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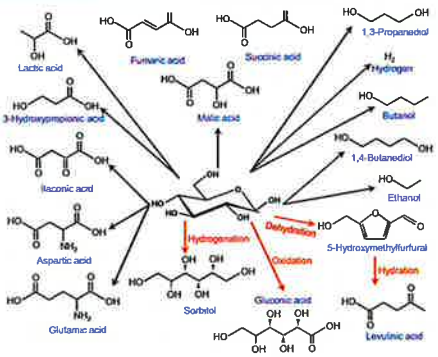
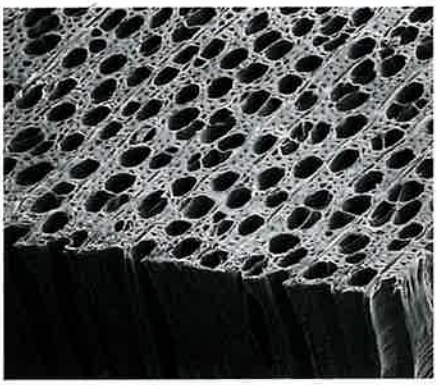
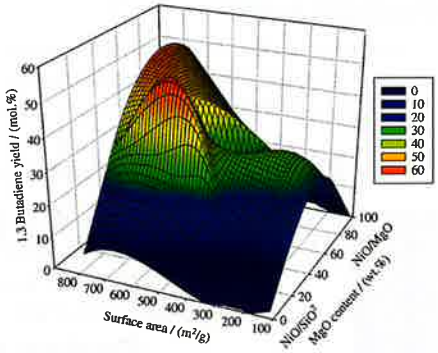


REVIEWS





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Optimization of a Poly-3-hydroxybutyrate Quantification Method for Rapid Detection in Plant Based Systems

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An established method for the extraction, detection, and quantification of poly-3-hydroxybutyrate in bacteria cells was adapted for use in higher plants and tested on hybrid poplar samples. Plant cell walls were degraded mechanically by grinding and interfering photosynthetic pigments were removed with ethanol before extracting the polyhydroxybutyrate in chloroform and converting it to crotonic acid in concentrated sulfuric acid. This allowed for detection and quantification by spectrophotometric analysis at 235 nm. This method showed strong correlation when compared with a gas chromatography based quantification method that has been used for polyhydroxybutyrate detection and quantification in plants. After the initial development of the assay, a second, biologically independent sample set was analyzed to confirm the repeatability and accuracy of the extraction and quantification method. The secondary study showed the same strong correlation with the gas chromatography quantification method as the initial study. This was followed by a series of assay sensitivity experiments in which potential procedural deviations were examined for their relative impact on assay results. Solvent extraction temperature and time had the greatest impact on the accuracy of the assay. While the effect of solvent to sample ratio was also significant, it could be partially accounted for by the calibration equations.

KEYWORDS: PHB, Poly-3-Hydroxybutyrate, Quantification, Assay, Detection, Crotonic Acid, Spectrophotometry.

1. INTRODUCTION

Environmental concerns about the sustainability of, and pollution created by petroleum-based thermoplastics have driven research on the production bio-plastics (Holmes, 1985). Poly-3-hydroxybutyrate (PHB) is a member of the polyhydroxyalkanoate (PHA) family, a group of biodegradable polyester polymers that may be able to compete commercially with non-degradable, fossil fuel derived plastics (Mergaert et al., 1995; Lee, 1996). These molecules are commonly produced by microorganisms in response to physiological stress and act as energy and carbon storage molecules, to be used when more common energy sources have been depleted (James et al., 1999). The high cost of PHB production in monoclonal, bacterial cultures has shifted research to focus on production of PHB in plant-based systems.

