Improvements in PHA Production, Control and Applications

Berezina Nathalie, Talon Olivier and Paternostre Laurent
NATISS R&D Centre, rue des Foudriers 1, 7822 Ghislenghien, Belgium
berezina.n@natiss.be; talon.o@natiss.be; paternostre.l@natiss.be

The productivity and copolymerisation control of PHBV were improved *via* specific feeding (sodium glutamate 10 g/L and sequential addition of valeric acid). The active monitoring of PHA content in cells was made possible through TGA analysis based on the large degradation temperature difference between PHAs and the rest of the cells. Finally a kinetic model for the depolymerisation of PHA was proposed and discussed.

1. Introduction

Industrial biotechnology is a well known tool for production of fine and specialty chemicals, mostly for pharmaceutical and cosmetic industries. Polymers are a new emerging domain for expansion of Industrial Biotechnology. Such polymers may be considered as well as material (Sudesh et al. 2000) as natural concentrates of chiral products (Seebach et al. 1987).

One of the most promising such obtained class of polymers is the poly(hydroxyalkanoates) (PHA) one. PHA are a huge family of bacterial polymers, they present interesting performances and chirality properties, but they suffer from too expensive and tricky production.

That is why a lot has to be done for improvements in PHA's production, analysis (control) and synthons application.

2. PHA production

In order to optimise the production of PHB and PHBV by *C. necator*, we made 2 experimental designs. We worked with experimental designs types 2^{k-p}, with k=3 and p=1. This allowed us to evaluate 6 factors in 8 experiments (Berezina et al. 2007).

First we wanted to enhance *C. necator* biomass. Indeed, as the PHA production depends on nutrient control, most of the authors (Potter et al. 2004, Mantzaris et al. 2002) use to allow the bacteria to grow under limited nutrient conditions: minimum mineral media with only carbon supply used for the PHA production (typically glucose, fructose, sodium gluconate etc.). On the other hand, it is known (Steinbüchel et al. 2003) that the PHA production is made in two steps: *C. necator* growth followed by PHA accumulation.

Different substrates were reported to be biomass enhancers such as citric acid (Wang et al. 1997), sodium glutamate (Elbahloul et al. 2005, Barclay et al. 2001, Sauer et al.

2004, Wang et al. 2001, Wang et al. 2000, Sonavane et al. 2003), aminolevulinic acid (Hungerer et al. 1995) or aspartate (Sonavane et al. 2003). The most efficient biomass enhancer seems to be sodium glutamate whereas citric acid is also supposed to influence valerate content of final PHBV (Akyiama et al. 1992, Steinbüchel et al. 2003); finally glucose concentration can also play a key role as well in biomass growth as in PHA production.

Thus in the first experimental design (Table 1) we evaluated: citric acid supply (0 or 2 g/L), sodium glutamate supply (10 g/L or 20 g/L) and glucose concentration during the accumulation step (10 g/L or 30 g/L).

Table 1 : Factor's effects of both experimental designs

Factors	DW	PHA	PHA/g$_{DW}$	PHA/g$_{glucose}$
Citric acid (2 g/L)	-3	6	-4	-1
Glutamate (10 g/L)	15	25	15	8
[G] 30 g/L at 48h	15	4	13	4
[G] 30 g/L at t_0	4	-3	0.4	0.9
$P(O_2)$ (30 %)	0.2	-1	-0.8	0.5
Valeric acid (4x5g/L)	-0.2	1	0.8	-0.5

During the second experimental design we evaluated: glucose concentration at t_0 (10 g/L or 30 g/L), $P(O_2)$ (3% or 30%) and valeric acid supply manner (4x5g/L or 1x20 g/L).

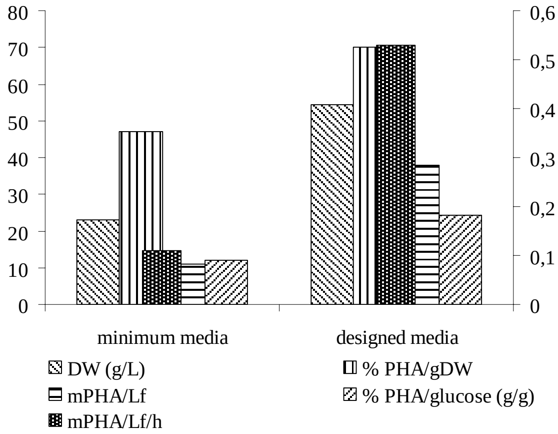

Figure 1 : Majors variables evolution before (minimum media) and after (designed media) the 2 experimental designs

The citric acid supply, P(O_2) variation and valeric acid supply manner resulted in no benefice on DW, PHA content, PHA production, PHA/glucose yield or the whole system productivity (Table 1), whereas an important beneficial effect of glucose concentration at 30 g/L (either at the beginning of the fermentation or during the accumulation step) and of glutamate supply at 10 g/L (at 20 g/L results were less interesting due to the extra ammonium supply present in glutamate) was observed (Table 1).

