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Optimal bioreactor inoculum preparation in shake flasks with automated seed inoculation, conditioning and online monitoring

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Abstract

While monitoring and control of inoculum characteristics at the seed fermenter stage are well established in most bioprocesses, the very first steps of culture expansion in shake flasks are usually neglected, even though they are influencing all following process steps. Especially for organisms with complex metabolic regulation or morphological variability, the utilization of suboptimal inocula can severely worsen the bioprocess outcome.

Using an *S. cerevisiae* ethanol production process as example, we present an integrated approach for optimal inoculum preparation in shake flasks with automated seed inoculation, conditioning and online monitoring, which allows the generation of reproducible inoculums and the flexibility to adjust the harvesting time according to the operator's requirements.

Introduction

The preparation of an optimal inoculum is crucial for the success of any bioprocess with regard to lag-times, growth kinetics, productivity and prevention of contaminations. Throughout the multiple inoculation steps of modern fermentation processes, from stock culture via shake flasks and multiple seed fermenters to the final production fermenters, the highest quality and integrity of each respective inoculum must be maintained with regard to the criteria listed in Table 1.

Criteria	Challenges in *S. cerevisiae* ethanol production bioprocesses
Inoculum must be in a healthy and active state.	Metabolic states influence growth rates, ethanol tolerance and, viability.
Inoculum must be available in sufficiently large volume at the planned inoculation time.	Metabolic and morphological states influence lag-time duration and growth rates.
Inoculum must be in suitable morphological state.	Morphological states influence growth rates and ethanol tolerance.
Inoculum must be free of contamination.	Contaminations must be identified as early as possible to reduce the cost of a total batch loss.
Inoculum must retain its product-forming capabilities.	Metabolic and morphological states influence productivity and ethanol tolerance.

Table 1: Criteria for an optimal bioreactor inoculum [1]

Especially in applications with organisms exhibiting complex metabolic regulation, diauxic growth behavior or strong morphological variability, the utilization of suboptimal early inocula can severely worsen the overall bioprocess outcome.

Fermentative ethanol production using the yeast *Saccharomyces cerevisiae* is a typical example for a bioprocess with high susceptibility to inoculum quality. Ethanol is one of the largest volume biotechnological products with a global production above 100 million tons [2], [3]. As for any other bulk chemical, ethanol production is cost-sensitive, so that suboptimal inocula considerably lower the profitability of the overall bioprocess due to lower fermentation yields. Several growth characteristics complicate the preparation of optimal *S. cerevisiae* inocula, first and foremost its diauxic growth behavior on glucose with an initial respiro-fermentative growth phase of desired ethanol production, followed by a respiratory growth phase of undesired ethanol consumption [4]. This diauxie comes along with growth phase dependent differences in the cell composition, storage carbohydrate utilization and morphology [5]–[8]. The underlying metabolic fluxes and their regulation persist over generations and shape the lag-phase [9]. Inoculum preparation is furthermore complicated by the fact, that *S. cerevisiae*'s growth rate and productivity considerably depends on the inoculation cell density [10]–[12].

Using *S. cerevisiae* as an example, we present an integrated approach for optimal inoculum preparation in shake flasks with automated seed inoculation, conditioning and online monitoring, which leverages the early stages of culture expansion to a level of bioprocess monitoring and control that has until today only been accessible in later stage stirred tank bioreactors.

Preconditioning	> Innova® S44i keeps the environment constant at 10 °C and 100 rpm. > YEP-based seed cultures of various cell densities in LIS cartridge adopts to environment.
Seed Inocultaion	> Pre-programmed Innova S44i sets the growth conditions to 30 °C and 200 rpm. > Pre-programmed Liquid Injection System (LIS) injects the seed culture into the shake flask containing fresh YEPD.
Growth Monitoring	> Cell growth is monitored in real-time by the CGQ through the shake flask bottom by optical back-scatter measurements. > The CGQ notifies the user as soon as a target growth rate is reached.
Inoculum Harvesting	> The user harvests an optimal inoculum under reproducible environmental and growth phase conditions and transfers it to the next seed-train step.

