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Probing control in *B. licheniformis* fermentations

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Abstract: This paper presents the implementation of the probing control technique in an industrially relevant fed-batch fermentation process utilizing the microorganism *Bacillus licheniformis*, which has been done in pilot-scale experiments. The aim of the work is firstly to find out whether it is possible to successfully implement probing control in *B. licheniformis* fermentations. Secondly, it is to find out whether probing control can improve process stability (defined as the ability to avoid process crashes) while achieving a yield at least as high as in the current process. In addition to presenting a successful outcome in regard to these aims, the paper also discusses possible process improvements and suggests a new type of probing control.

Keywords: Probing control; Process control; Fermentation processes; Industrial production systems; Biotechnology

1. INTRODUCTION

Bacillus licheniformis is a bacterium used in industrial production of enzymes. To achieve as high yield as possible in such processes the bacteria must be provided with an optimal environment at all times, which is the purpose of automatic control in fermentation processes. In fed-batch fermentation processes, the feed rate of substrate makes a suitable variable to manipulate in order to control the process.

To achieve efficient control of the feed rate in fed-batch fermentation processes several factors need to be taken into consideration, such as oxygen limitation, overfeeding and starvation. Overfeeding leads to overflow metabolism and thereby the production of by-products, such as acetate. These are typically harmful to the organism (Luli and Strohl [1990]) and it is believed that overflow metabolism causes occasional crashes in industrial *B. licheniformis* fermentations. The challenge in feed rate control is therefore to find a feed rate which gives optimal bacterial growth and enzyme production while avoiding these phenomena.

Probing control is a type of control for fed-batch bioprocesses which utilizes the characteristical saturation of the oxidative capacity in the system when the critical limit for overflow metabolism is reached (Fig. 1), to achieve a feed rate which is as high as possible without exceeding the critical limit. Probing control was originally developed in *Escherichia coli* cultivations (Åkesson [1999]) but has since been successfully implemented in cultivations utilizing various microorganisms, such as *Vibrio cholerae* (de Maré [2006]), *Saccharomyces cerevisiae* (Rudolf et al. [2007]) and *Pichia pastoris* (Henes and Sonnleitner [2007]). In this work, the possibilities for implementing probing control in an industrially relevant pilot scale protein production process utilizing the bacterium *B. licheniformis* have been investigated. The aim was to achieve a working controller which can avoid overflow metabolism and therefore decrease the risk of crashing while keeping a yield which is at least similar to what is achieved when using an existing control strategy.

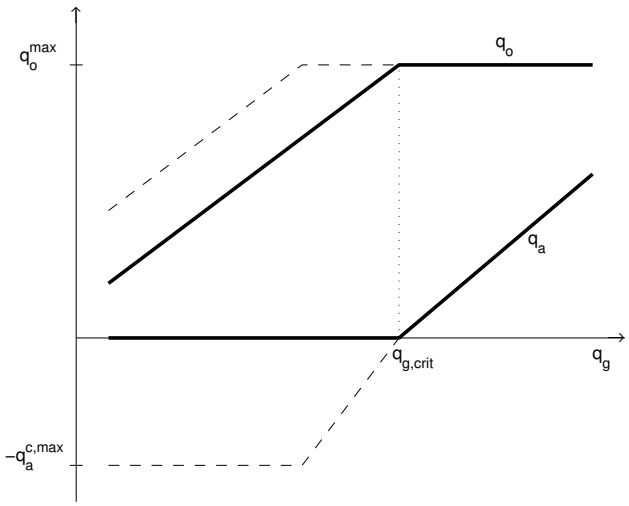

Fig. 1. Oxygen uptake rate q_o, acetate production rate q_a and acetate uptake rate q_a^c plotted against glucose uptake rate q_g. This shows the saturation in the oxidative capacity of the system which occurs when q_g reaches the critical glucose uptake rate for overflow $q_{g,crit}$. The dashed lines illustrate uptake of acetate.

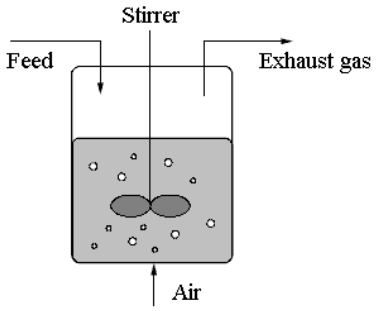

Fig. 2. A fed-batch bioprocess in a stirred tank with an in-flow of feed (Åkesson [1999]).

