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Production of Polyhydroxybutyrate from Lignocellulosic Hydrolysates—Optimization of *Bacillus sacchari* Fermentation and Scale Up from 2 L to 200 L

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Abstract

Biopolymers such as polyhydroxybutyrate (PHB) are considered to be carbon-neutral, and thus environment-friendly, replacements for fossil fuel-derived plastics. They are more expensive, however, and production process costs must be reduced to increase market acceptance. Alternative feedstocks offer a promising way to reduce costs.
This application note presents the process development and optimization of wheat straw hydrolysate fermentation to produce PHB in *B. sacchari*. Process engineers at Biotrend® (Portugal) evaluated various ratios of two sugar concentrations on a small scale, using an Eppendorf New Brunswick™ BioFlo®/CelliGen® 115. They established automated feed triggers for the New Brunswick BioFlo 415, 610 and Pro fermentation systems, allowing them to successfully scale up the process 100-fold.

Introduction

Research into polyhydroxyalkanoate (PHA) biopolymers (including PHB) has intensified. Since their mechanical, physical, and thermal characteristics are similar to many fossil-fuel-based plastics, such as polypropylene (PP) and polyethylene (PE), they have the potential to replace those high-volume products in certain applications. Naturally produced from sugars by various bacterial strains, they are an environmentally friendly alternative to plastics derived from petroleum and natural gas. PHAs are biodegradable, non-toxic, and can either be thermoplastic or elastomeric materials, making them suitable for applications in biomedicine, packaging, and many other fields.

In 2014, the global production of PHAs was estimated at 54 kilotonnes, with a more than 5-fold market increase expected by 2020 [1]. But although production costs have decreased substantially over the last several decades, PHA prices are still significantly higher than traditional plastics. Feedstocks account for 50 % of their average production costs [2]. Using cheaper feedstocks, such as lignocellulosic sugars, would be a major breakthrough in cutting costs.

The objective of the sequence of fermentation runs was to develop a robust process for production of polyhydroxybutyrate from wheat straw hydrolysate. The team at Biotrend evaluated different carbon sources to study the impact of hydrolysate composition on bacterial growth and PHB formation. They then validated the process by scaling up 100-fold, starting from a 2 L working volume, in a step-by-step approach using a constant tip speed-based scale up strategy.

Table 1: Feed compositions used in four (I-IV) 2 L fermentations; Hydr.: average composition of wheat straw hydrolysate

	Feed			
	Glucose [g/L]	**Xylose [g/L]**	**Total [g/L]**	**Xylose [%]**
I	573	270	843	32
II	532	315	847	37
III	491	360	851	42
IV	450	405	855	47
Hydr.	465	269	733	37

Material and Methods

Precultures

B. sacchari was repeatedly sub-cultured in shake flasks using a medium that contained sucrose as the sole carbon source to create a population rich in cells with improved sucrose uptake. After eight subcultures, a culture bank was prepared in cryogenic tubes and stored at -80°C.

The stored cells were used to inoculate shake flasks containing medium with sucrose as the carbon source and the growth was observed closely. The medium for the seed and flask cultures contained: 1.0 g/L $(NH_4)_2SO_4$, 4.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 1.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L yeast extract, and 0.1 % (v/v) trace elements solution [3], supplemented with 20 g/L sucrose. The cultures grew overnight, at 32°C and 150 rpm, in a New Brunswick Innova 44R shaker (Eppendorf AG, Germany).

Evaluation of feed composition at 2 L

A New Brunswick BioFlo/CelliGen 115 controlled the small-scale fermentation runs in a 2 L vessel (0.8 – 2.2 L working volume). The objective of these fermentations was to study the effect of feed composition on biomass and PHB formulation. The medium (1.3 L initial working volume) for the fed-batch cultures consisted of 4.0 g/L $(NH_4)_2SO_4$, 3.0 g/L KH_2PO_4, 1.7 g/L citric acid, 40 mg/L EDTA, 1 % (v/v) trace elements solution [3], 1.2 g/L $MgSO_4 \cdot 7H_2O$, and 20 g/L sucrose. The pH was adjusted to 6.8 with 5 N KOH. Temperature was controlled at 32°C. The culture was inoculated using 65 mL of the precultures grown on sucrose, with an optical density (OD_{600}) of 20. The fermentation protocol started with a batch phase using the sucrose as the sole carbon source. Once the initial sucrose was depleted in each 2 L fermentation run, it entered the fed-batch phase. Each culture was fed with one of four solutions that had different ratios of xylose to glucose (table 1). The New Brunswick BioFlo/CelliGen 115 automatically added 50 mL pulses of feed each time a sudden agitation rate decrease indicated a lack of carbon source. The reduced oxygen uptake rate (OUR) of the culture when sugars are exhausted causes the dissolved oxygen concentration (DO) to rapidly increase. The equipment, set to maintain the DO at 10 % by varying the stirrer speed, would then automatically decrease the speed, resulting in a detectable feed trigger. Up to 0.8 L of feed was added during the course of the entire fermentation run. The concentrations of the sugars in the feed were measured offline using HPLC. The concentration of the resulting PHB was measured by determining (also by HPLC) the crotonic acid produced by acid digestion of the biomass, assuming total hydrolysis of PHB into crotonic acid. In order to scale up effectively, the engineers maintained the same impeller tip speed at each scale. At larger fermentation scales, as the impeller radius increases, the rotational speed in revolutions per second (rps) must be reduced in order to maintain the same tip speed (in m/s) as in the smaller scale. The upper part of Figure 3 shows the relationship between impeller diameter and tip speed.

