		
Compound	M_1	M_2	M_3
K_2HPO_4	0.98	0.98	0.98
$MgSO_4$	0.40	0.40	0.40
CaCO3	0.40	0.40	0.40
Yam Peel	20.00	-	-
Banana Peel	-	20.00	-
Sugarcane Shaft	-	-	20.00
Inorganic Nitrogen source	2.00	2.00	2.00

Groundnut cake	20.00	20.00	20.00

The carbon sources and nitrogen source used i.e. yam peel, banana peel, sugar shaft were peeled, dried, blended and sieved to get a fine powder. They were weighed according to the quantity needed for the media preparations.

The media prepared was autoclaved at 121°C for 15mins and the fermentation was carried out in a 250ml of conical flasks. The flasks were inoculated with fresh culture of *P. fluorescens* and was incubated at 25°C under agitation (120rpm) for 72 hours.

3.5.2 *In vitro* antifungal activities of crude extract of *Pseudomonas fluorescens* fermentation broth

The screening for the *in vitro* antifungal activities of *P. fluorescens* was carried out by using agar well diffusion method. The cultures were centrifuged at 10,000rpm for 15mins to obtain the cell free filtrate (Tripathi and Johri, 2002). 0.1ml of the cell free extract was introduced into the well made on Sabourand Dextrose Agar plate flooded with the spores of *Aspergillus niger, Penicillium citrinum, Rhizopus stolonifer* and the plates was incubated at 25°C for 24h. this was done in duplicate and the zone of inhibition was measured and recorded.

3.6 Extraction of the crude extract metabolite from *Pseudomonas fluorescens* in the fermentation broth

The method used was described by Bonsali *et al*. (1997). Broth cultures including bacterial cells were acidified with 10% Trifluoroacetic acid (TFA) to pH 2.0 and then extracted with ethyl acetate.

The ethyl acetate and broth were shaken to achieve a homogenous mixture and to extract the metabolite from bacterial cell for 20 minutes. A separating funnel was used to remove the

aqueous phase from the organic phase. A rotary evaporator was used to remove the ethyl acetate and thus the extract was obtained.

3.7 Quantitative Measurement of Metabolite Substance in the Extract

The concentration of the unknown metabolite in the sample was determined by using a spectrophotometer. A standard solution of phloroglucinol was made in different concentrations (100, 50, 25, 12.5, 6.25 to 0.391mg/ml) with sterile distilled water as blank. The absorbance of

different concentrations of the standard phloroglucinol solution was measured using UV light spectrophotometer at wavelength of 200nm to 900nm to generate a standard curve. The standard curve was used to determine the concentration of the metabolite in the sample.

3.8 Determination of Minimum Inhibitory Concentration of the ethyl acetate fraction against test fungal spores

3.8.1 *In vitro* Minimum inhibitory concentration of ethyl acetate fraction against test fungal spores

Ten millilitres (10ml) volume of double strength SDA was melted and mixed aseptically with 10mls volume of varying concentration of the test antifungal crude extract metabolite. Each mixture was aseptically poured into sterile plates and allowed to set. The standardized spores of the test fungi was inoculated (10.0µl) in duplicates on sterile filter paper discs and was placed equidistance on the SDA test antifungal plates. The inoculated organisms were allowed pre-diffusion period of 30minutes. The plates were then incubated at 30°C for 48hours.

The first lowest concentration that showed no growth of the inoculated test fungi spores was considered as the MIC of the test antifungal metabolites.

3.8.2 *In vivo* minimum inhibitory concentration of ethyl acetate fraction against test fungal spores on yam tuber

This test was carried out on yam tuber. Healthy yam tubers were selected, surfaced sterilized with 70% ethanol and peeled with sterilized knife. The yam was sliced into cube shape and surface sterilized with 70% ethanol and rinsed with sterile distilled water and allowed to dry. The sliced yam was treated with different concentration of the crude extract metabolite by dipping the yam slice in a beaker containing prepared concentration of the extract. The sliced yam was allowed to dry and then a well was made using a sterile 10mm cork borer and this well was inoculated with 0.1ml of adjusted suspension (10^6 CFU/ml) of the phyto-pathogenic fungi *Aspergillus niger, Penicillium citrinum* and *Rhizopus stolonifer* using a micro pipette.

Yam slices treated with extracted metabolite with no organism inoculated served as negative control while yam slices treated with distilled water and inoculated with the organism served as positive control. The treated yam slices were placed in a desscicant cubic glass cabinet disinfected with 70%(v/v) alcohol. The cabinet was provided with 90% relative humidity by placing a sterile petri dish containing sterile normal saline. The treated yam slices were incubated for 7 days.

The minimum inhibitory concentration of the crude extract that inhibited visible rot was recorded as the *in vivo* MIC

3.9 Statistical analysis

All the data obtained from the studies were expressed as Mean ± SEM (Standard Error of Mean) and ANOVA was used to as the statistical tool. Data were also presented in the form of figures and tables.

CHAPTER FOUR

RESULTS

4.1 Isolation and identification of *Pseudomonas fluorescens*

The cultural, morphological and biochemical characteristics of *P. fluorescens* isolates from rhizosphere of sugarcane, rice and soybeans farms in Zaria was done and the result is in the Table 4.1 and 4.3. Out of six (6) soil samples of the rhizosphere of the plants such as 2 from sugarcane, 2 from rice and 2 soybeans, only the sugarcane and rice farm isolates were confirmed *Pseudomonas fluorescens* isolates.

4.2 Isolation and identification of fungi from rotten yam

The morphological characteristics of the fungi species isolated as seen on the plate is presented/shown in Table 4.3. The fungi organism was confirmed after microscopic identification using Bergey's Manual for identification.

