Optimize the measurement of Poly-hydroxy-alkanoates (PHA) in biomass

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Abstract: Poly-hydroxy-alkanoate (PHA) is an intracellular polymer that can be used as an energy and carbon source by microorganisms. Measuring PHA is important for understanding the microbial metabolism of enhanced biological phosphorus removal (EBPR) and aerobic granular sludge (AGS) systems. There is a commonly used method to measure PHA, which is based on organic solvent extraction and gas chromatography (GC). However, there are different versions of the same method with different parameters, but the role of some of these parameters is unclear. When different types of biomass are analyzed, there is a requirement to understand the parameters and obtain an optimal protocol. In this study, the effect of various digestion times, different alcohols and organic solvents, and acid concentrations were tested to obtain the optimal protocol. The results showed that a minimum digestion time was required to get the maximum yield of PHA, and the time might differ when using different types of biomass. Methanol was shown to be better for GC separation than propanol. Using different organic solvents didn't affect the final concentration, and an optimal acid concentration was required to determine by comparison. The GC temperature program optimization showed that lower oven temperature in GC is more beneficial for peak separation. From the analysis, it would be suggested to use methanol and chloroform for digestion and keep the digestion time for 24 hours.

Keywords: aerobic granular sludge, poly-hydroxy-alkanoates (PHAs), Gas Chromatography (GC), protocol optimization, GC temperature

Content

1 Introduction

Poly-hydroxy-alkanoate (PHA) is a category of intracellular polymers that can be used as energy and carbon source. Phosphate accumulating organisms (PAOs) play a major role in enhanced biological phosphorus removal (EBPR) processes in wastewater treatment, and they will synthesize and consume PHA in their metabolism (Mino et al., 1998). Therefore, the measurement of PHA is important to understand the metabolism of PAOs which affects the stability of EBPR process. The type of PHA produced is related to the substrate fed to the biomass. The common substrates for PAOs are acetate and propionate. PAOs mainly produce poly-hydroxy-butyrate (PHB) when feeding acetate, and mainly produce poly-hydroxy-valerate (PHV) and poly-hydroxy-2-methyl-valerate (PH2MV) when feeding propionate (Oehmen et al., 2005). If the substrate is more complex, for example, glycerol. The sludge will convert glycerol to propionate, then mainly synthesize PHV and PH2MV from propionate (Elahinik et al., 2022). Therefore, accurately quantifying different types of PHA is very important.

The first method to measure PHA was proposed by (Braunegg et al., 1978). The general principle of measuring PHA was to first break cell walls and release all the intracellular compounds. Then PHA polymers would be depolymerized into hydroxy-alkanoate (HA) monomers catalyzed by acid. After getting HA monomers, the alcohol added would form esters with the acid groups of HA at high temperature (100°C). The ester was dissolved in organic solvent along with other chemicals and would be analyzed later in gas chromatography (GC). This method was later modified by (Comeau et al., 1988) by adding distilled water as a reextraction step. The addition of water would cause two phases in the vials: organic phase (bottom) and aqueous phase (up). Acid and other hydrophilic chemicals will easily get into aqueous phase and the particulate debris will remain at the interface between aqueous and organic phase. To ensure less degradation of GC column, the organic phase needed to be dried with anhydrous sodium sulfate and passed through filter to get rid of acid and particulate matter. This modified method gave more reliable and reproducible results.

In the study of (Braunegg et al., 1978) and (Comeau et al., 1988), 3% H_2SO_4, methanol and chloroform were used in the digestion step, and the digestion time was 3.5 hours. However, in the study of (Smolders et al., 1994), 1-propanol, concentrated hydrochloric acid (4:l), and dichloroethane were used for digestion with the digestion time of 2 hours. From (Lageveen Roland et al., 1988), 15% H_2SO_4, methanol and chloroform were used with the digestion time of 2.5 hours. These protocols were the same principally, but the different parameters used made it confusing for researchers demanding an optimal PHA measurement protocol. Therefore, knowing the function of different parameters and how to obtain an optimal protocol becomes very important.

