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Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by *Ralstonia eutropha*

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Abstract We employed systematic mixture analysis to determine optimal levels of acetate, propionate, and butyrate for cell growth and polyhydroxyalkanoate (PHA) production by *Ralstonia eutropha* H16. Butyrate was the preferred acid for robust cell growth and high PHA production. The 3-hydroxyvalerate content in the resulting PHA depended on the proportion of propionate initially present in the growth medium. The proportion of acetate dramatically affected the final pH of the growth medium. A model was constructed using our data that predicts the effects of these acids, individually and in combination, on cell dry weight (CDW), PHA content (%CDW), PHA production, 3HV in the polymer, and final culture pH. Cell growth and PHA production improved approximately 1.5-fold over initial conditions when the proportion of butyrate was increased. Optimization of the phosphate buffer content in medium containing higher amounts of butyrate improved cell growth and PHA production more than 4-fold. The validated organic acid mixture analysis model can be used to optimize *R. eutropha* culture conditions, in order

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to meet targets for PHA production and/or polymer HV content. By modifying the growth medium made from treated industrial waste, such as palm oil mill effluent, more PHA can be produced.

Keywords Polyhydroxyalkanoate · *Ralstonia eutropha* · Organic acid · Mixture model

Introduction

Bioplastics are among the biomass-based products currently being developed to replace petroleum-based products (Frazzetto 2003; Young 2003). A large number of bioplastics have been discovered, with over 150 varieties (Williams et al. 1999), encompassing a wide range of thermoplastic properties (Cornibert and Marchessault 1972). Polyhydroxyalkanoates (PHAs) are naturally occurring biopolymers produced by a diverse group of bacteria (Madison and Huisman 1999; Sudesh et al. 2000) that possess characteristics similar to those of chemically synthesized polymers (Steinbüchel and Füchtenbusch 1998).

In general, the microbial biosynthesis of PHA results from the limitation of a nutrient, such as nitrogen, sulfur, oxygen, or phosphate (Anderson and Dawes 1990) in the presence of abundant carbon. These polymers are thought to serve in nature as storage molecules for carbon and reducing equivalents, and in some bacterial strains, PHA can account for more than 80% of CDW (Khanna and Srivastava 2005). The molecular weight and monomer composition of polymer can be controlled by utilizing specific PHA synthase enzymes and specific carbon substrates (Anderson and Dawes 1990; Sim et al. 1997). PHAs exhibit different thermal and mechanical properties depending on the types of monomers incorporated (Doi et al. 1995; Jendrossek and Handrick 2002). These polymers can be rapidly hydrolyzed in soil, sea water, and lake water by microorganisms (Mergaert et al. 1992; Khanna and Srivastava 2005). Many environmental factors can influence the polymer properties, which can, in turn, affect the rate at which the polymer degrades (Khanna and Srivastava 2005).

PHAs, which have better biocompatibility than petroleum-based plastics, have been used to develop medical devices, artificial organs, and therapeutic composites (Chen and Wu 2005; Misra et al. 2006; Wu et al. 2009a). Despite these advantages, production costs of bioplastics remain higher than those of conventional plastics and will need to be lowered for bioplastics to compete in the marketplace (Choi and Lee 2000). The overall cost of polymer production could be decreased by the efficient use of inexpensive carbon feedstocks (Nikel et al. 2006). Several studies have investigated the production of bioplastics from industrial waste streams (Ahn et al. 2000; Füchtenbusch et al. 2000; Marangoni et al. 2002; Chua et al. 2003; Liu et al. 2008), which have a potential as inexpensive carbon sources. The fermentation of these substrates yields favorable amounts of cell mass and bioplastic. However, few systematic or statistical approaches have been taken to enhance the production of the desired polymer products (Nikel et al. 2005).

To develop a cost-effective strategy to produce bioplastics from oil palm waste products (Hassan et al. 2002), the possibility of using anaerobically treated, clarified POME was examined. POME is an aqueous stream generated in large quantities during the milling of oil palm fruits. The oil palm tree, *Elaeis guineensis*, is the most productive oilseed plant under cultivation and is an important agricultural crop in Africa and Southeast Asia (Basiron 2007; Wu et al. 2009b). In Malaysia, approximately 46 million tons of POME are produced annually (http://Econ.mpob.gov.my/economy/Overview_2008_latest130109.htm). It is estimated that for every ton of crude palm oil produced in Malaysia, 2.5 tons of POME are generated.

