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Biotechnology Application in Starch/Natural Rubber Polymer Degradation: Panacea for Rubber Waste Disposal and Utilization

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Abstract

Reckless burning of expired automobile tyres and used rubber products and their disposal at refuse dumps pose serious waste disposal and pollution problems. Incorporation of starch bio-filler into natural rubber, NR opens up better structural surface in rubber that provides microorganisms a platform to act or feed on rubber molecule, cis-1,4 polyisoprene and subsequent breakdown into smaller low molecular weight components. Starch was extracted from Polynesian arrow root crop(amora), Tacaleonto petaloid. powdered, gelatinized and converted into starch/natural rubber blend by direct mixing and co-coagulation. It was thereafter compounded in a two-roll mill according to American Society of Testing and Materials (ASTM), D3184-80 and cured using sulphur and accelerators method of curing. The starch/ NR composite samples were subjected to the activities of isolated Pseudomonas aeruginosa, Bacillus sp, and Bacillus megaterium carried out under a closed system using mineral salt medium (MSM)procedure. Biodegradation activity was found to be at maximum in sample F0(cured rubber without starch filler). It was discovered that bacteria samples isolated from a botanical garden in North-east Nigeria could not act on the rubber samples under study while the bacteria isolated from the Rubber plantation at Rubber Research Institute, (RRIN) Iyanomo, Edo State acted on the starch/ rubber samples with positive breakdown products. These bacteria isolated from RRIN has exhibited selective adaptation in their ability to degrade rubber samples under study. Probably some bacterial enzymes were involved in this activity which are yet to be investigated and is open for further investigation. In this regard, biotechnology application is a potential panacea for rubber waste disposal and utilization.

Keywords: Natural rubber; Starch; Latex; Co-coagulation; Bacteria.

Abbreviations: NR: Natural Rubber; ASTM: American Society of Testing and Materials; MSM: Mineral Salt Medium; RRIN: Rubber Research Institute; PAR: Polynesian Arrow Root; TNTC: Too Numerous to Count; HPC: Heterotrophic Plate Count; IBR: Institution Based Research; TETFUND: Tertiary Education Trust Fund

Introduction

Recently, biopolymers have attracted widespread and global interest due to popular environmental concern and challenges posed by the inability of petroleum derived synthetic polymers to degrade after their useful service life. This development has motivated various researchers to develop novel environment friendly biomaterials. More so, degradable polymer composites have long been considered part of the solution to the environmental and waste management problems posed by extensive use of non-degradable polymer materials, [1].One of the most commonly used approaches to create such degradable polymers is to incorporate micro-sized fillers such as starch or cellulose into synthetic polymers or natural polymers with a view to enhance their biodegradability, [1,2].Starch, a semi-crystalline natural polymer stored in granules as a reserve in most plants is renewable, non-toxic and completely biodegradable, Robyt (1987). According to ISO definition, [3]; biodegradation is the degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of the exposed material and resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents [4]. Natural rubber (NR) is a macromolecular isoprenoid, poly (cis-1,4-isoprene) and is synthesized by more than 2000 plant species mostly belonging to the Euphorbiaceous and by some fungi. Despite many studies on microbial rubber degradation during the last 7 decades, only very little is known and understood about the distribution of NR-degrading bacteria and the biochemical mechanisms of NR degradation. In order to analyse the phenomenon of polyisoprene

degradation, two soil samples from Eucalyptus Garden in Science laboratory Complex, Federal Polytechnic, Mubi and another from Rubber tree plantation in Rubber Research Institute of Nigeria (RRIN) Iyanomo, Edo state were screened. Isolated bacteria and fungi cultures were used for benchscale biodegradation assay using mineral salt medium procedure in a closed system while the rubber vulcanizate samples served as the sole source of carbon and energy for the test microorganisms.

Materials and Method

Local variety of the starchy crop 'amora' (Polynesian arrow root), Taccaleontopetaloides Kunze was harvested from a Cassava farm in Paiko village, Gwagwalada FCT, Abuja. Natural rubber latex (dry rubber content, 30%) was obtained from Rubber Research Institute of Nigeria (RRIN) Iyanomo, Edo state. Calcium Chloride, (BDA) was purchased from chemical laboratory store along Sapele road, Benin, Edo state. Compounding chemicals and additives (Zinc oxide, Stearic acid, Mercaptobenzothiazole, Tetra methylquinoline, Sulphur and Carbon black (industrial grades) was supplied by Parchem fine and specialty chemicals, UK.

