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BIOENERGY/BIOFUELS/BIOCHEMICALS

Modeling and optimization of poly(3hydroxybutyrate-co-3hydroxyvalerate) production from cane molasses by *Azohydromonas lata* MTCC 2311 in a stirred-tank reactor: effect of agitation and aeration regimes

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Abstract The effects of agitation and aeration rates on copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] production by *Azohydromonas lata* MTCC 2311 using cane molasses supplemented with propionic acid in a bioreactor were investigated. The experiments were conducted in a three-level factorial design by varying the impeller (150–500 rev min^{-1}) and aeration (0.5–1.5 vvm) rates. Further, the data were fitted to mathematical models [quadratic polynomial equation and artificial neural network (ANN)] and process variables were optimized by genetic algorithm-coupled models. ANN and hybrid ANN-GA were found superior for modeling and optimization of process variables, respectively. The maximum copolymer concentration of 7.45 g l^{-1} with 21.50 mol% of 3HV was predicted at process variables: agitation speed, 287 rev min^{-1}; and aeration rate, 0.85 vvm, which upon validation gave 7.20 g l^{-1} of P(3HB-co-3HV) with 21 mol% of 3HV with the prediction error (%) of 3.38 and 2.32, respectively. Agitation speed established a relative high importance of 72.19% than of aeration rate (27.80%) for copolymer accumulation. The volumetric gas–liquid mass transfer coefficient (k_La) was strongly affected by agitation and aeration rates. The highest P(3HB-co-3HV) productivity of 0.163 g l^{-1} h^{-1} was achieved at 0.17 s^{-1} of k_La value. During the early phase of copolymer production process, 3HB monomers were accumulated, which were shifted to 3HV units (9–21%) during the cultivation period of 24–42 h. The enhancement of 7.5 and 34% were reported for P(3HB-co-3HV) production and 3HV content, respectively, by hybrid ANN-GA paradigm, which revealed the significant utilization of cane molasses for improved copolymer production.

Keywords Cane molasses · P(3HB-co-3HV) · Artificial neural network · Genetic algorithm · Volumetric oxygen transfer coefficient (k_La)

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Introduction

Many bacterial cells may accumulate a relatively massive amount of polyhydroxyalkanoates (PHAs), as an intracellular reservoir of carbon and energy sources under the condition of nutrients and oxygen imbalance. These biomaterials are biodegradable and biocompatible thermoplastic in nature and possess physical properties similar to that of petroleum-derived polymers [21]. PHAs have a widespread occurrence in both Gram-positive and Gram-negative bacteria and a number of investigations have been carried out on different aspects of PHAs production including the utilization of various raw materials such as sugarcane molasses, beet molasses, soy molasses, cheese whey, crude glycerol, and agricultural residues by selected microorganisms. A number of microorganisms such as *Alcaligens latus* (renamed as *Azohydromonas lata*), *Cupriavidus nector*, *Bacillus megaterium, Azotobacter beijerinckii*, and *Pseudomonas* sp. have been exploited for PHAs production on various renewable raw materials [2, 4, 7, 14, 17, 21, 25, 34, 37].

The material properties of PHAs can be improved by fine-tuning of their composition during the biosynthesis to facilitate their advance applications. The homopolymer poly(3-hydroxybutyrate) (PHB) is the most common representative of PHAs, which has a high degree of crystallinity and limited applications. Their processibity under

melt extrusion technology is pretty small due to a narrow difference between the decomposition (~270°C) and high melting (T_m) (~180°C) temperature. These properties can be enhanced by changing the PHB matrix through the incorporation of alternate building block monomers such as 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB), or 5-hydroxyvalerate (5HV). The material properties of short-chain-length (scl) PHAs are comparable to petro-plastic (polypropylene), even though with low copolymeric composition of 3HV, 4HB, or 5HV content. It is noteworthy that the increase in 3HV units in copolymer from 0 to 25 mol% decreases the T_m from 179 to 137°C; thus it increases the size of their processing window. Similarly, the crystalline temperature (T_g) also decreases from 10 to −6°C, allowing the use of materials at low temperature without embrittlement [17, 24]. The incorporation of different copolymeric units requires the addition of co-substrate (precursor) in the fermentation medium.

PHAs synthesis generally occurs when bacteria are grown aerobically and usually under stress, such as in limited nutrient and oxygen conditions. The effect of dissolved oxygen concentration on glucose metabolism for the PHAs accumulation and respiration of *A. beijerinckii* was first investigated by Carter and Dawes [6]. The supply of oxygen as oxygen transfer rate (OTR) is chosen as the controlling footstep in many industrial bioprocess systems and also in its scale-up [9, 22, 26, 30]. It is an important parameter for the design and operation of aeration and agitation systems of bioreactors. The aeration supplies the oxygen demand of microbial population, and its efficiency depends on oxygen solubilization and diffusion rate into the medium broth along with the bioreactor capacity. Oxygen transfer as a function of agitation and aeration to bioreactor vessel plays an important role, in order to obtain the appropriate volumetric oxygen transfer coefficient (K_La) that can correlate with the PHAs productivity in defined culture medium. The rationale of K_La values indicate a certain mass-transfer capability that can cope with the oxygen demand of the culture. It often serves as the scale-up criteria to compare the efficiency of bioreactors and mixing devices [3, 12].

Azohydromonas lata is a good candidate for PHAs synthesis since it accumulates biopolymers during both growth and non-growth phases. It prefers the consumption of sucrose as a carbon source than *C. nector*, indicating the possible utilization of industrial by-products beet or cane molasses for the production of PHAs [21, 46]. It is an aerobic, Gram-negative bacterium that requires oxygen for its growth and various metabolic activities. The study of agitation and aeration rates on growth and PHAs production is an important activity since oxygen is considered a limiting factor for PHAs accumulation inside many bacterial cells [1]. Agitation is required to sustain the homogeneous physico-chemical environment inside the culture broth through adequate mixing and mass transfer, whereas aeration is beneficial for growth performance of microorganism by improving the mass transfer characteristics with respect to substrate, product/byproduct, and oxygen. The physiological state of a bacterial cell can strongly influence the biopolymer accumulation with other by-products, and also affects the broth rheology, and mass/heat transfer capabilities [9, 11, 12].

In order to optimize the bioprocess parameters under the strategic analysis of a cost-effective PHA production process, several methodologies have been employed which range from conventional one-factor-at-a-time (laborious and time consuming) to a complex statistical based technique such as response surface methodology (RSM) [15, 20]. Under RSM, a model is usually constructed for the defined medium components by using quadratic polynomial equation, which depicts the interaction effects among the components. An artificial intelligence-based neural network model (ANNs) can also be used as an alternative to the polynomial regression-based modeling approach that overcome the non-linearity of bioprocess variable's interaction [13]. The stochastic search procedure based on genetic algorithm (GA) has been universally applied in an efficacious manner for the optimization of process variables with or without the need of statistical design and empirical models. GA is capable of exploring large input variables space through the search operators, viz. selection, crossing over, and mutation [40]. In recent years, hybrid RSM-GA [16, 39, 45] and hybrid ANN-GA [5, 10, 13, 33, 45] approaches have been successfully used for the optimization of the input variables of various bioprocess systems.

