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Full Length Research Paper

Utilization of kitchen waste for the production of green thermoplastic polyhydroxybutyrate (PHB) by *Cupriavidus necator* CCGUG 52238

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Polyhydroxybutyrate (PHB) was produced by *Cupriavidus necator* CCGUG 52238 using organic acids from fermented kitchen waste. HPLC and nuclear magnetic resonance (NMR) analyses revealed that the acid comprised mainly of lactic and acetic acids. In shake flask culture, the lactic acid concentration above 10 g/L inhibited both cell growth and polyhydroxybutyrate (PHB) production. The PHB production by the strain was achieved at the highest PHB content of 52.79% in batch fermentation using the kitchen-waste derived organic acids. The PHB yield and productivity were 0.38 g/g and 0.065 g/L/h, respectively. In fed-batch culture, about 4-fold increase in PHB productivity (0.242 g/L/h) was achieved by applying intermittent feeding strategy.

Key words: *Cupriavidus necator* CCGUG 52238, kitchen waste, organic acids, polyhydroxybutyrate (PHB).

INTRODUCTION

The growing interest in the development of biodegradable plastics with properties similar to synthetic thermoplastics for the management of the existing plastic waste has directed the attention towards bacterial polyhydroxyalkanoates (PHA). The production of PHAs from agriculture, food processing waste materials is an attractive approach for not only effectively decreasing PHA production cost but also constructing a process for effective utilization of waste. Considering that PHA content and productivity are usually lower for bacteria grown in crude and inexpensive substrates, the development of efficient processes for the successful bioconversion remain a challenge to be pursued (Castilho et al., 2009).

In Peninsular Malaysia, generation of solid waste has increased from 17.000 tonnes per day in 2003 to 19,100 tonnes in 2005 with an average of 0.8 kilogram per capita per day. As of 2008, 23,000 tonnes of waste is produced each day in Malaysia, the generation in Kuala Lumpur alone accounted to 3,000 tonnes per day. This will continue to increase in coming years and is expected to reach 30,000 tons per day in 2020 (GEC). The waste usually consists of 45% food waste, 24% plastic, 7% paper and 6% iron. In the current practice of waste management, approximately 95 to 97% of waste collected is taken to landfill for disposals and less than 5% of the waste is being recycled. However, considering the landfill shortage and contamination, government aimed to have 22% of the waste recycled by 2020. While composting the kitchen refuse can recycle back the nutrients and energy to soil and reduce almost 50% of the waste; anaerobic digestion can also be applied to convert the food waste into organic acids which are preferable substrate for PHA production. Producing PHAs from kitchen waste can further reduce the inevitable usage of conventional plastics when biodegradable PHA as packaging material would take place in the market.

Owing to higher moisture content, kitchen waste is more suitable to undergo anaerobic digestion than thermo-chemical conversion. Significant research has been focused on using organic acids from fermented food wastes as raw materials for PHA production (Hafuka et

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Table 1. Characteristics of the kitchen waste used for PHB production.

Characteristics	Value
pH	4.0 – 5.5
C/N	30
Total Kjeldahl nitrogen (%)	1.2
Ammonium Nitrogen (mg/L)	286
Total suspended solids (g/L)	70.12

al., 2011; Du and Yu, 2002; Du et al., 2004). However, food waste in developing countries is usually denser than in developed areas, and its composition is an indicator of the socio-economic situation of the population (Holmes, 1984). Nevertheless, the composition of organic acids in the fermented kitchen waste plays a major role in the overall performance of the PHA fermentation.

Considering the potential of using fermented kitchen waste as feedstocks for PHB production, this study was carried out to produce PHB in batch and fed batch fermentation by *Cupriavidus necator* CCGUG 52238, using mixture of lactic and acetic acids obtained from the kitchen waste.

