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Research Article

PHA Productivity and Yield of *Ralstonia eutropha* When Intermittently or Continuously Fed a Mixture of Short Chain Fatty Acids

Panchali Chakraborty,[1] Kasiviswanathan Muthukumarappan,[1] and William R. Gibbons[2]

[1] *Department of Agricultural and Biosystems Engineering, SAE 225/Box 2120, South Dakota State University, Brookings, SD 57006, USA*
[2] *Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57006, USA*

Correspondence should be addressed to Kasiviswanathan Muthukumarappan, muthukum@sdstate.edu

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The research described in this present study was part of a larger effort focused on developing a dual substrate, dual fermentation process to produce Polyhydroxyalkanoate (PHA). The focus of this study was developing and optimizing a strategy for feeding a mixture of SCFAs (simulated ARF) and maximizing PHA production in a cost-effective way. Three different feeding strategies were examined in this study. The substrate evaluated in this study for the growth phase of *R. eutropha* was condensed corn solubles, a low-value byproduct of the dry-mill, corn ethanol industry. The culture was grown to high cell densities in nitrogen-supplemented condensed corn solubles media in 5 L bioreactors. The overall growth rate of *R. eutropha* was 0.2 h^{-1}. The 20 mL ARF feeding every 3 h from 48 to 109 h strategy gave the best results in terms of PHA production. PHA productivity (0.0697 g L^{-1} h^{-1}), PHA concentration (8.37 g L^{-1}), and PHA content (39.52%) were the highest when ARF was fed every 3 h for 61 h. This study proved that condensed corn solubles can be potentially used as a growth medium to boost PHA production by *R. eutropha* thus reducing the overall cost of biopolymer production.

1. Introduction

Biodegradable polymers made from renewable resources such as agricultural wastes, corn, cassava, tapioca, whey, and so forth, do not lead to depletion of finite resources. The most studied of the biodegradable polymers include polyesters, polylactides, aliphatic polyesters, polysaccharides, and various copolymers [1]. These biopolymers have many of the desirable physical and chemical properties of conventional synthetic polymers [2]. To this point, high production costs have limited the use of biopolymers. However if these costs can be reduced, there would be widespread economic interest [3].

The focus of this project was production of the biopolymer Polyhydroxyalkanoate (PHA) at a low cost. PHA is actually a term used to describe a diverse family of polymers that are composed of 3-hydroxy fatty acid monomers. The carboxyl group of one monomer forms an ester bond with hydroxyl group of the neighboring monomer. Polyhydroxybutyrate (PHB) has been studied in most detail. PHB has good oxygen impermeability, moisture resistance, water insolubility, and optical purity [4, 5]. Young's modulus and tensile strength of PHB are similar to polypropylene, but elongation at break is 6% as opposed to that of 400% for polypropylene [6]. It has good UV resistance, but poor resistance to acids and bases [7]. The oxygen permeability is very low, making PHB a suitable material for use in packaging oxygen-sensitive products. PHB has low water vapor permeability compared to other bio-based polymers but higher than most standard polyolefins and synthetic

polyesters [5, 8]. Since PHB is toxicologically safe, it can be used for articles which come into contact with skin, feed, or food [9].

In the food industry, PHA has a wide application as edible packaging material, coating agent, flavor delivery agent, and as dairy cream substitute [10, 11]. It can also be used for making bottles, cosmetics, containers, pens, golf tees, films, adhesives and nonwoven fabrics, toner, and developer compositions, ion-conducting polymers, and as latex for paper coating applications [12, 13]. It can be used to make laminates with other polymers such as polyvinyl alcohol. The degradation products of PHB are found in large concentrations in human-blood plasma, so is not toxic for human use [14].

PHA production by *Ralstonia eutropha (R. eutropha)* generally occurs during stationary phase. Hence cells are first grown to high density, after which a key nutrient is limited to trigger PHA synthesis [14]. Because of this dual phase process, PHA production lends itself to fed-batch, as well as, continuous operation. This follows Pontryagin's maximum principle, which is an optimal feeding strategy for fed-batch fermentation [15]. The key to this principle is determining the optimum switching time (t_c). The maximum growth rate ($\mu_{\max}$) should be initially maintained, then switched to the critical growth rate (μ_c) at t_c to maximize the specific product production rate ($\rho_{\max}$). Since growth rate is affected by Carbon : Nitrogen (C : N) ratio, it should also be changed at t_c.

