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# Polyhydroxyalkanoate Production by Cultivating *Hydrogenophaga pseudoflava* in Fed Batch Culture

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**Abstract:** In current research, we investigated PHB production by *Hydrogenophaga pseudoflava* DSMZ 103 4under fed-batch cultivation. Fed-batch fermentation, conducted by two different strategies of feeding nitrogen ($NH_4CL$) (10 g/l) and glucose (300g/l). In first strategy, fed-batch fermentation conducted by constant feeding nitrogen and glucose at the rate of 7 ml/h yielded 8.6 g/l PHB in higher amount of biomass (17.2 g/l) in the end with an overall productivity of 0.46 g/l h. In another strategy, alternative feeding nitrogen and glucose was done at different rates of 6, 8.5 and 10 ml/h. These feeding conditions lead to PHB production about 11.5 g/l in biomass amount 23.7 g/l in the end with an overall productivity of 0.48 g/lh. Fed-batch cultivation with alternative feeding strategy resulted over production of PHB by *H. pseudoflava*.

**Key words:** Polyhydroxybutyrate (PHB) · Hydrogenophaga pseudoflava DSMZ 1034 · Fed-Batch cultivation

## INTRODUCTION

Polyhydroxyalkanoates are polymers of hydroxyalkanoic acids that are accumulated intracellularly as granule inclusions by prokaryotic microorganisms (eubacteria and archaea) as carbon and energy reserves or reducing-power storage materials [1-2]. They are synthesized in the presence of excess carbon, especially when another essential nutrient, such as nitrogen or phosphorus, is limiting Poly (β-hydroxybutyrate) from family of microbial energy/carbon storage compounds collectively known as polyhydroxyalkanoates (PHA). These can be accumulated as an intracellular carbon and energy storage material by many bacteria under unfavorable growth conditions [3-4]. PHB is a biodegradable thermoplastic polyester that can be considered analogous to many conventional petrochemical-derived plastics currently in use [5]. It is biocompatible, nontoxic and has a low immunogenicity and is suitable for medical applications [6]. At present, PHB and its copolymers and terpolymers can be used as bulk commodity plastics, e.g., in agricultural and medical applications, due to their biodegradability and biocompatibility, future applications are potentially more widespread. Polyhydroxyalkanoates (PHA) production by wild type bacterial strains occurs under nutrient depletion conditions.In the PHA production phase, the cell growth is limited owing to the depletion of essential nutrients such as nitrogen, phosphorus, magnesium, among others.

This depletion in the presence of excess carbon source triggers the metabolic shift from growth to PHA production phase [7]. Many organisms synthesize PHB from acetyl-CoA via *R*-(-)-3-hydroxybutyryl-CoA, employing a-three step pathway with β-ketothiolase, an NADPH- dependent acetoacetyl-CoA reductase and PHA synthase [8]. *Hydrogenophaga pseudoflava* DSMZ 1034 is the most widely used microorganism for the production of PHB since it is able to accumulate large amounts of PHB [9]. Fed-batch fermentation is the most common method to achieve a high cell density and induce desired nutrient limitation, which are often necessary for high yield and productivity of the final product in certain cultivations. In literature studies, nutrients were usually fed in the reactor intermittently or constantly in response to dissolved oxygen (DO) or pH [10] as a feedback parameter. In present work we compared batch and fed -batch cultivation in PHB production. This research aimed to optimize bacterial growth and PHB production under suitable conditions (e.g. carbon and nitrogen sources, C/N ratio, limitation of nitrogen, DO, pH and feeding strategies) in fed batch culture system.

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## MATRIALS AND METHODS

**Microorganism:** The microorganism used in the present study was *Hydrogenophaga pseudoflava DSMZ 1034* (Deutsche Sammlung von Mikroorganismen und Zellkulturen) for culture propagation. The stock culture was stored and maintained on Luria Agar slants at 4°C. The organism was sub-cultured every 15 days to maintain its viability.

**Media:** Glucose was used as standard substrate. The mineral solution for batch culture experiments design was prepared by mixing the following chemicals: $Na_2HPO_4$, 3.57 g/l; $KH_2PO_4$, 1.5 g/l; $(NH_4)_2SO_4$, 1.35 g/l. Trace element content of the solution were $MgSO_4$, 2.2 g; $FeSO_4$, 0.1 g; $MnSO_4$, 0.1 g; $K_2SO_4$, 2.2 g; $H_3BO_3$, 0.02 g; $CuSO_4$, 0.08 g added to 1 liter of distilled water [11].

