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Growth of *Pseudomonas fluorescens* on Cassava Starch hydrolysate for Polyhydroxybutyrate production

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ABSTRACT: The potential of local strains of microorganism (*Pseudomonas fluorescens*) in polyhydroxbutyrate production was investigated in this study. This was with a view to establishing the capabilities of local strains of microorganisms on utilizing renewable and locally available substrates in polyhydroxybutyrate production. This involved hydrolysis of starch extracted from freshly harvested cassava tubers using enzyme-enzyme method of hydrolysis, followed by aerobic fermentation of *Pseudomonas fluorescens* on a mixture of the hydrolysate and nutrient media in a fermentor in batch cultures. The reducing sugar hydrolysate served as the carbon source and di-ammonium sulphate as the limiting nutrient. The reaction temperature, pH and agitation rate in the fermentor were maintained at 30 ^{o}C, 7.5 and 400 rpm respectively. The biomass growth was measured by cell dry weight and the polyhydroxybutyrate content measured by gas chromatography. When the fermentation process was shut down after 84 hour, the substrate consumption by the organism was 9.2 g/L to give a dry cell weight of 1.75 g/L resulting in a biomass yield on substrate ($Y_{x/s}$) of 0.1902 g/g (19.02 % wt/wt). The gas chromatographic analysis gave a final polyhydroxybutyrate value of 1.254 g/L with corresponding product yield on biomass ($Y_{p/x}$) of 0.7166 g g^{-1} [71.66% wt/wt] and product yield on substrate ($Y_{p/s}$) of 0.1363 g g^{-1} [13.63% wt/wt]. The results show that the organism accumulated polyhydroxybutyrate in excess of 50 % of the cell dry weight by giving a final polyhydroxybutyrate yield on biomass ($Y_{p/x}$) of 0.7166 g g^{-1} [71.66% wt/wt] which agrees with the general trend in polyhydroxybutyrate production. @ JASEM

Poly-3-hydroxybutyrate (PHB) is a biodegradable polymer material that accumulates in numerous microorganisms under unbalanced growth conditions (Anderson and Dawes, 1990; Brandl *et al*., 1990; Doi, 1990) and has been produced on an industrial scale by a company called ZENECA using *Alcagenes eutrophus* (Byrom, 1987; Holmes,1985). Polyhydroxybutyrate is synthesized and accumulated as granules in the cytoplasm of bacteria cells. They are very attractive because they can be produced effectively from renewable resources (Braunegg *et al*., 1998). The demand for the use of biopolymers involves their biodegradable and bio-compatible properties suitable for disposable products and their potential use as bio-materials for medical applications such as surgical sutures, long-term carriers for drugs, moulded plastics and films (Holmes, 1985, Hahn *et al*., 1993). The cost of polyhydroxybutyrate production depend largely on the cost of the carbon sources, therefore if more expensive sources of carbon could be replaced with an inexpensive, locally available material as source of carbon, a significant cost reduction could be obtained (Pedros-Alio *et al*.,1985). This work therefore investigates the production of polyhydroxybutyrate from reducing sugar hydrolysate obtained from enzymatic hydrolysis of raw cassava starch. *Alcagenes eutrophus* is currently used for commercial poyhdroxybutyrate production (Byrom, 1987), but many other microorganisms accumulate polyhydroxybutyrate and can grow on more different carbon sources than can *Alcagenes eutrophus*. At least four microorganisms other than *Alcagenes eutrophus* have thus far been documented as being able to accumulate polyhydroxyalkanoate copolymers. These are *Bacillus megaterium* (Wallen and Rhowedder, 1974; Findlay and White, 1983), *Pseudomonas oleovorans* (de Smet *et al*., 1983; Brandl *et al*.,1988), *Norcadia* species (Davis,1964), and Cyanobacteria, including one species of the genus *Aphanothece* (Capon *et al*.,1983). Therefore, this study also seeks to investigate the polyhydroxybutyrate production capabilities of one of such microorganism, *Pseudomonas fluorescens* being a nutritionally versatile microorganism (Stanier *et al*., 1966). The most common method available at present for analysis of polyhydroxyalkanoates (PHA) in bacterial cells is gas chromatography (GC)(Braunegg *et al*.,1978; Huijberts *et al*., 1994; Riis and Mai, 1988). The GC method involves hydrolysis and subsequent methanolysis or propanolysis of the PHAs in whole cells in the presence of sulphuric acid and chloroform. This study therefore makes use of GC for polyhydroxybutyrate (PHB) analysis in the bacterial cells.