A variety of carbon sources have been used for production of PHA using different fermentation strategies. Carbohydrates, oils, alcohols, fatty acids, and hydrocarbons are potential carbon sources for PHA production. Ethanol byproducts, cane and beet molasses, cheese whey, plant oils, hydrolysates of corn, cellulose, hemicellulose, palm oil, soybean oil, tallow, corn steep liquor, casamino acids, and food scraps had been used as substrates to produce PHA using different organisms [16–21].

The substrate evaluated in this study for the growth phase of *R. eutropha* was condensed corn solubles (CCS), which is a low-value byproduct of the dry-mill, corn ethanol industry. In the dry mill process, the whole corn is milled, mixed with water, and enzymatically hydrolyzed to convert starch to glucose, which is converted to ethanol by fermentation. After distillation to remove ethanol, the larger corn particles are recovered by centrifugation as distiller's wet grains. The supernatant is condensed in multiple-effect evaporators to give condensed corn solubles [22].

The composition of CCS is shown in Table 1. Corn-milling byproducts are typically marketed as animal feed because of their protein content. However, these byproducts may also contain residual carbohydrates, which might be utilized by microbial fermentation to produce industrial biopolymers. CCS is an excellent source of vitamins and minerals, including phosphorous and potassium.

The objective of this study was to determine the effects of adding artificial rumen fluid (ARF) on cell viability, growth as well as PHA production to a 48 h culture of *R. eutropha*. Different strategies of feeding simulated ARF into the bioreactors were assessed to maximize PHA production.

Table 1: CCS composition from a dry mill ethanol plant.

Components	CCS
Dry matter %	34.9
Crude protein %	13.7
Crude fat (ether extract) %	16.2
Ash %	9.5
Crude fiber %	0.8
Copper ppm	8.3
Sodium ppm	5,620
Calcium ppm	487
Magnesium ppm	6,850
Zinc ppm	49
Phosphorus ppm	15,400
Potassium ppm	22,900

Composition is on dry matter basis.

2. Materials and Methods

2.1. Culture, Maintenance, and Inoculum Propagation. The ATCC 17699 type strain of *R. eutropha* was used. The culture was routinely transferred to nutrient broth and incubated on a reciprocating shaker (250 rpm) at 30°C for 24 h. For short-term maintenance, the culture was stored on Tryptic Soy Agar (TSA) slants covered with mineral oil and stored in the refrigerator. Inoculum for all trials was prepared in a stepwise manner, by transferring the culture from TSA plates into 100 ml of the CCS medium (described below), then incubating for 24 h on a rotary shaker (250 rpm) at 30°C. The inoculum rate for all bioreactor trials was 1% ($v\,v^{-1}$) from a 24 h grown culture to an average OD of 1.04.

2.2. Medium. A low-cost medium based on CCS was developed in a prior study [23]. This medium, containing 240 g CCS L^{-1}, with a C : N ratio of 50 : 1 was the best medium for the growth of *R. eutropha*. The medium was prepared by mixing 1,370 mL CCS with 4,630 mL deionized water, adjusting the pH to 6.5 using 10 M sodium hydroxide (NaOH), then centrifuging at 11,000 rpm for 7 min at 15–25°C. The supernatant was then filtered through Whatman filter paper #113 and autoclaved. A filter sterilized 178 g L^{-1} ammonium bicarbonate (NH_4HCO_3) stock solution was prepared and then 20.4 mL of this solution was added to each liter of CCS medium to adjust the C : N ratio to 50 : 1. The pH was further adjusted to 7.0 by adding 10 N sulfuric acid (H_2SO_4) before inoculation.

