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RESEARCH ARTICLE

EFFECT OF STARCH ACETYLATION ON BIOPLASTICS- DEGRADING MICROORGANISMS: A POINTER TO ASSESSING BIODEGRADATION

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ABSTRACT

The substitution of the hydroxyl groups with acetyl groups into starch moiety to increase hydrophobicity is geared toward enhancing the functional properties of starch for bioplastics production. The fear for reduced biodegradability of the products however, informed the isolation and identification of starch acetate based-bioplastics degraders in soil as a pointer for acetylation effect on degradation. Native starch and starch acetates from cassava, maize and potato were used for the production of bioplastics according to the composition of 45%, 60%, 75% and 90% respectively. Bioplastics measuring 2.5cm x 1.5cm x 0.5cm from each bioplastics composition was buried. Rate of degradation was estimated at intervals of 1week. Soil from the decomposed bioplastics were placed on nutrient and potato dextrose agars for bacteria and fungi isolation and incubated at 30^0C for 2hrs. Discrete colonies were transferred to nutrient and potato dextrose agars for 24 and 72hrs to produce pure cultures of bacteria and fungi. Gram staining and biochemical tests were done while fungi mycelia were stained with lactophenol blue for microscopy. Our results revealed that there was significant effect ($P < 0.05$) of starch acetylation on the degradation of bioplastics. *Pseudomonas spp, Bacillus anthrax, Corynebacterium diphtheria, Micrococcus, Klebsiella spp and Clostridium spp* were bacteria species isolated while the isolated fungi species were *Aspergillus niger, Mucor spp, Alternaria spp, Chanophora cucurbitarum* during the preliminary soil analysis. After the burial test, bacteria identified as colonizing the bioplastics were as follows; *Pseudomonas spp, Bacillus anthrax, Micrococcus, Klebsiella spp and Clostridium spp* while *Aspergillus niger, Mucor spp, Alternaria spp, Chanophora cucurbitarum* were the identified fungi and implicated for the degradation, which was confirmed through *in vitro*. The delayed degradation observed in our result may not mean jettisoning the idea of acetylating starch, especially *in planta* of starch-producing plant for bioplastics production comparing the degradation problems of synthetic plastics, which had led to finding alternatives. Our results explicitly suggest that though using acetylated starch for bioplastics production might have a little degradation problem, the microorganisms isolated had the capacity to degrade them in a very short interval of weeks.

INTRODUCTION

The development of innovative biopolymers has been underway for years, and is currently attracting much attention from both researchers and scientists. Biopolymers are important source of materials, which possess high chemical versatility and have great potential for a wide range of industrial applications. In the recent past, many research trials have reported biopolymers of natural origin that degrade effectively over controlled period of time producing degradation products with low toxicities. The production of biodegradable plastics from biopolymers which can easily be decomposed into carbon dioxide, methane and biomass following enzymatic action of microorganisms when disposed is thus quite significant (Udensi *et al*., 2009).

However, biopolymers like starch present some drawbacks such as the strong hydrophilic behaviour (poor water barrier) and poorer mechanical properties than the conventional non-biodegradable plastic films used in the food packaging industries (Hernandez- Muaoz & Kanavouras, 2003; Halley, 2002). It then means that if starch must meet the desired quality, modification such as acetylation becomes imperative. The whole essence of starch acetylation is geared towards attaching acetyl groups to the α-glucan into starch either *in situ* or *ex situ* through genetic or chemical substitution of hydroxyl groups with acetyl groups in order to synthesize starch acetates. Though there are fears in different quarters pertaining to the chemicals used during acetylation, the rigours involved in plant transgenesis and the subsequent production of transformants carrying the desired genes is worth considering before embarking on pilot experiments in *in planta* acetylation. The whole essence of acetylation is geared towards attaching acetyl groups to the α-glucan in starch-producing plants via genes encoding enzymes (*in planta* acetylation) or through chemical substitution of hydroxyl group with acetyl groups from acetic anhydride into starch, in order to produce starch acetates. Firouzabadi *et al.*

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(2007) reported that there may be opportunities for *in planta* starch acetylation but there are several limiting factors that may clog the successful process. However, the fear here is the possibility of delayed microbial degradation of the produced products occasioned by acetyl group incorporation thereby making nonsense the purpose of bioplastics production.

