

Simulation of Bioreactors for Poly (3-hydroxybutyrate) Production from Natural Gas

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ABSTRACT: *Recently, many economic studies of poly(3-hydroxybutyrate) PHB production on an industrial scale, and the impact of replacing petrochemical polymers by PHB were carried out, clearly indicating that the most crucial factors to reduce the cost of producing biopolymers are allotted to the application of microbial production strains capable of high productivity in inexpensive carbon sources, high cell density cultivation methods, cheap yet effective methods for the extraction of PHB and other polyhydroxyalkanoates (PHAs), and gene transfer from bacteria to plants. We present current strategies to reduce the production price of biological PHA. Because an important part of the PHA production cost is related to the cost of carbon source, the article focuses on the use of natural gas as an inexpensive and readily available C1-carbon source. Since the first and foremost point in PHA production is biomass growth, we discuss different types of bioreactors to be potentially used for efficient biomass production from natural gas, which facilitates the subsequent selection of the ideal bioreactor for PHA production from this substrate. Nowadays, process simulation software can be used as a powerful tool for analysis, optimization, design*

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and scale up of bioprocesses. Controlling the process design by *in silico* simulations instead of performing an excessive number of lab-scale experiments to optimize various factors to save in time, material, and equipment. Simulation of PHA production processes to find the optimal conditions can play a decisive role in increasing production efficiency. Computational fluid dynamics and mathematical modeling helps us to achieve a better understanding of the role of different nutrients, flow parameters of gaseous substrates, efficient feeding strategies, etc. This finding leads to higher productivity by prediction of parameters e.g. nutrient supply and biomass concentration-time profile and their respective yields.

KEYWORDS: Poly(3-hydroxybutyrate); Production; Bioreactor; Simulation.

INTRODUCTION

Plastics are utilized in almost every manufacturing industrial branch, ranging from automobiles to medicine. They are highly advantageous because as synthetic polymers, their structure can be chemically manipulated to obtain a wide range of physical properties, strengths, and shapes, such as fibers or thin films. Plastics generally display molecular weights ranging from 50,000 to 1,000,000. The synthetic polymers poly(ethylene), poly(vinyl chloride) and poly(styrene) are largely used in the manufacture of plastics. They display high chemical resistance and, dependent on their composition, certain elasticity, hence, they are popular in many durable disposal goods and as low-density packaging materials [1]. Due to growing piles of plastic waste, efforts to better understand the mechanisms for degradation of these polymers is growing increasingly. So far, several types of biodegradable polymers are described, with a steadily increasing number of such materials [2].

Among all biodegradable polymers, polyesters constitute the major group and are accessible by biological or chemical methods. Such biodegradable polyesters are grouped into polyhydroxyalkanoates (PHA), polycapro-lactone, polylactic acid, polyurethane (PU). Many factors like the chemical structure and environmental conditions affect the biopolymer accessibility towards biodegradation [3, 4].

PHAs are produced in the form of granules in the cytoplasm of many Gram-positive and Gram-negative bacteria and a range of extremophile representatives of the Archaea from at least 75 different genera and more than 300 species. This includes wild-type isolated from different environments such as *Cupriavidus necator*,

Azohydromonas lata (former *Alcaligenes lata*), *Azoto-bacter vinelandii*, *Pseudomonas* sp., or *Haloferax mediterranei*, and a range of genetically engineered organisms, especially recombinant *Escherichia coli*. Microorganisms able to accumulate PHA are easily detectable by simply staining with Sudan Black or the Blue Nile (blue) [5]. More sophisticated methods for detection and classification of PHA-accumulating species, such as fluorescence *in situ* hybridization were developed as recently summarized [6].

PHA has already been successfully used to produce biodegradable plastics [2, 7]. These polymers are accumulated intracellularly to levels up to more than 90% of the Cell Dry Mass (CDM) under conditions of nutrient stress and parallel availability of exogenous carbon source; predominantly, they act as a microbial carbon and energy reserve [1].

PHA have sufficiently high molecular mass in the range of 2×10^5 to 4×10^6 Da, depending on the type of microorganism, cultivation conditions, and on several factors such as the carbon source, and the activity of the enzymatic machinery. Molecular mass of PHA can be drastically increased by modern tools like CRISPRi, as recently demonstrated [8]. This high molecular mass provides them with material characteristics that are similar to conventional plastics, especially poly(propyl-ene) (PP) [1].

PHA degradation under aerobic conditions is less well described and of rather complex nature, whereas anaerobic degradation of PHAs from *Azotobacter vinelandii* UWD cultures was thoroughly investigated in activated sludge; here, PHA conversion yields to methane and

carbon dioxide were reported between 83 and 96% [9]. However, biodegradation performance under experimental conditions can strongly differ to the outcomes under real environmental conditions due to several complications such as the relative ease of parameter control under experimental conditions compared to real situation which have to be considered [10].

Several methods were suggested to monitor PHA biodegradation, including the control of cell mass or changing number of cells, the measuring of resulting changes in both composition and molecular mass of PHA, product analysis, consumption and CO₂ production, and reduction of the polymer mass [11]. Research done on PHA biodegradation indicates its expedient biodegradability, *in vivo* compatibility with critical natural materials such as natural polyesters and the auspicious thermoplastic properties of this polymer. Moreover, PHAs are produced from renewable resources.

Due to these characteristics, PHA can be applied in various fields of medical and veterinary sciences, pharmaceutical and packaging industries, agriculture, environmental remediation, animal and human health. Given the myriad effects of their use within the area of science, the number of investigative efforts, especially in industrialized countries, for the generation of knowledge in this field, is currently increasing exponentially [5]. PHA biodegradability and biocompatibility have caused application in the controlled release of drugs, tissue engineering and veterinary medicine [12-14]. Both on a lab scale and on (semi)industrial scale, PHA production can occur in batch, fed-batch and continuous systems, and can resort to different types of bioreactors, as detailed later in this article [15, 16].

POLY(3-HYDROXYBUTYRATE) (PHB) AND CUPRIAUDUS NECATOR

Poly(3-hydroxybutyrate) (PHB) and the copolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) are the best known and most widely occurring members of the PHA group [5, 17] PHB is a fully biodegradable polyester with optical activity, piezoelectricity and very good barrier properties for volatile compounds and diverse gases such as O₂, H₂O and CO₂ and flavors [18]. Its general structure is shown in Figure 1. PHB is a crystalline material with a high melting temperature and a high degree of crystallinity, making it stiff and brittle.

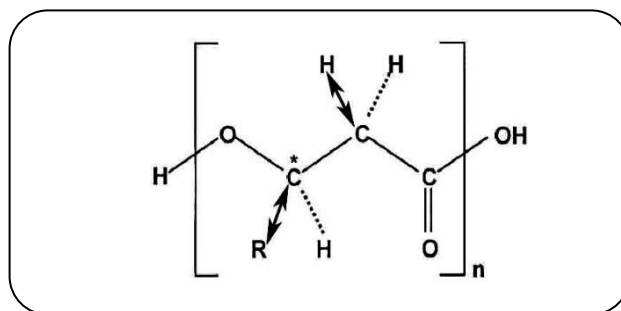


Fig. 1: General structure of PHB.

The degree of brittleness depends on the degree of crystallinity, glass transition temperature and microstructure. The longer it is stored at room temperature, the more brittle it becomes. Its physical and mechanical properties are comparable to those of its major petrochemical competitor, namely isotactic PP (iPP) [18]. In contrast to synthetic polymers of petrochemical origin, PHB does not contain any residues of catalysts. The bacterium *C. necator* is among the most famous microorganisms producing this polymer, with PHB fractions in biomass exceeding 75%. The number of polymer granules per *C. necator* cell amounts to 8 to 13, with a diameter of approximately 0.2–0.5 μm. The distribution and number of granules per *C. necator* cell highly depends on the environmental conditions and is a response to balanced, fluctuating, or nutrient-limited conditions, respectively [19]. This bacterium is known since a long time, originally termed as *Alcaligenes eutrophus*; after being classified into the *Ralstonia* lineage, and later named as *Wautersia eutropha*, it now is finally known as *C. necator*. Normally, heterotrophic PHB biosynthesis by *C. necator* occurs via the utilization of expensive carbon sources, which, at the same time, constitute materials with high importance for human nutrition, hence 1st generation substrates like sugars or edible oils. Therefore, the interest of scientists is devoted to find inexpensive substrates, most preferably carbon-rich waste streams, which are easily convertible towards PHB; in parallel, new powerful microorganisms are currently screened and investigated, together with the assessment of new, sustainable technologies for PHA recovery from biomass. Together, these efforts should minimize PHA and PHB production and extraction costs, and make these polymers competitive with petroleum-based polymers [5]. Table 4 presents examples of PHB producing bacteria and various carbon sources used by them for PHB biosynthesis [20-23].

