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RESEARCH ARTICLE

COMPARATIVE STUDIES OF MICROBIAL PRODUCTION OF POLY B-HYDROXY BUTYRATES AS BIOPOLYMER USING *BACILLUS* SP *MEGATERIUM* SP, *ALKALIGEN* SP AND *CLOSTRIDIUM* SP ISOLATED FROM TANNERY SLUDGE RESERVOIR

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ABSTRACT

An investigation was undertaken to isolate poly β hydroxylbutyrate (PHB) producing bacteria from Tannery waste sludge reservoir In Mario Jose Kano. A total of six isolates were purified, only three sp were selected for the production of PHB viz: *Alkaligen* sp *Bacillus megategarium* and *Clostridium* sp. PHB extraction was carried out by sodium hypochlorides digestion method. The effect of mutant on different bacteria using different UV – light radiation exposure were carried out on three bacteria sp base on time duration (5,10, 15, 20 and 30 min) which shows a significant reduction of growth with increase in time of UV light exposure. The culture conditions have been investigated at optimum conditions. *Alkaligen* sp and *Bacillus megaterium* were found to be most efficient PHB producers. Maximum production of PHB yellow purple "Dorowa" (*Pakia biglobosa*) was shown by *Alkaligen* sp (2.02 g/g using dried cell weight at 2.80 g/l) and *Bacillus megaterium* (1.52 g/g using dried cell weight at 1.93 g/l) yield and dried cell weight respectively. Thus, the present data indicate that yellow purple (*Pakia biglobosa*) could be alternatively used for PHB production. Use of bioplestic produced by these organisms, will help to reduce environmental pollution.

INTRODUCTION

Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non – biodegradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic material (Porier *et al.,* 1995). One option is to produce truly biodegradable polymers, which may be used in the same application as the existing synthetic polymers. These materials, however, must be processible, impervious to water and retain their integrity during normal use but readily degradable in a biologically rich environment. A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganism, to carbon dioxide, water and humic material. Biodegradable material under development viz; polylactides polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co – polymers and/ or blends (Steinbuchel 1991). Amongst these PHAs are of particular interest because they posses thermoplastic characteristic and resemble synthetic polymers to a PHAs are among the most investigated biodegradable polymers in recent years. They are superior to other biodegradable polymers because of the large number of different monomers constituents that can be incorporated. At present, about 150 different hydroxyl alkanoate units have been identified. The most common is poly hydroxybutyrate (PHB) (Steinbuchel and Valertin, 1995). Polyhydroxy butyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatible in the medical (Mona *et al*., 2001). The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastic with properties similar or superior to petrochemical plastic (Apostolis *et al.,* 2006).

Poly β – hydroxybutyrate (PHB) is an intracellular reserve polymeric compound found in many bacteria. The polymeric ester was originally discovered by Lemoigne in (1926) as a major component of *Bacillus mega terium* (Aderson and Dawes, 1990). It serves as a source of carbon and as principles substrates for the endogenous respiration of certain aerobes (Chandrashekhaiah, 2005). PHB and other poly (β – hydroxyl

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alkanoates) (PHAS) are of industrial interest because of their possible use as biodegradable plastic. In fact, a copolymer of PHB, β - hydroxy bytyrate and B- hydroxyl valerate are being produced by ICT Ltd USA under the trade name Bipol using eubacterium *A. eutrophus* (Dol, 2001). The commercial production of PHB has been using relatively cheap substrates such as methanol (Suzuk *et al*., 1986). Feed molasses ethanol (Alderet *et al.,* 1993; Senthil Kumar and Prabhakaran, 2006), starch and why (Kim, 2000). Cane molesse as a sole carbon source. (Mona *et al.,* 2001) wheat hydrolysate and fungal extract (Apostolis *et al.,* 2006) or soy cake. (Fabiane *et al*., 2006). *Parkia biglobosa* can be found in a belt stretching from Atlantic coast in Senegal to southern Sudan and northern Uganda (Thiombiano *et al*., 2012), the tree currently exist within a wide range of natural communities but is most abundant in anthropic communities places where cultivation is semi-permanent (Janck, 2008). Annual production of seeds in northern Nigeria is estimated at around 200,000t, while the products of the trees are not common in international trade (Ntui *et al*., 2012)