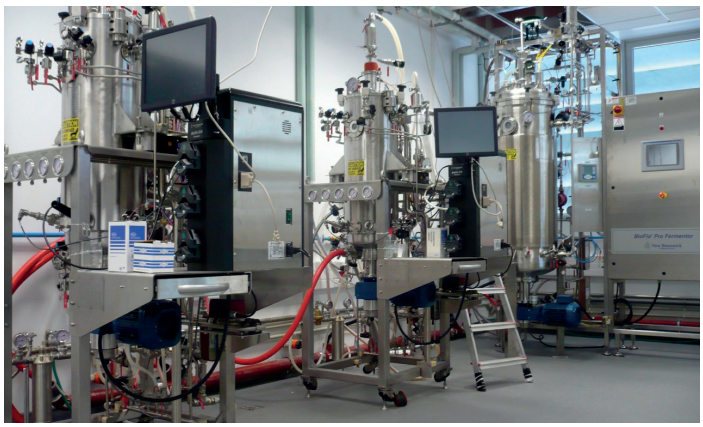

Figure 1: Biotrend facility in Cantanhede, Portugal.

Optimization of feed control at 10 L and 50 L scale

Several fermentations were carried out in 10 L vessels by adapting the optimized fermentation protocol developed at 2 L volume to the larger scale.

A New Brunswick BioFlo 415 benchtop sterilize-in-place unit was used with a 10 L vessel (4.0 – 10.5 L working volume). All fermentations were initiated with 4 L of broth containing 20 g/L of sucrose and 200 mL of inoculum. Feeding started once the initial sucrose had been consumed, using the same automated feeding strategy. All feeds contained 1.5 g/L of phosphate. Another fermentation was performed with phosphate added to the wheat straw hydrolysate. The lignocellulosic hydrolysates were prepared by Biorefinery.de GmbH (Teltow, Germany) from ground wheat straw, using the ammonia fiber expansion (AFEX) process as pre-treatment, followed by enzymatic hydrolysis of the cellulose and hemicellulose fraction [4]. 4 L of feed was added to the vessel.

For the fed-batch phase, the process engineers targeted optimizing the feed rate control switching from an agitation-based feeding to a DO trigger. To avoid the problem of "false" feed triggers caused by momentary increases of the DO readings (e.g., due to accumulated bubbles), they used an algorithm that calculated a moving average of the DO value. It activated feeding, adding a preset volume, when the

moving average DO exceeded 30 %.

They then applied this feeding control mechanism to a 50 L fermentation vessel (16 – 50 L working volume), controlled by a New Brunswick BioFlo 610 (figure 1). They used a simulated hydrolysate containing 472 g/L glucose and 302 g/L xylose as feed and applied the fermentation protocol, using a batch phase with 20 g/L of sucrose as carbon source, inoculated with sucrose-grown precultures. The batch phase volume was 20 L, and a total of 20 L of sterilized feed was added on demand, controlled by the DO trigger.

Scale up to 200 L fermentations

After integrating all the information gathered from the earlier experiments, the engineers devised a 200 L fermentation protocol for use in a New Brunswick BioFlo Pro with 240 L vessel (75.5 – 240 L working volume). The seed train for the fermentation required preparation of a preculture grown in shake flasks. A 6 L inoculum was grown in a 10 L fermentor and transferred to the larger scale production vessel. The working volume during the initial batch phase was 100 L. Increases in the moving average DO concentration beyond the 30 % threshold triggered feeding of the sugar mix (520 g/L glucose and 300 g/L xylose) during fermentation. Each feeding pulse lasted for 15 min, and added about 2.7 kg of sugars to the fermentor, for a total of approximately 90 L of feed during the fermentation run.