PHB applications

In medicine, PHB is compatible with the blood and tissues of mammals. As one of the ketone bodies, the monomer of PHB, 3HB, is a normal metabolite in the human blood. As the body reabsorbs PHB, it might be used as a surgical implant, in surgery, as seam threads for the healing of wounds and for production of artificial blood vessels [24, 25]. In pharmacology, PHB can be used to produce microcapsules to be applied therapeutically or as materials for cell and tablet packaging. In the packaging field, the polymer finds application for deep drawing articles in the food industry [26]. Further examples of application encompass the production of bottles, laminated foils, fishnets, potted flower, hygiene, fast food, one-way cups, agricultural foils and fibers in textile [18].

The history of research on PHB

PHB was for the first time discovered in 1925 at the Institute Pasteur in Paris by Lemoigne in the cytoplasm of *Bacillus megaterium*. At the same time, its general formula $(C_4H_6O_2)_n$ was elucidated [17, 28]. In 1952, the melting point of the polymer was determined with 180 °C. In 1958, Williamson and Wilkinson reported molecular mass and physical properties of PHB. In the same year, Macrae and Wilkinson observed that the accumulation of PHB in the cells increases with nitrogen limitation in the medium [29]. In the early 1960ies, Waarber and Baptist began producing PHB in two separate companies with clear business goals, and won the license for production and isolation of PHB. Of course, the fermentation method of that time was characterized by low productivity, and the applied technique for PHB extraction was based on harmful solvents; hence, the process was by far too expensive. In addition, the obtained PHB was not completely pure, so the project of these companies was abandoned because of the problems mentioned above, and industrial production of PHA was postponed for one decade [29]. In 1968, the technical information about large-scale fermentation and skills in the field of biopolymers increased significantly. At that time, ICI introduced the bacterium *Alcaligenes eutrophus* (which in 1997 was re-named to *Ralstonia eutropha*) able to produce and accumulate PHB to more than 70% of CDM. In parallel, it turned out that PHA copolymers different to PHB display properties even

closer to PP, and, due to reduced crystallinity and brittleness, were more suitable for processing and industrial applications [17]. In 1973 and 1975, PHB granules with a diameter of 240 to 720 nm, produced by the bacteria *Bacillus megaterium* and *Bacillus cereus*, were evaluated and purified. In this study, spectroscopy was used to identify and quantify PHB after acidic conversion to crotonic acid at concentrations as low as 50 µg/L [29]. In 1976, PHB was obtained by cultivating the bacterium *Azotobacter beijerinckii* in medium without ammonium, with glucose acting as sole carbon source [30]. As a result of the increasing need to rapidly and accurately determine PHA in biomass, a gas chromatography (GC) method was published in 1978, which is still in use in most laboratories worldwide dealing with PHA research [31]. This method, based on acid methanolysis of PHA towards volatile methyl esters of the monomers, performs faster and by far more accurately than previous methods, identifies and quantifies monomers other than 3HB, and is sensitive enough to trace PHA at levels as low as 1 µg/mL.

In 1981, Holmes and colleagues presented a biological process to produce PHB and its copolymers from different carbon sources by *R. eutropha* at ICI. The obtained copolymers were called BIOPOL. This method was based on changing the ratio of propionic acid (precursor for the monomer 3-hydroxyvalerate-3HV) and glucose as the major factor to adapt the ratio of the copolymer components 3HB and 3HV, resulting in BIOPOL copolymers of tailored melting point, mechanical properties and thermoplasticity [9].

In 1986, PHB was produced for the first time in a fed batch system by methanotrophic bacteria [32]. Two years later, studies were carried out to identify the structure of a new copolymer with the formula $[OCH(CH_3)CH_2C(O)]_x [OCH_2CH_2C(O)]_y$ with fascinating properties in terms of low crystallinity and outstanding elongation at break. Using nuclear magnetic resonance, it turned out that this new copolyester consisted of 3HB and the achiral monomer 4-hydroxybutyrate (4HB) [33]. Finally, in 1990, the first company started to produce the first commercial BIOPOL products as a biodegradable bottling material for shampoos [17].

Today, various research strategies to reduce the production price of PHB are done that can be classified in several groups: Isolation and breeding of bacteria capable

of high PHA productivity and suitable growth rate on inexpensive carbon sources, research on optimization of fermentation conditions and nutrient medium [34-36], cheap but effective extraction methods [34, 37], modeling and simulation of growth and production [38, 39], switching to cultivation methods enabling high cell density [15], and transfer of bacterial genes into plants for autotrophic PHA production [17, 40]. The optimization of the culture media is key to economic methods of experimental design. In order to optimize the continuous production process of PHA, *Khosravi-Darani et al.* used the two statistical design strategies presented by Plackett-Burman and Bremen and Taguchi to compare the efficiency of polymer production from the carbon source fructose by *R. eutropha* under different conditions in terms of optimizing the nutrient medium and process parameters [34, 36]

Results of these studies on shaking flask scale revealed the optimum conditions of studied variables as follows: initial concentration of fructose 5 g/L, C/N ratio 7/4, shaking at 200 rpm, cultivation time 40 h, temperature 30°C and seed age 15 h. Under these optimum conditions, the final concentration of cells in the culture medium and mass fraction of PHA in CDM amounted to 18 g/L and 85%, respectively.

BIOREACTORS

Horizontal tubular loop bioreactor

A single-walled forced-liquid horizontal tubular loop bioreactor with a dissolved methane detector is reported. The experiments were carried out in a bioreactor with a diameter, height and length of 3, 40, and 220 cm, respectively. Air and natural gas were distributed by a perforated tube through a gas pump in different zones within the horizontal section, and the liquid medium was circulated by a peristaltic pump. The horizontal section contained two Komax static mixers, which are used to generate a swirling motion (vortex) as the liquid passes through. Vorticity is a mathematical concept used in fluid dynamics related to the amount of circulation or rotation in a fluid. The vorticity at a point is a vector that is defined as the curl of the velocity. Following measurement with a gas flow meter, the gas was injected through annular holes in front of the static mixer.

Airlift bioreactor

Airlift bioreactors are a special class of pneumatic contactors that do not have any mechanical components,

like impellers or seals. Their main characteristics are low shear stress, simplicity of design and construction and low energy requirements for transport rates, besides a better definition of internal flow, and a good aseptic control. The performance of airlift reactors becomes limited when the cells used in the process consume high amounts of oxygen. In such cases, special changes on the configuration of the sparger and even high compression of air would be necessary. In concentric draught-tube airlift reactors, air is introduced into the culture from a sparger at the bottom of the central tube. This causes a reduction in the bulk density of the liquid in the draught tube ("riser") compared to the outer zone of the vessel ("down comer", "downer"), which sets the culture into circulation. Continuous production of beer, vinegar, citric acid, and biomass from yeast, bacteria, microalgae, and fungi has been successfully carried out in airlift vessels at different working capacities. These bioreactors have also been widely used for animal cell cultures, especially for antibody production, reaching high productivity. Some authors stressed that airlift bioreactors combine the benefits of high loading of solid particles and good mass transfer inherent for three-phase fluidized beds [41].

Bubble column bioreactor

Bubble column reactors belong to the general class of multiphase reactors, which consist of three main categories, namely the trickle bed reactor (fixed or packed bed), fluidized bed reactor, and the bubble column reactor. A bubble column reactor constitutes a cylindrical vessel with a gas distributor at the bottom. The gas is sparged in the form of bubbles into either a liquid phase or a liquid–solid suspension. These reactors are generally referred to as slurry bubble column reactors when a solid phase exists. Bubble columns are intensively utilized as multiphase contactors and reactors in chemical, petrochemical, biochemical and metallurgical industries [42].

Bubble column reactors are widely used in chemical and allied industry for gas–liquid and gas–liquid–solid contacting or chemical reactions. This involves reactions such as oxidation, chlorination, alkylation, polymerization and hydrogenation, as important in manufacturing synthetic fuels by gas conversion processes, and in biochemical processes such as fermentation and biological wastewater treatment because of their simple construction and ease of operation [43-45].

Due to their industrial importance and wide application area, the design and scale-up of bubble column reactors, hydrodynamic and operational parameters characterizing their operation have gained considerable attention during the past 20 years.