Methodology

Preparation of Starch/Natural Rubber Blends

Starch was extracted from the 'amora' crop, Polynesian arrow root (PAR) according to the method of Vasanthan [5]. The gelatinized starch was prepared according to the method of [6] with starch content at 5-90 part per hundred rubbers (phr). The starch aqueous suspension was stirred at 900C in a water bath for 30 minutes until the solution became transparent. When the solution cooled to ambient temperature, a starch paste (gelatinized starch) was obtained. The gelatinized starch and the rubber latex (30% DRC) were mixed and stirred vigorously for 30 minutes to harmonize properly and then 10%wt Calcium Chloride aqueous solution was added to co-coagulate the rubber latex and gelatinized starch mixture. The coagulum was washed in water and passed through the rollers in the Lohashilpi sheeting machine and compressed into thin sheets with embossed ribbed pattern. The coagulum sheets were washed in water and allowed to drain off water droplets and then cut into thin shreds (short strands) with scissors, labelled and placed in a dry oven at 800C for 18 hours and prepared for compounding and vulcanization.

Compounding procedure

The formulation for the compounding of the starch/natural rubber blend is shown in table 1. The compounding was carried out in accordance with American society for testing and materials (ASTM) D3184-80 in a two-roll mill and was cured using Sulphur as curing agent, according to the formulation in (Table 1).

Table 1: Typical formulation for the starch/rubber blends in part per hundred rubbers (Phr).

Components	Quantity (Phr)
Natural rubber	100
Starch	0,5,10-90.
Zinc oxide	5
Stearic acid	2
MBT	3
TMQ	1
Sulphur	3
Carbon black	30

Key: MBT= Mercaptobenzothiazole (Captax), TMQ = Tetra methylquinoline.

Microbial degradation tests

Microbial degradation test was conducted according to the method described by [7,8]. Soil samples were collected from under the soil from Eucalyptus tree garden behind Science Laboratory Complex, Federal Polytechnic, Mubi, Adamawa State as a control and from *Hevea brasiliensis* Rubber tree plantation at Rubber Research Institute of Nigeria (RRIN), Iyanomo, with reference number, RRIN/NG 900, series 0001. These soil samples were used to isolate microorganisms from the soil which are then used for biodegradation assays. The starch/ rubber composites made from amora filler at 5 phr, 30 phr and 90 phr plus an unfilled rubber (Control) were used as test specimens for the biodegradation test and are identified as F5, F30, F90 and Fo respectively.

Preparation of mineral salt media (MSM)

Mineral salt media was prepared according to [9,10]. The composition is as follows: KH_2PO_4-1.0g, K_2HPO_4-1.0g, NH_4NO_3-1.0g, $MgSO_4.7H_2O$ -g0.2g, $FeCl_3$- 0.05 g, $CaCl_2.2H_2O$- 0.02 g. They were incubated at 37°C for 24 hours and observed for colony formation. A control was set up; a test tube with a 9cm^3 of MSM without any carbon source (rubber specimens) but inoculated with the bacterial strains and incubated for 24 hours and then was used to inoculate an agar-agar plate (neutral agar) and incubated at 37°C for 24 hours and observed for colony formation.

Serial Dilution Procedures

Serial dilution procedures were employed according to [9], ISO 4833, (1991). The serial dilution was carried out at two consecutive dilutions according to (ISO 4833, 1991). The plates containing fewer than 300 colonies at two consecutive dilutions were used to calculate the results from a weighted mean. The number (N) of colony forming unit per ml (Cfu/cm^3) of test samples was calculated from the formula

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N = C/V (n1 + 0.1n2) d.

Where: C is the sum of colonies on all plates counted

V is the volume of inoculum (bacteria strain) applied to each plate

n1 is the number of plates counted at the first dilution

n2 is the number of plates counted at the second dilution

d is the dilution from which the first count was obtained.

The number (N) gives the viable number of cells in the colonies (ISO 4833,1991).

Gram-Stain Differential Technique

Gram-stain differential technique was employed according to [11] to differentiate the bacteria into Gram-positive and Gram-negative bacteria. The slide was finally observed in an oil immersion objective microscope (Olympus) at 100x magnification.