MATERIALS AND METHODS

Microorganisms: The local strain of *Pseudomonas fluorescens* used for this study was obtained from the stock culture of Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The microorganism was grown on 2.0% agar slants (pH 7.0) containing the following (per litre): 5g of yeast extract, 2.5g of beef extract, 5g of peptone, and 5g of $(NH_4)_2SO_4$. (Du *et al.,* 2001a) at 30^{o}C for 4 days and stored at 4^{o}C with regular sub-culturing.

Mineral salts medium: The mineral salts medium consisted of 1.5g of $(NH_4)_2SO_4$, 5.8g of K_2HPO_4, 3.7g of KH_2PO_4, 0.4g of $MgSO_4$ and 1ml of a microelement solution. The microelement solution

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was prepared such that one litre of it contained 2.78g $FeSO_4.7H_2O$, 1.98g of $MnCl_2.4H_2O$, 2.81g of $CoSO_4.7H_2O$, 1.67g of $CaCl_2.2H_2O$, 0.17g of $CuCl_2.2H_2O$, and 0.29g of $ZnSO_4$ (Du *et al.,* 2001a).

Preparation of substrate solution: Twenty-five grams of raw cassava starch (obtained from cassava tubers, freshly harvested from a farm in Ile-Ife, Nigeria) was made into slurry by dissolving in appropriate quantity of distilled water containing 40 ppm Ca^{2+} to form 25 % starch slurry. This starch slurry was gelatinised at 105 ^oC for 10 min. The gelatinised starch was liquefied at a lower temperature of 95 ^oC and pH of 6.5 by adding α – amylase (5 ml enzyme kg^{-1} of Novo Termamyl obtained from *Bacillus licheniformis*) with continuous stirring for 2 h. The liquefied starch was saccharified at a pH of 4.5 and 60 ^oC for 72 h by adding Novo amyloglucosidase (AMG) obtained from *Aspergillus niger* (5 ml enzyme kg^{-1} starch), with continuous agitation throughout the period of hydrolysis. After saccharification, the mixture was heated for 15 min in boiling water to stop the activities of the enzymes and filtered to get the syrup used as substrate for the production of polyhydroxybutyrate.

Preparation of inoculums: The inoculums was prepared in eight different 250 ml Erlenmeyer conical flasks containing 80 ml of the reducing sugar hydrolysate and 20 ml of mineral salts medium each. The flasks were cotton plugged and autoclaved at 121 ^oC for 15 min. The sterilized medium was allowed to cool to ambient temperature and large amount of the microorganisms (*Pseudomonas fluorescens*) was scraped from the cultured slant and added aseptically to each of the medium in the flasks to make 100 ml of the seed medium in each of the flasks. The flasks were transferred to the gyratory incubator shaker (New Brunswick Scientific Co., USA) and growth was achieved at a temperature of 30 ^oC at agitation rate of 200 rpm and for 48 hrs for proper adaptation of the microorganisms to the medium.

Preparation of modified 3, 5 – Dinitrosalicylic acid (DNS) reagent: This contained 1% (w/v) 3,5 – Dinitrosalicylic acid, 0.2%, (w/v) Phenol; 0.05% sodium bisulphite, 20% (w/v) Rochelle salt (Potassium sodium tartarate) and 1% (w/v) sodium hydroxide. To prepare 1 – litre of solution, 10 g of sodium hydroxide pellet was dissolved in 1- litre of distilled water. To this was added 10 g of powdered 3,5 – Di-nitrosalicylic acid, 0.5 g of sodium bisulphite, 2.52 ml of 80% Phenol and stirred to complete dissolution. Then 200 g of Rochelle salt was added to the solution and stirred to dissolve. The solution was then stored under refrigeration in a brown bottle (Miller 1959).

*Preparation of 0.1% (w/v) glucose standard solution***:** This was prepared by dissolving 1g of D-glucose in 1 litre of distilled water to give 1 g/L standard glucose solution.

