Advanced continuous cultivation methods for systems microbiology

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Increasing the throughput of systems biology-based experimental characterization of in silico-designed strains has great potential for accelerating the development of cell factories. For this, analysis of metabolism in the steady state is essential as only this enables the unequivocal definition of the physiological state of cells, which is needed for the complete description and in silico reconstruction of their phenotypes. In this review, we show that for a systems microbiology approach, high-resolution characterization of metabolism in the steady state – growth space analysis (GSA) – can be achieved by using advanced continuous cultivation methods termed changestats. In changestats, an environmental parameter is continuously changed at a constant rate within one experiment whilst maintaining cells in the physiological steady state similar to chemostats. This increases the resolution and throughput of GSA compared with chemostats, and, moreover, enables following of the dynamics of metabolism and detection of metabolic switch-points and optimal growth conditions. We also describe the concept, challenge and necessary criteria of the systematic analysis of steady-state metabolism. Finally, we propose that such systematic characterization of the steady-state growth space of cells using changestats has value not only for fundamental studies of metabolism, but also for systems biology-based metabolic engineering of cell factories.

Introduction

An increasing number of genetically engineered recombinant cell factories with superior characteristics or totally novel functions has led to the successful industrial-scale production of food supplements, bulk chemicals and pharmaceuticals (Becker et al., 2011; Huang et al., 2012; Jantama et al., 2008; Lee et al., 2012; Nakamura & Whited, 2003; Paddon et al., 2013; Sun & Alper, 2015; Yim et al., 2011). The development of such bioprocesses mainly involves metabolic engineering of cells based on the knowledge of their metabolism, use of genome engineering technologies and optimization of bioprocess conditions (e.g. pH, aerobicity, cultivation mode). Additionally, various metabolic modelling techniques, mainly constraint-based stoichiometric models, are increasingly and successfully contributing to the creation of superior recombinant cells (Bordbar et al., 2014; McCloskey et al., 2013). However, even for the bacterium whose metabolism is best described, Escherichia coli, the number of potentially superior producer cells designed in silico exceeds the experimental throughput for comprehensive characterization of their metabolism, which is the best proof for validating in silico designs. Reduction of the gap between throughput of in silico cell design and experimental validation is important for accelerating systems microbiology-based bioprocess optimization. This could potentially be achieved by a more accurate and quantitative understanding of metabolism at the whole-cell level through high-resolution experimental characterization of steady-state metabolism of the in silico-designed cells.

Analysis of metabolism in the steady state is essential as only this enables the unequivocal definition of the physiological state of cells (see below; Hoskisson & Hobbs, 2005), which is needed for the complete description and in silico reconstruction of their phenotypes. Steady-state cell culture can be defined as the state of unchanging concentrations of different molecules inside and outside of the cells, which
results from a one-to-one correspondence between biochemical processes and environmental conditions. Steady-state metabolism could, in theory, be studied using batch cultures, but in practice the rapid transient changes of substrate and product concentrations do not allow an accurate definition of the physiological state of cells (Hoskisson & Hobbs, 2005), and thus cannot be used to validate *in silico* designs. Therefore, continuous culture methods chemostat and turbidostat are commonly used for steady-state cell physiology studies (Bryson & Szybalski, 1952; Monod, 1950; Novick & Szilard, 1950). However, these methods are too resource-exhaustive for high-resolution systems microbiology. In this review, we describe the systematic analysis of steady-state metabolism and summarize the research highlighting that high-resolution characterization of steady-state metabolism can be achieved by using advanced continuous cultivation methods termed changestats, such as the accelerostat (A-stat; Paalme et al., 1995) and dilution rate stat (D-stat; Kasemets et al., 2003). We will not focus on the specific details of control algorithms, which were presented earlier in Kasemets et al. (2003).

**Steady-state growth space analysis (GSA)**

An accurate definition of the physiological state of cells is essential for a complete description and successful reproduction of their phenotypes *in silico*. The physiological state of cells is essentially determined by the concerted effect of numerous different environmental parameters, e.g. temperature, pH, oxygen concentration and nutrient availability, and the near-infinite combinations of their values (Málek, 1958; Stephano-poulos et al., 2002). The effects of several environmental parameters on cell growth have been conceptually organized into growth and non-growth spaces (Koseki, 2009; Le Marc et al., 2005). In this review, we define the set of aforementioned combinations, i.e. physiological states, where cells show growth [specific growth rate ($\mu > 0 \text{ h}^{-1}$)] as the growth space. Importantly, such growth space can accurately be described by steady-state analysis as only this enables the unequivocal definition of the physiological state of cells by providing a one-to-one correspondence between biochemical processes and environmental conditions (Hoskisson & Hobbs, 2005). We propose that a more complete and quantitative understanding of cell metabolism can be achieved by steady-state GSA: systematic high-resolution characterization of the steady-state growth space of cells through collecting systems-level data in different physiological states and *in silico* analysis of quantitative relationships between the combinations of environmental parameters and characteristics of cell metabolism. In our view, GSA should form an important part of systems microbiology research.