PHB biosynthesis genes from *Ralstonia eutropha* (formerly *Alcaligenes*), a bacterium found to produce PHB in nature (Peoples and Sinskey, 1989), have been transformed into several plant species including *Populus trichocarpa* (Dalton et al., 2011), *Arabidopsis thaliana* (Kourtz et al., 2007), *Medicago sativa L.* (Saruul et al., 2001), and *Saccharum* spp. Hybrids (Petrasovits et al., 2007). Further work is being completed to optimize PHB production in these systems and to expand the library of plants transformed to produce the polymer (Poirier et al., 1992). One of the challenges in this context are complex PHB detection methods which are often optimized for use in microorganisms. Gas chromatography is the standard method of measuring PHB in plants (Braunegg et al., 1978). The process involves extracting and converting the PHB into 3-OH butyrate before the sample can by separated by gas chromatography and analyzed. While this method yields accurate results, gas chromatographs are costly and samples undergo a lengthy derivatization prior to analysis. An alternative method used for determining PHB content in bacteria involves extracting and converting PHB to crotonic acid using concentrated sulfuric acid (Law and Slepecky, 1961). This conversion can be carried out in 10 minutes. Crotonic acid can then be quantified based on its absorbance maximum at 235 nm via spectrophotometry and used to estimate the PHB content. This method uses less expensive equipment, can provide rapid results, and produces accurate results that are comparable to the results from GC (Braunegg et al., 1978; Nakashita et al., 1999). Unfortunately this method had not been shown to work on plants

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because photosynthetic pigments extracted with the PHB create high, inconsistent background signals that mask the absorbency signal created by the crotonic acid.

In this paper we describe the development and sensitivity analyses of a modified PHB detection and quantification protocol based on the classic crotonic acid assay. This method was designed to overcome the difficulties posed by plant-based systems, namely interference by photosynthetic pigments and incomplete extraction caused by the robust cell wall. This method requiring minimal product transformation and equipment access is faster than current techniques while producing results comparable to GC quantification. The protocol has been optimized for use with *Populus trichocarpa*, but could be applicable for use in a much wider variety of plant-based PHB biosynthesis systems that pose similar difficulties for traditional detection methods.

2. MATERIAL AND METHODS

2.1. Sample Preparation

Samples were produced by co-cultivating poplar explants with *Agrobacterium* that had been transformed to contain the PHB biosynthesis genes (PHBabc) linked to an inducible promoter designed to respond to Intrepid (Busov et al., 2010). A subset of theses produced shoots and roots in tissue culture that were confirmed to contain the transgenes for the PHB biosynthesis pathway via PCR. Two plants with the highest levels of PHB were selected for experiments and were treated for 7–8 weeks with .05, .1, .5, or 1 mM concentrations of Intrepid (Dalton et al., 2011). Control samples without the engineered plasmid were treated with the same inducing agent at the same levels. Plants were also cultivated in the absence of Intrepid. Leaf samples were harvested, dried in conventional oven at 60 °C, and stored in ambient conditions until further processing.

Dried samples were ground in a knife mill, until the entire sample passed through a 2 mm screen in the bottom of the mill. The samples were then stored in ambient conditions until analysis.

2.2. Conventional Crotonic PHB Quantification Method

One conventional protocol for determination of PHB content in bacteria is based on conversion of PHB to crotonic acid (trans-2-butenoic acid, $CH_3CH{=}CHCO_2H$) when heated in concentrated H_2SO_4. Crotonic acid has an absorbance maximum at 235 nm that can then be analyzed to determine the PHB content (Law and Slepecky, 1961). In this method samples are extracted with chloroform at 55–60 °C for 3 h and the extract is filtered over Whatman filter paper. A small volume of extract (0.05–0.1 mL depending on the amount of estimated PHB concentration in sample) is added to a test tube and the chloroform is evaporated to dryness before addition of 7.5 mL of concentrated H_2SO_4. The mixture is heated at 100 °C for 10 min for complete conversion of PHB into crotonic acid, which is quantified by measuring the absorbance of the cooled mixture at 235 nm using concentrated H_2SO_4 as a blank.