The potential of *A. lata* for the accumulation of PHB has been widely studied [15, 38], but few studies have been reported on the production of copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] in the last decade [2, 37]. In our previous study, cane molasses has been utilized for the production of P(3HB-co-3HV) by *A. lata* MTCC 2311 with the supplementation of sodium propionate [46]. In the present study, the operational parameters of a bioreactor such as agitation speed and aeration rate have been optimized with the objectives to enhance the accumulation of P(3HB-co-3HV) concentration and incorporation of 3HV units (mol%) in copolymer. The experiments have been conducted in a three-level factorial design to identify the optimum combination of airflow and agitation rates that favor the maximum possible accumulation of P(3HB-co-3HV) inside the bacterial cells. Subsequently, the volumetric oxygen transfer coefficients (k_La) have been estimated and its effects on P(3HB-co-3HV) productivity and 3HV content (mol%) have been

examined. Further, mathematical (quadratic polynomial equation) and artificial intelligence (artificial neural network)-based models have been used to navigate the experimental data and goodness-of-fit of the models has been determined by some statistical/mathematical constants. In order to optimize the process variables (agitation and aeration rates) for maximum P(3HB-co-3HV) production, these models have been integrated with a global search tool, (GA). The predicted optimal solutions of both the approaches have also been validated by conducting the separate experiments in a 3-l bioreactor.

Materials and methods

Microorganism and inoculum preparation

The lyophilized culture of *Alcaligenes latus* MTCC 2311 was procured from the Institute of Microbial Technology, Chandigarh, India, which is reclassified as *A. lata* [42]. The culture was revived in nutrient broth for 24 h followed by inoculum preparation in the AL_2 medium (pH 7.0) by using 50 ml of medium in a 250-ml capacity conical flask kept at 30°C and 180 rev min^{-1} for 24 h in an orbital shaking incubator [38].

Bioreactor setup and design of experiments

The batch experiments were conducted in a stirred 3-l bioreactor (2-l working volume) equipped with ez-control (Applikon, The Netherlands). The production medium of copolymer P(3HB-co-3HV) contained the following ingredients (previously optimized): cane molasses, 3.96% (total sugar, 20 g l^{-1}); urea, 0.53 g l^{-1}; and sodium propionate, 15.00 mmol l^{-1}; C/N ratio, 30 [46]. Sodium propionate was used as a precursor of 3HV units in copolymer P(3HB-co-3HV). The feeding of propionate was carried out in three equal pulses of 10 mmol (at 12, 24, and 36 h), in order to overcome the inhibitory effects of its high doses on bacterial growth. The first feeding of propionate was made after the onset of exponential phase, which was used subsequently by consumption of reducing sugars and urea in the medium. The initial pH of the solution was maintained at 6.8 by using 2 M NaOH/HCl solution. The experiments were conducted in three-level factorial designs for varying aeration and agitation rates (Table 1). The culture medium was inoculated with 5% (v/v, approximately 1×10^6 cells ml^{-1}) of the prepared seed medium and cultivated for 72 h at 30°C. The samples were taken at regular intervals to estimate the total sugar, $(NH_4)^+$ ions, propionic acid, biomass, and P(3HB-co-3HV) concentrations.

Analytical procedures

The biomass concentration was estimated turbidimetrically at 600 nm using a UV-Vis spectrophotometer (Lambda 35, PerkinElmer, MA, USA). In addition, the bacterial cell pellet was collected by centrifugation of 5-ml culture samples drawn at regular interval at $8{,}000 \times g$ for 10 min. 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 copolymer P(3HB-co-3HV) was carried out by propanolysis method proposed by Riis and Mai (1988) with little modification using gas chromatograph (Thermo, USA) [31, 46]. The ratio of 3HB and 3HV units of copolymer was calculated from the area under peak of propyl ester derivatives of these *n*-alkanoic acids [41]. The sugar content of the cane molasses solution was analyzed by a high-performance liquid chromatograph (HPLC) apparatus (Waters, USA), equipped with sugar-pak column (6.5 × 250 mm length, Waters, USA) and refractive index (RI) detector (model 24140, Waters). The deionized-water at 90°C was used as eluent with a flow rate of 0.5 ml min^{-1}. The initial and residual concentrations of propionate were quantified by HPLC system equipped with IC pak ion-exclusion column (300 × 7.8 mm length, Waters) and photodiode array (PDA, model 2998, Waters) detector. The eluent composed of 16 mN sulfuric acid in 10% methanol supplemented with the deionized water (Millipore system, USA) was prepared daily and was vacuum filtered through a 0.45-μm nylon filter (Pall Corporation, MI, USA). The PDA detector was operated at 210 nm and with the flow rate of eluent at 0.6 ml min^{-1}. The standard of organic acids (1–10 ml l^{-1}) and diluted culture samples were filtered through a 0.45-μm nylon syringe filter prior to the injection. The cell-free supernatant was analyzed for residual $(NH_4)^+$ ion concentration by phenol-hypochloride method [35].

Determination of volumetric oxygen mass transfer coefficient (K_La)

The volumetric oxygen mass transfer coefficient (K_La) in an aerobic bioprocess depends on the hydrodynamic conditions around the gas bubbles provided by sparger of the bioreactor system. The experiments were conducted in a three-level factorial design with varying aeration and agitation rates and the K_La for each experimental run was estimated by using dynamic method [11].

$$K_La = \ln\left(\left(C'_{AL} - C_{AL_1}\right)/\left(C'_{AL} - C_{AL_2}\right)\right)/(t_2 - t_1) \qquad (1)$$

where, C'_{AL} is the steady-state value of dissolved oxygen level after re-oxygenation (% air saturation), C_{AL_1} and C_{AL_2} are two oxygen concentrations (% air saturation)

Table 1 Observed and model-derived P(3HB-co-3HV) concentration and k_La along with the 3HV content (mol%) and copolymer productivity on a set of experiments conducted in full-factorial design

Run	Process variables		P(3HB-co-3HV) concentration (g l^{-1})			Volumetric oxygen transfer coefficient (s^{-1})			% P(3HB-co-3HV) (g biomass^{-1})	3HV content (mol %)	Copolymer productivity (g l^{-1} h^{-1})[a]
	Agitation (rev min^{-1})	Aeration (vvm)	Y_{exp}	Y_{rsm}	Y_{ann}	k_La_{exp}	k_La_{rsm}	k_La_{ann}			
1	150	0.5	5.48	5.25	5.45	0.027	0.017	0.028	69.80	20.50	0.137
2	500	0.5	3.84	3.96	3.85	0.207	0.250	0.212	63.15	14.65	0.096
3	150	1.5	5.52	5.26	5.52	0.152	0.140	0.152	49.11	18.35	0.138
4	500	1.5	3.80	3.89	3.80	0.726	0.770	0.728	43.08	12.50	0.095
5	325	0.5	5.80	5.91	5.80	0.054	0.020	0.049	60.73	20.00	0.145
6	325	1.5	5.70	5.87	5.70	0.368	0.340	0.369	51.72	14.00	0.142
7	150	1.0	5.28	5.77	5.31	0.048	0.070	0.048	63.16	20.20	0.132
8	500	1.0	4.66	4.45	4.65	0.584	0.500	0.575	63.40	13.20	0.116
9	325	1.0	6.48	6.41	6.51	0.155	0.170	0.161	56.25	18.25	0.162
10	325	1.0	6.54	6.41	6.51	0.168	0.170	0.161	55.65	18.25	0.163
RMSE				0.220	0.019		0.039	0.005			
SEP(%)				4.170	0.353		15.028	1.823			
MPE(%)				3.570	0.251		33.237	2.556			
A_f				1.036	1.002		1.286	1.025			
B_f				1.002	1.000		0.866	0.994			

[a] P(3HB-co-3HV) productivity was calculated for 40 h at which maximum copolymer accumulation was observed

measured during re-oxygenation at time t_1 and t_2, respectively.

Extraction and determination of material properties of P(3HB-co-3HV)

The intracellular P(3HB-co-3HV) was extracted from bacterial cell pellets using chloroform (200 ml) in a Soxhlet extractor for 12 h. The mixture was filtered and treated with 4 volumes of methanol in order to precipitate the copolymer, which was filtered off and dried at room temperature.