Table 4.1: Cultural and Morphological Characteristics of *Pseudomonas fluorescens* isolates on Cetrimide agar

Soil samples	Growth on Cetrimide	Elevation	Surface	Gram staining	Shape	Growth at	
						4ºC	41ºC
Sugarcane A1	+	Flat	Circular	-	Rod	+	-
Sugarcane A_2	+	Flat	Circular	-	Rod	+	-
Rice B_1	+	Flat	Circular	-	Rod	+	-
Rice B_2	+	Flat	Circular	-	Rod	+	-
Soybeans C_1	-	-	-	-	-	-	-
Soybeans C_2	+	Flat	Circular	+	Rod	-	-

Key: + growth, - no growth, Gram staining; + Gram positive, - Gram negative

Table 4.2: Biochemical characterization of *Pseudomonas fluorescens* isolates from rice & sugarcane rhizosphere

Isolates	Oxidase test	Catalase test	Indole test	Gelatin hydrolysis	Nitrate reduction	Arginine dihydrolase test	Sugar fermentation test			
							Glu	Lac	Suc	Mal
Sugarcane A1	+	+	-	+	+	+	+	-	-	+
Sugarcane A2	+	+	-	+	+	+	+	-	+	-
Rice B1	+	+	-	+	+	+	+	-	+	+
Rice B2	+	+	-	+	+	+	+	-	+	+

Key: + positive to test, - Negative to test, Glu- Glucose, Lac- Lactose, Suc- Sucrose, Mal- Maltose

Table 4.3: Morphological characteristics of isolated fungi species

Source of fungi	Mycelial colour	Reverse pigmentation	Organism
Rotten yam	Initially white, quickly becoming black when matured with conidial production	Pale yellow	*Aspergillus niger*
Rotten yam	Initially white when matured has a central gray colour with a white outer edge	Pale yellow	*Penicillium citrinum*
Rotten yam	Has a white fluffy cottony growth covering the entire plate	Cream	*Rhizopus stolonifer*

4.3: Antifungal activities of crude extract of *Pseudomonas fluorescens* fermentation broth against the test fungi spores

The antifungal activities of crude extract of *P. fluorescens* isolates from rhizosphere of sugarcane and rice farms against phyto-pathogenic fungi from rotten yam tubers using the formulated media (M_1, M_2 and M_3) is as shown in Table 4.4.

Table 4.4: Antifungal activities against test fungi spores by the crude extract from *Pseudomonas fluorescens* fermentation broth

Keys: M_1= Medium 1 containing same quantity of yam peel and groundnut cake as carbon & nitrogen sources

M_2= medium 2 containing same quantity of banana peel and groundnut cake as carbon & nitrogen sources

M_3= medium 3 containing same quantity of sugarcane shaft and groundnut cake as carbon & nitrogen sources

There was statistically significant difference ($p < 0.05$) in antifungal activities of the crude extract of *Pseudomonas fluorescens* isolate on the test organisms in M_1. The crude extract of isolate SGA_1 had the lowest mean of activity with no activity against the three (3) test organisms. Either the organism

Diameter of Zones of Inhibition (Mean±SEM) (mm)

	Aspergillus niger			***Penicillium citrinum***			***Rhizopus stolonifer***		
	M_1	**M_2**	**M_3**	**M_1**	**M_2**	**M_3**	**M_1**	**M_2**	**M_3**
***Pseudomonas fluorescens* isolates**									
Sugarcane A1	0	0	0	0	20.50±0.71	27.00±1.41	0	0	0
Sugarcane A2	20.50±0.71	24.50±0.71	0	19.00±1.41	25.00±1.41	24.50±0.71	14.00±1.41	31.50±2.12	0
Rice B1	34.00±1.41	0	19.00±1.41	24.00±1.41	0	29.50±0.71	20.50±0.71	39.00±1.41	20.50±0.71
Rice B2	24.50±0.71	30.50±0.71	0	29.00±1.41	33.50±2.12	20.50±0.71	21.50±2.12	31.00±1.41	0

doesn't produce metabolite substance or the metabolite substance produced is too small to inhibit the test organisms. Rice B1 had the best mean of activity against the test organisms (Appendix 3)

There was no statistically significant difference ($p > 0.05$) in the antifungal activities of the crude extract metabolite of *P. fluoresens* isolates on the test organism in M_2. SGA_1 had the lowest activity compared to the remaining *P. fluorecens* isolates. Rice B_2 had the best mean activity against the test organism (Appendix 4)

There was statistically significant difference ($p < 0.05$) in the antifungal activities of the crude extract of *P. fluorescens* isolates on the test organism in M_3. Rice B_1 had the best mean activity against the test organisms (Appendix 5).

4.4 Antifungal activities of the ethyl acetate fraction of fermentation broth of *Pseudomonas fluorescens*

Only three *Pseudomonas fluorescens* isolates i.e. sugarcane A_2, rice B_1 rice B_2 were used with two media formulation (M_1 &M_2) for the extraction of the crude extract metabolite. A total of 6 crude metabolite extract were collected after the complete removal of the aqueous phase with a separating funnel and the solvent ethyl-acetate with rotary evaporator.

The results of their antifungal test are presented in Tables 4.5 & 4.6.

Table 4.5: Antifungal activities of ethyl acetate fraction of M_1 fermentation broth culture of *Pseudomonas fluorescens*

	Diameter of Zone of Inhibition (mm) (Mean ±SEM)		
domonas fluorescens *ites*	*ergillus niger*	cillium citrinum	opus stolonifer
2	*0±0.71*	*0±2.12*	*0±1.41*
B1	*0±0.71*	*0±1.41*	*0±1.41*
B2	*0±1.41*	*0±1.41*	*0±1.41*

:M1= Medium 1 containing same quantity of yam peel and groundnut cake as carbon and plex nitrogen sources

There was no statistical significant difference ($p>0.05$) in antifungal activities of the crude extract metabolite of *Pseudomonas fluorescens* isolate on the test organism in M_1 and crude extract from Rice B_1 had the highest mean antifungal activity against the test organisms (Appendix 9).

Table 4.6: Antifungal activities of ethyl acetate fraction of M_2 fermentation broth culture of *Pseudomonas fluorescens*

Diameter of Zone of Inhibition (mm) (Mean ±SEM)

		ergillus niger	cillium citrinum	opus stolonifer
domonas *ites*	fluorescens			
2		*0±0.71*	*0±0.71*	*0±1.41*
B1		*0±0.71*	*0±2.12*	*0±2.12*
B2		*0±2.12*	*0±1.41*	*0±1.41*

s:M2= Medium 2 containing same quantity of yam peel and groundnut cake as carbon and complex nitrogen source respectively

There was no statistically significant difference ($p>0.05$) in antifungal activities of the crude extract metabolite of *Pseudomonas fluorescens* isolate on the test organism in M_2 and crude extract from SGA_2 had the highest mean antifungal activity against the test organisms (Appendix 10).