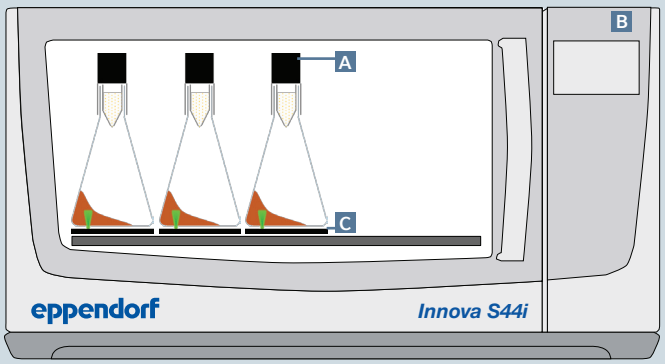

Figure 1: Schematic representation of the integrated approach for optimal inoculum preparation in shake flasks with:
A: automated seed inoculation (LIS)
B: conditioning (Innova S44i)
C: online monitoring (CGQ)

Materials and Methods

The integrated inoculum preparation approach on the basis of the Eppendorf programmable Innova® S44i shaker and aquila biolabs' liquid injection system LIS- and cell growth quantifying system CGQ is depicted in Figure 1.

Summarizing the criteria listed in Table 1, an optimal inoculum preparation process must ensure, that the culture is healthy, productive and in the correct metabolic and morphologic state at the time of harvesting.

The integrated inoculum preparation process presented here aims to fulfill these criteria by:

1. Preconditioning the seed inocula at low temperatures.
2. Controlling the harvesting time point with preprogrammed seed inoculation.
3. Monitoring the cell growth in real-time, to identify optimal harvesting times as well as potentially growing contaminations.
4. Harvesting time elongation on the basis of multiple inoculation setups.

Finally using this process, the user should be allowed to plan and prepare inoculation processes reproducibly and to harvest optimal inocula at predefined time windows.
Three cultivation setups were mounted on the universal shaker platform, each setup consisting of a shake flask with a LIS cartridge and LIS drive mounted on top of the flask and a CGQ sensor plate placed inside a 1000 mL universal adapter between the flask and the respective clamp. All CGQ sensor plates were connected via the CGQ base station to a PC running the biomass monitoring software CGQuant. The Innova S44i (shaking throw of 50 mm) was pre-programmed to 10 °C and 100 rpm for a preconditioning phase of about two hours with a subsequent shift to optimal growth conditions of 30 °C and 200 rpm. *S. cerevisiae* seeding solutions of 10 mL were prepared from lyophilized cells in autoclaved YEP (10 g/L yeast extract, 20 g/L peptone, dest. water) with final biomass concentrations of 12.5 g/L, 25.0 g/L and 50.0 g/L, respectively.

The seeding solutions were filled into LIS cartridges and mounted together with LIS drives on top of 1000 mL non-baffled glass shake flasks, each filled with 100 mL YEPD (YEP + 20 g/L glucose). The Innova S44i was programmed to rise in temperature and shaking speed to 30 °C and 200 rpm after the preconditioning phase and then to hold these growth conditions. The LIS drives were programmed to inject 9.5 mL of their seeding solution into the flasks one hour after Innova S44i set the growth conditions. Subsequently, cell growth was monitored in real-time by CGQ backscatter measurements through the shake flask bottom, thus providing the data for selection and harvesting of the optimal inoculum. Further information on the handling of CGQ and LIS is available on the aquila biolabs websites www.aquila-biolabs.de. Materials used from aquila biolabs are listed below:

> **Shake Flask:** 1000 mL shake flask (38 mm neck) with aluminum cap (FLAC1000)
> **LIS Drive:** Wireless programmable liquid injection system for shake flasks (LIDI)
> **LIS Software and Coordinator:** LIS software incl. wireless communication accessories (LISO)
> **LIS cartridges:** 50 sterile packed LIS cartridges incl. accessories (LICA50)
> **CGQ Sensor Plate:** for cell growth monitoring in a 100 mL shake flask (SPOD100IF)
> **Universal Adapter:** Adapter for application of a 100 mL sensor plate in a 1000 mL clamp (ADAP1000)
> **Clamp Adapter:** Adapter for a 1000 mL shake flask (STCA1000)
> **CGQ Base Station:** Base station for up to 8 or 16 sensor plates (BASE8 or BASE16)
> **CGQuant:** PC-Software for cell growth monitoring and analysis (CGQS)

Results and Discussion

An optimal inoculum for ethanol production with *S. cerevisiae* should be harvested during the respiro-fermentative growth phase, where the cells are growing on glucose while producing ethanol. In order to keep lag-phases in the stirred tank bioreactor as short as possible and to enable optimal growth rates, the inoculum should be harvested at maximal growth rates of e.g. 90 % of the target growth rate or above.

Results of a representative inoculum generation for the bioprocess of ethanol production with *S. cerevisiae* are shown in Figure 2. At time 0 h, the three cultivation setups were mounted inside the Innova S44i shaker. From 0 – 2 h with the shaker chamber set to 10 °C and shaking speed to 100 rpm, the resolubilized cells inside the cartridge were preconditioned in a glucose-free cultivation medium, to prevent cell growth, but to allow the cells to slowly adopt their metabolism to the other medium contents. Following this 2 hour preconditioning phase, the Innova S44i was preprogrammed to automatically increase temperature and shaking speed to the desired growth phase conditions of 30 °C and 200 rpm. Within approx. 20 min. the shaker chamber reached the growth phase temperature, while the warming-up of the CGQ and the cultivation liquids took 50 min. The injection of the preconditioned seed inoculation by the LIS system was preprogrammed to one hour after the shaker ramped up to 30 °C and 200 rpm (time 3 h) and is clearly visible in the CGQ backscatter intensities at 3.6 h for the different seed biomass concentrations. From the time point of seed injection, the cultures started growing with almost no lag-phase (only 15 min. according to the growth rate curves), which demonstrates the usefulness of a controlled preconditioning phase. In good accordance with literature [10]–[12], different acceleration phase lengths and maximum cell densities were observed depending on the seed inoculum biomass concentrations, with the densest seed inoculum of 50 g/L biomass concentration yielding the shortest growth acceleration phase (1 h) compared to 25.0 g/L (2 h) and 12.5 g/L (2.5 h). The maximal growth rates exhibited a similar trend with higher rates for higher seed inoculation densities.

