

Periodic Appraisal of Cholesterol Degradation by Two Bacteria Isolates from 'Iru' (Fermented *Parkia Biglobosa*)

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ABSTRACT

This study was carried out to evaluate the incubation period on cholesterol degrading ability of bacteria isolated from 'iru' (fermented *Parkia biglobosa* seeds). The total bacterial count after culturing 'iru' (*Parkia biglobosa*) on nutrient agar were 7.30, 8.13, 7.28 and 8.11 respectively. The isolates were identified as *Bacillus cereus* and *Bacillus subtilis*. The isolates were screened on a salt agar of 1% cholesterol. Some of the isolates utilized cholesterol and grew well. IRUA and IRUC had the highest growth on the minimal salt cholesterol agar, followed by IRUB. The growth of 2IRUB was very low while IRUD, 2IRUA, 2IRUC, 2IRUD had no growth on the nutrient agar. *Bacillus subtilis* and *Bacillus cereus* degraded cholesterol maximally as 59.57mmol/L and 43.51mmol/L respectively. The cholesterol concentration degraded greatly with incubation period showing that there were high activities of the microorganisms and maximum cholesterol degradation was recorded on the 7th day. This study showed that the cholesterol degrading bacteria from *Parkia biglobosa* are endowed with the capability to degrade cholesterol and are good sources of cholesterol oxidase exploited for potential biomedical and industrial applications.

Keywords

Cholesterol, Incubation, *Bacillus cereus*, *Bacillus subtilis*, 'iru' (*Parkia biglobosa*).

Introduction

Cholesterol is a lipidic, waxy alcohol found in the cell membranes and transported in the blood plasma to establish proper membrane permeability and fluidity. Cholesterol is the principal sterol synthesized by animals, plants and fungi which responsible for causing atherosclerosis. Recently, cholesterol has also been implicated in cell signaling processes, assisting in the formation of lipid rafts in the plasma membrane and it is the precursor molecule for many biochemical pathways. The level of cholesterol in the body is regulated and maintained by cholesterol production and absorption, which have a shared relationship, and by elimination of cholesterol into the bile [1].

Most ingested cholesterol is esterified, which causes it to be poorly absorbed by the gut. The body also compensates for absorption

of ingested cholesterol by reducing its own cholesterol synthesis [2]. For these reasons, cholesterol in food, seven to ten hours after ingestion, has little, if any effect on concentrations of cholesterol in the blood [3]. However, during the first seven hours after ingestion of cholesterol, as absorbed fats are being distributed around the body within extracellular water by the various lipoproteins (which transport all fats in the water outside cells), the concentrations increase [4].

High cholesterol in the body will develop deposit of fat in the blood vessels, which will eventually make it difficult for enough blood to flow through the arteries. The deposits can break suddenly and form a clot causing a heart attack or stroke. The brain requires some cholesterol to function optimally but could be damaging when it is too much (Wikipedia).

The popularly used drug for lowering hypercholesterolemia are the statins which inhibit HMG-CoA reductase-enzyme, thereby up-regulating low density lipoprotein (LDL) receptors reduces LDL-

cholesterol level [5] but they are very expensive and expresses side effects like skin rashes, heart burns, muscle aches, inordinate liver function, low sexual desire, constipation, dizziness and abnormal pain [6]. Hyperlipidemia has a contributory risk factor to the severity and prevalence of coronary heart disease. Hyperlipidemia is characterized by increased serum total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) level [7]. Aronow W.S 2013, submitted that recurrent coronary event is a risk factor that emanated from LDL or serum total cholesterol (TC). Another drug that can be used to lower cholesterol in the body is Nicotinic acid, which reduces the flux of free fatty acid (FFA) by inhibiting adipose tissue lipolysis, thereby inhibiting very low-density lipoprotein (VLDL) production in the liver, which in large doses may produce fatty liver. Pro-adifen HCl is another drug that will block the pathway between mevalonate to squalene but it is not often used due to its side effects [5].

Consequently, it may be necessary to research into the natural means of reducing the body's cholesterol, which probably may be a cheaper remedy for hypercholesterolemia or an enroute to lowering of cholesterol in the body of animals without leaving any side effect. One of the popular ethnomedicine applied in the treatment of cardiovascular related diseases is parkia biglobosa.

