

**EFFECT OF DIFFERENT CARBON SOURCES ON
THE GROWTH OF ANTIMICROBIAL PRODUCING
Bacillus Species IN *Citrillus vulgaris*.**

BY

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ENUGU.**

AUGUST, 2012.

TITLE PAGE

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GROWTH OF ANTIMICROBIAL PRODUCING BACILLUS
*Species IN Citrillus vulgaris (Ogiri-egusi).***

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MB/2008/383

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BIOTECHNOLOGY**

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**CARITAS UNIVERSITY, AMORJI-NIKE
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AUGUST, 2012

CERTIFICATION PAGE

This is to certify that this research project work was carried out by Ushie Joan Ochuole (MB/2008/383) In the department of Microbiology Biotechnology, faculty of Natural sciences, Caritas university, Amorji- Nike Enugu. The Department recognized that Ushie Joan Ochuole (MB/2008/383) bears full responsibility for this work.

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Above all persons, My God Almighty who brought me to this world with love for a successful completion of this work. All praise and honor be unto his name.

DEDICATION

This work is dedicated to my parents, late Mr. Hilary Ushie for his moral and financial support towards my upbringing and my mother Mrs. Maria Ushie for her great care and encouragement, and for their guidance and financial support towards my educational background and welfare.

Above all, to God almighty who is my rock, refuge and stronghold for his divine love and mercies towards my academic journey.

ABSTRACT

Effect of different carbon sources on the growth of antimicrobial producing *Bacillus* species in ogiri-egusi sold in Ogbeta, Orie, Artisian, Kenyatta, Enugu State were evaluated using standard microbiological and analytical methods. 12 bacteria were isolated and identified such as *Bacillus subtilis*, (33.33), *Bacillus pumilis* (41.66), *Bacillus licheniformis* (25) were identified based on their colony biochemical characteristics. The isolates of the highest occurrences is *Bacillus pumilis*. Agar well diffusion method was used to determine the antimicrobial activity against two pathogenic organisms, *Escherichia coli*, *Staphylococcus aureus*. *Bacillus subtilis* and *Bacillus pumilis*, gave a zone of inhibition *Escherichia coli* 0.6mm and 0.8mm after 24 hours of incubation also carbon sources of 5% and 10% sucrose, Glucose and soluble starch were used to check their different carbon sources on the isolates using the spectrophotometer. 5% had the highest optimum growth of 1.400 for glucose 1.545 for sucrose, 2.376 for soluble starch which can be added in maximum quantity to a medium to produce antibiotics.

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CHAPTER ONE

1.0 INTRODUCTION

Traditional fermented condiments (OGIRI-EGUSI) based on vegetable proteins are consumed by different ethnic groups in Nigeria, have been the pride of culinary traditions for centuries. It is evident that these products have played a major role in the food habits of communities in the rural regions serving not only as nutritious non-meat proteins substitute but also as condiment and flavoring agents in soup.

Traditional methods of manufacture should take advantage of biotechnological progress to assure reasonable quality and at the same time assure safety of these products. The requirements for a sustainable biotechnological development of Nigerian condiments are discussed in the scope of the microbiology and biochemical changes of the raw materials. Fermented vegetables, proteins have potential food uses as protein supplements and as functional ingredients in the fabricated food (Achi. 2005).

Seeds of legumes may account for up to 80% of dietary protein and maybe the only source of protein for some groups. Their cooked forms are eaten as meals and are commonly used as fermented form as meals and are commonly used in fermented forms as condiments to enhance the flavors of food (Odunfa, 1985). With high content of protein, legume condiments can serve as a tasty condiment to sauce and soups and can substitute for the food flavoring condiments are prepared by traditional methods of uncontrolled solid substrate fermentation resulting in extensive hydrolysis of the protein and carbohydrate components (Fetuga et al. 1973).

Fermented foods are essential parts of the world, particularly African (Odunfa. 1985). Fermentation is one of the oldest and most economical methods of producing and preserving foods in developed countries (David and Aderibigbe 2010). In Africa, many proteineous oily seeds such as cotton seed (*Gossypium hirsutum*), African locust bean (*Parkia*) and melon seed (*Citrillus vulgaris*) are fermented to produce soup condiments (Odunfa, 1981), which give pleasant aroma to soups and sauces. In many countries especially Nigeria and India

where protein calories. Malnutrition is a major problem, these condiments serve as food source of energy, low cost protein and fatty acids in diets (Odumodu. 2007).Ogiri is an oily paste produced by fermented melon seeds (*Citrillus vulgaris*) in the western part of Nigeria. Oyenuga (1986) have the composition of melon seed.

A melon seed has high protein and low Carbohydrate content. *Citrullus vulgaris* is a member of the family Cucurbitaceae (Alfred, 1986). Ogiri is characterized with very strong pungent odour. Among the consumers, there are preferences fir Ogiri produced from specific locality. The production process being a local art makes the quality varies. The fermented products are also stored at ambient temperature (28+2) °C. For varied length of time (days or weeks). The population and types of micro organisms involved during fermentation and storage could have affected the quality of the product.

