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Production of Polyhydroxybutyrate (PHB) by Pseudomonas Putida Strain KT2440 on Cassava Hydrolysate Medium

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Abstract

The potential of local strain of Pseudomonas putida strain KT2440 in polyhydroxbutyrate production was investigated in this study. This was done to establish the capabilities of local strains to utilize renewable and locally available substrates in polyhydroxybutyrate production. It involved hydrolysis of extracted starch from freshly harvested cassava tubers using enzyme-enzyme hydrolysis method, followed by aerobic fermentation using Pseudomonas putida in batch cultures on a mixture of the hydrolysate and nutrient media. The reducing sugar hydrolysate served as the carbon source while di- ammonium sulphate was the source of Nitrogen. 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 chromatographic method. The results obtained showed that the medium supported the growth of the organism. After 72 h fermentation, the substrate consumption by the organism was 8.88 g/l to give a dry cell weight of 0.91 g/l, resulting in a biomass yield on substrate ($Y_{x/s}$) of 0.1025 g g^{-1}. The gas chromatographic analysis gave a final polyhydroxybutyrate value of 0.2285 g/l with corresponding product yield on biomass ($Y_{p/x}$) of 0.2511 g g^{-1} (25.11%) and product yield on substrate ($Y_{p/s}$) of 0.0257 g g^{-1}. It can be concluded that Pseudomonas putida strain KT2440 has capability to utilize cheap, renewable and locally available substrates in polyhydroxybutyrate production. Also, comparing the result of the present study with those from the previous ones showed that although Pseudomonas putida strain KT2440 accumulated polyhydroxybutyrate lower than the usual value in excess of 50 % of the cell dry weight given by various strains of microorganisms during polyhydroxybutyrate production it is a potential candidate for polyhydroxybutyrate production.

Keywords: Reducing sugar hydrolysate, local strains, renewable, fermentation, enzyme-enzyme hydrolysis, gas chromatographic.

Introduction

A polymer is a large molecule composed of repeating structural units typically connected by covalent chemical bonds.[12] Synthetic and semi-synthetic polymers are frequently used to produce ephemeral commercial goods in many industrial, domestic and environmental applications[15,20]. However, those features that made these synthetic polymeric materials so convenient and useful to human life, have contributed to creating a serious polymer waste burden on the environment. The increased costs of solid waste disposal as well as the potential hazards from synthetic polymer waste incineration, such as dioxin emission from polyvinyl-chloride has made synthetic polymer source of waste a management problem. The currently adopted technologies such as burial in landfill operations, incineration with energy recovery and mechanical or chemical recycling, all

have their shortcomings[14] and can be expensive. The need to eliminate the problem of waste management posed by these synthetic polymers and the quest to preserve the limited mineral oil from which they are sourced led to the development of natural polymers called biopolymers. Biopolymers are formed from renewable sources and can be recycled or composted effectively. Polyhydroxybutyrate (PHB) which constitutes the most important and widely studied representative of the polyhydroxyalkanoates (PHAs) is a biodegradable polymer derived from renewable resource[16]. It is produced by many bacteria as carbon and energy storage material, apparently in response to conditions of physiological stress. It is a biopolymer with mechanical properties similar to conventional commercial polymers like polypropylene or polyethylene[16,25]. As its accumulation by bacteria is a response to the imbalance in growth environment, where suitable carbon sources are in excess and other nutrient is limiting, this physiological condition can be exploited in the production process to achieve high yields and productivity[26]. Biopolymers are beneficial in that they are environmentally friendly, non toxic and renewable. In contrast to production of toxic compounds such as polychlorofied dioxins and furans from the burning of synthetic polymers, biopolymers have ecologically neutral CO_2 balance since their burning does not release more CO_2 into the atmosphere than is withdrawn during growth of the raw materials. Hence they cause less damage to the environment even when burned[11]. However, a major obstacle to their commercialization is that their production costs are much higher than those of conventional petrochemical based polymers[7]. Therefore, there is an emerging need to reduce the overall cost of polyhydroxybutyrate production by designing novel processes and product separation/purification procedures in order to maximize its yield and productivity. However, process economics reveal that the use of inexpensive and renewable substrates for polyhydroxybutyrate production can contribute to as much as 40 - 50% reduction in the overall cost[7], for this work, the main carbon source was reducing sugar hydrolysate obtained from hydrolysed raw cassava starch as cheap and renewable substrates while the organism employed was a local strain of *Pseudomonas putida.* The objective of the study was to establish the capabilities of *Pseudomonas putida* to utilize cassava hydrolysate in polyhydroxybutyrate production.