Many bacterial species have been reported to be involved in biodegradation of cholesterol by means of bi-functional, Flavin Adenine Dinucleotide (FAD) containing cholesterol oxidase, which oxidizes the cholesterol and produces 4-cholesten-3-one, with reduction of oxygen to hydrogen peroxide [8,9]. The degradation of cholesterol by *Mycobacterium*, *Rhodococcus*, *Brevibacterium*, *Streptomyces* and some other Gram positive as well as Gram negative genera including *Comamonas*, *Burkholderia*, *Pseudomonas*, and *Chromobacterium* has been reported [10]. Several bacterial genera, such as *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Pseudomonas* reportedly mineralize cholesterol in the presence of molecular oxygen. Several species of *Mycobacterium* could use cholesterol as the sole source of carbon and energy. Genome sequence analyses revealed that diverse actinobacteria, including members of the *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Streptomyces* genera, harbour mce transporter operons involved in steroid uptake.

Fermented *Parkia biglobosa* (African locust bean) seed is a popular food condiment in Nigeria and other West African countries. It is a nutritious source of food since it is rich in protein and some beneficial health components. It will serve as a cheap source of protein for most of the people whose protein intake is low due to high costs of animal protein sources. This fermented condiment is highly appreciated for its sensory attributes and have been contributing to the nutritional diets of people in many regions of the world. Studies show that locust bean is highly digestible (74% - 97%) with pepsin followed by trypsin. This is coupled with its high commercial values as food and medicinal agent. Lipolysis and proteolysis are very important for the quality of African locust bean-based condiments fermented by *Bacillus*

spp. Proteolysis has been reported as the main metabolic activity during the fermentation of African locust bean. There are two types of iru, iru woro (harder fermented product) and iru pete (the softer and pastier product with mashed cotyledons) which is the fermented form. The spice also thickens and adds flavor to soup. This fermented condiment has also play a significant socio-economic role in Nigeria. The aim of this study is to determine the effect of incubation period on cholesterol degrading ability of bacteria isolated from 'iru' (fermented *Parkia biglobosa*).

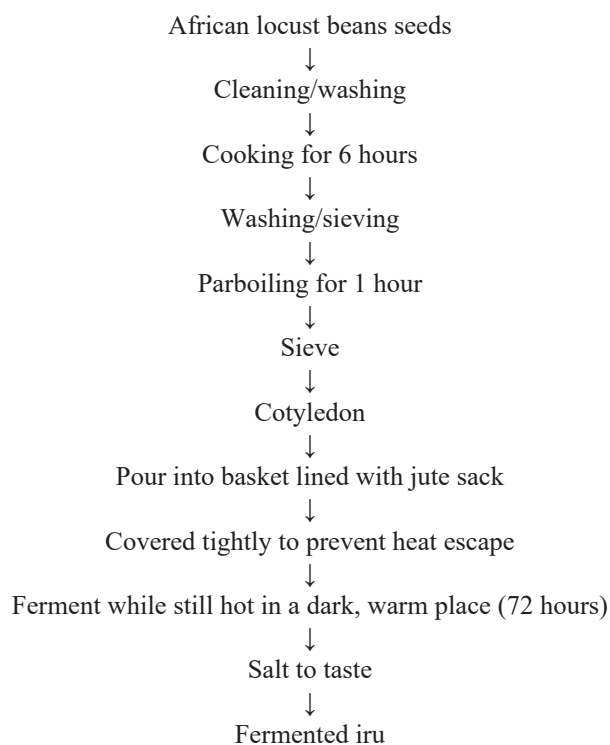
Materials and Methods

Sources of Materials

The *Parkia biglobosa* seeds were purchased from Oja Oba in Ado-Ekiti.

Laboratory production of 'iru' from African locust bean (*Parkia biglobosa*) seeds

The method described by Omodara and Olowomofe, (2015), on the production of 'iru' from *Parkia biglobosa* seeds was adopted. Five hundred grams (500g) of dried African locust bean seeds were soaked in 4litres of water for 15 minutes and boiled using pressure pot for two hours (2h). The cooked seeds were dehulled and washed thoroughly to remove the testa. The cotyledons were boiled for the second time under pressure for 45mins with the addition of 5g of 'kuuru'; a local softening agent for the production of 'iru-pete'. The boiled cotyledon was drained poured into fermenting can and fermented in an incubator at 35°C for 96h.



Traditional processing of 'iru' (*Parkia biglobosa*)

Isolation and Identification of Microorganisms

Microbial isolate was determined using serial dilution and plating

technique on nutrient agar (NA) plates.

