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PRODUCTION OF BIODEGRADEABLE PLASTIC BY *Bacillus* sp. USING SUGARCANE BAGGASE

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ABSTRACT
Plastic materials originated from petrochemicals causes serious environmental problems due to their non-degradable nature. Bio based polymers or biodegradable polymers are found to be better solution in terms of protecting the environment from petroleum based plastic products. Polyhydroxybutyrates (PHBs) are synthesized and deposited as cytoplasmic inclusions in various bacteria like Bacillus sp. Biodegradable plastic have been available for many years in the market, however, their high cost has meant they have not replaced the traditional non-degradable plastics. The aim of this research is to produce biodegradable plastic by Bacillus sp. using sugarcane bagasse. Pure culture of different isolates obtained from soil, were screened by sub culturing in a modified nutrient agar medium containing glucose and Nile Blue A stain to confirm that the isolated strain were capable of producing PHB. The strain was identified as Bacillus velezensis when subjected to molecular characterization. Optimization of growth parameter such as substrate concentration (0.5, 1, 2, 3 and 4%) and temperature (30, 35, 40, 45 and 50) for production of bioplastic, was further conducted. The maximum PHB accumulation when pretreated sugar cane bagasse was used as carbon source was at 30°C and pH 8.0 after 48h incubations with total yield of 50.23%. Biodegradability test showed that soil borne organism are capable of utilizing the PHB. The results of this study confirmed that cheaply available agro-residues can be used for the production of PHB.
Key words: Biodegradation, Nile Blue, Polyhydroxybutyrates, Sugar-cane bagasse.

INTRODUCTION

Plastic materials originated from petrochemicals cause serious environmental problems due to their non-degradable nature (Getachew and Woldesenbet, 2016). Since synthetic plastics marked their debut in the 1950s, they have emerged to be among the most needed material in our daily life (Khandpur *et al.,* 2012). Approximately 25 million tons of plastics are produced by the plastics industry every year (Sharma. 2019), due to their relatively low cost, ease of manufacture and flexibility, the demand of plastics is ever-growing (Thapa *et al.,* 2018) but their persistence has a significant environmental impact (Brandl *et al.,* 1990) The consistent dependency on commercial non-biodegradable plastics causes an adverse impact on environment viz. crude oil reduction (Sharma 2019), severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas (Khandpur *et al.,* 2012). With the imminent fossil fuel crisis, the alarming rate of petroleum prices and environmental impact associated with the products, the search for alternatives is essential in reducing mankind's dependencies in non-renewable resources (Giin-Yu *et al.,* 2014). Disposal of plastic waste through incineration may generate toxic products and is expensive, thermal process and recycling can be done but it is time-consuming process (Sharma 2019) and due to high cost of recycling, plastics are rarely recycled leading to crammed up landfills. For eradication of these and various other problems such as carbon emission during incineration, biodegradation of plastic is a must (Christina *et al.,* 2018). The industries and researchers are in search of alternative products such as biodegradable polymers to combat the essential need of the world population (Bala *et al.,* 2017).

Justification of the research are due to increase in petroleum prices, adverse environmental impact and forthcoming fossil fuel crisis, the industries and researchers are in search of alternative products such as biodegradable polymers to combat the essential need of the world population (Giin-Yu *et al.,* 2014). Bio based polymers or biodegradable polymers are found to be better solution in terms of protecting the environment from petroleum based plastic products which are potentially toxic (Galia 2010). Biodegradable plastics are seeing some use and they have been

available for many years on the market. However, their high cost has meant they have not replaced the traditional non-degradable plastics (Yu *et al.,* 1998).

The aim of this research is to produce biodegradable plastic by *Bacillus* sp*.* using agricultural waste.