POME is a colloidal suspension containing 95–96.5% water, 0.6–0.7% oil, and 4–5% total solids that has high chemical oxygen demand and biological oxygen demand, making it a severe pollutant that must be treated (Hassan et al. 2002; Ahmad et al. 2003). POME can be fermented anaerobically by wild-type bacteria to produce the volatile organic acids acetate, propionate, and butyrate in the mass ratio of 3:1:1, respectively (Yee et al. 2003). The feasibility of utilizing this treated POME as a carbon source for the production of PHAs by *Ralstonia eutropha* has been demonstrated (Hassan et al. 2002). In order to confirm and extend these preliminary findings, we have used a mixture analysis model to optimize the concentrations of volatile organic acids in minimal medium. We have determined the effects that altering the ratio of these organic acids has on microbial cell growth, PHA production, and monomer composition of the polymer. We report here the steps we have taken toward the goals of enhancing bioplastic productivity and lowering production costs.

Materials and methods

Bacterial strains and growth media

R. eutropha H16 (Wilde 1962) was precultured in 3 mL of dextrose free tryptic soy broth (TSB) for 24 h at 30°C. The cells were harvested and washed twice with sterile water, then used to inoculate 5 mL of PHB minimal medium, prepared as previously described (York et al. 2003) with a final NH_4Cl concentration of 0.1 g/L. Cell growth was monitored by measuring optical density (OD) at 600 nm, starting from an initial OD_{600} of 0.05. For media containing

high phosphate buffer (10×), we used 67 mM of Na_2HPO_4, 62.5 mM of NaH_2PO_4, 26 mM of K_2SO_4, and 10 mM of NaOH. Cells for high phosphate media growth experiments were grown initially in 5 mL PHB minimal medium in a test tube for 48 h on a roller drum or shaker. For larger volume mixed acid experiments with high phosphate media, 1 mL of cells from an overnight culture grown in TSB was used to inoculate 50 mL of high phosphate minimal medium in 250-mL flasks and then grown for 48 h at 30°C. For 1-L cultures with mixed acids, 1 mL of cells from an overnight culture grown in TSB was used to inoculate 50 mL of TSB in a 250 mL flask, which was then incubated for 24 h at 30°C. These cells were harvested, washed with sterile water twice, used to inoculate 1 L of high phosphate minimal medium, and then grown for 72 h at 30°C before harvesting. The cells were harvested, washed twice with cold water, and lyophilized for 48 h. Note that when describing acid concentrations, all values refer to the percent (*w*/*v*) of the organic acid sodium salt added to the media. All media used in this study were supplemented with 10 μg/mL gentamicin.

Design of experiments and mixture analysis

To develop a strategy for optimizing cell growth and PHA production, a mixture analysis model of three acids in the minimal medium was developed and populated using a standard mixture analysis methodology (Nowruzi et al. 2008) and the Minitab V14 program (http://www.minitab.com). For the design of mixture analysis experiments to populate the model, we used a simplex lattice method augmented by the design of axial points (0.17:0.17:0.67, 0.17:0.67:0.17, and 0.67:0.17:0.17 for acetate/propionate/butyrate). The degree of lattice for this mixture analysis is 3; therefore, the experimental design contains the set of all 13 combinations, where each value of organic acid is 0.00, 0.33, 0.67, and 1.00 (Table 1). The experimental data for response trace plots are also shown in Table 1. All experiments were performed using 50-mL cultures with 0.3% total organic acid content. To plot mixture contours, a mixture regression using the model fitting method was applied with full quadratic component terms initially included. In the data analysis, the coefficients with p value below 0.1 were used as parameters (Online Resource 1).

Analytical methods

PHA quantity and composition were determined by gas chromatography using a slight modification of a method described previously (Brandl et al. 1988). Approximately 10 mg of freeze-dried cells from each experiment was weighed and placed in Teflon-stoppered glass vials. For methanolysis, 1 mL $CHCl_3$ and 1 mL CH_3OH/H_2SO_4 (85:15 *v*/*v*) were added to the samples, which were then incubated at 105°C for 2 h, cooled to room temperature, and incubated on ice for 10 min. After adding 0.5 mL of cold water, the samples were vortexed for 1 min, then centrifuged at 2,000×*g*. The organic phase was extracted by pipette and moved to clean borosilicate glass tubes containing Na_2SO_4. These samples were then filtered and injected into a gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a DB-Wax capillary column (Agilent, 30 m×0.32 mm×0.5 μm). The volatile acids were monitored by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 50°C with a diode array detector at 210 nm. The mobile phase was 5 mM sulfuric acid, with a flow rate of 0.6 mL/min.

