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BUTANEXT
Next Generation Biobutanol

D3.4 Performance Data on Cellulosic Sugar with GBL BEST system. Butanol productivities > 0.75g/L/h.

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1. EXECUTIVE SUMMARY

In work package 3 GBL's aim was to improve fermentation performance by optimising the medium formulation and process control parameters. JMP™ Design of Experiments software (SAS Institute Inc.) was used to optimise the performance of GBL6095 on a representative lignocellulosic feedstock. All fermentations were performed at 1.5L scale with a 70:30 blend of C6/C5 sugars from the representative feedstock.

In the first block of DoE, gas stripped fermentations were run to establish the optimum conditions for reduction of the initial batch phase. The factors evaluated were seed type, initial sugar concentration and initial medium pH; the measured response was sugar uptake rate. A statistically significant negative correlation between initial sugar concentration and sugar uptake rate was validated in the AFP™ (formally known as BEST). Reducing the initial sugar concentration, reduced the initial batch phase duration (time before feed initiation) by 77.3% and the overall fermentation time by 22.5% which, assuming comparable solvent production, could lead to significant increases in volumetric ABE productivity. Seed type and initial medium pH had no significant effect on sugar uptake rate and thus the length of the initial batch phase.

The factors in the second block of DoE were: pH set point, temperature and nutrient concentration in the feed. The responses were: ABE yield (g/g), ABE productivity (g/L.h) and butanol ratio (%). The optimum conditions from the first round of experiments were implemented i.e. lower initial sugar concentration. However, the yield and productivity results obtained were substantially lower than those from the first block of DoE. This was attributed to a change within the batch of feedstock (increased inhibitor concentration) due to prolonged storage at low pH.

In May 2016, GBL received samples of a miscanthus enzyme hydrolysate and a wheat straw enzyme hydrolysate from CENER. A bottle screen was carried out to determine at what sugar concentration the hydrolysates began to be inhibitory and determine the rate of sugar consumption for the initial batch stage of the AFP™. Two GBL production strains were evaluated: GBL6095 and GBL6225 (inhibitor tolerant strain). In both hydrolysates GBL6225 had a higher sugar uptake rate than GBL6095 at an industry relevant sugar concentration, indicating possible higher inhibitor tolerance than the parent strain.

Due to concerns over how the change in the quality of the representative feedstock may have influenced the results obtained from the optimisation work, the AFP™ fermentations with the miscanthus and wheat straw hydrolysates were run under GBL's standard conditions. In GBL's AFP™ the project target for butanol productivity was met by GBL6095 on both hydrolysates and by GBL6225 on miscanthus. Butanol productivity on miscanthus hydrolysate was similar for both GBL6095 and 6225. In contrast, the two strains showed a substantial but not statistically significant difference in productivity on the wheat straw hydrolysate, with GBL6095 giving the best performance. The data shows that with these hydrolysate samples GBL6225 offered no real advantage over its parental strain, with GBL6095 giving more consistent and comparable performance on both hydrolysates.

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2. GLOSSARY

ABE – Acetone Butanol Ethanol

AFP – Advanced Fermentation Process (formally known as BEST)

DoE - Design of Experiments

GBL – Green Biologics Ltd.

RCM - Reinforced Clostridial Medium

SBR – Sequential Batch Reactor

SUR - Sugar Uptake Rate

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3. INTRODUCTION

This report covers the technical work performed by GBL during work package 3 of the project (see project plan). In work package 3, GBL's aim was to improve solvent productivity in the AFP™ on a representative lignocellulosic feedstock by optimising the fermentation conditions and control parameters, using DoE to develop an AFP™ model for a GBL production strain on this feedstock. This model would then be used to ferment miscanthus and wheat straw enzyme hydrolysates received from CENER. The aim was to achieve project targets for butanol productivity and butanol yield.

3.1 Aims of the Project

The ButaNexT project aims to develop and validate a more economically and environmentally sustainable process for biobutanol production. It will link technology by using several biomass sources (wheat straw, organic fibre from municipal solid waste and miscanthus), a two-step pre-treatment process (TR) and enzyme cocktails to work synergistically with Clostridium (DYADIC/MetGen). GBL will develop improved butanol and inhibitor tolerant strains and VITO will develop *in situ* product recovery (ISPR) through pervaporation. The technology will then be transferred to CENER for scale up work. The design will underpin the economic and sustainability measures performed in work package 6 (E4TECH).