**Cell Dry Weight:** The cell concentration in the cultured media was determined by the cell optical density at wavelength of 620 nm using spectrophotometer (UNICO2100, USA) with distilled water for suitable dilution rate. The cell dry weight was also measured based on standard calibration curve. The standard curve was experimentally defined by filtration of exact volume of the broth contained *C. necator*. The cell optical density is defined as light absorbance of the culture as a function of cell dry weight was based on pure culture of *C. necator*. The cell dry weight measurements were quite accurate (mg cells.$l^{-1}$). The mean value of triplicate weight measurements had a standard deviation of less 3%.

**Glucose and Nitrogen Concentration:** The supernatant obtained from centrifuged solution was used for residual nutrient analysis and The concentration of glucose, ammonium were measured by glucose assay kit, ammonium assay kit (Chem Enzyme Co.).

**Biopolymer Analysis:** For PHB quantification, 5ml of culture broth was centrifuged at 3600rpm for 20min. A 2 ml solution of chloroform and 2ml of acidified methanol (3% sulfuric acid) were added to the cell pellet in a vial with Teflon screw cap and heated at 100°C for 3.5h. The developed extraction method was based on experimental method developed by [12].

Gas chromatography (GC) was performed using a gas chromatograph (Philips PU4400, US) equipped with flame ionization detector (FID) and data acquisition system with computer software (Clarity 4.2, Data Apex, Czech Republic). The GC was used for the methyl ester of

Fig. 1: Schematic of bioreactor for batch and fed- batch cultivation

3-hydroxybutyric acid (3HB) analysis. The GC was equipped with capillary column (BP20 SGE, Australia), 0.33 mm internal diameter and, 25m length. The column temperature was initially maintained at 80°C for 4 min, followed by the temperature programming at a rate of 8°C/min till it reached to 160°C, maintained for 3 min and then at a rate of 30°C/min increased to 200°C. The detector and injector temperatures were 280 and 250°C, respectively. The carrier gas used was helium with a flow rate of 1.5ml/min. Hydrogen and air flow rates were 30 and 300 ml/min, respectively. The injection volume size was 1il of the prepared samples.

**Experimental Conditions:** Stock culture in slants of *C. necator* was incubated at 30°C for 24h. The resultant cultures were inoculated into 5 l bioreactor (Figure 1) containing 2 l medium. The inzoculum size was 5% of the medium. For the above experimental conditions the dry cell weight (DCW) and PHBs accumulation inside the cells were investigated [13].

**Fed-Batch Cultivation:** Fed-batch bioreactor was conducted with the same operating condition in batch cultivation. According to the cell specific growth rate, cell dry weight and dissolved oxygen amount, when nitrogen concentration approached zero level in batch fermentation, feeding glucose and nitrogen was started. Feeding amount was concluded from glucose, nitrogen mass balance in bioreactor during the fermentation.

**Fed-Batch Fermentation with Constant Feeding Glucose and Nitrogen:** The fed-batch fermentation was initiated as a batch with an initial glucose and nitrogen concentration of 50 and 1 g/l and a working volume of 2 l. At 24 h when

the exponential phase of growth and a Optical density of about 8 were established, nitrogen (as ammonium chloride) feeding (10g/l) and glucose feeding (300 g/l) were initiated at a constant rate of 7 ml/h. Feeding was continued around 36 h. The feeding was stopped at 60 h. After completion of nitrogen and glucose feed, the fermentation was continued as a batch for the next 30 h to consume the residual glucose in the media.

**Fed-Batch Fermentation with Alternative Feeding Glucose and Nitrogen:** In the same fermentation condition, Fed-batch cultivation with alternative feeding glucose and nitrogen was conducted. At 18 h, Nitrogen (as ammonium chloride) feeding (10g/l) and glucose feeding (300 g/l)were initiated at a rate of 6.5 ml/h and increased to 8 and 10 ml/h after 6 and 12 h fermentation time respectively. The feeding was stopped at 54 h. After completion of nitrogen and glucose feed, the fermentation was continued as a batch to consume the residual glucose in the media.

## RESULT AND DISCUSSION

**Fed-Batch Cultivation of *H. pseudoflava* in Bioreactor with Constant Feeding Strategy:** A fed-batch process was initiated to supply the nitrogen concentration and enhance the biomass accumulation. However, according to the cell specific growth rate and cell dry weight, Glucose and ammonium chloride feeding were done at a flow rate of 7 ml/h with a feeding solution containing 300 g/l and 10 g/l respectively. A simple constant feeding of nitrogen was attempted, starting from the time when nitrogen concentration approached zero level in batch fermentation. Glucose and nitrogen Feeding started from 24 h until 60 hr (Figure 2). The feeding of nitrogen and glucose was done to increase the biomass so that PHB accumulation (8.6 g/l) was induced in higher amount of biomass (17.2 g/l). Glucose feeding prepare excess carbon source in media where lead to maximum PHB production in cell body.