Material and Methods

Microorganism: *Pseudomonas putida* strain KT2440 used for this study was obtained from Institute of Agricultural Research and Training (IAR and T) Ibadan, Nigeria. The microorganism was regularly grown on an agar slant containing yeast extract, beef extract, peptone and $(NH_4)_2SO_4$ at 30^oC for 48 h and then stored at 4^oC with regular sub-culturing 8,9.

Preparation of Reducing Sugar Hydrolysate solution: 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 in a water bath by boiling up to 105^oC and held at this temperature for 10 minutes at a pH of 6.5. 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. The syrup obtained contained the reducing sugar hydrolysate[3] used as substrate for the production of polyhydroxybutyrate.

Mineral Salts Preparation: The mineral salts solution was prepared according to the method of Du *et al.,* 2001a such that one litre of solution contained 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 was prepared such that one litre 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 $ZnSO_4$[8]. All chemicals used for the mineral salts preparation are all of analytical grade.

Preparation of Growth Medium and Inoculum: The medium 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^{o}C for 15 min. The sterile medium was allowed to cool to ambient temperature and the microorganism (*Pseudomonas putida*) was scraped from the agar 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 30^{o}C and agitation rate of 200 rpm after incubation for 48 h.

Fermentation: The fermentation was carried out aerobically in a bench scale fermentor manufactured by New Brunswick Scientific Co., USA**.** The fermentor vessel containing 1 litre of the mineral salts solution and 2.4 liter of the substrate solution was sterilized in the autoclave at 121^{o}C for 15 min. The solution in the fermentor vessel was allowed to cool and inoculated with 800 ml of actively growing inocula. The fermentor was agitated at 400 rpm with an airflow rate of 2 vvm (volume of air per volume medium per unit) and fermentation was conducted at 30^{o}C and pH of 7.5. Samples were taken at 6 h intervals. Each of the samples collected was centrifuged and the supernatant analyzed for reducing sugar and nitrogen concentration. The pellet was analyzed for biomass and polyhydroxybutyrate 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[18].

Ammonia Nitrogen Analysis: The free ammonia nitrogen content of the broth was analyzed by an Orion specific ion electrode (Model 95-12) using the method of Marchessault (1996).

Turbidity: The turbidity of the culture medium served as a parameter to follow cell growth during fermentation. To do this 5 ml sample were withdrawn every 6 h interval, diluted and the absorbance measured at 600 nm on the Novo spec II spectrophotometer by Pharmacia Biotech.

Biomass Concentration: Twenty millilitre samples were withdrawn every 6 h interval and centrifuged at 10,000 rpm for 10 min. The supernatant was removed and the pellet re-washed twice by re-suspending in an equal amount of distilled water and repeating the centrifugation process. The washed cells were poured on a pre-weighed filter paper and dried to constant weight in an oven at 60^{o}C, allowed to cool in a dessicator and the final weight recorded. The biomass weight was determined by subtracting the weight of the filter paper from the weight of the filter paper plus the cells 10.

Polyhydroxybutyrate (PHB) Concentration: One hundred millilitre sample was withdrawn every 6 h interval throughout the period of fermentation and analyzed for polyhydroxybutyrate concentration using the method of 22. The polyhydroxybutyrate content of the sample was 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 500ml of air, 30ml of hydrogen and 30ml 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 22.

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 %.