2.3. Method Development

The standard crotonic acid method was used on dried and ground poplar leaves to determine its shortcomings. Initially, the background caused by contamination of the extracted PHB with photosynthetic pigments was accounted for by the background subtraction method. In the background subtraction method, the assay was performed on a sample without PHB and absorbance measured in that sample was subtracted from all experimental samples. This method allowed for a rough determination of PHB content, but it was unreliable, and resulted in PHB content estimates with high standard deviations.

To optimize the assay for plant-based systems a primary extraction and wash step was developed to remove chlorophyll and other interfering pigments while avoiding the removal of PHB. Samples from a poplar line that had been identified to contain high PHB levels with the background subtraction crotonic acid method were chosen for the experiments. These samples were extracted with ethanol, hexane, chloroform, or acetone for 2 h prior to being analyzed with the standard crotonic acid method. The results generated from this test, along with knowledge of the chemical properties of PHB, chlorophyll, and the solvents, was used to select an extraction solvent used in the primary extraction and wash steps.

2.4. Modified Crotonic Acid Method

The standard extraction method was modified (Fig. 1) by adding a primary extraction and wash step prior to chloroform extraction to reduce the interference from chlorophyll

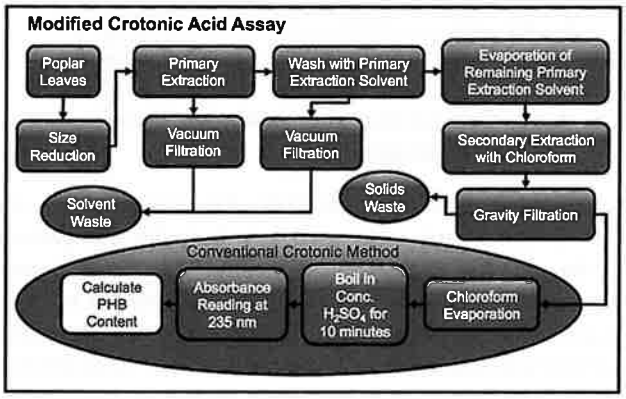

Fig. 1. Modified crotonic acid method for PHB determination of poplar leaves.

in hybrid poplar leaves. Ethanol, acetone, hexane and chloroform were tested for their suitability in the first extraction step. Based on the results from these experiments, ethanol was chosen as the primary extraction solvent used for during the primary extraction and washing. A 1:30 solids to solvent ratio was used during the 2 h extraction in pure ethanol at 65 °C. This was followed by vacuum filtration over Whatman filter paper and washing the residual solids two times with one volume of ethanol each time. The solids were then dried at 85 °C for 25 minutes to remove residual ethanol.

From this point forward the chloroform extraction was carried out as per Law and Slepecky's method (Law and Slepecky, 1961) with a 1:30 solids to solvent ratio at 55 °C. The conversion to crotonic acid was carried out with a 1:100 ratio of evaporated chloroform to H_2SO_4.

The calibration curve was developed by adding known amounts of PHB to the hybrid poplar leaf samples and following the modified crotonic acid protocol described above.

2.5. Calculations

Initially, a simple calculation of PHB percent by weight was derived directly from the best linear fit of the calibration curve. This calculation functioned well as long as all ratios of solvent to sample remained consistent between the calibration samples and the experimental samples, but scaling the offset ($B \rightarrow \hat{B}$) in accordance with actual solvent to sample ratios allowed for more consistent results (Eq. (1)). Equation 1: The equation used to convert absorbance at 235 nm to percent PHB w/w and the equation.