Biodegradable plastics offer the best solution to protect the environment from hazards caused by conventional petroleum based plastics as they are 'eco-friendly' in nature. There are many types of biodegradable plastics with different degrees of biodegradability. Among them polyhydroxybutyrate (PHBs) are the only 100% biodegradable ones. Polyhydroxybutyrate are macromolecules produced majorly by bacteria and they are found as inclusion bodies accumulated as reserve material during stress condition in the growth medium (Giin-Yu *et al.,* 2014). These polymers possess properties which are similar to various synthetic based polymers like polypropylene which makes a good alternative for petroleum based products which can also be produced commercially (Verlinden *et al.,* 2007).

Up to 50% of PHB production costs are dependent on the precursor substrate materials, mainly the carbon source (Urtuvia *et al.,* 2014) and therefore, intense researcher is underway on investigating the utilization of more cost-effective substrates. For example sugarcane molasses and vinasse were used as substrate for PHB production (Dalsasso *et al.,* 2019). Sugarcane bagasse is biomass resulting from the cleaning, preparation and extraction of sugarcane juice (Kamel *et al.,* 2021). For biomass conversion, several pretreatment techniques are used prior to the actual fermentation, such as milling, liquid hot water, steam explosion, CO2 explosion, wet oxidation, AFEX, diluted acid, alkali, organosolv, ozonolysis, and biological pretreatment (Maurya *et al*., 2015).

MATERIALS AND METHODS

Isolation and screening

Soil samples were collected aseptically from topsoil of different dumping sites of Samegu, Kumbotso local government Kano state, Nigeria. Ten grams of each sample was dispersed in 90ml of sterile distilled water and heated at 80^{o}C for 15 minutes to isolate only endospore forming bacteria. Serial dilution of the samples was done, followed by spread plating of 100µl diluted samples on nutrient agar plates (Christina *et al,.* 2018). Thereafter, the plates were incubated at 35°C for 24 hours (Singh *et al.,* 2011).

Pure cultures of morphologically distinct colonies were grown on a nitrogen free agar plate plates for 48 hours. Detection for PHB production was employed by using lipophilic stain Sudan Black B (Panigrahi and Badveli, 2013). Stain was prepared by dissolution of 0.3 gm powdered stain in 100 ml of 70% ethanol. For microscopic studies, smears of colonies were heat-fixed on clean, grease-free glass slides, followed by staining with 0.3% solution of the Sudan Black B. After leaving the slides undisturbed for 15 minutes, immersion in Xylene and counterstaining with Safranin (5% w/v in sterile distilled water) was performed. Cells appearing blue-black under microscope were accredited as PHB positive strains (Thapa *et al.,* 2018). .

Sudan black B positive isolates were further screened with Nile blue A staining on carbon rich nutrient agar medium containing 0.0005g Nile blue, colonies containing PHB accumulating strains were stained with Nile blue A showed a pink fluorescence on irradiation with UV light. The isolates which showed pink fluorescence were considered PHB positive (El-Hamshary *et al., 2019*). PHB positive strains were preserved on two vials, for working and stock vials, containing agar slants with 2% glycerol for preservation

Molecular characterization

The strain was subjected to molecular identification by analyzing 16S r-RNA sequencing, genomic DNA was isolated For genomic DNA isolation, all the isolates were grown overnight under submerged conditions and proceeded for DNA isolation. The DNA samples were run in 1.2% agarose gel under 100 V. The DNA bands were visualized with the help of ethidium bromide dye. All the isolates were having DNA of more than 1 Kb size. purified genomic purified genomic DNA of these isolates were amplified (Ausubel *et al.,* 1987) and amplification was done by PCR using universal primer.

Production of PHB by selected isolates using mineral salt medium

Mineral salts medium (MSM) [composition (g/L): Urea (1.0), Yeast extract (0.16), KH2PO4 (1.52), Na2HPO4 (4.0), MgSO4·7H2O (0.52), CaCl2 (0.02), Glucose (40), and trace element solution 0.1 ml] was used for the production of PHB by the selected isolates. The trace element solution contained (g/L): ZnSO4·7H2O (0.13), FeSO4·7H2O (0.02), (NH4)6MO7O24. 4H2O (0.06) and H3BO3 (0.06). Both glucose and trace element solution was autoclaved separately, and reconstituted prior to inoculation. The production medium (100mls) was inoculated with 2mls of standardized inoculum and incubated at 37 °C and 150 rpm for 48 h. (Getachew and Woldesenbet, 2016).