4.5 *In vitro* Minimum inhibitory concentration (M.I.C.) values for the ethyl acetate fractions of *Pseudomonas fluorescens* fermentation broth on the test fungi spores

The *in vitro* minimum inhibitory concentrations (M.I.C) of the crude extract metabolite on *Aspergillus niger*, *Penicillium citrinum* and *Rhizopus stolonifer* were found and the results are shown in the Tables 4.7 & 4.8

The Six (6) crude extract metabolite that was extracted were used for the *in vitro* MIC. *Rhizopus stolonifer* was inhibited at lower concentrations by all the ethyl acetate fractions.

Table: 4.7 *In vitro* MIC of ethyl acetate fraction of M_1 fermentation broth culture of *Pseudomonas fluorescens* on the test fungi spores

In vitro *Minimum Inhibitory Concentration (mg/ml)*			
ergillus niger	cillium citrinum	opus stolonifer	domonas fluorescens
tes			

.2

B₁

B₂

ys:M₁= Medium 1 containing same quantity of yam peel and groundnut cake as carbon and complex nitrogen sources

Table: 4.8 *In vitro* MIC of ethyl acetate fraction of M_2 fermentation broth culture of *Pseudomonas fluorescens* on the test fungi spores

In vitro *Minimum Inhibitory Concentration (mg/ml)*

domonas fluorescens ergillus niger cillium citrinum opus stolonifer
ites

2

B1

B2 *00*

ys:M2= Medium 2 containing same quantity of banana peel and groundnut cake as carbon and complex nitrogen sources

4.6 *In vivo* Minimum inhibitory concentration values for the ethyl acetate fractions of fermentation broth of *Pseudomonas fluorescens* on the test fungi isolates on yam tuber.

The *in vivo* minimum inhibitory concentrations of the ethyl acetate fractions were carried out on yam tuber inoculated with the test fungi spores and the results are shown in the Tables 4.9 & 4.10

Based on the results obtained, all the crude extract had active against *Rhizopus stolonifer* isolate at different concentrations as compared to *Aspergillus niger and Penicillium citrinum* isolates.

Table 4.9: *In vivo* MIC of ethyl acetate fractions of M_1 fermentation broth culture of *Pseudomonas fluorescens*

In vivo *Minimum Inhibitory Concentration (mg/ml)*				
	ergillus niger	cillium citrinum	opus stolonifer	domonas fluorescens
				tes

2	*00*	*00*
B1		
B2		

ys:M1= Medium 1 containing same quantity of yam peel and groundnut cake as carbon and complex nitrogen sources

4.10: *In vivo* MIC of ethyl acetate fraction of M_2 fermentation broth culture of *Pseudomonas fluorescens*

In vivo *Minimum Inhibitory Concentration (mg/ml)*

ergillus niger cillium citrinum opus stolonifer domonas fluorescens
tes

.2			
B₁	*00*	*.00*	
B₂	*00*	*.00*	*0*

ys:M_2= Medium 2 containing same quantity of banana peel and groundnut cake as carbon and complex nitrogen sources

CHAPTER FIVE

DISCUSSION

Pseudomonas fluorescens are aggressive colonizers of the rhizosphere of plants and have been isolated from many rhizospheric plant soils from diverse geographical regions. Jayashree *et al* (2000) isolated fluorescent pseudomonads from the rhizosphere of black gram, carrot, banana, pepper, rice & forest trees grown in several geographical areas of Tamil nadu. In this study, *Pseudomonas*

fluorescens strains were isolated from the rhizosphere of rice and sugar cane farms in Samaru, Zaria of Kaduna state. This is in agreement with the work of Nkene (2015) who isolated *Pseudomonas fluorescens* strains from rice, soybeans and groundnut farms in Keffi, Nassarawa state. Also, (Prakash *et al*.,2011; Reddy & Reddy 2009) both isolated *Pseudomonas flourescens* from rhizosphere of rice plants.

Pseudomonas fluorescens has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources (Palleroni, 1984) and waste materials contain minerals and nutrients that can meet the nutritional requirements of microorganism, Hence an alternative media using agro-waste consisting of yam peel, banana peel, sugarcane shaft as sources of carbon and groundnut cake as source of nitrogen were studied in this work for antifungal activities of the isolated *Pseudomonas fluorescens* strains for the production of metabolite substance. Although previous studies on activities of bioactive metabolites of *P. fluorescens* against fungal pathogens of other plants using lactose, glucose, molasses and malt extract as carbon source as well as yeast extract, peptone, corn steep liquor and soy bean extract as complex nitrogen source has been reported by (Heidari-Tajabadi *et al.,* 2013).

The antimicrobial activity of *P. fluorescens* have been reported against several phytopathogens. *P. fluorescens* was shown to effectively inhibit *R. solani* and *P. oryzae* by agar plate method (Rosales *et al* 1995). The results in this study showed that crude extract from the fermentation broth of four strains of *Pseudomonas fluorescens* in three formulated media (M_1, M_2 and M_3) inhibited mycelial growth of *Aspergillus niger, Penicillium citrinum* and *Rhizopus stolonifer* isolates from rotten yam. A total of 36 crude extracts from fermentation broth of *Pseudomonas fluorescens* were obtained and screened for antifungal activities (Table 4.4). 24 crude extract presented antifungal activities against the fungi test organisms showing inhibition range of (19.00mm-39.00mm) (Table 4.4.). the results show that the crude extract of SGA_2, $RiceB_1$, Rice B_2 had antifungal activities against at least two (2) of the test fungal organisms. However, crude extract of SGA1 showed no antifungal activity

against *Aspergillus niger* and *Rhizopus stolonifer* in M_1, M_2 and M_3. This finding agrees with a study conducted by Raajimaker *et al.* (2002) that many antibiotics produced by *P. fluorescens* isolate has varying activity against different phyto-pathogenic micro-organism. On the other hand, *Penicillium citrinium* was the most susceptible test organism as it was inhibited by almost all the crude extracts (Table 4.4). Based on these results, only three (3) *Pseudomonas fluorescens* isolates SGA_1, Rice B_1 and Rice B_2 were chosen for the extraction of metabolites.