$$\begin{aligned} \mathrm{PHB}_{\%w/w} = ((M \times Abs_{@235\ \mathrm{nm}} + \hat{B}) \\ \times \left(\frac{V_{\mathrm{CHCl_3}} \times V_{\mathrm{H_2SO_4}}}{V_{\mathrm{CHCl_3\ Evaporated}}} \right) \\ \times \left(\frac{1}{10000 \times Wt_{\mathrm{Sample}}} \right) \end{aligned} \quad (1)$$

$$\hat{B} = B \left(\frac{V^{\mathrm{Cal}}_{\mathrm{CHCl_3}} \times V^{\mathrm{Cal}}_{\mathrm{H_2SO_4}} \times V^{\mathrm{Actual}}_{\mathrm{CHCl_3\ Evap}} \times Wt^{\mathrm{Actual}}_{\mathrm{Sample}}}{V^{\mathrm{Actual}}_{\mathrm{CHCl_3}} \times V^{\mathrm{Actual}}_{\mathrm{H_2SO_4}} \times V^{\mathrm{Cal}}_{\mathrm{CHCl_3\ Evap}} \times Wt^{\mathrm{Cal}}_{\mathrm{Sample}}} \right)$$

2.6. Assay Comparison and Validation Using Gas Chromatography

An established protocol for PHB detection in plants via gas chromatography was used as a basis for comparison to the modified crotonic acid method. The ground sample material was weighed into aliquots of 40–80 mg and placed into microfuge tubes. To this 800 μl of extraction solvent was added (90% 1-butanol and 10% concentrated HCL). The tubes were then flash frozen by submersion in liquid nitrogen and the samples were further pulverized with a small plastic pestle attached to an electric drill until the sample thawed. The tube contents were transferred to a 20-mL round bottom flask and the tube was rinsed with an additional 2 mL of extraction solvent, which was added to the flask. The sample was gently refluxed for 60 minutes to convert the PHB into 3-OH butyrate. The sample was transferred to 15 mL centrifuge tube and the volume was adjusted to 3.0 mL with extraction solvent. Three mL of aqueous 5% Na_2CO_3 was added to neutralize the acid before the tubes were briefly shaken and then centrifuged at 1000 g for 5 minutes to quicken phase separation. The upper (organic) phase was analyzed with a Hewlitt-Packard model 5890 gas chromatograph equipped with a 6 ft × 1/8 in stainless column of 10% Rtx-1 100/120 silcoport. The column temperature was maintained at 150 °C for the first 1.5 minutes of analysis and was then raised at a rate of 45 °C/min to 270 °C. Both the injector and detector were held at 200 °C (Dalton et al., 2011).

2.7. Assay Verification

To further validate the assay the amount of PHB in a second set of biologically independent samples was determined. These samples were produced from the same high PHB producing events used in the development of the method, but each plant was induced with 1 mM Intrepid and samples were harvested over a two-month period. This provided a set of biologically independent from samples used for assay development and ruled out the possibility that the method was registering falsely high PHB levels based on the varied levels of the Intrepid used in the first set.

2.8. Sensitivity Analysis

Potential variations in the assay likely to be encountered in practice were analyzed and the extent of assay sensitivity to these variations were quantified. During this testing, baseline PHB content values were determined for a control sample without PHB and a sample with known PHB content using the complete extraction and quantification method described above. The method was repeated with deviations in individual steps to study sensitivity of the assay to the particular step (Table I). Extraction times, solvent to sample ratios, and the number of primary solvent

Table I. The variables explored during sensitivity analysis.

Method of deviation (normal)	Deviation above protocol	Deviation below protocol
Chloroform: Sample (30:1)	50:1	10:1
Ethanol: Sample (30:1)	60:1	10:1
Ethariol washes (2)	4	1
Chloroform contact time (3 h)	5 h	1 h
Ethanol contact time (2 h)	4 h	1 h
Chloroform extraction temp. (60 °C)	Not explored	22 °C
Ethanol extraction temp. (65 °C)		22 °C

washes were examined at levels both above and below the recommended procedure, but extraction temperatures were only examined below the recommended set point as temperatures above the recommended temperatures were near to the boiling point of the solvents and evaporative loss became difficult to control.