MATERIALS AND METHODS

Bacterial strain and cultural conditions

C. necator CCGUG 52238, obtained from Culture Collection, University of Goteberg, Sweden was used throughout in this study. The wild type strain was grown on nutrient agar plate at 30°C for 24 h. The cells were then inoculated into a growth medium containing (in g/L) 8.0 nutrient broth, 5.0 yeast extract, 5.0 peptone, and 2.5 sodium lactate with pH 6.8 to 7.0. The cells were cultivated under rotational agitation at 150 rpm using incubator shaker (Lab Line, Environment Shaker) for 24 h at 30°C in 500 mL Erlenmeyer flasks with 200 mL of the working volume. The cells were harvested by centrifuging it aseptically at 4000 rpm for 15 min and the supernatant was discarded. The pellet was then re-suspended in distilled water and was transferred into a 2 L bioreactor containing PHB production medium. The production medium used for PHB production contained (in g/L): 2 K_2HPO_4, 6.7 KH_2PO_4, 1.0 $(NH_4)_2SO_4$, 0.4 $MgSO_4$. $7H_2O$, 0.1 $CaCl_2$, 0.04 $FeSO_4$, 2.0 Lactic acid and 1.0 mL of trace elements (Hassan et al., 1997a). The pH of the production medium was controlled at 6.8 to 7.0 using 2M H_2SO_4 and 2M NaOH.

Substrate for PHB fermentation

Kitchen waste was collected from several food courts in UPM, Serdang, Malaysia. Acidogenic fermentation of kitchen waste was carried out to produce organic acids until 8th day at 37°C with initial pH adjusted to 7.0 as reported by Omar et al. (2009). The separation of the solids from the fermentation broth was achieved by spinning the slurry at 10,000 rpm in 50 mL centrifuge tube for 30 min. The pellets were discarded and organic acids in the supernatant were concentrated to ten-folds using rotary vacuum evaporator (Eyela) at 70°C. The final concentration of organic acids after evaporation process ranged from 200 to 280 g/L. The samples were then kept at -20°C prior to use as carbon substrate for PHB production.

PHB biosynthesis by *C. necator* CCGUG 52238

Shake-flask experiments

In order to examine the potential of the organic acids from kitchen waste, a series of batch experiments were conducted to examine the effect of 5, 10, 15 and 20 g/L of organic acid mixtures on the PHA production. The flasks containing mineral media were inoculated with 24 h old culture of *C. necator* CCGUG 52238 and were incubated at 30°C, 200 rpm for 24 h. The cells were harvested and examined for biomass and PHB concentration. Parallel set of experiments were conducted using technical grade organic acids in the range of 5 to 20 g/L for comparison.

PHB fermentation in bioreactors

Batch and fed batch fermentations were performed in a 2 L bioreactor (Biostat ® MD B.Braun Biotechnology International) with the working volume of 1 L. Agitation was achieved by six-blade rushton type impeller and pH was maintained at 7.0 by using 2 N NaOH and 2 N H_2SO_4. The dissolved oxygen level (DO) was maintained above 30% air saturation by sequential cascade control of fixed air flow and variable rotation speed. Silicone based antifoam (10% v/v) was used to control foaming and temperature was maintained at 30°C.

Fed-batch studies were carried out in 7 L bioreactor (Bioengineering AG, Switzerland) with 5 L of working volume in order to improve the PHB production and increase the productivity from batch studies. The initial batch fermentation contained 10 g/L of organic acids from kitchen waste and after 10 h of fermentation; concentrated acid solution (200 to 280 g/L) was added intermittently to keep the residual acid concentration in the reactor below 3 g/L (Figure 4). The organic acids were sterilized and used without any nutrient supplementation. Samples were withdrawn every 5 h to examine biomass, residual organic acid and PHB concentration. The fermentation was terminated at 40 h.