Further, the thermal characteristics of P(3HB-co-3HV) were measured by thermo gravimetric analyzer (Pyris Diamond, PerkinElmer Inc, USA) with simultaneous recording of differential thermal analysis (DTA), and thermogravimetry (TG) curves. The analysis was carried out in an inert gas atmosphere of nitrogen of flow rate 100 ml min^{-1} in the temperature range of 25–400°C at a heating rate of 10°C per min. The temperatures of melting and decomposition were determined as the temperature of corresponding endothermic maxima.

The molecular mass of extracted P(3HB-co-3HV) sample was determined by gel permeation chromatograph (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 an RI detector. A narrow dispersed polystyrene and tetrahydrofuran with a flow rate of 0.6 ml min^{-1} were used as the MW standard and mobile phase, respectively.

Modeling of experimental data

The relationship between aeration and agitation rates is expressed mathematically in the form of a quadratic polynomial equation (Eq. 2) under the RSM methodology. The numerical data obtained for three-level factorial designs were subjected to the regression analysis and fitted to the following quadratic polynomial equation using the software Design-Expert v 6.0.10 (Stat-Ease Inc., Minneapolis, MN, USA).

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where, Y is the predicted response, β_o is the constant, β_i are the linear coefficients, β_{ii} are the quadratic coefficients, β_{ij} are the second order interaction coefficients, and X_i, X_j ($i = 1, 2$; $j = 1, 2$) are the concentrations of the independent medium variables in the coded form. The significance of model terms (linear, squared, and quadratic) was examined by performing analysis of variance (ANOVA).

A feed-forward architecture of ANN model, which is also known as multilayer perception (MLP), has been used

with the back propagation (BP) algorithm to build the predictive model with scaled values of aeration and agitation rates as input and the P(3HB-co-3HV) concentration as output of network (Fig. 1). All the inputs and output are normalized within a uniform range of (0.1–0.9) in order to ensure the uniform attention during the training process. The new scaled variables (X^*) are calculated by Eq. (3) for different inputs (X). After the training process, the optimized value of variable (X) is rescaled by Eq. (4) using X^* values.

$$X^* = 0.8\frac{X - X_{\min}}{X_{\max} - X_{\min}} + 0.1 \tag{3}$$

$$X = \frac{(X_{\max} - X_{\min})(X^* - 0.1)}{0.8} + X_{\min} \tag{4}$$

where, X is the input variable in a group of variables to be scaled. Likewise $X_{\min}$ and $X_{\max}$ are the minimum and maximum values of variables, respectively, and X^* is the corresponding scaled variable.

The first step in the training of a neural network model is to design the topology of the network. The number of neurons in input and output layers of neural model is fixed to the total number of selected input parameters and output response of the bioprocess, respectively [39]. Further, the determination of the number of neurons in the hidden layer of the network is the critical step, and is determined by varying the number of neurons from 1 to 6 in the hidden layer. During the training process, the mean square error (MSE) between the experimental and predicted values is calculated, and propagated backward through the network using the well-known resilient back propagation algorithm (trainrp). The back propagation algorithm adjusts the weights in each successive layer to reduce the MSE. This procedure is repeated until the MSE between experimental and corresponding predicted values satisfy the pre-specified error criteria.

In order to evaluate the fitting and prediction accuracy of constructed models, root mean square error (RMSE), standard error of prediction (SEP), and model predictive error (MPE) are employed along with the bias (B_f), and accuracy (A_f) factors [18].

$$\mathrm{RMSE} = \sqrt{\sum_{i=1}^{n} (Y_{i,e} - Y_{i,p})^2}/n \tag{5}$$

$$\mathrm{SEP}(\%) = (\mathrm{RMSE}/Y_e) \times 100 \tag{6}$$

$$B_f = 10^{\left(\sum_{i=1}^{n} \log(Y_{i,p}/Y_{i,e})/n\right)} \tag{7}$$

$$A_f = 10^{\left(\sum_{i=1}^{n} |\log(Y_{i,p}/Y_{i,e})|/n\right)} \tag{8}$$

$$\mathrm{MPE}(\%) = \frac{100}{n}\sum_{i=1}^{n} \left|(Y_{i,e} - Y_{i,p})/Y_{i,p}\right| \tag{9}$$

where, $Y_{i,e}$ is the experimental value of ith experiment, $Y_{i,p}$ is the corresponding predicted value by model of ith experiment, Y_e is the mean value of experimental data of PHA concentration or 3HV content (mol%), and n is the number of experiments.

Optimization of process variables for P3(HB-co-3HV) production

A stochastic-based GA is used to search the optimum input space (X) representing process variables, by using the RSM and ANN models as a fitness function. The details of optimization steps have been described in our previous work [44]. In the present study, GA-based optimization processes are performed using second-order quadratic polynomial and feed-forward ANN (network of weights and bias in each layer) models as the fitness functions to give the global optimal solutions [28]. The data simulations

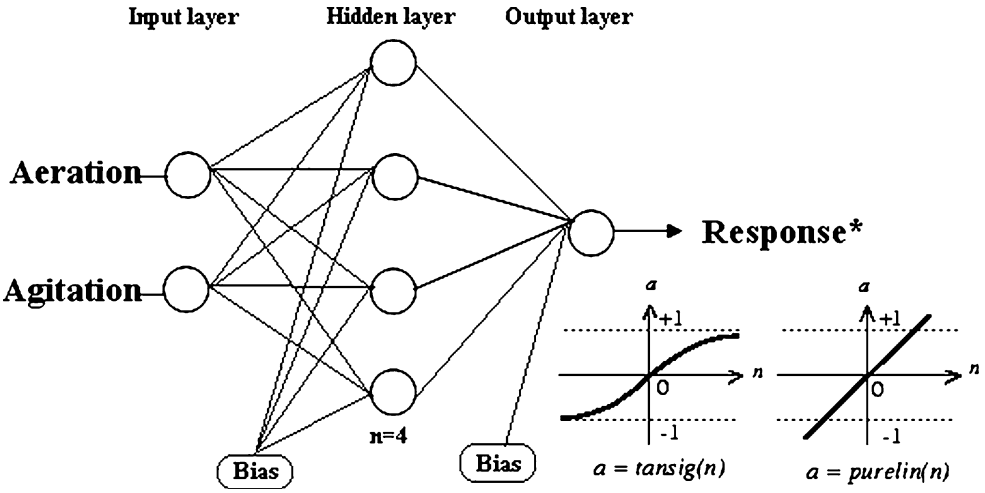

Fig. 1 Architecture of artificial neural network model along with the transfer functions

were performed using GA and ANN toolboxes of MATLAB 7.8 (MathWorks, Natick, MA, USA).

Experimental validation of optimized solutions

The optimized solutions given by both hybrid RSM-GA and ANN-GA approaches were externally validated by conducting the separate experiments in a 3-l bioreactor, in duplicate.

Results and discussion

Effect of agitation speed and aeration rate of P(3HB-co-3HV) production

In order to study the effect of agitation and aeration rates on P(3HB-co-3HV) production by *A. lata* MTCC 2311, the experiments were conducted in a full-factorial design ($2^n = 8 + 2$ (center point) $= 10$ experiments) by varying each variables at three (n) levels (Table 1). The selected agitation (rev min^{-1}) and aeration (vvm) rates were varied from 150 to 500 and 0.5 to 1.5, respectively. In a bioreactor system, the gradients between medium broth and the interior of a microbial cells is sustained by better mixing (agitation) and aeration rates. Likewise, the better diffusion of oxygen in the broth assists the supply of sugars and other nutrients to the microbial cells and facilitate the removal of gases and by-products of catabolic reactions from the microenvironment of microbial cells [22].