2. THE PROCESS

The process was run in stirred-tank fermentors, operated in fed-batch mode with an inlet of feed (glucose), which is the typical mode of operation for a modern bioprocess. For an illustration of a typical stirred-tank fed-batch process, see figure 2. 20 l fermentors were used during the development of the controller, while during the evaluation of the controller the process was scaled up to use 2.5 m^3 fermentors.

The product of the process is an enzyme produced by the bacteria. No batch phase was utilized, meaning that the glucose feed was started just after inoculation. The starting medium contained all nutritients needed by the bacteria, except for glucose and nitrogen. Nitrogen was added through pH regulation and existed in excess throughout the whole fermentation. Air was continuously sparged through the fermentor vessel to maintain aerobic conditions while the addition of glucose was regulated by the probing controller.

3. THE CONTROLLER

The controller must be capable of controlling a fed-batch fermentation throughout its whole duration. To allow this in a simple way, it consists of several different parts acting separately and at different times. At the start of the process a static feed ramp is employed to control the feed rate (F) until the dissolved oxygen saturation level (DO) in the fermentation medium has reached the setpoint used by the probing controller, at which point the probing controller starts acting. After the probing controller has increased F to a point where the oxygen transfer capacity of the fermentor becomes limiting, a safety net acts to continuously decrease the feed rate when DO is below a minimum allowed level, so that oxygen limitation is avoided.

3.1 Probing controller

The controller used in this work is based on the probing controller created by Mats Åkesson in 1999. Although some improvements of the probing control strategy have been suggested since then, such as only using up or down pulses (Åkesson [2001] and Henes and Sonnleitner [2007]) and combining probing control with the temperature limited fed-batch technique (Velut [2005]), most of these were either considered less useful or simply not feasible in this implementation.

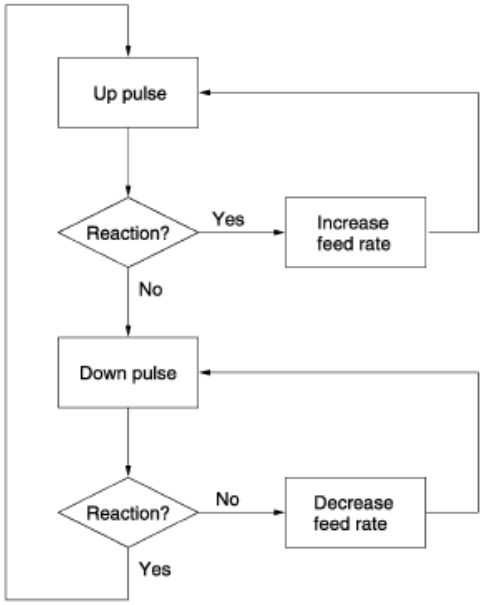

Fig. 3. Illustration of the algorithm used to determine the direction of pulses and changes in F after pulses (Åkesson [1999]).

The controller inserts probing pulses into F and the DO response to pulses are studied and determine how F is changed after each pulse. The principle behind this is that any change in F which occurs above the value corresponding to $q_{g,crit}$, which is labelled F_{crit}, will not contribute to the oxidative metabolism and therefore not cause a change in q_o (as seen in figure 1), whereas any change in F which occurs below F_{crit} will lead to a corresponding change in q_o and thereby a change in DO.

The amplitude of a pulse, F_{pulse}, is proportional to F. If the greatest change in DO during a pulse, DO_{pulse}, exceeds a predefined level DO_{reac}, the pulse is considered as having given a reaction. An algorithm is used to switch between up and down pulses and determine how F should be changed after a pulse (Fig. 3). An illustration of pulses and pulse responses is shown in figure 4, where almost all of the first pulse contributes to the oxidative metabolism and causes a notable change in DO, whereas only a small part of the second pulse contributes to it and the change in DO therefore becomes lower than DO_{reac} in that case.

