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PRODUCTION OF RHIZOBIUM BIO-FERTILIZER CARRIER FROM TANNERY SOLID WASTES

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ABSTRACT
The indiscriminate disposal of chrome shavings, splits and trimmings on land and close to water bodies leads to high concentration of chromium within such environments. Therefore, a treatment process which was able to reduce 99% of the chromium concentration in the waste was adopted, and the fiber left after the de-chroming process was investigated as a potential carrier for nitrogen-fixing bacteria. The product of the bacteria and the carrier is used as rhizobium bio-fertilizer. De-chromed tannery solid wastes with a pH of 4.88 and eggshell (a natural source of calcium carbonate) with a pH of 9.36 were mixed at ratio 3:2 to attain a desired pH of 6.71. This pH value was found to be suitable for the growth of Bradyrhizobiumjaponicum strain (USDA 110), nitrogen-fixing bacteria often used as bio-fertilizer inoculant. The mixture was sterilized using gamma irradiation and autoclaving. The strain was obtained from the Soil Microbiology Laboratory of the Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria. A fresh culture of the strain was prepared on Yeast Mannitol Agar (YMA) plates. The sterile carrier was inoculated with the cultured inoculum at 50% and 55% moisture content and cured for 21days at a temperature of 28^oC for maturation in an orbital incubator. The survival and growth of the strains after the maturation period confirmed the suitability of the developed carrier for the growth of rhizobium bio-fertilizer. The strain was re-isolated from the developed carrier using serial dilutionat 10^{-10} dilution factor on YMA plates. Gram stained isolates from serial dilution were observed under microscope at 100x magnification and found to be gram negative short rods which is a typical morphology of rhizobium cells.
***Keywords**: Tannery Wastes, Sterilization, Bradyrhizobiumjaponicum, Bio-fertilizer, Carrier.*

INTRODUCTION
Tanning is an age long technology that has transcended generations. It consists of series of processes that lead to the conversion of raw hides and skin to leather, which is used for the manufacture of shoes, bags, upholstery etc. During tanning, different categories of wastes such as solid, liquid and gaseous are generated, which if not properly managed pose great threat to the environment. Out of the solid wastes category, chrome shavings, splits and trimmings account for 34-40% (Kanagaraj *et al*., 2006). The indiscriminate disposal of chrome shavings, splits and trimmings on land and close to water bodies leads to high concentration of chromium within such environments. Therefore, a treatment process was developed which was able to reduce 99% of the chromium concentration in the waste, and the fiber left after the de-chroming process was investigated as a potential carrier for nitrogen-fixing bacteria. The product of the bacteria and the carrier is used as rhizobium bio-fertilizer.

Dry inoculants can be produced using different kinds of materials such as peat, coal, clays and inorganic soil. In addition, organic materials such as composts, soybean meal, wheat bran, sawdust, etc. or inert materials such as vermiculite, perlite, kaolin, bentonite and silicates with good moisture absorption capacity (Hedge *et al.*, 1992; Smith, 1992; Keyser *et al.*, 1993) can also be used. Though peat is the most widely used, its unavailability and high cost even when it is accessed poses a limitation to its continuous use and has triggered research to source for other readily available and less expensive substitutes.

Bio-fertilizer is a substance containing living micro-organisms with the ability to convert available or supplied nutrient in the atmosphere and/or soil to suitable forms that can be easily absorbed by plants. When the substance is applied to seeds, plant surfaces or soil, the micro-organisms colonize the rhizosphere or the interior of the plants and promote growth by increasing the supply and availability of primary nutrients to the host plant (Vessey, 2003; Bi *et al*., 2003; Rajendran and Deverag, 2004; Gaur, 2010).

As the world undergoes green revolution (i.e. research and development of new processes that are environmentally safe and products that are biodegradable, which cause little or no threats to the environment), interest in the application of bio-fertilizers is increasing due to enhancement in nutrient uptake efficiency of plants (Adesemoye *et al.*, 2008; Malusà *et al.*, 2008 and Lempert *et al.*, 2003). The introduction of bio-fertilizers as a complement to organic fertilizers as well as a potential substitute to chemical fertilizers is a welcome development. Therefore, this research work is aimed at producing a carrier for bio-fertilizers form tannery solid wastes (an industrial environmental pollutant) that is suitable for rhizobium microbial inoculants. This is to be achieved by producing bio-fertilizer carrier using de-chromed tannery solid wastes as well as testing the survival of cultured microbial inoculant (bio-fertilizers) in the newly produced carrier. This is simply converting wastes to wealth while securing the health of living organisms and the environment.