Results

Optimizations of mixed acid concentration, buffer concentration, and C/N ratio

Initial experiments were performed to determine the experimental range of organic acid concentrations to be explored for the mixture analysis. Specifically, we examined the growth of *R. eutropha* H16 in minimal media containing individual organic acids or combinations of mixed acids as sole carbon sources, various buffer concentrations, and various carbon to nitrogen (C/N) ratios. We tested utilization of the individual acids and found that *R. eutropha* exhibited maximal growth in defined minimal medium containing 0.5% acetate, 0.4% propionate, or 0.5% butyrate as the sole carbon source (Fig. 1). When *R. eutropha* H16 was grown on the 3:1:1 (acetate/propionate/butyrate), maximum growth was observed with a 0.3% total acid concentration (Fig. 2a).

The growth of *R. eutropha* in the standard minimal medium was accompanied by an increase in the final pH to values as high as 9.5. Therefore, experiments were performed to study cell growth on the mixed acid carbon source using different concentrations of phosphate buffer added to control pH. A range of phosphate buffer concentrations from 10 to 500 mM was examined, and media with up to 130 mM phosphate buffer exhibited an increase in final culture optical density that was proportional to the amount of buffer added (Fig. 2b). The buffer concentration of 130 mM phosphate (i.e., high phosphate media, compared to our initial media) was therefore employed in all further mixed acid culture experiments. Various C/N ratios were examined by adding different levels of mixed acids to the standard minimal medium. We found that 40 C/N allowed for the most robust cell growth using mixed acids as the carbon source (Fig. 2c), while the percent PHA of CDW was highest at 80 C/N (Fig. 2d).

Table 1 Experimental design points chosen through a simplex lattice methodology for the mixture analysis model and their experimental results

ID #	Acetate	Propionate	Butyrate	CDW (mg/50mL)	PHA content (wt%)	3HV%
1	1.00	0.00	0.00	38.6±1.3	66.5±0.5	0.0±0.0
2	0.67	0.33	0.00	46.9±0.1	71.1±1.9	7.7±1.7
3	0.67	0.00	0.33	57.5±0.5	76.8±0.5	0.0±0.0
4	0.33	0.67	0.00	51.7±2.7	72.9±1.4	17.1±3.2
5	0.33	0.33	0.33	61.3±2.2	77.6±0.8	8.5±1.3
6	0.33	0.00	0.67	71.7±1.5	83.0±3.3	0.0±0.0
7	0.00	1.00	0.00	43.0±3.9	70.0±3.9	35.1±2.7
8	0.00	0.67	0.33	60.2±1.0	76.8±2.2	15.4±2.8
9	0.00	0.33	0.67	66.0±1.9	83.7±0.2	4.6±0.7
10	0.00	0.00	1.00	66.1±4.9	82.2±0.3	0.0±0.0
11	0.67	0.17	0.17	49.1±0.6	77.7±3.7	4.7±0.8
12	0.17	0.67	0.17	53.2±1.5	74.6±0.9	18.4±3.6
13	0.17	0.17	0.67	70.0±0.1	78.1±2.6	2.0±0.3

Although 80 C/N provided the highest PHA content, the low cell densities of these cultures made them difficult to analyze. We therefore used 40 C/N in all further mixed acid experiments.

The initial pH of the culture medium also affected cell growth, especially with propionate and acetate as carbon sources. With increases in the initial pH of the growth medium, *R. eutropha* showed a lower final OD_{600} on acetate as the main carbon source. However, when cells were grown on propionate, a different trend emerged: the final optical density increased as the initial pH was increased up to 7.3, then went down with further increases in pH (Fig. 3a). The increase in pH of the cultures was greater when acetate was used as the carbon source than when the other organic acids were used (Fig. 3b). The reason why acetate consumption increased the pH of the growth medium was not clear, and no significant production of by-product was detected by HPLC analysis (data not shown). A similar pH increase was observed in *R. eutropha* cultures in another study (Wang and Yu 2001), but no explanation for this phenomenon was given.

