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MAINTENANCE-ENERGY-DEPENDENT DYNAMICS OF GROWTH AND POLY(3-HYDROXYBUTYRATE) [P(3HB)] PRODUCTION BY *Azohydromonas lata* MTCC 2311 USING SIMPLE AND RENEWABLE CARBON SUBSTRATES

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Abstract - The dynamics of microbial growth and poly(3-hydroxybutyrate) [P(3HB)] production in growth/ non-growth phases of *Azhohydromonas lata* MTCC 2311 were studied using a maintenance-energy-dependent mathematical model. The values of calculated model kinetic parameters were: m_{s1} = 0.0005 h^{-1}, k = 0.0965, μ_{max} = 0.25 h^{-1} for glucose; m_{s1} = 0.003 h^{-1}, k = 0.1229, μ_{max} = 0.27 h^{-1} for fructose; and m_{s1} = 0.0076 h^{-1}, k = 0.0694, μ_{max} = 0.25 h^{-1} for sucrose. The experimental data of biomass growth, substrate consumption, and P(3HB) production on different carbon substrates were mathematically fitted using non-linear least square optimization technique and similar trends, but different levels were observed at varying initial carbon substrate concentration. Further, on the basis of substrate assimilation potential, cane molasses was used as an inexpensive and renewable carbon source for P(3HB) production. Besides, the physico-chemical, thermal, and material properties of synthesized P(3HB) were determined which reveal its suitability in various applications.
Keywords: Poly(3-hydroxybutyrate); Microbial dynamics; Cane molasses; Maintenance energy; *Azohydromonas lata*.

INTRODUCTION

In response to the problems associated with the application of synthetic oil-based polymers, the scientific communities are searching for alternative materials and their sustainable process development. The main concern of polymer industries and decision makers is to minimize the dependency on non-degradable polymers under the present scenario of environmental awareness (Akaraonye *et al.*, 2010). It is of prime importance to assess the biodegradable polymer production potential of plants, microbes, and the archaea domain of life to reduce the environmental impact of petroleum-based polymers. The microbial production of polyhydroxyalkanoates (PHAs) is considered to be a 'green bio-refinery process', which protects the environment by reducing the CO_2 emission in the atmosphere (Chen, 2011). The microbial

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PHAs have a broader diversity in terms of monomer units, i.e., about 180 types of different monomers exist in nature. Poly(3-hydroxybutyrate) (P3HB) is a type of bacterial PHA that possesses almost similar thermo-plasticity and low O_2 permeability to that of petro-plastic. It is synthesized and accumulates intracellularly as carbon and energy granules up to 90% of the cellular dry weight and possesses unique thermal and material properties which make it suitable for various applications in processing industries, agriculture, and medical fields (Gouda *et al*., 2001; Chen, 2011).

Many bacterial species such as *Alcaligenes sp., Pseudomonas sp*., *Aeromonas hydrophila*, *Rhodopseudomonas*, *Methylobacterium*, *Saccharophagus degradans*, *Bacillus sp.,* and *Azotobacter sp.*, have been used extensively in the last decade for the production of PHAs (Castilho *et al.*, 2009; Gouda *et al.*, 2001; Annuar *et al.*, 2006; Chen *et al*., 1991; Chen, 2011). The industrial production of biopolymer takes place under an excess of carbon source and a limited amount of certain nutrients such as nitrogen (*A. latus*, *P. oleovorans*, *Ralstonia eutropha*), carbon (*Spirillum sp*., *Hyphomicrobium sp*.), iron, magnesium (*Pseudomonas sp*.), oxygen (*Azotobacter vinelandii*, *Rhizobium* ORS 571), phosphate (*Rhodobacter rubrum*, *Caulobacter crecentus*), and potassium sulfate (*Bacillus, Rhodospirillum sp*.). The industrial production of PHAs is expensive due to the associated high cost of carbon sources, which can account for up to 29% of the overall production cost (Kim and Lenz, 2000). Therefore, it is essential to investigate the assimilation potential of bacterial strain towards simple carbon substrates such as glucose, fructose, sucrose, lactose, and xylose and subsequently renewable and inexpensive sources like cane molasses, cheese whey, and lignocellulosic materials.

It is essential to describe the dynamics of microbial growth and growth-associated production of PHAs in order to sustain the accumulation potential of microorganism. The suitable mathematical models have been used to describe the dynamic behavior of microorganisms using kinetic approaches based on growth parameters. Some of the researchers have incorporated 'maintenance expenditure' for painstaking explanations of microbial growth/product kinetics (Pirt, 1965, 1982; Bodegom, 2007; Nancib *et al*., 1993; Nielsen *et al*., 2005). The term physiological maintenance is used for endogenous metabolism which comprises the energy expenditure for osmo-regulation, cell-motility, active transport of bio-molecules, defense mechanisms, and proofreading/turnover of macromolecules (Bodegom, 2007; Nielsen *et al.*, 2005). The physiological maintenance does not include the energy expenditures of metabolic pathways, storage of polymers (PHAs), and extracellular losses (Bodegom,2007). The maintenance expenditure is not constant throughout the fermentation process and varies with the substrate dependent growth with respect to specific growth rate (μ) and yield coefficient. Thus, in order to assess the economic feasibility of the use of carbon substrates, it is essential to evaluate the energy expenditures in terms of growth, maintenance, and P(3HB) formation.