METHODS
The summary of the process for the production of bio-fertilizer carrier from tannery solid wastes is shown in Figure 1.

However, details of the stages involved are explained in the next section.

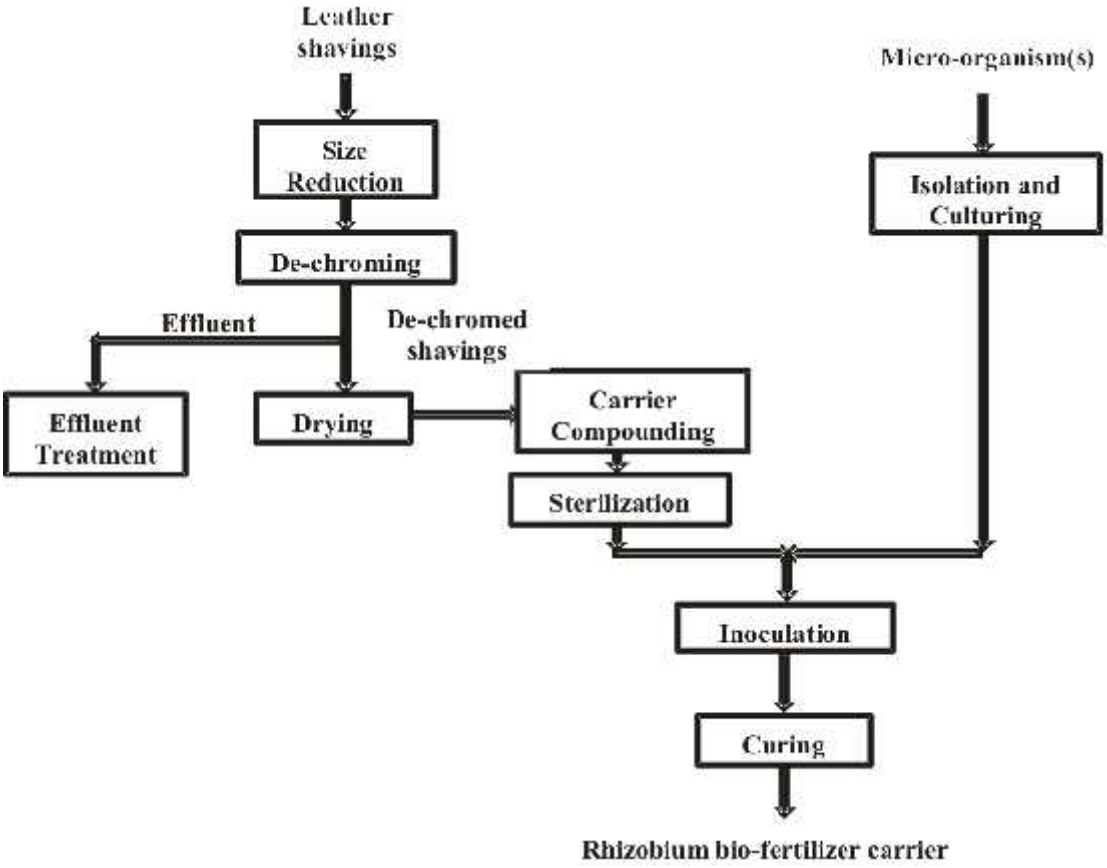

Figure 1: Flow Diagram for Rhizobium Bio-fertilizer carrier production from tannery solid wastes

De-chroming process

The de-chroming process established by Adeoye *et al.* (2014) was used because of its ability to remove the major pollutant which is chromium from the collagen fiber without the destruction of the fiber. The fiber is a potential fertilizing material because it contains primary and secondary nutrients such as carbon, sulphur, and nitrogen among others required for plant growth (Sethuraman *et al.*, 2013).

Carrier compounding

Poultry eggshells from National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria, with a pH of 9.36 was milled and mixed at ratio 3:2 with the de-chromed tannery solid wastes which had a pH of 4.88 to attain the desired pH that fell within the pH range of 6.5-7.5 required for rhizobia growth (Lei *et al.*, 2011). The compounded carrier was further milled to 0.5mm.