The capacity of microorganisms to degrade and assimilate organic matter depends on their ability to produce enzymes needed for degradation of the substrates. Wide range of prokaryotes produces amylases, which enable them, degrade starch. Modelli *et al.* (2004) investigated the extent and rate of degradation of flax fibers after acetylation. In vitro biodegradation was undertaken by exposing the fibers to a pure culture of *Cellvibrio fibrovorans*. The degradation rate of acetylated fibers in soil nearly equals that of unmodified fibers, whereas in the pure culture, acetylated fibers biodegrade more slowly than native fibers.

The thrust of this research paper is hinged on assessing the impact of starch acetylation on degrading bacteria and fungi and the rate of degradation of bioplastics produced from these acetates, which will help in furthering the protocol for *in planta* acetylation

MATERIALS AND METHODS

Acetic anhydride, glacial acetic acid, tetraoxosulphate (vi) acid (H_2SO_4) of technical grade were purchased from BOH, Ltd. Poole, England. Cast iron fillings were obtained from the Nigerian Metallurgical Institute, Onitsha, Anambra State, Nigeria. Tubers of cassava (TMS 30555) were obtained from the Agricultural Development Programme (ADP) Calabar while maize and potato were bought from Watt market, Calabar, Nigeria.

Preparation of starch acetates (acetylation of starch)

The starches were processed according to Udensi *et al.* (2009) while acetylation protocol was according to Aziz *et al.* , 2004. About six thousand five hundred grams of glacial acetic acid was mixed with 400g of acetic anhydride in a fume cupboard. Two hundred gram of concentrated tetraoxosulphate (vi) acid (H_2SO_4) was then added to the mixture before transferring into a glass lined cast iron agitated acetylator. The mixture was cooled to 70^0C and about 1400g of each starch sample was added separately at each round of reaction. The whole mixture was allowed to stand for 8 hours while keeping the temperature at 45^0C. The resulting viscous fluid was diluted with equal parts of concentrated acetic acid and10% H_2SO_4, and allowed to stand for 15 hours at a temperature of 38^0C. During this process, hydration of some of the acetic groups occurred. This was controlled by pouring the resulting mixture in large volume of water and the precipitate obtained was centrifuged for 3000 revolution per minutes (rpm) for 15 minutes to separate the liquid further. The precipitate was washed, dried and kept safely for further analyses. The acetylation process was carried out using acetic anhydride at 68% and 89% concentrations, respectively. To ascertain whether there was complete acetylation of the starches, about 20g of the different starches was added to 100ml of acetone separately. Complete dissolution indicated complete acetylation.

Protocol for bioplastics production

The formulations and protocols for the production of biodegradable plastics were according to Udensi *et al.* (2009) with few modifications (Table 1). The formulations contained 45%, 60%, 75% and 90% native starch and starch acetates from the different starch sources. Gelatin, agar and sorbitol were weighed according to the specific composition using a Mettler balance and then dissolved in 70ml of distilled water. 100ml and 150ml of glycerol from the stock solution was added into the mixture and stirred thoroughly for complete dissolution for each round of production. The mixture was loaded into an oven (Continent, MW800G) at a regulated temperature of 80˚C. At intervals of about 30 seconds, the process was interrupted while the contents were stirred to forestall solidification at the bottom. It was removed from the oven when it began to simmer. The plastics produced were poured into a non- stick baking pan and allowed to dry in an oven (Multioven, Japan) at about 150˚C. This protocol was followed for all the compositions and different starch samples.

Table 1: Bioplastics recipes

Bioplastics Additives	45% starch composition	60% starch composition	75% starch composition	90% starch composition
Starch (g)	8.0	14.6	29.2	83.0
Gelatin (g)	3.0	3.0	3.0	3.0
Agar (g)	3.2	3.2	3.2	3.2
Sorbitol (g)	3.5	3.5	3.5	3.5
Glycerol (ml)	100;150	100;150	100;150	100;150
Water (ml)	70	70	70	70

Evaluation of the rate of degradation of the bioplastics (Soil burial test)

The rate of degradation was evaluated according to the method of Udensi *et al*. (2009). The bioplastics produced were divided into tiny squares of $1mm^2$ each. A rectangular hole of 1.4cm x 0.5cm was dug on the ground where the bioplastics were placed and covered completely with soil. The soil was first analyzed before the burial test. The percentage degradation was determined as the number of degraded portion per week/ total number of small squares multiplied by 100.