In this study, fermentation of *Pseudomonas fluorescens* isolate in M_1 and M_2 with carbon source yam peel and banana peel supported the production of secondary metabolite as both media had higher inhibition averages (17.20mm and 19.60mm) against the test fungi organisms compared to those obtained in M_3 (11.75mm) which sugarcane shaft was carbon source. It was observed that mycelial growth was reduced due to the production of secondary metabolites which inhibited growth of the test organisms, and production of secondary metabolites depends mostly on nutrients available in the medium (Higgs *et al*., 2001). This makes it possible for different species of Pseudomonas to generate secondary metabolite of different composition during their growth (Robles-Huízar *et al*., 2017). Polanski *et al*. (2013) found that the production of extracellular antibiotics produced by *P. fluorescens* CL0145A against *B. subtilis* depends on the content of carbon sources as well as the presence of amino acids and vitamins, since when glycerol, hydrolyzed soy, tryptophan, glutamine, biotin and riboflavin were added to the medium, the antibiotic was generated. However only carbon sources were varied in this media formulated as other components were the same.

In vitro evaluation of antifungal activities of ethyl acetate fractions of fermentation broth of *Pseudomonas fluorescens* in formulated media M_1 reveal that the ethyl acetate crude fraction completely inhibited the growth of *A. niger, P. citrinum, R. stolonifer*. Maximum inhibition was observed in extracts of Rice B_1 (29.00mm) against *P. citrinum* and SGA_2 (29.00mm) against *R. stolonifer* (Table 4.5). Crude fraction from Rice B_1 had the highest mean antifungal activities against the test fungi organisms (Appendix 9). Similarly, *in vitro* evaluation of antifungal activities of ethyl

acetate fractions of fermentation broth of *Pseudomonas fluorescens* in formulated media M_2 also reveal that the ethyl acetate crude fraction completely inhibited the growth of *A. niger, P. citrinum, R. stolonifer*. Maximum inhibition was observed in extracts of Rice B_1 (33.50mm) against *P. citrinum* and SGA_2 (31.00mm) against *R. stolonifer* (Table 4.6). Crude fraction from SGA_2 had the highest mean antifungal activities against the test fungi organisms (Appendix 10). *P. flourescens* was shown to have highest antifungal activity against *Penicillium italicum* (94%) and was moderately effective against *Aspergillus niger* (61%) (Mushtaq *et al*., 2010). Reddy *et al*. (2007) reported that the crude compounds from *P. fluorescens* isolates metabolites completely inhibited the growth of *Magnaporthe grisea*, *Dreschelaria oryzae*, *R. solani* and *Sarocladium oryzae*. The antifungal activity of the three solvent extracts of secondary metabolites of Pf 1, Pf 2, Pf 4, Pf 5, Pf 6, Pf 7, Pf 9, Pf 10 and Pf 11 showed that ethyl acetate extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with ethyl acetate than methanol and hexane (Alemu and Alemu, 2013). Similarly, the metabolite extracted from *P. fluorescens* with ethyl acetate effectively inhibited *P. oryzae* and *R. solani* (Battu and Reddy, 2009).

Observations from the *In vitro* MIC of the ethyl acetate fraction of fermentation broth of *Pseudomonas fluorescens* in M_1 against the test fungi organisms in this study reveal that the ethyl acetate crude fraction inhibited all the test organism at different concentrations (0.15mg/ml-6.49mg/ml). *Rhizopus stolonifer* was the most susceptible organism as it was inhibited at lower concentrations compared to *A. niger* and *P. citrinum. A. niger* needed a x4 concentrations to be inhibited by all the crude fractions (Table 4.7). Similarly, the *in vitro* MIC of the ethyl acetate fraction of fermentation broth of *Pseudomonas fluorescens* in M_2 show that *Rhizopus stolonifer* was inhibited at lower concentrations. However, ethyl acetate fraction from Rice B_2 didn't have activity against *A. niger* even at the highest prepared concentration (>12.00mg/ml) (Table 4.8). In most cases, antibiotics showing *in vitro* activity are generally active *in vivo* against plant diseases.

Results from the *in vivo* MIC of the ethyl acetate fraction of fermentation broth of *Pseudomonas fluorescens* in M_1 show that ethyl acetate fraction from SGA_2 had no activity against *A. niger* and *P. citrinum* at the highest prepared concentrations (>20.00mg/ml) expect against *R. stolonifer* (5.00mg/ml) while the ethyl acetate fraction of Rice B_1 and Rice B_2 had activity against *R. stolonifer*, *P. citrinum* and *A. niger* ranging from (1.00mg/ml-5.00mg/ml) (Table 4.9). the *in vivo* MIC of the ethyl acetate fraction from SGA_2 in M_2 had activity against *A. niger*, *P. citrinum*, *R. stolonifer* (4.00mg/ml-5.00mg/ml). However, ethyl acetate fraction from Rice B_1 and Rice B_2 had no activity against *A. niger* and *P. citrinum* at the highest prepared concentrations (>20.00mg/ml and >30.00mg/ml) respectively but had *in vivo* activity against *R. stolonifer* (3.37mg/ml and 20.00mg/ml) respectively. The result from this study show that *P. fluorescens* strain from zaria has great potential for the biocontrol of yam rot fungi *in vitro* and *in vivo* conditions.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Pseudomonas fluorescens strains were isolated from the rhizosphere of rice & sugarcane farms in samaru, Zaria. Alternative media using agro waste consisting of yam peel, sugarcane shaft as sources of carbon and groundnut cake as source of nitrogen was studied in this work for antifungal activities

of the isolated *P. fluorescens* strains for the production of metabolite substance. Observation from this study showed that *Rhizopus stolonifer* was the most susceptible test organism as it was inhibited at a lower MIC in vitro (0.15-2.01 mg/ml) & in vivo (1-20 mg/ml) irrespective of the crude extract metabolite compared to the other test organisms.