Sterilization

In this paper, two sterilization methods were experimented:

Gamma irradiation sterilization

This is suitable for carrier sterilization because the process makes almost no change in physical and/or chemical properties of the material (Tejima *et al.*, 2012). Some of the advantages of gamma over competitive procedures include high penetration power, isothermal character (small temperature rise), and no residues. It also provides a better assurance of product sterility. It is capable of killing microorganisms by breaking their chemical bonds, producing free radicals that attack the nucleic acid of the microorganism. Sterility by gamma irradiation is achieved mainly by the alteration of nucleic acid and preventing the cellular division.

The carrier was packed in thin-walled polyethylene bags and sterilized by gamma irradiation with the use of Cobalt 60 as the sterilizing/irradiation source. The sterilizing doses were varied to determine the optimum sterilization dosage at which the integrity of the sample will not be compromised, and the dosage that favoured complete or near complete elimination of pathogenic micro-organisms from the carrier produced was used.

Autoclave sterilization

The carrier was packed in thin-walled polyethylene bags, wrapped in foil paper to inhibit the penetration of moisture during the autoclaving process and placed in an autoclave to sterilize for 900 second (15 minutes) at temperature of 121^{o}C and pressure of 1.5 MPa.

Isolation of Micro-organisms

Nitrogen-fixing bacteria, *Bradyrhizobiumjaponicum* (USDA 110) strain was isolated from a pre-formed peat-based inoculant in the Soil Microbiology Laboratory of the Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria. One gram of the inoculant was weighed and transferred aseptically into 9ml sterile distilled water to give 10^{-1} dilution ratio. One millilitre of stock was transferred into a test tube containing 9ml sterile distilled water to obtain serial dilution of 10^{-2}. This was continued till a dilution of 10^{-6} was attained.0.1ml of 10^{-5} and 10^{-6} serial dilution ratios were pipetted and transferred aseptically onto sterile Yeast Mannitol Agar (YMA) plates which was produced in duplicate for each dilution ratio. A sterile bent glass rod (spreader) was used to spread the inoculum on the culture media using the spread plate method. The inoculated YMA plates were incubated at room temperature for 5-7 days. Isolates from colonies formed were aseptically collected and streaked on fresh YMA plates, produced in duplicate (i.e. two YMA plates for dilution ratio 10^{-5} and 10^{-6}) to reduce contamination and produce pure rhizobia strains.

Culturing process

500 ml of yeast mannitol broth was prepared without the addition of Congo red (pigment used while preparing YMA plates) and agar powder to prevent solidification. The broth was sterilized by autoclaving at temperature and pressure of 121^{o}C and 1.5 MP a respectively for 900 second, cooled and aseptically inoculated with strains of *Bradyrhizobiumjaponicum* (USDA 110), and kept in an orbital incubator for 8 -10 days at 65rev/min for proper agitation and growth of the strains.

Inoculation of Carrier

0.03 kg each of sterile carrier was packaged in duplicates; the moisture content was varied at 50%and 55% to determine the best moisture content required for the growth of inoculum in the carrier. The broth containing inoculum was introduced aseptically into the carrier by using 50ml sterile syringe; the pierced point of the syringe on the packaged carrier bag was sealed with stickers to prevent contamination. Thereafter, the broth-carrier mixture was agitated with hand for proper mixing.

Curing and Maturation

The properly mixed samples were kept in a sterile orbital incubator at 28^{o}C for 21days for maturation (Organic Fertilizers and Bio-ferments, 2010)

Re-isolation of Microorganism from the Newly Developed Inoculant

After curing and maturation period of 21 days, serial dilution (as explained in 2.4) at 10^{-10} dilution factor was carried out to ascertain and determine the growth of *Bradyrhizobiumjaponicum* (USDA 110) in the developed carrier.(Organic Fertilizers and Bio-ferments, 2010)

RESULTS AND DISCUSSION

Results

The tables below show the results obtained for various analyses carried out in the course of this research.