Results of biodegradation assays

Table 2,3, Figure (1-3)

Table 2: Viable Number of Cells (N) CFU/mLs

Duration phase	24 hours (Lag phase)	48 hours	72 hours
F_o	320	280,909	427,272.7
F_5	340	454,545.5	380,000
F30	300	453,636.4	340,909
F90	150	274,545.5	418,181.8

Key: CFU = Colony forming unit

Table 3: Viable Number of Cells (N) in Logarithm Value

Duration Phase	24 hours (Lag Phase)	48 hours	72 hours
F_o	2.51	5.45	5.63
F_5	2.53	5.66	5.58
F30	2.48	5.66	5.53
F90	2.18	5.44	5.62

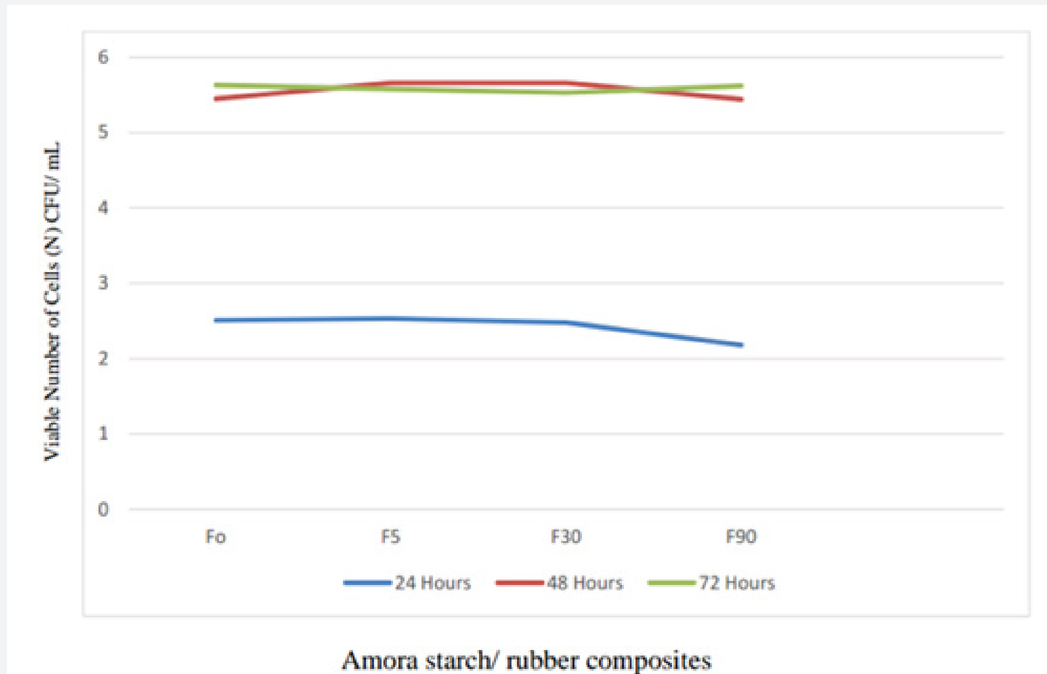

Figure 1: Plot of Viable Number of Cells (N) CFU/mL against No. of Hours

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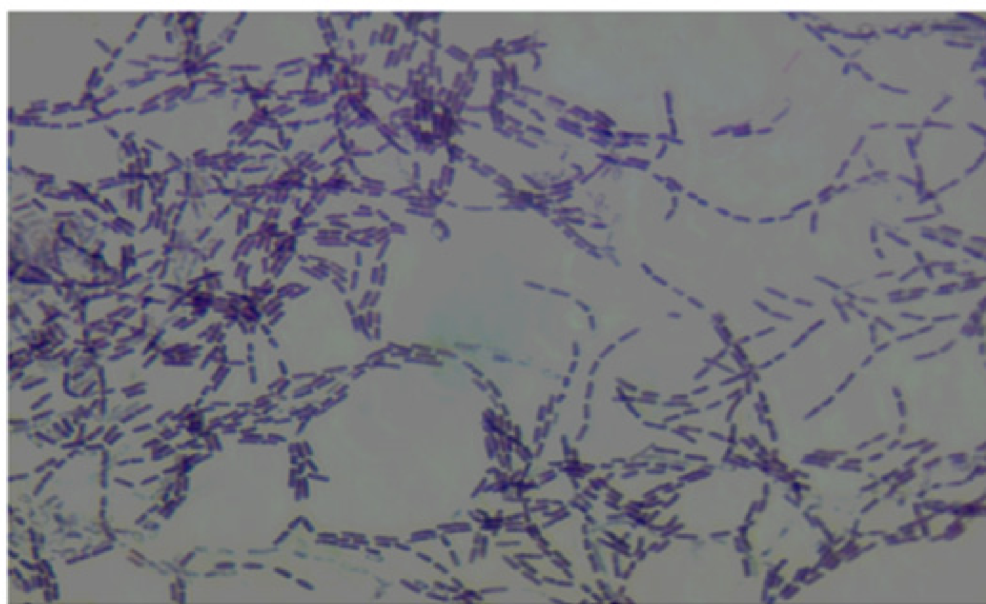

