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Redox Potential Monitoring for Improved Anaerobic Fermentation Using the BioFlo® 120 Bioprocess Control Station and BioBLU® 3f Single-Use Vessels

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Abstract

In fermentation, redox potential is an important physiochemical factor that measures the tendency of the culture medium to acquire electrons. It can directly influence bioprocess efficiency. For example, in the anaerobic fermentation of *Clostridium* to produce industrial solvents, the redox potential relates to the intracellular ratio of $NAD(P)^+$ to NAD(P)H, which in turn influences the production of solvents, including butanol.

In this study, we cultivated *C. beijerinckii* under anaerobic conditions in BioBLU 3f Single-Use Vessels. We monitored the redox potential of the fermentation broth online using ISM® redox sensors and observed that the redox potential varied throughout the process. When we kept it close to –500 mV by adding sodium sulfide, bacterial growth and butanol production drastically increased compared to a process without redox potential control.

The study demonstrates the suitability of the BioFlo 120 bioprocess control station and the BioBLU 3f Single-Use Vessel for anaerobic fermentation and shows the advantages of redox potential monitoring during *C. beijerinckii* fermentation.

Introduction

Butanol is an important industrial solvent. It can be produced by fermenting sugars using the obligate anaerobic *Clostridium* species. As a biofuel, butanol has advantages over ethanol due to its greater heating value, flexibility in blending with gasoline, and compatibility with the current pipeline infrastructure [1]. Historically, butanol bioproduction was largely performed by *Clostridium acetobutylicum* through acetone/butanol/ethanol (ABE) fermentation. *Clostridium beijerinckii* can further convert acetone into isopropanol, another useful organic solvent widely used in laboratory research and the printing industry [2]. Solvent production by *Clostridium* species follows two consecutive metabolic steps, which relate to the growth phase. In an early culture, acidogenesis produces mainly acetic and butyric acid. In the late exponential growth phase, the metabolism switches to solventogenesis, which produces largely organic solvents including butanol [3]. To increase the efficiency of butanol bioproduction, researchers aim to establish culture conditions that favor robust solventogenesis. To direct the electron flow towards butanol production, regeneration of the $NAD(P)^+$ pool in the *Clostridium* species is essential [3]. The intracellular ratio of NAD(P)H and $NAD(P)^+$ is highly related to the redox potential. Therefore, control of the redox potential can be used to alter biomass accumulation and solvent production [4].

The objective of this study was to test the suitability of the BioFlo 120 control station equipped with BioBLU 3f Single-Use Vessels for anaerobic fermentation. Furthermore, we demonstrated how to monitor the redox potential with this control system and tested its influence on *C. beijerinckii* growth and butanol production.

Material and Methods

Strain and medium

In this study, we used the anaerobic bacterium *Clostridium beijerinckii* (ATCC® 6014).

We used two types of cultivation media for inoculum preparation and fermentation in the bioreactor [1]. The inoculation medium contained 5 g/L glucose, 5 g/L BD Bacto™ proteose peptone No. 3, 5 g/L yeast extract, 5 g/L sodium thioglycolate, 5 g/L potassium phosphate monobasic, and 0.002 g/L methylene blue as oxygen indicator. We adjusted the pH to 6.5. The fermentation P2 medium contained 30 g/L glucose, 1 g/L yeast extract, 1 g/L tryptone, and 0.002 g/L methylene blue as oxygen indicator. We adjusted the pH to 6.5. After sterilization by autoclaving, we added 1 % (v/v) sterile nutrient stock solution to the P2 medium. The 100x nutrient stock solution contained 50 g/L potassium phosphate monobasic, 50 g/L potassium phosphate dibasic, 220 g/L ammonium acetate, 0.1 g/L 4-aminobenzoic acid, 0.1 g/L thiamin, 0.001 g/L biotin, 20 g/L magnesium sulfate heptahydrate, 1 g/L manganese(II) sulfate monohydrate, 1 g/L iron(II) sulfate heptahydrate, and 1 g/L sodium chloride. It was sterilized by membrane filtration.

We purchased all medium components from Sigma Aldrich® (USA), except the BD Bacto proteose peptone No. 3, which was from Becton Dickinson® (USA).