2.3. Fermentation Conditions for Cultivation of R. eutropha in Bioreactor. Experimental trials were conducted in 5 L New Brunswick, Bioflo III, Edison, NJ bioreactor that contained 4 L of CCS medium. Filter sterilized air (1 L L^{-1} min^{-1}) was sparged into the bioreactor, and 2-3 mL of antifoam (Cognis Clerol FBA 5059, Cognis, Cincinnati, OH) were added to the medium before inoculation. Fermentation medium was incubated at 30°C and 500 rpm for 48 h, since prior research had shown *R. eutropha* to reach its

maximum population by this time/temperature combination [23].

At 48 h we began feeding the fermenter a mixed short-chain fatty acid (SCFA) solution, using any of the three different strategies. This SCFA solution, referred to as ARF, contained 10 parts acetic, 2 parts butyric, 15 parts lactic, and 20 parts propionic acids ($v\,v^{-1}$). The composition of ARF was based on a separate study evaluating fermentation of biomass with rumen consortia. A total volume of 372 mL of ARF was fed to the culture over a period of 48 h. In the first feeding strategy (24 h feeding), 124 mL of ARF was added at 48, 72, and 96 h. In the second feeding strategy (3 h feeding), 20 mL ARF was added at 48 h and then every 3 h until 109 h. In the third feeding strategy (continuous feeding), ARF was continuously added from 48 to 96 h at a rate of 7.75 mL h^{-1}. All incubations were continued until 144 h. Two replications were performed for each feeding strategy to determine the effect of mixed fatty acids on cell viability, acid utilization, and PHA production.

2.4. Analytical Methods

2.4.1. Viable Counts, Cell Dry Weights, pH, HPLC, and Ammonium/Phosphate Analysis. Samples were collected every 12 h and viable cell counts were done with TSA. At 72, 96, and 144 h, 50 mL samples were collected to determine cell dry weights. Samples were centrifuged and the precipitate was dried in the hot air oven at 80°C for 2 days. pH was measured using an Acumet 950 pH meter (Thermo Fisher Scientific of Waltham, MA). Samples were also analyzed via a Waters HPLC system (Milford, MA) for sugars, organic acids and glycerol. These samples were first filtered through a nonsterile 0.2 μm filter to remove solids, and then frozen until analysis. An Aminex HPX 87H column (Bio-Rad Laboratories, Hercules, CA), operated at 65°C with a helium-degassed, 4 mM H_2SO_4 mobile phase at a flow rate of 0.6 mL min^{-1} was used. Peaks were detected using a refractive index detector. Standard solutions of maltose, glucose, lactic acid, butyric acid, acetic acid, propionic acid, succinic acid, and glycerol (at 3 and 30 g L^{-1}) were used to calibrate the integrator. Samples collected at 0, 72, and 120 h were also tested for ammonia and phosphate using Hach Ammonium and Phosphate Unicell tests (Hach Company, Loveland, Colorado).

2.4.2. PHA Analysis. To measure PHA, 50 mL samples of broth were collected at 72, 96, and 144 h and centrifuged. The pellets were then lyophilized and ground using a mortar and pestle. The method developed by Braunegg et al. [24] was used to simultaneously extract and derivatize PHA to the 3-hydroxyalkanoate methyl esters of the monomers. In this method, 20–30 mg of ground cells were digested by adding 5 mL of digest solution and incubating at 90–100°C for 4 h. The digest solution contained 50% chloroform, 42.5% methanol, 7.5% H_2SO_4 ($v\,v^{-1}$). After cooling, sample was washed with 2 mL of water, and the bottom layer (containing the chloroform and methyl esters of PHA) was collected and placed in a Gas Chromatography (GC) vial with anhydrous sodium sulfate (to remove residual water). Vials were frozen until analysis.

PHA was quantified using a Hewlett-Packard 5890 Series II (Palo Alto, CA) GC with a flame ionization detector (GC-FID) [24, 25]. Split injection was used onto a Supelco SSP-2380 (Park Bellefonte, PA) capillary column (30 m × 0.25 mm I.D. with 0.20 um film). The inlet head pressure was maintained at 28 psi, and the temperature program started at 50°C for 4 min, then increased by 3°C min^{-1} to a final temperature of 146°C for 4 min. The injector and detector temperatures were 230°C and 240°C, respectively. Purified poly (3-hydroxybutyric acid co-3-hydroxyvaleric acid) (P(HB-HV)) obtained from Sigma-Aldrich was used for a standard calibration. The copolymer consisted of 88% 3-hydroxybutyric acid (3-HB) and 12% 3-hydroxyvaleric acid (3-HV). Copolymer concentrations of 2–10 mg mL^{-1} were digested as above, and then analyzed by GC-FID. Retention times were 14.9 min for methylated 3-HB, and 17.8 min for methylated 3-HV.