3.2 The GBL Advanced Fermentation Process (AFP™)

AFP™ is an advanced process in which fermentation, cell recycling and *in situ* solvent removal take place simultaneously. Maintaining solvents below inhibitory levels allows fermentation duration to be extended with improved ABE productivity. The system involves tight control of sugar concentration in the fermenters, which is achieved by the careful and continuous addition of feed.

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4. Baseline AFP™ Data for the Production Strain

This block of experiments was performed in the AFP™ systems. Two GBL strains were tested and compared: GBL6095 and GBL6046.

4.1 Results

The baseline data obtained for the strains tested show that GBL6046 gave higher solvent yield than GBL6095 however spore formation was observed at the end of fermentation, which may diminish the strain's performance in extended runs and give lower than desired results (GBL6095 showed no evidence of spore formation). Both GBL6046 and GBL6095 gave similar butanol ratios. Based on these results GBL6095 was selected for further optimisation work.

5. AFP™ Optimisation with GBL 6095 – 1st Block of DoE

A block of eight fermentations were run to establish the optimum conditions for reduction of the initial batch phase of fermentation. JMP™ statistical software was used to develop a full factorial experimental design to examine the effects of 3 factors: initial sugar concentration in the fermentation medium, initial pH of the medium and the type of seed used to inoculate the production fermenters. The response for comparison was sugar uptake rate (SUR).

The optimum conditions for reduction of the initial batch phase found from the first block of DoE were then validated in duplicate AFP™ systems.

5.1 Results

5.1.1 Optimisation of Initial Batch Phase Fermentation Conditions

Statistical analysis of the data gathered showed that of the three factors tested only initial sugar concentration had a statistically significant effect on sugar uptake rate.

As expected, a lower initial sugar concentration led to earlier initiation of feeding. The feed was started when glucose concentration in the fermenters was below a pre-determined set point. It was observed that by reducing the initial sugar concentration, the duration of the initial batch phase (time before feed initiation) was shortened by 77.3%, regardless of the initial pH or type of seed used. In fermentations with a higher initial sugar concentration, feeding was started later after inoculation. The difference in feed starting point significantly reduced the overall fermentation time by 22.5% without compromising the duration of the fed-batch phase. Overall sugar consumption rate was substantially higher in the fermentations with lower initial sugar concentration.

There was substantial sugar accumulation observed in all fermentations with higher initial sugar concentration. The runs with lower initial sugar concentration had much lower residual sugars at the end of the fermentation. This could suggest that younger cultures i.e. where feeding was started earlier, have better capability of utilising the sugar fed. This observation might be attributed to lower feedstock stress (lower sugar and inhibitor concentrations) on the cells in the fermentations with lower initial sugar and/or the different physiological stage of the cultures at initiation of and during the fed batch phase.

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5.1.2 AFP™ Validation of Optimised Conditions

Validation of the first block of DoE in the AFP™ systems confirmed the results obtained from the gas stripped fermentations i.e. the performance of GBL6095 can be improved by reducing initial sugar concentration in the fermentation medium. As expected, low initial sugar concentration allowed for prompter start of the feed and reduced the overall fermentation time by ~25%. There was 14.8% improvement in ABE yield and a 21.6% increase in productivity compared to the baseline data as a result of reducing initial sugar concentration in the fermenters. No significant increase in butanol ratio was observed.

6. AFP™ Optimisation – 2nd Block of DoE

The next step in process optimisation was a second block of DoE aiming to further develop and optimise AFP™ conditions in order to improve solvent production. JMP™ statistical software was used to develop a 3 level response surface design (Box-Behnken) to examine the effects of and interactions between 3 factors: pH set point, temperature and nutrient concentration in the feed. The responses for comparison were ABE yield on sugar fed (g/g), solvent (ABE) productivity (g/L.h) and butanol ratio. A block of 13 fermentations was run as per the design with implementation of the optimum conditions from the 1st block of DoE i.e. lower initial sugar concentration.