Inoculum preparation

We obtained *C. beijerinckii* spores from ATCC. We suspended the spores in 1.5 mL sterile inoculation medium and evenly transferred the suspension to three or four 1.5 mL Eppendorf Safe-Lock Tubes. The spores were activated by heating at 80 °C for 10 min in a water bath, and then cooled to room temperature on ice. We transferred 150 µl to a 15 mL Eppendorf conical tube containing 10 mL sterile inoculation medium. Usually, 10 conical tubes were prepared for inoculation. The conical tubes were loosely capped, placed on a culture tube holder, and transferred to an anaerobic jar (BD GasPak® 100 System, Becton Dickinson, USA) with a fresh Oxoid™ AnaeroGen® 2.5 L sachet (Oxoid GmbH, Germany). We placed the jar in a New Brunswick™ Excella® E24 Incubator Shaker (Eppendorf, Germany) at 37 °C. With the establishment of anaerobic conditions, the blue color from the anaerobic indicator dye, methylene blue, gradually disappeared. After 3 to 5 days, when the medium began turning turbid and gas bubbles formed, 400 µL of the *C. beijerinckii* suspension were transferred to a 50 mL Eppendorf conical tube containing 40 mL sterilized fermentation P2 medium. The 50 mL conical tubes were loosely capped, transferred to another anaerobic jar (Sigma-Aldrich, USA) with a fresh AnaeroGen 2.5 L sachet, and placed in the incubator at 37 °C. Two to three days later we used this *C. beijerinckii* suspension to inoculate the culture in the BioBLU 3f vessel at a ratio of 1:100 (v/v). The preparation of inoculum is illustrated in Figure 1A. Figure 1B shows the *C. beijerinckii* cultures in the 50 mL conical tubes within the anaerobic jar.

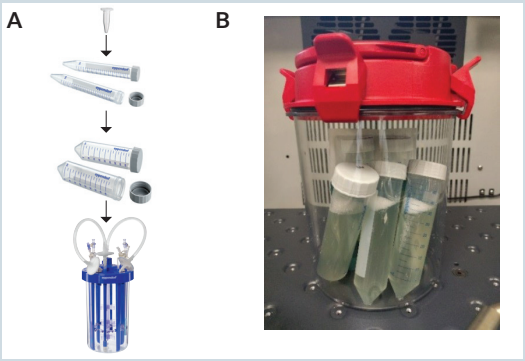

Fig. 1:
A: Schematic of the *C. beijerinckii expansion process*
B: Anaerobic jar

Fermentation in BioBLU 3f Single-Use Vessels

We performed batch fermentations using an Eppendorf BioFlo 120 bioprocess control station equipped with BioBLU 3f Single-Use Vessels (Figure 2). We filled each of the vessels with three liters of fermentation medium. One port on the bioreactor head plate was replaced by a single-use septum (Eppendorf, Germany). After autoclaving and cooling of the medium to 37° C, we added 30 mL of the 100x

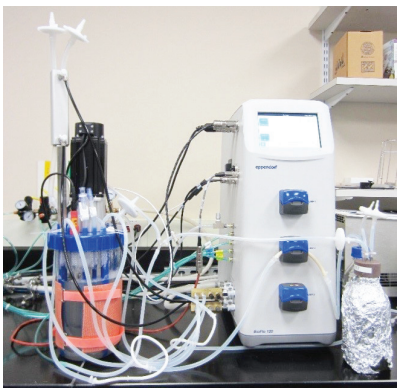

Fig. 2:
Bioprocess equipment

nutrient stock solution and 30 mL of the inoculum, through the septum.

We cultivated *C. beijerinckii* under anaerobic conditions, as described below. The temperature was controlled at 37 °C, and the cultures were agitated at 50 rpm. We conducted two batch fermentation runs. In the first run we did not control the redox potential. In the second run, we controlled the redox potential to a relatively low and stable redox potential of –500 mV as described below. The -500 mV level was chosen based on the base line data generated from the first run.

The process parameters and setpoints common to both runs are summarized in Table 1.

Table 1: Overview of process parameters and setpoints

Parameter	Device/setpoint
Inoculation density	1:100 (v/v)
Working volume	3 L
Sparger	Macrosparger
Gassing control	100 % constant nitrogen flow at 0.1 vvm (0.3 SLPM), first 4 hours through sparging, after 4 hours through overlay
Agitation	Magnetic drive; 50 rpm
Temperature	37 °C; controlled by heat blanket and cooling baffles

Anaerobic conditions

We established anaerobic conditions by constantly gassing with nitrogen at 0.1 vvm. In a test run, we monitored the dissolved oxygen concentration using a DO sensor. DO remained zero throughout the process. In the following runs, we did not measure DO with a sensor, but monitored the anaerobic state with the indicator dye methylene blue.