After the completion of control fermentations, which consisted in the combination of best factors, we obtained the following improvements (Figure 1): Dry Weight enhancement on 250%, PHA content on 30%, Volumetric production on 400%, PHA/glucose yield on 200% and Productivity on 500%.

Table 2 : PHBV analysis

Valeric acid supply[1]	HV (mol %)		DSC		SEC	
	GC	NMR	T_g	T_m	$\overline{M}_n$	Ip
4 times	7	9	3	168[2]	120000	1.9
1 time	4	5	0	171[3]	220000	1.4

1. final valeric acid amount is the same in both cases
2. bimodal pick
3. monomodal pick

The manner of valeric acid supply has poor influence on growth, but deeply influences as well the valerate content as the polymer structure (Table 2). Valerate content grows from 4% (unique addition) to 8% (multiple additions). The results are even more interesting when comparing other analysis: SEC clearly indicates 2 different types of polymers and DSC suggests a block system for multiple additions and a statistic system for single addition.

These results are consistent with Srienc's group suggestions (Kelley et al. 2001, Mantzaris et al. 2001, Pederson et al. 2006): switching of carbon source results in increasing of valerate content and block copolymer structure.

3. PHA analysis

The parameters usually followed in a fermentation process, such as optical density, dry weight or glucose concentration do not give any direct information about the yield of PHBV production.

The control of the bacterial production of poly(hydroxyl alkanotes) (PHA) is usually (Sudesh et al. 2000, Steinbüchel et al. 2003) done by an extraction method. A large sample (ca 10 mL) is withdrawn from the fermentation reactor. The sample is then centrifuged before to undergo a freeze-drying step. The lyophilizate is then immersed in chloroform for the extraction of the PHA from the cells, and filtered. After precipitation of the filtrate and drying of the PHA such obtained, one can at last, after several days, have an idea of the yield of the process at the time the sampling was made. It is obvious that, for a more or less one week long fermentation process, this method is unsuitable for a reactive monitoring, not to mention the large use of solvents.

We improved and developed the method previously reported by Hahn et al. (1995) (Talon et al. 2007). TGA performed on pure PHBV and on PHBV still enclosed in bacteria showed that a significant gap exists between the thermal degradation temperatures of the PHA and of the cells. It appears indeed that the PHA is fully degraded at 210-220 °C, whereas the weight loss due to the degradation of the cells only begins at about 250-260 °C (Figure 2).

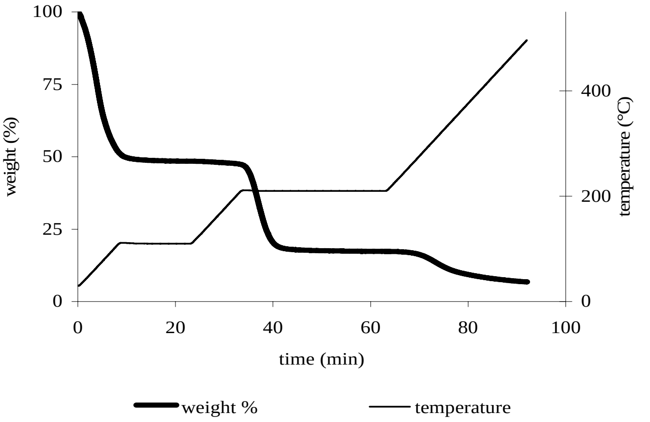

Figure 2 : time course evolution of temperature program and weight (%) evolution

When used to follow two fermentations done in identical conditions, the results of the thermogravimetric method show (Figure 3) that both the fermentation process and the method of assay are reproducible.

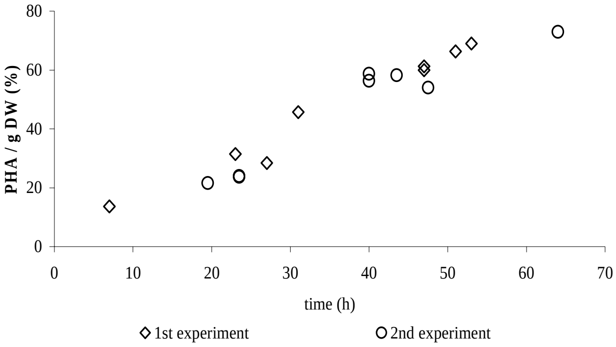

Figure 3 : Reproducibility of TGA analysis between 2 similar experiments

In order to confirm the validity of the method, a fermentation experiment was followed according to the two methods (extraction and TGA methods). The results are shown in Figure 4, where it can be seen that the results provided by the two methods are quite comparable. Moreover, it seems that the thermogravimetric method would give more exact results, since they are a little higher than those obtained with a single extraction step, and it is well known that the yield of only one extraction is not quantitative.