The PHA measurement protocol currently used in our lab was designed for measuring PHB in flocculant biomass (Riis & Mai, 1988). But as we are growing granular biomass and using different substrates, the protocol to get optimal measurement of PHA might be different. Therefore, we would like to test various digestion times, different alcohols and organic solvents, and acid concentrations to understand the PHA measurement and optimize the PHA protocol.

2 Methods

2.1 Chemicals and Biomass

The standards (Sigma-Aldrich, USA) used for PHA quantification: the HB standard is 3-hydroxybutyric acid (95%), the HV standard is methyl-(R)-3-hydroxyvalerate (98%), and the H2MV standard is 2-hydroxyhexanoic acid (98%). Internal standard was made by adding 1 mg of benzoic acid into 50 mL of 1-propanol. The biomass used was mainly from aerobic granular sludge fed with glycerol and NaCl (expected to produce PHV and PH2MV) and aerobic granular sludge fed with acetate and seawater (expected to produce PHB and PHV) in the lab.

2.2 Protocol used

PHA was measured through the following procedure (Table 2-1).

Table 2-1 PHA measurement protocol steps

Step	Description
1	Biomass samples were taken out of bioreactors.
2	A few drops of formaldehyde (37%) were added into the biomass samples and mixed.
3	The biomass samples were centrifuged, and the supernatant was removed.
4	Demi-water was added into the biomass samples for washing, then the samples were centrifuged and the supernatant was removed again
5	The biomass pellets were put into -80℃ freezer until completely frozen.
6	The frozen samples were put into the freeze dryer (operated at -57℃ and 0.050 mbar) until completely dry
7	The granular sludge samples needed to be smashed into powders in the porter for homogeneity and better digestion (either in dry or wet state)
8	About 30 mg of dry biomass was added into a glass tube and the exact weight was measured and recorded
9	50 μL of Internal Standard was added into the glass tube and the weight was recorded
10	1.5 mL of Propanol + HCl (4:1) was added into the glass tube
11	1.5 mL of Dichloroethane was added into the glass tube
12	Digestion: the tube was put in the heater (100℃) and mixed every 30-60 mins
13	The digestion duration varied depending on the type of PHA (PHB and PHV for 3 hours, PH2MV for 20 hours)
14	The tube was cooled to room temperature, then 3 mL of milli-Q water (ultrapure water) was added into the tube for extraction
15	The tube was mixed completely and centrifuged (5 mins, 2500 rpm) to get separate phases
16	1 mL of organic (bottom) phase was dried with about 0.5 g of anhydrous sodium sulfite and passed through a tip with filter to remove particles
17	The 1 mL organic phase was transferred into a GC vial and ready for GC analysis
18	Ran GC analysis with certain operation parameters (e.g., temperature)

The GC system (6890N, Agilent, USA) used was equipped with OPTIMA column (60 m length × 0.25 mm I.D. × 0.25 μm film). The system was operated with a split injection ratio of 1:30 and helium as the carrier gas (1.0 mL/min). The flame ionization detector (FID) unit was operated at 250℃ with an injection port temperature of 230℃. The oven temperature was programed to start with 120℃ for 4 mins, increased at 30℃/min to 180℃, maintained at 180℃ for 6 mins, then increased to 240℃ at 40℃/min and held for 16 mins.

2.3 Optimizing protocol

2.3.1 Digestion time effect

For biomass samples, kept all the steps in Table 2-1 as the same except step 13. The digestion time was set to be 3, 6, 10, 15, 20, and 25 hours. For PHA standard samples, PHA standard was used instead of dry biomass in step 8, and the same digestion time setting as biomass samples were used.