6.2 Conclusion

The result in this work showed that *Pseudomonas fluorescens* produce antifungal substances which justify their use as biocontrol agents against fungi pathogens of different crops in the field. This secondary metabolite when probably engaged will promote/enhance food security by reducing the loss of yam tubers to rot in Nigeria.

This study showed that fermentation with medium containing yam and banana peels as carbon source led to the production of more bioactive secondary metabolite by *Pseudomonas fluorescens* isolates from rice and sugar cane rhizospheres.

Furthermore, the use of waste products like yam and banana peel with groundnut cake to produce useful antibiotics demonstrated in this work has shown that local waste can indeed be utilized as laboratory media.

6.3 Recommendations

Antibiotic production by fluorescent *Pseudomonas* spp. is now recognized as an important feature in plant disease suppression by some strains and the presented data exhibit the antifungal activities of Pseudomonas strains and indicate the possibility of using *Pseudomonas fluorescens* as a biological control agent against some plant pathogenic fungi of yam

it is however recommended that;

i. There should be further screening of a large number of Pseudomonas strains from different region in Nigeria that can produce active antifungal agents.

ii. Further studies on the isolation and characterization of the secondary metabolites of *P. fluorescens* from the crude extract of the formulated media should be carried out.

iii. Since *Pseudomonas fluorescens* are known to produce more than one bioactive metabolite, further studies should be carried out to know which of the secondary metabolite is responsible for the biocontrol.

iv. Also continuous application of these organism to disease control will help select and produce better candidates for reliable commercial products.

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APPENDICES

Appendix 1: Antifungal activities against test fungi spores by the crude extract from *Pseudomonas fluorescens* isolates in three (3) fermentation media

Zone of inhibition (mm) in duplicate for medium 1: yam peel + groundnut cake

***Pseudomonas fluorescens* isolates**	***Aspergillus niger***		***Penicillium citrinum***		***Rhizopus stolonifer***	
SGA_1	0	0	0	0	0	0
SGA_2	20	21	20	18	15	13
Rice B_1	35	33	25	23	20	21
Rice B_2	25	24	30	28	20	23

Zone of inhibition (mm) in duplicate for medium 2: Banana peel + groundnut cake

***Pseudomonas fluorescens* isolates**	***Aspergillus niger***		***Penicillium citrinum***		***Rhizopus stolonifer***	

SGA_1	0	0	20	21	0	0
SGA_2	25	24	26	24	30	33
Rice B_1	0	0	0	0	40	38
Rice B_2	30	31	35	32	30	32

Zone of inhibition (mm) in duplicate for medium 3: sugarcane shaft + groundnut cake

	Aspergillus niger		***Penicillium citrinum***		***Rhizopus stolonifer***	
***Pseudomonas fluorescens* isolates**						
SGA_1	0	0	28	26	0	0
SGA_2	0	0	24	25	0	0
Rice B_1	20	18	30	29	20	21
Rice B_2	0	0	20	21	0	0

Appendix 2: Antifungal activities of ethyl acetate fractions of fermentation broth culture of *Pseudomonas fluorescens*

Zone of inhibition (mm) in duplicate for SGA_2

	Aspergillus niger		***Penicillium citrinum***		***Rhizopus stolonifer***	
Media						
M_1	20	19	25	22	30	28
M_2	30	31	30	31	30	32

Zone of inhibition (mm) in duplicate for RiceB$_1$

Media	*Aspergillus niger*		*Penicillium citrinum*		*Rhizopus stolonifer*	
M_1	25	26	30	28	25	23
M_2	25	24	35	32	30	27

Zone of inhibition (mm) in duplicate for RiceB$_2$

Media	*Aspergillus niger*		*Penicillium citrinum*		*Rhizopus stolonifer*	
M_1	20	22	25	27	30	27
M_2	20	23	21	23	30	28

Appendix 3: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates against the three test organism in medium 1

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
SGA1	3	0	0	0		
SGA2	3	53.5	17.83333	11.58333		
Rice B1	3	78.5	26.16667	49.08333		
Rice B2	3	75	25	14.25		
A. niger	4	79	19.75	205.4167		
P. citrinum	4	72	18	160.6667		
R. stolonifer	4	56	14	98.16667		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1312.417	3	437.4722	32.67427	0.000411	4.757063
Columns	69.5	2	34.75	2.595436	0.154121	5.143253
Error	80.33333	6	13.38889			

Appendix 4: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates against the three test organism in medium 2

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
SGA1	3	20.5	6.833333	140.0833		
SGA2	3	81	27	15.25		
Rice B1	3	39	13	507		
Rice B2	3	95	31.66667	2.583333		
A. niger	4	55	13.75	258.0833		
P. citrinum	4	79	19.75	202.4167		
R. stolonifer	4	101.5	25.375	299.5625		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1220.729	3	406.9097	2.30444	0.17671	4.757063
Columns	270.375	2	135.1875	0.765603	0.505662	5.143253
Error	1059.458	6	176.5764			
Total	2550.563	11				

Appendix 5: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates against the three test organism in medium 3

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
SGA1	3	27	9	243		
SGA2	3	24.5	8.166667	200.0833		
Rice B1	3	69	23	32.25		
Rice B2	3	20.5	6.833333	140.0833		
A. niger	4	19	4.75	90.25		
P. citrinum	4	101.5	25.375	14.72917		
R. stolonifer	4	20.5	5.125	105.0625		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	513.4167	3	171.1389	8.798286	0.012895	4.757063
Columns	1114.125	2	557.0625	28.6387	0.000853	5.143253
Error	116.7083	6	19.45139			
Total	1744.25	11				

Appendix 6: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates in the three formulated media against *Aspergillus niger*.

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
M1	4	79	19.75	205.4167		
M2	4	55	13.75	258.0833		
M3	4	19	4.75	90.25		
SGA1	3	0	0	0		
SGA2	3	45	15	172.75		
Rice B1	3	53	17.66667	290.3333		
Rice B2	3	55	18.33333	261.0833		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	456	2	228	1.378569	0.321638	5.143253
Columns	668.9167	3	222.9722	1.348169	0.344609	4.757063
Error	992.3333	6	165.3889			

Appendix 7: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates in the three formulated media against *Penicillium citrinum*.