Fermented foods are essential parts of diets in all parts of the world particularly Africa (Odunfa, 1985). Fruits, vegetables, cereals, root crops, legumes and oil seeds are used in the production of fermented food. Fermentation is one of the oldest and most economical methods

of producing and preserving foods in developing countries (David and Aderibigbe, 2010). In Africa, many proteinaceous oily seeds such as cotton seeds (*Gossypium hirsutum*), castor bean (*Parkia biblobosa*) and melon seed (*Citrullus vulgaris*) are fermented to produce food condiments (Odunfa, 1981), which gave pleasant aromas to soups and sauces. In many countries especially Nigeria and India where protein/calories malnutrition is a major problem, these condiments serve as good source of energy, low cost protein and fatty acids in diets (Odumodu, 2007). Thereby, supplement the nutritive quality of the respective diets where they consumed

Ogiri is one of the condiments consumed in the Eastern and Western parts of Nigeria especially by the Ibos. Ogiri is an oily paste produced by fermenting melon seeds (*Citrullus vulgaris*) in the Eastern and Western parts of Nigeria. Oyenuga (1988) have the composition of melon seed to be dry weight (88.9%); crude protein (32.6%); ether extract (50.2%); crude fibre (3.7%); silica free ash (3.45%). Minerals (mg\100g) content of shelled melon seed were Calcium(112); Phosphorus (1777); Magnesium (578); Potassium

(538); Sodium (5); Chlorine (32); Vitamins (N/g); A (30.65); D (11.20) and E (0.25). Melon seed has high protein and low Carbohydrate content. *Citrullus vulgaris* is a member of the family cucurbitacea (Alfred, 1986).

Ogiri is characterized with very strong pungent odour. Among the consumer, there are preferences for Ogiri produced from specific locality. The production process being a local art makes the quality of the product varies. The fermented products are also stored at ambient temperature $(28.2 \pm 2)^{\circ}\text{C}$ for varied length of time (days or weeks),(David and Aderibigbe, 2010). The population and types of micro organism, involved in fermentation can result in food poisoning. Others are responsible for producing antibiotics (Obeta, 1983).

1.1 AIM AND OBJECTIVE OF THE STUDY

- 1) Isolation of antimicrobial producing *Bacillus* species in *Citrullus vulgaris*

- 2) To elucidate reasons data for preferences in 'Ogiri' from the four different market in Enugu town.
- 3) To identify the characteristic ability of microorganisms responsible for fermentation of *Citrillus vulgaris* to produce Ogiri
- 4) To identify the potential microorganisms and to study the effect of different carbon sources on isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

Fermentation is a process where food substrate is transformed by micro organisms to obtain a better final product: nutrition, hygiene, stability, odor, color, taste, digestibility, safety. (Jacobsen et al. 2007).

Food is fermented for many reasons which include;

- a) Improvement of sensory characteristics by development of diverse flavor and aroma compounds.
- b) Improvement of safety; absence of toxins and partial and/or complete elimination of antimicrobial factors.
- c) Increase of nutritive value through the breakdown of proteins, carbohydrates and lipids to essential amino acids easily, digested sugars and essential fatty acids. It also brings about synthesis of some vitamins.
- d) Decrease in cooking time and thereby fuel requirement.(Jacobson et al. 2007)

2.1 TRADITIONAL FERMENTED FOODS

Fermented foods are defined as potable products, which are prepared from raw or heated materials and which acquire their characteristic properties by a process that involves Microorganisms micro-organisms (Bucken Huskes, 1993). Fermented condiments give pleasant aroma to soups and sauces in many countries especially in Africa and India where protein calorie malnutrition is a major problem (Sarker et al. 1993).

They also have great potential as key protein and fatty acid sauces and are good sources of gross energy.

**Table 1: SOME TRADITIONAL NIGERIAN
FERMENTED FOODS.**

Iru (Dawadawa)	Africa locust bean (<i>Parkia biglobosa</i>)	<i>Bacillus</i>
Ogiri(Ogili)	Melon seed(<i>Citrullus vulgaris</i>).Fluted pumpkin (<i>Telfaira Occidentalis</i>) Castor oil seed (<i>Ricina Commuris</i>)	<i>Bacillus spp</i> , <i>Pediococcus spp</i> <i>Escherichia sp.</i>
Ugba (Ukpaka)	Africa oil bean (<i>Pentacletha macrophylla</i>)	<i>Bacillus licheniformis.</i> <i>Micrococcus sp.</i> <i>Staphylococcus sp.</i>

Source; Achi, 1991.

Traditional diets in West Africa often lack variety and consist of large quantities of the staple food(Cassava, yam, maize) with supplement of plantain, cocoyam, rice and beans depending on the availability and season (Achi, 1999). Soups eaten with the staples are an essential component of the diet and may contain a variety of seeds, nuts, pulses, and leaves (Campbell-platt, 1980).The staple foods

provide the calories but are poor in other nutrients. Soups are the main sources of protein and minerals and one of the ways to improve the seeds and legumes may account for up to 80% of dietary protein and may be the only source of protein for some groups. Their cooked forms are eaten as meals and are commonly used in fermented form as condiments to enhance the flavors of foods (Odunfa, 1985). With high contents of protein, legume condiments can serve as tasty complement to sauces and soup and can substitute for fish or meat.

The food flavoring condiment is prepared by traditional methods of uncontrolled solid substrate fermentation resulting in extensive hydrolysis of the protein and carbohydrate complements (Fetuga, et al.1973). Apart from increasing the shelf life, and a reduction in the anti-nutritional factors (Odunfa, 1985b, Reddy 1999). Fermentation markedly improves the digestibility, nutritive value and flavors of the raw seeds.

Although fermented food condiments have constituted a significant proportion of the diet of many people, Nigerians have exhibited an ambivalent attitude in terms of consumer tastes and

preferences for such foods (Achi, 2005).The introduction of foreign high technology products of orderly processed ones because of the globalization and liberalization of the economy radically changed the Nigerian food culture into a mixed grill of both foreign and local dishes (Ogo, 1991).

Fermented products remain of interest since they do not require refrigeration during distribution and storage. According to Campbell, 1987, many developing countries are still preparing traditional condiments have not attained commercial statues due to the very short shelf life. Objectionable packaging materials stickiness and the characteristics putrid odors (Arogba et al. 1995). Fermented condiments often have a stigma attached to them, they are often considered as food for the poor.

According to Odunfa, 1981a. 1985 1985, the production of fermented vegetable proteins for use as food condiment is craft-based. Remarkably, in many areas of Nigeria today they are still made in traditional ways with success depending on the observance of good manufacturing phase. Starter cultures are normally used and therefore

variations in the quality and stability of the products are often observed (Sanni et al. 1997).