Table 1: Sample Microbial Contaminant Determination

Sample type	Unsterile (Cfu/ml)	Gamma Sterilized (2.5kGy)	Autoclaved
Bacterial Bioburden @ 10^{-3} serial dilution (Colonies)	38	26	0
Bacterial Bioburden @ 10^{-4} serial dilution (Colonies)	30	10	0
Bacterial Bioburden @ 10^{-5} serial dilution (Colonies)	22	5	0
Fungal Bioburden @ 10^{-3} serial dilution (Colonies)	14	7	0
Fungal Bioburden @ 10^{-4} serial dilution (Colonies)	8	3	4
Fungal Bioburden @ 10^{-5} serial dilution (Colonies)	6	2	0

Table 2: Rhizobium Growth in Sterile Peat and Developed Carrier

B. japonicum colonies Moisture Content	Sterile Peat (cfu/g)	Gamma Irradiated Carrier (cfu/g)	Autoclaved Carrier (cfu/g)
50% Moisture Content	$20x10^{10}$	$12x10^{10}$	$16x10^{10}$
55% Moisture Content	$35x10^{10}$	$25x10^{10}$	$30x10^{10}$

Discussion

After sterilization using two distinct methods, serial dilution was carried out on the samples to ascertain the sterility of the samples. Table 1 shows the bacterial and fungal count for unsterile, gamma-sterilized and autoclaved samples at dilution ratios 10^{-3}, 10^{-4} and 10^{-5} respectively.

From Table 1, at 25 kGy gamma irradiation dose, the bacterial and fungal contamination concentration was least; while for autoclaving, except for fungal contaminants of four colonies at dilution factor 10^{-4}, which could be due to external contaminations, the samples autoclaved were sterile. The gamma irradiation dose was not increased beyond 25 kGy so that the integrity of the sample would not be compromised considering the fact that it is a biological substance. The result of the serial dilution at 10^{-10} dilution factor carried out on inoculated samples after 21 days curing and maturation period is shown in Table 2.

Gram staining was carried out on isolates from each plate to authenticate the type of micro-organism that grew on the plate. All isolates after gram staining were gram negative**,** short rods when viewed under the microscope at 100x magnifications. This proved that the microbial growth on the plates and in the carrier were rhizobia. Results from gram staining of isolates

from the newly developed inoculant in comparison with the sterile peat are displayed in Plates 1-6.

Plate 1a: Peat Inoculant at 50% Moisture content

Plate 2a: Peat Inoculant at 55% Moisture content

Plate 3a: Gamma irradiated new inoculant at 50% moisture content

Plate 4a: Gamma irradiated new inoculant at 55% moisture content

Plates 1a and 2a show sterile peat inoculated with rhizobium culture of 50% and 55% moisture content respectively, while Plates 2a and 2b show the gram stained rhizobium colonies at 100 magnification.