2.5. Statistical Analysis. All trials were performed in duplicates. Various fermentation parameters (maximum cell populations, SCFA utilization rates, PHA productivity, etc.) were analyzed to determine least significant differences between treatments using randomized complete block design. Fermentation efficiency (FE) was calculated as the percentage of substrate supplied to that was consumed. PHA productivity was calculated as the concentration of PHA produced per hour whereas PHA content was determined as a percentage of PHA concentration over cell dry weight. Data were analyzed using the PROC GLM procedure of SAS software to determine F values and least squares (LS) means. Exponential regression equations were used to determine growth rates and acid utilization rates for each replication, from which means were calculated. They were statistically analyzed by ANCOVA to test homogeneity of slopes. Statistical data were analyzed at the significant level of $P < 0.05$.

3. Results

In all bioreactor trials, the organism was incubated under similar conditions (30°C, 500 rpm, and aeration at 1 L L^{-1} min^{-1}) for the first 48 h, and this data was relatively uniform. Figure 1 and Table 2 show the average cell population, growth rate, acid utilization, and ammonia and phosphate uptake rates during this growth period.

Compared to shake flasks trials (Table 3), the maximum cell population in bioreactor trials was almost 10-fold higher (2.3×10^{10} cfu mL^{-1}). Likewise, the growth rate of *R. eutropha* was also higher in the bioreactor (0.20 h^{-1}) compared to the aerated shake flasks (0.13 h^{-1}). In the shake flask trials, lactic acid was consumed at the fastest rate followed by acetic, butyric, succinic, and propionic acids. In the bioreactors, the organic acid utilization rates in the growth phase were generally similar to shake flask trials except for lactic acid. It was consumed slower than acetic acid. Overall acid utilization rates were higher in the bioreactors than in shake flasks probably due to the higher cell population. Percentage

Table 2: Average growth and nutrient utilization rates of *R. eutropha* in CCS medium through 48 h in biorector.

Maximum cell population (cfu mL^{-1})		Maximum growth rate (h^{-1})	Ammonia utilization rate (mg L^{-1} h^{-1})	Phosphate utilization rate (mg L^{-1} h^{-1})
2.3×10^{10}		0.20	2.3	0.023
SCFA utilization rate (g L^{-1} h^{-1})				
Acetic	Butyric	Lactic	Propionic	Succinic
0.051	0.026	0.046	0.023	0.027
SCFA FE (%)				
Acetic	Butyric	Lactic	Propionic	Succinic
100	100	81.25	65.5	71.2

Table 3: Average growth and nutrient utilization rates of *R. eutropha* in CCS medium through 48 h in shake flasks.

Maximum cell population (cfu mL^{-1})		Maximum growth rate (h^{-1})	Ammonia utilization rate (mg L^{-1} h^{-1})	Phosphate utilization rate (mg L^{-1} h^{-1})
2.6×10^{9}		0.13	1.7	0.022
SCFA utilization rate (g L^{-1} h^{-1})				
Acetic	Butyric	Lactic	Propionic	Succinic
0.033	0.026	0.054	0.010	0.021
SCFA FE (%)				
Acetic	Butyric	Lactic	Propionic	Succinic
77.6	76.2	94.2	35.6	62.7

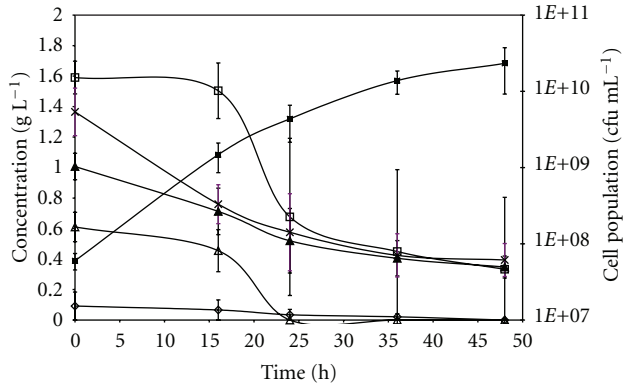

Figure 1: Average growth rate and organic acid utilization during the initial 48 h incubation in the CCS medium. The values for average cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□) propionic acid utilization, (▲) and succinic acid utilization (×) are indicated. Values are the means of two replications with standard deviation showed by error bars.