In this study, the dynamics of microbial growth and P(3HB) production by *A. lata* MTCC 2311 using different carbon sources, namely glucose, fructose, and sucrose is assessed. The kinetics of growth and P(3HB) production are evaluated by including the growth-dependent-maintenance energy expenditure. A thorough discussion has been provided for a better insight of maintenance energy expenditure during growth and P(3HB) production. The experimental data derived for these carbon sources were fitted by mathematical models developed to represent the biomass growth, substrate consumption, and P(3HB) production profiles. Besides, the potential of *A. lata* for P(3HB) production using cane molasses as an inexpensive and renewable carbon source is also examined with its physico-chemical characterization.

MATERIALS AND METHODS

Microorganism and Seed Culture Preparation

The lyophilized culture of *Alcaligenes latus* MTCC 2311 was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India, which is reclassified as *Azohydromonas lata* (Xie and Yokota, 2005). The culture was first revived in nutrient broth for 24 h followed by inoculum preparation in AL_2 medium (pH 7.0) which contained (g/L): Sucrose, 20.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.6; $Na_2HPO_4.12H_2O$, 3.6; $MgSO_4.7H_2O$, 1.0; $CaCl_2.2H_2O$, 0.1; citric acid, 0.1; and trace element solution 3 mL/L (Wang and Lee 1997). The inoculum was prepared by using 50 ml of AL_2 medium in 250 mL capacity conical flasks kept at 30 °C and 180 rpm for 24 h in an orbital shaking incubator. The culture was maintained at 4 °C on slants of AL_2 agar for further applications.

P(3HB) Production on Different Carbon Sources

In order to promote the P(3HB) production, 5% inoculum containing approximately 10^6 cells/ml of medium was used to inoculate the P(3HB) production

medium, which contained (g/L): carbon substrate (5 to 40), $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 1.0, citric acid 0.1, $CaCl_2.2H_2O$ 0.1, $Na_2HPO_4.12\ H_2O$ 3.6, KH_2PO_4 0.6, and Trace Element (TE) solution 3 mL/L, and pH 6.8. The different pure carbon substrates such as glucose, fructose, and sucrose were used. The P(3HB) production was carried out in AL_2 medium with varying C/N ratio at 30 ºC and agitation speed of 180 rpm for 72 h. The samples were collected at regular intervals for determination of biomass growth, P(3HB) concentration, and residual sugar concentration.

Analytical Procedures

The biomass concentration was estimated turbidimetrically at 600 nm using a UV-VIS Spectrophotometer (Lambda 35, Perkin Elmer, MA, USA). In addition, the bacterial cell pellet was collected by centrifugation at 8000×g for 10 min of 5 mL culture samples drawn at regular interval. The centrifuged cell pellet was washed twice with distilled water and dried at 80 °C in a hot air oven to a constant weight. The quantification of P(3HB) was carried out by the propanolysis method with little modification using a gas chromatograph (Thermo, USA) (Riis and Mai 1988). The analysis of sugars was carried out on a high performance liquid chromatograph (HPLC) (Waters, USA), equipped with a sugar-pak column (6.5×250 mm length, Waters, USA) and a refractive index (RI) detector (model 24140, Waters). Deionized-water at 90 ºC was used as eluent with a flow rate of 0.5 ml/min. The cell-free supernatant was analyzed for residual $(NH_4)^+$ ion concentration by the phenol-hypochlorite method (Solozano 1969).

Physico-Chemical Characterization of Synthesized P(3HB)

Fourier transform infrared (FTIR) spectra of extracted P(3HB) were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, USA) between 4000 and 500 cm^{-1}. The ^{13}C Nuclear magnetic resonance (NMR) spectra of samples were recorded at 75.4 MHz on a model Av 500 MHz NMR spectrometer (Bruker Inc., USA) using deuterated chloroform ($CDCl_3$) as solvent. The thermal characteristics of P(3HB) samples were measured with a Pyris Diamond Thermogravimetric Analyser (Perkin Elmer Inc, Wellesley, MA, USA). The analysis was carried out in an inert gas atmosphere of nitrogen at a 100 mL/min flow rate in the temperature range from 25 to 250 °C at a heating rate of 10 °C/min. The molecular mass of the extracted P(3HB) sample was determined with a gel permeation chromatography (GPC) system (Waters Inc., MA, USA) at 40 °C. Two columns in series (high resolution HSP gel HR 2.5 and HSP gel HR 3.0, 6.0×15 cm length, Waters) were used with a RI detector. A narrow dispersion polystyrene and tetrahydrofuran (THF) with a flow rate of 0.6 mL/min were used as the MW standard and mobile phase, respectively.