2.3.2 Alcohol effect

Kept all the steps in in Table 2-1 as the same except step 10. Methanol dissolved in sulfuric acid (3%) is used to replace Propanol + HCl (4:1), the volume of 1.5mL doesn't change. A new internal standard was made by adding 1 mg of benzoic acid into 50 mL of methanol to ensure methanol is the only alcohol used.

2.3.3 Organic solvent effect

The test was done after switching to using sulfuric acid and methanol (step 10). Both chloroform and dichloroethane (DCE) are used to test the organic solvent effect. The digestion time is controlled at 24 hours (step 13), both biomass samples and PHA standard are used (step 8), and the rest of the protocol stays the same.

2.3.4 Acid concentration effect

The test was done after switching to using sulfuric acid, methanol, and chloroform (step 10 and 11). Different acid concentration (1, 3, 6, 10, 15, 25% acid) is used to test the acid concentration effect (step 10). The digestion time is controlled at 20 hours (step 13), the rest of the protocol stays the same.

2.3.5 Oven temperature program

Kept all the steps in in Table 2-1 as the same except step 18. Different temperature programs were tested to understand the role of temperature value, holding time and slope and get better separation of PHA peaks.

Table 2-2 GC temperature programs that were used to test

Number	Temperature program
Program 1	Started with 80℃ for 4 mins, increased at 5℃/min to 120℃, maintained at 120℃ for 10 mins, then increased to 240℃ at 20℃/min and held for 10 mins
Program 2	Started with 80℃ for 4 mins, increased at 10℃/min to 110℃, maintained at 110℃ for 15 mins, then increased to 140℃ at 10℃/min and held for 10 mins, finally increased to 240℃ at 20℃/min and held for 4 mins
Program 3	Started with 130℃ for 14 mins, increased at 100℃/min to 180℃, maintained at 180℃ for 2 mins, then increased to 240℃ at 100℃/min and held for 5 mins
Program 4	Started with 130℃ for 14 mins, increased at 100℃/min to 180℃, maintained at 180℃ for 2 mins, then increased to 240℃ at 100℃/min and held for 5 mins

2.4 Calculation

Normalized PHA signal: From the chromatography, we could get different peaks for different chemicals, then the integrated peak area of PHA can be used to determine the amount of PHA. Moreover, the PHA area is dependent on the amount of biomass added, so the mass of biomass will be used for normalization. The mass and peak area of internal standard (IS) is known, and the ratio of them can be used to reduce error brought by volume. Therefore, normalized PHA signal can be calculated as Equation (1). The result reflects extracted PHA concentration regardless of the amount of biomass and internal standard added, which can be used to compare the effect of digestion time, organic solvent, and acid concentration.

$$\text{Normalized PHA signal} = \frac{\text{PHA peak area}}{\text{IS peak area}} \times \frac{\text{IS mass}}{\text{biomass or standard mass}} \quad (1)$$

PHA concentration in biomass samples: The calibration line was made with PHA standards (at least 4 samples were used), with the ratio of PHA mass added and IS mass as the x-axis and the ratio of PHA area and IS area as the y-axis. Similar to calculating normalized PHA signal, we can calculate the concentration in mg/mg by using the slope and intercept value obtained from the calibration line (Equation (2)).

$$\text{PHA concentration} = \left(\frac{\text{PHA peak area}}{\text{IS peak area}} - Intercept\right) \times \frac{1}{Slope} \times \frac{\text{IS mass}}{\text{biomass or standard mass}} \quad (2)$$

3 Results and Discussion

3.1 Digestion time effect

The change of PHA yield over digestion time using PHA standards is shown in Figure 3-1. From the results, the PHA standards didn't degrade with longer digestion time, and that the maximum yield was reached within 3 hours. The PHA amount at 25 hours was more different than others, which seemed to be

an outliner, but the overall difference was still small. The trend of PHA change can be explained by esterification which is complete and stable after certain amount of digestion time, so the yield wouldn't increase or decrease after longer time.