Extraction of PHB

(a) Pre-treatment: The biomass was harvested by centrifugation at 3000 rpm for 20 minutes after

48 hours of fermentation. It was frieze dried at -40° with a lyophilization machine.

(b) Extraction: Biomass powder was treated using equal volume of 6% sodium hypochlorite and chloroform. The mixture was heated at 80°C for 1hr. After the heat treatment, the dispersion was centrifuged at 3000 rpm for 20minutes. The three separate phases were obtained. The upper phase was the hypochlorite solution, the middle phase contained NPCM (non polyhydroxybutyrate cellular mater) and undisrupted cells, and the bottom phase consisted of the chloroform layer containing PHB. The chloroform layer was obtained by filtration. Separation of PHB-enriched solvent was achieved by using syringe from the bottom part of the centrifuge tube. Then, the PHB material was precipitated by mixing methanol with the concentrated chloroform (methanol: chloroform = 9:1). Finally, the PHB-precipitate was filtered by simple filtration and then dried by evaporation, in hot air oven at 60°C and dry weight of extracted PHB was measured with a weighing machine. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHB. The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the Dry Cell Weight (DCW) (Zakaria*et al.,* 2010).

Residual biomass (g/ml) = DCW (g/ml) – Dry weight of extracted PHB (g/ml)

PHB accumulation (%) = Dry weight of extracted PHB (g/ml) × 100 / DCW (g/ml)

Agricultural sample collection

Locally collected sugarcane bagasse was shredded into pieces, dried in oven at 60 °C for about 1 week to remove the moisture content and pulverized into fine particles.

Acid pretreatment and hydrolysis of sugarcane bagasse

Pulverized sugarcane bagasse was pretreated by suspending in weak acid, containing about 5% v/v sulphuric acid in distilled water, followed by autoclaving at 121°C for 30 min. The pretreated samples were then filtered and the supernatants was further neutralized using 6N sodium hydroxide (Ramadas, Singh and Pandey. 2009).

Optimization of production parameters

Optimization studies was carried out using different parameters such as different sugarcane bagasse concentration (0.5, 1, 2,3 and 4 %) and incubation temperature (30, 35, 40, 45 and 50°C) on PHB production medium was investigated using mineral salt medium with sugarcane bagasse as carbon source at 120 rpm (Getachew and Woldesenbet, 2016).

RESULTS AND DISCUSSION

Nile blue A staining is the most suitable and precise method for screening PHB producers, the use of Sudan black b is effective only when the organism is grown in a medium containing small amount of nitrogen source, with excess carbon. Depletion in the nitrogen source induces stress, therefore the organism accumulates the excess carbon source to form intracellular granules for energy reservation (Bernard, 2014) the granule are visible when the cell is stained at this stage, this contradicts the findings of Anteneh *et al*. (2016) where the isolates stained were grown on nutrient agar plates.

Carbon source concentration of 0.5, 1, 2, 3 and 4% sugarcane bagasse was incorporated into the production medium and yield of PHB was estimated after 48 hours of incubation. The result obtained in figure 1. Indicate that the concentration of carbon source affects the PHB accumulation and growth of the test organism. The best growth and maximum yield of PHB was obtained at 2 and 3% sugarcane bagasse concentration with DCW of 1.810g and 1.835g respectively and PHB concentration of 40.16 and 34.03% respectively. The effect of different concentration of carbon source has been studied by Moha *et al.* (2001) which showed that the production of PHB was high at 2g/l. However the level of cell dry weight was higher at 3% carbon source.