Fermentation studies: After proper dilution and mixing with nutrient medium, the substrate gave initial reducing sugar concentration of 12.2 g L^{-1} while the ammonium sulphate provided the medium with free amino nitrogen concentration of 0.073gL^{-1}. The initial total biomass concentration in the medium was measured to be 0.18(±0.02)g/l and the initial polyhydroxybutyrate concentration was measured to be 0.00 g/l.

Biomass and Polyhydroxybutyrate Production: Figure 1 shows a plot of the dry cell mass value obtained against fermentation time for the cell growth during fermentation while figure 2 shows a plot of the polyhydroxybutyrate value obtained against fermentation time. The results show that the substrate (reducing sugar hydrolysate) was able to support the growth of (*P. putida*) since the biomass concentration increased over time. In figure1, the biomass concentration increased as the substrate consumption by the microorganism increased while figure 2 shows that the polyhydroxybutyrate production increased with increase in biomass. The biomass yield on substrate $Y_{(x/s)}$ obtained was 0.1025 g g^{-1}, while the product yield on substrate $Y_{(p/s)}$ was 0.0257 g g^{-1} and the corresponding product yield on biomass was $Y_{(p/x)}$ 0.2511 g g^{-1}.

Hydrolysis of raw cassava starch by enzyme has been previously reported 11,19,2. The result of 81.6 % hydrolysis obtained in this study compare favourably with those previously reported.11 reported a conversion efficiency of 72 % for enzymatic hydrolysis of cassava starch to maltose. In the study of 19, hydrolysis of raw cassava starch by amylase of *Aspergillus niger* was reported to give 27.17 % conversion efficiency while 2 reported a conversion efficiency of 87.3 % for the enzyme-enzyme hydrolysis of cassava starch to reducing sugar hydrolysate.

For biomass growth, biomass concentration increased with time up to 70th h. This can be observed in the curve of biomass growth which is in the exponential phase. It was observed that earlier in the fermentation process, the substrate was consumed in a progressive manner by the microorganism, thereby enhancing biomass growth but with a very little amount of polyhydroxybutyrate accumulation until after 18th hour of fermentation when polyhydroxybutyrate accumulation started appreciating considerably probably due to the exhaustion of nitrogen in the medium. This observation agrees with the previous works of 6,1 and 23 who all reported that most of the polyhydroxybutyrate accumulation in conventional batch cultures using microorganisms occur after the medium has reached the point of nitrogen limitation. When the fermentation was shut-down after 72 h, a polyhydroxybutyrate yield of 0.2285 g L^{-1} was obtained in a biomass of 0.91 g L^{-1} resulting in a polyhydroxybutyrate yield on biomass $Y_{(p/x)}$ of 0.2511 g g^{-1} which translates to 25.1 % of cell dry weight. This translates to a PHB production of 25.1% of the cell biomass.

The PHA recorded in this study was low when compared with some previous studies. 24 reported 43.44 ± 0.25 % PHA of the cell dry weight in *Bacillus sp*. strain COL 1/A6 when grown on commercially available soluble starch; 27 reported a maximum accumulation of 35 – 40 % PHA of the dry cell weight in *Bacillus sp*. strain when grown on molasses as the carbon source, 5 reported an accumulation of 44 % PHA of the cell dry weight in *Alcaligenes eutrophus* DSM 545 after 75 h of fermentation in medium containing 0.5 g L^{-1} molasses, 4 reported 57.7% PHB of the cell dry weight in *Pseudomonas aeruginosa* NCIB 950 when grown on reducing sugar hydrolysate obtained from cassava starch, 28 reported 49%, 55% and 60% PHO (Poly-3-hydroxyoctanoic acid) respectively for three separate fed-batch cultivation of *Pseudomonas putida* GP01 in mineral salts medium containing

initially 20mM Sodium octanoate as the carbon source. The highest reported accumulation so far was 76.9 % PHA of the cell dry weight by *Alcaligenes eutrophus* DSM 545 when grown on hydrolysed potato processing waste 23.