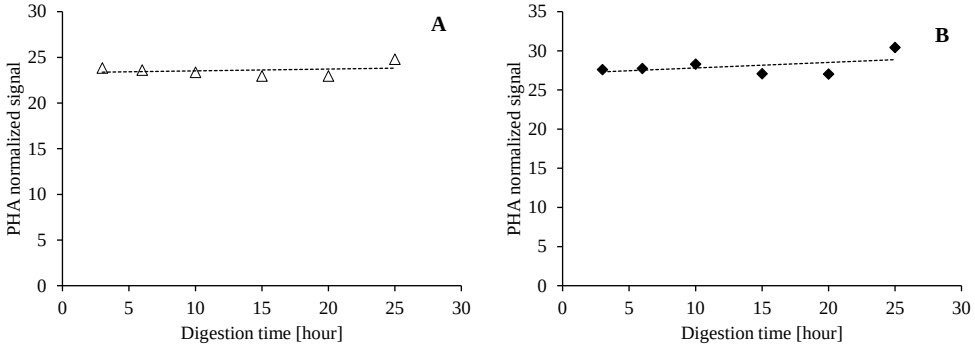


Figure 3-1 PHA yield change with different digestion time using HB standard (Figure A) and H2MV standard (Figure B)

The change of PHA yield over digestion time using biomass samples is shown in Figure 3-2. This shows a minimum amount of digestion time was required to obtain the maximum yield. For PHB, the minimum digestion time required was 10 hours, and after that the yield became more or less stable. For PH2MV, longer digestion time is required (>20 h) since the yield increased with increasing digestion time. However, since the slope of the curve decreased and reached a plateau after 10 hours of digestion time, we speculate that the yield does not increase significantly with more digestion time. Compared to the PHA standards, the biomass samples need more digestion time. This is likely due to processes of breaking cells and depolymerization in biomass samples which does not happen with the standards.

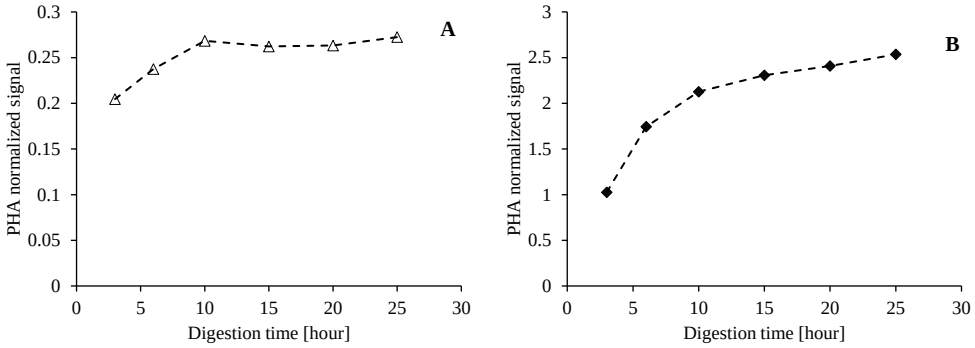


Figure 3-2 PHA yield change in different digestion time in biomass samples (Figure A: HB; Figure B: H2MV)

The study of (Huijberts et al., 1994) gave a very similar conclusion about the effect of digestion time. Furthermore, the study of (Jan et al., 1995) showed that longer digestion time wouldn't produce degradation products from PHA. Meanwhile, the minimum digestion time from these studies were different, which were 2 hours and 6 hours respectively. This suggested that different types of biomass would need different minimum digestion time. The time needs to be verified when using different biomass.

3.2 Alcohol effect

A general chromatography of mixture of HB, HV and H2MV standard is shown in Figure 3-3. This shows the separation of HV and H2MV peak was not complete in this case. Then methanol is used to replace propanol in the digestion step, the chromatography result is shown in Figure 3-4. The separation of HV and H2MV peak is almost complete after using methanol for digestion.