Yellow purple or yellow pericarp which contains the seeds it is naturally" sweet" and is processed into a valuable carbohydrate food known as "Sikomu and dodawa" among the Yoruba and Hausa people of Nigeria respectively (Olaniji, 2013). The yellow pericarp which is known as waste from *Parkia biglobosa* is naturally and highly rich in glucose (69%), while the most valuable parts of the locust bean are the seeds themselves which are high in lipid (29%), protein (35%) and carbohydrate (16%) and is a good source of fat and calcium for rural dwellers (Gbolagunte, 2003) and (Ntui,2012) respectively. The aim of this study is to used yellow purple (Dorowa) which is available in Nigeria with regard to its high natural glucose content, cheaper and affordable as an alternative to the conventional glucose in production of PHB.

MATERIALS AND METHODS

Collection of sample

Samples were collected from effluent sludge waste Mario Jose tannery within Chalawa industrial estate located at kumbotso local Government area of Kano state Nigeria. Investigations were carried out at Nigerian Institute of leather and Science Technology (NILEST) and National Research Institute for Chemical Technology (NARICT) Basawa Zaria respectively. On the isolation of poly β-hydroxybytyrate PHB producing bacteria from different sources and their screening for maximum PHB production. The culture parameters were optimized for the selected effluent strains

Isolation of predominant bacteria species from effluent sludge waste

Ten ml of the effluent sludge waste was transferred in 90 ml of distilled water and aseptically serial dilution was performed. Ten ml of the effluent sludge suspension was pepitted into 90 ml water blank number 2 to make up 1:100 (10^{-2}) and another 10 ml of soil suspension was pipette into 90 ml water blank number 3 to make up 1: 1000 (10^{-3}). Further dilution were repeatedly carried out for (10^{-4}) to 10^{-6} respectively. 0.1 ml aliquot was aseptically transferred into freshly prepared nutrient agar medium. The plate were incubated at 32^{o}C for 48 h. emerging bacterial colonies were picked and aseptically sub cultured into freshly prepared nutrient agar (NA) and incubate to obtain pure isolates. These isolates were then kept on (NA) slant at 4^{o}C for further use (Adawiah, 2008).

Colony characterization

i The colony characters viz – shape, colour and polysaccharide production were observed on agar medium, simple staining, Gram staining spore staining II.
ii. Biochemical Tests: Starch hydrolysis, Gelatin liquefaction, casein hydrolysis hydrogen sulfide test catalase test, urease test.
iii physiological characterization: growth at 40 ^{o}C, Growth at 41 ^{o}C citrate utilization test and indole test production were carried to ascertain the probable respective organisms (Adawiah, 2008).

Fluorescent staining method

Detection of PHB production in the isolates was also done the following fluorescent staining method using acridine orange, as suggested by Senthilkumar and Prabhakaran, (2006). Ten µ1 of 48 hr old culture of the isolate was transferred to an eppendorf tube containing 50 µ1 of acridine orange (Himedia) and incubated for 30 minutes at 30^{o}C. After the incubation period, the culture was centrifuged at 4000 rpm, for 15 min. the pellet was collected and re suspended in distilled water. A smear was prepared on a clean microscopic slide and observed in a fluorescent microscope at 460 nm. The appearance of yellow granules inside the cell indicate PHB production

Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using sudan black B dye (Juan *et al*., 1998). For rapid screening of PHB produces, Nutrient agar medium supplemented with 1% glucose was sterilized by autoclaving at 121^{o}C for 20 minutes and cooled to 40^{o}C. The medium was poured into sterile Petridis plates and allowed for solidification. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plate was incubated at 30^{o}C for 24 hours. Ethanolic solution of (0.02%) sudan black was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark coloured colonies were taken as positive for PHB production. All the positive isolates were assigned the code number based on their source of isolation.