Ideally, GSA should determine all the necessary cell characteristics (e.g. $\mu$, substrate and product flows, cellular composition) to understand the metabolism behind the physiological states forming the surface of the growth space. Fig. 1 illustrates an example of a 3D growth space related to $\mu$ as a function of pH and residual substrate concentration, simply generated based on the Monod equation (see below; Monod, 1950) and near-to-real effects of $\mu$ and substrate concentration on $\mu$. The illustration is just one possible case out of many and certainly more complex growth space surfaces based on more than three parameters can be envisaged. Although their visualization is very challenging, these surfaces can be analysed *in silico*. Ultimately, systematic GSA should contribute to the acceleration of systems biology-based metabolic engineering of an organism into a cell factory.

**Current challenge of steady-state GSA**

GSA under defined steady-state conditions can be achieved by application of continuous cultivation methods (e.g. chemostat and changestats), which enables the study of cells at strictly defined physiological steady states with unchanging concentrations of intra- and extracellular molecules. Whilst continuous cultivation methods have been around for decades, analytical methods for high-throughput measurement of the molecules relevant for quantitatively defining steady states of metabolism have emerged quite recently. *-omics methods enable the measurement, for instance, of mRNAs (transcriptomics), proteins (proteomics), and intracellular metabolites and extracellular compounds (metabolomics). Whilst these methods are generally used for relative comparison between two growth conditions, absolute quantification of intracellular molecule abundances/concentrations is now also possible (Arike et al., 2012; Esquerré et al., 2014; Ishii et al., 2007), thus enabling

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Fig. 1. High-resolution steady-state GSA using different changestats. The surface was generated based on the Monod equation (see main text; Monod, 1950) and near-to-real effects of pH and substrate concentration on $\mu$. Note: residual substrate concentration starts inhibiting $\mu_{\text{max}}$ above a certain concentration due to osmotic stress. Blue and green points represent chemostat and turbidostat experiments, respectively, which can be carried out during one A-stat or auxo-accelerostat experiment, respectively. Refer to text for details of the methods.
GSA when coupled with continuous cultures. However, the current challenge of steady-state GSA is increasing the throughput of experimental characterization of the growth space to match that of in silico cell design, strain construction and -omics analyses.

Three criteria for steady-state GSA

Ideally, continuous cultivation methods should meet three basic criteria for the desired quality of steady-state GSA described above: (i) be able to conduct experiments in strictly defined physiological steady states through controlling environmental conditions (e.g. temperature, pH, limiting substrate concentration), (ii) be able to change environmental conditions within one experiment to cover a region, instead of a single point, of the growth space, and (iii) be able to conduct GSA within a reasonable time frame and with reasonable resources.

The first criterion is a priori achieved if a cultivation system enabling the control of environmental parameters (e.g. temperature, pH) and substrate feeding rates (for both fluids and gases) together with culture outflow is available. The first application of such a system was the development of the chemostat continuous cultivation method 65 years ago allowing the study of cell physiology under steady-state conditions (Monod, 1950; Novick & Szilard, 1950). Whilst Novick & Szilard (1950) developed the chemostat for following the emergence of spontaneous mutations under constant conditions over a long period of time (weeks and months), Monod (1950) designed his chemostat mainly for studying growth kinetics of bacterial populations. Over decades, chemostats have been widely used for various applications, which have been excellently reviewed previously (Bull, 2010; Ferenci, 2007; Hoskisson & Hobbs, 2005) and thus will not be discussed further in this review. Briefly, steady-state metabolism is achieved in continuous cultures by adding fresh medium and removing cultivation broth from the cultivation vessel at a constant rate, historically defined as the dilution rate ($D$, h$^{-1}$): $D=F/V$, where $F$ is the feed rate (l h$^{-1}$) and $V$ the volume of cultivation broth (l). Most importantly, one can control $\mu$ of the cells through $D$ as under steady-state conditions the concentration of the growth-limiting substrate is in a unique relationship with $\mu$. This can be described by the Monod equation: $\mu=\mu_{\text{max}} \times \bar{s}/(\bar{s} + K_s)$, where $\bar{s}$ is the residual substrate concentration in the cultivation broth and $K_s$ is the affinity constant to the particular substrate (Monod, 1950).