Fermentation: The fermentation was carried out aerobically in a bench scale bioreactor manufactured by New Brunswick Scientific Co., USA**.** The fermentor vessel containing 1.5 liter of the mineral salts solution and 3 liter of the feedstock (glucose solution) was sterilized in the autoclave at 121 ^oC for 15 min. The solution in the fermentor vessel was inoculated with actively growing inoculums with a volume of 800 ml and was properly agitated and aerated for about 30 min before making the initial withdrawal. The dissolved oxygen concentration was maintained above 20% of air saturation by maintaining an aeration rate of 2 vvm and agitation speed of 400 rpm. The pH of the system was maintained at 7.5 and controlled by addition of 0.5 M HCl or 0.5 M NaOH solution as desired. The temperature was controlled and maintained at 30 ^oC by circulating water at that temperature from a water bath through the cooling water pot of the fermentor. Samples were withdrawn at 6 hr interval. Each of the samples collected was centrifuged and the supernatant analyzed for reducing sugar and nitrogen concentration.

Sample Analysis.

Reducing sugar concentration: The reducing sugar concentration of the fermentation broth was estimated by analyzing the glucose in the supernatant using the DNS method of Miller (1959).

Ammonia nitrogen analysis: The nitrogen content of the broth was analyzed by using the method of Marchessault (1996). 5 ml sample of the supernatant was diluted to 50 ml in order to be within the range of 1.4 to 140 ppm NH_3 (Marchessault, 1996). 0.5M NaOH was used to adjust the solution to a pH of 13 to shift the balance of equilibrium from NH_4^+ to NH_3 for direct quantification. The nitrogen content of the samples was then quantified using an Orion specific ion electrode (Model 95-12). Samples were read immediately after NaOH adjustment to minimize error of NH_3 disappearance from the solution since in its gaseous form it tends not to remain in solution over time (Marchessault, 1996).

Biomass concentration: To do this, 20 ml samples were withdrawn every 6 hr interval and centrifuged at 15,000 rpm for 10 min. The supernatant was removed and the precipitate re-washed twice by re-suspending in an equal amount of distilled water and repeating the centrifugation process. The residue was poured on a pre-weighed filter paper and dried to constant weight in an oven (model EP120SSF100D1E by Leader Engineering, Eng.) at 60 ^oC , allowed to cool in a dessicator and the final weight measured with an analytical balance (model AB54 by Mettler Toledo). The biomass weight was determined by subtracting

the weight of the filter paper from the weight of the filter paper plus the cells (El- Holi and Al- Delaimy, 2003).

Polyhydroxybutyrate (PHB) concentration: Here 100 ml of the culture medium was withdrawn every 6 hr interval throughout the period of fermentation. The sample was prepared according to the method of Riis and Mai (1988). The sample was centrifuged at 10,000 rpm for 20 min in screw cap tubes. The supernatant was removed and 2 ml of 1, 2 dichloroethane was added to the contents of the tubes. To this, 2 ml of a solution containing 4 volumes of propanol to 1 volume of concentrated HCL was added with 0.2 ml of an internal standard solution of 2.0 g of benzoic acid in 50 ml propanol. The tubes were vortexed and placed in an oven at 105 ^{o}C for 3 hr. They were removed every half hour, vortexed and replaced.

After 3 hr, the tubes were removed and allowed to cool. After the tubes had cooled, 4 ml of distilled water was added and the tubes were vortexed again for 30 seconds after which phase separation occurred. The organic (bottom phase) was removed with Pasteur pippetes for analysis. An external standard solution was prepared in the same way with 0.02 g of polyhydroxyalkanoate containing 78% (wt/wt) polyhydroxybutyrate. The polyhydroxybutyrate content of the sample was then quantified using an SRI 8610 gas chromatograph. The gas chromatograph was fitted with an automatic injector and a flame ionization detector which was supplied with 500 ml of air, 30 ml of hydrogen and 30 ml of helium per minute. The injection split ratio was 100:1 with a helium flow of 0.9 ml / min through the 25 m long HP5 capillary column. The injector port temperature was 120 ^{o}C and increased by 8 ^{o}C per min to a final temperature of 210 ^{o}C. Injections of 5 µl were made and the retention times for the methyl ester of 3-hydroxybutyrate and benzoic acid were 4.1 and 7.0 min respectively.