2.2 OGIRI

Ogiri is an oily paste produced mainly from melon seeds and consumed within the West African countries (Odunfa, 1981). The production is still a traditional family act and fermentation is by chance inoculation (Odunfa, 1985). ‘Ogiri serves as cheap soup condiments particularly among the poor rural dwellers. In the South-East, Nigeria, Ogiri can also be produced from castor oil seeds (*Ricinus communis*) (Enujiugha, 2003) and fluted pumpkin (*Telferia Occidentalis* Odibo, et al 1990; Omafuvbe and oyedapo 2000). Obizoba and Atti (1991) studied the chemical properties of fluted pumpkin, as mostly used food condiments in some part of Nigeria.

Apart from *Citrullus Lanatus* which is the regular substrate used for the production of ‘Ogiri’, there are other varieties of melon seeds which are readily available in the South-West part of Nigeria. These other melon seeds which are under-utilized by fermentation can serve

as alternative substrate for the production of Ogiri. Contamination of food by pathogenic organisms remains one of the major public health problems worldwide (Nestal et al, 1998). Food borne diseases are endemic in many developing countries and constitute a major cause of mortality in these areas (Adams and Moss 1999).

2.3 DETAILS OF SOME TRADITIONAL METHODS EMPLOYED TO MANUFACTURE ‘OGIRI

In production of ogiri, the unit operations listed below are found to be the most difficult and tedious during local or traditional processing of ogiri egusi.

- (i) Cooking of the seed (Adewuyi, 1983).
- (ii) Dehulling and hull separation.
- (iii) fermentation/conditioning
- (iv) Post fermentation treatment.

These unit operations are carried out traditionally as follows;

- (i) Cooking; traditionally, this takes about 2-3 hours averagely for the seeds to be softened, additional water is required when the initial water evaporates. At times, softening agents like potash may be introduced, but the whole rigor has been reduced to 2hrs by the pressure cooker (Benchat, 1978).
- (ii) Dehulling and hull separation; the boiled seeds are put in a mortar which could either be pressed with food or pounded with pestle to remove the testa. Sometimes sands wood ash may be added to assist in the testa is removed using sieve or basket in large quantity of water or done in a nearby stream to hasten the process.
- (iii) Fermentation/conditioning; It is usually carried out in a calabash tray stacked together and then put in a preparation hole in underground insulated with local materials and allowed the fermentation process to take place for another 36 hours or more (Benchat, 1978).

(iv) Post fermentation treatment; in western Nigerian Ogiri is salted, rolled into balls and displayed in the market for sales either in show case or calabash covered with raffia flat trays. If not disposed off within 3 days, it will start losing its food value (Odunafa, 1986). However in northern Nigerian Ogiri is usually sun-dried on a cemented floor surface and later pounded molded into balls before sales/consumption.

In summary (melon seeds *Citrullus vulgaris*). Dehulled melon seeds boiled for 2-3 hour. The seeds are ground into paste. Ash from burnt palm bough is added which imparts a grey colour to the paste. The paste is wrapped in small portions with leaves and left in a warm place until the characteristics aroma of the condiment is developed. (Odunfa, 1981)

2.4 MICROBIAL ECOLOGY

The dynamic fermentation in any food matrix is a complete microbiological process involving interaction between quite different micro organisms (Daeschel, 1987). The contribution of the accompanying flora of fermenting substrates is determined by the

composition and hygiene during production. During fermentation, the micro organisms use their nutritional components of seeds, converting them into products that contribute to the chemical composition and taste of the condiments. A number of *Bacillus* species have been isolated from various fermented food condiments (Achi, 1992).

Yeasts and other bacteria can also be seen, only part of them can be considered to play a substantial role in fermentation process. Odunfa (1981) reported that non-fermenting species may just be ubiquitous contaminants although they may affect the flavor of the final product when occurring in high numbers. Other bacteria present include *Staphylococcus* specie, *Bacillus subtilis* and *Bacillus lichenformis* have been identified as the major bacteria present. The predominance of *Bacillus* species has been demonstrated in other fermenting legume proteins (Achi 1992; Odunfa 2002). The co dominance of *staphylococcus Bacillus* Spp. Was typically of the micro flora of fermenting begins (Obeta, et al, 1983). In the view of the fact that the major constituents of vegetable seeds are proteins, the

organisms responsible for ferment them must be capable of utilizing these constituents (Antai et al,1986).

Bacillus species isolated from variable sources has been reported to be proteolytic and are able to breakdown oils (Frazier, 1967 and Forgy et al, 2003).

It is evidence that production of fermented condiments is initially mediated by a diverse microbial flora which eventually becomes Gram- positive flora (Odunfa, 1985c). The contribution of this accompanying flora of bacteria is only partly understood (Eke, 1996).Most probably, they play a role in flavor development and influence the chemical composition through substrate modification and synthesis of vitamins (Nout and Rombouts, 1995).

2.5 MICROBIOLOGY OF OGIRI-EGUSI:

The bacterial isolates from ogiri - egusi are *megatarium*, *Bacillus cerus*, *Bacillus licheniforms*, *Bacillus subtilis*, *Lactobacillus brevis*, and *Lactobacillus plantarum* and *bacillus casei*.

They are all used for starter culture fermentation (Odunfa and Adewuyi, 1985).

2.6 BIOCHEMICAL CHANGES ACCOMPANYING THE FERMENTATION OF OGIRI - EGUSI (CITRULLUS VULGARIS)

Ogiri is a product of fermentation of polar food condiments in southern Nigeria. During its production, melon seeds are boiled till they are very soft.

