Groundnut shell infusion agar as a culture medium for moulds

ABSTRACT

Comparative mould analysis was carried out using groundnut shell infusion agar (GSA) and potato dextrose agar (PDA), which was the control. GSA was prepared by weighing 28g of blended gari and 15g of agar powder into 1L of groundnut shells filtrate. Potato dextrose agar, a conventional medium was prepared according to the manufacturer's specifications. The mean mould counts on each of the media from the different locations ranged from 3.7×10^7cfu/ml to 7.8×10^7cfu/ml on GSA and 3.7×10^7cfu/ml to 1.5×10^9cfu/ml on PDA following incubation at room temperature (27^0c ± 2) for 3-5 days. The moulds identified were *Aspergillus niger, Aspergillus flavus, Trichoderma viride, Rhizopus* sp. *Mucor* sp. *Botrytis* sp. *Helminthosporium caryopsidum, and Penicillum* sp. From the results obtained, it showed that GSA can now be used successfully for quantitative mould counts and other mycological studies. This would proffer solution to the high cost of conventional media used for moulds as well as agro waste pollution in the environment.

Introduction

As a legume, groundnut also known as peanut, belongs to the genus *Arachis* and species *hypogaea,* of the family Fabaceae (also known as Leguminosaea, and commonly known as the bean or pea family) (Isleib *et al.,* 1994). Like most other legumes, peanuts harbour symbiotic nitrogen-fixing bacteria in root nodules (Gwilyn *et al.,* 2005). It is widely grown in the tropics and subtropics and classified both as a grain legume and because of its high oil content, an oil crop (Hymowitz, 1990).

Peanuts are similar in taste and nutritional profile to tree nuts such as walnuts and almonds and are often served in similar ways in western cuisines. It is particularly important that peanuts are dried properly and stored in dry conditions. If they are too high in moisture or if storage conditions are poor, they may become infected by the mould, *Aspergillus flavus.*

The groundnut husks are shells that are discarded after processing or shelling of groundnut seeds. Groundnut is a good protein source and has a high lysine content which makes it a good complement for cereal protein, which is low in lysine (Okaka, 2005).

Global peanut production has increased to 35.88 million metric tons in year 2011 (USDA, 2012), which led to concern in Agricultural waste management. One of the best alternatives in curbing agricultural waste problems is to transform agricultural wastes to other utilized materials (Dongmeza *et al.*, 2009). Thus, using groundnut shell agar as culture medium for moulds is essential in the reduction of agricultural waste problems as well as cost of purchasing other expensive media.

Gari is rich in carbohydrate therefore, suitable for fungal growth. Moulds such as *Aspergillus, Penicillium, Fusarium, Rhizopus, Cladosporium*and *Mucor* have been associated with gari during storage and distribution (Ogugbue *et al.*, 2011).

Therefore, groundnut shell infusion agar, when processed by obtaining filtrate from blended groundnut shell and mixed with gari and agar, in some respect is similar to agar media and could be used to isolate and identify microorganisms that utilize it.

Potato dextrose agar is a general purpose medium for yeasts and moulds that can be supplemented with acid or antibiotics to inhibit bacterial growth. It is used in plate count methods when testing food, dairy products and cosmetics (USDA,2012).

The USP lists PDA as one of the recommended media for use in the Microbial Enumeration Test when testing non-sterile pharmaceutical products.

In addition, this medium is used to stimulate sporulation (slide preparations), maintain stock cultures of certain dermatophytes and differentiate atypical varieties of dermatophytes by pigment production (MacFaddin,1985).The uses of groundnut are diverse; all parts of the plant can be used. The nut (kernel) is a rich source of edible oil containing 36-54% oil and 25-32% protein (Knauftand Ozias-Akins, 1995).

Non-food products such as soaps, medicines, cosmetics, pharmaceuticals, emulsions for insect control, lubricant and fuel for diesel engines can be made from groundnut. The haulms are excellent high protein hay for horses and ruminant livestock. Groundnut shells may be used for fuel (fireplace "logs"), as a soil conditioner, for building trade as blocks or hardboard and as a raw source of organic chemicals (Gibbons, 1980).

According to analysis by the Animal science Department, Aidabase, Zimbabwe, groundnut shells contain an average of 68% organic matter, 6.8% crude protein, 18.2% crude fibre and 7.1% ash. Another nutritional composition analysis of groundnut shells indicate that the shells contain 65.7% cellulose, 21.2% carbohydrate, 7.3% protein, 4.5% minerals and 1.2% lipids. Since the processed shells contain bits and skins of nuts, the actual protein and lipid contents of this waste material are probably much higher.

Previous studies have shown the feasibility of using agricultural products such as gari as culture medium for moulds (Okorondu *et. al.,* 2011). The present study is to investigate the growth characteristics of moulds on groundnut shell infusion agar, which might serve as an inexpensive but also effective alternative culture medium in comparison to potato dextrose agar.