Table 1 provides the experimental and predicted values of P(3HB-co-3HV) and k_La values at various agitation and aeration rates. We observed that a decrease in P(3HB-co-3HV) content is also observed when the agitation speed increases over 325 rev min^{-1}. Though, the high values of these process variables encourage the consumption of sugars present in molasses solution for the biomass growth, it is noticed that the consumption of propionate (a precursor of 3HV unit) is slow at initial stage of fermentation (12 h) and increases significantly after the depletion of NH_4^+ ions in the solution. It is noteworthy that during PHAs metabolism, propionate is firstly converted into propionyl-CoA and further, a portion of propionyl-CoA is condensed with acetyl-CoA into five carbon monomers of PHAs by the action of β-ketothiolase. The yield of 3HV content is generally less than 50% due to the decarboxylation of some of propionate before the monomer synthesis [17]. It is also observed that the growth of *A. lata* is comparatively slow on molasses as compared to growth on simple sugars such as glucose, fructose, and sucrose (lag phase 12 h). After the onset of the exponential phase, the biopolymer accumulation is initiated in terms of 3HB monomers by sugar consumption during this phase. Consequently, the consumption of propionic acid is initiated after 20 h of cultivation period when the growth becomes slow (with depletion of nitrogen source). Further, the growth is observed but at a slow rate with simultaneous consumption of residual sugars and propionic acid for the incorporation of 3HV units in copolymer. Thus, it is noted that the incorporation of 3HB and 3HV unit in copolymer take place both during growth and non/slow growth phase of *A. lata* MTCC 2311.

These findings show that the high agitation speed and aeration rate increase the dissolved oxygen concentration in the fermentation broth, and are conducive to microbial growth whereas the moderate agitation and aeration rates lead to the accumulation of copolymer in the fermentation broth containing cane molasses and propionic acid as a source of 3HB and 3HV units of copolymer, respectively. It is also reported that the high copolymer production is obtained under the combined conditions of low shear stress and optimal dissolved oxygen concentration [1].

Effect of agitation and aeration rates on gas–liquid mass transfer (k_La)

The k_La values are increased with the increase in agitation and aeration rates (Table 1). During the growth phase, with varying agitation speed (150–500 rev min^{-1}) and aeration rate (0.5–1.5 vvm) the k_La values are established in the range of 0.048–0.584 s^{-1} and 0.054–0.368 s^{-1}, respectively. The higher values of k_La are observed with the increase in agitation rate than the increase in aeration rate. A similar trend for k_La values (9.72–19.10 h^{-1}) has been observed with agitation range of 200–800 rev min^{-1} for exopolysaccharide production from *Enterobacter cloacae* WD7 [3].

Further, the second-order polynomial equation (Eq. 2) has been used for the stepwise regression analysis to estimate the effect of agitation and aeration rates on k_La. The main, interaction and quadratic effects of variables are considered and are represented as:

$$Y_{k_La} = 0.24569 + 0.002316\,X_1 - 0.11659\,X_2 + 0.0000037145\,X_1^2 + 0.035634\,X_2^2 + 0.0011257\,X_1X_2 \quad (10)$$

where, X_1 and X_2 are the process variables, agitation speed (rev min^{-1}) and aeration rate (vvm), respectively.

The correlation coefficient (R^2) value of 97.30 indicates that the above model is well fitted to the experimental data. In addition, the adj. R^2 value of 95.36 is also close to R^2 value and suggests the good agreement of model prediction with experimental data. A contour is also plotted between model-derived k_La values and agitation and aeration rates, which represent the positive association between these process variables for k_La values (Fig. 2). It is noteworthy that in addition to agitation and aeration rates, many other factors such as mixing, broth

viscosity, product formation, and biomass may also influence the k_La values during the bioprocess. The regression analysis between dependent (k_La value) and independent variables (agitation and aeration rates) reveals that the k_La value is strongly affected by linear effects of agitation speed, aeration rate, and quadratic effect of agitation rate (p value < 0.05). The interaction between aeration and agitation (p value < 0.05) rates also affects the k_La value (Table 2).

Correlation of k_La with P(3HB-co-3HV) productivity and 3HV content (mol%)

Cane molasses is a by-product of the sugar industry, which contains reducing sugars such as glucose, fructose, and sucrose in abundance. In addition, it also contains the various ions, minerals, and vitamins that may have detrimental effects on microbial growth and product synthesis. These components are required to remove from the molasses solution in order to increase the yield of desired product. This leads to the development of various pre-treatment techniques such as treatment with potassium ferrocyanide (PFC), EDTA, sulfuric acid, and tricalcium phosphate. These have been used for molasses pretreatment during production of citric acid, ethanol, and gluconic acid [29]. In our laboratory experimental study, we have also used PFC-treated molasses for the production of P(3HB-co-3HV) with the supplementation of propionic acid (data not shown). However, the maximum copolymer accumulation is observed in the medium containing untreated molasses than the PFC-treated molasses solution. The possible reason may be that the trace metal ions are essential for the copolymer metabolism, which could have been removed during treatment with PFC. Similar observation has been reported by Purushothaman et al. [27] during PHB production from *A. beijerinckii*. It is also observed that the higher concentration (>4% solution) of cane molasses has detrimental effects on copolymer accumulation [46]. Further, it is noted that the copolymer productivity may enhance by controlling the agitation and aeration rates which indirectly facilitate the assimilation of sugar inside the bacterial cells.

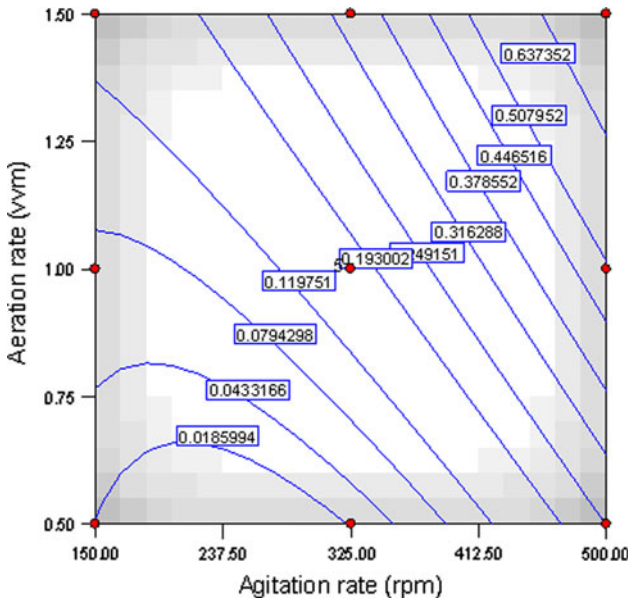

Fig. 2 Contour plot of model-derived k_La values as a function of agitation and aerations rates of bioreactor system

It is found that the copolymer productivity initially fluctuates with the increase in k_La values from 0.027 to 0.207 s^{-1}, achieves a maximum of 0.163 g l^{-1} h^{-1} at 0.170 s^{-1} k_La value and then decreases at higher values of k_La (Fig. 3). The fluctuation in copolymer productivity might be occurred due to the change in the physiological state of bacteria by changing the microenvironment in bioreactor system. The productivity of copolymer is also associated with the incorporation of 3HB and 3HV monomers in P(3HB-co-3HV) structure. At high k_La values, the accumulation of growth-associated 3HB monomers units is observed whereas the incorporation of 3HV

Table 2 Analysis of variance of fitted model and estimated regression coefficients for P(3HB-co-3HV) concentration and k_La values

Variable terms	P(3HB-co-3HV) concentration				k_La value			
	Coefficient	MS	F value	p value	Coefficient	MS	F value	p value
Intercept/model	1.04326	1.73	84.34	<0.0001	0.24569	0.10	50.36	<0.0001
Agitation (X_1)	0.02401	2.64	128.93	<0.0001[a]	−0.00232	0.28	135.96	<0.0001[b]
Aeration (X_2)	4.19268	0.20	9.85	0.0164[a]	−0.11659	0.15	74.98	<0.0001[b]
X_1^2	−0.00004	2.34	114.07	<0.0001[a]	0.000004	0.036	17.52	0.0041[b]
X_2^2	−2.07586	1.13	55.19	0.0001[a]	0.035034	0.0002	0.10	0.7567
X_1X_2	−0.00023	0.29	14.24	0.0070[a]	0.00112	0.039	19.02	0.0033[b]
R^2 (%)	98.37				97.30			
R^2_{adj} (%)	97.20				95.36			