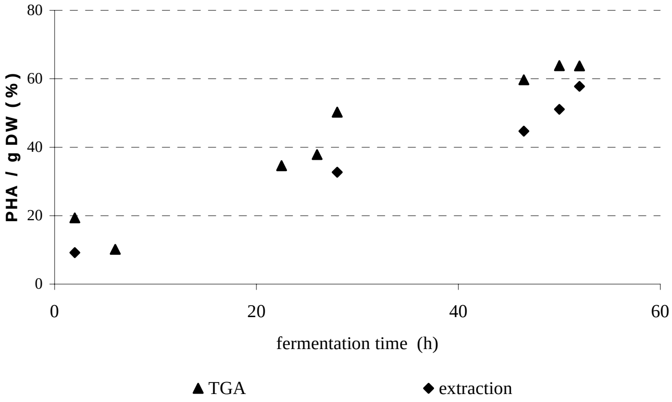

Figure 4 : Comparison between 2 methods of PHA content's measurement

4. PHA Applications

Across different applications of PHA one can be interested by using their chiral monomers, i.e. β-hydroxyacids. Indeed, PHA are perfectly chiral polymers, as well their monomers are, and β-hydroxyacids are very useful products. β-hydroxyacids can be used by themselves (mostly in cosmetic industry) and as chiral synthons for a range of products such as lactones, β-lactams, dioxanones, (Seebach et al. 1987) or even proven antibiotics as tienamycin (Chiba et al. 1985) or elaiophylin (Seebach et al. 1986, Evans et al. 1997).

The depolymerisation of PHA has been of interest for a long time. Pioneering work on the PHB depolymerisation was performed by Merrick et al. (1964) and consisted on enzymatic depolymerisation of non purified polymer. Pyrolysis approach developed by Morikawa et al. (1980) produced mostly crotonic acid. More recent results (Lee et al. 2000, Lee et al. 1999) concerned the alcoolysis approach, finally Ren et al. (2005) worked on *in situ* depolymerisation of PHA_{MCL} (medium chain length PHA).

Herzog et al. (2006) proposed a kinetic model for polyester depolymerisation by lipases, but no kinetic model is yet available for the PHA depolymerisation, to our knowledge.

We wanted to make a simple kinetic model for the depolymerisation of the non purified PHA, i.e. still contained inside the cells. This means that we have to treat 2 reactions: the transfer across the cells' membranes and the depolymerisation of the free PHA.

$$PHA_c \xrightarrow{k_1} PHA_f \xrightarrow{k_2} \beta\text{-hydroxybutyric acid} \qquad (1)$$

PHA_c represents PHA inside of bacterial cells
PHA_f represents free PHA in the reaction media

On first approximation we can assume that the percentage of depolymerisation will essentially depend on PHA transfer across cell membrane. The second reaction is supposed to be much faster than the PHA transfer (ester hydrolysis in aqueous basic media), $k_2 >> k_1$. In that case the only important parameter to be measured will be the PHA content in cells and we can express the depolymerisation process as following:

$$\%\ \text{depolymerisation} = \frac{\text{PHAi}}{\text{PHA (t)}} \qquad (2)$$

PHAi being initial content of PHA in cells
PHA (t) being the content of PHA in cells at instant t

In order to check this kinetic model, we made an experiment with 10 molar NaOH. We compared results obtained by HPLC analysis of produced β-hydroxybutyric acid with remained PHA content in cells measured by previously described TGA method (Figure 5). We can observe quiet a correct correlation between the theoretic model and the experimental data.

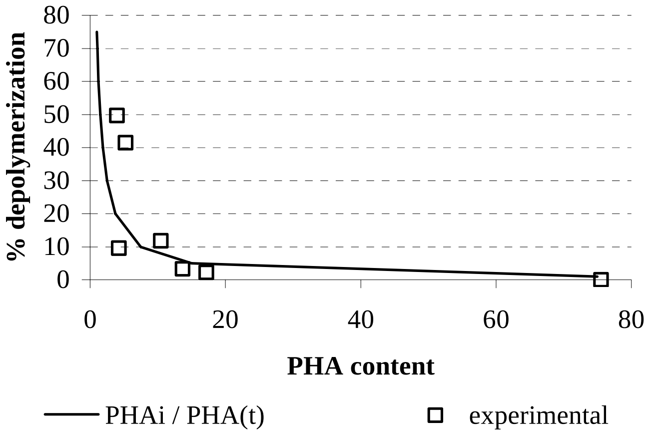

Figure 5 : Comparison of the kinetic model with the experimental depolymerisation

However, we may assume that that weaker the base the wronger the hypothesis that the depolymerisation step can be neglected in regard of the diffusion step. Therefore, even if this kinetic model is satisfactory at the first stage it should be précised for being more universal for this system.