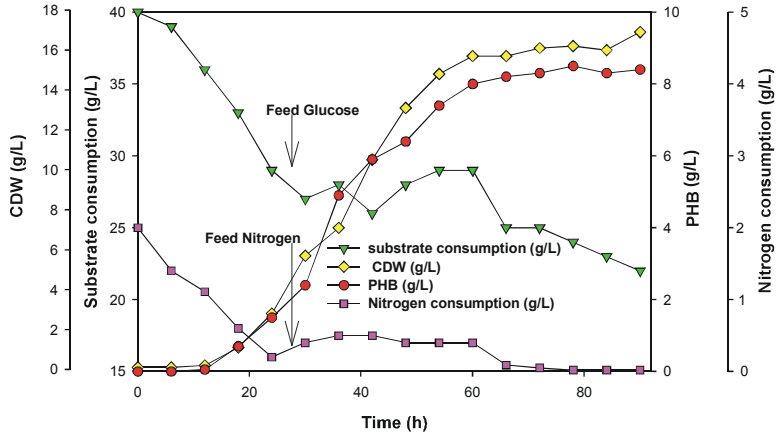


Fig. 2: Fed batch cultivation with constant feeding of glucose and nitrogen

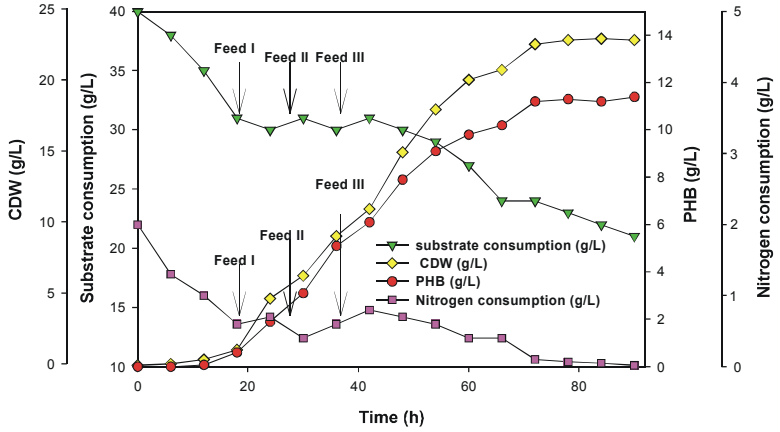


Fig. 3: Fed batch cultivation with alternative feeding of glucose and nitrogen

**Fed-Batch Cultivation of *H. pseudoflava* in Bioreactor with Alternative Feeding Strategy:** A fed-batch process was initiated to supply the nitrogen concentration and enhance the biomass accumulation. At 18 h, according to the cell specific growth rate and cell dry weight, when nitrogen concentration approached zero level in batch fermentation, Nitrogen (as ammonium chloride) feeding (10g/l) and glucose feeding (300 g/l) were initiated at a rate of 6.5 ml/h and increased to 8 and 10 ml/h after 6 and 12 h fermentation time respectively. The feeding was stopped at 54 h. After completion of nitrogen and glucose feed, the fermentation was continued as a batch to consume the residual glucose in the media. The feeding of nitrogen and glucose was done to increase the biomass so that PHB accumulation (11.5 g/l) was induced in higher amount of biomass (23.7 g/l).

## CONCLUSION

In overall summary, the total cell biomass and PHB production parameters attained with batch culture of *C. necator* with a C/N ratio of 30 was compared to that derived from fed-batch culture. Fed-batch culture exhibited a higher cell mass and PHB concentration, content and productivity. Nutrient feed, which contained 300 g/l of total sugar at a C/N ratio of 30 clearly supported the highest cell growth and PHB production and attained, after 60 h of cultivation, a maximal PHB concentration of 8.6 g/l, which is equivalent to 48% DCW. Overall, these results suggest that H. pseudoflava requires carbon and nitrogen sources as well as minerals for optimal growth and PHB production. However, DCW and PHB production increased around 100% in fed- batch fermentation. In literature study, there is not any reports of such induce in PHB concentration from batch cultivation compare to fed-batch cultivation.

Gotten results from fed-batch fermentation process with two different feeding strategies indicated that the fed-batch cultivation with alternative feeding strategy could improve biomass and PHB production amount 23.7 and 11.5 g/l respectively.

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