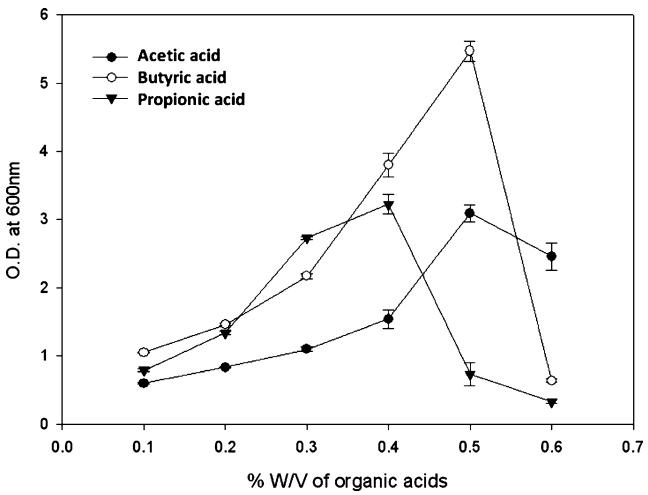

Fig. 1 Growth of *R. eutropha* in minimal medium with acetate, propionate, or butyrate as the sole carbon source. Cultures were inoculated to an initial OD_{600} of 0.05 and the reported values were measured after 48 h of growth

Modelling the effects on cell growth, pH, amount of PHA, and 3HV content

To populate the mixture analysis model of the effect of the three acids on cell growth, PHA production, and 3HV content, *R. eutropha* was grown with 13 culture compositions of mixed acids, as described in "Materials and methods" (Table 1). Each culture condition was grown for 48 h and tested in duplicate. For each culture, we measured the cell growth, medium pH, amount of PHA per 50 mL culture, PHA content in the cells (percent of CDW), residual CDW (CDW minus PHA), and the 3HV content of PHA.

Contour plots of each variable, generated by the modelling software, were used to predict growth and PHA production over the range of compositions tested (Fig. 4). To examine the accuracy of these models, three additional mixture compositions, which had not been included in the first set of 13 conditions, were selected and tested. The three conditions used for the model validation tests were (acetate/propionate/butyrate) 0.1:0.1:0.8, 0.1:0.4:0.5, and 0.6:0.3:0.1. The results of these cultures validated the predictive power of the model for CDW, PHA content, total amount of PHA per volume of culture, and 3HV%. The results indicated that the model contour plots accurately reflected each of the empirical trends (Fig. 5) and that the model could be used to reliably predict the results of growth on various acid mixtures. From the results outlined in Fig. 4, an increase in the initial concentration of butyrate augmented cell growth and PHA production. Interestingly,