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
M1	4	72	18	160.6667		
M2	4	79	19.75	202.4167		
M3	4	101.5	25.375	14.72917		
SGA1	3	47.5	15.83333	198.5833		
SGA2	3	68.5	22.83333	11.08333		
RiceB1	3	53.5	17.83333	246.0833		
Rice B2	3	83	27.66667	43.58333		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	118.7917	2	59.39583	0.405029	0.683914	5.143253
Columns	253.5625	3	84.52083	0.57636	0.651421	4.757063
Error	879.875	6	146.6458			
Total	1252.229	11				

Appendix 8: Two-way ANOVA for the antifungal activities of the crude extract of *Pseudomonas fluorescens* isolates in the three formulated media against *Rhizopus stolonifer*

Anova: Two-Factor Without Replication						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
M1	4	56	14	98.16667		
M2	4	101.5	25.375	299.5625		
M3	4	20.5	5.125	105.0625		
SGA1	3	0	0	0		
SGA2	3	45.5	15.16667	249.0833		
Rice B1	3	80	26.66667	114.0833		
RiceB2	3	52.5	17.5	252.25		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	824.2917	2	412.1458	6.08271	0.036034	5.143253
Columns	1101.833	3	367.2778	5.420519	0.038236	4.757063
Error	406.5417	6	67.75694			
Total	2332.667	11				

Appendix 9: Two-way ANOVA for the antifungal activities of ethyl acetate fraction of M_1 fermentation broth culture of *Pseudomonas fluorescens*

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
SGA_2	3	72	24	22.75

Rice B_1	3	78.5	26.16667	6.583333
Rice B_2	3	74.5	24.83333	11.58333
A. niger	3	66	22	9.75
P. citrinum	3	78.5	26.16667	7.583333
R. stolonifer	3	80.5	26.83333	6.583333

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	7.166667	2	3.583333	0.352459	0.722796	6.944272
Columns	41.16667	2	20.58333	2.02459	0.246954	6.944272
Error	40.66667	4	10.16667			
Total	89	8				

Appendix 10: Two-way ANOVA for the antifungal activities of ethyl acetate fraction of M_2 fermentation broth culture of *Pseudomonas fluorescens*

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
SGA2	3	92	30.66666667	0.083333
Rice B1	3	86.5	28.83333333	20.33333
Rice B2	3	72.5	24.16666667	17.58333
A. niger	3	76.5	25.5	21
P. citrinum	3	86	28.66666667	35.58333
R. stolonifer	3	88.5	29.5	1.75

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	67.38888889	2	33.69444444	2.735062	0.178406	6.944272
Columns	26.72222222	2	13.36111111	1.084555	0.420412	6.944272
Error	49.27777778	4	12.31944444			
Total	143.3888889	8				

Appendix 11: Zones of inhibition of ethyl acetate fraction of *Pseudomonas fluorescens* fermentation broth in M_2

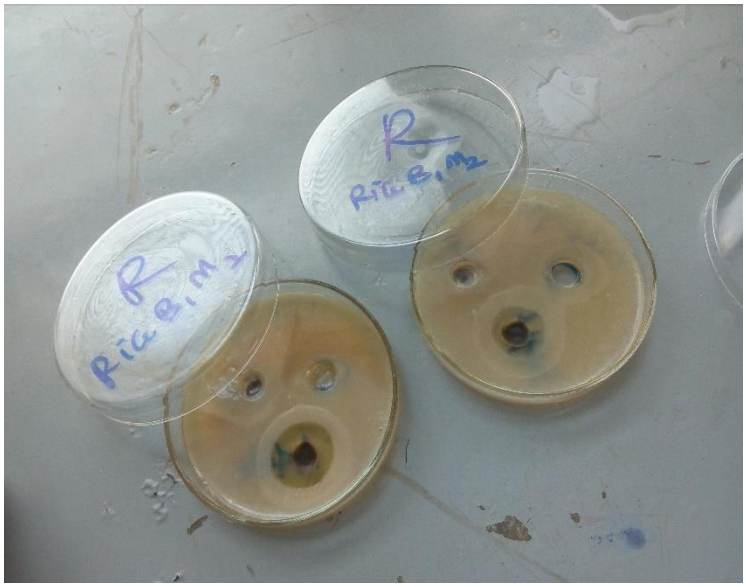
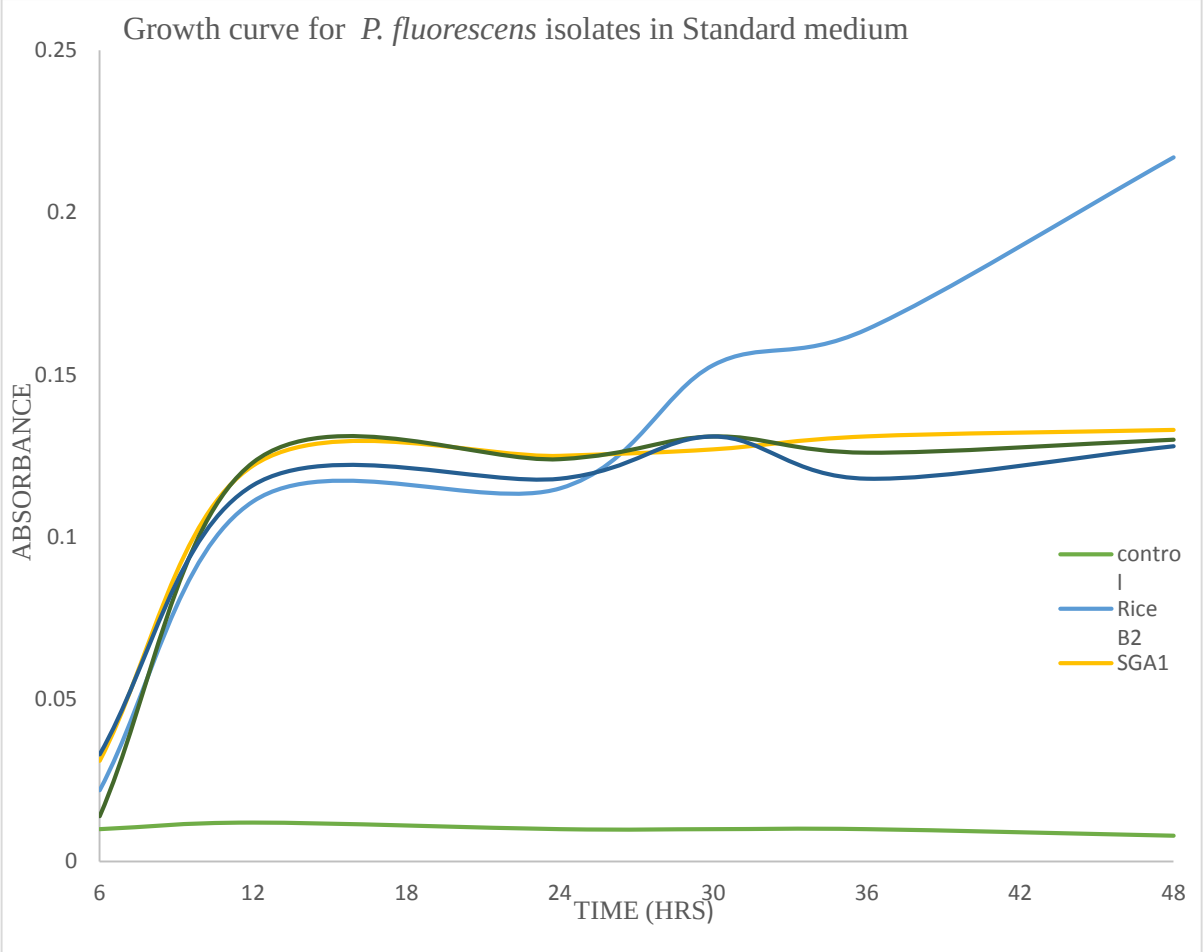