If F is changed after a pulse, the size of the change in F, labelled ΔF, is determined by the size of DO_{pulse} as shown in equation 1 (up pulses) and 2 (down pulses). $DO^\star$ is the dissolved oxygen saturation at equilibrium with the gas phase and DO_{sp} is the setpoint for the DO controller and hence the baseline value for DO between pulses. κ and γ_p are constants, where a κ value of 1 or less ensures controller stability in the model for *E. coli* fermentations used in Åkesson [1999] and γ_p is the decrement factor for a pulse which gives no change in DO. A saturation of ΔF as shown in equation 3 is also employed.

$$\Delta F = \kappa \cdot \frac{|DO_{pulse}|}{DO^\star - DO_{sp}} \cdot F \tag{1}$$

$$\Delta F = \left(\kappa \cdot \frac{|DO_{pulse}|}{DO^\star - DO_{sp}} - \gamma_p \right) \cdot F \tag{2}$$

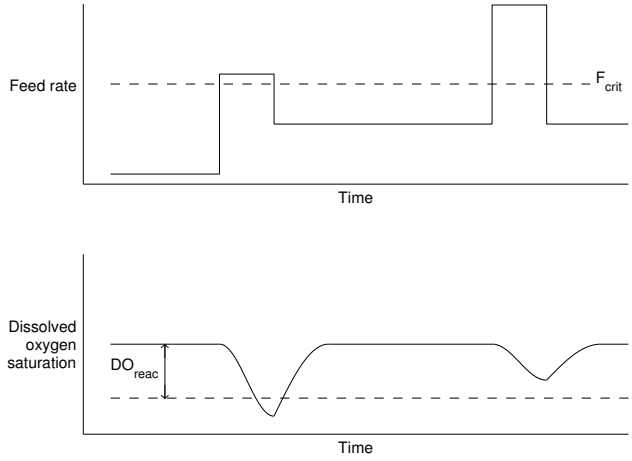

Fig. 4. Illustration of pulses and DO responses to pulses. The first pulse response exceeds DO_{reac} and the pulse therefore counts as having given a reaction, whereas the second pulse does not.

$$|\Delta F| \leq |F_{pulse}| \tag{3}$$

To achieve a steady DO baseline between pulses, which is required to ensure that only the change in F during a pulse will influence DO, a PI controller is used. This controller manipulates the stirrer speed, N, and aeration rate, $Q_{gas,in}$, of the fermentor, switching to aeration rate control when the stirrer speed has reached its maximum and back again should the aeration rate reach its minimum. Gain scheduling of the PI controller is necessary when manipulating the stirrer speed, as the process dynamics vary greatly with time due to the biomass growth and increase in liquid volume. Four gain scheduling points are used and suitable gains for these are estimated from experiments run for this purpose. In order to not interfere with DO responses, the DO controller is switched off during pulses.

In the process, the time constant of the feed rate dynamics is large enough that the actual peak of a probing pulse occurs after the pulse has ended. For this reason an observation time is used after each pulse, with a duration equal to that of a pulse. During the observation time the setpoint for F returns to its value before the pulse. DO_{pulse} then becomes the greatest change in DO during the pulse and the observation time.

3.2 Safety net

The safety net is set to act only when the oxygen transfer capacity of the fermentor is saturated, meaning that the stirrer speed and aeration rate are at their maxima. It acts when DO is below 10 %, decreasing F by 2 % once every 15 minutes until DO is above the level of its baseline, DO_{sp}, so that DO limitation is avoided and new probing pulses can be performed. This facilitates a continuation of the probing control scheme even though the stirrer speed and aeration rate are at their respective maxima, and ensures that no metabolic shift leading to overflow metabolism has occurred.

4. CONTROLLER EVALUATION

The success of the controller is evaluated with respect to process yield and stability.

After a successful implementation of the probing controller had been achieved in experiments using 20 l fermentors, the controller was used to control the process in a 2.5 m^3 fermentor. After ensuring that the controller behaved as desired in this scale as well, an evaluation experiment was run in 2.5 m^3 scale. As a reference, a similar experiment was performed in 20 l scale using a standard control strategy. Earlier experience of processes in these two fermentor scales had shown that similar results were achieved in both and scaling issues were therefore considered a minor problem. The yield in regard to product (enzyme) activity for both processes was measured and compared. Process robustness was evaluated by measuring acetate and glucose levels.

5. RESULTS AND DISCUSSION

5.1 Probing controller behaviour

The behaviour of the probing controller during the evaluation experiment is illustrated in figure 5, which shows some of the most important process parameters during the probing control phase. As seen there, probing pulses have a distance of 15-30 minutes between them. The main limitation in regard to performing pulses more frequently is the feed rate dynamics, as F must reach its new setpoint before new pulses are performed. The probing controller acts very much as desired, with clear DO responses to each pulse. Not all pulses give a response which exceeds DO_{reac} and some down pulses are therefore performed as well, but as the probing controller acts during the outgrowth phase of the fermentation there is no need for the probing controller to decrease F at any time. After 11 h the maximum allowed F base level has been reached, meaning that F is not increased despite up pulses achieving a DO response which exceeds DO_{reac}.