The changes in the principal constituents of melon during ogiri production by fermentation were investigated. The nitrogen decreased in the fermented ogiri. The activities of proteases increased during the fermentation as well as the amounts of amino acids. The amylase activities also increased with fermentation, but the soluble sugars showed a remarkable fluctuation culminating in a peak at 120 hours of fermentation. Lipase activity was minimal in the fermentation mash.

The results of the enzymatic activities in ogiri are compared with the fermentation of similar vegetable protein (Odunfa, 1999).

Changes in carbohydrate: *Bacillus* species have been reported as producers of certain enzymes such as amylase, galactanase, galactosidase, nglucosidase and fructofuranosidase, which are involved in the degradation of carbohydrates (Adenibigbe, et al,1990). Microbial amylases hydrolyze carbohydrates into sugars, which are then readily digestible humans, 1997).

Changes in amino acids: In most fermented high-protein products, the extent of protein hydrolysis is one of the most important factors in the changes in texture and flavor (Whitaker, 1978)

Soluble low molecular weight peptides and amino acids that contribute to flavor are produced through enzymatic breakdown of proteins (Odunfa, 1985, Njoku 1989). Condiment of a good quality has a characteristic strong smell of ammonia, a dark color and is semi hard. There is a gradual increase in PH (Barimalla et al, 1982). Soluble products increased during fermentation of melon seeds resulting in high digestibility of the fermented product. Alanine, lysine and glutamic acid were the predominant amino acids with arginine and proline occurring in small amounts. (Odunfa, 1983,

Aidoo, 1986).The improved nutritive values are attributed to the increase in amino acid profiles due to fermentation.

Fermented condiments have a characteristic organanalyptic quality which probably is the most important factors for consumers (Dakara et al, 2005).

2.7 ANTIMICROBIAL ACTIVITIES ON BACILLUS SPECIES.

Bacillus is a genus of a gram positive, rod shaped bacteria and a member of the division FIRMICUTES(Antair et al, 1986).*Bacillus* species can be obligate aerobes or facultative test positive for the enzyme catalase (Adenye et al, 2003). Ubiquitous in nature, *bacillus* includes both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval end spores that can stay dormant for extended periods (Ogbadu et al, 1988).

Many *Bacillus* species are able to secrete large quantities of enzymes.

Bacillus amyloliquefaciens is the source of a natural antibiotic protein (Barnase Rib nuclease). Alpha amylase used in starch hydrolysis. The protease *Subtilis* used with detergents and the Bam H1 restriction enzyme used in DNA research (Ogbadu et al, 1988). Apposition of *B. thuringiensis* genome was incorporated into iron (and cotton) crops. The resulting GMOS are therefore resistant to some insect pest (Ogbadu et al, 1988).

Bacillus subtilis an important model organism is one of the best understood prokaryotes in terms of molecular biology and cell biology. Its super amenability and relatively large size have provided the powerful tools required to investigate a bacterium from all possible aspects (Harrigan et al, 1966). Research on *Bacillus subtilis* has been at the fore frontal of bacterial molecular biology and cytology and the organism is a model for differential gene protein and cell cycle events in bacteria (Ogbadu et al, 1988). Scientist have demonstrated that *Bacillus subtilis* concurrently produces antibiotics and spores.

Antibiotic production increases *Bacillus subtilis* chances survival as the organism produces spores and a toxin that might kill surrounding gram positive microbes that compete for the same nutrients. Examples of antibiotic that *Bacillus* specie can produce are; polymyxin, difficin, subtilin, and *mycobacillin*. *Bacillus subtilis* bacteria are non pathogenic, though they can contaminate food, the seldom result in food poisoning.

Some *Bacillus species* can cause food poisoning, such as *Bacillus licheniformis*, *Bacillus cereus* *Bacillus subtilis* has the ability to produce antimicrobial compound which are active against *Lactococcus latis*, *Saccharomyces uvaru* and others (Sarkar et al,1994).

Matrix is a complex microbiological process involving interactions between quite different determine by the composition and hygiene during production.

During fermentation, the microorganisms use the nutritional components of seeds, converting them into products that contribute to the chemical composition and taste of the condiment. A number of

Bacillus species have isolated from various fermented food condiment (Achi, 1992). Yeast and other bacterial can also be seen; only part of them can be considered to play a substantial role in fermentation process. Odunfa reported that non fermenting machine may just be ubiquitous contaminants although they may affect the flavor product when occurring in high numbers. Other bacteria present include *Staphylococcus* sp., *Bacillus subtilis* and *Bacillus lichenformis* have been identified as the major bacteria protein (Achi 1992, Barber et al. 1998 and Omafurbe et a., 2000). The co dominance of staphylococcus and *Bacillus* spp. was typical of the micro flora of fermenting beans (Obeta, 1983, Antai and Ibrahim, 1986 Achi. 1992).

In the view of the fact that the major constituents of vegetables seeds are proteins, the organisms responsible for fermenting them must be capable of utilizing these constituents (Antai et al., 1986).

Bacillus specie isolated from variable sources has been reported to be proteolytic and are able to break down oils (Frazier et al, 1967). It is evident that production of fermented condiment is initially mediated by a Gram- positive flora (Odunfa, 1985c). The

contribution of the accompanying flora of bacteria is only partly understood (Iwudna *et al*, 1996). Most probably, they play a role in flavour development and influence the chemical composition through substrate modification and synthesis of vitamins (Nout *et al* 1995).

Members of the *Bacillus* group *sensu lato* are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and spore formation capacity confer *Bacillus* strains with a double advantage in terms their survival in different habitats. The presence of *Bacillus* species in food poisoning, and some species or strains are even used in human and animal food production such as, for example, *Bacillus subtilis* strains that are used in Natto, an East Asian fermented *Citrullus vulgaris* (Hosoi and Kiuchi, 2003), production.