Plate I: Zone of inhibition of the ethyl acetate fraction from Rice B_1 in medium 2 against *Rhizopus stolonifer*

Plate II: Zone of inhibition of the ethyl acetate fraction from Rice B_1 in medium 2 against *Penicillium citrinum*

Plate III: Zone of inhibition of the ethyl acetate fraction from Rice B_1 in medium 2 against *Aspergillus niger*

Appendix 12: Growth curve for *Pseudomonas fluorescens* isolates in a standard medium containing glucose as carbon source, NH_4NO_3 as nitrogen source.

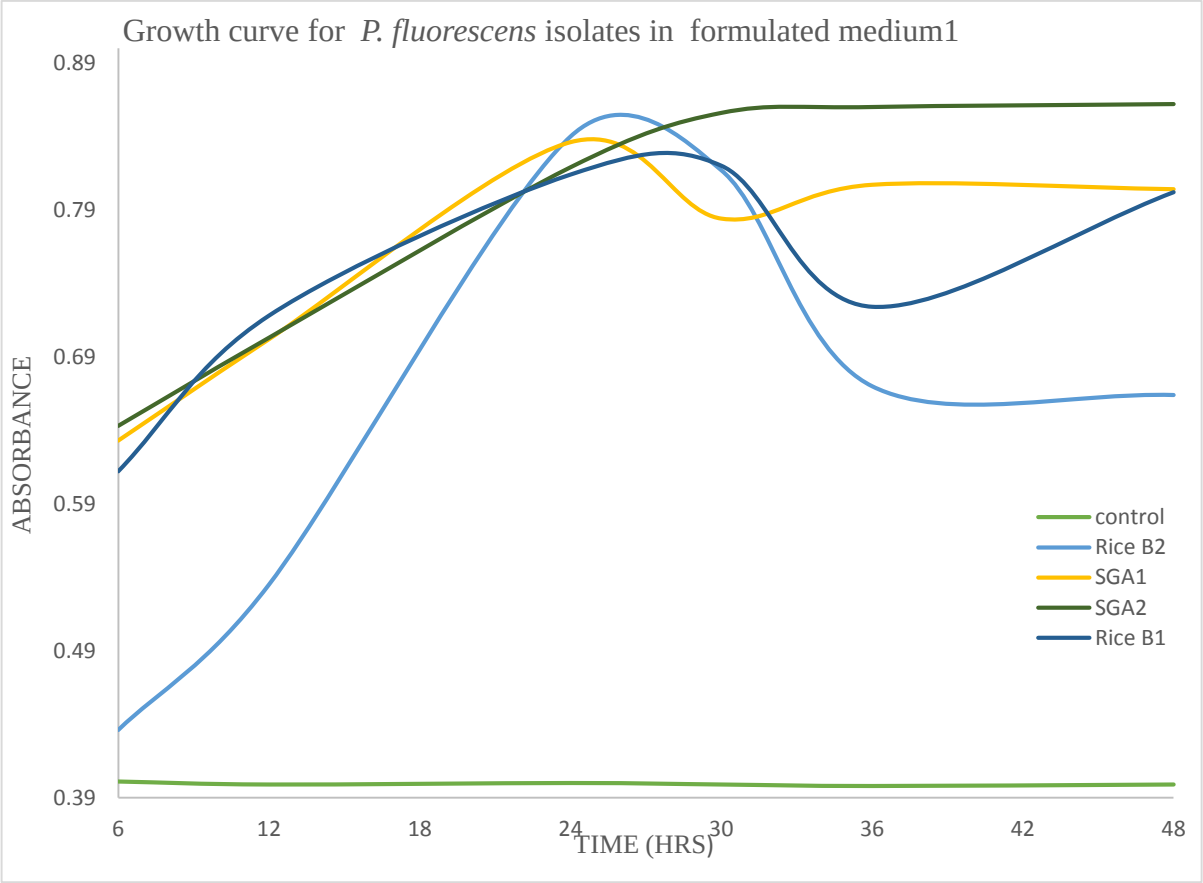


Optical density values for *Pseudomonas fluorescens* isolates in standard medium

Time (hrs)	*Absorbance*				
	Control	*SGA$_1$*	*SGA$_2$*	*RICE B$_1$*	*RICE B$_2$*
6	*0.01*	*0.031*	*0.014*	*0.033*	*0.022*
12	*0.012*	*0.122*	*0.123*	*0.116*	*0.111*
24	*0.01*	*0.125*	*0.124*	*0.118*	*0.115*
30	*0.01*	*0.127*	*0.131*	*0.131*	*0.153*