3. RESULTS AND DISCUSSION

3.1. Primary Solvent Choice

Several factors were considered in selecting a primary extraction and wash solvent. The first criterion was that the modified crotonic acid assay using the selected primary solvent produces results comparable result to those produced with the background subtraction method with lower standard deviation. When a given primary extraction solvent produced PHB concentration estimates significantly different from the results generated with the background subtraction method the solvent was reasoned to either not fully extract the pigments (resulting in higher than expected values) or to partially extract PHB (resulting in lower than expected values). The comparison between a sample's PHB concentration as determined by the background subtraction method and the same sample's PHB concentration as determined by the modified crotonic acid method with four different wash solvents is shown in Figure 2.

Based on the comparison in Figure 2, ethanol and acetone received the most consideration as a primary extraction and wash solvents. The higher boiling point of ethanol (78.4 °C) as compared to acetone (56.53 °C) was an advantage in that it allowed both extraction steps to be performed near the chloroform's boiling point of 61.2 °C. It was noted that PHB estimates were slightly higher when the wash was performed with ethanol than with acetone. This was likely because while PHB is partially soluble in acetone, it is insoluble in ethanol (Anderson and Dawes, 1990). The acetone was likely extracting a small amount of PHB that was then not available for detection after the secondary chloroform extraction. Hence ethanol was chosen as the solvent for the primary extraction and wash steps in the modified crotonic acid assay.

3.1.1. Calibration

The calibration curve generated for the modified chloroform extraction protocol is shown in Figure 3. The relationship between absorbance and PHB concentration was linear in the range of 0–5.5 μg/mL PHB concentrations and up to an absorbance of 0.7. Therefore, samples with absorbance >0.7 were diluted and the concentration was measured to assure a linear operating range. While this dilution was accounted for at least in part by the calibration equations, it would be advisable to use same dilution for all the samples.

It should be noted that calibration curves need to be made with the equipment used to run experimental samples. During our research, when attempts were made to calculate PHB concentrations using data produced using one set of equipment and a calibration curve produced using another the agreement between the crotonic acid assay and PHB quantification by GC was not strong (not reported).

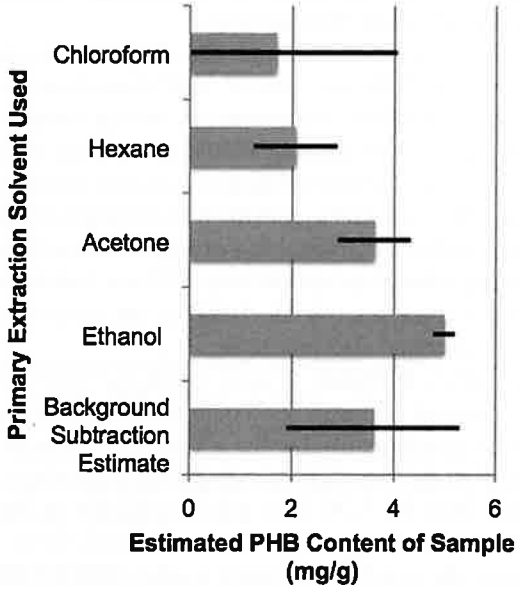

Fig. 2. A comparison of PHB concentration of a sample by the background subtraction method and using four different solvents in the modified crotonic acid assay.

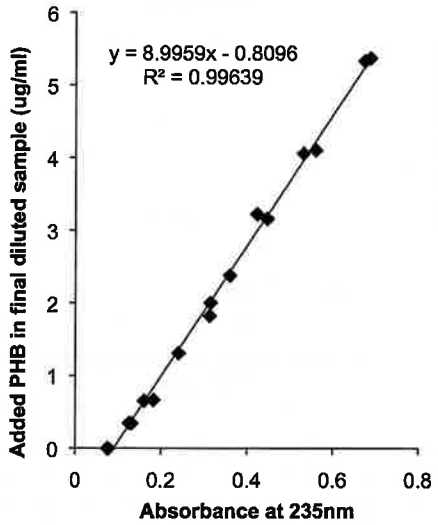

Fig. 3. The PHB calibration curve for crotonic acid method was created by adding known amounts of PHB crystals to leaf material that did not contain the PHB synthesis pathway.