Analytical procedures

Organic acid in the broth and the PHB content in the cell mass was measured by using HPLC (SPD-10A, UV-Vis Detector; LC-10 AS Liquid Chromatograph Shimadzu) with HPLC Acid Analysis Column, 300 × 7.8 mm (Aminex HPX-87H Ion Exclusion Column, Bio-Rad Laboratories) as described previously (Hassan et al., 1997b). Cell dry weight was measured gravimetrically. The organic acid composition in the kitchen waste organic acid was further verified using nuclear magnetic resonance (NMR) analysis. The 400 MHz ^1H NMR spectra recorded at 27°C on a $CDCl_3$ solution. Peak areas were determined by spectrometer integration by using tetramethylsilane (TMS) as an internal reference.

RESULTS AND DISCUSSION

Characteristics of substrate for PHB fermentation

The characteristics of the kitchen waste used in this study are shown in Table 1. The kitchen waste had an acidic pH range of 4.5 to 5.5. The range is similar to the

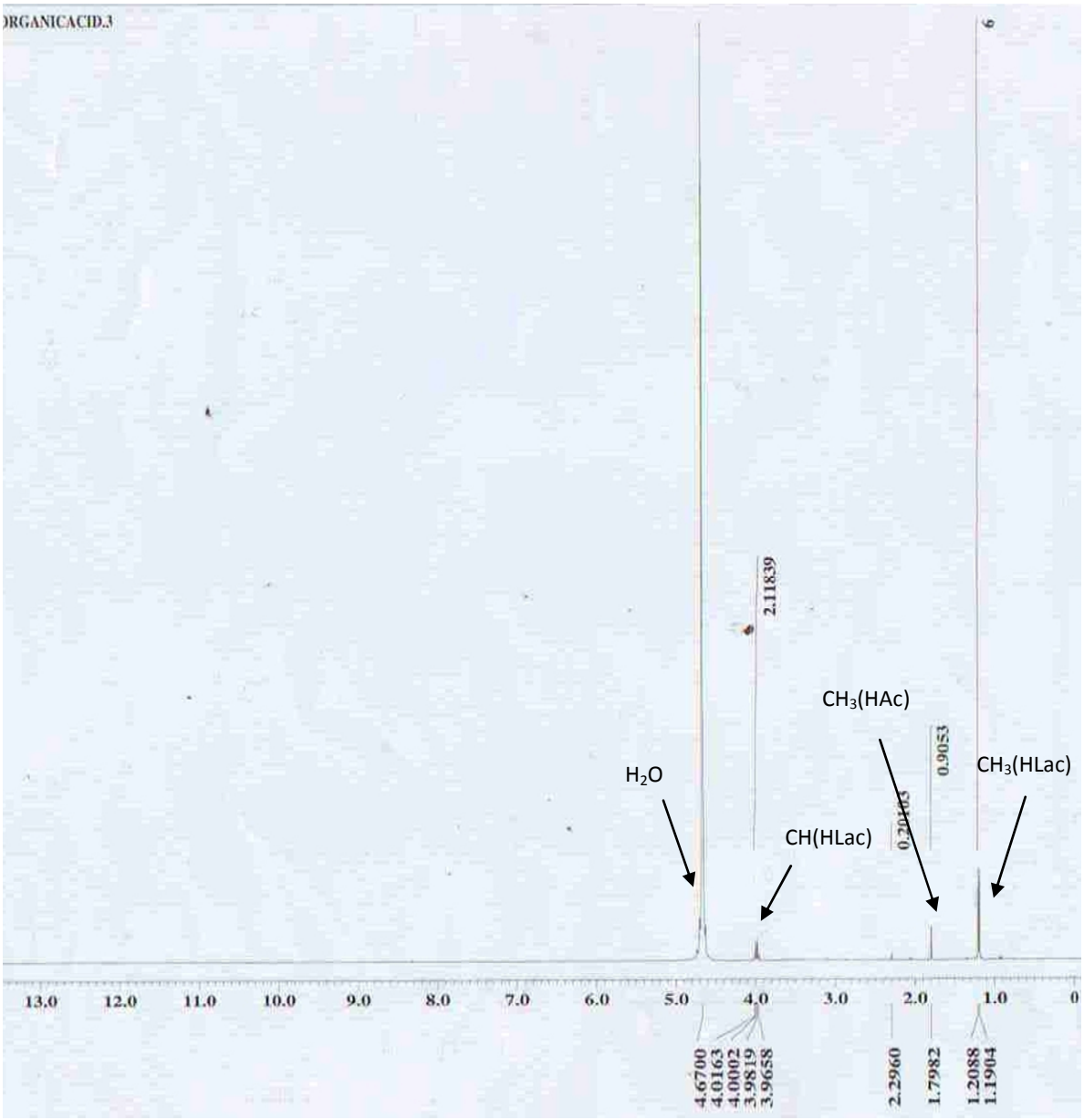

Figure 1. The 400 MHz ^{1}H NMR spectra of organic acids produced from fermented kitchen waste used as PHB substrate in this study. HLac- Lactic acid, HAc- Acetic acid.

reported value of 4.6 and 5.1 by Kim et al. (2004) and Stabnikova et al. (2008), respectively. This is due to the action of indigenous microbes in the fermented kitchen wastes that break down the complex organic materials into organic acids and contributed to the acidity of the solution. The concentrated organic acid was brown in colour and was consisted of 210 g/L lactic