6.1 Results

The 2nd block of DoE was designed to improve and optimise solvent production but an unforeseen change in feedstock quality had a major effect on fermentation performance negating the results. A new batch of feedstock was used in the 2nd block of DoE experiments. This was from the same drum of material as the batch used in the 1st block but had been stored longer by the supplier. HPLC analysis showed a substantial increase in the inhibitor concentration in the 2nd batch, a likely consequence of the extended hold at low pH. Statistical analysis of the AFP™ experimental data from the two batches showed that the 2nd batch of feedstock gave consistently lower solvent productivities.

7. Bottle Screen Evaluation of Miscanthus and Wheat Straw Hydrolysates

On the 12th May 2016, GBL received 15L each of miscanthus (GBL ref: F-CEN1) and wheat straw (GBL ref: F-CEN2) enzyme hydrolysates (MetZyme SUNO V005) from CENER. These hydrolysates were centrifuged to remove the particulates and the supernatant (8L of each) stored at -20°C until required. To provide initial information about fermentation performance on these two hydrolysates, they were screened in bottles using GBL production strains 6095 and 6225 (inhibitor tolerant strain produced in WP3). A further aim was to understand the growth characteristics for the initial batch stage of the AFP™. Three different sugar concentrations of each hydrolysate were screened in 100 mL serum bottles (60 mL working volume); the experimental control was a 2:1 mix of pure glucose and xylose. Throughout the bottle screening, samples were taken for sugar and solvent quantification by HPLC, from which sugar uptake rates, solvent productivities and yields were calculated.

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7.1 Results

Peak sugar uptake rates and solvent productivities were higher for both strains on Wheat Straw than on Miscanthus with good growth and productivity at intermediate sugar concentration (both hydrolysates were slightly inhibitory at high sugar concentration). The HPLC analysis of the hydrolysates support these observations as Wheat Straw contained a lower concentration of inhibitors than Miscanthus. In both hydrolysates GBL6225 had a higher sugar uptake rate than GBL6095, which may indicate higher inhibitor tolerance than the parent strain.

8. AFP™ Evaluation of Miscanthus & Wheat Straw Hydrolysates

The miscanthus (F-CEN1) and wheat straw (F-CEN2) hydrolysates provided by CENER were run in duplicate 1.5L AFP™ fermentations to determine the performance of GBL production strains 6095 and 6225. The fermenters were inoculated with secondary seed from the standard seed train. The aim was to meet project targets for butanol productivity and yield.

8.1 Results

The target for overall butanol productivity was exceeded by both strains on the miscanthus hydrolysate and by GBL6095 on wheat straw hydrolysate. GBL6225 failed to meet the project butanol productivity target on wheat straw hydrolysate. Butanol productivity on miscanthus hydrolysate was similar for both GBL6095 and 6225. Overall, better butanol productivity was achieved on the miscanthus hydrolysate with GBL6225. In contrast, the two strains showed a substantial but not statistically significant difference in butanol productivity on the wheat straw hydrolysate. The performance of GBL6095 was consistent on both hydrolysates, with similar butanol productivities achieved on both hydrolysates and less variability between the duplicate fermentations than GBL6225. The butanol yield target was only achieved by GBL6095 on miscanthus based on sugar fed (based on sugar used the target was also met by GBL6225 on miscanthus but neither strain reached the target on the wheat straw hydrolysate). Based on this data, the adapted strain (GBL6225) seems to offer little benefit over the parent strain (GBL6095) and there were issues with performance consistency.

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9. Conclusion

In work package 3 we were able to significantly reduce the initial batch and thus overall fermentation time by reducing the initial sugar (and thus inhibitor) concentration. In the first round of optimisation, a 14.8% improvement in ABE yield and a 21.6% increase in ABE productivity was achieved compared to the baseline data. Unfortunately the second round of optimisation was adversely affected by intra-batch variation of the representative lignocellulosic feedstock; prolonged storage at low pH had generated a substantial increase in the concentration of inhibitors present.

Miscanthus and wheat straw enzyme hydrolysates provided by CENER were tested and we demonstrated that we were able to meet the project targets for butanol productivity and butanol yield with GBL6095 on miscanthus hydrolysate. The inhibitor tolerant strain developed in this work package was not demonstrated to offer consistent performance improvement over its parent strain (GBL6095) on these hydrolysates in GBL's AFP™ but it has been shown to offer improved performance on other cellulosic feedstocks.

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