Figure 2: A slide of Gram stain reaction showing two Gram positive bacteria in a mixed culture, Bacillus species (+), Bacillus megaterium (+)

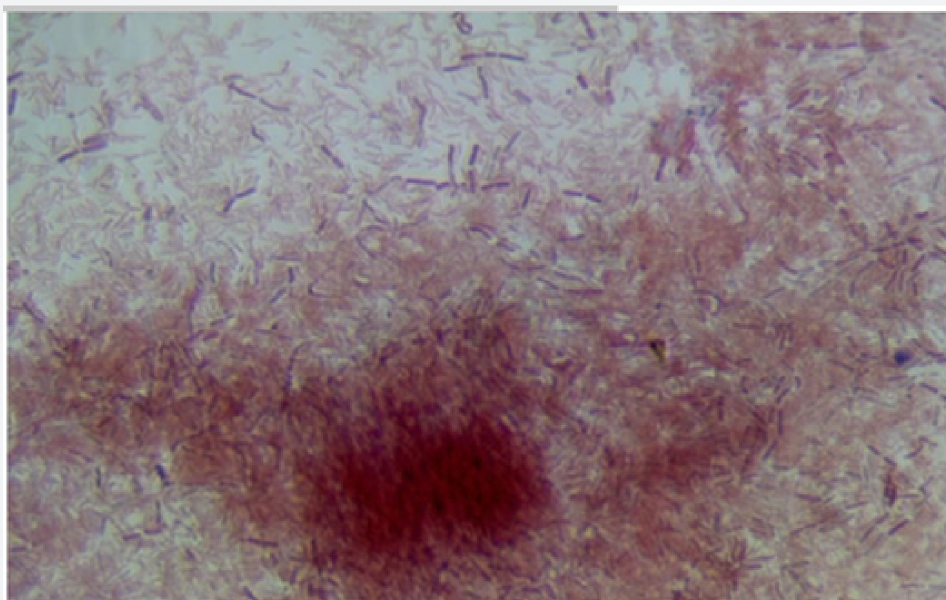

Figure 3: A slide of Gram stain reaction showing two bacteria in a mixed culture, Pseudomonas aeruginosa (–), Bacillus megaterium (+)

Microbial Degradation Assays

The two mixed cultures, spherical colony bacteria and filamentous colony bacteria were introduced to the starch-filled rubber, F5, F30, F90 and unfilled rubber, Fo in a mineral salt medium as a sole source of carbon and energy. Direct inoculation and serial dilution with 0.1ml inoculum on agar plates produced bacteria colony by both mixed cultures. The spherical colony bacteria type produced colonies above 300 at 24 hours after incubation (too numerous to count, TNTC) and therefore its growth pattern could not be monitored and was not used but rather filamentous colony type with less than 100 colony counts at 24 hours inoculation was used to monitor the colony growth pattern, Heterotrophic Plate Count (HPC) (ISO 4833, 1991). Table 2 shows the colony counts calculations for the bacteria growth. Unfilled rubber composite (without starch filler) Fo and amora starch/ natural rubber composite at 5phr (F5), 30 phr (F30) and 90 phr (F90) filler addition were used as test starch/ rubber

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specimen for biodegradation test. According to Heterotrophic plate count (HPC) ISO 4833 (1991), unfilled rubber, Fo (cured rubber without starch) yielded viable number of cells (N) which increased steadily from 320 at the lag phase to 427,272.7 after 72hours under serial dilution. The presence of protein and lipids in the unfilled cured rubber sample Fo served as nutrient source for the bacteria in addition to the rubber molecule. Since there was no starch in this specimen, the microorganism will likely exhaust the protein and lipids before feeding on the rubber which is a much heavier molecule of high molecular mass, hence the observed cell multiplication. More so, the bacteria are still in their exponential phase in the closed system after 72hours under serial dilution. Specimen F5 (rubber filled with 5phr starch) yielded growth of cells from 340 at lag phase to 454,545.5 after 48hours and then declined after 72hrs to 380,000. This decline is probably since the bacteria has entered its stationary phase having exhausted the available nutrients in the medium probably from lipids, protein and starch in the rubber composite [12-20].