Maintenance-Energy-Dependent Kinetics and Development of Mathematical Models

Several batch studies were conducted to estimate the kinetic parameters of biological processes with particular attention to the substrate utilization for cell maintenance (Minkevich *et al*., 2000; Nielson *et al*., 2005; Nancib *et al.*, 1993; Djavan and Jones, 1980). But little attention has been focused on the maintenance energy related kinetics during PHAs production (Liu *et al*., 2005; Wang *et al*., 2007). The microorganisms need energy for growth, intracellular/extracellular product formation, and for maintenance functions such as transportation of cellular materials, osmotic regulation, defense against O_2 stress, cell motility, and for proofreading, synthesis, and turnover of cellular macromolecules, *viz.* enzyme, and RNA (Bodegom *et al.*, 2007; Oliveira *et al*., 1992; Pirt, 1982). The kinetic approach to microbial dynamics requires the incorporation of maintenance energy expenditure with biomass growth under various types of bioprocesses such as primary/secondary metabolite production, bioremediation, bioaccumulation and biosorption (Bodegom *et al.*, 2007).

During the fermentation process, substrates are utilized by microbial cells for biomass production, product formation, and for maintenance expenditure. The exponential growth of bacterial cell biomass is expressed as:

$$\frac{dX}{dt} = \mu\ X \tag{1}$$

in which μ is the specific growth rate (h^{-1}) according to Monod:

$$\mu\ = \mu_{max}\left(\frac{S}{K_s + S}\right) \tag{2}$$

where μ_{max} is the maximum specific growth rate (h^{-1}), K_s is the substrate saturation constant (g/L), and S is the substrate concentration (g/L).

Here, the intracellular P(3HB) accumulation is considered as a part of the biomass and both a growth and non-growth associated product (Wang

and Lee, 1997). It is assumed that substrate inhibition of biomass growth is not observed. Thus, the microbial growth and growth-associated P(3HB) accumulation are associated with the types of substrate and their concentration.

During fermentative production of P(3HB), the initial microbial concentration (inoculum) and substrate are considered as reactants which, upon biocatalytic reaction, lead to an increase in the biomass growth as well as in P(3HB) accumulation. Thus, the microbial growth dynamics are determined by both the microbial specific growth rate and the substrate consumption rate.

The rate of substrate consumption, analogous to the biomass growth rate as a function of biomass concentration is given by:

$$\frac{dS}{dt} = -q_s X \tag{3}$$

where q_s is the specific substrate consumption rate and can be expressed in terms of the true growth yield and maintenance coefficients as:

$$q_s = \frac{1}{Y_{X/S}}\mu + m_s \tag{4}$$

where $Y_{X/S}$ is maximum growth yield coefficient (g/g) and m_s is the maintenance energy coefficient (h^{-1}).

Upon substitution of Eq. (2) into Eq. (4)

$$q_s = \frac{\mu_{\max}}{Y_{X/S}}\left(\frac{S}{K_s + S}\right) + m_s \tag{5}$$

When the product formation (P(3HB)) is related to carbon source consumption:

$$q_{P(3HB)} = Y_{P/S} q_s \tag{6}$$

Upon substitution of Eq. (4) into Eq. (6)

$$q_{P(3HB)} = \frac{Y_{P/S}}{Y_{X/S}}\mu + Y_{P/S} m_s \tag{7}$$

where $Y_{P/S}$ is the maximum P(3HB) yield coefficient (g/g).

In addition, the P(3HB) production by bacterial cells is described as:

$$q_{P(3HB)} = \frac{1}{X}\left[\frac{dP}{dt}\right] \tag{8}$$

$$q_{P(3HB)} = K_1\mu + K_2 \tag{9}$$

where $q_{P(3HB)}$ is the product formation rate, and K_1 and K_2 represent the growth and non-growth associated product constant, respectively.

The straight line for $q_{P(3HB)}$ verses μ gives the slope K_1 and intercept K_2 and reveals the type of P(3HB) production. If the straight line passes through the origin, K_2 is zero and hence the fermentation is growth-associated, otherwise it is of the mixed type, i.e., both growth and non-growth associated.

The free energy change during catabolic reaction is slightly coupled with the anabolic cellular biomass and biopolymer accumulation and the total energy flux is partitioned into biomass and the maintenance function (Wang *et al.*, 2007).

The total maintenance energy expenditure is not constant throughout the bioprocess and can be divided into two components; one is a constant which is required throughout the cultivation process and the other component is growth dependent (Pirt, 1982). The second component of maintenance energy depends on the specific growth rate (μ) and decreases hyperbolically as a function of the specific growth rate. The maintenance is considered to be the consumption phenomenon, corresponding to energy wastage, which is increased under unfavorable environmental conditions. In general, a low maintenance value (m_s) is observed at high specific growth rate that favors biomass growth and metabolite production (Pirt, 1982).

According to Pirt, 1982:

$$m_s = m_{s1} + m_{s2} \tag{10}$$

where, m_s is maintenance energy expenditure, m_{s1} is the constant and m_{s2} is the growth-dependent component of the maintenance coefficient.

$$m_s = m_{s1} + k\left(1 - \frac{\mu}{\mu_{\max}}\right) \tag{11}$$

where k is a positive quantity that depends on the substrate-microorganism system. The above relationship describes the cellular metabolic efficiency under specific environmental conditions. It is observed that the maintenance is high when the specific growth rate is low and vice versa.