acid and 13.9 g/L acetic acid. As shown in Table 1, the organic acids contained 1.2% of total Kjeldahl nitrogen (TKN) and about 286 mg/L of ammonical nitrogen. This value is about three times lower than that reported by Zhang et al. (2007) where total N was 3.16% and ammonical nitrogen content was 973 mg/L. Comparatively lower fraction (20%) of protein-rich materials in the original food waste as reported by Hafid et al. (2010) may be responsible for the lower amount of ammonium nitrogen in our sample. The C/N ratio in our sample was found to be 30 whereas studies carried out by Malakahmad et al. (2008) showed slightly higher C/N ratio (38.2). However, in both cases, the higher C/N ratio reflects the lower nitrogen content in the kitchen waste which makes this substrate suitable for PHA production.

Figure 1 shows the NMR spectra of concentrated organic acids from kitchen waste. The peak assignments were performed on the basis of chemical shift comparisons with data in the literature (Figueiredo et al.,

Table 2. Comparison of shake flasks fermentation for PHB production utilizing organic acids from kitchen wastes and technical grade organic acids at different initial concentrations.

	Technical grade organic acids (g/L)				Kitchen waste organic acids (g/L)			
Initial concentrations	5	10	15	20	5	10	15	20
DCW (g/L)	2.2	3.1	1.5	0.7	2.6	2.9	1.2	0.79
PHB (g/L)	0.682	1.364	0.18	0.07	0.91	1.218	0.12	0.09
PHB (%)	31	44	12	10	35	42	10	11
Productivity(g/ L/h)	0.028	0.057	0.007	0.003	0.038	0.051	0.005	0.004