Furthermore, specific, *B. subtilis* strains also used as a starter culture for fermenting soybeans into the traditional West African condiment *Citrullus vulgaris* (Narkie *et al*, 2007). A nontoxigenic *Bacillus cereus* spp. Toyoi with spore-forming properties is also used as an animal feed additive (Lode man *et al*. 2008). On the other hand,

Bacillus species or strains have been implicated in food poisoning *Bacillus cereus*, *Bacillus coagulans*, *Bacillus pumilus*, *Bacillus thuringiensis* and *Bacillus sphaericus* (Jay et al,2005).

2.8 BIOTECHNOLOGY IN NIGERIA’S FERMENTED LOCAL CONDIMENTS

Biotechnology as defined at the United Nations conference on biological diversity (Earth summit) as ‘any technological application that uses biological system, to make or modify products or processes for specific use’ (Okafor, 2007).

Members of the *Bacillus* group *sensu lato* are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and speculation capacity confer *Bacillus* strains with a double advantage in terms their survival in different habitats. The presence of *Bacillus* species in food poisoning, and some species or strains are even used in human and animal food production such as, for example, *Bacillus subtilis* stains that are used in Natto, an East Asian fermented *Citrullus vulgaris* production (Hosoi et al. 2003).

Furthermore, specific, *B. subtilis* strains also used as a starter culture for fermenting soybeans into the traditional West African condiment *Citrullus vulgaris* (Narkie, et al. 2007). A nontoxinogenic *Bacillus cerus* sp. Toyoi with sprobiotic properties is also used as an animal feed additive(Lode man et al. 2008).On the other *hand*, *Bacillus* species or strains have been implicated in food poisoning B.cerus, *Bacillus* coagulants, *Bacillus pumilus*, *Bacillus thuringiensis* and *Bacillus sphaericus* (Jay, et al,2005).

2.8.1 Effect of Different Carbon Sources on Isolates.

Carbon as a part of ingredient in the medium is required for bacterial growth and to enhance the production of antimicrobial substances. Antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources (EL- Banna et al. 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

Incubators, plate count agar (PCA biotech), nutrient agar, Nutrient broth, agar Autoclave, and Test tubes Petri dishes. 'ogiri' samples, conical flask, glass slides, microscope, glass rod.

3.2 SAMPLE COLLECTION.

'Ogiri' sample were bought from four different local market located in Enugu town namely: Orié Emene, Eke Obinagu, Artisan market and Ogbete main market. The sample were bought and kept in cellophane bags, and were stored in the refrigerator at 4°C. The samples were later transferred into the laboratory for microbial analysis.

3.3 PREPARATION OF SAMPLES

The sample was prepared through serial dilution of sample with 10ml of distilled water was added to 1 gram of sample in a test tube,

and it was called stock'' from the stock dilution, 1ml was collected and transferred to another test tube (i) containing 10ml of solution in test tube (ii) to test tube (iii) same procedure where repeated for 3 more time and it was done for the four available sample of ogiri-egusi. These was done to reduce the microbial population sufficient to obtain separate colonies. When plating.

3.3.1 Isolation Techniques: Pour Plate Method

After the nutrient agar was prepared, small volumes of several the liquid nutrient agars that have been cooled for about 45 and the mixture poured immediately into sterile culture dishes. This was done by slightly opening the side of the plate and poured the mixed media slightly and carefully, it was then shake slightly and avoided it touching the cover. It was then turned upside down and incubated.

3.3.2 Streak Plate Method

This method was used to obtain pure colonies by subculturing in each of a similar colonies of the plate was transferred to the edge of an agar plate with a loop containing nutrient agar, it is called primary

inoculating loop the surface. After the first sector was streaked, the inoculating loop was sterilized and inoculums for the second sector is obtained from the first sector.

Sterilization of the inoculating loop was done by passing it through a burning flame to reduce the microbial growth.

3.4 IDENTIFICATION OF ISOLATE: CULTURAL CHARACTERISTICS

When the sub culturing of each different colony type was done and the pure cultures were obtained, the colony, cell morphology and standard biochemical test of each pure culture were determined according to bacterial taxonomical methods (Holding et al. 1972).

3.5. GRAM STAINING

The method used was that described by (Barker et al.1976) and (Thomas, 1973). Smears of the isolates were prepared and heat fixed on clean grease free slides. The smears were stained for one minute with crystal violet. This was washed out with a gentle running tap

water. The slides were flooded with dilute Gram's iodine solution. This was washed off with water and the smears were decolorized with 95% alcohol till the blue colour no more and dripped out (about 30 Seconds). The smears were then counter stained with saffranine solution for about 10 seconds. Finally, the slides were washed with tap water, air dried and observed under oil immersion objective.

3.5.1 Starch Hydrolysis

Using aseptic technique, an inoculums from (Nutrient agar). The inoculated plate was incubated at 37°C for 24-48hours. Then a small amount of Gram's iodine was added to flood the starch growth. A clear zone around the bacterial growth was observed.

3.5.2 Oxidase Enzyme Activity

Using aseptic techniques, tryptic soy (Ts) agar plates were inoculated with the inoculums. The inoculated plates were incubated at 37°C for 24-48hours then 2-3 drops of p- aminodimethylaniline presence or absence of colour change was observed within 10-30seconds after adding the reagent.

Presence of colour change from pink to purple indicates positive (+ve) while absence of colour change indicates negative (-ve).

Presence of clear zone around the bacterial growth was observed. Presence of clear zone indicates (+ve) positive while absence of clear zone indicates (-ve).

3.5.3 Voges-Proskauer Test (V.P Test)

This test was used to detect which of the isolates were able to produce a neutral red end point acetyl methyl carbinol (acetoin) from glucose fermentation or its reductive product butylene glycerol. The test is usually used to differentiate between Gram negative organisms especially members of the *Enterobacteriaceae* (Bakeret, et al. 1976). The test was carried out as described by(Kirk, et al. 1975). Tubes of buffered glucose peptone broths were lightly inoculated with a young culture of the isolates. The tubes were incubated at 37⁰c for not less than 48hours. A Burrits reagent was used for the test. 0.6% w/v of solution A and 0.2ml of solution B were added into 1ml of the culture in turns. The mixtures were shaken well after each addition.