In addition to the specific growth rate, the maintenance energy expenditure also depends on some physical factors such as temperature and salt concentration in the medium.

Upon substituting Eq. (11) into Eq. (4)

$$q_s = \frac{\mu}{Y_{X/S}} + m_{s1} + k\left(1 - \frac{\mu}{\mu_{\max}}\right) \tag{12}$$

where $k\left(1-\mu\ /\mu_{\max}\right)$ is growth dependent and m_{s1} is the constant component of maintenance energy.

During the exponential growth of the microorganism m_{s2} is required for the cell growth and cellular function in addition to m_{s1}. The amount of m_{s2} decreases with the increase in growth rate and approaches zero at the maximum specific growth rate (μ_{max}). Eq. (12) can be rewritten as:

$$q_s = \left(\frac{1}{Y_{X/S}} - \frac{k}{\mu_{\max}}\right)\mu\ + \left(k + m_{s1}\right) \tag{13}$$

where $Y_{X/S}$, $\mu_{\max}$, k, and m_{s1} are constant.

$$q_s = A\mu\ + B \tag{14}$$

where $A = \left(\frac{1}{Y_{X/S}} - \frac{k}{\mu_{\max}}\right)$ and $B = \left(k + m_{s1}\right)$

Eq. (14) is not exactly applicable in the case of cell dormancy resulting in a very slow specific growth rate (Pirt, 1982). In our study, *Azohydromonas lata* efficiently utilized the simple carbon substrate without any lag phase.

The percent of the total substrate (carbon source) consumption used for cell energy maintenance varies with the specific growth rate of microorganism and calculated as (Djavan and James, 1980):

$$\%\ \text{carbon as maintenance} = (m_s / q_s) \times 100 \tag{15}$$

where m_s (h^{-1}) is total maintenance expenditure $(m_{s1} + m_{s2})$ and q_s is the specific substrate consumption rate (g/g).

RESULTS AND DISCUSSION

Maintenance-Energy-Dependent Kinetics and Mathematical Modeling

The dynamics of biomass growth and P(3HB) production by *A. lata* MTCC 2311 were studied in defined AL_2 medium supplemented with varying concentrations of glucose, fructose, and sucrose. About 65% of the initial concentration of these carbon sources (30 g/L) were metabolically assimilated and high values of biomass and P(3HB) concentrations of 8.35 and 3.60 g/L were observed with fructose followed by sucrose (7.92 and 3.50 g/L) and glucose (7.55 and 3.40 g/L), respectively. It is noteworthy that the simple carbohydrates such as glucose, fructose and sucrose are mostly processed through the EMP pathway to yield pyruvate and acetate as the main products. Acetate is partially converted into P(3HB) through synthesis of acetyl CoA and further consumed during the sporulation process of bacteria. It is also documented that the glucose in the medium represses the expression of the phospotransbutylase (Ptb) gene that is needed for PHB production (Shamala *et al.*, 2009). This may be a possible reason due to which fructose gave a little higher yield than glucose and sucrose in the medium. Table 1 shows the calculated growth and P(3HB) kinetic parameters, along with the maintenance expenditure during microbial metabolism on various carbon substrates. Almost similar kinetic constants were observed for all tested sugars with slightly higher values for fructose: $\mu_{max} = 0.27\ h^{-1}$; $Y_{X/S} = 0.45$; $Y_{P/S} = 0.20$; $q_s = 0.60$; $q_p = 0.12$, and Y_c (carbon yield) = 0.24.

The maintenance expenditure is an important biological kinetic parameter, that is not constant throughout the bacterial metabolic processes. It may vary with the increase in substrate concentration and growth rate of the microorganism. The growth-rate-dependent maintenance energy expenditure is represented by Eq. (11). In order to calculate both the constant and growth-dependent parts of the maintenance expenditure, the experimental data for biomass growth derived from varying sugar concentrations were fitted to Eq. (11) and can be represented as:

$$m_{s(\text{fructose})} = 0.0031 + 0.1229\left(1 - \frac{\mu}{0.27}\right) \tag{16}$$

$$m_{s(\text{glucose})} = 0.0005 + 0.0965\left(1 - \frac{\mu}{0.25}\right) \tag{17}$$

$$m_{s(\text{sucrose})} = 0.0076 + 0.0695\left(1 - \frac{\mu}{0.26}\right) \tag{18}$$

Eqs. (16) – (18) revealed that the maintenance energy requirement of microbial cells during the substrate assimilation and metabolic activities was associated with the growth and P(3HB) production. The maximum maintenance expenditure was observed with sucrose (0.01294 h^{-1}) followed by fructose (0.01218 h^{-1}) and glucose (0.00436 h^{-1}) in the medium (Table 1). This means that, in order to pro-

Table 1: Kinetic parameters derived for various carbon substrates, along with the maintenance coefficients.