However, this result compares favourably well with that of the study of 21 in which *Azotobacter beijerinckii* DSM 1041 was reported to accumulate 25 % PHA of the dry cell weight when grown on a mixture of molasses and corn steep liquor (mixture 65.4 g L^{-1} and 13.2 g L^{-1}). Therefore, *Pseudomonas putida* KT2440 grown on cheap and renewable reducing sugar hydrolysate obtained from cassava starch is a potential candidate for polyhydroxybutyrate production.

Conclusion

Pseudomonas putida KT2440 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 is a means of upgrading local cassava starch. This 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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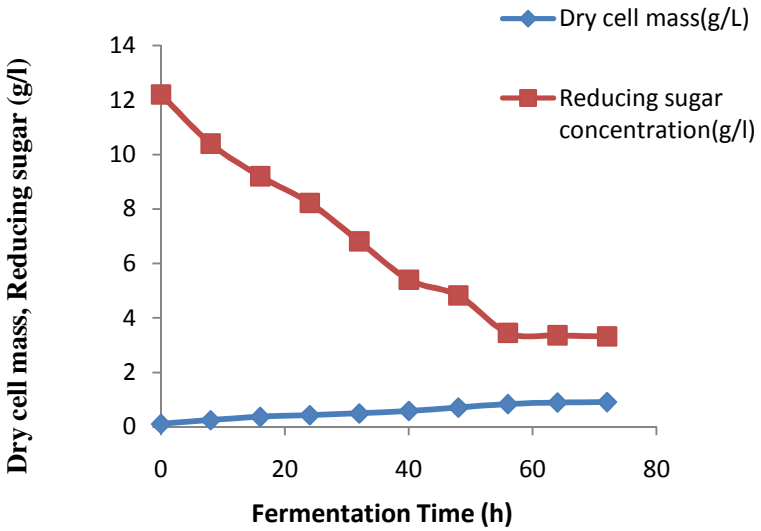

Figure-1: Plot of Dry cell mass and Reducing sugar concentrations against Fermentation Time for aerobic growth of *Pseudomonas putida* KT2440

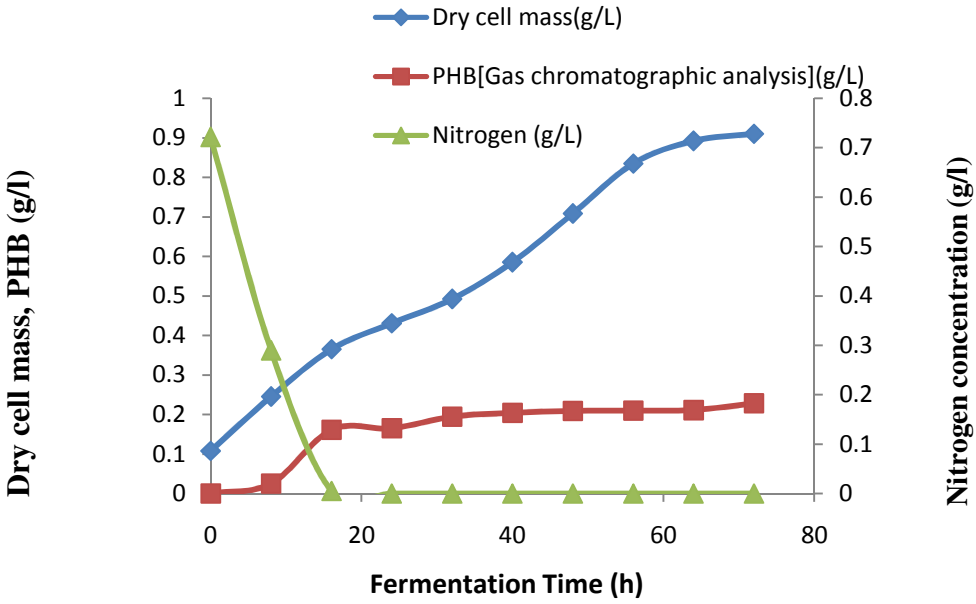

Figure-2: Plot of Dry cell mass, PHB and Nitrogen concentrations against Fermentation Time for aerobic growth of *Pseudomonas putida* KT2440