Recent research with bubble columns frequently focuses on the following topics: gas holdup studies [46-55], bubble characteristics [44, 56-60], flow regime investigations and computational fluid dynamics studies [61-66], local and average heat transfer measurements [67-71], and mass transfer studies [72-76].

An important application area of bubble columns is their use as bioreactors in which microorganisms are utilized to produce industrially valuable products such as enzymes, proteins, antibiotics, etc. Using *Streptomyces cattleya*, Arcuri et al. [77] studied the production of thienamycin in a continuously operated bubble column bioreactor. Federici et al. [78] performed the production of glucoamylase by *Aureobasidium pullulans*. Sun and Furusaki [79] investigated the production of acetic acid in a bubble column by using Acetobacteraceae. Rodrigues et al. [80] reported that the cultivation of hybridoma cells in a bubble column reactor resulted in a high monoclonal antibody productivity of 503 mg/(L d). Bordonaro and Curtis [81] designed a 15 L bubble column reactor to produce root cultures of *Hyoscyamus muticus* which in turn produces secondary plant metabolites. Son et al. [82] developed a novel bubble column bioreactor to produce taxol by *Taxus cuspidate* and inoculated the cells in various types of bioreactors to test growth performance. Chang et al. [83] cultivated *Eubacterium limosum* on carbon monoxide to produce organic acids in a bubble column reactor.

Shiao et al. [84] investigated the tolerance of *Arabidopsis thaliana* hairy roots to low oxygen conditions in a bubble column reactor. A recent study that was not aimed to produce a bio product but, instead, investigated the hydrodynamic and heat transfer characteristics of the bubble column in the presence of microorganisms, has been carried out by Prakash et al. [44]. These authors utilized a suspension of yeast cells (*Saccharomyces cerevisiae*) as the solid phase in an air-water system. Another study carried out by Ogbonna et al. [85] was based on the potential of producing fuel ethanol from sugar beet juice in a bubble column. In this study, *S. cerevisiae* was used in order to investigate the feasibility of scaling up the process.

PHB PRODUCTION IN BIOREACTORS

PHB production in airlift bioreactor

Tavares et al. used the time constants as tools to evaluate the influence of the oxygen supply on PHB production by *C. necator* DSM 545 during the accumulation phase in an airlift bioreactor. Further, the suitability of different superficial gas velocities applied to the riser section for better PHB production performances was assessed. All these experiments were performed in a concentric draught-tube airlift bioreactor with a working capacity of 10 L and automatic temperature and pH controls. The gas sparger was a stainless steel perforated ring located at the bottom of the riser section. For comparison, a stirred tank fermenter experiment was performed. The stirred tank fermenter had 7 L of working volume, an inner diameter of 188 mm and was equipped with four baffles, three standard flat blade turbines with a diameter of 75 mm and automatic temperature- and pH controls. The gas sparger was a stainless steel perforated ring located at the bottom of the tank. Lyophilized stock culture of the production strain was maintained at -80 °C. For microbial PHB production, the cells were cultivated in a two-stage method. First, a suspension of the stock culture in water was inoculated in Erlenmeyer flasks (0.1 ml l⁻¹) with nutrient broth medium (Table 1) and incubated for 15 h at 30 °C and 250 rpm. Then, an amount of 10% (v/v) of this culture was used to inoculate the seed culture medium and incubated for 15 h at 30 °C and 250 rpm. A volume of about 1.5 L of this culture, large enough to guarantee an initial biomass concentration of 0.4 g/L, was transferred to the airlift bioreactor containing about 8 L of initial medium (Table 1). Into the stirred vessel, 1 L of the seed culture and about 6 L of initial medium were transferred. The temperature was maintained at 32 °C, and the pH-value was controlled at 7.0 by addition of H₂SO₄ (4N) and NH₄OH (v/v 1:5) solutions. Dissolved oxygen concentration (pO₂) was maintained at 20% of air saturation by increasing air flow in the airlift reactor and by increasing the agitation speed in the stirred vessel. An abrupt pO₂ increase indicated the depletion of glucose and fructose in the culture medium at the end of the growth stage. At that time, PHB accumulation was induced by ammonium limitation, which was carried out by the replacement of the NH₄OH pH control flask by a NaOH (4N) pH control flask, and by the addition

Table 1: Comparison between three grid resolutions.

Case number	Number of nodes	Grid size	Air volume fraction	Air velocity	Time (s)
1	89235	361696	0.070267	0.18	200
2	112793	470428	0.08398	0.19	200
3	137395	591377	0.086932	0.196	200

of a glucose and fructose solution (190 g/L) into the fermenter at a constant rate. PHB production in the airlift reactor was examined by carrying out six different runs under six different values of aeration during the accumulation phase. Cell concentration was determined by measuring the CDM after centrifugation at 9220×g for 10 min at 4 °C and subsequent filtration using 0.45 µm membrane and drying at 104 °C until constant mass was obtained. Residual biomass (X_r) was defined as total cell mass minus PHB mass. The PHB content (PHB%) was defined as the ratio of PHB concentration to total cell mass. pO₂ was measured with a polarographic dissolved oxygen (DO) probe (Ingold, Switzerland). The probe was calibrated after the sterilization process. The 0% oxygen point was achieved by sparging pure nitrogen into the culture medium to displace all oxygen in solution, whereas the 100% oxygen calibration point was achieved before inoculation when the broth was fully aerated at Q_{air} = 20 L min⁻¹. The PHB concentration was determined by GC [38]. Measurements of glucose and fructose concentrations in the medium were carried out with HPLC. The outlet gas composition was analyzed by a paramagnetic and an infrared analyzer. The inlet airflow rate was measured by a mass flowmeter[41].

PHB production in a 7 L lab scale bioreactor

In 2004, Shilpi Khanna et al. devoted efforts to optimize the growth of *Ralstonia eutropha* NRRL B14690 in the presence of nutrients, which would not only decrease the production cost of PHB but also help in increasing the productivity. Using fructose and ammonium sulphate as carbon and nitrogen source in a nutrient media reported in literature, *R. eutropha* exhibited a maximum biomass of 3.25 g/L with a PHB concentration of 1.4 g/L in 48 h. To determine the maximum growth potential of *R. eutropha*, the organisms was cultivated on different carbon sources, among which

fructose, lactic acid, sucrose and glucose yielded good growth and PHB production. In order to incorporate cheaper nitrogen source and growth factors in media, ammonium sulphate was substituted by ammonium nitrate, urea and ammonium chloride. Urea featured highest PHB accumulation of 3.84 g/L after 60 h of growth. Instead of yeast extract as a rather expensive source of minerals and vitamins, corn steep liquor was used which yielded a PHB concentration of 2.66 g/L. Statistical media optimization design was then used to optimize the composition of culture medium for maximizing the productivity of PHB. A maximum of 6.65 g/L residual biomass and 6.75 g/L PHB was obtained using optimized concentrations. Growth kinetics of the culture was then studied in a lab-scale bioreactor [86]. Batch cultivation was carried out at 30 °C in a 7 L bioreactor containing 4 L of media. Culture pH was maintained at 7.0. DO concentration was maintained at 30% saturation value by manually adjusting the speed of the agitator and/or the airflow rate. Growth, PHB production and the nutrient utilization profile were studied.

Fig. 2. shows the growth kinetics of *R. eutropha* on 40 g/L fructose as carbon source and 1 g/L urea as nitrogen source. 0.5 g/L of CSL was used as mineral source. After a lag phase of approximately 10 h, the biomass increased to 20.73 g/L in 60 h. In this period, 0.37 g/L nitrogen was consumed from an initial value of 0.55 g/L; at the same time, approximately 30 g/L of fructose was metabolized. The synthesis of PHB, product of interest, started after 15 h and reached a final concentration of 9.35 g/L. The yield of fructose to PHB (Y_{P/S}) was 0.24 g/g, and a volumetric productivity of 0.15 g/(L·h) was obtained. This is a remarkable improvement in comparison to 3.8 g/L (productivity: 0.08 g/(L·h)) obtained previously [87] in batch cultivation of *A. eutrophus* (*R. eutropha*, *C. necator*) using 40 g/L fructose.

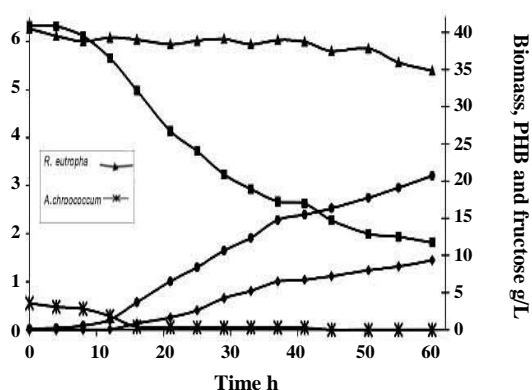


Fig. 2: Bioreactor (7 L) batch kinetics ((&) fructose; (^) PHB [86].