Sensors

We monitored pH and redox potential online using two ISM pH/redox sensors (Mettler Toledo®, Switzerland). The sensors were installed with a single-use compression fitting (Eppendorf, Germany) before sterilization of the medium-filled vessel. The same sensor type can be utilized to measure either pH or the redox potential, and the choice can be made in the setup section of the bioprocess control software (Figure 3).

Sensor calibration

We calibrated the pH and redox sensors outside the vessel, using 2-point calibration methods. For pH sensor

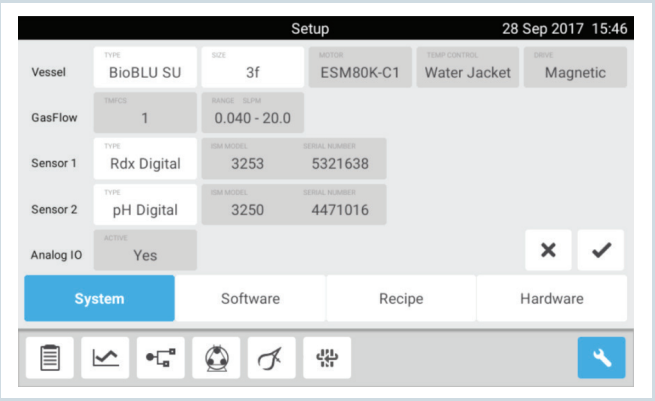

Fig. 3: The screenshot shows the setup page of the BioFlo 120 bioprocess control station monitoring a BioBLU 3f Single-Use Vessel equipped with ISM pH/redox sensors.

calibration, we used a buffer at pH 7.00 to set ZERO, and a buffer at pH 4.00 to set SPAN (please refer to the BioFlo 120 operating manual for details). As standards for redox sensor calibration, we prepared two solutions, saturated with respect to quinhydrone (2 g/L, Sigma-Aldrich, USA), in pH 7.00 and pH 4.00 buffer. We used the quinhydrone solution at pH 7.00 to set ZERO at a redox potential of 86 mV, and the quinhydrone solution at pH 4.00 to set SPAN at a redox potential of 263 mV. A commercial redox buffer solution (e.g. from Mettler Toledo, 51350060) can be used to validate the calibration. For this buffer, a calibrated sensor should read a redox potential of around 220 mV.

Adjustment of redox potential

We adjusted the redox potential of the fermentation broth with 35 g/L sodium sulfide nonahydrate (Sigma-Aldrich, USA). We prepared the solution freshly at the beginning of the fermentation run, filtered it through a 0.2 µm membrane filter, and kept in a 1 L foil-wrapped Pyrex® bottle. To avoid oxygen introduction we pre-sparged the solution with nitrogen for 15 min before pumping it into the vessel. The pH of the solution was 12.84.

To maintain the redox potential at approximately –500 mV, 24 h, 32 h, 48 h, and 120 h after inoculation we turned on the pre-calibrated pump to add 2 – 3 mL sterile filtered sodium sulfide solution to the broth.

Analytical methods

We measured pH and redox potential online using the ISM pH/redox sensors.

To monitor bacterial growth, we took 1 mL samples of the

fermentation suspension and measured the optical density at 600 nm (OD_{600}) using an Eppendorf BioSpectrometer®.
To quantify the concentrations of glucose and butanol, we took two 1 mL samples every 24 hours. We pelleted the bacteria by centrifugation in a MiniSpin® plus microcentrifuge at 14,000 rpm for 90 s and used the supernatants for further analysis.

We quantified the glucose concentrations with a Cedex® Bio Analyzer (Roche Diagnostics®, Germany).

We determined the butanol concentration with the NAD-ADH Reagent Multiple Test Vial (Sigma-Aldrich, USA) according to the manufacturer's instructions [5].
We measured the optical density at 340 nm using an Eppendorf BioSpectrometer. To calculate the butanol concentration of the sample based on a given OD_{340} value, we created a standard curve to correlate the optical density at 340 nm with the concentration of butanol between 0 and 10 g/L [6]. The equation was $OD_{340} = -0.0045 \times (C_{butanol})^2 + 0.0825\ C_{butanol}$ ($C_{butanol}$ in g/L, R^2=0.9726).