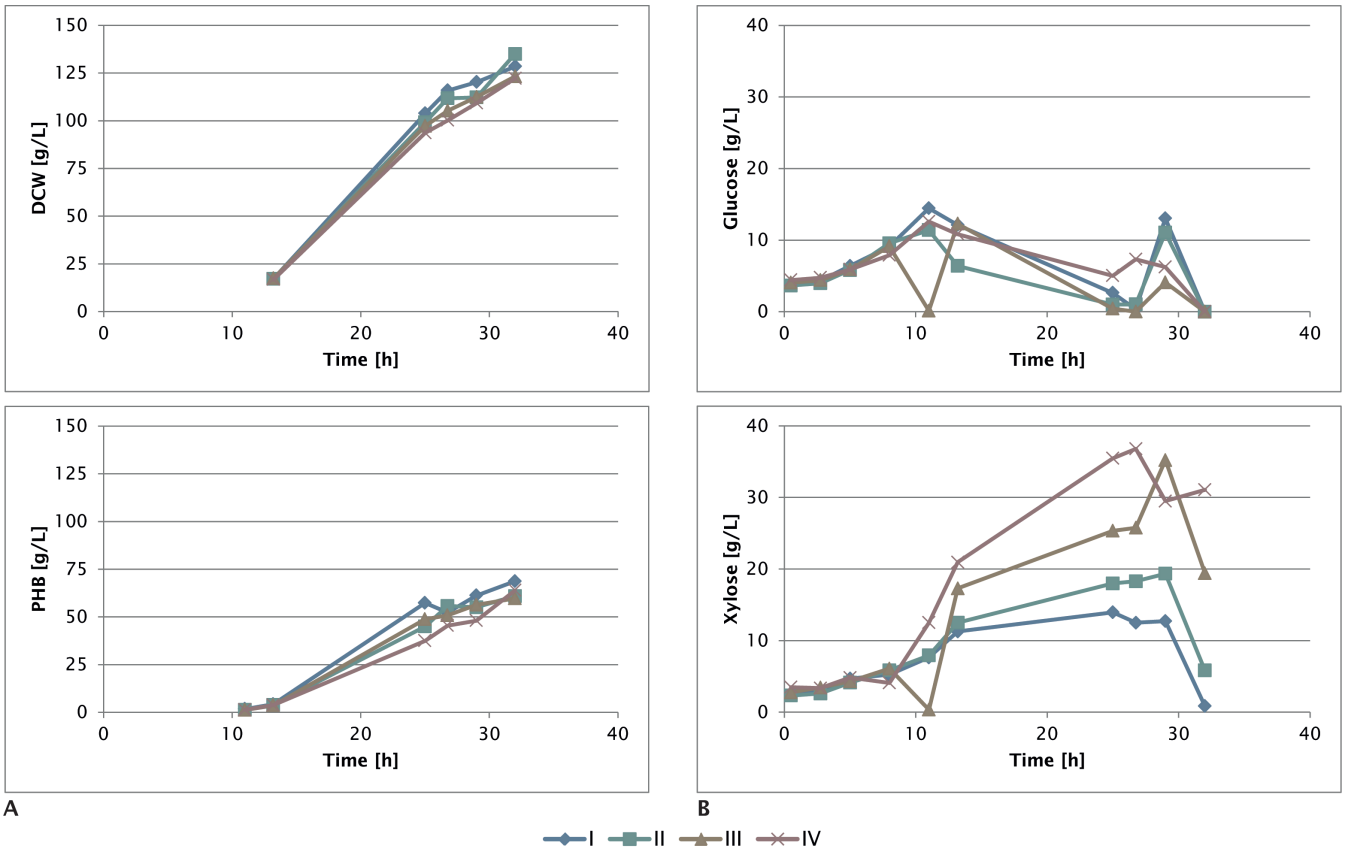

Figure 2: Growth and PHB formation in four 2 L fermentations (I – IV, see table 1) fed with feeding solutions containing varying glucose/xylose ratio. **A**: Dry cell weight (DCW) and PHB concentration; **B**: Glucose and xylose concentrations

Results and Discussion

All 2 L fermentations showed similar dynamics in biomass and PHB formation, and feeding proceeded at almost identical rates (Figure 2). Broth xylose concentrations increased with increasing concentrations of xylose in the feed. Once feeding was stopped at the end of the fermentation, all xylose was consumed. Apparently the accumulated xylose (up to 35 g/L) did not inhibit the metabolic activity of the cells, consistent with previously