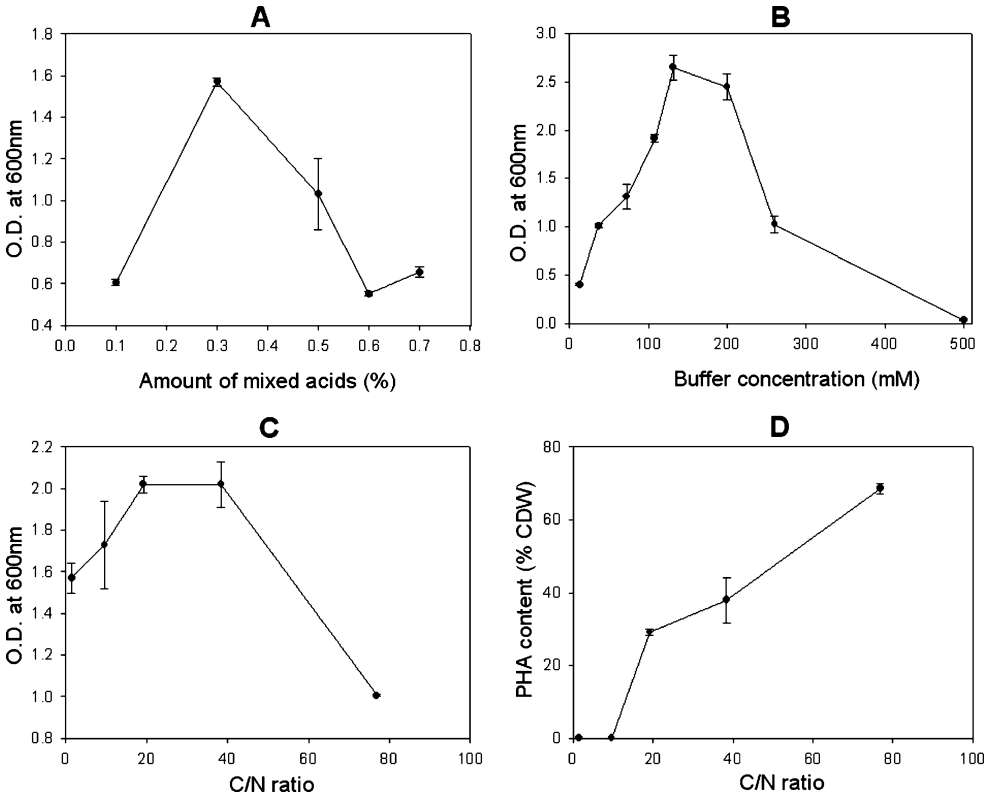

Fig. 2 Optimizations of total mixed acid concentration (**a**), buffer concentration (**b**), C/N ratio (**c**), and PHA production (**d**). Measurements were made after 48 h of growth. For **a**, media was buffered with 70 mM phosphate buffer. In all experiments, a ratio of 3:1:1 (acetate/propionate/butyrate) was used. For **b**–**d**, 0.5% total mixed acids were used

the optimal acid composition for total PHA production predicted by the model is not pure butyrate but rather 0.2:0.0:0.8. The reason that a small fraction of acetate in the medium is beneficial is not currently understood. Butyrate concentration correlated positively with cell weight and amount of PHA, while propionate concentration correlated positively with 3HV content in the final PHA.

We determined that the effect of butyrate on cell growth and PHA production was not due simply to an increase in total carbon available in the media. When we normalized carbon levels to 124 mM of total carbon and examined differences in growth between conditions of 0.3% (3:1:6), 0.34% (3.6:1:1), and 0.33% (3:1.5:1), the culture with the highest concentration of butyrate reached the highest OD_{600} (Online Resource 2).

The amount of 3HV in the final PHA was proportional to the amount of propionate added to the culture, as previously reported (Bhubalan et al. 2008). With a constant amount of propionate in the media and different acetate/butyrate ratios, combinations with higher acetate led to PHAs containing more 3HV. For example, polymer from the 0.67:0.33:0 (acetate/propionate/butyrate) cultures contained 7.7% 3HV, while the polymer from the 0:0.33:0.67 (acetate/propionate/butyrate) culture contained 4.6% 3HV. However, cultures containing higher acetate yielded less PHA and total biomass (Table 1). These results, which are based on model

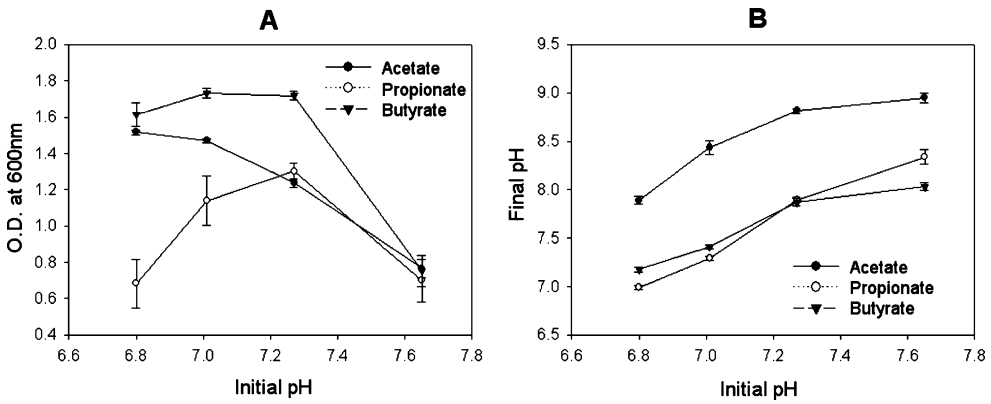

Fig. 3 Effect of initial pH on cell growth. OD_{600} (**a**) and final pH (**b**) were measured after 48 h of growth as functions of initial pH. Cultures contained 0.5% (*w*/*v*) acetate (*filled circles*), 0.4% (*w*/*v*) propionate (*open circles*), or 0.5% (*w*/*v*) butyrate (*closed triangles*)