Positive reaction was indicated by a pink colour that appears immediately or within 5 minutes at the top most part of the tube, Solution A Contains 5g of - naphthol100ml absolute ethyl alcohol Solution B contains 100ml Distilled water 40g potassium hydroxide. The alkalis oxidize the acetyl methyl carbonyl (acetoin) to diacetyl which gives the pink colour.

3.5.4 Citrate Utilization Test

This test was used to identify which of the isolates can utilize citrate as the sole sources of carbon for metabolism. The test is usually used as an aid in the differentiation of organisms in the *Enterobacteriaceae* and most to the genera (Baker, 1976). The medium used for this test was the Simon's citrate agar.

Slant tubes of Simon's citrate agar were inoculated with young culture of the isolates, the inoculation was done by stabbing medium on the tubes using sterile straight inoculating wire loop containing the culture. The tubes were then incubated at 37⁰C for about 24 hours

A change in colour from green to blue after about 24 hours of incubation indicated positive result.

3.5.5 Sugar Fermentation

Each of the isolates was tested for its ability to ferment a given sugar with the production of acid and gas or acid only. Since most bacteria especially gram negative bacteria utilize different sugar as source of carbon and energy with the production of either acid and gas or acid only. The test is used as an aid in their differentiation.

The growth medium used was peptone water and the method used was that described by(Kirk, et al. 1975). Peptone water was prepared in a conical flask and the indicators, bromocresol purple was added. The mixture was dispensed into test tubes containing Durham's tube. The tubes with their content were sterilized by autoclaving at 121°C for 15minutes. 1% solution of the sugar was prepared and sterilized separately at 115°C for 10 minutes. This was then aseptically dispensed in 5ml aliquote volume into the tube containing the peptone water and indicator.

The tubes were incubated at 37°C. Acid and gas production or acid only were observed after about 24 hours of incubation. Acid production was indicated by the change of the medium from light green to yellow colour while gas production was indicated by the presence of gas in the Durham's tubes.

The control tubes were not incubated.

Tubes of buffered glucose- peptone broth were lightly inoculated with the isolates. The tubes were incubated at 37°C for not less than 48 hours. About 5 drops of the methyl red reagent was added into 5ml of the culture. The production of a bright red colour immediately on the addition of the reagent showed a positive test.

3.5.6 Methyl Red Test

This test was used to detect which of the isolates could produce and maintain sufficiently a stable acid product from glucose fermentation. The test is usually used as an aid in the identification and differentiation of the *Enterobacteriaceae* (Baker, 1976). This test was carried out as described by (Kirk, et al. 1975).

Tubes of buffered glucose- peptone broth were lightly inoculated with the isolates. The tubes were incubated at 37⁰c for not less than 48 hours. About 5 drops of the methyl red reagent was added into 5ml of the culture. The production of a bright red colour immediately on the addition of the reagent showed a positive test,

Methyl red test indicator consist of

0.1g methyl Red

300ml of 95% ethyl alcohol.

3.6 EFFECT OF DIFFERENT CARBON SOURCES

The ratio 5% and 10% Glucose, sucrose, soluble starch were added into nutrient broth medium as carbon sources. 5ml of the mixed solutions were dispensed into different test tubes and were sterilized at 121⁰c for 15 minutes. The isolates were inoculated into the test tubes containing the mixed solution and incubated for 37⁰C for 72hours.

CHAPTER FOUR

RESULTS

Twelve (12) bacteria strains were isolated from a local condiment ogiri-egusi. The isolates were identified based on their morphological appearance, biochemical tests which include; Gram staining, Catalase, Oxidase, Voges.proskeur, Methyl red, Citrate, Starch hydrolysis, Sugar fermentation test. The isolates were identified as *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*. All the isolates were Gram- positive forming rods.

The frequency and occurrence of *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis* isolated are shown in table 4. *Bacillus subtilis* had the highest occurrence of 25% While *Bacillus pumilus* and *Bacillus licheniformis* had the lowest 16.66% occurrence.

Table 5 showed the zone of inhibition. *Bacillus subtilis* and *Bacillus pumilus* inhibited the growth of *Escherichia coli*, *Bacillus subtilis* showed 0.6mm zone of inhibition While *Bacillus pumilus* showed 0.8mm zone of inhibition against *Escherichia coli*.

Table 6 shows the effect of different carbon sources on isolates in which 5%, 10% Glucose, 5%, 10% Sucrose, 5%, 10% Soluble starch were used, 5% had greater effect on the isolates.

Table 2: Cultural characteristics of the Bacteria isolated from Ogiri- egusi

Isolates Code	Colony Morphology	Probable Identification
A1	Cream, circular, long opaque rod.	<i>Bacillus subtilis</i>
A2	Cream, circular, opaque rough.	<i>Bacillus pumilus</i>
B1	Cream, circular, flat, rough and lobate.	<i>Bacillus subtilis</i>
C1	Small, round, grayish, blustering, smooth, edge, raised, opaque.	<i>Bacillus pumilus.</i>
C2	Small, round, grayish, blustering, smooth, raised, opaque.	<i>Bacillus pumilis</i>
C3	Cream, circular, long rod, opaque.	<i>Bacillus pumilus</i>
C4	Small, round, grayish, blustering, smooth edge, raised raised, opaque.	<i>Bacillus subtilis</i>
D1	Cream, flat, dull, opaque, smooth, small.	<i>Bacillus licheniformis.</i>
D2	Small, round, grayish, blustering, smooth, raised, opaque	<i>Bacillus pumilus.</i>
D3	Cream, circular, smooth, entire.	<i>Bacillus subtilis.</i>
D4	Cream, opaque, raised, lobate, smooth.	<i>Bacillus licheniformis .</i>
D5	Cream, circular, flat, rough, and lobate.	<i>Bacillus licheniformis</i>

Table 3: Biochemical characteristics of isolates.