[a] Significant model terms for P(3HB-co-3HV)

[b] Significant model terms for K_La value

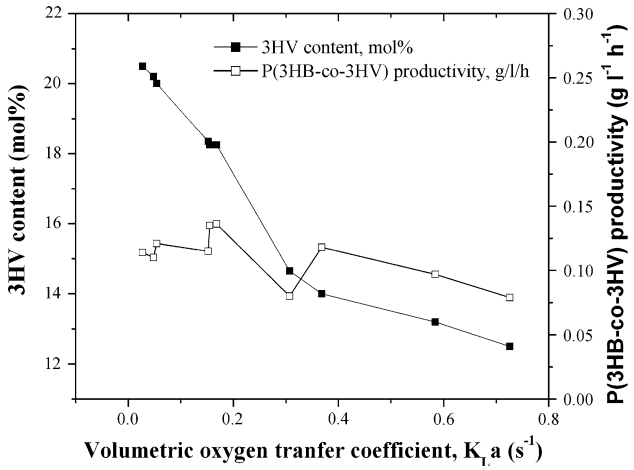

Fig. 3 Effect of volumetric oxygen transfer coefficient (k_La) on 3HV content (mol%) and P(3HB-co-3HV) productivity

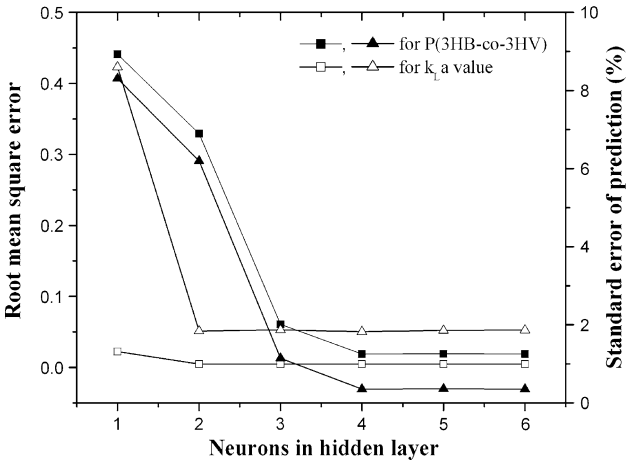

Fig. 4 Effect of number of neurons in the hidden layer on the root mean square error (*filled square*/*open square*) and standard error of prediction (*filled triangle*/*open triangle*) during the training of ANN models of P(3HB-co-3HV) (g l^{-1}) and k_La (s^{-1})

units is accelerated at low k_La values with the utilization of propionate.

Comparison of modeling ability of RSM and ANN for P(3HB-co-3HV) production

In order to study the effect of process variable's agitation and aeration rates on copolymer P(3HB-co-3HV) production, experiments were conducted in full-factorial design (Table 1). These experimental data were fitted by using RSM and ANN approaches. Under RSM approach, the quadratic polynomial equation, which represents the empirical relationship between the response and process variables, is given as follows:

$$Y_{\text{copol}} = 1.04326 + 0.024008\,X_1 + 4.19268X_2 - 0.000042\,X_1^2 - 2.07586X_2^2 - 0.000228\,X_1X_2 \quad (11)$$

where, Y_{copol} is predicted P(3HB-co-3HV) concentration, X_1 and X_2 represent the agitation and aeration rates, respectively.

Further, the statistical testing of the model is performed by using Fisher's test (F test) for analysis of variance (ANOVA) and the results are given in Table 2. The model F value of 84.34 indicates that the above model is well fitted to the experimental data. The determination of correlation coefficient (R^2) value of 98.37%, indicates that only 1.67% of the variability in response is not explained by the model. The adj. R^2 value, which corrects the R^2 value with respect to sample size and number of model terms is 97.20% (close to R^2 value), suggests good agreement of prediction with experimental data.

The regression coefficients obtained from non-linear regression analysis between response and process variables are also listed in Table 2. A p value of less than 0.05 indicates that the model terms are significant. It is found that the aeration and agitation had a strong positive linear effect on copolymer production (p value < 0.05). Moreover, a significant negative quadratic effect of agitation speed (p value < 0.05) and aeration rate (p value < 0.05) on copolymer production is reported. Finally, significant negative interaction is noticed among the aeration and aeration rates indicating that the P(3HB-co-3HV) production increases initially with the increasing agitation and aeration rates, reaches a maximum, and then decreases at high values of these variables.

Under the ANN approach, a feed-forward neural network trained by resilient backpropagation algorithm with one hidden layer is constructed (Fig. 1). The inputs of the network are agitation and aeration rates whereas the outputs are P(3HB-co-3HV) and k_La values (for two separate model). The number of nodes in the hidden layer is varied from one to six and correspondingly the RMSE (Eq. 5) and SEP (Eq. 6) between experimental and predicted values at varying neurons are calculated (Fig. 4). For the P(3HB-co-3HV) model, it is observed that the RMSE and SEP values decrease with the increase in neurons in hidden layer from one to four and become constant from five to six neurons in the hidden layer. Likewise, for k_La, RMSE and SEP values decrease sharply with the increase in neurons from one to three, further decrease slightly for four neurons, and become constant for five and six neurons in the hidden layer. Thus, four neurons are chosen in the hidden layer for the training of experimental data. The primary goal of training is to minimize the error function (MSE) by searching a set of connection weight and bias values that produce outputs equal or close to the target value.

Further, the experimental data of bioreactor are used to feed the constructed neural network and divided into training (eight data sets) and testing (two data sets). After successful training, the weight and bias values of each layer of network are determined (Table 3) and are used to correlate the inputs of network to the output responses of network ((P(3HB-co-3HV) content and k_La values) as:

$$\begin{aligned}\text{ANN} = {} & \text{purelin}(\text{act.LW}\{3,2\} * \tan\text{sig}(\text{act.LW}\{2,1\} \\ & * \text{purlin}(\text{act.IW}\{1,1\} * \text{p} + \text{act.b}\{1\}) \\ & + \text{act.b}\{2\}) + \text{act.b}\{3\})\end{aligned} \quad (12)$$

where, act.IW{1,1}, act.LW{2,1}, and act.LW{3,2} represents the weights (N_i, i = 1–4, i.e., number of neurons) of input, hidden, and output layers, respectively, with the purelin and tan sig transfer functions. Similarly, act.b {1}, act.b {2}, and act.b {3} represent bias in input, hidden, and output layers, respectively.

The RMSE, SEP, bias (B_f), accuracy (A_f), and MPE (Δ) for the RSM and ANN models are calculated by Eqs. (5–9). For P(3HB-co-3HV) concentration, as shown in Table 1, the RMSE (0.22) and SEP (4.17%) for the RSM model are larger than those for the ANN model, 0.019 and 0.353%, indicating that the ANN has higher modeling ability than the RSM for copolymer P(3HB-co-3HV) production. Furthermore, the B_f and A_f are close to unity for both RSM and ANN models, indicating a good concordance between the experimental and predicted values. Better accuracy for predictions has been observed by ANN model with 0.25% prediction error as compared to 3.57% prediction error by RSM. For k_La, the RMSE and SEP values are 0.005 and 1.823%, respectively, for ANN model as compared to 0.039 and 15.03%, respectively, for the RSM model. These values indicate a good fit of the experimental data to ANN model in comparison to RSM model. The B_f and A_f values, also closed to unity for the ANN model, indicate good harmony between the experimental and predicted values. A very low value (2.50%) of prediction error is also indicative of better modeling ability of the ANN model than RSM model with a predictive error of 33.24%. A three-dimensional response surface plot is generated in MATLAB 7.8.1 (MathWorks Inc., MA, USA), which represents the interaction between agitation and aeration rates for the copolymer production by using quadratic polynomial Eq. (11) (Fig. 5). It is found that the increase in both the process variables at a certain level results in maximum copolymer accumulation; whereas further increase in its concentrations beyond the optimum value decreases the copolymer accumulation.