Carbon source	Specific growth rate, μ (h^{-1})	Biomass yield ($Y_{X/S}$)[a]	P(3HB) yield ($Y_{P/S}$)[b]	q_S	q_P[c]	Y_C[d]	Maintenance coefficient (h^{-1})		
							m_{s1}	m_{s2}	m_s
Glucose	0.25	0.40	0.17	0.62	0.11	0.23	0.0005	0.0039	0.00436
Fructose	0.27	0.45	0.20	0.60	0.12	0.24	0.0031	0.0091	0.01218
Sucrose	0.26	0.42	0.17	0.61	0.10	0.23	0.0076	0.0053	0.01294

[a]$Y_{x/s}$= g biomass/g substrate; [b]$Y_{p/s}$= g P(3HB)/ g total sugar; [c]Maximum P(3HB) content was reported at 40 h of cultivation period
[d]Y_C, Carbon yield= carbon moles in P(3HB)/ carbon moles in substrate

duce one gram of biomass by *A. lata*, about 12.94, 12.18, and 4.36 mg h^{-1} of sucrose, fructose, and glucose, respectively, are needed just to meet the demands of cell energy maintenance. The assimilation of sucrose and fructose takes place through a branch metabolic pathway of glycolysis to convert them into pyruvate, which subsequently results in 3HB monomers through the β-oxidation pathway. Some additional maintenance energy is required during the expression of specific genes which lead to the synthesis of specific proteins, i.e., enzymes that participate in these metabolic pathways.

The maintenance energy was not constant throughout the fermentation process and changed with the specific growth rate of *A. lata*. Similarly, the percent of carbon used as maintenance energy decreased with the increase in specific growth rate (Fig. 1). It has been reported that the maintenance energy consumption corresponds to the energy wastage and increases under adverse environmental conditions (Nancib *et al*., 1993). At a low specific growth rate of 0.12 h^{-1}, about 22% of fructose was used as maintenance energy. However, only 2.14% of fructose expenditure was observed as maintenance energy at the high specific growth rate of 0.25 h^{-1}.

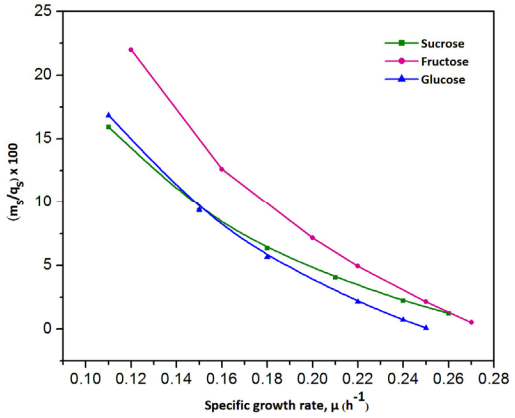

Figure 1: Effect of specific growth rate (μ) on the percent of carbon used as maintenance energy with varying concentrations of various sugars.

Similarly, about 16.85% and 15.93% of glucose and sucrose, respectively, were consumed as maintenance requirement at the low specific growth rate of 0.11 h^{-1}. These expenditures were decreased to 0.73% and 2.22% of glucose and sucrose, respectively, at the high specific growth rate of 0.24 h^{-1}. The high specific growth rate indicates a favorable environment for bacterial growth at which the maintenance requirement is comparatively low.

The varying concentration of carbon substrates such as glucose, fructose, and sucrose have significant effects on the observed specific microbial growth, specific substrate consumption, and P(3HB) production rates [Figs. 2(a)-(c)]. It can be seen that Eqs.(2), (5), and (8) have appropriately modeled the experimental data for biomass growth, substrate consumption, and P(3HB) formation, respectively. Fig. 2(a) shows the effect of fructose concentration on the specific growth rate, substrate consumption, and P(3HB) production rates, as well as satisfactory modeling of experimental data with determination of correlation coefficients (R^2) of 0.98, 0.99, and 0.96, respectively. The maximum specific biomass growth rate of 0.27 h^{-1} was reported at the concentration of 30 g/L. Similarly, the maximum substrate consumption (q_p) and P(3HB) production rates ($q_{P(3HB)}$) of 0.60 and 0.12 h^{-1}, respectively, were reported at the same concentration of fructose. Very similar trends were observed with glucose and sucrose in the medium, but to different extents (Fig. 2(b)-(c) and Table 1).

The calculated specific growth and specific P(3HB) production rates were fitted using Eq. (10) to predict the nature of P(3HB) synthesis. A straight line correlation was observed between μ and $q_{P(3HB)}$ with a positive intercept on the y-axis (K_2) and slope (K_1) for all simple carbon sources, viz., glucose, fructose, and sucrose (Fig. 3). This indicates that the P(3HB) production is of the mixed type, i.e., both growth and non-growth associated. The higher value of K_1 than K_2 also indicates that the accumulation of P(3HB) inside the microbial cells is more growth-associated than non-growth associated (stationary phase).