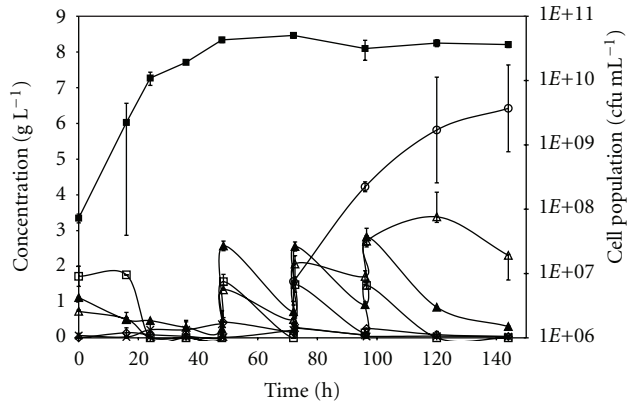

Figure 2: Acid utilization and growth of *Ralstonia eutropha* with 24 h interval additions of ARF. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation showed by error bars.

acid consumptions were also higher in the bioreactor with the exception of lactic acid. Ammonia and phosphate usage rates were higher in bioreactor trials again due to the higher cell population.

Three ARF-feeding strategies were compared in this study. Figures 2, 3, and 4 show that ARF feeding resulted in a slight increase in cell populations. The effects of different feeding strategies on maximum cell population, and ammonia and phosphate utilization rates are shown in Table 4. The maximum cell populations were not significantly different between the three treatments. In all cases, the cell population continued to rise after ARF additions began, suggesting that the acid levels were not inhibitory. The rates of ammonia utilization and phosphate utilization were similar for all feeding strategies.

As expected, the 24 h addition method (Figure 2) resulted in the highest spikes in SCFA concentration, with acid levels then falling as *R. eutropha* metabolized the acids to

Table 4: Comparison of all the key parameters under different ARF feeding strategies.

Key parameters	Feeding strategies		
	24 h addition	3 h addition	Continuous addition
Cell population at 48 h (CFU^{-1} mL)	4.28×10^{10a}	1.70×10^{10b}	1.01×10^{10b}
Maximum cell population (CFU^{-1} mL)	5.03×10^{10a}	5.68×10^{10a}	5.47×10^{10a}
Ammonia utilization rate (g^{-1} L^{-1} h)	1.2^a	1.0^a	1.1^a
Phosphate utilization rate (g^{-1} L^{-1} h)	0.010^a	0.013^a	0.011^a
Acid utilization (g^{-1} L^{-1} h)			
Acetic	0.029^a	0.052^b	0.047^b
Butyric	0.009^a	0.010^a	0.014^a
Lactic	0.074^a	0.080^a	0.078^a
Propionic	0.077^a	0.083^a	0.080^a
Combined	0.20^a	0.25^b	0.24^b
Fermentation efficiency (%)			
Acetic	58.8^a	100^b	98.7^b
Butyric	94.7^a	100^b	100^b
Lactic	100^a	100^a	100^a
Propionic	95.7^a	100^b	95^a
Combined	82.7^a	100^b	97.8^b
PHA production			
Cell dry weight (g^{-1} L)	17.6^a	21.13^a	17.3^a
PHA concentration (g^{-1} L)	6.42^a	8.37^a	6.67^a
PHA productivity (g^{-1} L^{-1} h)	0.0537^a	0.0697^b	0.056^a
PHA content (%)	36.23^a	39.52^a	37.78^a

[a,b,c] Means within column not sharing common superscript differ significantly ($P < 0.05$).