Results

To test for changes in the redox potential throughout the duration of the culture, we monitored it using redox sensors. We compared bacterial growth and butanol production in two processes. In one process we kept the redox potential relatively stable at –500 mV by adding sodium sulfide nonahydrate 24 h, 32 h, 48 h, and 120 h after inoculation. In the other process we did not experimentally alter the redox potential.

Redox trends

At the beginning of the fermentation run, the redox potential was around 0 mV. During the first 24 h, and with the exponential growth of *C. beijerinckii*, it dropped drastically to –500 mV. When we did not experimentally alter the redox potential during the fermentation run, it varied between –600 and –300 mV from 24 h after inoculation onward (Figure 4). When we added sodium sulfide nonahydrate to the culture as needed, starting 24 h after inoculation, a relatively stable redox potential of –500 to –400 mV was maintained. Interestingly, the redox potential increased immediately by 10 – 50 mV every time that 2 – 3 mL sodium sulfide solution was pumped into the broth. As fermentation progressed, the redox potential dropped below the initial value. It appeared that the addition of sodium sulfide helped to create a better buffered system for maintaining a reduced redox potential in *C. beijerinckii* fermentation. Variation over this wide range could affect the electron flow in the microbial metabolic pathway. The redox potential is closely correlated with the accumulation of $NAD(P)^+$ in *C. beijerinckii*, which directs butanol production [3]. Therefore, setting the redox potential to enable continuous regeneration of the $NAD(P)^+$ pool proved to be an appropriate method to manipulate the *C. beijerinckii* metabolism [4].

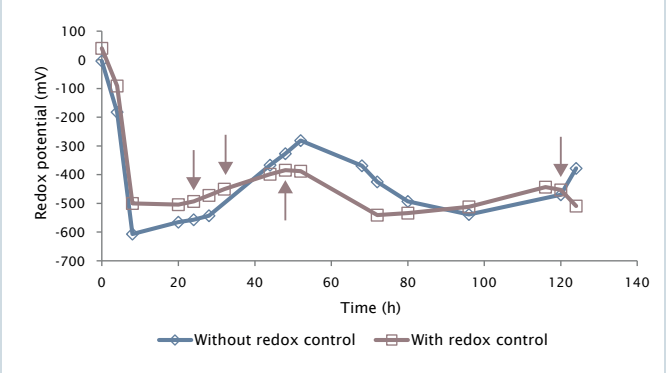

Fig. 4: Redox potential trends. In the run with redox control, sodium sulfide nonahydrate was added 24 h, 32 h, 48 h, and 120 h after inoculation (arrows).

pH trends

The development of the pH in both cultures is compared in Figure 5. During the exponential growth phase in the first 24 h of the culture, the pH decreased from 6.5 to 5.1 in both runs. It is likely that acidogenesis took place, and acetic and butyric acid were produced. In the run without redox control, pH stayed relatively low at 5.0-5.1 with no trend toward rebound throughout the entire culture period, indicating the lack of a dominant solventogenesis phase. By contrast, in the redox-controlled fermentation the pH began to recover after 24 h, approached a value of 6.0 after 70 h, and then gradually dropped to 5.5 until the end. It is likely that the curved pH trend was caused by a shift in the microbial metabolism. Butanol production may have required the