3.2. Comparisons of Modified Crotonic Method and Gas Chromatography

Portions of the ground samples used for development and testing of the modified crotonic acid quantification method were treated to convert PHB to 3-OH butyrate and analyzed by gas chromatography. The results were compared with the results generated by the modified crotonic acid assay in Figure 4(A). GC analysis of PHB has been used for detection and quantification in both bacteria (Braunegg et al., 1978) and plants such as tobacco (Nakashita et al., 1999) and was considered the standard for this study.

The correlation between the GC and modified crotonic acid assay was high ($R^2 = 0.67$). It should be noted that while the two methods did produce well-correlated results, the correlation was not one to one. This may be attributed to minor differences in the calibration curves or differences in extraction efficiency. If both methods are to be used in a study a correction factor may need to be implemented to make the methods align. These results demonstrate the utility of the method for developing a high throughput screening methods and clone selection.

To verify this correlation, the assay was repeated on a set of samples independent from those used in the development of the assay. These samples were from plants treated with 1 mM Intrepid and were harvested over a 2-month period. The resulting PHB values were again compared with PHB quantification values obtained by gas chromatography. The results of this comparison (Fig. 4(B)) showed a similarly strong correlation ($R^2 = 0.63$) to that of the initial trial. The differences in regression between the two trials were not significant ($p = 0.13$).

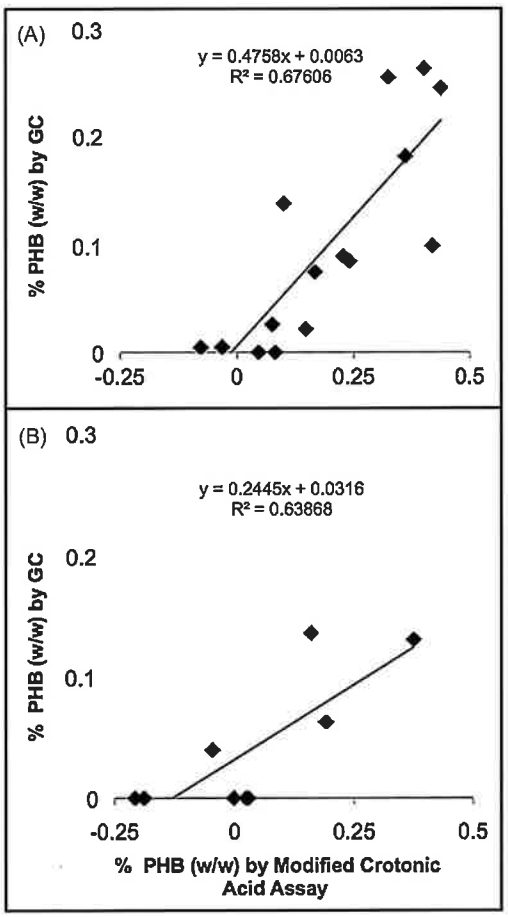

Fig. 4. A comparison of the spectrophotometric method (X-axis) based on the conversion of PHB to crotonic versus the gas chromatographic method based on derivatization of PHB into the butyl ester of 3-OH butyrate performed on (A) samples induced with Intrepid concentrations of 0, .05, .1, .5, or 1 mM harvested in bulk, and (B) samples induced with 1 mM Intrepid harvested over a 2 month period.

3.3. Assay Sensitivities Testing

The sensitivities of the assay were examined through the comparison of PHB levels determined using the recommended modified crotonic acid assay procedure and using the same method with variations made to individual steps. Solvent to sample ratios, extraction temperature, extraction duration, and the number of ethanol washes were examined. Each deviation was tested in triplicate on both control samples without PHB and samples with PHB. Figure 5 shows the resulting discrepancies and indicates that importance of optimum extraction temperature and time.