Materials and Methods

Study location

The study was carried out in the microbiology laboratory, Rivers State University of Science and Technology, Nkpolu-Oroworukwo Port Harcourt Rivers State, South-south Nigeria.

Media used

1. Potato dextrose agar (PDA)

2. Groundnut shell infusion agar (GSA)

Collection of samples

Garden soil sample

The garden soil samples were collected in sterile closed containers from different locations(how many locations?) in Rivers State university of Science and Technology (RSUST).

Groundnut shells sample

Groundnut was purchased from hawkers in RSUST. The nuts were sorted and the shells were recovered. Some grams were weighed and dried in an oven to a constant weight. These were then

blended with distilled water and sieved to obtain the filtrate. The filtrate was stored in a refrigerator prior to analysis.

Gari sample

Gari was bought from mile 1 market in Port-Harcourt, Rivers State, Nigeria. The gari was ground with a blender to give very fine particles. It was then meshed to obtain the fine and smooth particles.

Media preparation

Potato dextrose agar

Potato dextrose agar was prepared according to Manufacturer's specifications.

Explain how PDA was prepared like GSA in L98-100 below, up to autoclaving,

Groundnut shell infusion agar

Groundnut shell infusion agar was prepared by weighing 15g of agar powder into 1000ml of the groundnut shell filtrate and 28g of gari was also added to the mixture and then shaken for proper mixing before being sterilized.

Analysis of samples

After preparation of the groundnut shell infusion agar, analysis of the soil sample was carried out. A set-up for serial 10 fold dilutions was done, in which 10g of garden soil was mixed with 90ml of sterile distilled water in an Erlenmeyer flask. This was agitated thoroughly in order for the microorganisms present in the sample to be dislodged into the water. This was labelled as stock, from which serial dilutions up to 10^{-8} were made.

Normal saline was dispensed into test tubes (9ml each). These were sterilized by autoclaving for 15minutes and then allowed to cool. Some of the stock (1ml) was pipetted into the first test tube (10^{-2}), containing 9ml of normal saline. Further serial 10 fold dilutions up to 10^{-8} were made, using a 1ml sterile pipette for each dilution.

Mould count

Moulds in the samples were enumerated by pipetting aliquots (0.1ml) of each dilution (10^{-2} -10^{-8}) into already prepared GSA and PDA plates in triplicate.

A sterile bent glass spreader was used to spread the sample evenly on the plate, and it was then incubated at 25^0C($25\ ^0$C) for 3-5 days. Colonies which appeared on the plates were counted and expressed as colony forming units per gram (cfu/g). The moulds were isolated and sub-cultured to obtain pure cultures.

Isolation of pure cultures

Mixed colonies of isolates were observed after about 5days of inoculation of fungi at 25^0c($25\ ^0$C) and the respective plates were further examined morphologically and individual distinct colonies were subcultured for further characterization. Distinct colonies of fungal isolates were isolated using inoculating needle and inoculated into freshly prepared GSA and PDA plates.

Identification of moulds: Gari slide culture

Gari granules were placed in a glass petri dish and sterilized by autoclaving at 121^0c for 15mins. Individual fairly large grains of the sterile gari were transferred using a flamed forceps onto clean microscope slides and then placed in different petri dishes, cushioned with moistened cotton wool. Pure isolates of moulds were inoculated using inoculating needle, onto separate grains and covered with cover slips then the petri dishes were covered and incubated at ambient temperature for 2-3 days. The slide cultures obtained were stained with lactophenol cotton blue to enhance structures of growth and then viewed under the microscope. These were observed

under the low power first and then the high power of the microscope and the observations were recorded.

Results

Total viable mould counts on the media from sample location 1 are shown on Table 1 below. On GSA, the counts were within the range of 1.0×10^7 and 2.0×10^6 cfu/g while PDA had counts between 3.0×10^7 and 5.0×10^6 cfu/g.

Table 1. Total viable mould count from location 1 on GSA and PDA

Sample code	Viable count (Cfu/g)
L1 GSA4	2.0×10^5
L1 GSA5	2.0×10^6
L1 GSA6	1.0×10^7
L1 GSA7	1.0×10^8
L1 PDA4	5.0×10^6
LI PDA5	3.0×10^7
L1 PDA6	4.0×10^8
L1 PDA7	4.0×10^9

Key: L1 GSA 4= Dilution 10^{-4} on groundnut shell infusion agar from location 1

L1 PDA 4= Dilution 10^{-4} on potato dextrose agar from location 1

Total viable mould counts on the media from sample location 2 are shown on Table 2 below. On GSA, the counts were within the range of 1.0×10^7 and 2.0×10^8 cfu/g while PDA had counts between 1.0×10^6 and 1.0×10^8 cfu/g.