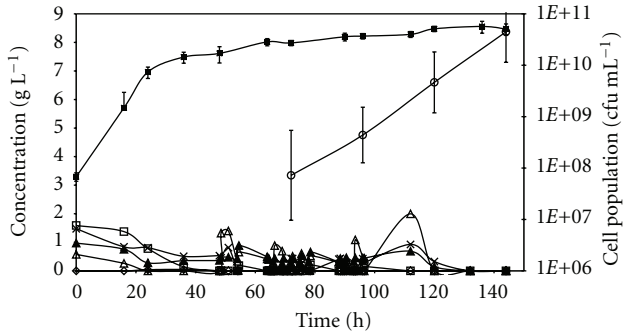

Figure 3: Acid utilization and growth of *Ralstonia eutropha* with 3 h interval additions of ARF. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation showed by error bars.

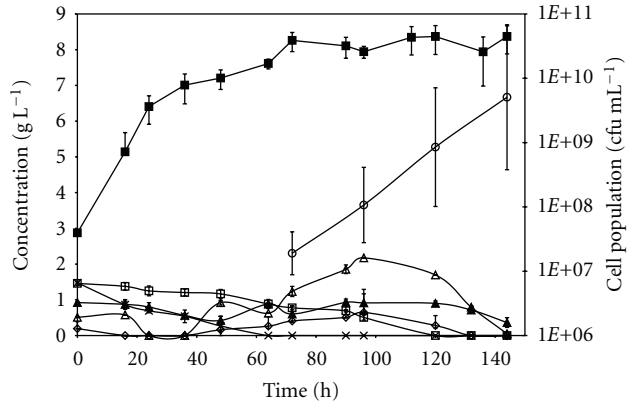

Figure 4: Acid utilization and growth of *Ralstonia eutropha* with continuous ARF addition. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation shown by error bars.

PHA. There was no apparent accumulation of lactic, butyric, or succinic acids for the 24 h feeding strategy. However, acetic acid (2.3 g L^{-1}), and to a lesser extent propionic acid (0.5 g L^{-1}), had accumulated over time. All the SCFAs were completely utilized by 144 h for the 3h feeding strategy. For the continuous-feeding strategy, all the SCFAs were consumed by 144 h with the exception of propionic acid (0.5 g L^{-1}).

Utilization rates of the individual SCFAs during the final 96 h of fermentation (48 to 144 h), along with the combined acid utilization rates, are shown in Table 4. The combined utilization rate also included consumption of succinic acid that was already present in the CCS medium. The highest combined acid utilization rates were observed in the 3 h and

Table 5: Comparison of combined short fatty acid feeding of *Ralstonia eutropha* in shake flasks.

Parameters	Volatile fatty acid			
	Acetic (5 g L^{-1})	Butyric (5 g L^{-1})	Lactic (8 g L^{-1})	Propionic (5 g L^{-1})
Maximum cell population (cfu mL^{-1})	5.70×10^{9a}	6.17×10^{9ab}	5.32×10^{9a}	6.67×10^{9b}
Fermentation efficiency (%)	70.6^{a}	95.6^{b}	70.7^{a}	68.6^{a}
Acid utilization rate (g L^{-1} h^{-1})	0.048^{a}	0.041^{a}	0.080^{b}	0.046^{a}
PHA concentration (g L^{-1})	2.9^{ab}	4.6^{a}	2.4^{b}	4.3^{a}
Cell dry weight (g L^{-1})	9.9^{ab}	14.5^{b}	6.0^{a}	14.7^{b}
PHA productivity (g L^{-1} h^{-1})	0.024^{ab}	0.037^{a}	0.020^{b}	0.036^{a}
PHA content (%)	29.2^{a}	31.9^{a}	30.7^{a}	29.3^{a}

[a,b] Means within column not sharing common superscript differ significantly ($P < 0.05$).

continuous feeding strategies, with the lowest rate in the 24 h feeding method.

The trend of higher acid utilization rates with the 3 h and continuous feeding methods also evident for the individual acids. However, the difference was only significant for acetic acid. Propionic and lactic acids were used most rapidly followed by acetic and butyric acid.