RESULTS AND DISCUSSION

*Reducing sugar hydrolysate production***:** The result obtained from the enzyme-enzyme hydrolysis of raw cassava starch show that 25 % starch slurry gave reducing sugar concentration of 20.4 % with starch conversion efficiency of 81.6 %. After proper dilution and mixing with nutrient medium, the substrate gave initial reducing sugar concentration of 13.4 g L^{-1} while the ammonium sulphate provided the medium with free amino nitrogen concentration of 0.073 g L^{-1} (Table 1).

Table 1: Initial conditions for the batch fermentation of *Pseudomonas fluorescens* in a stirred fermentor.

Fermentation Mode	Working Volume(L)	Initial Reducing Sugar conc.(g/L)	Initial nitrogen conc.(g/L)	Initial Total Biomass(g/L)	Initial PHB Conc.(g/L)
Batch	4.2	13.4	0.073	0.18(±0.02)	0.00

Fermentation studies: Figure 1 shows a plot of the dry cell mass value against fermentation time for the cell growth during fermentation. The results show that the substrate (reducing sugar hydrolysate) was able to support the growth of the microorganism (*Pseudomonas fluorescens*) since the biomass concentration increases throughout the period of fermentation.

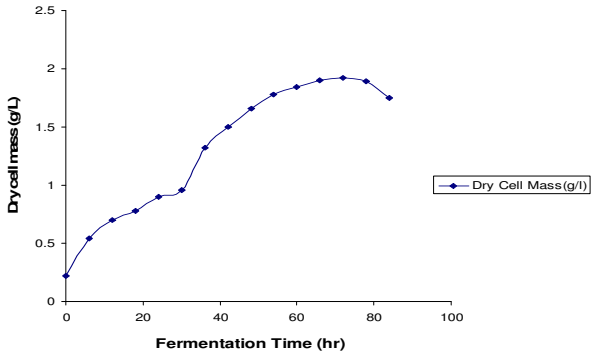

Fig.1:Plot of Dry Cell Mass against FermentationTime for *Pseudomonas fluorescens.*

Figure 2 shows the hydrolysate consumption pattern of *Pseudomonas fluorescens* during fermentation. The results showed that the organism really utilize the substrate as carbon source for its metabolic needs by consuming 9.20 g/L of the reducing sugar hydrolysate over the 84 hr fermentation period**.** Figure 4 illustrates biomass and polyhydroxybutyrate accumulation by *Pseudomonas fluorescens* in relation to nitrogen utilization. From the results, it was observed that the starting point of high value polyhydroxybutyrate accumulation coincided with the point of nitrogen limitation.

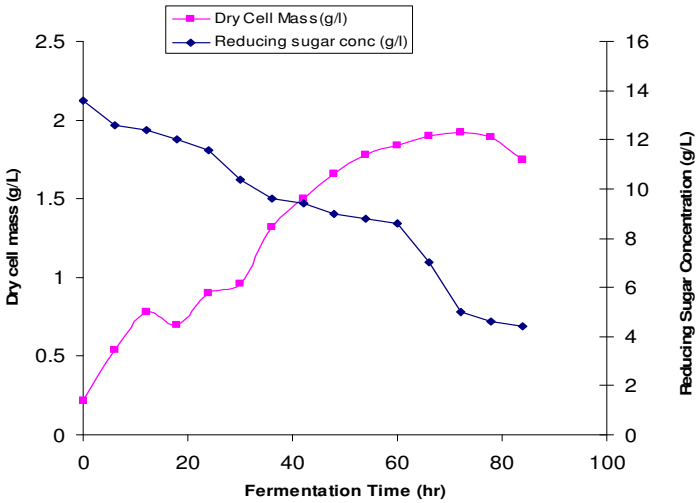

Fig. 2: Plot of Reducing Sugar Concentration and Dry cell mass against Fermentation Time for *Pseudomonas fluorescens*

This research was undertaken with the aim of producing polyhydroxybutyrate from cheap, locally available and renewable resources using local strains of microorganism.