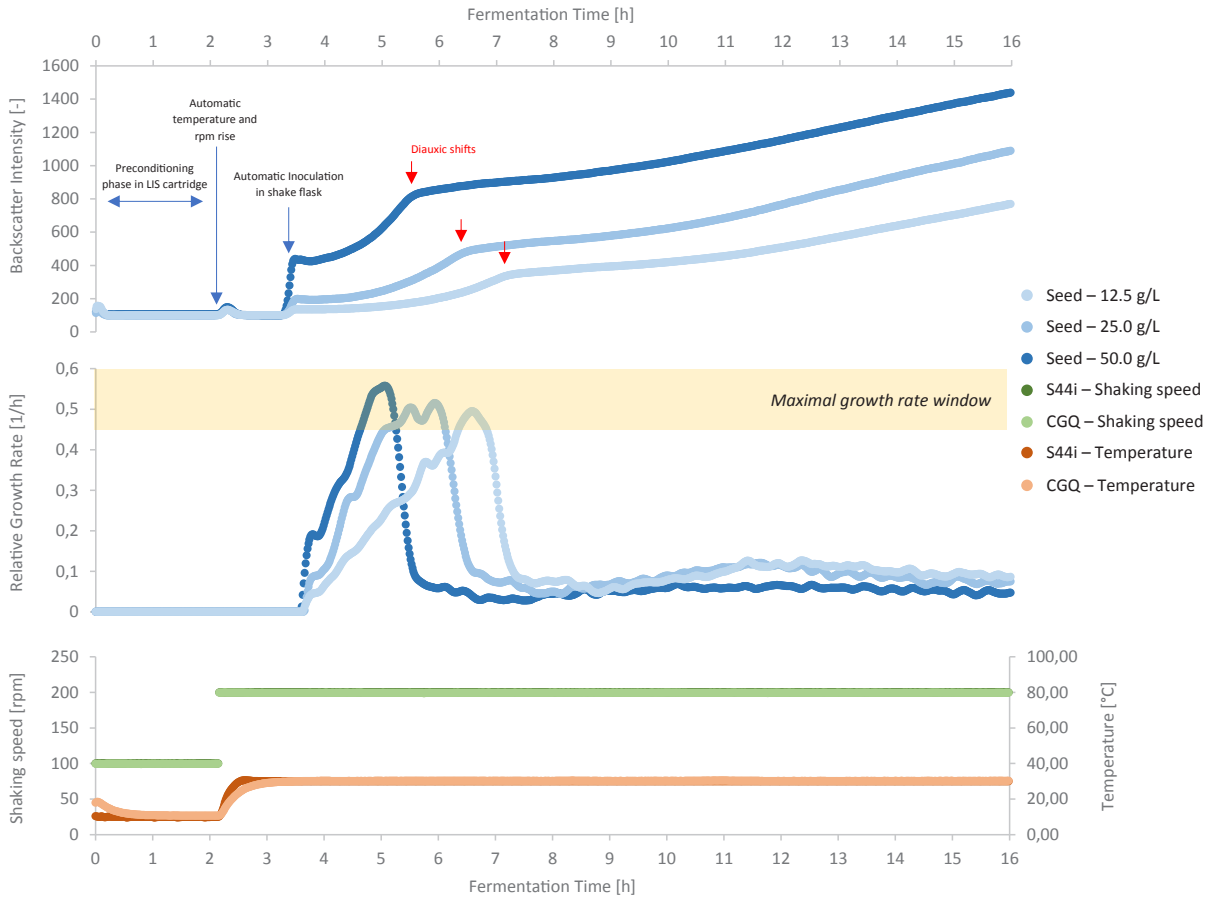

Figure 2: Process data for the preparation of *S. cerevisiae* inocula using an integrated approach in shake flasks with automated seed inoculation (LIS), conditioning (Innova S44i) and online monitoring (CGQ)

By using the approach of three different seed inoculation biomass concentrations with accordingly different growth acceleration phases, it was possible to prolong the optimal harvesting time with growth rates in the maximal growth rate window from ca. 0.6 h for a single cultivation to more than 2 h for the combination of cultivations, thus giving the bioprocess operator more flexibility in the inoculation schedule.

Instead of harvesting the cultures at the optimal time point regarding productivity, metabolic state and growth rate, the cells were grown further to demonstrate the diauxic shift, which occurs in *S. cerevisiae* cultures as soon as glucose is exhausted. This diauxic shift towards respiratory growth on the accumulated ethanol is clearly visible in the CGQ backscatter and the growth rate curves with a sudden drop in growth rate, and a subsequent slower growth on ethanol, which resembles the typical *S. cerevisiae* growth curve as described in the literature [4], [5], [7]. When comparing the time of desired respiro-fermentative growth (1.5 – 3.5 h), the optimum harvesting period (0.5 – 1.0 h) and the length of the undesired respiratory growth phase (> 8 h), it becomes obvious, that the chance of harvesting the inoculum at a suboptimal time point in the morning is extremely high. In other words, with the typical unsupervised and uncontrolled process of precultivation in shake flasks, the bioprocess operator will almost always generate a suboptimal inoculum, characterized by cells of low growth rate with wrong metabolic pathways being activated, thus leading to prolonged lag-phases and reduced productivity.