Qualitative Screening of Bacteria Isolated

All the bacterial isolates from *Parkia biglobosa* were screened on the minimal salt agar plates containing 1% cholesterol as the only carbon source. Cholesterol plates were streaked with cultures and incubated at 37°C for 7 days. The potentiality of bacteria to utilize cholesterol was evaluated via the growth of bacteria on these plates.

Quantitative Screening of Bacteria Isolated

Enzymatic colorimetric cholesterol oxidase peroxidase method was used to calculate the cholesterol degradation. The minimal salt medium prepared was supplemented with 1% cholesterol. Ten millimetres of the medium was pipetted into test tubes and the isolates were inoculated into the medium and incubated for 7 days. After 7 days, the residual concentration of cholesterol in the medium was checked using Randox kit (CH200). The absorbance of the samples and the standard were checked against the blank at 500nm using spectrophotometer. The concentration of the cholesterol was determined by:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

The value was multiplied by dilution factor to get the concentration.

Effect of Incubation Period on Cholesterol Degradation

Enzymatic colorimetric cholesterol oxidase peroxidase method was used to calculate the cholesterol degradation. The growth was monitored at 500nm using spectrophotometer according to the incubation period at intervals. The minimal salt medium prepared was supplemented with 1% cholesterol. Ten millimeters of the medium was pipetted into test tubes and the isolates were inoculated into the medium and incubated for 7 days. After 7 days, the residual concentration of cholesterol in the medium was checked using Randox kit (CH200). The absorbance of the samples and the standard were checked against the blank at 500nm using spectrophotometer. The concentration of the cholesterol was determined by:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

The value was now being multiplied by dilution factor to get the concentration.

Results

Identification of Bacteria Isolated

The isolates were identified and confirmed to be *Bacillus cereus* and *Bacillus subtilis*. The isolates were identified and confirmed to be *Bacillus cereus* and *Bacillus subtilis*. The identification of the isolates was confirmed with the biochemical characterization and morphological characteristics in which *Bacillus cereus* is a

gram positive bacterium, motile, catalase positive, indole negative, citrate positive, methyl red positive, Voges Proskauer positive, gives out sodium chloride and glucose positive in haemolysis on blood agar. *Bacillus subtilis* is a gram-positive bacterium, motile, catalase positive, citrate positive, methyl red positive, Voges proskauer positive, does not give out sodium chloride.

Isolation of Microorganisms

After 24 hours of incubation, different colonies were seen which were counted and converted into log (CFU/mL). The plates has the total bacterial count of 7.30, 8.13, 7.28, 8.11 respectively after being converted to the log value as shown in Table 1.

Table 1: Total Bacteria Count.

Isolate code	Total colony count [Log(CFU/mL)]
IRUA	7.30
IRUB	8.13
2IRUA	7.28
2IRUB	8.11

Qualitative Screening of Bacteria Isolated

Several bacteria strains were checked for their growth on minimal salt media. Some bacterial isolates utilized cholesterol and grew well on minimal salt cholesterol agar media as shown in Table 2.

Table 2: Qualitative Screening of Bacteria Isolates from 'IRU' (*Parkia biglobosa*).

Isolate code	Bacterial growth in MSC agar
IRUA	++++
IRUB	+++
IRUC	++++
IRUD	-
2IRUA	-
2IRUB	++
2IRUC	-
2IRUD	-

Quantitative Screening of Bacteria Isolated

The cholesterol degraded gradually and after seven days, the cholesterol concentration was checked at 500nm and compared with the control as shown in Table 3.

Table 3: Quantitative Screening of Bacteria Isolates from 'IRU' (*Parkia biglobosa*).

Organism	Absorbance value (500nm)	Cholesterol concentration (mmol/L)
<i>Bacillus subtilis</i>	0.170	88.06
<i>Bacillus subtilis</i>	0.115	59.57
<i>Bacillus cereus</i>	0.084	43.51
<i>Bacillus cereus</i>	0.186	96.34
Control	0.204	105.66

Effect of Incubation Time on Cholesterol Degradation

The effect of incubation on cholesterol degradation after each day is shown in Table 5. At 168th hour, the highest level of cholesterol degradation was observed.

Table 4: Effect of Incubation Time on Cholesterol Degradation.