PHB is carbon reserve by cells when there is limited nitrogen source and excess carbon source in the production medium. The optimum temperature for growth and production of PHB was found to be 30°C, yielding 0.748g/l of PHB. However there was high growth but lower PHB production at temperature 40 and 45^{o}C, this is because increase in growth cause decrease in production due to disruption of cell wall and also due to alternation of metabolic activity. Increase in the temperature (50^{o}C) causes decrease in the production this is due to the disruption of cytoplasmic membrane. Therefore the cytoplasm content diffuses out and PHB cannot exist from the cell (Barathi *et al.,* 2016).

The 16S rDNA sequencing was performed using commercial service. These sequences were compared using NCBI BLAST (Basic Local Alignment Search Tool) service which was found to have 100% homology with previously reported sequence of *Bacillus velezensis.*

Extracted PHB subjected to biodegradation studies was utilized as carbon source by soil borne microorganisms on nitrogen free mineral agar medium, this was ascertain by the presence of growth, The plastic nature and biodegradability of the extracted polymer was confirmed by preparing sample plastic film and the clear zone formed by soil born bacteria PHBs are degraded by the action of microbial enzyme into water-soluble forms (Mergaert *et al.,* 1994).

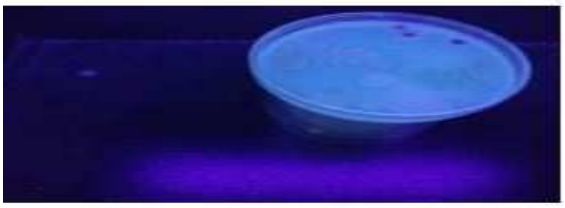

Plate 1 Pinkish/purple colonies indicates PHB producers, colorless colonies indicates non-PHB producers

Plate 2 Plate 3 Pinkish/purple colonies indicates PHB producers, colorless colonies indicates non-PHB producers

Plate 3 Plate 4 Pinkish/purple colonies indicates PHB producers, colorless colonies indicates non-PHB producers

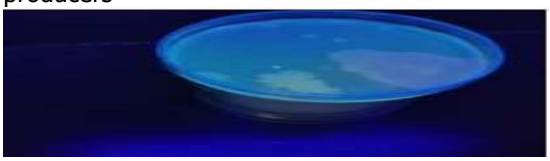

Plate 4 Plate 5 Pinkish/purple colonies indicates PHB producers, colorless colonies indicates non-PHB producers