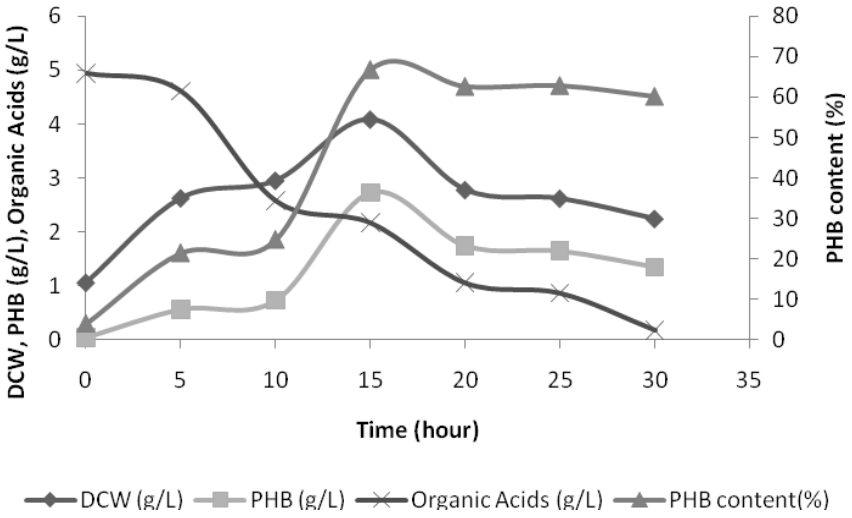

Figure 2. Time course of PHB production by *Cupriavidus necator* CCUG 52238 using 5 g/L technical grade organic acid in batch culture.

2006; Espartero et al., 1996). The most intense signals were assigned to CH (4.02 ppm) and CH_3 (1.21 ppm) of L-lactic acid, while peak assigned to CH_3 (1.79 ppm) was for acetic acid. Spectral analysis revealed that the predominant organic acid species in the fermented kitchen waste in this study were lactic acid and acetic acid (Figure 1).

PHB biosynthesis by *C. necator* CCGUG 52238

Shake-flask experiments

The biomass concentration, PHB concentration, PHB content and productivity using different concentration of organic acid in shake flask experiments are shown in Table 2. It can be seen that the concentration of cells decreased as the initial concentration of lactic acid in the medium increased from 10 g/L until 20 g/L. Similar trend was observed by Tanaka et al. (1993) in shake flask cultures of *Alcaligenes eutrophus* ATCC 17697 using mixture of lactic acid and acetic acid from xylose. More interestingly, the inhibitory effect of these acids to the cells of *C. necator* CCGUG 52238 were found to be similar regardless of their origin (either kitchen waste derived or technical grade) under identical culture conditions and acid concentrations. In both cases, concentration of 10 g/L of organic acids was found to be suitable for the batch production of PHB by *C. necator* CCGUG 52238 in the shake flask condition. The higher concentration (above 10 g/L) reduced the cell growth as well as the PHB production probably due to the inhibitory effect of lactic acid to the cells. Hence, it was important to keep the concentration of lactic acid at low level throughout the cultivation process to obtain optimum PHB production in batch culture. Since shake flask experiments could not provide the ideal condition of mixing and pH control, further studies were carried out in 2 L bioreactor.

Batch cultivation in bioreactor for PHB production

The time course of PHB accumulation in pH-controlled batch cultivation of *C. necator* CCUG 52238 using 5 g/L technical grade organic acid as carbon source is shown in Figure 2. A sharp decline in the concentration of organic acid was noticed in the first 10 h after which the

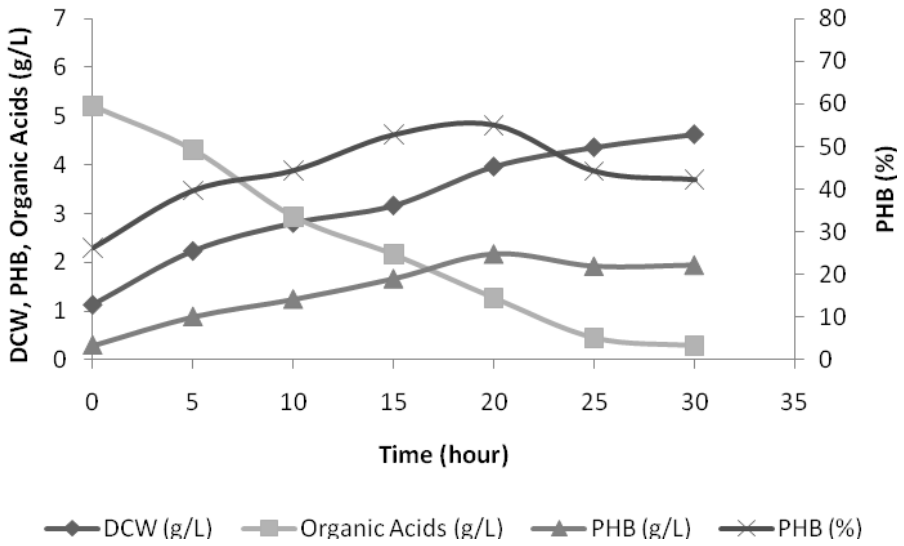

Figure 3. Time course of PHB production by *Cupriavidus necator* CCUG 52238 using 5 g/L organic acids from kitchen waste in batch culture.