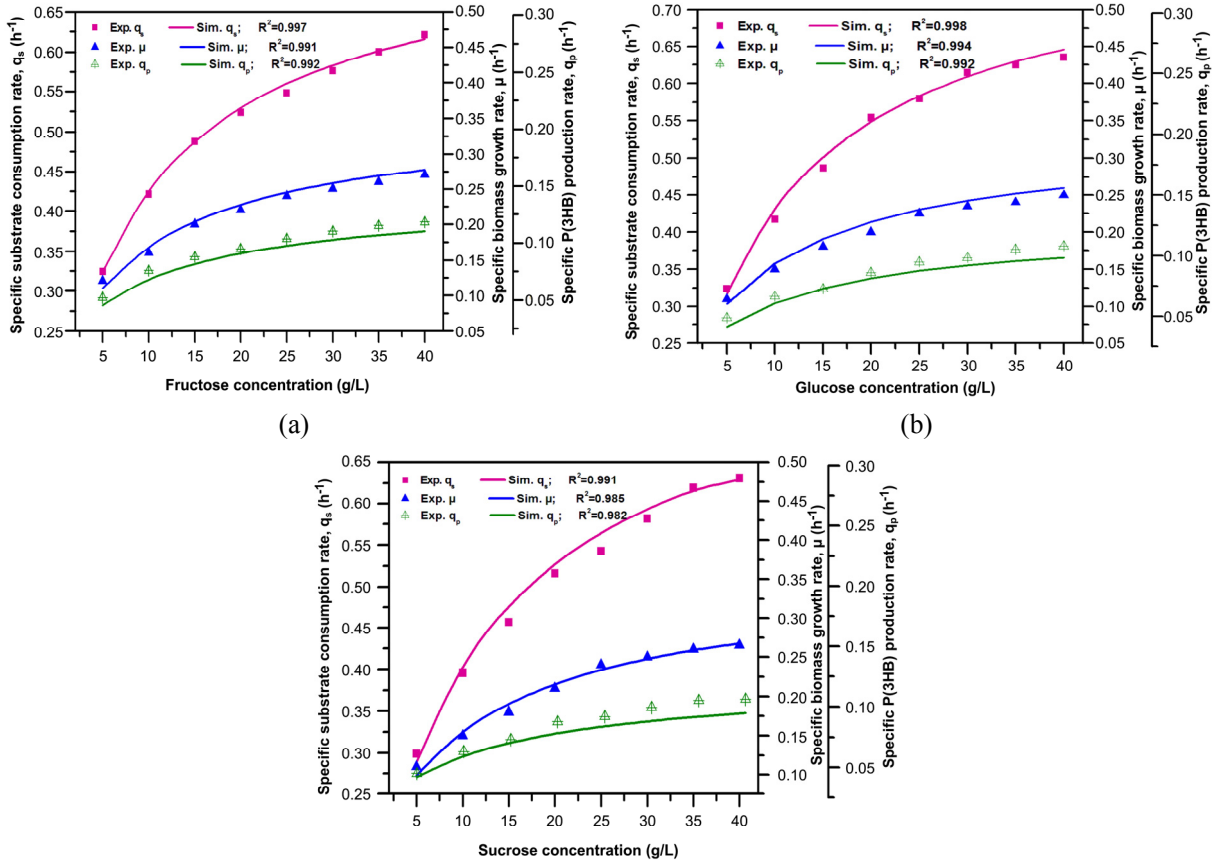

Figure 2: Effect of (a) fructose concentration in the medium, (b) glucose concentration in the medium, and (c) sucrose concentration in the medium on the specific substrate consumption rate (■), specific biomass growth rate (▲), and specific P(3HB) production rate (⛢).

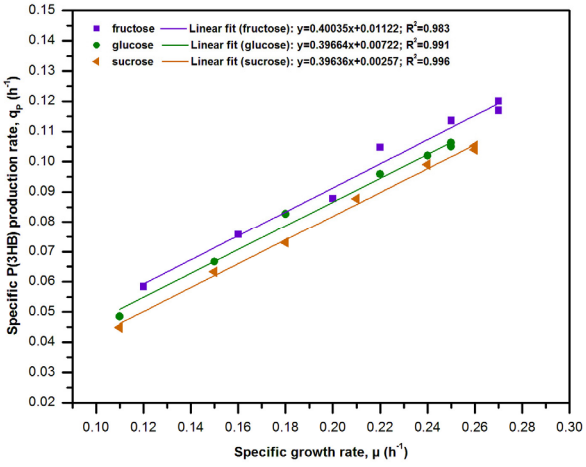

Figure 3: Relationship between specific P(3HB) production rate (q_P) and specific growth rate (μ) for fructose, glucose, and sucrose.

P(3HB) Production Using Inexpensive Cane Molasses

Sugarcane molasses is an inexpensive and renewable carbon substrate derived from the massive sugar industry in India. The cane molasses obtained from a sugar industry (Uttam Sugar Mills Ltd., Roorkee, India) contains reducing sugars (46.8%) such as glucose (5.50%), fructose (9.54%), and sucrose (31.75%) in addition to some growth promoters, ions, minerals, and vitamins in abundance. The experiments were conducted at different concentrations (1.5 to 7.5%, w/v) of cane molasses to promote biomass growth and P(3HB) accumulation by *A. lata.* The course of dry cell weight, P(3HB) concentration, and the specific P(3HB) productivity is illustrated in Fig. 4. The optimum dry cell weight and P(3HB) of 7.85 and 3.75 g/L, respectively, were observed at 4.5% (w/v) of cane molasses and no significant further increase in biomass growth and P(3HB) was observed at high concentrations of cane molasses.