Treatment of yellow purple "Dorowa" as carbon source (glucose)

Yellow purple (Dorowa) a waste from agriculture was procured from Sabon Gari and Sarkin power market at a cheaper rate was brought to the Laboratory for analysis. The waste purple was sieved to obtain a fine texture. Two hundred and fifty

grams at the fine texture was weight and added to 1000 ml of based medium with the following ingredients g/l (Na_2H PO_4.7H20 – 6.7; KH_2PO_4 -1.5; $(NH4)_2SO_4$ – 1.0; $MgSO_4$ – 7 H_2O- 0.2; Ferrous ammonium citrate – 6.0 mg; Ca Cl_2. $2H_2O$ – 1mg) was then mixed and boiled to a semi solid slurry so that the complex organic compound are broken down to simpler substances. The paste slurry was later allowed to cool and place in a freeze drier or spread on a dried sterile tray and oven dried at 40°C for 7 days. The pH of the paste was brought down using *Tamarindus indica* (Tsamia) to a neutral pH (7.2) and kept for further analysis.

Growth Medium

The probable organisms isolated and screened for the production of PHB in this study were *Bacillus megaterium*, *Alkaligen* sp and *Clostridium* sp respectively. Twenty grams of the modified yellow purple "Dorowa" medium was added to each different volume (50, 65, 25, 85 and 100) of distilled water to pH 7.2 and sterilized in an autoclave at 121°C for 15 minutes. The solution was allowed to cool before the flask were incubated and incubated for fermentation for 72 h at 30 °C

Mutation studies

The liquid culture (16 hours) grown in nutrient both (0.1) = 0.6 at 660 nm was diluted ($4x10^{-4}$ and $8x10^{-6}$ time) with sterile saline and 1 ml was taken in sterile Petridis plates. The plates were exposed to ultralviolet light in a UV chamber (locally fabricated for varying time intervals (5, 10, 15, 30 and 60 min).10 ml was pour and thoroughly mixed with culture. The plates were incubated overnight at 37°C mutant colonies were isolated on the basis of clearance zone with black paper to protect the cells from photo – reaction and were incubated at 30°C for 24 hr. (Sudha, 1996). All the sudan Black B. positive isolates were subjected to quantification of PHB production as per the method of (Juan *el al.,* 1998).

The bacterial cells containing the polymer were estimated by harvesting the cells in a cooling centrifuge (5000 x g) of 50 ml sample. The pellets were then suspended in 5 ml of 0.1% (W/V) sodium hypochloride solution. After one hour of incubation at 37°C to allow the total lysis of the cells suspension, cells with PHB granules were centrifuged and the pellets were washed with distilled water acetone and alcohol successively, finally the polymer was dissolve in small portions of boiling chloroform. The chlorogenated solution was filtered and the filtrate was hydrolysed and de – hydrochlorogenated with concentrated sulphuric acid. The solution was cooked and after through mixing absorbance of the sample was measured at 235 nm (Hahn *et al*., 1994).