Specimen F30 (rubber filled with 30 phr starch) yielded growth of cells from 300 at lag phase to 453,636.4 at 48hrs and then declined to 340,909 at 72hrs. This decline after the exponential phase indicates that the bacteria has entered its stationary phase having exhausted the available nutrients, starch, lipids, and protein, in the rubber composite or that the presence of waste product generated has made conditions of growth unfavourable.

Specimen F90 (rubber filled with 90 phr starch) yielded growth of cells 150 at lag phase and increased to 274,545.5 at 48hrs and then continued to increase till 418,181.8 at 72hrs according to Table 2. In this situation, starch is in the continuous phase and rubber in dispersed phase while the bacteria are still in the exponential phase of growth having enough nutrients to feed on like starch, protein and lipids which are more easier to digest than high molecular mass rubber molecule. Alternatively, the viable number of cells(N) in logarithm value are also displayed in Table 3 showing similar growth pattern.

According to the graph in figure 3, there was increase in the growth of the microorganism according to colony count from lag phase hours to 48 hours and then to 72 hours respectively for F0 and F90 and then with a decline at 72 hours for F5 and F30. The control set up containing the inoculum inside the mineral salt medium (MSM) only without any carbon source (rubber vulcanizates) did not produce any bacteria colony on the agar plate showing that rubber vulcanizates is the only source of carbon and energy in the test experimental setup. (ISO 4833,1991).

Bacterial Degradation/ Identification Tests

The test bacteria were identified to be in a mixed culture in both figure 1 & 2 and was identified by biochemical tests as indicated in (Table 4). The organisms are as follows:

- *Pseudomonas aeruginos a* (-), Gram negative bacterium
- *Bacillus megaterium* (+), Gram positive bacterium
- *Bacillus spp.* (+), Gram positive bacterium

Table 4: Biochemical characterization of isolates from nutrient agar slants

	Bacterial Isolates		
Tests	***Bacillus megaterium***	***Bacillus* spp.**	***Pseudomonas aeruginosa***
Gram reaction	+	+	-
Motility	Motile	Motile	Motile
Spore shape	Oval	Oval	NA
Spore position	Central spore	Central spore	NA
Swelling of bacillary body	-	-	NA
Growth at 45°C	-	+ +	
Growth in 7 % NaCl	+ +	-	
Utilization of Citrate	+ +	-	+ +
Glucose	+ +	+ +	+ +
Arabinose	-	-	
Mannitol	-	-	+ +
Xylose	-	-	+ +
Indole	-	-	-
Urease	+ +	-	+ +
Gelatin hydrolysis	+ +	-	+ +
Oxidase			+ +
Pigment			+ +

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Growth at 5°C			-
Growth at 42°C			+ +
Growth on MacConkey			+ +
Lactose			-
Maltose			-
Salicin			-
Sucrose			-

Key: (+) Gram positive bacteria

(-) Gram negative bacteria

+ + Positive growth

- - No growth

t4The bacteria worked together in a mixed culture to feed on the starch/ rubber composites as indicated by the colony growth calculations that showed the viable number of cells (N) (ISO 4833,1991) in Table 2 & 3 respectively. In figure 3, *Pseudomonas aeruginosa*, a Gram-negative rod and *Bacillus megaterium*, a Gram-positive rod worked in a mixed culture to feed on the starch/ rubber composite while *Bacillus megaterium* and *Bacillus sp* in figure 4 worked together in a mixed culture to feed on the starch/ rubber composite in the mineral salt medium as indicated by the colony growth calculations showing the viable number of cells (N) in the colonies. This observation is supported by the work of [7] where *Pseudomonas aeruginosa* produced holes in polymer films when used as the sole source of carbon and energy on a solid mineral sal ts medium.

Biochemical characterization of isolates.

Results of biochemical characterization of bacterial isolates are displayed in Table 4 above. Bacillus megaterium and Bacillus spp are both Gram positive bacteria while Pseudomonas aeruginosa is Gram negative. All the three bacteria are motile. The spores of Bacillus megaterium and Bacillus spp are Oval in shape, except for Pseudomonas aeruginosa. Three bacteria species showed positive reaction in glucose. The distinguishing test of positive reaction(growth) in MacConkey agar was confirmed for Pseudomonas aeruginosa as a Gram-negative bacterium [21-27].

Conclusion

Biodegradation activity of the mixed bacterial culture of Pseudomonas aeruginosa, Bacillus megaterium, and Bacillus spp in the degraded residue of rubber product having been established, has opened a new application for utilizing biotechnological processes in rubber waste disposal and control. The newly formulated PAR starch/natural rubber biopolymer can with little fortification be utilized as a tire thread material in automobile tire manufacturing.

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