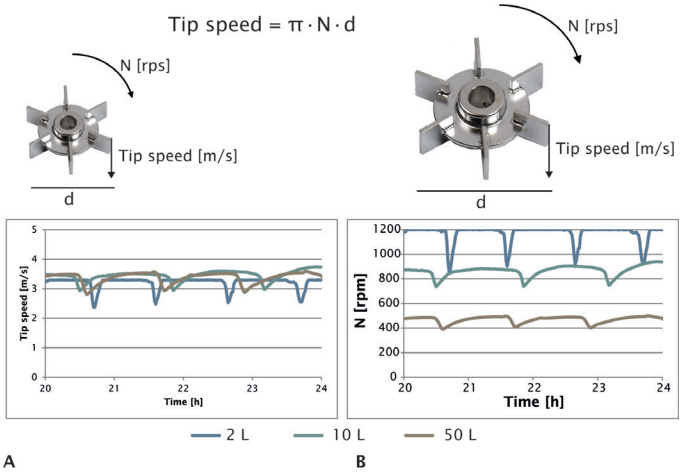

Figure 3: Comparison of turbine tip speed and stirring speeds when controlling the dissolved oxygen concentration at 10% in fermentations at three different scales

reported results. Earlier experiments had shown that xylose concentrations higher than 40 g/L did inhibit growth [5]. The results suppose that the developed fermentation strategy will work without problems with real hydrolysates even if their ratio of xylose to glucose varies.

Figure 3 shows the tip speeds calculated from the rotational stirring speeds obtained in fermentations at 2 L, 10 L, and 50 L scales when controlling the fermentations at the same dissolved oxygen level (10 % of saturation). The oxygen transfer required to maintain a specific dissolved oxygen concentration is highly correlated to the tip speed of the impellers. What is more, the figure clearly indicates that the sudden decrease of stirring speed that occurs at the 2 L scale in response to DO increases is less dramatic at the larger scales.

When transferring the 2 L protocol to the 10 L scale, the fermentation resulted in a high concentration of biomass, but the PHB content in the cells was relatively low (data not shown). This led to speculate that higher PHB yields might be obtained by lowering the phosphate content of the feed.

In a next step, a DO-based feed mechanism was implemented. At higher fermentation scales, stirring speeds need to be lowered in order to maintain the same tip speed as in the smaller scale. As the impeller diameter increases, the range of stirring speeds during the fermentation will decrease.

When sugar was present during the fed-batch phase of the 10 L fermentation, the average DO value fluctuated between 0 % and 10 %. It increased rapidly to 30 – 40 % when the sugar in the broth was spent. At this point, a pre-set feed volume was added to the fermentation. The strategy worked flawlessly at the 10 L scale, using a glucose/xylose mixture as feed, providing a reliable means for feed control. In fact, the responsiveness in returning DO levels to the normal range was faster than the agitation-controlled feed used for the 2 L fermentation, with DO-triggered feeding (primary response) responding within 3 – 4 minutes while the agitation trigger (secondary response) took 5 – 10 minutes (data not shown).

The next fermentation, at 50 L scale, accumulated amounts of biomass and PHB equivalent to those produced

Table 2: Comparison of biomass and polyhydroxybutyrate (PHB) attained at 2 L, 10 L, 50 L, and 200 L fermentation scales; DCW = dry cell weight, $Y_{X/S}$ = yield coefficient for biomass production, $Y_{P/S}$ = yield coefficient for PHB production

Working volume	Feed	DCW [g/L]	PHB [g/L]	PHB [%]	$Y_{X/S}$ [g/g]	$Y_{P/S}$ [g/g]
2 L	Hydrolysate	123.4	63.6	51.6	0.42	0.23
10 L	Hydrolysate	123.0	64.6	52.5	0.40	0.19
50 L	Sugar mix	101.6	56.3	55.4	0.42	0.25
200 L	Sugar mix	129.0	71.1	56.2	0.45	0.22