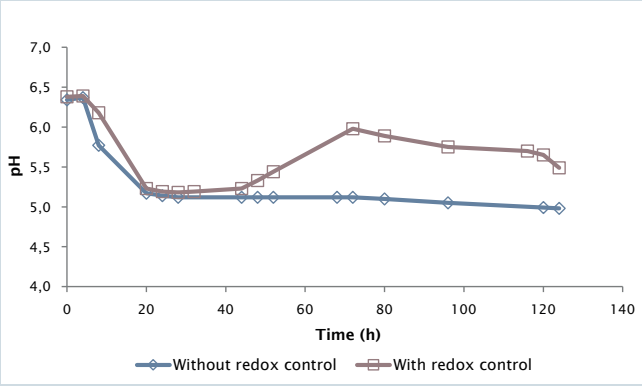

Fig. 5: pH trends

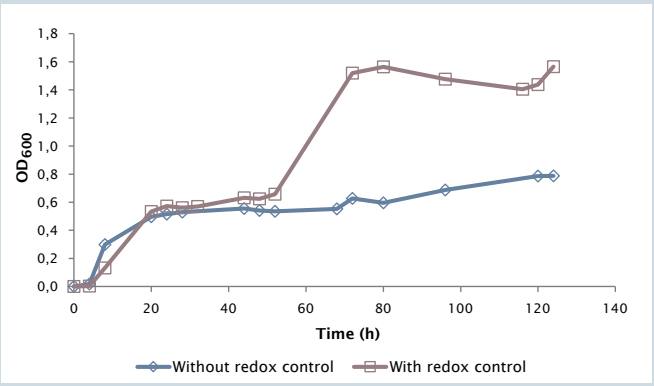

Fig. 6: *C. beijerinckii* growth curves

consumption of acetic acid, leading to a product shift from butyric acid to butanol. The decrease in acid concentration likely caused the pH increase.

Bacterial growth

We measured the growth of *C. beijerinckii* by quantifying the optical density at 600 nm (Figure 6).

During the first 24 h, we observed comparable exponential growth in both cultures. The OD_{600} increased from 0 to about 0.5. Meanwhile, the blue color of the medium disappeared with the establishment of an anaerobic environment in the vessel. In the run without redox control, the growth slowed down significantly after 24 h and finally ended at an OD_{600} of 0.788 at 124 h. In contrast, *C. beijerinckii* continued to grow robustly when we maintained the redox potential at –500 mV, especially between 48 h and 72 h after inoculation. The OD_{600} increased 2.4 fold from 0.625 to 1.519 during this time. We observed foam accumulation during the first 72 h. The final OD_{600} at 124 h was 1.565. This is two-times higher than in the run without redox control.

Glucose consumption and butanol production

Glucose consumption and butanol production trends are summarized in Figure 7. When we did not experimentally alter the redox potential, the culture consumed 33 % of the initially supplied glucose, the majority during the first 24 h. The butanol concentration at the end of the run was 0.83 g/L. When we controlled the redox potential at –500 mV, the culture consumed 51 % of the initially supplied glucose and the consumption rate was relatively stable over the entire fermentation. Butanol was also continuously produced from t = 8 h, reaching a final concentration of 2.43 g/L. This is approximately three times more than in the run without control of the redox potential. The increase of butanol production toward the late stationary growth phase further indicated that robust solventogenesis took place when redox potential was maintained close to –500 mV.

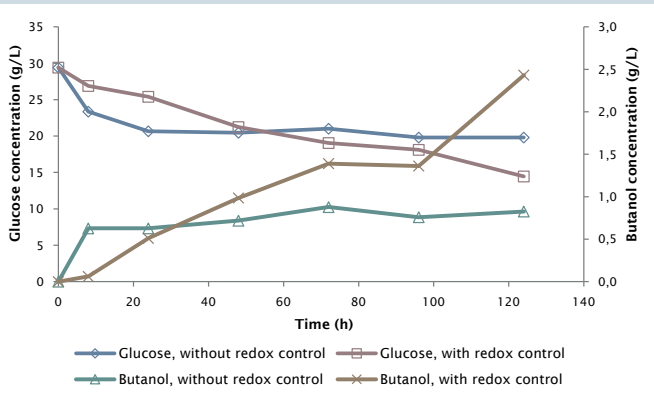

Fig. 7: Glucose and butanol concentrations

Conclusion

In this study we have demonstrated the suitability of the Eppendorf BioFlo 120 bioprocess control station equipped with BioBLU 3f Single-Use Vessels for anaerobic fermentation of *C. beijerinckii*.

The redox potential of the fermentation broth is a critical process parameter in butanol bioproduction by *C. beijerinckii*. We have shown how to monitor the redox potential in the fermentation broth using an ISM pH/redox sensor and the control software of the BioFlo 120 bioprocess control station.

When we experimentally adjusted the redox potential to a relatively stable value of –500 mV, the final biomass was two times, and the butanol yield three times higher than in the run without redox control. This gives an example for bioprocess optimization through monitoring and adjustment of the redox potential throughout the duration of the culture.

Literature

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