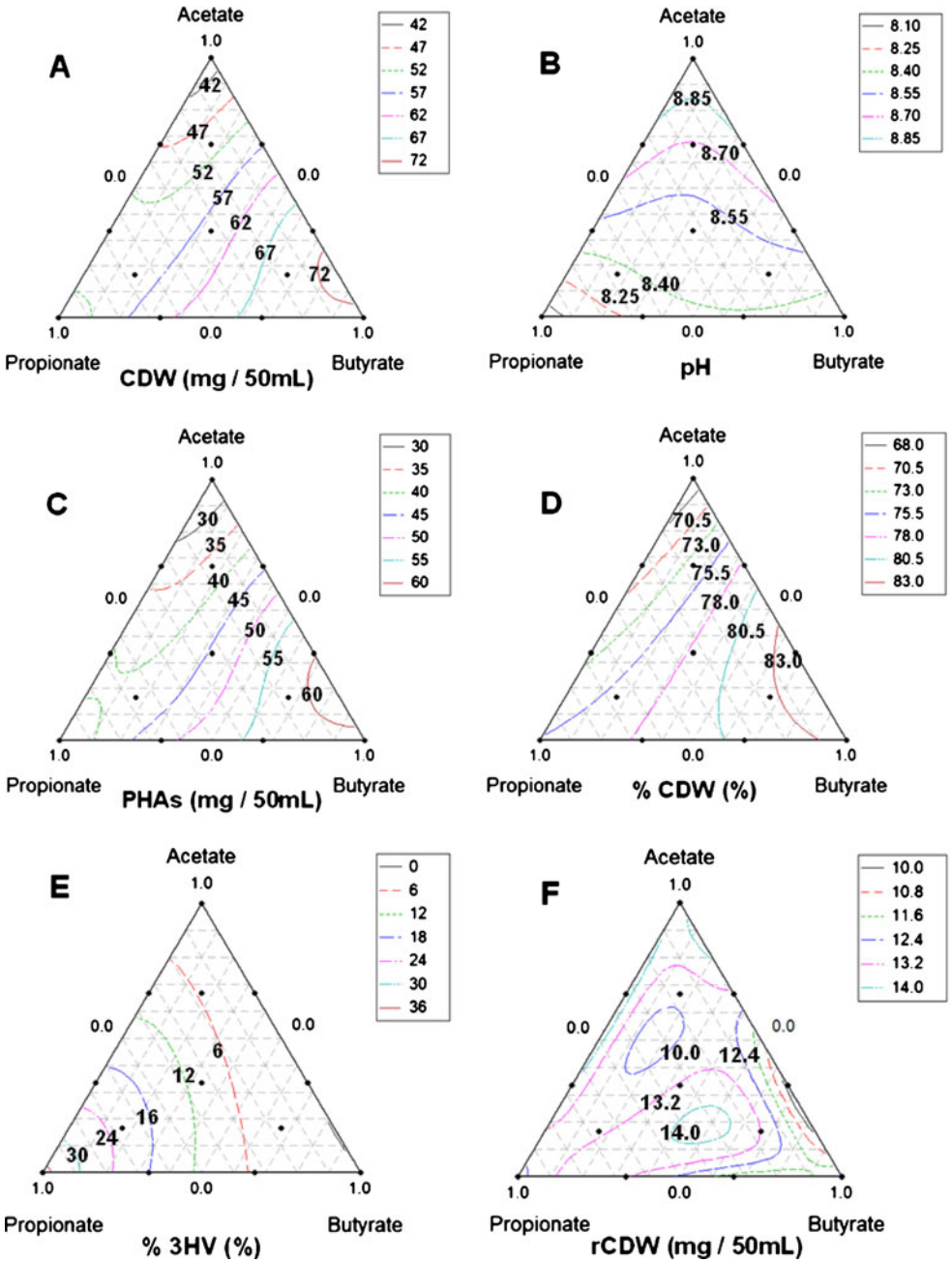

Fig. 4 Optimization of mixed acid composition. The effects on CDW (**a**), final pH (**b**), amount of PHA produced (**c**), PHA content (wt%) (**d**), 3HV content (wt%) (**e**), and residual CDW (**f**) of different mixed acid compositions

predictions and empirical experiments, can be applied to adjust conditions to optimize cell growth and polymer production. The model can also be used to maximize polymer production with a given monomer composition (i.e., culture conditions can be identified that will maximize polymer accumulation with a minimum 3HV content).