Table 2. Total viable mould counts from location 2 on GSA and PDA

Sample code	Viable counts (Cfu/g)
L2 GSA 4	4.0×10^5
L2 GSA 5	4.0×10^6
L2 GSA 6	1.0×10^7
L2 GSA 7	2.0×10^8
L2 PDA 4	3.0×10^5
L2 PDA 5	1.0×10^6
L2 PDA 6	1.0×10^7
L2 PDA 7	1.0×10^8

Key: L2 GSA 4= Dilution 10^{-4} on groundnut shell infusion agar from location 2

L2 PDA 4= Dilution 10^{-4} on potato dextrose agar from location 2

Total viable mould counts on the media from sample location 3 are shown on Table 3 below. On GSA, the counts were within the range of 4.0×10^5 and 2.0×10^8 cfu/g while PDA had counts between 4.0×10^5 and 2.0×10^8 cfu/g.

Table 3. Total viable mould count from location 3 on GSA and PDA

Sample code	Viable counts (Cfu/g)
L3 GSA 4	4.0×10^5
L3 GSA 5	5.0×10^6
L3 GSA 6	3.0×10^7
L3 GSA 7	2.0×10^8
L3 PDA 4	4.0×10^5
L3 PDA 5	4.0×10^6
L3 PDA 6	1.0×10^7
L3 PDA 7	2.0×10^8

Key: L3 GSA 4= Dilution 10^{-4} on groundnut shell infusion agar from location 3

L3 PDA 4= Dilution 10^{-4} on potato dextrose agar from location 3

Mean mould counts on the media from different sample locations are shown on Table 4 below. On GSA, the counts were within the range of 3.7×10^7 and 7.8×10^7 cfu/g while PDA had counts between 3.7×10^7 and 1.5×10^9 cfu/g.

Table 4. Mean mould counts from the different locations on GSA and PDA

Media	Location 1 (Cfu/g)	Location 2 (Cfu/g)	Location 3 (Cfu/g)
GSA	3.7×10^7	7.1×10^7	7.8×10^7

PDA	1.5×10^9	3.7×10^7	7.1×10^7

The morphological and microscopic characteristics of isolates obtained from groundnut shell infusion agar are presented on table 5 below. Some of the isolates were *Aspergillus niger*, *Mucor sp*, *Rhizopus* sp

Table 5. Morphological and microscopic characteristics of moulds isolated from groundnut shell infusion agar medium

Isolate code	Cultural morphology	Microscopic observation	Probable organism
GSA M1	White and scattered greenish patches on the surface	Septate hyphae with conidiophores and globose conidia	*Trichoderma viride*
GSA M2	Dark colony with compact white and yellow on the reverse	Smooth-walled erect conidiophores with globose conidia	*Aspergillus niger*
GSA M3	Powdery masses of yellow-green spores on the upper surface	Septate hyphae with rather long conidiophores. Conidia are globose	*Aspergillus flavus*

GSA M4	Fluffy white appearance	Non-septate or sparsely septate with short erect sporangiophores	*Mucor* sp
GSA M5	Dense cotton white growth becoming grey with sporulation	Non-septate unicellular sporangiophores	*Rhizopus* sp

Cite the authorities used for identification of the probable organisms.

The morphological and microscopic characteristics of isolates obtained from potato dextrose agar are presented on table 6 below. Some of the isolates were *Penicillium* sp., *Botrytis* sp., *Aspergillus niger, Mucor sp, Rhizopus* sp

Table 6. Morphological and microscopic characteristics of moulds isolated from potato dextrose agar medium

Isolate code	Cultural morphology	Microscopic observation	Probable organism
PDA M1	Grey-greenish colony	Slender phialides with cylindrical conidia and septate hyphae	*Penicillium* sp.
PDA M2	Dark colony with compact white yellow	Smooth-walled erect conidiophores with	*Aspergillus niger*

	on the reverse	globose conidia	
PDA M3	Dense cotton white growth becoming grey with sporulation	Non-septate unicellular sporangiophores	*Rhizopus* sp.
PDA M4	Very dark mycelium	Septate hyphae with tall conidiophores in clusters and porospores	*Helminthosporium caryopsidum*
PDA M5	Hyaline mycelium becoming grey to greyish brown	Abundant short conidiophores with septate hyphae	*Botrytis* sp.

Cite the authorities used for identification

Table 7 shows the frequency of occurrence of mould isolates from GSA and PDA media with *Aspergillus niger* having the highest % frequency of occurrence of 14 on PDA and *Mucor* sp. having the least frequency of occurrence of 0 on GSA.