RESULTS AND DISCUSSION

Six different bacteria were isolated viz; *Bacillus megaterium, Alkaligen* sp *Proteus* sp, *Clostridium* sp, *Kilebsiella* sp and *Staphylococcus* sp from tannery effluent sludge reservoir for production of PHB. These bacteria were screened and characterized owing to the nature of their survival, mode of carbon source assimilation, their morphology (Chandrashekhraiah, 2005) as seen in Table 1. Rapid screaming of native bacterial isolates for PHB production was also done specifically to ascertain the bacterial of interest. Three out of six were identified viz; *Alkaligen* sp, *Bacillus megaterium* and *Clostridium* sp, in the case of this study. Rapid screening of native bacterial isolates for PHB production was also done specifically to ascertain the bacterial of interest. As shown in the Table 2, 3, and 4 represent the enrichment for PHB product ion by UV – irradiation. As an evident, the increase in PHB production by UV – irradiation was very low in terms of percentage. The percentage mutants for PHB production among the survivors ranged from (52.3 – 10.20%) and UV light irradiation from (5-30 min) for *Bacillus mageterium*, (30.2-3.10%) and UV light irradiuation from 5-30 min) for *Alkaligen* sp and (42.31-9.30%), UV light irradiation from (5-30 min) for *Clostridium* sp. The *Bacillus megaterium* has the highest percentages range of the UV light exposure with (52.3-10.20%) followed by *Clostridium* sp (42.31 -9.30%) and the least was recorded on *Alkaligen* sp (30.2-3.10%). However going through by the data trend, it shows that the rate of increase in UV light irradiation exposure with time increased led to considerable decrease in the percentage of the three organisms. Similarly the number of isolates showed increase in growth and number of viable cell per m/l x 10^4). One could conclude that the UV light exposure contributed to the production of PHB at longer duration of time. It's possible that the UV light exposure at minimal time of duration could possibly transformed natural wild types of PHB at low rate to higher production of PHB using the mutant under a conducive and glucose available so as to booster the production of PHB (Sasmita *et al.,* 1998).

Comparative studies of the three selected bacterial sp viz; *Alkaligen* sp, *Bacillus megaterium* and *Clostridium* sp as seen in Table 5, shows the production yield and dried cell weight from *Alkaligene* sp was recorded the highest yield (2.02 g/g and 2.80g/l) respectively followed by *Bacillus megaterium* with (1.52g/g and 1.93g/l) and the least was recorded from *Clostridium* sp (52g/g and 1.02 g/l) respectively; *Alkaligene* sp could ensured PHB up to 80% under normal growth condition, therefore, one step PHB production process could be used with this organism (Hrabak, 1992). According to (Do, 1990) two stages fed batch culture is the most widely used technique to minimized high concentration of both cell and PHB. In first stage of growth phase, optimum nutritional condition were used to develop a high biomass concentration than a selected nutrient was limited to stimulate PHB production in the second stage or accumulation phase (Kim 2002).

Bacillus megaterium has in minimal medium without any added growth factors. The majority are mesophiles, with temperature optima between 30 and 45°C. (Kenneth, 2005) indeed, the ability of *B. megaterium* to accumulate PHB is so dominant that the PHB content in the cells could reach up to 32% of the cell dry weight (Hori *et al*., 2002). PHB provides a reserve of carbon and energy, accumulated as intracellular granules which can be extracted from a wide range of bacterial. The average molecular mass of PHB is also affected on the molecular mass of the polymer (Hori *et al*., 2002). In addition condition such as pH temperature duration biomes to aqueous

Table 1. **Morphological and Biochemical Characterization of PHB producing Bacteria**

S/No	Character	*Bacillus Megaterium*	*Alkaligen* Sp	*Proteus* Sp	*Clostridium* Sp	*Klebsiella* sp	*Staphylococcus* sp
Morphological Test	Colony colour pigmentation	White	White	Milky	White	White	
	Shape						+
		-	+	-	-	-	+
		diplococcic	diplococcic	Rod small	Drumstick	Rods	-
	Gram reaction			-			+
	Fluorescent	+	-	-	+	-	+
	Motility	+	+	+	-	-	+
	Swarming	-	-	+	+	-	
		-	-		-	-	
Physiological test	Geletine ligute			+			
	Starch hydrolysis	+	-		+	+	
	Casin hydrolysis			+			
	Citrate utilization	+	-		+	+	
				-			
					+	-	
		+	-	+	+	+	
	Indole production						
	Catalase test	-	-	+	-	+	-
	Urease Test	+	-	+	+	+	
	MR Test	-	-	+	+	+	+
	VP Test	+	-	+	+	+	
	VP Test	+	-	+	+	+	-
		+	-	+	-	+	+
Biochemical Test	Growth at 40ºC						+
	Growth at 41ºC	+	+	-	-	-	
	Spore formation						+
	Capsule test	+	+	+	+	+	+
	Growth at 12% Nacl						-
		+	-	-	+	-	
		-	-	-	-	+	
		-	ND	ND	+	-	
	Glucose	+	+	+	+	A/g +	A/g
	Fructose	+	+	+	+	+	
	Sucrose		+			+	
	Lactose	+	-	-	+		