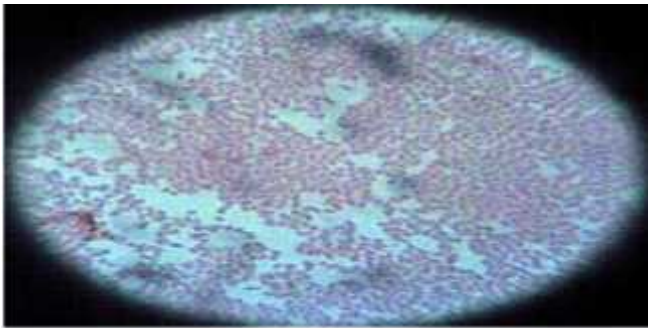

Plate 1b: Microscopic image of Rhizobium growth from 1a at 100 magnifications

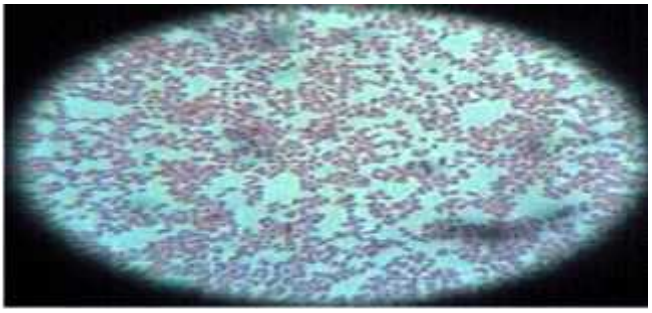

Plate 2b: Microscopic image of Rhizobium growth from 2a at 100 magnifications

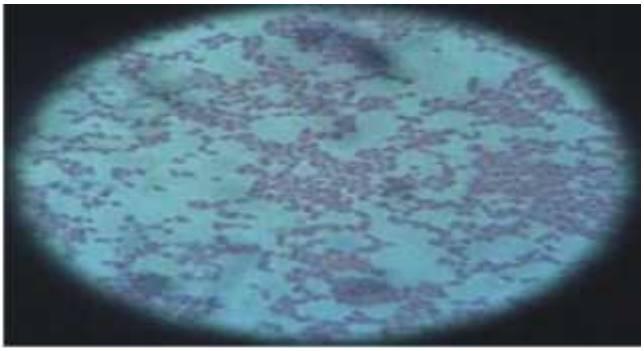

Plate 3b: Microscopic image of Rhizobium growth from 3a at 100 magnifications

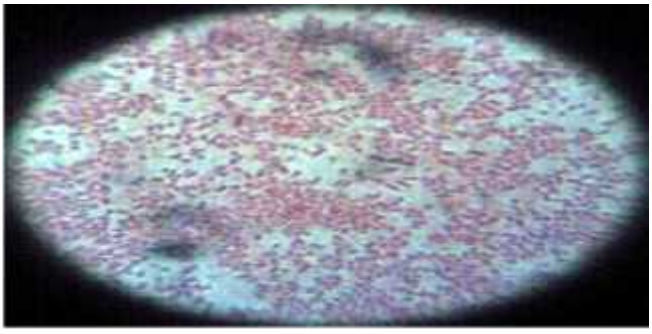

Plate 4b: Microscopic image of rhizobium growth from 4a at 100 magnifications

Plate 5a: Autoclaved New Inoculant at 50% Moisture content

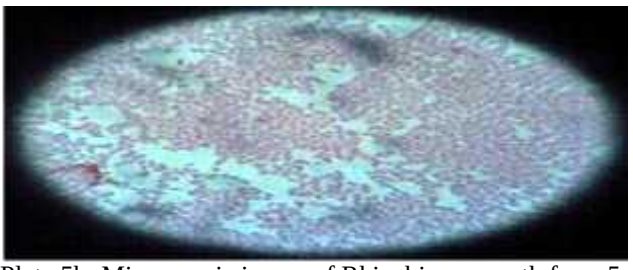

Plate 5b: Microscopic image of Rhizobium growth from 5a at 100 magnifications

Plate 6a: Autoclaved New Inoculant At 55% Moisture content

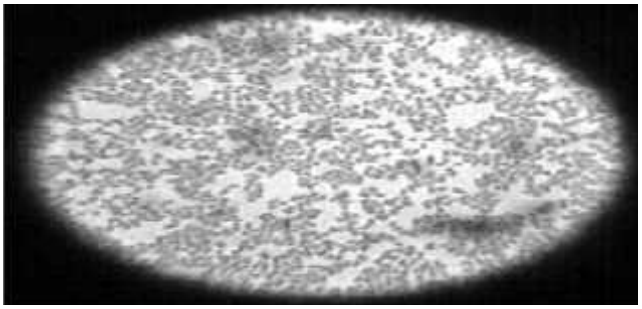

Plate 6b: Microscopic image of Rhizobium growth from 6a at 100 magnifications

Plates 3a and 4a show gamma-irradiated carrier inoculated with rhizobium culture of 50% and 55% moisture respectively, while Plates 3b and 4b show gram stained rhizobium colonies at 100 magnifications. Plates 5a and 6a show autoclaved carrier inoculated with rhizobium culture of 50% and 55% moisture respectively, while Plates 5b and 6b show gram stained rhizobium colonies at 100 magnifications.

The moisture content was varied between 50% and 55%. A significant difference in the rhizobium growth as depicted in the number of colonies counted in Table 2 was observed. Therefore, a moisture content of 55% is recommended while using this developed carrier.

CONCLUSION

Rhizobium bio-fertilizer carrier, which is a valuable product of economic importance for the agricultural sub-sector was produced using tannery solid wastes after removal of 99% chromium from the wastes. This new technology has the potential to eliminate the concept of chrome tanned leather shavings and trimmings wastes. In addition, it has exposed a potential income generating source for the tanning industry. The de-chroming process could be incorporated in tanneries as a treatment process for the solid wastes; thereby propelling the tanning industry towards near-zero waste and generating additional income.

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