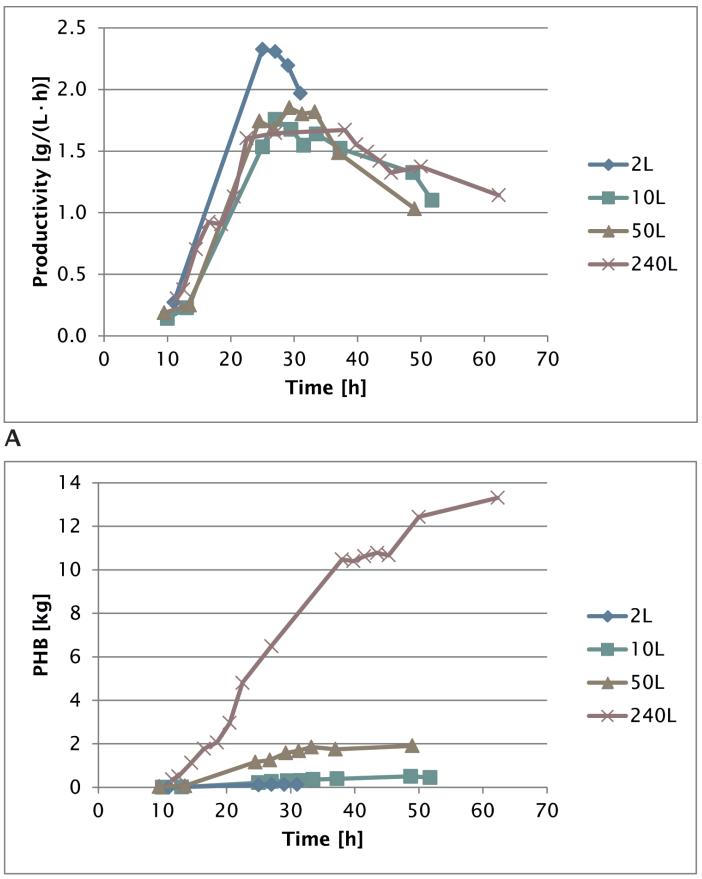

Figure 4: Comparison of productivity and total PHB produced in 2 L, 10 L, 50 L, and 240 L fermentation vessels

at smaller scales (table 2). The dynamics of biomass and PHB levels were similar to those at smaller scales, showing similar glucose and xylose profiles (data not shown). The DO increase in response to exhaustion of glucose diminished towards the end of the fermentation, and the DO trigger ceased to function. At this point, a suboptimal constant feed rate was imposed. It resulted in a decrease of the amount of accumulated xylose in the broth and a decrease in biomass and PHB yields. In the fermentation trials carried out, it was observed that whenever a period of a couple of hours was allowed to elapse between the exhaustion of glucose and the beginning of the feed—forcing the strain to grow exclusively on xylose—the metabolic activity of the culture never fully recovered. Hence, the time between glucose exhaustion and onset of feeding should be minimized.

The concentrations of biomass and PHB obtained in the 200 L fermentation were comparable and even in the higher range of those obtained at lower scale. Indeed, the concentration of PHB was the highest obtained, as was the PHB content of the cells (Table 2).

Figure 4 compares the productivities that were attained at the different scales. The highest productivity was obtained at 2 L scale, but the low feed volume (0.8 L) vs. initial batch volume (0.8 L) did not allow to feed the fermentation during long periods of time. As such the productivity dropped after it reached the maximum. Interestingly, the productivity of the 200 L fermentation started very similarly to the one of the 2 L fermentation, while the 10 L and 50 L fermentations showed time lags. This was caused by the delayed feed described above. The short inflexion of the 200 L productivity curve at about 1 g/(L·h) at 200 L scale suggests that there is further room for optimization of the feeding. But the long productivity plateau at the maximum value of about 1.7 g/(L·h) indicates that during that time the system kept producing PHB in such quantities that it allowed the productivity (PHB produced per unit of volume and unit of time) to stay constant at its maximum value. This is especially remarkable, because the volume of the fermentation broth was continuously increasing, suggesting that the product concentration would drop. Maintaining high productivity is a primary goal when optimizing a bioprocess.

Conclusion

The results confirm that the process engineers at Biotrend achieved a successful fermentation scale up and validated the scalability of the process. Despite the challenges of scaling up bioprocesses, fine-tuning the fermentation protocol in smaller scale transitions resulted in robust and scalable processes. Scale up based on maintaining a constant tip speed is a common strategy, but adjustments may be necessary to manage a scalable yield. The careful, rational, and step-by-step approach, using 5-fold scale increases worked well. The sequence of fermentation trials at successively larger scales was crucial to gathering enough information to successfully deploy a productive process at the largest scale on its first attempt. Overall, the scientists achieved a 100-fold scale increase. Though further improvements are possible, and some opportunities for further optimization have been identified, they have shown that the process is low risk and robust as it scales. It suggests that the same protocol would work at even higher scales in commercial production.

Literature

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Ordering information

Description	Order no.
New Brunswick™ BioFlo® 115, Configured Master Control Station with TMFC	Contact us for details
New Brunswick™ BioFlo® 115, 2 L Advanced Fermentation Vessel Kit	M1369-1602
New Brunswick™ BioFlo® 415	Contact us for details
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