PHA production

We have found that increasing the ratio of butyrate to the other organic acids increased total biomass and PHA content of *R. eutropha*, consistent with previous reports (Chakraborty et al. 2009). Based on our model predictions and experiments, the reported acid composition from treated POME (3:1:1 acetate/propionate/butyrate) is not an optimal ratio for maximizing cell growth and PHA production (Hassan et al. 2002). Our model shows that supplementing the original 3:1:1 mixture to change the ratios of the volatile acids could yield several alternative media compositions that are more favourable for cell growth and production of PHAs (Online Resource 3). To test some of the predictions listed in Online Resource 3, we compared 3:1:1 (acetate/propionate/butyrate, Fig. 6a) to 3:1:6 (acetate/propionate/butyrate, Fig. 6b) conditions using larger (1 L) culture volumes. The concentration of each volatile acid was monitored by HPLC. CDW and PHA

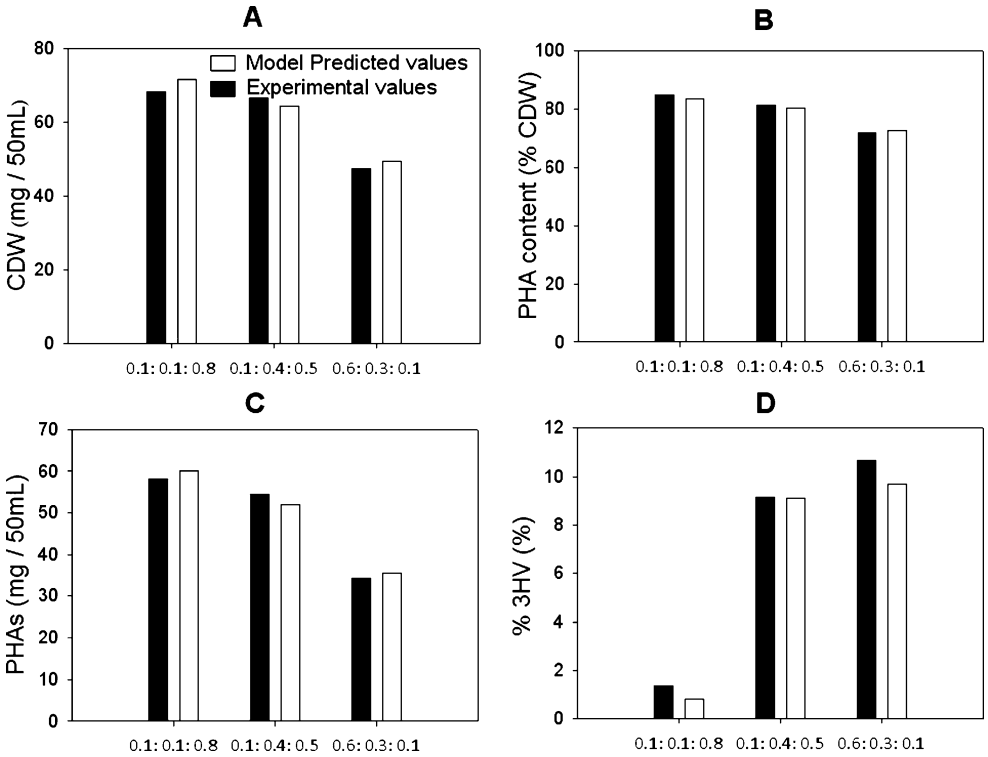

Fig. 5 Confirmation of mixed acid model by comparison of experimental values (*solid bars*) and predicted values (*white bars*) at 0.1:0.1:0.8 (acetate/propionate/butyrate), 0.1:0.4:0.5, and, 0.6:0.3:0.1 using high phosphate buffer

content increased continuously until 41 h (Fig. 6a, b). When the results in Fig. 6a and b are compared, it can be seen that the maximum PHA obtained from 3:1:6 (41 h, CDW 1.33 g/L, PHA 0.86 g/L) is significantly higher than that obtained from 3:1:1 (41 h, CDW 1.10 g/L, PHA 0.64 g/L). In addition, measuring the consumption of volatile acids showed that butyrate was depleted first, regardless of its initial concentration in the medium (Fig. 6a, b). This observation was confirmed using a culture medium containing a uniform percentage of each volatile acid (1:1:1 acetate/propionate/butyrate) (Online Resource 4). The rapid depletion of butyrate is consistent with a report, which suggested that *R. eutropha* can direct 67% of butyrate into the tricarboxylic acid cycle (TCA) cycle, whereas only 33% of acetate can enter the TCA cycle (Chakraborty et al. 2009). Our experiments clearly show that butyrate increased PHA accumulation more effectively than acetate. This may be related to the observation that butyrate can be incorporated into PHA without decomposition of the molecule (Doi et al. 1988).