Isolate code	Charaterizat ion	CT	OX I	SH	CA	MR	VP	Sugar tests			Probable organisms
								GL U	SU	LA C	
A1	Gram +ve, long rod.	+	+	+	+	+	-	AG	A	A	<i>Bacillus subtilis</i>
A2	Gram +ve long rod in chain.	+	+	-	+	+	-	AG	A	A	<i>Bacillus pumilus</i>
B1	Gram +ve, long rod.	+	+	-	+	+	-	AG	A	A	<i>Bacillus subtilis</i>
C1	Gram +ve, rods in cluster	+	+	+	+	+	-	AG	A	A	<i>Bacillus pumilus</i>
C2	Gram +ve, rods in cluster	+	+	+	+	+	-	AG	A	A	<i>Bacillus pumilus</i>
C3	Gram +ve, rods in chains.	+	-	+	+	+	-	AG	A	A	<i>Bacillus pumilus</i>
C4	Gram +ve,	+	+	+	+	+	-	AG	A	A	<i>Bacillus subtilis</i>

	rods in cluster.										
D1	Gram +ve, rods in chains	+	+	+	+	+	-	AG	A	A	<i>Bacillus lichneformis</i>
D2	Gram +ve rods in cluster.	+	+	+	+	+	-	AG	A	A	<i>Bacillus pumullis</i>
D3	Gram +ve, long rod.	+	+	+	+	+	-	AG	A	A	<i>Bacillus lichneformis</i>
D4	Gram +ve long rod in chain.	+	+	+	-	+	-	AG	A	A	<i>Bacillus pumilus</i>
D5	Gram +ve, long rod.	+	+	+	-	+	-	AG	A	A	<i>Bacillus pumilius</i>

Foot note: += positive, -=Negative, AG= Acid and gas, A=Acid only..

CT= Citrate, SH=Starch, OX= Oxidase, CA= Catalase, M.R= Mrthyl

Red, V.P=Voges Proskeur, SU= Sucrose, GLU=Glucose,

LAC=Lactose.

Table 4: Percentage occurrence of isolates

Isolates	Number of Isolates	% Occurrence
<i>Bacillus subtilis</i>	4	33.33%
<i>Bacillus pumilus</i>	5	41.66%
<i>Bacillus licheniformis</i>	3	25%
	Total	100%

Table: 5 Antimicrobial activity of Bacillus species.

Isolate code	Zone of inhibition (mm)	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
A1	0	0
A2	0	0
B1	0	0
C1	0.6	0
C2	0	0
C3	0.8	0
C4	0	0
D1	0	0
D2	0	0
D3	0	0
D4	0	0
D5	0	0

Foot Note: 0= No inhibition zone.

Figure 1: Antimicrobial activity of *Bacillus subtilis* against *Escherichia coli* showing 0.6mm zone of inhibition.

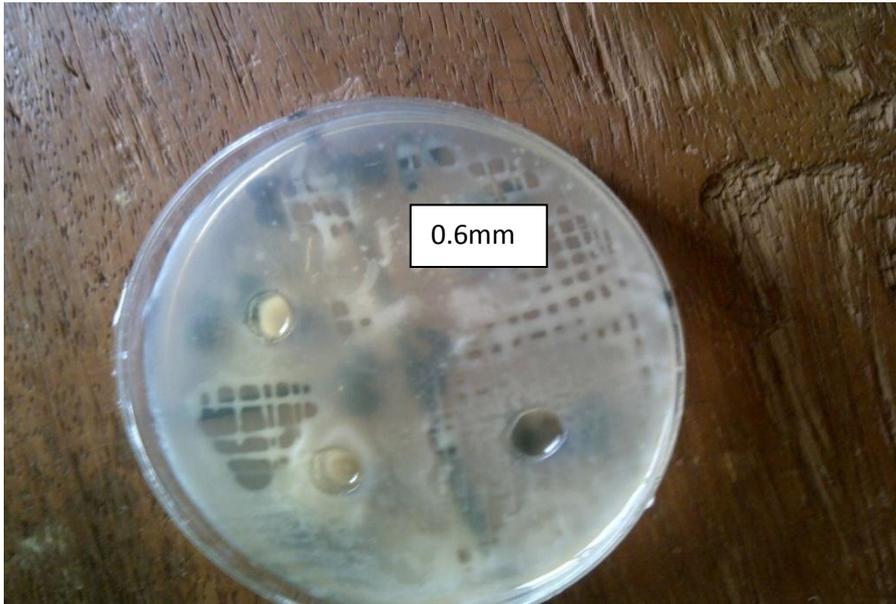


Figure 2: Antimicrobial activity of *Bacillus pumilus* against *Escherichia coli* showing 0.8mm zone of inhibition.