Perdos-Alio et al. [88] obtained only approximately 1.9 g/L of the polymer by *A. eutrophus* with fructose as carbon source but the polymer content of the cell dry matter was very high (96%). The results obtained in another study using *R. eutropha* on fructose, presented by Lino et al., are higher regarding a maximum polymer concentration of 6.9 g/L and a productivity of 0.14 g/(L·h) [89]. *Pseudomonas cepacia*, when grown on xylose as sole carbon source, gave a PHB concentration of 1.55 g/L after 60 h of batch cultivation [90]. The same culture, when grown on lactose, achieved a PHB concentration of 2.0 g/L in 120 h [91]. A total biomass concentration of 1.17 g/L with a productivity of 0.014 g/(L·h) was obtained in batch cultivation of *Azotobacter chroococcum* with starch as carbon source [92]. Thus, the results obtained in the study of Shilpi Khanna are significantly better than the previous investigations reported in literature.

This significant improvement in PHB and biomass formation may be due to the accomplished optimization of the nutrient media, which had been missing in the earlier studies. An important feature of the present study was the relatively cheap corn steep liquor (CSL) being used instead of expensive yeast extract as a source of vitamins and growth factors; while CSL is a promising nutrient component for large-scale applications, the use of yeast extract on larger scale is impeded due to economic reasons [86].

PHB production in bubble column bioreactor

In order to avoid mechanical stirring, which puts stress on growing biomass by the generation of shear forces,

special types of bioreactors have been proposed, the most popular of which are loop bioreactors [93-95]. Loop bioreactors are characterized by a defined directed circulation flow, which can be driven in fluid or fluidized systems by a propeller or jet drive and most typically in gas-liquid systems by an airlift drive or liquid pump. They are particularly suitable for fluid systems requiring high dispersion priority. On the other hand, their simple constructions and operation result in low investment and operational costs [96]. In bio-reactions where poorly water soluble gases are to be dissolved in an aqueous reaction medium, it is required that the gas-liquid contact occurs in plug flow. Otherwise the driving force is too small, and a large portion of the gas is lost unless the reactor is very big. When one of the substrates is supplied via a gas phase that is sparged to the reactor, there are two rate-determining factors possible, namely the gas to liquid transport and the bio-reaction in the liquid phase [94]. Loop bioreactors have shown an acceptable level of performance for the production of biomass from natural gas due to their unique hydrodynamic characteristics [93, 96, 97]. Rahnama et al. produced PHB in a bubble column bioreactor by *Methylocystis .hirsuta*. For this purpose, a 1 L glass column of 51.0 cm height, 5.0 cm internal diameter and 200 mL working volume, as shown in Fig. 3, was used. Natural gas and air streams entered through separate lines, mixed at the bottom of the reactor, and were fed into the column by a sparger having 40 fine holes. To prevent evaporation, a condenser was installed on the top of the column. For all experiments, reactor temperature and pH-value were adjusted at 30 °C and 7.0, respectively, by a heat controllable water bath and 1.0 N HCl/NaOH solution. 20 mL of shaking flask culture was used to inoculate 180 mL of the fresh medium, which was incubated at 30 °C under continuous aeration of a natural gas/air mixture in a bubble-column bioreactor according to a full factorial design. All cultivations were performed in two stages as follows: In a first stage, cells were grown in liquid medium under a natural gas/air mixture in the bubble column bioreactor at 30 °C. In the second stage, cells were harvested by centrifugation at 5,000 rpm for 20 min, and the pellets were re-suspended in a nitrogen-deficient medium.

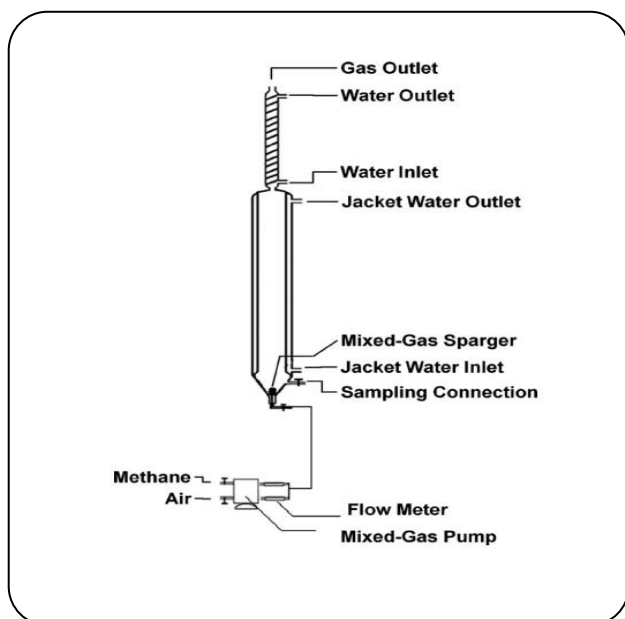


Fig. 3: A schematic diagram of a bubble column bioreactor [95].

The capability of *M. hirsuta* for growth in the two culture media using a bubble column bioreactor is shown in Fig. 3. A culture medium modified by Mokhtari *et al.* was used for the cultivation [98].

PHB production in a forced-liquid vertical loop bioreactor

A forced-liquid vertical loop bioreactor [94] made of glass with an on-line dissolved methane detector was used for experiments carried out by Yazdian *et al.* A schematic diagram of the bioreactor is illustrated in Fig. 4. The bioreactor consisted of a single-wall looped-glass tube of diameter 0.03 m and height 2 m. Air and natural gas (mostly methane; 90%) were distributed through a perforated tube by means of a gas pump within a vertical section. The methane content of the exit stream from the separator was continuously monitored. The liquid medium was circulated by a liquid magnetic pump. A Komax static mixer was installed in the discharge part (0.1 m in length). The liquid passed through the static mixer, which induced a swirling motion. The gas was injected through two spargers (each with six holes) before the static mixer. Because of using a well-designed sparger on the suction side, the static mixer could be omitted. To compensate for loss

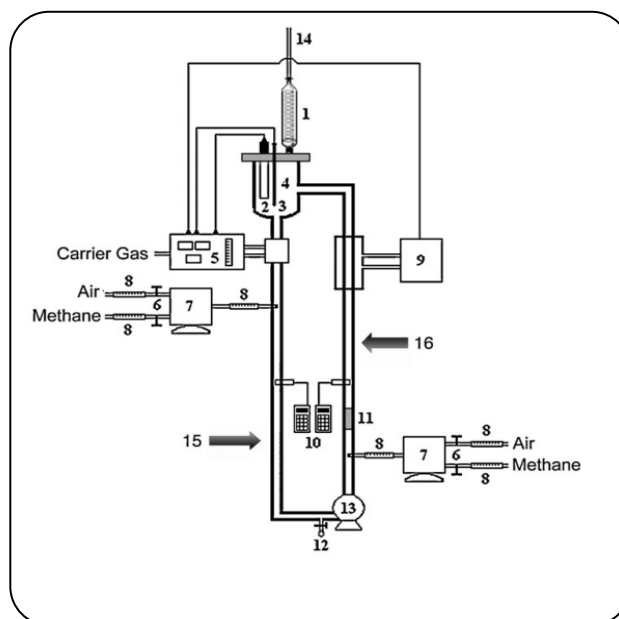


Fig. 4: A schematic diagram of a forced-liquid vertical tubular loop bioreactor [95].

of substrate due to evaporation, a water-cooled condenser was mounted on top of the fermenter. In addition to this water-cooled condenser, one side of the loop was jacketed to supply cooling. All experiments were carried out at $30 \pm 0.5^\circ\text{C}$. This temperature was maintained by a Temperature Loop Controller (TLC) placed inside the detector and connected to an electrical heater positioned at the top of the VTLB. The pH-value of medium was measured with an online pH probe.