Discussion

Many efforts have been undertaken to seek renewable sources of carbon for the production of PHA (Tan et al. 1997; Loo et al. 2005; Nikel et al. 2005). Industrial and agricultural effluents are promising because these aqueous streams contain various sugars, organic acids, and other organic compounds that can be recovered and used as substrates for fermentation. Despite the advantages of turning waste streams into carbon feedstocks, several

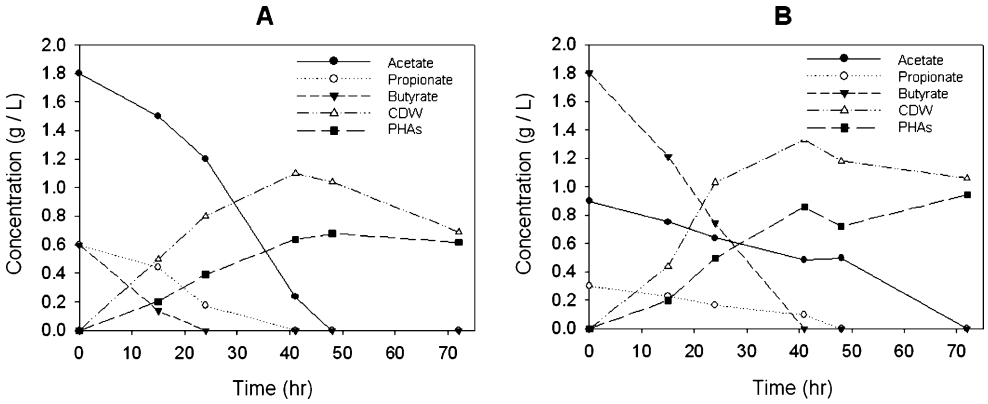

Fig. 6 Production of PHAs from 3:1:1 (**a**) and 3:1:6 (**b**) organic acids (acetate/propionate/butyrate) in high phosphate buffer media. Acetate (*filled circle*), propionate (*empty circle*), butyrate (*filled inverted triangle*), CDW (*empty triangle*), and PHA (*filled square*) concentrations were monitored. Depletion of NH_4^+ occurred within 12 h

problems remain to be solved regarding the use of industrial wastes as carbon sources for PHA production. First and foremost, the carbon composition of an untreated waste stream is unlikely to be optimal for biological polymer production. Untreated waste streams may contain harmful components, which could inhibit bacterial growth and, in turn, limit PHA production. Furthermore, the composition of these streams may not be easy to control and reproduce (Zaldivar et al. 2001). One strategy that can therefore be employed is to treat a waste stream in order to generate a more desirable feedstock, as has been demonstrated by using anaerobic fermentation of POME to produce organic acids (Hassan et al. 1996).

In this study, we applied systematic optimization modelling of mixed acid ratios to establish the effect of each volatile fatty acid and to maximize cell growth and PHA production. We first reformulated our minimal medium, raising the phosphate buffer concentration so as to increase buffering capacity and changing the C/N ratio. We then populated a model using data from cultures with various mixed acid compositions and confirmed the predicted results with additional mixed acid cultures. With the validated model, we can predict growth and PHA production of *R. eutropha* on any combination of acetate, propionate, and butyrate. We also observed that the initial pH of the growth medium resulted in differences in cell growth: depending on the type and concentration of each organic acid, different final pH values were observed, suggesting that fermentation proceeds by different routes and different metabolites are produced. These results suggest several strategies to make treated POME a better substrate for cell growth and PHA production. One way to stimulate biomass production, starting with a 3:1:1 (acetate/propionate/butyrate) mixture, is to increase the amount of butyrate, which is consumed by *R. eutropha* faster than the other volatile acids tested in this work. More butyrate would increase CDW as well as PHA content. To increase the proportion of 3HV in the resulting PHA, cells can be grown with more propionate and higher initial pH. Previous work has shown that introducing 3HV into a PHB chain reduces the melting temperature and increases the toughness of the resulting plastic, making it a more useful material (Anderson and Dawes 1990). Copolymers of 3HB and 3HV synthesized by *R. eutropha* are also known to have lower molecular weights, compared to the PHB homopolymer (Kunioka and Doi 1990; Scandola et al. 1992). The mixed acid model allows one to formulate a medium for production of PHA with a desired 3HV content.

Furthermore, the validated mixture optimization model can also guide the development of alternative procedures for upstream treatment of POME, in order to improve the proportions of certain acids in the final composition. For example, the composition of organic acids generated from POME has been shown to be affected by controlling the pH of the treatment conditions (Hassan et al. 1996).

In this work, a model designed to improve PHA production from mixed organic acids by *R. eutropha* H16 was constructed and validated. The results discussed here represent a significant step toward the production of low-cost bioplastic using organic waste streams as a carbon feedstock.

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