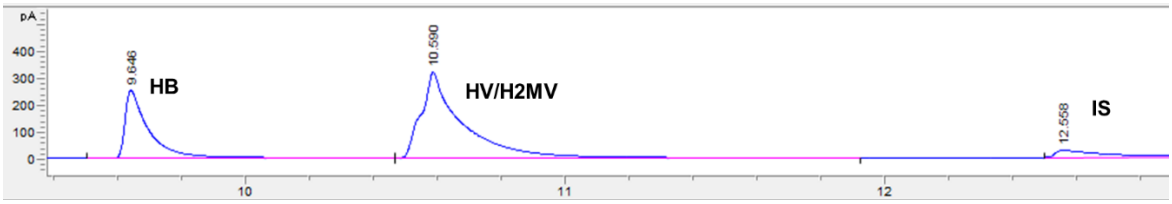


Figure 3-3 Gas Chromatography of mixture of HB, HV and H2MV standard (HB: 3-hydroxy butyrate peak; HV: hydroxy valerate peak; H2MV: hydroxy-2-methyl-valerate standard peak; IS: internal standard peak)

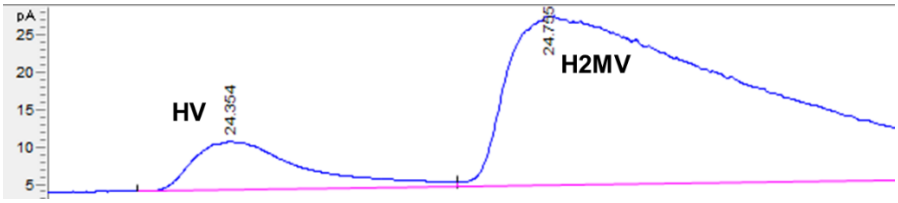
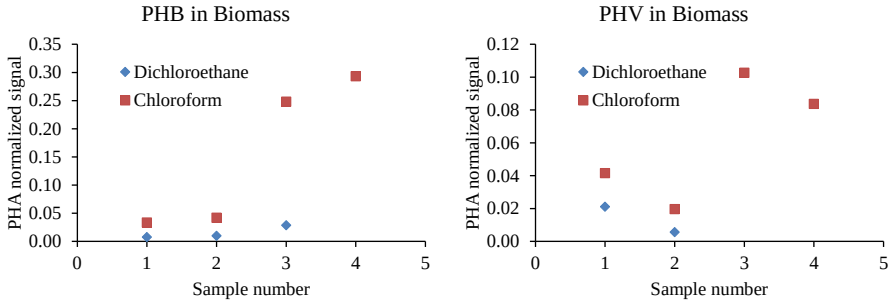


Figure 3-4 Better separation of HV and H2MV standard on GC after using methanol

This is because the use of methanol makes shorter carbon chain during the esterification, thus the relative difference of the two esters become larger and the separation of the two peaks becomes better on GC.

3.3 Organic solvent effect

The difference of using chloroform and dichloroethane on PHA yield was shown in Figure 3-5. For PHA standards, chloroform is shown to have about 3 to 4 times higher yield than dichloroethane. For biomass samples, higher yield was shown for samples using chloroform as well. The difference in biomass samples was lower might be due to lower concentration of PHA.



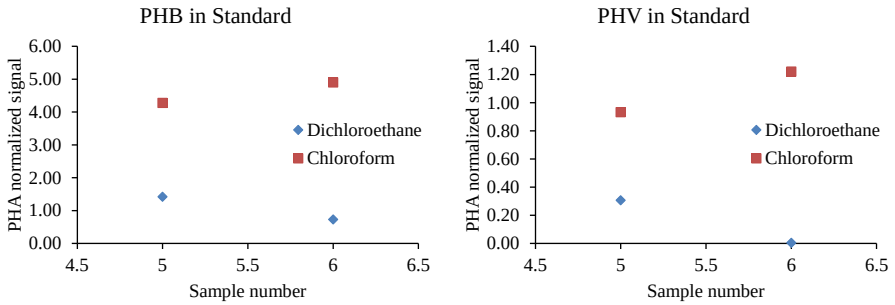


Figure 3-5 The PHA yield of PHA standards and biomass samples using chloroform and dichloroethane (sample 1 to 4 were biomass samples, and sample 5 and 6 were PHA standards; the data of sample 4 using dichloroethane was missing due to evaporation)