PHB production was carried out at the same optimum condition, determined in the bubble column bioreactor described above, in a well-mixed VTLB. Only the results of VTLB are presented, and no variables were studied for this bioreactor. Only the experiments with favorable hydrodynamic and mass transfer characteristics were carried out at the optimum conditions determined in the bubble column bioreactor. According to Yazdian *et al.*, the highest mass-transfer coefficient ($k_{La} = 0.034 \text{ s}^{-1}$) was obtained at $S = 0.61$, $H/D = 67$, $Us_L = 0.35 \text{ m/s}$, and $Us_G = 0.035 \text{ m/s}$ whereas the bubbly flow regime was observed in the VTLB. S , H/D , Us_L and Us_G indicate the separator to bioreactor volume ratio, height to diameter ratio of VTLB, superficial liquid velocity (m/s) and superficial gas velocity (m/s), respectively. In order to investigate the performance of the VTLB, a series

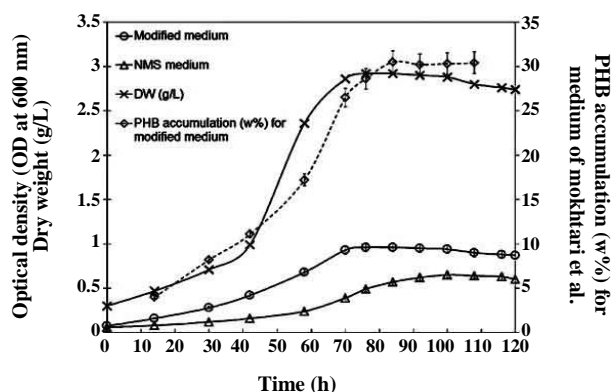


Fig. 5: Comparison of NMS and modified media for cell growth and PHB accumulation of *M. hirsuta* in a bubble column bioreactor [95].

of experiments based on k_{La} , was performed by Yazdian *et al.* [94], previously. In all of these experiments, S , H/D , UsL , and UsG were fixed at the values mentioned above. Methane was monitored by a dissolved methane sensor and values are 2.35 mg/L, 5.87 mg/L and 9.40 mg/L, while the methane-to-air ratios were 20/80, 50/50 and 80/20, respectively. The fermentation was carried out for about 8 h after induction of nitrogen limitation. The average PHB content of CDM was as high as 51.6% (w/w) in the VTLB (optical density is about 1).

M. hirsuta was used for the first time to produce PHB from natural gas in loop bioreactors such as bubble column and vertical loop bioreactors. It was found that PHB accumulation by this microorganism occurs in a growth associated way. A statistical design of experiments was applied to correlate PHB production with process variables. It was found that this bacterium could accumulate PHB up to 51.6% w/w of CDM in the VTLB at optimum conditions (Fig. 5). Further research is under way to enhance PHB production by this methanotrophic bacterium using natural gas as a cheap substrate for development of a cost effective bioprocess. C. Zúniga *et al.* (2011) [99].

SIMULATION

Simulation of airlift bioreactor

Today Computational Fluid Dynamics (CFD) has emerged as a powerful tool for simulation of flow behavior [100, 101] and local hydrodynamics in bubble columns, membrane bioreactors, and airlift and stirred

tank reactors [102-105]. This is an approach based on first principles in which the governing equations of continuity, momentum, and energy for each phase are solved. An important advantage of the CFD approach is that column geometry and scale effects are automatically accounted for. High cost of experiments, inaccessibility to all locations in the system and turbulent multiphase flow lead to using CFD simulation, which is a cost-effective tool. So, CFD can shed light on investigation and prediction of various characteristics [104].

Computational fluid dynamics is a powerful tool to analyze the bubble columns as well as PHB producing airlift bioreactors. CFD can be used to analyze different characteristics in airlift reactors including gas hold up, shear stress, mass transfer, and flow dynamics. [106-110] Mousavi *et al.* [111] investigated ferrous biooxidation in a bubble column bioreactor by CFD simulation in the Eulerian framework to describe the hydrodynamics of the bioreactor and ferrous biological oxidation in a two-phase system. In 2010, Jia *et al.* used CFD modeling to investigate phenol biodegradation by immobilized *Candida tropicalis* in a three-phase bubble column to develop a 3D transient CFD model for simulating the dynamic behaviors of batch phenol biodegradation. Wang *et al.* [112] studied bio-treatment of toluene waste gas in a three-phase airlift loop reactor by immobilized *Pseudomonas putida* using a transient CFD modeling. Xu *et al.* [113] used a two-dimensional axisymmetric CFD model to optimize the inner structure of a cylindrical airlift bioreactor. Liu *et al.* [114] developed a two-dimensional CFD model to optimize structure design of an airlift bioreactor for hairy root culture. Bannri *et al.* [108] used CFD to simulate a new design of airlift bioreactor. The results showed a significant improvement in the performance of the bioreactor by the increase of mass transfer and the decrease of the shear stress.

The main objective in simulate an airlift bioreactor producing PHB is to describe various hydrodynamic characteristics including gas holdup, volumetric oxygen transfer coefficient, and shear stress as well as PHB biosynthesis reaction using CFD in the Eulerian framework. A metabolic model of PHB biosynthesis was applied to examine the PHB biosynthesis reaction and used in the simulation. Furthermore, optimizing kinetic parameters to predict the experimental data in the

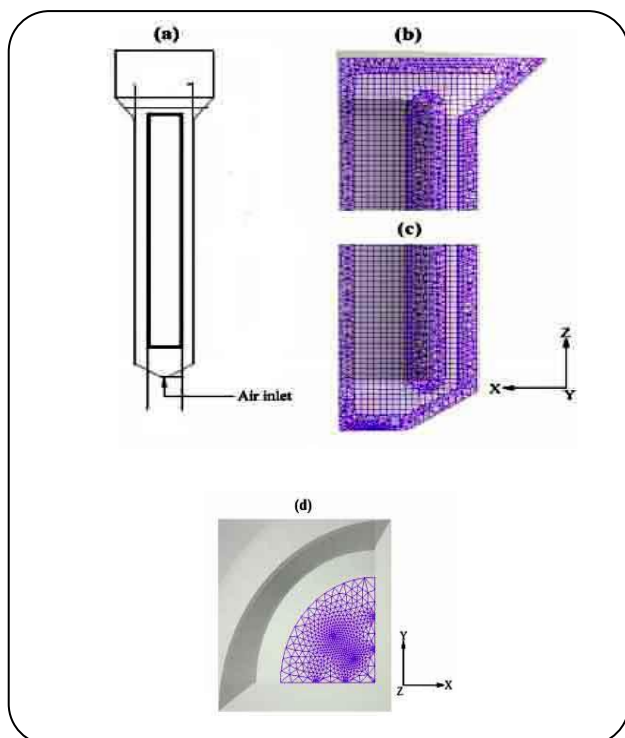


Fig 6: (a) Schematic diagram of the simulated bioreactor, [105] and the generated mesh of the bioreactor at (b) upper side view, (c) lower side view, and (d) bottom cross view (Sparger).

bioreactor was carried out According to *Tavares et al.*, the PHB production process consisted of a growth and an accumulation phases [41]. After the initial cell growth phase in the bioreactor, PHB growth was induced by ammonium limitation, and residual biomass (total cell mass minus PHB mass) remained constant during the accumulation phase.

The PHB pathway of the bacterium *C. necator* consists of three major steps including the catalytic action of the key enzymes 3-ketothiolase, reductase and PHA synthase. For investigation of the PHB production in the bioreactor, a model proposed by Leaf and Srienc was used based on equation (1).

$$\frac{d[\text{PHB}]}{dt} = R_{\text{synthase}} - \mu_s [\text{PHB}] \quad (1)$$

In the experiment, the feed solution contained glucose and fructose. Because both compounds are converted to glucose-6-phosphate, for simplicity, glucose was assumed as the only substrate in the simulation.

Eulerian model with two-phase flow (air dispersed in liquid phase) was applied in this study. In this model,

continuity, momentum, and energy equations are solved for each phase. The continuity equation is written as:

$$\frac{\partial}{\partial t} (\alpha_j \rho_j) + \nabla \cdot (\alpha_j \rho_j \bar{u}_j) \quad (2)$$

Where α_j is the volume fraction, ρ_j is density, and \bar{u}_j is the average velocity for the j phase.