substrate was gradually consumed and completely depleted within 30 h of incubation. The cells and PHB concentration started to increase gradually and reached maximum value at 15 h of cultivation. The final cell concentration reached up to 2.25 g/L at 30th h. The highest production of PHB obtained was about 2.73 g/L with 4.09 g/L of cell dry weight at 15 h of incubation corresponding to 0.47 g/g of PHB yield and 0.53 g/g of cell yield.

When organic acids from kitchen waste was used as sole carbon source, on the other hand, the biomass and PHB concentration reached 4.6 and 1.9 g/L, respectively at the end of fermentation (Figure 3). Cell and PHB yield were 0.65 and 0.38 g/g respectively. The PHB content was 60% with PHB productivity of 0.11 g/L/h.

It is interesting to note that in the fermentation with technical organic acids, maximum biomass was achieved at earlier stage, that is, 15 h of fermentation after which the cell dry weight as well PHB concentration were reduced probably due to the complete exhaustion of nitrogen source in the medium. In contrast, in the case of kitchen waste-derived organic acids, the cell mass increased gradually until 30 h at the end of fermentation. PHB also started to accumulate gradually and reached 55% by weight at 20 h after which the concentration remained almost same. The biomass continued to increase until end of the cultivation, whereas PHB biosynthesis slowed down. The residual acid in the broth was below 1 g/L at this moment and the depletion of carbon sources may result into re-utilization of PHB inside the cell.

Generally, technical grade organic acids (98% purity) give higher values for most parameters measured including PHB yield and productivity whereas the organic acids from kitchen waste may contain dissolved solids or other impurities. Zhang et al. (2008) stated that the non-sterile kitchen wastes fermentation system gave low purity due to the complex natural microbial composition. However, in this study, prior to fermentation, organic acids recovered from fermented kitchen waste was concentrated using rotary evaporator and was sterilized at 121℃ for 15 min in the autoclave. Compared to technical grade organic acids, batch studies using organic acid from kitchen waste showed superior results in *C. necator* probably due to the additional components in the acid that improve the fermentation performance. Moreover, the presence of nitrogen may act as a buffering agent in the culture media and provides optimal condition for PHB fermentation. The existence of characteristic peaks of acetic acid and lactic acid in NMR spectra further confirmed that only acetic acid and lactic acid were present in the organic acid from fermented kitchen waste (Figure 1).

Fed-batch cultivation in bioreactor for PHB production

In order to achieve higher cell density and higher productivity of PHB from that obtained in batch production, fed batch fermentation in 7 L bioreactor was carried out with 5 L of working volume using organic acids from kitchen wastes. A typical time course for growth and PHB accumulation of *C. necator* CCGUG 52238 in fermentation process is shown in Figure 4. The cell and PHB concentration at 25 h were 14.44 and 12.20 g/L, respectively, resulting in a PHB content of 84.54%. The yield of cells ($Y_{x/s}$) and PHB ($Y_{p/s}$) were 1.08 and 0.79 g/g, respectively. At the end of the fermentation, about