Isolation and identification of bioplastics-degrading bacteria and fungi

Soil samples were collected from the experimental sites where the bioplastics were to be buried and analyzed. About 10g of the soil samples was suspended in 90ml of sterile distilled water and mixed properly. Serial dilution was done 3 times with sterile distilled water. 0.1ml of the diluted soil samples were spread on nutrient agar plates and incubated at 30^0C for 2 hours for culturing bacteria while fungi isolation was done through direct plating where 2g of the soil samples were placed on potato dextrose agar (PDA) plates fortified with 0.1mg/ml streptomycin sulfate, using a sterilized glass spreader. The samples were incubated at room temperature for about 3 days. Discrete colonies were transferred to nutrient agar slant for bacteria and potato dextrose agar for fungi to produce pure cultures for 24 and 72 hours, respectively. After the isolation of bacteria and fungi in the different experimental sites, the bioplastics were cut into shape of 2.5cm x 1.5cm x 0.5cm and buried in the different sites. The buried bioplastics were observed for decomposition. At the point decomposition, the bioplastics were retrieved and plated directly on nutrient and potato dextrose agars for the isolation and identification of bacteria and fungi, respectively.

Gram staining was done for the isolated bacteria species and confirmatory biochemical tests: Catalase, citrate utilization, indole, oxidase, sugar fermentation and coagulase tests were carried out according to Cheesbrough, (2005) to confirm the species of bacteria isolated. *In vitro* experiment was mounted where the produced bioplastics were incubated with pure cultures of the isolated microorganisms.

Data collection and analysis

The total bacteria and fungi counts (TBC and TFC) were collected at the end of the experiment and subjected to the analysis of variance (ANOVA) while Least Significant Difference (LSD) was used to separate significant means (Obi, 2002).

RESULTS

Rate of degradation of the bioplastics

Soil analysis results tests revealed that the soil had a pH value of 7.42, a nitrogen content of 0.15%, carbon content of 2.32%, and

Pseudomonas spp, Bacillus anthrax, Corynebacterium diphtheria, Micrococcus, Klebsiella spp and Clostridium spp were bacteria species isolated while the isolated fungi species

Fig. 1: Total bacterial count (TBC) of bioplastics produced from native and acetylated starches during bioplastics burial test (5 weeks). (A = Acetylated; N = Native; S = Starch; C = Cassava; M = Maize; P = Potato).

Fig. 2: Total fungal count (TFC) of bioplastics produced from native and acetylated starches during a 5 weeks burial test. (A = Acetylated; N = Native; S = Starch; C = Cassava; M = Maize; P = Potato).

was 23.10% at a temperature of 25˚C. It was observed after the first week of burial test that the percentage starch composition and the level of plasticization of the bioplastics produced significantly affected ($P < 0.05$) the rate of biodegradation in soil. As the percentage of starch and level of glycerol in the bioplastics increased, the rate of biodegradation in soil also increased. The rate of biodegradation for the other weeks was almost the same as in the first week. There was significant ($P < 0.05$) effect of starch acetylation on rate of biodegradation of bioplastics which was evidenced in the delayed degradation process of bioplastics produced from starch acetate (Figure 3).

Total bacteria and fungi counts (TBC & TFC).

FIG. 3: Rate of biodegradability of bioplastics produced from native and starch acetates during a 5 weeks burial test. (A = Acetylated; N = Native; S = Starch; C = Cassava; M = Maize; P = Potato).

cucurbitarum during the preliminary soil analysis. After the burial test, bacteria identified as colonizing the bioplastics were as follows; *Pseudomonas spp, Bacillus anthrax, Micrococcus, Klebsiella spp and Clostridium spp* while *Aspergillus niger, Mucor spp, Alternaria spp, Chanophora cucurbitarum* were the identified fungi. During *in vitro* result showed that the biodegradable plastics produced from the native starch and acetates were observed to undergo deformation, deterioration and subsequent decomposition, which however delayed in the acetate produced bioplastics.