The bioreactor configuration investigated by *Tavares et al.* [41] is shown in Fig.6 (a). Considering the massive required computations, to decrease the time of simulation, only one quarter of the bioreactor was simulated; this approach was reasonable due to its symmetry. In this study, GAMBIT 2.3.16 was used to create the geometry and the grids. For simulation, Fluent 6.3.26 was utilized. An unstructured mesh with the type of Tetra-Hybrid/Hex Core was selected. The flow pattern around the air diffusers is important for the reactor performance. Therefore, they were meshed using size function method to create a very fine mesh near the holes to make sure that the convergence is reached (Fig. 6b, c, and d). The SIMPLE method of pressure-velocity coupling was used. Further, the used time step was 0.01 seconds. In this paper, a 24 core Super Micro system computer was used. Under relaxations factors including pressure, body force, density, and momentum are equal to 0.3, 1, 1, and 0.7, respectively. The convergence criterion was 103. The first order upwind method was employed for discretization of fundamental equations. To determine the sensitivity analysis of the system, aeration rate was changed and a significant variation in gas holdup was not observed. Air was used as the gas phase, whereas the liquid phase was assumed as mixture of glucose, water, H^+ , CO_2 , and PHB monomers, according to the simplified reaction. In reality, PHB is an intercellular biopolymer, but in the simulation with the Fluent software, PHB-containing cells were assumed as a material with a density and molecular weight (molecular weight of a 3HB monomer) of 1250 kg/m^3 [115] and 86.09 g/mol respectively. The authors did not report the gas holdup of the bioreactor, therefore, to validate the obtained gas holdup of the bioreactor by simulation, some correlation for calculation of gas holdup in bubble columns and airlift reactors were used for comparison of the results. To investigate the production of PHB in the bioreactor in the accumulation phase, the model equations can be written to describe the hydrodynamics of bubbly flows, and were solved simultaneously by a MATLAB program

using the presented information, and through an optimization. the V_1 values of thiolase, reductase and synthase step were determined as V_1 (thiolase) = 1.8 mM/min, V_1 (reductase) = 400 mM/min, and V_1 (synthase) = 380 mM/min to suitably predict the experimental PHB production rate [41]. V_1 values of thiolase, reductase, and synthase steps were unknown. In the optimization, all possible combinations of V_1 of the three steps were used to solve the mathematical model equations presented by Leaf and Srienc [116], and their results were compared with the experimental results of Tavares et al. [41] and the set of combination of V_1 with which lowest difference was obtained was chosen as the optimized values. A wide range of data has been reported for V_1 by various authors using different assays [116], and it was not possible to exactly determine this parameter for the desired case; however, the determined values for V_1 are located within the proposed data. As result, a PHB production rate (3.436 $\mu\text{M/s}$), and production of 1 mole PHB monomer per 1 mole glucose substrate consumption according to the simplified reaction stoichiometry, variations of substrate and PHB were obtained and presented in Fig. 7, in addition to experimental data by Tavares et al. [41]. Data are related to an aeration rate of 30 L/min, as a nearly optimized value. At higher aeration rates, no significant difference in performance of production reported was observed, indicating that the oxygen transfer was not a limiting factor at higher aeration rates than 30 L/min.

In Fig. 7, the accumulation phase has been taken into account with the initial time of 10.8 h and 612 g substrate and 29.5 g PHB according to Tavares et al. [41]. It should be noted that all of the substrate was almost consumed according to experimental results by Tavares et al. [42]. This difference can be explained by considering the consumption of substrate for cells maintenance and supply of other probable metabolic paths. In addition, it can be inferred that a major part of the consumed substrate has been used by the cells for production of PHB. In this paper, hydrodynamics and reactions of an airlift bioreactor producing PHB were investigated using CFD. The results show the ability of CFD to gain new insights in biochemical processes. The Eulerian approach was used to predict the hydrodynamics of the airlift bioreactor. A PBE was implemented, and gas holdup, liquid velocity vectors, shear stress and volumetric

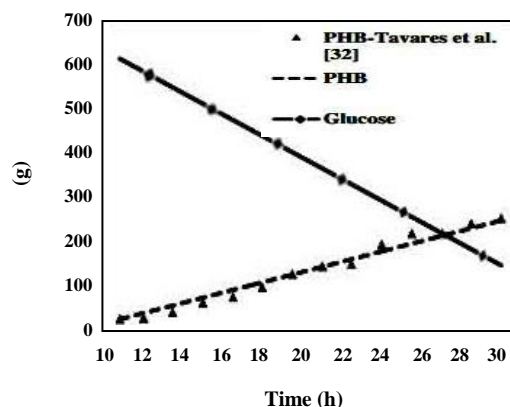


Fig. 7: Variation of the modeled polyhydroxybutyrate and glucose and experimental data by Tavares et al. [105].

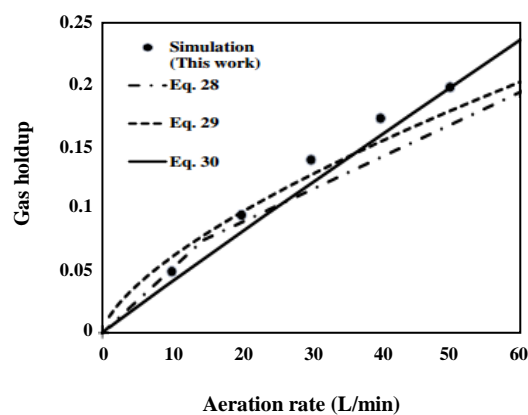


Fig. 8: Gas holdup obtained by simulation and various correlations vs aeration rate [105].

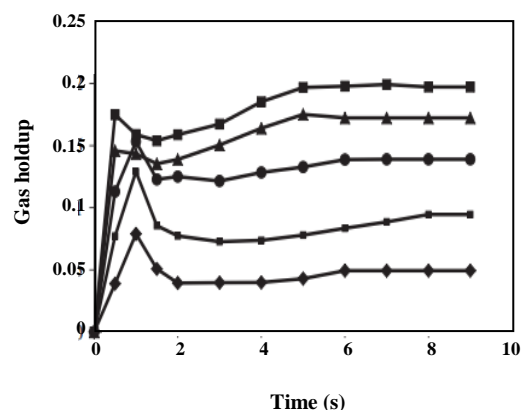


Fig. 9: Gas holdup vs time for five different aeration rates [105].

oxygen transfer coefficient of the bioreactor were examined and various correlations were presented (Fig. 8). The simulated gas holdup and the experimental volumetric oxygen transfer coefficient were compared with various correlations (Fig. 9).

By using the metabolic modeling of PHB by Leaf and Srienc, [116] the experimental PHB production rate by Tavares *et al.* [41] was predicted suitably by the V_1 (maximum forward reaction rate) values of thiolase, reductase and synthase steps as V_1 (thiolase)=1.8 mM/min, V_1 (reductase)=400 mM/min, and V_1 (synthase)=380 mM/min. A simplified reaction was implemented in the software as a zero order reaction with a rate of 3.436 $\mu\text{M/L}$ by assuming PHB-containing as a matter with a density equal to 1250 kg/m^3 [115] and a molecular weight equal to 86.09 (molecular weight of a PHB monomer). The molar concentrations of PHB and glucose were determined at 2 and 10 min with a mild accumulation of matters at the top of the riser. CFD simulation of biochemical reactions is not as easy as common chemical reactions; however, it may increase the understanding of process and help to visualize it, and also it can be used to improve or scale up the process.

Simulation of tubular loop bioreactor

Loop reactors, which are characterized by a defined directed circulation flow that can be driven in fluid or fluidized systems, have shown an acceptable performance in the production of biomass from natural gas because of their unique hydrodynamic characteristics. Gas holdup is an important hydrodynamic characteristic of multiphase systems not only for its effect on the liquid circulation rate, but also for its effect on gas residence time, oxygen transfer, heat transfer, mass transfer and liquid mixing [11]. Thus, understanding Gas holdup is essential for a reliable description of a loop bioreactor.

Because multiphase mixtures occur very frequently in processing industries, modeling methods need to be extended to deal with these multiphase flows. Due to the inherent complexity of two-phase flows from both a physical and numerical point of view, "general"-use CFD codes do not exist.

The main reasons for this absence include the physical complexity of two-phase flow phenomena, complex interactions between the gas and the liquid phases and the complexity of the governing equation

numerics. Taguchi design of experimental methods are extensively used to optimize the critical parameters of any process. They largely minimize the number of experiments required to achieve an optimal set of performance characteristics for a process by incorporating readily available fractional factorial matrices or orthogonal arrays (OA). This approach is a combination of mathematical and statistical techniques used in empirical studies. In the Taguchi approach, OA and analysis of variance are used as the analytical tools. OA can minimize the number of experimental replicates, and ANOVA can estimate the effect of a factor on the characteristic properties. A conventional statistical experimental design uses measured values of the characteristic properties to enable the determination of the optimal conditions, whereas the Taguchi method identifies the experimental condition having the least variability to determine the optimal condition.