The connection weights of networks obtained after training can resemble the regression coefficients of polynomial model (Table 3). These connection weights with the corresponding bias terms are used to estimate the relative importance of each input variable on the output variables. The relative importance of each input term (I_j) on the output response can be determined using Eq. (13), which is based on the partitioning of connecting weights:

$$I_j = \frac{\sum_{m=1}^{m=N_h} \left(\left(\left| W_{jm}^{ih} \right| / \sum_{k=1}^{N_i} \left| W_{km}^{ih} \right| \right) \times \left| W_{mn}^{ho} \right| \right)}{\sum_{k=1}^{k=N_i} \left\{ \sum_{m=1}^{m=N_h} \left(\left| W_{km}^{ih} \right| / \sum_{k=1}^{N_i} \left| W_{km}^{ih} \right| \right) \times \left| W_{mn}^{ho} \right| \right\}} \quad (13)$$

where, I_j is the relative importance of the jth input variable on the output variable, and N_i and N_h are the numbers of input and hidden neurons, respectively. W is the connection weight with the superscripts 'i', 'h' and 'o' referring to input, hidden and output layers, respectively, and the subscript 'k', 'm' and 'n' referring to input, hidden and output neurons, respectively.

On the basis of the above calculations, it has been observed that the agitation speed with relative importance of 72.19% is comparatively more influential process variable for copolymer accumulation than the aeration rate with a relative importance of 27.80%. A similar trend is observed for the relative influence of agitation speed (64.44%) and aeration rate (35.55%) on volumetric oxygen transfer coefficient in the bioreactor.

Table 3 Structure of trained neural network models for the prediction of P(3HB-co-3HV) and k_La estimations

Neuron	NN architecture: P(3HB-co-3HV)				NN architecture: K_La estimation			
	N_1	N_2	N_3	N_4	N_1	N_2	N_3	N_4
Input-hidden layer								
Agitation (I_1)	4.3072	−10.5640	4.1504	−0.6839	−1.1228	0.6204	4.4068	2.7741
Aeration (I_2)	0.8584	−0.6898	−2.6174	−3.6973	−2.0492	1.4651	0.9393	−0.9119
Bias in hidden layer	−2.3714	1.2322	5.6735	−2.8315	3.1418	0.2999	−0.0682	2.6555
Hidden-output layer								
Weights	0.1674	0.2067	0.4346	−0.3887	−0.5244	−0.1343	−0.3378	0.6408
Bias in output layer	0.0297				0.3937			

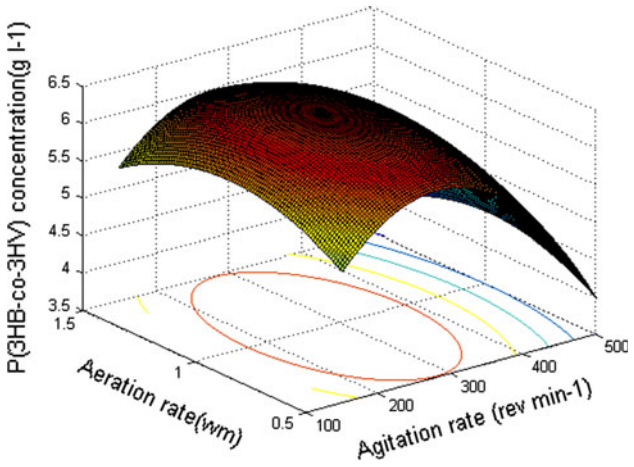

Fig. 5 Response surface plot showing the model-derived P(3HB-co-3HV) production as a function of agitation and aerations rates of the bioreactor system

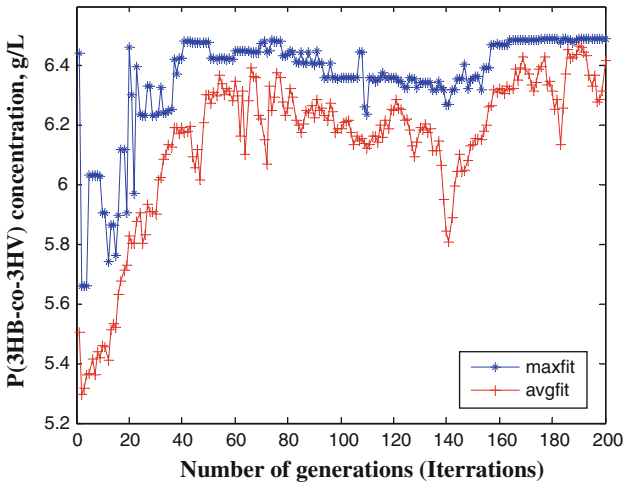

Fig. 6 Graphical representations of the maximum and average fitness of P(3HB-co-3HV) production (g l^{-1}) over a number of iterations of GA operators

Comparison of GA-based optimization using RSM and ANN models

Once the satisfactory RSM- and ANN-based models with good prediction accuracy were developed, then the optimization of input space (variables) was performed by genetic algorithm (a stochastic search engine). The different parameters of GA-based optimization were set as: chromosome length (l_{chr}) 10; population size (N_{pop}) 10; crossover probability (P_{cr}) 0.9; and mutation probability (P_{mut}) 0.01 as reported in the literature [10, 28, 40]. The optimum conditions were identified after the evaluation of GA for 200 iterations ($N_g^{max} = 200$) to achieve maximum copolymer P(3HB-co-3HV) production by using Eq. (11). The maximum and average fitness response of P(3HB-co-3HV) is plotted in Fig. 6.

The hybrid RSM-GA approach predicts the maximum P(3HB-co-3HV) concentration of 6.43 g l^{-1} with 19.80% 3HV content (mol%) at agitation and aeration rates of 279 rev min^{-1} and 1.15 vvm, respectively. The algebraic form of Eq. (12), which represents the network of weight and bias values, was used as fitness function in the hybrid ANN-GA approach. After successful conversion of responses in neural architecture, the maximum P(3HB-co-3HV) concentration of 7.45 g l^{-1} with 21.50% 3HV content (mol%) was predicted at optimum concentration of agitation and aeration rates of 287 rev min^{-1} and 0.85 vvm, respectively (Table 4). It is observed that the hybrid ANN-GA has performed better and predicts a higher response than hydrid-GA approach. The application of stochastic technique, GA is well suited for searching the global optima in multi-dimensional search spaces in contrast to statistical approaches, which are restricted to local optima [40]. The ANN is flexible and able to model the non-linear phenomena without mathematical description whereas RSM is based on mathematically derived quadratic polynomial equation with regression analysis and test of significance [28]. ANN mimics the biological processing system during the non-linear mapping of a set of inputs to the outputs. Thus, with the superiority of modeling ability, ANN is successfully integrated with GA paradigm to give the global optimal solution for maximum production of copolymer P(3HB-co-3HV) and incorporation of 3HV units.

Validation of predictive responses

Cane molasses is a by-product derived from the sugar industry and is generally sold at about 33–50% of the price of pure sugars. In the present study, the process parameters, agitation and aeration rates, are optimized for maximum P(3HB-co-3HV) production using previously designed molasses medium [46]. Upon validation in bioreactor, with optimum agitation and aeration rates (RSM-GA solution 1), 5.70 g l^{-1} of P(3HB-co-3HV) with 20 mol% 3HV was observed with the prediction error (%) of 11.28 and 6.38, respectively. In contrast, a slightly higher yield of P(3HB-co-3HV) of 7.20 g l^{-1} with 21 mol% of 3HV units was observed with prediction error of 3.38 and 2.32, respectively, at the optimum process variable level (ANN-GA 1): agitation speed 287 rev min^{-1}, and aeration rate 0.85 vvm. The above findings reveal a comparatively higher prediction ability of hybrid ANN-GA paradigm due to the better modeling ability of ANN than RSM model (Table 5).