Key:
ND=Not done
A/g=Acid gas

Table 2. Effect of U.V Irradiation on *Bacillus megaterium*

Duration of Exposure to U.V light (min)	Survivor	No of isolates showing Increase growth	No of viable Cell per m/l $Y10^4$
5	52.3	1	9
10	43.2	1	6
15	31.3	2	4
20	15.0	-	2
25	10.20	-	1
30	7.6	-	1

Table 3. Effect of U.V Irradiation on *Alkaligen* sp

Duration of Exposure to U.V light (min)	% survivor	No of isolates showing Increase growth	No of viable Cell per m/l $Y10^4$
5	30.2	1	5
10	25.2	1	4
15	10.3	-	2
20	9.6	-	1
25	5.2	-	1
30	3.1	-	1

Table 4. Effect of U.V Irradiation on *Clostridium* sp

Duration of Exposure to U.V light (min)	% survivor	No of isolates showing Increase growth	No of viable Cell per ml $Y10^4$
5	42.31	1	8
10	32.21	1	7
15	25.21	2	5
20	15.25	2	1
25	11.20	1	1
30	9.30	1	1

Others show no response to high exposure at UV light hence they are not counted for living

Table 5. Comparative Studies of Fermented Production of PHB from locally available Yellow Purple (*Parkia biglobosa*) as natural Carbon Source using three Bacteria sp

Carbon Source	Mutant Strains	PHB Concentration	Product PHB granules g/L	PHB yield g/g	Cell dry Weight g/L
Yellows Purple (dorowa) (*Pakia globulosa*)	*Alkaligens* Sp	3.96	0.820	2.02	2.800
	Bacillus megaterium	1.82	0.903	1.52	1.930
	Clostridium sp	0.82	0.803	0.52	1.026

phase could reduce degradation (Berger *et al*., 1989). Chloroform was also used for recovery of PHB from 12 *Eutropha* (Hahn *et al*., 1994). PHB is highly crystalline. Thermoplastic polymer with relatively high melting temperature in the range of 170 -180^{o}C) and a glass transition temperature in the range of (0-5^{o}C) it undergoes thermal degradation at temperature around the melting temperature. Currently, the cost of production of PHB is higher mainly due to the use of synthetic (glucose) as substrate, and thereby making the use of these (PHB) materials in commercial production difficult. At present, the industrial productions of PHBs are carried out from conventional glucose as substrate. If cheaper material could be used in the form of highly enriched locally available yellow purple "Dorowa" to produce PHB, then this will reduce cost in comparison with the expensive convention one (glucose).

Conclusion

The proportion of carbon to nitrogen in the initial medium was affected on amount of bacterial growth and PHB accumulation. The survivor percent decreased with the extension of duration of UV irradiation. This showed that *Alkaligene* sp *B. megaterium* and *Clostridium* sp strains cells are very sensitive to UV ray which kill the cells resulting in low growth frequency. An alternative way to increase the PHB production, enrichment of RNA and Amino acid synthesis of the wild type bacteria cell is the chemical mutagenesis in the present work, a low cost of raw materials as yellow purple "Dorowa" could improve the economics of the process and distain high PHB production. Thus, substrate from renewable resources with low cost for commercial PHB production was a target to verity the chemical structure and thermal properties of polymers from fermentation process.

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