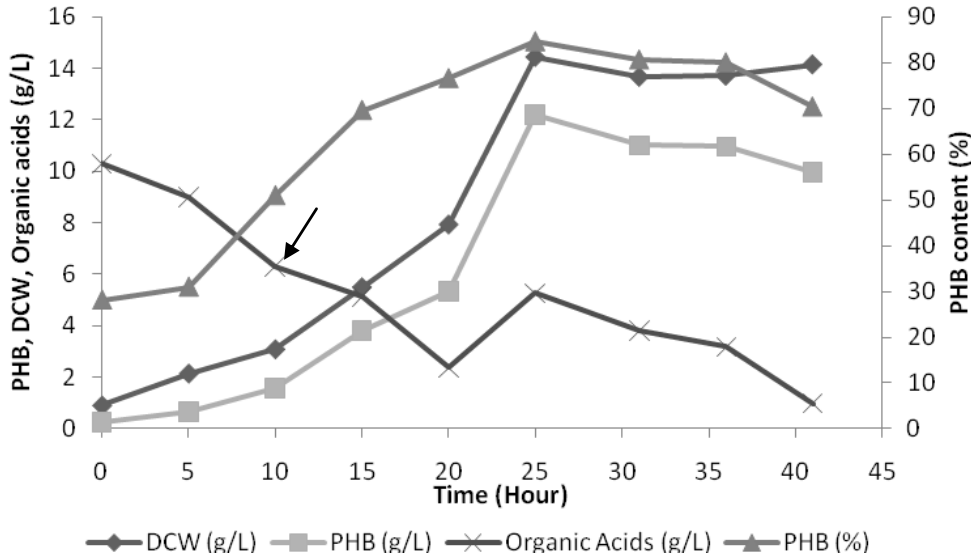

Figure 4. Time course of PHB production by *Cupriavidus necator* CCUG 52238 using organic acids from kitchen waste by fed-batch culture in 7L bioreactor (Arrow indicates the initiation of feeding).

0.98 g/L of organic acids remained in the fermentation broth. Substrate utilization rate and PHB production rate were 0.3 and 0.24 g/L/h, respectively. Du et al. (2004) also reported PHB productivity of 0.23g/L/h by *C. necator* using short chain fatty acids from fermented food scraps.

In this study, the carbon to nitrogen (C/N) ratio after 10 h was changed to 30 in order to promote PHB biosynthesis. Interestingly, the organic acid from kitchen wastes used in this study had C/N 30, so the requirement for additional nitrogen source was minimized. The DO was maintained at 30% giving a little pressure for the cells to accumulate the PHB intracellularly. It is well known that when *C. necator* is cultivated under oxidative growth conditions, the biopolymer synthesis can be enhanced (Jung and Lee, 2000).

In order to maintain the cell growth and also to keep the PHB accumulated in the cells, residual concentration of organic acids in the broth need to be kept at lower concentration. While, pH-stat feeding has been employed by most of the researchers to feed short chain fatty acids (Kobayashi et al., 2000; Sugimoto et al., 1999; Tsuge et al., 1999), for lactic acid and a mixture of lactic and acetic acids, feeding at different time intervals that is intermittent feeding strategy has been found to be more suitable (Tsuge et al., 2001; Linko et al., 1993; Tanaka et al., 1993). Very recently, Hafuka et al. (2011) reported high PHB content (~87%) by continuous feeding of filtered fermented food wastes in *C. necator*. However, the biomass and PHB production rate was quite low as compared to others. In this study, attempt to produce PHB by pH-stat feeding strategy was not successful. However, by applying intermittent feeding strategy, similar PHB productivity (0.24 g/L/h) and PHB content (~86%) as reported in the literature were obtained.

Conclusions

In this study, fed-batch fermentation resulted into 5-fold increase in the PHB production as compared to batch production. Accumulation of PHB as high as 84.54% w/w in the cells of *C. necator* CGUG 52238 was achieved by intermittent feeding of lactic and acetic acid mixture (~ 230 g/L, 15:1 ratio) obtained from fermented kitchen waste. This high accumulation is favourable for cost-effective recovery and can be compared with the glucose fermentation. The results also demonstrated that the organic acids from kitchen waste can be utilized as a suitable, cheap substrate for PHB production thereby increasing the possibility of recycling the major portion of food waste into biodegradable green plastics.

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