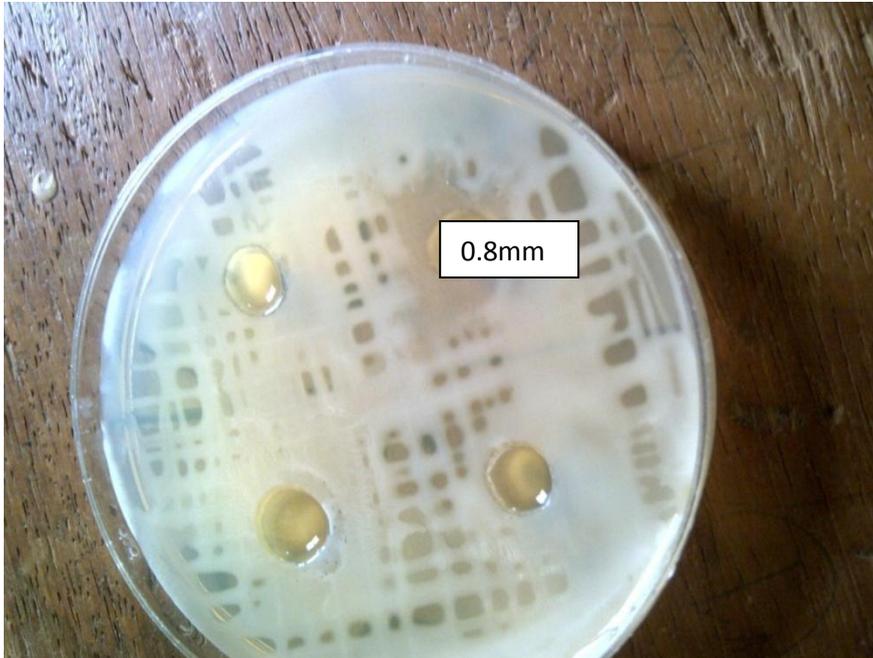


Table 6: effects of carbon sources on the isolates. (Cod 540nm)

Carbon sources	Control	5%	10%
Glucose	0.477	0.614	0.473
Glucose	1.644	1.400	1.388
Sucrose	1.677	1.545	1.340
Sucrose	1.500	1.450	1.400
Soluble starch	1.144	2.376	1.290
Soluble starch	1.644	2.327	2.065

CHAPTER FIVE

5.0 DISCUSSION

Many, organisms are reported to be isolated from traditionally prepared ogiri-egusi. Most of these organisms according to Obeta 1982, may have been introduced through air, banana leaves, or by handling during the preparation.

Bacillus species were isolated from ogiri-egusi a Nigerian condiment. This agrees with the work of Sarker, (1993) who have equally isolated *Bacillus* species from this source. The cultural and biochemical characteristics of the *Bacillus* species isolated in this work were confirmed with the Bergey's manual of systematic bacteriology (Sneath et al. 1986). The *Bacillus* species isolated in this work are, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*. The occurrence of *Bacillus subtilis* in higher proportion than other species as shown in table 4 in the isolates may be due to the fact that *Bacillus subtilis* is more adaptable to a wider varying environment than other species.

According to Ogbadu (1988), the antimicrobial activity of *Bacillus subtilis* and *Bacillus pumilus* against *Escherichia coli* and *Stapylococcus* (Test Organism) using agar well diffusion method, *Bacillus subtilis* and *Bacillus pumilus* showed zone of inhibition of inhibition against *Escherichia coli* as shown in table 5, *Bacillus subtilis* showed 0.6mm zone of inhibition against *Escherichia coli* and *Bacillus pumilus* showed 0.8mm zone of inhibition against *Escherichia coli*, *Stapylococcus* was resistant that is they was zone of inhibition.

The result of the *Bacillus* species isolated in this work had their highest growth of antimicrobial in the medium containing 5% and 10% Glucose, 5%(0.614) and 10% (0.473), Sucrose, 5% (1.545) and 10% (1.400) soluble starch used as the sources of carbon as shown in table 6.and was read using spectrophotometer. Between the 5%(2.376) and 10% (1.290) concentration, it showed 5% had the highest growth of antimicrobial, therefore I can now say the lower the concentration, the higher the growth of antimicrobial and the higher the concentration, the lower the growth of antimicrobial. This research

work has shown that *Bacillus* species can be use in the production of ogiri-egusi.

5.1 CONCLUSION

In conclusion, the effect of different carbon sources on isolates was analyzed which showed ogiri- egusi is normal and not harmful to the health in the microbiological properties, microorganisms were isolated as shown in table 1-6, Therefore it could be concluded that *Bacillus* sp. is the predominate organism responsible for the fermentation of melon seeds to give the fermented products. The effect of the different carbon sources is used to know the level or concentration of the growth of antimicrobial in the carbon sources .In conclusion, 5 percent (%) of the concentration should be in a medium to produce antimicrobial.

5.2 RECOMMENDATION

Since most microorganisms isolated from ogiri-egusi have been known to be harmless, I recommend that pregnant women should increase the rate of eating ogiri -egusi because it contains pro-biotic potentials which balance the intestinal wall of the stomach. The high concentration of the antimicrobial in the carbon sources can be used in the production of antibiotics.

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APPENDIX 1

MEDIA PREPARATION

The following media were used;

1) NUTRIENT AGAR (NA)

This medium was used for the enumeration of bacteria cells and to maintain pure cultures.

Nutrient agar is a general medium. It was therefore used here on the assumption that as many organism as were on the samples will grow.

Composition

The medium is composed of the following.

Lab-lemco power	1g
Yeast Extract	200g
Peptone	50g
Sodium Chloride	5.0g
Agar No.3	15g
pH	7.4

The powdered form was used and it was prepared as directed by the manufacturer.

Twenty eight gram (28g) of the powdered nutrient agar (oxid) was suspended into 1000ml of freshly prepared distilled water and was made to dissolve by heating. This was autoclaved at 121°C for 15minutes. The sterilized medium was allowed to cool down to about 45°C and then poured into sterile Petri dishes in about 20ml aliquots. The medium was allowed to solidify on these plates and were thereafter used.

PEPTONE WATER

Dispense 15g in 1L of deionised H₂O, soak for 10minutes, swirl to mix, then dispense final containers. Sterilize by autoclaving for 15minutes at 121°C.

Carbohydrate and a pH indicator can be added for studying fermentation reaction.

Composition:

Peptone-5.0

Tryptone - 5.0

Sodium Chloride - 5.0

NUTRIENT BROTH

Composition:

Beef – 1.0

Yeast – 2.5

Sodium chloride – 5.0