Considering there was a step of water extraction, where PHA ester was mixed with water and chloroform together, then PHA ester would dissolve in both water and chloroform but with different concentration. The concentration depended on the PHA ester's solubility in different phases, which can be considered as partition effect (Jan et al., 1995). Chloroform is a stronger organic solvent than dichloroethane, so it has a higher solubility of PHA ester. Then chloroform is able to have higher PHA ester concentration than dichloroethane, which leads to higher PHA normalized signal. The partition effect was due to solvent, so the higher concentration would exist in both biomass and standard samples, which explained the results from Figure 3-5. Since the partition effect will be on PHA standard, the calibration line based on PHA standard will also be different for chloroform and dichloroethane.

After making a calibration line on both solvents, the same biomass sample was tested to see whether the concentration in mg/mg would be the same. The results showed that the sample using dichloroethane had a concentration of 0.027 mg PHA/mg biomass, while the sample using chloroform had a concentration of 0.024 mg PHA/mg biomass. This proved that the partition effect from solvents could be eliminated by making calibration lines separately. A similar conclusion could be found from (van Loosdrecht et al., 2002) as well, where using methanol and chloroform had similar results as using propanol and dichloroethane. Even though the concentration wouldn't be different, using chloroform will have stronger signal and larger peak in GC, which was better for quantification when analyzing samples with small concentration of PHA.

3.4 Acid concentration effect

Figure 3-6 showed the difference of PHA yield using two types of biomass in different acid concentration. For PHB, the highest yield for using biomass1 was in 1% acid condition while the highest yield for using biomass2 was in 6% acid condition. This showed that different types of biomass might have different optimal acid concentration, but it could also be due to low PHB concentration in biomass2 (no statistical difference). For PHV, the highest yield for both biomass was in 10% acid concentration.