Using the above described integrated approach for optimal inoculum preparation in shake flasks, not only the reproducibility and quality of the inoculum can be enhanced, also the harvesting time can be adjusted according to the operator's requirements by setting the lengths of the preconditioning phase. Due to the low preconditioning temperature and the absence of a growth enabling carbon source in the preconditioning medium, the cells can be stored for many hours inside the LIS cartridge under light agitation, without negatively influencing the cultivation after injection into the fresh medium. As depicted in Figure 3, the growth rate curves show no significant differences, regardless of the preconditioning period length. Programming the Innova S44i preconditioning and growth phases as well as the LIS injection time allows for optimized seed inoculation timing and turns nights and weekends into productive times for the preparation of optimal inocula, which are then available for harvesting in the morning or whenever the fermenters are prepared for inoculation, not earlier or later, just right in time.

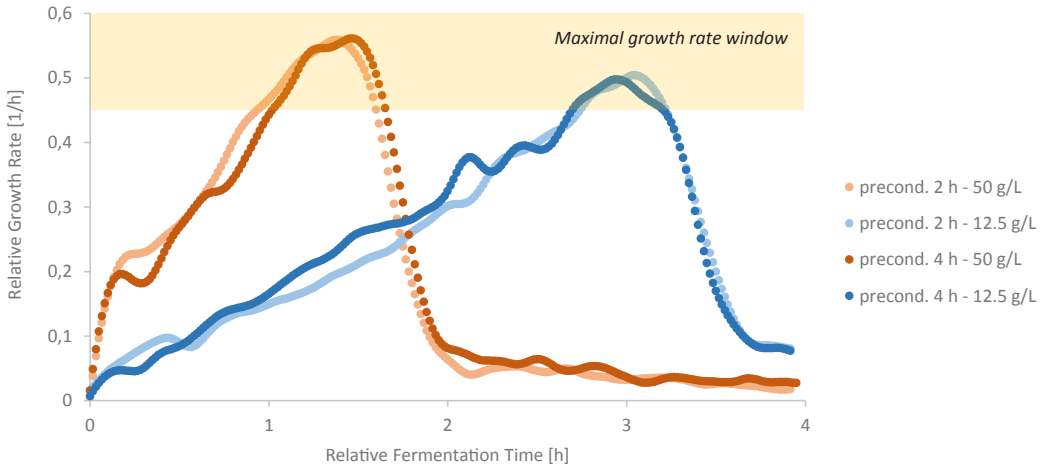

Figure 3: Growth rates with 2 h and 4 h of preconditioning at 10 °C and 100 rpm. The fermentation times are adjusted relative to the start of seed inoculum injection.

Conclusion

Using an ethanol production process with *S. cerevisiae* as an example, we presented an integrated approach for optimal inoculum preparation in shake flasks with automated seed inoculation, conditioning and online monitoring, which broadens the optimal inoculum harvesting time window. Furthermore, the reproducible identification on the basis of real-time backscatter measurements and growth rate curves enables an optimized seed inoculation timing by programmatically defining the length of the preconditioning phase. The integrated inoculum preparation approach on the basis of the programmable shaker Innova S44i and aquila biolabs' LIS- and CGQ-system turns nights and weekends into productive times for the preparation of optimal inocula and for the first time leverages the early stages of culture expansion to a level of bioprocess monitoring and control that has until today only been accessible in later stage stirred tank bioreactors.

Acknowledgement

Data acquisition has been done in the facilities of the FermFactory in the Institute of Applied Microbiology (iAMB) of the RWTH Aachen University. The FermFactory is a lab collaboration and networking innovation space between companies and academic science dedicated to build a work environment and platform for future innovative bioprocess solutions, for more information please visit:
http://www.apz-rl.de/FERM-FACTORY/

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