The carbon yield is calculated as the ratio of the C moles in the product P(3HB-co-3HV) to the number of C moles in the substrate(s), total reducing sugar and propionate. Here,

Table 4 Optimal solutions predicted by hybrid RSM-GA and hybrid ANN-GA approaches along with their validated results in a 3-l bioreactor

Optimization method	Process variables		Predicted response		Validation				P(3HV-co-3HB) carbon yield[b]
	Agitation (rev min^{-1})	Aeration (vvm)	P(3HV-co-3HB) conc. (g l^{-1})	3HV (mol %)	P(3HV-co-3HB) conc. (g l^{-1})	PE[a]	3HV (mol %)	PE[a]	
RSM-GA 1	279	1.15	6.425	18.80	5.70	11.28	20	6.38	0.48
RSM-GA 2	277	1.15	6.324	19.20	5.75	9.08	20	4.17	0.48
ANN-GA 1	287	0.85	7.452	21.50	7.20	3.38	21	2.32	0.58
ANN-GA 2	333	0.99	6.921	20.50	6.80	1.75	21	2.44	0.55

[a] Prediction error (PE) $= \left| {}^{(\text{Experimental - Predicted})}/_{\text{Predicted}} \right| \times 100$

[b] Carbon yield (Y_{CPHBV}) = carbon mole in P(3HB-co-3HV)/carbon mole in substrates (total sugar + propionic acid)

Table 5 A brief comparative study on PHAs production using various pure/raw substrates along with the findings of present study

S. no.	Microorganism	Carbon/VFA sources	Cultivation vessel	Process parameters				PHAs conc. (g l^{-1})	% PHA (dcw^{-1})	Productivity (g l^{-1} h^{-1})	References
				Temp. (°C)	pH	DO (vvm)	Agitation (rpm)				
1	Recombinant *Escherichia coli*	Glucose	5.6-l bioreactor	30	–	1.5	125	PHB: 0.91	20.40	0.019	[1]
		Glucose	5.6-l bioreactor	30	–	1.5	500	PHB: 3.51	37.20	0.073	
		Glycerol	5.6-l bioreactor	30	–	1.5	125	PHB: 1.43	30.10	0.030	
		Glycerol	5.6-l bioreactor	30	–	1.5	500	PHB: 1.63	16.90	0.034	
2	*Alcaligenes eutrophus* DSM 545	Cane molasses 0.3% (w/w)	500-ml flask	30	7.0	–	300	PHB: 8.9	39.00	0.12	[4]
3	*Alcaligenes latus* DSM 1122	Sucrose/Na-propionate	5-l bioreactor	35	7.0	40–50% (air sat.)	640	PHB: 3.4 PHV: 1.3	85.00	q_p(B): 0.17 q_p(V): 0.01	[8]
4	*Bacillus megaterium*	Cane molasses 2% (w/w)	250-ml flask	30	7.0	–	130	PHB: 2.53	46.00	0.053	[14]
5	*Ralstonia eutropha*	Whey/Inverted sugar/ Propionic acid	5-l bioreactor	30	7.0	–	–	PHB: 2.47 PHV: 1.46	37.00	0.07	[23]
6	*Azotobacter beijerinckii*	Molasses/corn steep liquor	500-ml flask	30	7.0	–	–	PHB: 3.73	24.55	0.15	[27]
7	*Rhodobacter sphaeroides*	Acetate/ $(NH_4)_2SO_4$	5-l bioreactor	33.5	7.0	1.0	150	PHB: 8.76	95.40	0.15	[32]
8	*Azohydromonas lata* MTCC 2311	Cane molasses/ Na-propionate	3-l bioreactor	30	6.8	0.85	287	PHB: 5.65 PHV: 1.55	67.92	0.16	This work

the carbon yields of 0.58 and 0.48 were calculated on optimized solution given by hybrid ANN-GA and RSM-GA, respectively. These calculated values are comparable to the theoretical carbon yield of 0.48 and 0.50 on pure substrates glucose and sucrose [43] and suggest that the optimized process parameters favor the cost-effective production of P(3HB-co-3HV) from cane molasses.

The production of copolymer P(3HB-co-3HV) is initiated after the onset of exponential growth and reaches a maximum of 70.75 and 60.08% of dry cell weight at 40 h

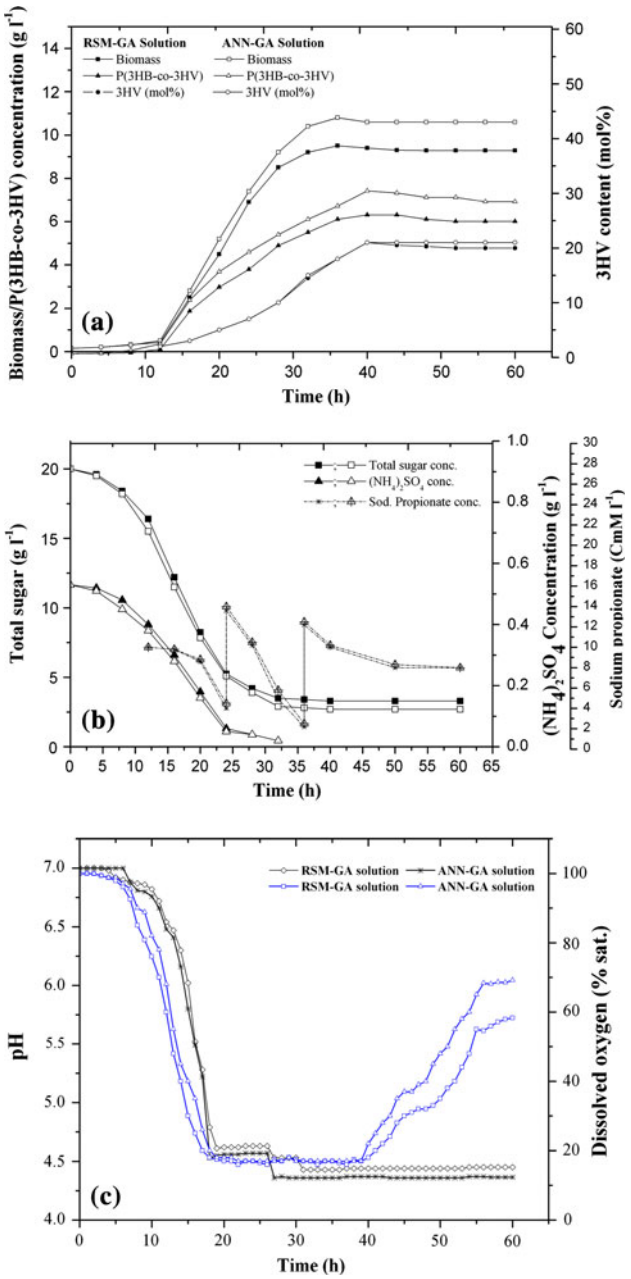

Fig. 7 **a** Validated responses of biomass, P(3HB-co-3HV), and 3HV content derived on optimal solutions (RSM and ANN) over time. **b** Utilization patterns of total sugar, NH_4^+ ions, and propionate concentration over time during validation of RSM-GA solution (*filled square/filled triangle/asterisk*) and ANN-GA solution (*open square/open triangle/triangle with plus*). **c** Change in pH (*asterisk/diamond*) and dissolved oxygen (%saturation) (*open square/open triangle*) during validation of optimal solutions

of cultivation on ANN-GA and RSM-GA based solutions, respectively (Fig. 7a). Thereafter, the copolymer concentrations are maintained at 7.20 (ANN-GA) and 6.20 (RSM-GA) g l^{-1} until the end of fermentation (60 h). During the copolymer accumulation, monomer compositions of the synthesized copolymer are changed. At the start of copolymer accumulation, the principal monomer unit is 3HB, which is shifted to 3HV monomers later during the fermentation period from 24 to 42 h. During this period, the mole fraction of 3HV units was increased from 9 to 21%. The total sugar concentration declines from 20 to 5 g l^{-1} during the initial 24-h cultivation with a sharp decrease in NH_4^+ ion concentration also during validation process (Fig. 7b). This leads to the microbial growth and growth-associated 3HB accumulation inside the microbial cells. The first installment of propionic acid is commenced at 12 h of cultivation, but its assimilation is started after 20 h of cultivation. The consumption of propionic acid is accelerated during 24–36 h of cultivation with simultaneous accumulation of 3HV units in copolymer P(3HB-co-3HV). Studies reported by Chen et al. [8] showed the production of 3.4 g l^{-1}, and 1.3 g l^{-1} of 3HB and 3HV units, respectively, corresponding to 85% PHA accumulation in cell mass using sucrose supplemented with propionic acid.