Time (hours)	Cholesterol concentration (mmol/L)		CONTROL
	IRUC (<i>Bacillus subtilis</i>)	IRUA (<i>Bacillus cereus</i>)	
24	134.16	136.75	105.66
48	118.62	131.57	105.63
120	106.71	108.26	105.62
144	99.97	84.95	105.68
168	53.87	67.85	105.65

Discussion

In this study, 'iru' (*Parkia biglobosa*) was cultured in a nutrient agar and the colonies were counted which was converted to log value (LogCFU/mL). The total bacteria count of each plate are 7.30, 8.13, 7.28, and 8.11 respectively after being converted to the log value (Table 1). The cultures were duplicated and later isolated for identification and their morphological characteristics (Table 2). The organisms identified are *Bacillus cereus* and *Bacillus subtilis*. *Bacillus cereus*, characterized to be small, white dried colonies, gram positive bacterium, motile, catalase positive, indole negative, citrate positive, methyl red positive, Voges Proskauer positive, gives out sodium chloride and glucose positive in haemolysis on blood agar while *Bacillus subtilis* have large creamy, opaque colonies, a gram positive bacterium, motile, catalase positive, citrate positive, methyl red positive, Voges proskauer positive, does not give out sodium chloride.

The isolates were screened in a minimal salt agar containing 1% cholesterol as the carbon source. After incubation, some of the bacterial isolates utilized the cholesterol and grew well on the agar (Table 3). IRUA and IRUC had the highest growth on the minimal salt cholesterol agar, IRUB has a moderate growth, 2IRUB had a low growth while IRUD, 2IRUA, 2IRUC, 2IRUD had no growth on the agar. The isolates were further investigated for the ones with maximum cholesterol degradation where 2 isolates were screened for maximum concentration of cholesterol degradation. They were identified as *Bacillus subtilis* and *Bacillus cereus* having cholesterol concentration of 59.57mmol/L and 43.51mmol/L respectively. They are the strains in which their cholesterol level was greatly reduced which shows that these 2 bacteria have higher ability to degrade the cholesterol (Table 4). Comparatively, from table 4, the absorbance has a reciprocal relationship with the concentration of cholesterol in the media, which implies that the level of cloudiness of the sample in the spectrophotometer is a function of un-degraded cholesterol.

The effect of incubation period were later checked on the two isolates (Table 5). After 24 hours of incubation, the cholesterol concentration was yet very high signifying that the cholesterol has not really been degraded, the activities of the microorganisms at the optimum not yet high. This might probably be because cholesterol is a waxy organic compound in which bacteria like *Bacillus subtilis* and *Bacillus cereus* might not easily get adapted to metabolize it as in some other common media for culturing bacteria within the first few hours of inoculation.

However, similar trend was observed in other studies in which the maximum absorbance of cells was 4.75 at 600 nm after 24 hours of incubation [11]. The incubation time varies in different microorganisms for cholesterol decomposition. The maximum cholesterol utilization by *Bacillus cereus* was attained after 24 hours incubation [12], while others reported 3-7 days for the maximum degradation of cholesterol by most of *Rhodococcus* strains [13].

After 48 hours of incubation till the 168 hours of incubation (7th day), the cholesterol reduces greatly which also showed that there was a high activity of the microorganisms indicating maximum cholesterol degradation in the media (Table 5). Other studies also show that as the incubation time increases the cholesterol decreases. Maximum cholesterol degradation was recorded as the activities of the microorganisms increased. The residual cholesterol content decreased during growth of the microorganism depicting a rapid reduction in cholesterol content observed through 36th hour of incubation. Incubation for 24 hours resulted in the degradation of about 31% of the cholesterol in the medium and at a time after 60th hour of incubation, 1.0 mg ml of cholesterol was detected in the culture broth, suggesting that 50% of the cholesterol was degraded [11]. Consequently, it is noteworthy that *Bacillus subtilis* will be a better microorganism for the degradation of cholesterol juxtaposing *Bacillus cereus*.

Conclusion

Aside iru being a good condiment playing a multi-role in human diet and performing some therapeutic functions in animal body, it has been shown by this study that iru is a good agent for degrading cholesterol in the body of animal when time is considered. The study also revealed that *Bacillus subtilis* is the best microorganism for cholesterol degradation.

Recommendations

- There should be advocacy/sensitization programme for consumption of iru by the patients with hypercholesterolemia.
- Government and corporate organisations should realize the potentials of iru and endeavour to engage in its large production to meet the teeming population of the world.
- The agricultural sectors should engage in massive planting of iru tree and guard jealously its afforestation.
- Microbiologists should engross in culturing of *Bacillus subtilis* that can produce cholesterol oxidase in large quantity and devise a good storage system to prolong its shelf life.
- Medical personels/Health Officials in Hospitals and Health Centres should create interest in recommending iru as a natural remedy against synthetic drugs for hypercholesterolemia and allied diseases.

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