The outcome of this experiment shows clearly that it is possible to implement probing control in *B. licheniformis* fermentations and achieve the same controller behaviour as in fermentations using for instance *E. coli*. Although there is still room for improvement of for instance the gain scheduling of DO control, the implementation itself must be considered successful.

5.2 Process stability

As can be seen in figure 7, low levels of acetate were accumulated during the first few hours of the evaluation experiment when the static feed ramp acted but as the probing controller started the accumulated acetate was consumed, as would be expected, and the acetate levels are then kept low throughout the entire fermentation. Glucose and acetate levels at the start of the fermentation are particularly critical and as can be seen in figures 6 and 7, they are low in the evaluation experiment when compared to the reference experiment.

The safety net starts acting at a point around 14 h into the experiment, putting an end to the probing control

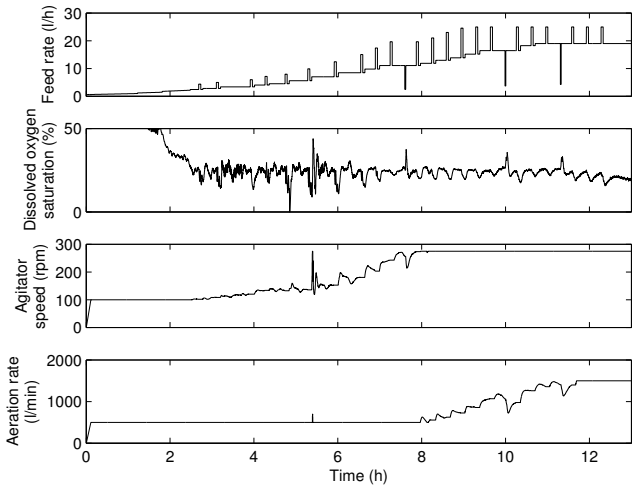

Fig. 5. Process parameters in the evaluation experiment, between 0 and 13 h.

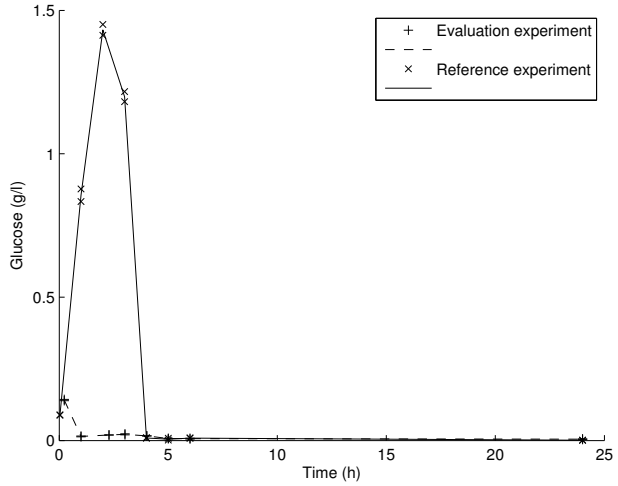

Fig. 6. Comparison of glucose levels in the evaluation experiment and the reference experiment.

phase. The probing controller still acts occasionally after this point however, to ensure that F is not decreased too far or that a metabolic shift in the bacteria would result in overflow metabolism even at the decreased levels of F which are present late in the fermentation.

These observations are in themselves not proof that the probing controller is capable of actively avoiding overflow metabolism and process crashes caused by accumulation of acetate. However, already at an early stage in the development of the probing controller, it showed itself capable of detecting and avoiding both overflow metabolism and unnecessarily low feed rates in the process, similarly to what would be expected based on implementations of probing control in other systems using different bacteria. The development experiment illustrated in figure 8 demonstrates this clearly, as detailed below.

A disturbance in F was introduced at 29 h into the experiment, however the probing controller detected this and returned F to a level close to that before the disturbance.

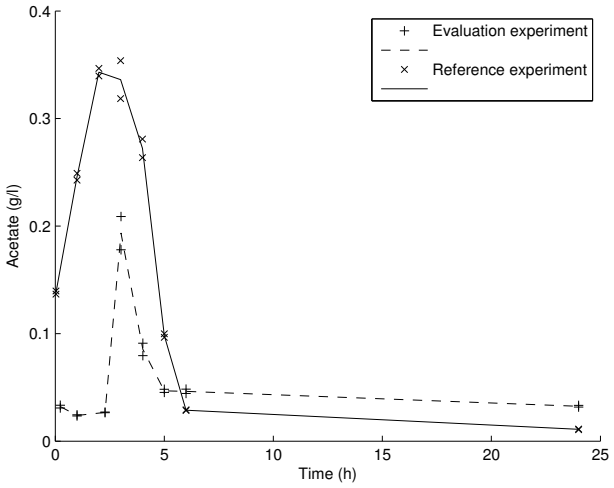

Fig. 7. Comparison of acetate levels in the evaluation experiment and the reference experiment.