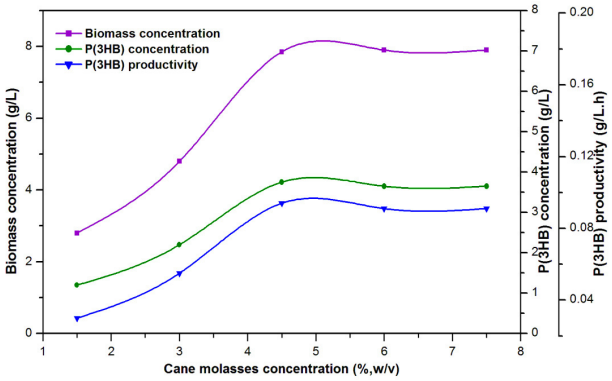

Figure 4: Effect of concentration of cane molasses on biomass concentration, P(3HB) concentration, and P(3HB) productivity.

The maximum specific P(3HB) formation rate of 0.094 g/L.h was found at 4.5% (w/v) of cane molasses. The maximum specific growth rate (μ_{max}) of 0.29 h^{-1} and substrate (cane molasses) saturation constant (K_s) of 1.67% (w/v) were estimated by the least square technique using the curve fitting toolbox of MATLAB 7.8.1 (The Mathworks Inc., MA, USA). The production of P(3HB) using cane molasses has been reported by some researchers (Beaulieu *et al*., 1995; Gouda *et al*., 2001). Beaulieu *et al*. (1995) reported a maximum accumulation of 26% (w/w) of dry weight by *Alcaligenes eutropha* DSM 545 on different concentrations of cane molasses. Similarly, Gouda *et al*. (2001) reported a maximum P(3HB) concentration of 46.20% per mg cell dry weight with 2% molasses, while best growth was obtained for *Bacillus megaterium* with 3% molasses.

Physico-Chemical, Thermal and Material Properties of Produced P(3HB)

The qualitative evaluation of the structural composition of P(3HB) produced by *A.lata* on cane molasses was analyzed by FTIR and NMR spectroscopy. The FTIR spectrum revealed the band at 1450 cm^{-1} corresponding to the asymmetrical deformation of the C-H bond in the CH_2 group, and the equivalent at 1380 cm^{-1} for CH_3 group (Fig. 5). The bands at 1728 and 1280 cm^{-1} correspond to the stretching of the C=O bond, while a series of intense bands located between 1280 to 1000 cm^{-1} represent the C-O bond stretch of the ester group and characterize the valence vibration of the carboxyl group. The ^{13}C NMR spectrum represents the P(3HB) structural composition as shown in Fig. 6. The spectrum reveals the presence of different types of carbon atoms in the P(3HB) structure. The chemical shift signals at 19.75, 40.66, 67.45, and 169.10 ppm represent the different carbons such as CH_3, CH_2, CH and C=O, respectively. The FTIR absorption bands of extracted P(3HB) are in close agreement with those observed by Rozsa *et al*. (1996) and Oliveira *et al*. (2007), who characterized the PHB produced by *Bacillus circulans* in submerged fermentation and by *Cupriavidus nector* in solid state fermentation, respectively. The ^{13}C NMR spectra of extracted P(3HB) illustrate the chemical shift signals and spectral signatures of the different functional groups of P(3HB) and are similar to the findings of other researchers (Doi *et al*., 1986; Rozsa *et al*., 1996; Oliveira *et al*., 2007). The thermal degradation of synthesized P(3HB) and standard P(3HB) were observed between 200 to 359 °C and 200 to 246 °C, respectively (Fig. 7). The DTA thermogram reveals the melting behavior (T_m) of the polymer; the T_m of P(3HB) is lower (163 °C) than that of standard P(3HB) (169 °C). The GPC analysis of extracted P(3HB) revealed a weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity index (D) of 2.384×10^4 g/mol, 1.304×10^4 g/mol, and 1.828, respectively. Sudesh *et al*. (2000) reported that the M_w of P(3HB) synthesized by wild bacteria ranges from 1×10^4 to 3×10^6 g/mol with a polydispersity of around two.

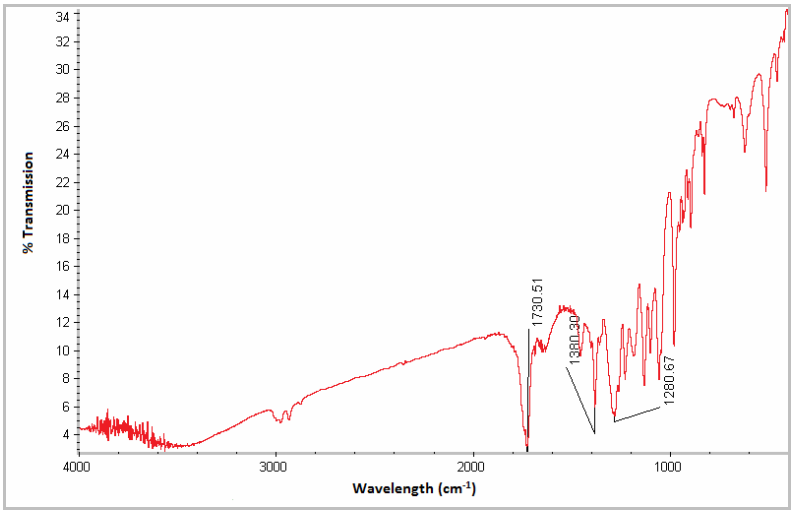

Figure 5: FTIR Spectra of P(3HB) isolated from *Azohydromonas lata* cells.