5. Conclusion

In this work we applied the experimental design system to investigate 6 different factors for PHA production. We succeeded to identify the most important benefit factors which allowed us to enhance the whole system PHB productivity for 5 times. We also succeeded to control the PHBV structure (block or statistical) by valeric acid supply manner.

Further developed thermogravimetric analysis enables a rapid control of the evolution of a fermentation process, and therefore can offer the opportunity of being reactive enough to adapt the process parameters when something seems to go wrong. This method of assay provides good and reproducible results, and it is solvent free and hugely time-saving.

Finally, we turned our interest to the PHA's depolymerisation and established a satisfactory kinetic model for the combination of membrane transfer and depolymerisation reactions.

6. References

Akiyama, M., Taima, Y. and Doi, Y., 1992, Appl. Microbiol. Biotech., 37, 698.

Barclay, S.S., Woodley J.M., Lilly M.D., Spargo P.L. and Pettman A.J., 2001, Biotech. Lett., 23, 385.

Berezina N., Colin T., Bensaid A., Paternostre L, in European Bioperspectives Proceedings, Cologne, 2007

Chiba, T. and Nakai, T., 1985, Chemistry Letters, 651.

Elbahloul Y., Krehenbrink M., Reichelt R., Steinbüchel A., 2005, Appl. Environ. Microbiol., 71-2, 858

Evans D.A., Fitch D.M., 1997, J. Org. Chem., 62, 454

Hahn S.K.; Y.K. Chang, Biotech. Tech., 1995, 9, 873.

Herzog, K., Muller, R. J. and Deckwer, W. D., 2006, Polym. Degrad. Stability, 91, 2486-2498.

Hungerer C., Troup B., Romling U., Jahn D., 1995, J. Bacteriol., 177-6, 1435

Kelley, A. S., Mantzaris, N. V., Daoutidis, P. and Srienc, F., 2004, Nano Lett., 1, 481.

Lee, S., Lee, Y. and Wang, F., 1999, Biotech. Bioeng., 65, 363.

Lee, Y., Park, S. H., Lim, I. T., Han, K. and Lee, S., 2000, Enz. Microbiol. Tech., 27, 33.

Mantzaris, N. V., Kelley, A. S., Srienc, F. and Daoutidis, P., 2001, AIChE Journal, 47, 727.

Mantzaris, N. V., A. S. Kelley, et al., 2002, Chem. Eng. Sci., 57, 4643.

Merrick, J. M. and Doudoroff, M. ,1964, J. Bacteriol., 88, 60-71.

Pederson, E. N., McChalicher, C. W. J. and Srienc, F., 2006, Biomacromolecules, 7, 1904.

Pötter M., Muller H., Reinecke F., Wieczorek R., Fricke F., Bowien B., Friedrich B., Steinbüchel A., Microbiology, 2004, 150 2301.

Ren, Q., Grubelnik, A., Hoerler, M., Ruth, K., Hartmann, R., Felber, H. and Zinn, M., 2005, Biomacromolecules, 6, 2290.

Sauer K., Cullen M.C., Rickard A.H., Zeef L.A.H., Davies D.G., Gilbert P., 2004, J. Bacteriol., 186-21, 7312

Seebach, D., Chow, H. F., Jackson, R. F. W., Sutter, M. A., Thaisrivongs, S. and Zimmermann, J., 1986, Liebigs Ann. Chem., 1281.

Seebach, D., Imwinkelried, R. and Stucky, G., 1987, Helv. Chim. Acta, 70, 448.

Sonawane, A., Kloppner, U., Derst, C. and Rohm, K. H., 2003, Arch. Microbiol, 179, 151.

Steinbüchel, A. and Lutke-Eversloh, T., 2003, Biochem. Eng. J., 16, 81.

Sudesh K., Abe H., Doi Y., 2000, Prog. Polym. Sci., 25, 1503

Talon. O, Berezina N., Lewillon P., Allert F., Dombrun E., Paternostre L., in Bioplastics 2007 proceedings, Montreal

Wang, S. J. and Loh, K. C., 2000, Biotech. Bioeng., 68, 437.

Wang F., Lee S.Y., 1997, Appl. Environ. Microbiol., 63-9, 3703.

Wang, S. J. and Loh, K. C., 2001, Biodegradation, 12, 189.