Starch source (cassava, maize and potato) did not significantly ($P > 0.05$) affect microbial degradation rather percentage starch composition significantly ($P < 0.05$) enhanced the rate of microbial colonization and subsequent degradation. This is evidenced in the total bacteria and fungi counts (TBC & TFC) result (Figures 1 & 2). Plasticizing bioplastics with 100ml of glycerol did not significantly enhanced degradation rate. However, when the bioplastics were plasticized with 150ml of glycerol , degradation rate was significantly affected ($P < 0.05$)

DISCUSSION

Rate of degradation and bioplastics degraders

Our result revealed that microbial colonization was much lower on starch acetate-produced bioplastics than bioplastics from native starch. This seems to suggest that substitution of hydroxyl radicals with acetyl groups into starch moiety affected microbial colonization, which might have affected the rate of biodegradation of bioplastics in soil. The present study is a further confirmation of the report of Udensi *et al.* (2009) that starch source does not play any significant role in the determination of biodegradability rate. It was the fear that since acetylation of starch is presumably to increase its hydrophobicity; it might affect the rate of biodegradation. Expectedly, the result showed that acetylation did significantly ($P < 0.05$) affected the rate of biodegradation. It is probable that though the substitution of acetyl groups into glucose moiety in the acetates may have been weakly bonded due to the degree of substitution, leading to the ease with which microbes dislodged

the acetyl attachment, degradation of bioplastics was however retarded. The mechanism of attack is such that after the colonization by microorganisms, depending on the moisture level in the degrading environment, bioplastics degrader's enzymes are secreted on the bioplastics surface, which commenced the digestion process with the resultant effect of loosening the weakly bonded acetyl groups; leading to increased degradation.

The isolated bacteria and fungi species in this research corroborated with the reports of Nishide *et al*. (1991) and Mergaert *et al.* (1993). Though the species isolated and identified were not exactly the same, the differences might have been due to environmental factors inherent in the domain of research and the base of the bioplastics. Incidentally, there were some fungi such as *Alternaria spp,* and *Chanophora cucurbitarum* that are known to cause leaf spot disease of *Telfairia occidentalis* but possess degradation potential. It does imply that any microorganisms having the genes encoding the degradation enzymes for bioplastics and other plastics will inevitably degrade them, the environment of isolation notwithstanding. The higher the percentage of starch composition in the bioplastics, the more substrates the microbes see as energy source thus increasing the influx of microorganisms to them. These according to Mergaert *et al*. (1993), Poirier *et al*. (1994), and Lee (1996) might have been responsible for the increased rate of bioplastics degradation observed in the 90% bioplastics materials. The mechanism of attack especially for bacteria, is such that after the colonization by microorganisms, depending on the moisture level in the degrading environment, bioplastics-degrading enzymes are secreted on the bioplastics surface, which commenced the digestion process with the resultant effect of loosening the weakly bonded acetyl groups; leading to degradation. For the fungi, the mycelia envelope the bioplastics surface like blood capillaries on the skin thereby secreting bioplastics-degrading enzymes, probably amylases on the surface. This enzyme digests the substrate, absorb and assimilate the nutrients saprophytically, leading to their growth and development. Doi *et al*. (1994) and Mergaert and Swing (1995) asserted that significant degradation occurred only after colonization of the bioplastics by microbes. This underscores why 90% starch composed bioplastics had the highest TBC and TFC (Figs. 1 & 2). Plasticization increased microbial colonization with the concomitant increase in degradation. This is in agreement with the report of Stevens (2002). It was revealed that microbial colonization was much lower on starch acetates-produced bioplastics than on bioplastics produced from native starch (Figure 2). It seems to suggest that substitution of acetyl groups for hydroxyl radicals in the starch moiety due to acetylation affected microbial colonization, which also might have affected the rate of biodegradability of bioplastics in soil.

The delayed degradation observed in our result may not mean jettisoning the idea of acetylating starch, especially *in planta* from starch-producing plant for bioplastics production comparing the degradation problems of synthetic plastics, which had led to finding alternatives. Our results explicitly suggest that though using acetylated starch for bioplastics production might have a little degradation problem, the microorganisms isolated had the capacity to degrade them in a very short interval of weeks.

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