Table 4 also shows the FEs of the four SCFAs, along with the combined FE. The 3 h and continuous-feeding strategies resulted in the highest combined FE, while the lowest occurred with the 24 h feeding method. This is consistent with the lower acid utilization rates observed with the 24 h feeding strategy.

In comparing the individual acids, lactic acid was consumed completely, with greater than 95% utilization of propionic and butyric acids. FEs were higher for all the acids in the bioreactor trials compared to the prior shake-flask trials (Table 5).

Cell dry weights and PHA production parameters for the different ARF-feeding strategies are provided in Table 4. The 3 h feeding strategy resulted in the highest cell dry weight, PHA concentration, and PHA content, but the values were not significantly different from the other feeding strategies. Only the PHA productivity for the 3 h feeding strategy was significantly higher. Cell dry weight and PHA production were much higher than that obtained during the shake-flask trials. The highest PHA concentration in the cells in the shake-flask trials was 4.6 g L^{-1} (Table 5), whereas in the fermenter trials, the PHA concentration of the cells was about 1.82 times higher [23].

4. Discussion

Typically, PHA production occurs during stationary phase. Hence cells are first grown through exponential phase in a balanced medium to maximize growth rate. This medium is formulated to run out of a key nutrient when the maximum cell population is achieved, then additional carbon is added by fed-batch or continuous mode to maximize PHA production [26].

In this study, nitrogen deficiency was used to trigger PHA production in the presence of excess carbon. Nitrogen-supplemented CCS medium resulted in better growth of *R. eutropha* in the bioreactors as compared to the shake flasks due to the improved aeration and agitation provided in the bioreactor, along with more consistent pH control. The organism continued to grow after the SCFAs were added at 48 h. This growth was supported by the residual ammonia and phosphorus present in the medium. The organism reached its stationary phase by 96 h when most of the ammonia and phosphorous present in the medium were consumed. Ideally, one or both of these nutrients becomes limiting at the end of exponential phase, to trigger the shift from reproductive metabolism to PHA synthesis [26]. Because nitrogen and phosphate were not depleted until 72 h, this could have contributed to the continued increase in cell numbers observed after 48 h. It is likely that at least some of the SCFAs fed at 48 h were utilized for growth, until the point at which nitrogen became limiting. Researchers have found that the complete lack of nitrogen may suppress enzyme activity in PHA synthesis [27]. Thus, a small amount of ammonia in the media might be necessary to trigger PHA synthesis.

The utilization rate of lactic acid in the initial 48 h was lower than that of acetic acid probably due to higher concentration of lactic acid (1.6 g L^{-1}) in the bioreactor media compared to the shake-flask media (1 g L^{-1}). This variation was due to the different batches of CCS obtained from the ethanol plant.

In this study, fed-batch (24 h feeding) and continuous-feeding strategies were chosen to determine their effect on PHA production. The lowest acid utilization rates and FEs were observed for the 24 h feeding strategy due to the periodic spikes in SCFA concentrations, that might have disrupted the acid utilization and decreased cell activity. At high SCFA concentration the pH of the medium can reach below the pKas for SCFAs (lactic 3.86, acetic 4.76, butyric 4.83, and propionic 4.87). At low pHs the undissociated form predominates, and the SCFAs readily cross the cell membrane. Once inside, they rapidly dissociate and acidify the cytoplasm [28]. As a result, the proton gradient cannot be maintained as desired, and energy generation and transport systems dependent on proton gradient are disrupted [29]. This can also cause an increase in osmotic pressure due to the accumulation of anions [30]. At pH levels closer to the optimum for *R eutropha* (~7.0), SCFAs would be in the dissociated form in the medium. While the anions wouldn't

be transported as readily, once inside the cell they would not cause the adverse effects of the undissociated form. Therefore, SCFAs can only be effective carbon sources when pH and SCFA concentrations are carefully regulated. Acid utilization rates might have also been reduced by depletion of certain acids at the end of each 24 h phase.

For the continuous strategy, small volumes of the SCFAs were fed continuously. Though FEs of the SCFAs were higher than that of the 24 h feeding strategy, there were slight accumulations in SCFAs towards the end of the fermentation.