Table 7. Frequency of occurrence of mould isolates from GSA and PDA media

Isolates	No. of occurrence	On GSA (%)	On PDA (%)
Trichoderma viride	5	7.0	1.7

Aspergillus niger	14	10.5	14.0
Aspergillus flavus	4	3.5	3.5
*Rhizopus*sp	9	7.0	8.7
Mucor sp	4	0	7.0
Helminthosporium caryopsidum	9	12.0	3.5
Botrytis sp	4	3.5	3.5
Penicillium sp	8	7.0	7.0

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190 Plate 1below showsa 3-4-day old *Aspergillus niger (*mould) culture grown on GSA while plate 2**, a** 6-7-
191 day old *Aspergillus niger* grown on PDA

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Plate 1: A 3-4-day old *Aspergillus niger* culture grown on GSA

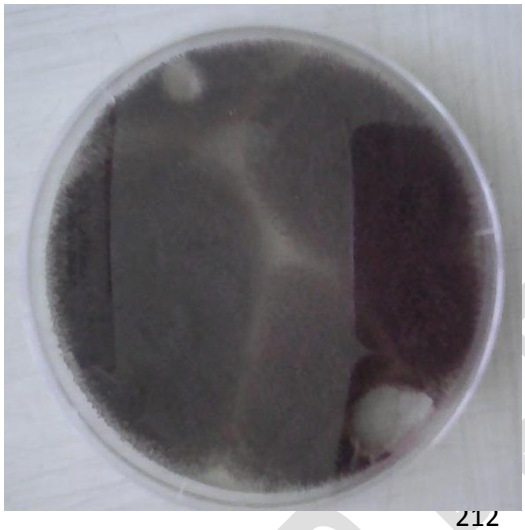

Plate 2: A 6-7-day old *Aspergillus niger*grown on PDA

Discussion

In this study, it was observed that both GSA and PDA supported the growth of moulds as indicated in the mould counts (Tables 1, 2 and 3.) The results of the study also revealed that GSA supported only the growth of moulds and not that of bacteria. This could be due to the high percentage of cellulose contained in the shells.(Ref)

The pH of the media could also be the reason why bacteria were not able to grow on the media since GSA has a pH of 4.3-5.6 which is acidic, thus, prevents bacterial growth while supporting that of moulds.

Groundnut shell infusion agar mixed with gari produced healthy moulds probably because of additional nutrients from the gari, and it also aided in making the pH of the medium acidic, since it has a pH that lies within 3.5-4.2 and it supports the growth of fungi but inhibits the growth of bacteria (Okoronduet *al.*, 2011).

The moulds isolated from the soil include *Aspergillus niger, Rhizopussp.,Aspergillus flavus, Mucor*sp, *Penicillium*sp, *Botrytis* sp, *Helminthosporiumcaryopsidum, Trychodermaviride*. The growth of *Trichoderma*and *Aspergillus* were impressive on the both media. Although *Mucor*sp. did not grow on GSA, but it did on PDA, as shown in Table 7. See Table 5 which showed that *Mucor* sp was identified on GSA

Quantitative counts on GSA showed discrete colonies as is the case with conventional media, PDA, that was used. As indicated in Table1, the mould count was greater on PDA (5.0×10^6- 4.0×10^9) than on GSA (2.0×10^5- 1.0×10^8). This might be due to insufficient carbohydrate content in the GSA medium, as this medium was not enriched with gari, a rich source of carbohydrate when it was used for the sample from the first location. However, the mould counts in tables 2 and 3 ($4.0 \times 10^5 - 2.0 \times 10^8$) showed that the growth of moulds had increased on GSA, probably because of the gari that was used to enrich the medium.

The growth of moulds was impressive on PDA, although this medium was found to support the growth of bacteria when no antibiotic was added.

According to Okoronduet. *al., (*2011), proximate analysis of gari showed 1% crude protein, 2% fats, 1% ash, 3% fibre, 12% moisture content and 81% carbohydrate while that of groundnut shells according to Abdulrazaket *al.,*(2014) showed 4.43% crude protein, 0.50% lipids, 2.50% ash, 59.0% fibre, 8.0% moisture content and 25.57% carbohydrate. This revealed that GSA is nutritious and has all it takes to support the growth of fungi.

Conclusion and Recommendation

From the results of this study, it can be concluded that groundnut shell infusion agar possesses the ability to support the growth of moulds and it also inhibited the growth of

bacteria. Growth characteristics compared favourably with that on potato dextrose agar (a conventional media). Hence, GSA can be used successfully for quantitative mould counts in any laboratory experiment and it has provided a less expensive alternative to other expensive conventional media used to culture moulds.

It is therefore recommended that for the quantitative count of moulds and other mycological studies, GSA should (could)always be employed.

Further studies on the use of groundnut shell infusion agar as culture medium should be carried out.

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