Also, Marangoni et al. [23] used dairy waste whey for the production of copolymer P(3HB-co-3HV) with the supplementation of inverted sugar and propionic acid. They have observed 1.46 g l^{-1} of 3HV content in 3.93 g l^{-1} of accumulated polymer, corresponding to 37% of the dry cell weight. Beaulieu et al. [4] described the maximum accumulation of 5.75 g l^{-1} of PHB at optimal level of molasses of 0.3% by *A. eutrophus* DSM 545. Gouda and colleagues [14] observed the maximum PHB concentration of 46.20% per mg cell dry weight with 2% molasses, while best growth was obtained with 3% molasses using *Bacillus megaterium*.

Similarly, Purushothaman et al. [27] used the cane molasses with corn steep liquor and reported the 3.7 g l^{-1} of PHB after 24 h of cultivation of *A. beijerinckii*.

During validation, the initial pH (7) was decreased sharply during the exponential growth with feeding of propionic acid and simultaneous production of copolymer (Fig. 7c). With a slight decrease in pH during accumulation phase, the pH become constant to 4.35 and 4.45 until the end of the fermentation process solution given by ANN-GA and RSM-GA approaches, respectively. Similarly, the dissolved oxygen consumption is started rapidly after 12 h of cultivation (during growth phase) and exhausted from 100 to 22% within the next 10 h. However, during copolymer accumulation, oxygen limitation (<20% air sat.) is encountered and conquered after the cessation of growth and copolymer accumulation at 40 h of cultivation. Almeida et al. [1] studied the effect of aeration on the PHB production from glycerol and glucose by recombinant *E. coli*. They have reported the maximum PHB concentration of 1.43 (30.1% of dcw) and 1.62 g l^{-1} (20.4% of dcw) on glycerol and glucose, respectively, with moderate agitation (125 rev min^{-1}). In glycerol culture, a low-agitation

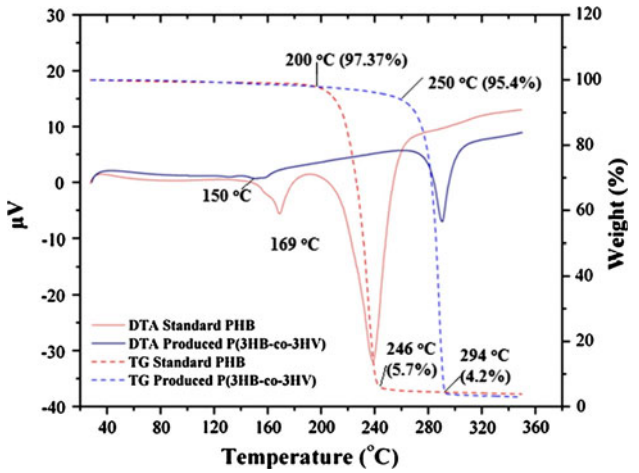

Fig. 8 TG curves of thermal degradation of produced copolymer P(3HB-co-3HV) and standard PHB

condition favors more PHB accumulation while a decrease in PHB formation was observed in glucose culture. Similarly, the maximum PHB concentration of 8.79 g l^{-1} (95.4% of dcw) has been reported on agitation and aeration rates of 150 rpm and 1.0 vvm, respectively, with optimized acetate medium [32].

Analysis of material properties of copolymer P(3HB-co-3HV)

The thermal behavior of produced copolymer is analyzed by simultaneous thermogravimetry–differential thermal analysis (TG–DTA) curves illustrated in Fig. 8. The thermal degradation of copolymer P(3HB-co-3HV) and standard PHB were observed between 250–294°C and 200–246°C, respectively. This lead to the weight losses of about 91% for both biopolymer with endothermic DTA peaks at 290 and 238°C for copolymer and standard PHB, respectively. The DTA thermogram also revealed the melting behavior (T_m) of polymer and noted that the T_m of P(3HB-co-3HV) is significantly lower (150°C) than the T_m of standard PHB (169°C). For copolymer P(3HB-co-3HV), the difference between T_m and decomposition temperature (200°C) is high enough to increase its processing window under various applications.

GPC analysis of copolymer P(3HB-co-3HV) extracted from *A. lata* cells revealed that weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (D) defined as M_w/M_n were 2.0616×10^4 g mol^{-1}, 1.2117×10^4 g mol^{-1}, and 1.701, respectively. These values are comparable with the standard P(3HB-co-3HV) (12 mol%) as M_w 6.54×10^5, M_n 3.55×10^5, and D as 1.84 [19]. Sudesh et al. [36] reported that the M_w of P(3HB) produced from wild bacteria ranges from 1×10^4 to 3×10^6 g mol^{-1} with a polydispersity of around two.

Conclusions

The feeding of propionic acid in cane molasses solution has been successfully applied for the production of copolymer P(3HB-co-3HV) by *A. lata* MTCC 2311. Further, significant improvement has been observed under the strategic optimization of process variables such as agitation and aeration rates. The following conclusions have been made:

1. The performance of P(3HB-co-3HV) production process has been modeled by a three-layered neural network with four neurons in hidden layer in a better way (RMSE: 0.019; SEP:0.353%). Again, the genetic algorithm has been employed successfully to search the global domain of agitation and aeration range to predict the maximum copolymer concentration (7.45 g l^{-1}) with 21.50% 3HV content at process variables: agitation speed, 287 rev min^{-1}; and aeration rate, 0.85 vvm. Upon validation, 7.20 g l^{-1} of P(3HB-co-3HV) and 21 mol% of 3HV content were observed with prediction error (%) of 3.38 and 2.32, respectively.
2. The observed k_La value has been found to be directly proportional to the agitation and aeration rates. It is also observed that the copolymer productivity is initially fluctuated with an increase in k_La value, achieves maximum of 0.163 g l^{-1} h^{-1} at 0.17 s^{-1} of k_La value, and then decreases at its higher values. In contrast, a sharp decrease in 3HV content has been reported on an increased k_La value.
3. Agitation speed was observed to be a more influential process parameter with a relative importance of 72.19% for copolymer production than aeration rate with a relative importance of 27.80%.
4. During the fermentative production of P(3HB-co-3HV), the monomeric composition of accumulated polymer changed with time. During the initial copolymer synthesis, the 3HB units accumulate, which is shifted to 3HV units (9–21%) later during the cultivation period of 24–42 h.
5. The increase in copolymer content enhanced the thermal and material properties; such as T_m, degradation temperature profile, and M_w of produced P(3HB-co-3HV) significantly in order to increase its processing windows.

Hence, the production of cost-effective copolymer P(3HB-co-3HV) with desired 3HV content (mol%) and improved material properties has been observed from cane molasses upon optimization of process parameters, agitation and aeration rates of a bioreactor.

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