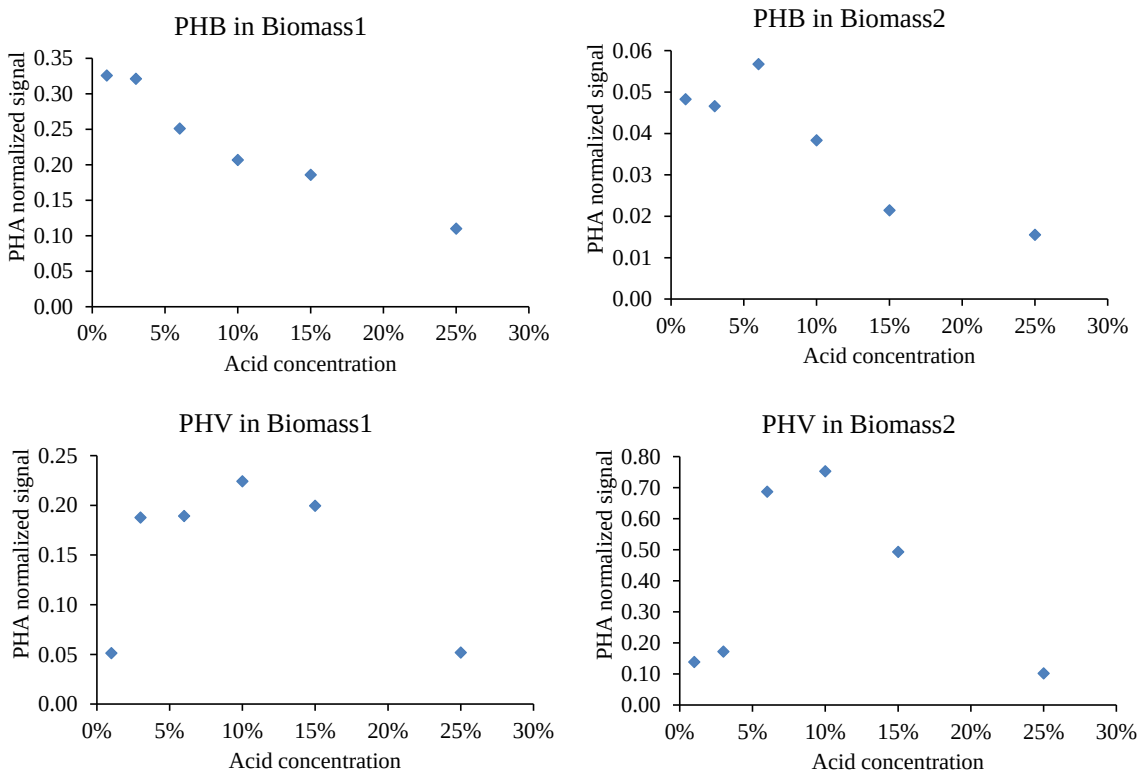


Figure 3-6 The PHA yield in different acid concentration with two types of biomass (biomass 1: fed with acetate; biomass 2: fed with propionate)

The results were based on normalized PHA signal without applying calibration lines, so they might have partition effect as discussed about organic solvent. According to (Lanham et al., 2013), acid concentration also led to partition effect and this might be due to different pH. The effect should be equivalent to both samples and standards, so making the calibration lines in the same condition as the samples would correct the effect. But different than organic solvent, high acid concentration can cause degradation of PHA ester. From the results, higher acid concentration condition will lead to very low yield of PHA ester, which might be difficult to correct by applying calibration lines. There were also results showing that when acid concentration was higher than a critical point, the PHA yield would become lower with time increasing (Braunegg et al., 1978; Oehmen et al., 2005). Therefore, it was better to determine an optimal acid concentration based on the type of biomass used and type of PHA targeted.

3.5 GC temperature program optimization

Program 1 was adjusted by the method from (Oehmen et al., 2005). As a comparison, Program 2 changed to a lower temperature in the middle and added another platform at 140°C. The result showed that HV and H2MV peak appeared during the platform of 140°C and separated better than Program 1, indicating lower temperature will give better separation. Moreover, holding temperature will give a smooth baseline while increasing temperature will make the baseline shift, which showed holding temperature while targeted peaks appeared was important for identification and separation. Program 3

showed a similar separation of HV and H2MV peak as Program 1, indicating holding a suitable temperature long wouldn't make a difference than keeping temperature increase very slowly. After realizing that lower temperature might be helpful to separate peaks better, Program 4 that added a decrease temperature part till 105℃ was tested. The results showed that it had much better separation than all the programs before, the time difference of HV and H2MV peak can reach 1.1 mins while other programs had the time difference between 0.4 and 0.6 mins. Therefore, the way to keep high temperature first then decrease temperature before the targeted peaks appearing can be a useful tip to enhance the targeted peaks separation.

4 Conclusion

- ✧ A minimum digestion time is required for obtaining the maximum yield of PHA, longer digestion time wouldn't decrease the PHA yield. Different types of biomass might have different minimum digestion time, and this should be verified. Long digestion time (>20h) would be suggested to use to ensure highest yield.
- ✧ Using methanol can lead to better separation of PHA peak on GC than propanol because shorter esters are formed.
- ✧ Chloroform extraction will have higher PHA concentration and GC peak area than dichloroethane extraction, but the results for biomass samples would be the same after applying calibration line.
- ✧ High acid concentration will cause PHA degradation, and an optimal acid concentration needs to be determined based on the biomass used.
- ✧ A lower oven temperature of GC is more beneficial for PHA peak separation.
- ✧ Practical information: it would be suggested to use methanol and chloroform for digestion with time about 24 hours and determine an optimal acid concentration by comparing the results of different concentration.

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