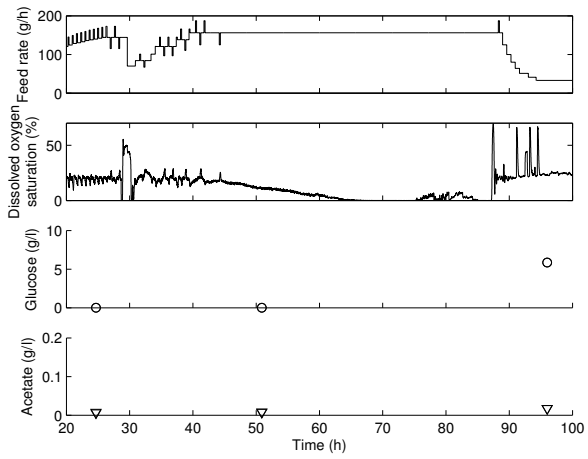

Fig. 8. Process parameters in a development experiment.

No safety net was implemented in this experiment which led to DO limitation for a long time towards the end of the experiment, causing the process to crash at 87 h. The crash was however not complete and some bacteria survived, meaning that DO could be returned to near its setpoint and the probing controller started again. At this point F was far above the level suitable for such low biomass concentrations, leading to an accumulation of glucose and hence to overflow metabolism. This was detected by the probing controller, which decreased F to its minimum value as fast as possible.

These events show that the probing controller has the desirable feature of being able to counteract both too high and too low levels of F in the process. The outcome of this development experiment indicates that the probing controller should be far more capable of avoiding process crashes caused by overflow metabolism, than the controller used in the reference experiment which does not employ any type of feedback control for avoiding overflow and is known to have a problem in this regard.

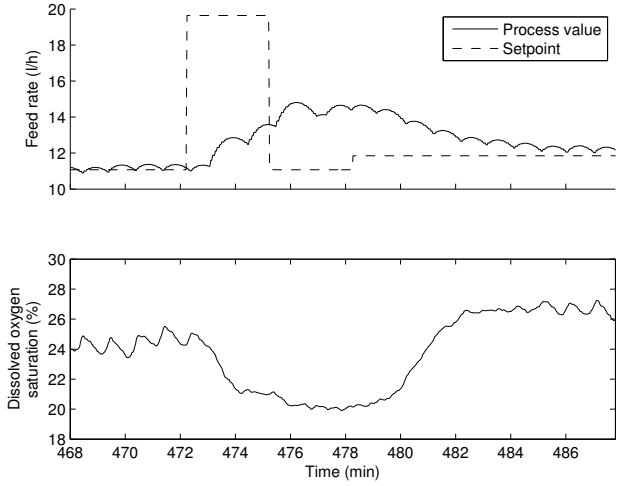

Fig. 9. Oscillations in F and DO during part of the evaluation experiment.

A clear indication that the probing controller finds a value of F just below F_{crit}, also in 2.5 m^3 fermentations, is the behaviour of the DO signal as shown in figure 9. In these experiments the feed of glucose was not continuous but was rather added in small "shots", where the size of or time between these depend on the desired feed rate. Although this can be regarded as an undesirable disturbance, it also gives information about the process as the "shots" can be seen as small probing pulses in themselves. As seen in figure 9, these give a response before and after a pulse but not during it. This is the type of behaviour which can be expected when F_{crit} is exceeded during a pulse and the oxidative capacity of the system becomes saturated, meaning that F lies only slightly below F_{crit} before the pulse. Similar DO behaviour can be seen throughout the whole probing control phase, indicating that the probing controller is at all times capable of setting F to a suitable value just below F_{crit}. To achieve complete experimental proof of improved process stability, a large number of fermentations must be run and the crash rates compared.

5.3 Product yield

A comparison of the product activity levels (Fig 10) indicates that the evaluation experiment using probing control achieves a higher product yield than the reference experiment. The most important conclusion which can be drawn here is however that the probing controller can achieve a yield in the same range as the reference controller.