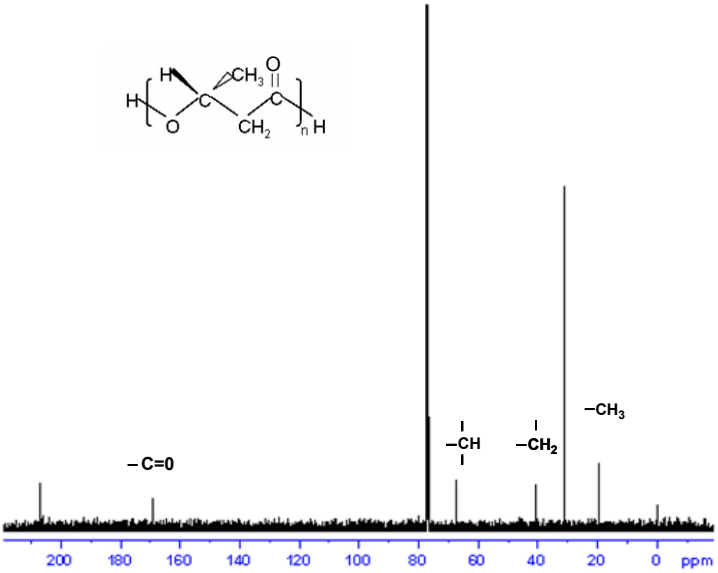

Figure 6: ^{13}C NMR Spectra of P(3HB) produced by *Azohydromonas lata* MTCC 2311.

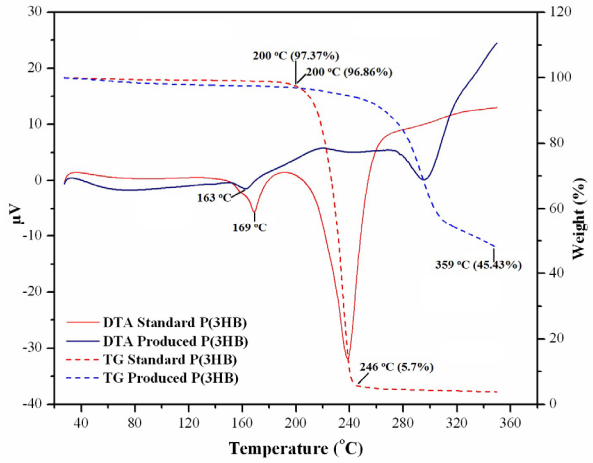

Figure 7: TG-DTA curves of thermal degradation of produced P(3HB) and standard P(3HB).

CONCLUSION

The dynamics of microbial growth and P(3HB) production on simple carbon substrates has been investigated with the incorporation of maintenance-energy-dependent kinetics and mathematical models. The changes in maintenance energy expenditure has been successfully correlated with the varying specific growth rate and yields of biomass and P(3HB) during growth/P(3HB) production processes. Besides, the assimilation potential of cane molasses as an inexpensive and renewable carbon source for biomass growth and P(3HB) production has also been examined. The physico-chemical, thermal, and material properties of synthesized P(3HB) have been found to be similar to those of standard P(3HB).

NOMENCLATURE

k	Constant that depends on the substrate-microorganism system	h^{-1}
K_1	Growth-associated product constant	(-)
K_2	Non-growth associated product constant	h^{-1}
K_s	Substrate saturation constant	g/L
M_n	Number average molecular weight	g/mol
m_s	Maintenance energy coefficient	h^{-1}
m_{s1}	Constant component of the maintenance energy expenditure	h^{-1}
m_{s2}	Growth dependent component of the maintenance energy expenditure	h^{-1}
M_w	Weight average molecular weight	g/mol
$q_{P(3HB)}$	Specific P(3HB) formation rate	h^{-1}
q_s	Specific substrate consumption rate	$g.g^{-1}$
S	Concentration of substrates, glucose, fructose, and sucrose	g/L
T_m	Melting Temperature	°C
$Y_{P/S}$	P(3HB) yield coefficient, w.r.t. substrate consumption	g/g

Greek Letters

μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}

Abbreviations

3HB	3-hydroxybutyrate
D	Polydispersity index
DTA	Differential Thermal Analysis
DTG	Differential Thermogravimetry
GPC	Gel Permeation Chromatography
HPLC	High performance liquid chromatography
MTCC	Microbial Type Culture Collection
P(3HB)	Poly(3-hydroxybutyrate)
PHA	Polyhydroxyalkanoate
PHAs	Polyhydroxyalkanoates
SEM	Scanning Electron Microscopy
TGA	Thermo-Gravimetric Analysis

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