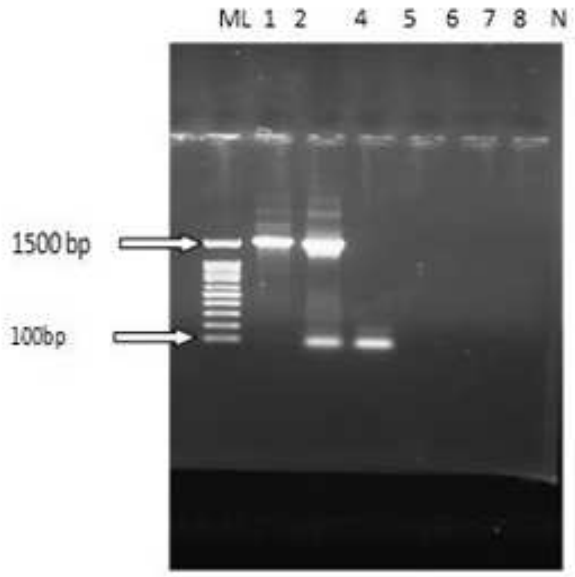

Figure 5 Polymerase chain reaction (PCR) amplification of 16S rRNA

PLATE 6 Extracted PHB

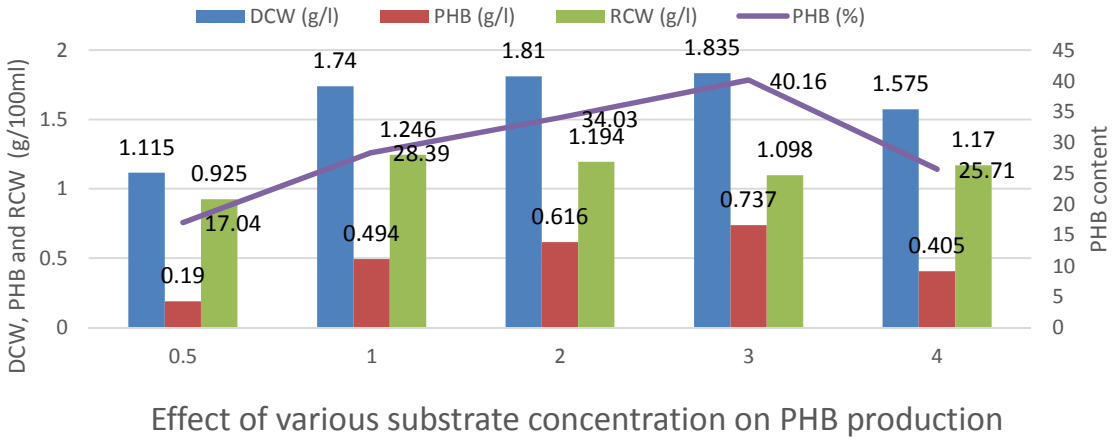

Figure 1. Effect of various substrate concentration on PHB production
KEYS: DCW-Dry Cell Weight, PHB- Polyhydroxybutyrate RCW- Residual Cell

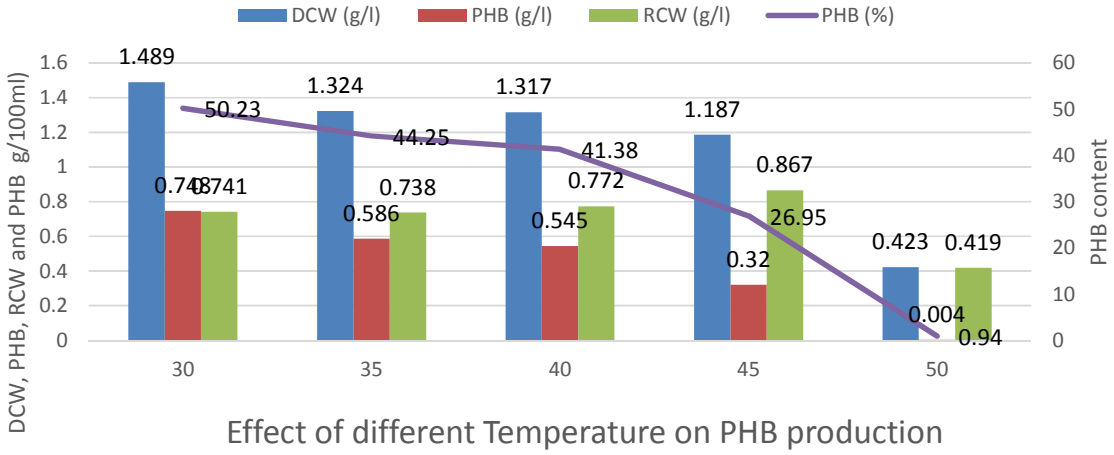

Figure 2. Effect of different temperature on PHB production
KEYS: DCW-Dry Cell Weight, PHB- Polyhydroxybutyrate RCW- Residual Cell Weight

CONCLUSION

From the above studies, it was concluded that the *Bacillus* sp. has a capability of producing PHB, from sugarcane bagasse as sole carbon substrates. The results of this study confirmed that cheaply available agro-residues can be used for the production of PHB serving triple purposes of reducing the cost of biodegradable plastics, reducing environmental pollution problems caused by conventional plastics and solving disposal problem of the agricultural wastes. Incubation parameters such as temperature and substrate concentration affect the overall yield of PHB production.

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