The microorganism (*Methylobacterium* sp.) used in the investigation done by Khosravi-Darani *et al.* [117] was isolated from samples obtained from an oil field in Iran. The bacteria were cultured on methane salt broth, a carbon-free salt broth medium. The equations were solved sequentially. The nonlinear governing equations were linearized to produce a system of equations for the dependent variables in every computational cell. The resulting system was then solved to yield an updated flow-field solution. A point implicit (Gauss-Seidel) linear equation solver was used in conjunction with an algebraic multigrid method to solve the resulting scalar system of equations for dependent variable in each cell.

To solve conservation equations for chemical species, software predicts the local mass fraction of each species, Y_i , through the solution of a convection-diffusion equation for the i^{th} species. This conservation equation takes the following general form:

$$\frac{\partial}{\partial t}(\rho Y_i) + \nabla \cdot (\rho \mathbf{v} Y_i) = \nabla \cdot \mathbf{i} + R_i S_i \quad (3)$$

Here, R_i is the net rate of production of species i by chemical reaction, and S_i is the rate of creation by addition from the dispersed phase plus any user-defined sources. An equation of this form will be solved for $N - 1$ species, where N is the total number of fluid phase chemical species present in the system. Since the mass fraction of the species must sum to unity, the N^{th} mass fraction is determined as one minus the sum of the $N - 1$ solved mass fractions. To minimize numerical error,

the N^{th} species should be selected as that species with the overall largest mass fraction, such as N_2 when the oxidizer is air.

J_i is the diffusion flux of species i , which arises due to concentration gradients. By default, FLUENT uses the dilute approximation, under which the diffusion flux can be written as:

$$J_i = -D_i \cdot m \nabla Y_i \quad (4)$$

Here D_i , m is the diffusion coefficient for species i in the mixture. In this work, a commercial grid-generation tool, GAMBIT 2.2 was used to create the geometry and generate the grids. The use of an adequate number of computational cells while numerically solving the governing equations over the solution domain is very important. To divide the geometry into discrete control volumes, more than 4.7×10^5 3D tetrahedral computational cells and 112,793 nodes were used. The following simplifications were assumed in the simulated geometry: (a) the effects of the liquid pump and the separator part of the bioreactor were negligible, (b) the structure of the static mixer was simplified, and (c) the four jet holes were considered to be four active holes in the spargers (Fig 9 & 10). The phase-coupled simple (PC-SIMPLE) algorithm, which extends the SIMPLE algorithm to multiphase flows, was applied to determine the pressure-velocity coupling in the simulation. The velocities were solved coupled by phases, but in a segregated fashion. The block algebraic multigrid scheme used by the coupled solver was used to solve a vector equation formed by the velocity components of all phases simultaneously. Then, a pressure correction equation was built based on total volume continuity rather than mass continuity. The pressure and velocities were then corrected to satisfy the continuity constraint. The volume fractions were obtained from the phase continuity equations. The results are grid independent. To select the optimized number of grids, a grid independence check was performed. The three mesh sizes that were examined included a total of 361.696, 470.428 and 591.377 cells, respectively. The data were recorded at 200 s, which was the point at which the system stabilized for all three cases. A comparison of the data generated at 200 s for these three grid sizes is given in Table 1. Based on the accuracy of resulting data, which relied on the air volume fraction and air velocity, case 2 was selected as

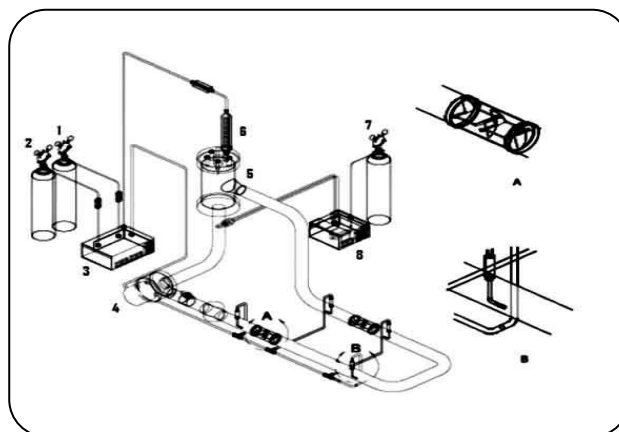


Fig. 10: Schematic diagram of the forced-liquid horizontal tubular loop bioreactor used for the experiments: (1) natural gas capsule; (2) air capsule; (3) gas pump; (4) liquid pump; (5) gas-liquid separator; (6) condenser; (7) helium capsule; (8) dissolved methane detector. Magnified views of the (A) static mixer and (B) sparger [101]

the optimum mesh size. Although the final volume fractions and velocities for cases 1 and 2 were very similar, case 2 was selected for the simulations due to the smaller number of meshes [101].

Modeling of a continuous 5-stage bioreactor cascade

Atlić *et al.* have experimentally tested a continuous five-step stirred tank bioreactor cascade system (5-CSTR) to produce PHB with high molecular mass and low polydispersity index. The five-stage system was designed to provide balanced biomass growth regarding the complete nutrient supply in the first reactor. Subsequently, the second vessel was intended to finalize consumption of nitrogen source so that in this stage growth-associated PHA synthesis should occur. Furthermore, in the last three reactors, the aim was to expose the cells to non-growth associated synthesis of PHA under nitrogen-free conditions in order to increase molecular mass of PHA and to reach a low polydispersity index as well as to collect information about the cellular ability to tolerate permanent long-term N-limitation without serious cell damage or loss of the ability of PHA synthesis. Under operating conditions (dilution rate for R_1 was $D_1 = 0.139 \text{ h}^{-1}$; the overall dilution rate amounted to 0.034 h^{-1}), a final concentration of $81.00 \pm 0.27 \text{ g/L}$ of CDM containing $77 \pm 7.5 \%$ w/w of PHB and overall volumetric PHB productivity 2.14 g/L.h were obtained [120].

In order to provide even better results considering the productivity and production costs, formal kinetic modelling was carried out by Horvat to find out whether the applied 5-stage system carries the potential to produce higher concentration of biomass with superior PHB content, along with maintaining the molecular mass at a constant and high level, as well as sustaining the favorably low polydispersity of PHB [119]. Optimization of the continuous 5-step PHA production system using formal-kinetic mathematical modelling, especially considering dilution rate, productivity, growth rate and nutrient (C and N source) concentrations may reduce the number of experiments on laboratory scale concerning strain treatment, technological set-up and process design which is needed to draw well-grounded conclusions about the ultimate potential of the 5-CSTR for PHA production aiming at higher productivity compared to the basic experiments reported by *Atlić et al.* [120].

They use of *C necator* as a microorganism for producing PHB was carried out in three different fermentations (FM1, FM2, FM3), which differ in operational characteristics, were modelled according to principles described below.

Modelling principles

(a) Residual biomass (non-PHB part of biomass) is synthesized from glucose and ammonia as C and N sources, respectively. The growth rate is determined by nitrogen and by glucose concentration; the nitrogen depletion causes termination of biomass growth and predominant PHA formation. (b) PHB is synthesized from glucose; this occurs partly during the phase of balanced biomass growth (growth associated synthesis) and predominantly in the non-growth phase (non-growth associated PHA synthesis). High nitrogen source concentration is applied as an inhibitory regulator of non-growth associated PHB synthesis. The Luedeking–Piret model [121] of partial growth associated product synthesis was chosen as the modelling strategy. (c) A part of the glucose is converted to CO₂ and other metabolites, such as excreted organic acids. (d) Previously conducted kinetic analysis was performed to determine the basic model parameters. (e) Two specific growth rates' relations to substrate concentrations (C; N) have been tested: "double Monod" term according to *Megee et al.* [122] and Mankad-Bungay relation for double substrate

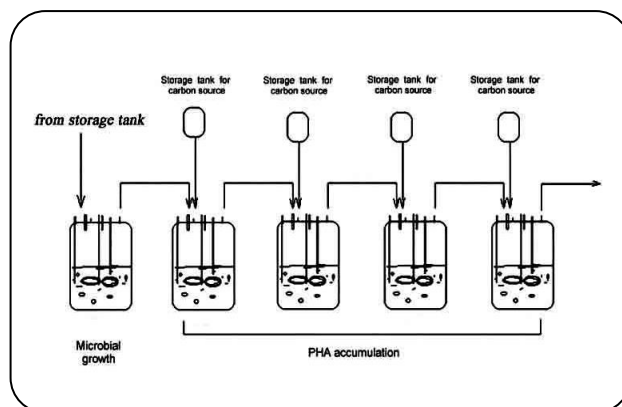


Fig. 11: Five-step bioreactor cascade for continuous PHB production as used in the experiments performed by *Atlić et al.* [120].

limited growth according to *Mankad and Bungay* [123] Specific non-growth associated PHB production rate:

$$a_n = a_{\max} \left(\frac{S_n}{S_n + k_{as}} \right) \left(\frac{k_{in}}{N_n + k_{in}} \right) \quad (5)$$

Mathematical model

The basic formal kinetic model with growth-associated and non-growth-associated PHB synthesis under nitrogen limitation was adopted from *Koller et al.* [124]. The model was restructured according to the applied 5-step continuous mode of fermentation (Fig. 11). The model consists of 20 differential mass balance equations related to substrate (S_n), product (P_n), nitrogen (N_n) and residual biomass (X_n) concentrations ($n=1-5$, position of reactor in the cascade).