Decreased temperature appeared to cause incomplete extraction during both primary ethanol and secondary chloroform extraction steps. Incomplete pigment extraction with ethanol led to higher than expected PHB estimates by addition of unaccounted-for background while incomplete PHB extraction by chloroform resulted in lower than expected PHB readings.

Deviations in extraction time also lead to assay inconsistencies for both the ethanol and chloroform extraction steps. Shortened chloroform extraction duration led to incomplete extraction of PHB and resulted in lower PHB estimates. Increased ethanol extraction time resulted in lower PHB estimates, perhaps due to increased extraction of interfering pigments. Increasing the chloroform and decreasing ethanol extraction times did not lead to deviations beyond the standard deviation of the samples tested with the recommended procedure.

The values in Figure 5 were calculated with Eq. (1) which was able to partially account for the different chloroform to sample ratios, but the accuracy of the assay was observed to decrease as the sample to chloroform ratio deviated from the 1:30 ratio used in creation of the calibration curve. Additionally, since extraction times, temperatures, the number of ethanol washes, and the ethanol to sample ratio were not incorporated into the calibration equations, these should be held constant across all experiments to generate consistent results.

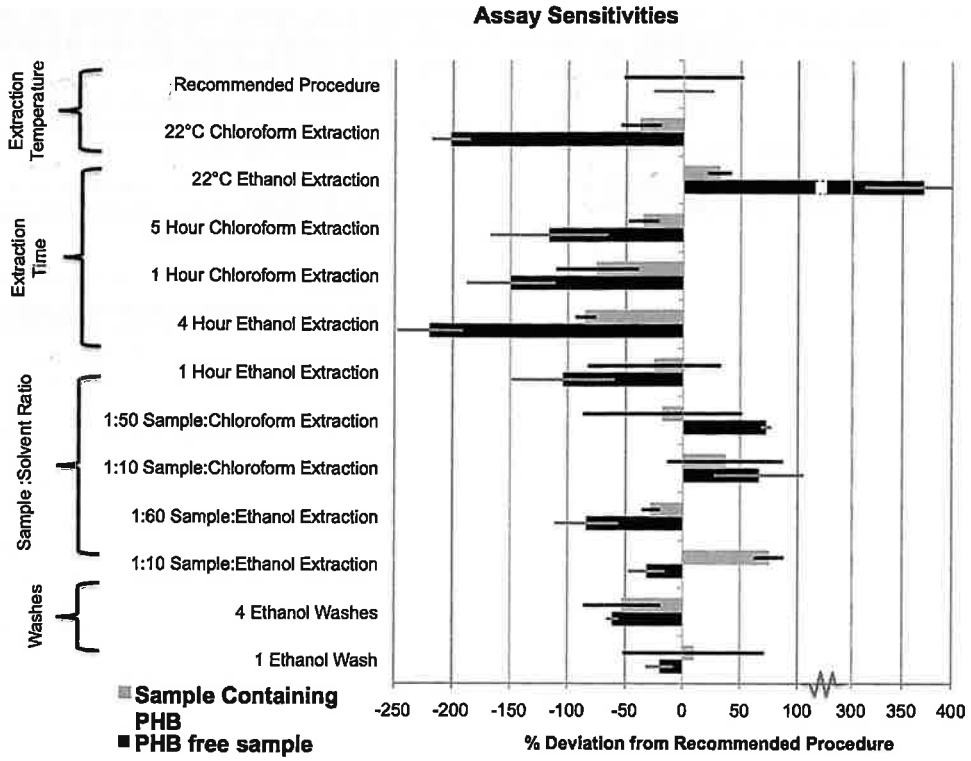

Fig. 5. Average percent deviations from controls determined using the recommended crotonic acid method (1:30 solvent solid ratio, 2 h ethanol extraction at 65 °C, 2 ethanol washes, 3 h chloroform extraction at 60 °C) and varied as described in the Sensitivity Analysis Methods section. PHB concentrations were calculated with equation 1 to limit error by changing background concentration, but for maximum accuracy it is important that conditions and dilution ratios used for generating the calibration curve be used for unknown samples.