Considering the result of analysis, Figure 4 showed that the polyhydroxybutyrate accumulation corresponds to the point of nitrogen exhaustion. The value of polyhydroxybutyrate rose to 0.207 g/L at 24th hr of fermentation from the initial value of 0.086 g/L at 18th hr of fermentation. This sharp increment occurs within 6 hr when the initial 71.34 mg/L of Nitrogen was exhausted after about 18th hr of fermentation period. At this point, about 2.0 g/L of the reducing sugar hydrolysate was consumed while the dry cell mass was 0.901 g/L resulting in a biomass yield on glucose of 0.451 g/g and polyhydroxybutyrate yield on biomass of 0.230 g/g. After this point, the cells kept on accumulating polyhydroxybutyrate due to the exhaustion of nitrogen in the medium until 72nd hour after which biomass concentration started decreasing. The decreasing biomass concentration affected the polyhydroxybutyrate accumulation which also started decreasing and when the fermentation process was shut-down after 84th hr period, a final polyhydroxybutyrate value of 1.254 g/L was obtained in 1.75 g/L biomass which corresponds to a polyhydroxybutyrate yield on biomass of 0.7166 g/g [71.66 % (wt/wt)].

As expected, the result of the study was in line with the general trend of results of experimentation in polyhydroxybutyrate production. According to literature, most of the accumulation of polyhydroxybutyrate in conventional batch cultures using microorganisms occur after the point of Nitrogen limitation (Merrick, 1978; Byrom, 1987; Brandl *et.al.*, 1988; Ramsay *et.al.*, 1989; Anderson and Dawes, 1990; Rusendi and Sheppard, 1996).

Also, the final polyhydroxybutyrate value of 71.66% (wt/wt) of the biomass dry weight given by the organism was comparable to those reported by Sonnleitner *et. al.* (1979), Holmes,(1985) and Rusendi and Sheppard (1996) of 78% (wt/wt), 80% (wt/wt) and 76.9% (wt/wt) in *Alcagenes eutrophus* respectively.

The slightly lower results could be attributed to the fact that the organism used was local strain and not genetically engineered organisms like *Alcagenes eutrophus*, *Pseudomonas cepacia, Pseudomonas oleovorans* e.t.c. that were used in most of the studies referred to above.

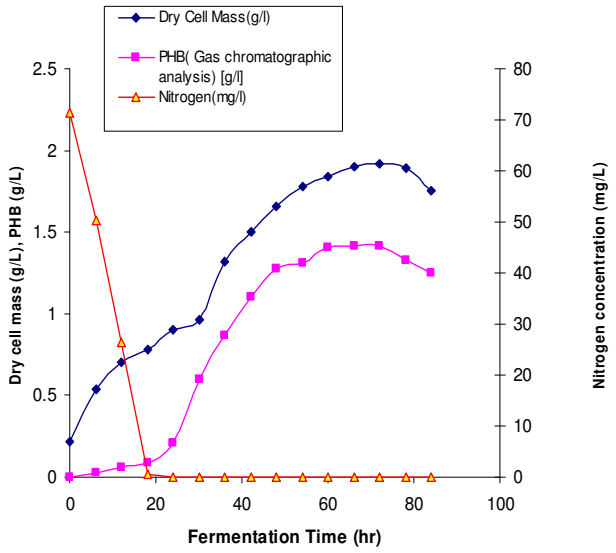

Fig.4: Plot of Dry cell mass, PHB and Nitrogen consumption against Time for Pseudomonas fluorescens (Gas chromatographic Analysis)

Finally, the yield was somewhat lower in terms of polyhydroxybutyrate produced to substrate consumed ($Y_{p/s}$) which was 0.14 g/g for this organism compared to the theoretical maximum of between 0.44 g/g and 0.48 g/g specified by Yamane (1993).

Conclusion: *Pseudomonas fluorescens* was found to utilize natural, cheap and renewable reducing sugar hydrolysate from cassava starch as substrate for polyhydroxybutyrate production in batch fermentation processes. This ability is an advantage because commercial production of value added products such as polyhydroxybutyrate from cassava starch will not only ensure reduction in manufacturing costs, but will also go a long way to solve the problem associated with management of synthetic polymer wastes.

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