It was thus necessary to develop an optimal feeding strategy which could potentially increase acid utilizations and FEs. The 3 h feeding strategy was thus chosen. Since the SCFAs were added in smaller doses at shorter intervals, pH of the medium was more efficiently regulated, which resulted in better utilization and 100% FE of the organic acids.

In comparison to the prior aerated shake-flasks trials (Table 5), which were fed with individual SCFAs, the addition of mixed acids to the bioreactor resulted in more rapid uptake of propionic acid and slower utilization of butyric acid. Propionate utilization rate rose from $0.046\,g\,L^{-1}\,h^{-1}$ in aerated shake flasks [23] to approximately $0.08\,g\,L^{-1}\,h^{-1}$ when added as a part of the ARF mixture in the bioreactor trials. This was likely due to the higher cell populations achieved in the bioreactor trials coupled with the fact that proprionate utilization by *R. eutropha* is energetically favorable [31]. Moreover, addition of propionic acid in small doses might have controlled the change in pH, and thus resulted in better utilization. The decline in the utilization rate of butyric acid, from $0.041\,g\,L^{-1}\,h^{-1}$ when fed individually in aerated shake flasks to $0.011\,g\,L^{-1}\,h^{-1}$ in bioreactor trials, may have been due to additional ATP needed to transport this acid [32, 33]. Thus utilization of other acids was preferred over butyric acid when fed as a mixture for growth.

The high FEs for lactic and propionic acids were consistent with the metabolic preference of *R. eutropha*, especially considering that the ARF contained 15 and $20\,g\,L^{-1}$ of these acids, respectively. The high FE of butyric acid may be due to the fact that ARF contained only $2\,g\,L^{-1}$ of this acid. While conducting the shake-flask trials, we had previously noted that *R. eutropha* did not prefer acetic acid, therefore its lower FE was not surprising.

Thus, it can be concluded that in the 24 h strategy the sudden rise in the SCFA concentrations must have lowered the pH by accumulation of acid. This might have caused decrease in acid consumption, FE, and PHA productivity. Though the dosages of acids were small for the continuous feeding, continuous addition might have lowered the efficiency of the process. The smaller dosage and the fed-batch mode of the 3 h feeding strategy might have resulted in better control of pH and of catabolite repression. So the optimized growth conditions (as discussed before) for the organism and 3h feeding strategy of ARF addition was considered the best to obtain optimum PHA production by the organism.

Yu et al. suggested [31] in their study that an average yield of PHA was $0.39\,g\,g^{-1}$ of carbon sources (acetic, butyric, and propionic acids). In the current study the highest yield of about $0.2\,g\,g^{-1}$ was observed when the 3 h feeding strategy was used. Investigators have reported that inexpensive carbon sources lead to low PHA productivity due to inefficient utilization of nutrients by organisms [34–36]. A majority of carbon sources was probably utilized to generate energy for cell maintenance.

Wild-type strain of *R. eutropha* used in this study was not able to use glucose and did not completely utilize the high amount of glycerol present in the CCS medium. Recombinant microorganisms can be used to increase growth rate and nutrient utilization. PHA biosynthesis genes can be inserted in organisms that have wider range of utilizable substrates to increase PHA productivity and PHA content. For example, the recombinant strains of *R. eutropha* (H16) containing glucose-utilizing genes of *Escherichia coli (E. coli)*, and *E. coli* harboring the *R. eutropha* genes had higher PHA productivity and concentration as compared to the wild-type strain [14, 34].

5. Conclusion

Since the carbon source is a major contributor to PHA cost, inexpensive sources of carbon are important. From an economical point of view, the use of purified media to increase PHA yield will significantly increase the production cost. This study showed that *R. eutropha* is capable of growing in CCS medium, a low-value byproduct of ethanol industry. We also concluded that mixture of SCFAs in the same compositional ratios of ARF was not inhibitory when added at stationary phase of growth. SCFAs can be diverted toward PHA production by *R. eutropha*. As the use of purified SCFAs is cost prohibitive, developing a mixed culture system to produce a mixture of SCFAs from another ethanol industry byproduct is highly cost-effective. Thus utilization of inexpensive carbon sources may lead to economically viable PHA production in future when superior genetics and fermentation strategies are applied together.

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