4. CONCLUSION

The method of detecting and quantifying PHB by conversion to crotonic acid followed by spectrophotometric analyses, which was developed for use in bacteria, was successfully modified for use with plant tissues by adding a primary ethanol extraction and wash step. Results show a strong and repeatable correlation between the developed method and the more conventional, but also more time consuming and expensive, method for detection and quantification by gas chromatography. The primary ethanol extraction and wash steps in the modified crotonic acid assay overcome the difficulties in PHB quantification that are likely to be present in many plant pant based systems. This makes it a good candidate for rapid PHB detection in future plant centered PHB research.

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REFERENCES

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiolofical Reviews* 54(4): 450–472.

Braunegg, G., Sonnleitner, B., and Lafferty, R. M. (1978). A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in bacterial biomass. *European Journal of Applied Microbiology and Biotechnology* 6: 29–37.

Busov, V. B., Strauss, S. H., and Pilate, G. (2010). Transformation as a tool for genetic analysis in populus. *Plant Genetics and Genomics* 8(2): 113–133.

Dalton, D., Ma, C., Shrestha, A., Kitin, P., and Strauss, S. (2011). Tradeoffs between biomass growth and inducible biosynthesis of polyhydroxybutyrate in transgenic poplar. *Plant Biotechnology Journal* 9(7): 759–767.

Holmes, P. A. (1985). Application of PHB—A microbially produced biodegradable thermoplastic. *Physics in Technology* 16: 32–36.

James, B. W., Mauchline, W. S., Dennis, P. J., Keevil, C. W., and Wait, R. (1999). Poly-3-hydroxybutyrate in legionella pneumophila, an energy source for survival in low-nutrient enviornments. *Applied and Environmental Microbiology* 65: 822–827.

Kourtz, L., Dillon, K., Daughtry, S., Peoples, O. P., and Snell, K. D. (2007). Chemically inducible expression of the PHB biosynthetic pathway in *Arabidopsis*. *Transgenic Research* 16: 759–769.

Law, J. H. and Slepecky, R. A. (1961). Assay of poly-β-hydroxybutyratic acid. *J. Bacteriol.* 82(1): 33–36.

Lee, S. Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends Biotechnol.* 14: 431–438.

Mergaert, J., Wouters, A., Anderson, C., and Swings, J. (1995). *In situ* biodegredation of poly(3-hydroxybutyrate) and poly(3-hydroxybutryate-co-3-hydroxyvalerate) in natural waters. *Can. J. Microbiol.* 41(1): 154–159.

Nakashita, H., Arai, Y., Yoshioka, K., Doi, Y., Usami, R., Horikoshi, K., and Yamaguchi, I. (1999). Production of biodegradable polyester by a transgenic tobacco. *Bioscience, Biotechnolology, and Biochemistry* 63(5): 870–874.

Peoples, O. P. and Sinskey, A. J. (1989). Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). *J. Biol. Chem.* 264: 15298–15303.

Petrasovits, L. A., Purnell, M. P., Nielsen, L. K., and Brumbley, S. M. (2007). Production of polyhydroxybutyrate in sugarcane. *Plant Biotechnology Journal* 5(1): 162–172.

Poirier, Y., Dennis, D. E., Klomparens, K., and Somerville, C. (1992). Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. *Science* 256: 520–523.

Saruul, P., Srienc, F., Somers, D. A., and Samac, D. A. (2001). Production of a biodegradable plastic polymer, poly-b-hydroxybutyrate, in transgenic alfalfa. *Crop Science* 42(3): 919–927.

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