36	*0.01*	*0.131*	*0.126*	*0.118*	*0.164*
48	*0.008*	*0.133*	*0.13*	*0.128*	*0.217*

Appendix 13: Growth curve for *Pseudomonas fluorescens* isolates in medium 1 containing yam peel as carbon source and groundnut cake as nitrogen source



Optical density values for *Pseudomonas fluorescens* isolates in medium 1

Time (hrs)	*Absorbance*				
	Control	*SGA1*	*SGA2*	*RICE B1*	*RICE B2*

6	*0.401*	*0.633*	*0.643*	*0.612*	*0.436*
12	*0.399*	*0.702*	*0.703*	*0.718*	*0.535*
24	*0.4*	*0.836*	*0.819*	*0.814*	*0.84*
30	*0.399*	*0.784*	*0.856*	*0.82*	*0.817*
36	*0.398*	*0.807*	*0.86*	*0.724*	*0.67*
48	*0.399*	*0.804*	*0.862*	*0.802*	*0.664*

Appendix 14: Growth curve for *Pseudomonas fluorescens* isolates in medium 2 containing banana peel as carbon source and groundnut cake as nitrogen source

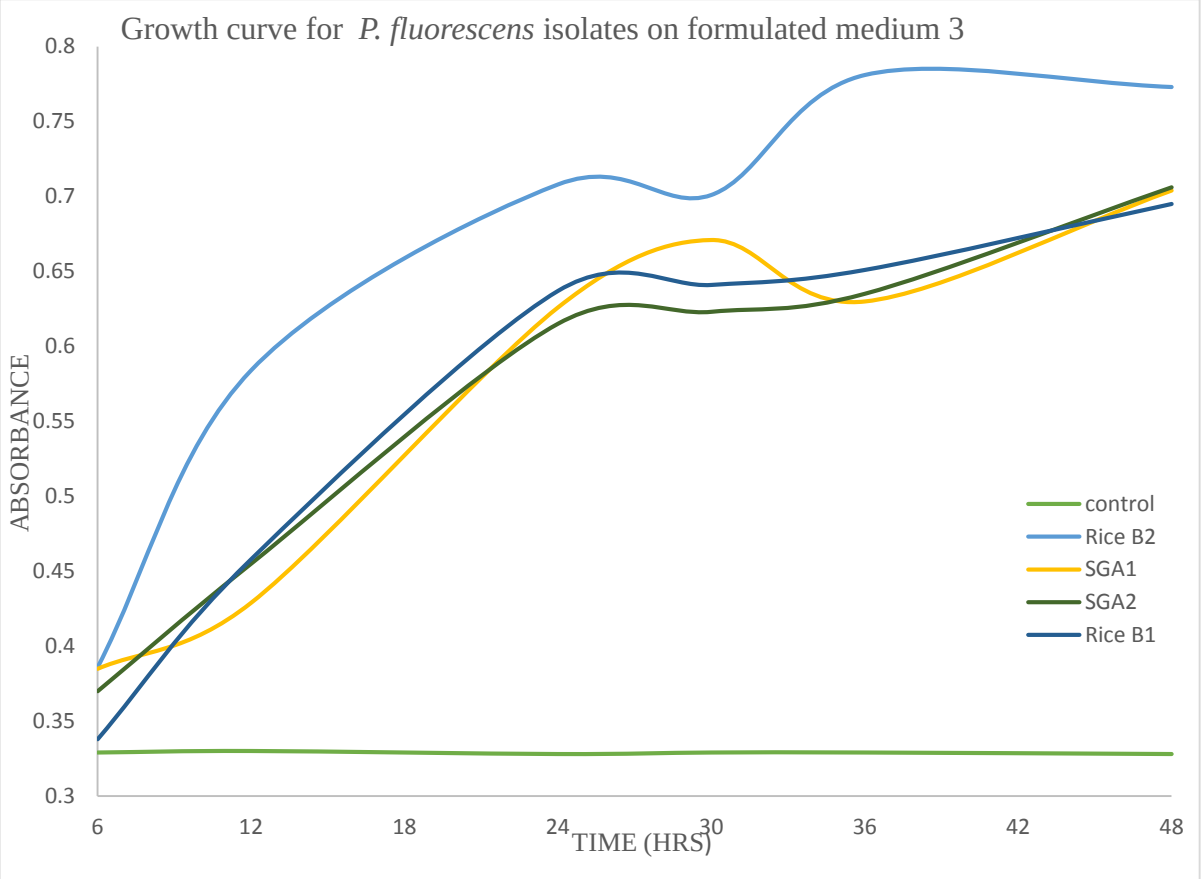
Optical density values for *Pseudomonas fluorescens* isolates in medium 2

Time (hrs) *Absorbance*

	Control	*SGA_1*	*SGA_2*	*RICE B_1*	*RICE B_2*
6	*0.31*	*0.351*	*0.433*	*0.334*	*0.408*
12	*0.311*	*0.467*	*0.544*	*0.409*	*0.534*
24	*0.311*	*0.615*	*0.613*	*0.608*	*0.833*
30	*0.309*	*0.607*	*0.698*	*0.614*	*0.943*

36	*0.308*	*0.582*	*0.632*	*0.599*	*0.907*
48	*0.309*	*0.637*	*0.649*	*0.621*	*0.965*

Appendix 15: Growth curve for *Pseudomonas fluorescens* isolates in medium 3 containing sugar cane shaft as carbon source and groundnut cake as nitrogen source



Optical density values for *Pseudomonas fluorescens* isolates in medium 3

Time (hrs)	*Absorbance*				
	Control	*SGA1*	*SGA2*	*RICE B1*	*RICE B2*

6	*0.329*	*0.385*	*0.37*	*0.338*	*0.386*
12	*0.33*	*0.429*	*0.455*	*0.458*	*0.584*
24	*0.328*	*0.626*	*0.615*	*0.637*	*0.708*
30	*0.329*	*0.671*	*0.623*	*0.641*	*0.701*
36	*0.329*	*0.63*	*0.635*	*0.651*	*0.781*
48	*0.328*	*0.704*	*0.706*	*0.695*	*0.773*