6. FUTURE IMPROVEMENT OF THE PROCESS

A limitation on how well probing control can control a process is how frequently new probing pulses can be performed. Even if every pulse places F just below F_{crit} at that point in time, the feed demand of the process is constantly changing and a too high granularity of F will mean a bad following of its optimum.

Before a new pulse can be performed, F and DO must both return to near their setpoints. In this process, it

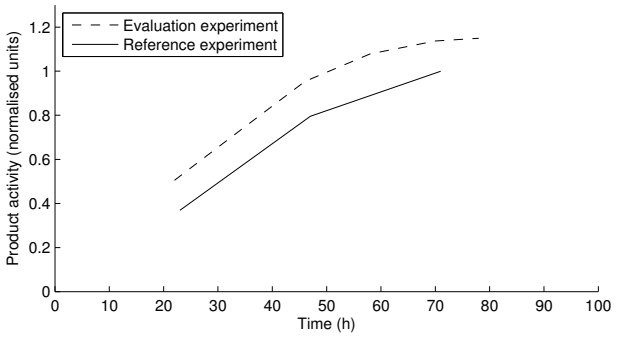

Fig. 10. Relative product activity levels in the evaluation experiment and reference experiment.

was noted that the dynamics of the stirrer speed were considerably slower than those of the aeration rate. To avoid unnecessarily slow DO control when the stirrer speed is used to control DO, mid-ranging control employing stirrer speed and aeration rate control at the same time could be used. However, this has to be coupled with fast feed dynamics in order to achieve an improvement of the process.

Mid-ranging control of DO would also have the benefit of increasing the aeration rate from its minimum value immediately from the start of the process, which is beneficial as it means a lower risk of too high CO_2 levels in the gas phase of the process which might otherwise inhibit microbial growth and decrease fermentation performance.

Another modification which may improve the outcome of the process would be to replace the simple DO safety net with a DO-controlled feed, that is, where F is regulated by a PID controller to achieve a DO near a given setpoint. Switching from probing control to this type of control would then occur when the oxygen transfer capacity of the fermentor is near or at its maximum and as no signs of metabolic shifts which can result in overflow metabolism late in the process have been observed in the experiments, this should be safe to do even though this type of control does not in itself avoid overflow metabolism.

7. FURTHER DEVELOPMENT OF THE PROBING CONTROL STRATEGY

As mentioned above, a limitation of the probing control strategy is that F and DO must reach their respective setpoints before a new probing pulse can be performed. It would however be possible to construct a probing controller where pulses caused by setpoint changes in F are not necessary and could, possibly, run in a more continuous manner rather than only evaluating DO responses at certain points in time.

The phenomenon which makes this possible is the non-continuous addition of feed to the fermentor used in the process described here, which can be considered as a constant stream of probing pulses. As mentioned above, figure 9 shows the oscillations in F and the resulting oscillations in DO, as well as how the pulse at 472 min increases F and causes the oscillations in DO to fade away, to return again when F is decreased after the pulse. The frequency of the feed "shots" into the fermentor can be

set to a desired value and the oscillations in *DO* caused by this effect when F is below F_{crit} have the same period as the oscillations in F. Therefore, it appears that the amplitude of the oscillations in *DO* would make a very suitable parameter to measure in order to determine if the system has reached or is close to overflow metabolism. Around the time probing control was first developed the dynamics of existing *DO* probes were not considered fast enough to measure these small pulses (Hagander [2010]), but modern, fast *DO* probes now allow this.

A controller based on this principle could either run continuously through the process, evaluating the amplitude of the oscillations in every data point, or could evaluate the values of *DO* at certain points similar to the probing control strategy used in this work. Having the controller run continuously appears as a more attractive option, however the action of the *DO* controller must then be taken into consideration and perhaps be changed.

8. CONCLUSION

The work described in this paper has shown experimentally that probing control can be implemented in an industrially relevant pilot scale *B. licheniformis* process and can achieve a product yield similar to that of the current controller. It is shown that the probing controller can achieve its goal of actively setting the feed rate of the process to a level just below the critical limit for overflow metabolism. Suggested future improvements of the process are mainly centered around allowing the probing controller to act more frequently. To that end a new type of probing control is also suggested, where small oscillations of the feed rate which occur without setpoint changes are used as pulses. This would mean that pulses caused by setpoint changes in the feed rate would not be necessary and presumably that the probing controller could act at all points in time rather than acting only at certain intervals.

9. ACKNOWLEDGEMENTS

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