Kinetic parameters were obtained through kinetic analysis of experimental data originated from cultivation FM1 reported earlier. Mathematical model was validated by simulation of two additional cultivations (FM₂ and FM₃) [119] operated with different operational characteristics. Afterwards, the continuous biotechnological system was simulated for the steady states (after 23, 29 and 32 h of batch mode for FM1, FM2 and FM3, respectively) by Berkeley-Madonna quick solver using four-step Runge–Kutta numerical integration method for solving differential equations. This software package was used for all modelling and simulation procedures in this work. Simulated results were adjusted for steady state conditions to the experimental data obtained by *Atlić et al.* [120]. Here, the least squares method was applied.

Simulation of PHB in fed-batch culture

In fed-batch, it is possible to keep the substrate concentration at a low level to prevent the undesired effects of high substrate concentration e. g. inhibition of microbial growth. However, in this system, higher productivity compared with that of batch culture, cannot be achieved without having a sophisticated feeding strategy. A proper feeding strategy can be designed in simulation of the environment and thus employing an appropriate model of the system. In 2006, *Shahhoseini et al.* [125] developed a mathematical model of fed-batch culture to simulate the process of PHB production during fed-batch culture.

For choosing the best kinetic model for bacterial growth, fed-batch experimental data extracted from literature were employed to estimate model parameters and then to evaluate simulation results. These experiments were conducted by *Suzuki and co-workers* to produce PHB by *Pseudomonas extorquens* (previously known as *Pseudomonas sp. K*) in fed-batch culture [126]. Kinetic models proposed by several researchers were investigated in order to select the one, which results in better performance of the process model. The criterion for this selection was $\delta 2$ as described in Eq. (1). An optimization routine was used to estimate model parameters.

$$\sigma^2 = \frac{\sum(\text{model data} - \text{experimental data})^2}{\text{degree of freedom}} \quad (6)$$

Where, degree of freedom is difference between number of experimental data and estimated parameters.

Suzuki and colleagues selected *P. extorquens* among 51 bacterial strains from soil due to its capability of accumulating intracellular PHB and good growth on methanol as a sole carbon source. Methanol concentration was kept between 0.2-0.5 g/L, and the initial concentration of nitrogen was 0.2 g/L [126].

A high concentration of methanol inhibits bacterial growth. Therefore, the Monod model as the most popular kinetic model, would not be appropriate for use in this process. Four different kinetic models that could fit into data involving growth rate versus methanol concentrations were investigated. The list of kinetic equations is shown in Table 2. An optimization program was written in order to calculate the values of $\delta 2$ for each model. These values are shown in Tables 3, 4. These results indicate that the Haldane model leads to the lowest

value for $\delta 2$. Therefore, this kinetic model was selected to be incorporated into the fed batch culture model of the PHB production process.

Fed-batch model

Mass Balance equations: In this section mass balance for nutrient sources and the product were written and Haldane kinetic model was added to the equations

Mass balance for residual biomass.

$$\frac{dX_r}{dt} = \left[\frac{\mu_m S}{(K_s + S) \left(1 + \frac{S}{K_i} \right)} - \frac{1}{V} \frac{dV}{dt} \right] \cdot X_r \quad (7)$$

Carbon source (methanol) mass balance.

$$\frac{ds}{dt} = -K_1 \frac{dX_r}{dt} - K_2 X_r + \frac{F_m}{V} S - \frac{dV}{V dt} \quad (8)$$

Nitrogen source mass balance.

$$\frac{dS_n}{dt} - K_3 \frac{dX_r}{dt} + \frac{F_N}{V} S_n - S_n \frac{dV}{V dt} \quad (9)$$

Product mass balance.

$$\frac{dP}{dt} = -K_4 \frac{dX_r}{dt} + K_5 X_r - \frac{PdV}{V dt} \quad (10)$$

Total feed rate to the bioreactor.

$$\frac{dV}{dt} = \text{Feed rate} - \text{Volume loss} \quad (11)$$

In equation 6 above, volume loss is equal to the sampling volume per hour plus the rate of volume loss due to the evaporation.

Simulation results of the first method

The first phase of this method involved growth when a nitrogen source was added to the reactor. The second phase was the PHB production stage, where no nitrogen was fed into the reactor. In the present work done by *Shahhoseini* [125], an optimization program was written in order to estimate the values of K1-K5 parameters by using experimental data from the first cultivation method. In each iteration of the optimization procedure, equations 2 to 6 were solved and the values of calculated biomass, The objective of the optimization was minimizing total error squares between the data and model predictions. The equations were solved numerically by employing a program, written in this work [125].

Table 2: Selected kinetic equations for PHB production modeling and estimated parameters for the kinetic models.

	equation	μ_m (h ⁻¹)	k_s (g/L)	k_i (g/L)	σ^2
Haldane	$\mu = \frac{\mu_m \cdot s}{(k_s + s) \left(1 + \frac{s}{k_i}\right)}$	0.2556	0.0251	10.4854	2.75×10 ⁻⁴
Edwards 1	$\mu = \frac{\mu_m \cdot s}{k_s + s} \exp\left(-\frac{s}{k_i}\right)$	0.2334	0.0149	19.784	4.51×10 ⁻⁴
Edwards 2	$\mu = \mu_m \left(\exp\left(\frac{s}{k_i}\right) - \exp\left(\frac{s}{k_s}\right) \right)$	0.2304	0.0469	20.16	3.78×10 ⁻⁴

Table 3: Estimated parameters for Mulchandani's model.

Equation	μ_m (h ⁻¹)	k_{sr} (g/L)	s_m (g/L)	n	σ^2
$\mu_i = \mu_m \frac{s}{k_{sr} + s} \left[1 - \left(\frac{s}{s_m} \right)^n \right]$	0.9357	0.0726	6.9379	0.1345	0.0012

Simulation results of the second method

Suzuki et al. applied the following strategy. In the first phase, cultivation was conducted by applying the pH-stat strategy. In the second phase, the feeding of ammonia was changed from the pH-stat mode to continuous feeding rate. In this phase, ammonia feeding rate was set at 0.02 g NH₃/h for the first experiment and 0.08, 0.26 and 0.53 g NH₃/h for the second to fourth experiments respectively [125].

$$q_{PHB} = (q_{PHB})_0 \exp(-k_d t) \quad (12)$$

Where q_{PHB} is specific production rate of PHB, t is the time when complete consumption of nitrogen source occurs. $(q_{PHB})_0$, is the value of q_{PHB} at $t=0$ and k_d is the specific reduction rate of PHB. Physical meaning of q_{PHB} is the same as K_s in Eq. 7 thus when simulating the 2nd method cultivation K_5 in Eq. 7 was substitute with q_{PHB} . So, the number of parameters were reduced to 4 leading to more accurate parameter estimation. The values of $(q_{PHB})_0$ and k_d were estimated by employing data from 4 experiments [125].

CONCLUSION

The first step in the production of PHB should be high-throughput production of catalytically active biomass. For this purpose, proper mixing and loop bioreactors, which enable a sufficiently high gas residence time compared with other reactors are suitable when using natural gas as an inexpensive, abundantly available

substrate. Simulation software tools are able to gain new insights in such biochemical processes. Gas holdup, liquid velocity vectors, shear stress and volumetric oxygen transfer coefficient of the bioreactor can be examined, and various correlations between the process parameters and the biotechnological output become clear.

Especially the simulated gas holdup and the experimental volumetric oxygen transfer coefficient can be compared with various correlations.

By using the tools of high-structured metabolic modeling of PHB biosynthesis, the experimental PHB production rate at varying experimental conditions can be predicted. Simulation of biochemical reactions as occurring in PHB biosynthesis is not as easy as common chemical reactions; however, it may increase the understanding of process and help to visualize it, and also it can be used to improve or scale up the process with a limited number of experiments on lab-scale.

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