The second criterion for analysing steady-state metabolism at various environmental conditions within one experiment could be fulfilled by automated stepwise control of environmental parameters in a chemostat culture (Fig. 2). However, such stepwise chemostats are very time-consuming due to the need to stabilize the culture after each step change, making them also prone to the emergence of unwanted mutations (Ferenci, 2007; Gresham & Hong, 2015; Harder et al., 1977; Helling et al., 1987; Novick & Szilard, 1950). Both of these issues can be circumvented by using changestat cultivation methods (e.g. the A-stat and D-stat), which enable the continuous change of one or several environmental parameters within a single experiment without needing the long stabilization phases after each change (Fig. 1). Importantly, cells grown in changestats are formally in a quasi steady state, defined here as a state where the cell culture is moving continuously from one steady state to another one. However, if the experiment is carried out properly, changestats describe steady-state physiology equally to chemostats (see below). Hence, changestats are more suitable than chemostats for covering a region of the growth space of interest.

The third criterion for conducting GSA within a reasonable time frame can be fulfilled by either the use of changestats or automation of small-scale continuous cultivation systems. As outlined above, changestats save time through not needing stabilization phases. The same can be achieved by conducting chemostats in a multiplexed micro-scale continuous culture platform, the development of which, especially for micro-organisms, has recently seen considerable progress (Dénervaud et al., 2013; Long et al., 2013; Moffitt et al., 2012). However, as all the three criteria for realizing the desired level of steady-state GSA can be achieved by using changestat methods, we conclude that changestats are currently the most suitable for GSA.

Advanced continuous cultivation methods – changestats

The unifying concept behind all different changestats, which also differentiates them from a classical chemostat or turbidostat, is the continuous change of one or several environmental parameters within a single experiment, whilst maintaining the physiological state comparable with a chemostat or turbidostat. The ‘family’ of changestat
methods can be divided into two groups: chemostat-based and turbidostat-based methods. Whilst the former enables the study of cells at various \( \mu \) under nutrient limitation, cells are grown at \( \mu_{\text{max}} \) and in substrate excess conditions in the latter. Thus, steady-state growth both under nutrient limitation and excess can be studied using changestat continuous cultivation methods, making them superior to batch cultures for studying metabolism.

The most important benefit of using changestats for GSA is following dynamic changes of steady-state metabolism with the accurate detection of metabolic switch-points and optimal growth conditions. For instance, in the shift of metabolism from respiratory to respiro-fermentative growth, environmental conditions providing the best target product formation from respiratory to respiro-fermentative growth, environmental conditions can be determined reliably (see below for examples).

It was stated above that changestats describe steady-state cell physiology equally well to chemostats if the experiments are carried out properly. In this particular context, this means that the rate of change (acceleration/deceleration) of the environmental parameter of interest has to maintain the cells in a state comparable with the steady state (quasi steady state), meaning that cells are able to adapt to changing environmental conditions. This can be validated during a changestat experiment most easily by stopping the acceleration/deceleration and setting the culture into chemostat mode: if growth characteristics do not change then changestat data are equal to that of the chemostat. The best evidence for changestat and chemostat equally describing steady-state metabolism is provided by the comparison of data from independent experiments of various organisms, including transcriptome and proteome data (Albergaria et al., 2000, 2003; Barbosa et al., 2003, 2005; Girbal et al., 2000; Nahku, 2012; Valgepea et al., 2010, 2011; van der Sluis et al., 2001; van Dijken et al., 2000). It is also important to note that the A-stat and D-stat have been shown to be very reproducible (Barbosa et al., 2005; Lahtvee et al., 2011; Valgepea et al., 2010, 2011; van der Sluis et al., 2001; van Dijken et al., 2000).

Two factors have to be considered when planning a changestat experiment because stress responses might be induced with the change of environmental conditions. If the rate of change of an environmental parameter is too fast, metabolism will be disrupted and the quasi steady state lost. However, if the rate of change is too slow, cells can specifically adapt to changing conditions leading to phenotypes not seen in chemostats under identical environmental conditions. The latter was seen in D-stats of \( L. \) lactis where pH was first decreased from 6.3 to 5.4 and then increased back to 6.3 with a very slow rate of change (0.02 U h\(^{-1}\)) resulting in hysteresis of metabolism: activation of arginine degradation with a decrease of pH to 5.4 was not deactivated at the end of the experiment after pH had been increased back to the initial pH of 6.3 (Lahtvee et al., 2009). Additionally, a very long changestat experiment due to slow acceleration risks the of emergence of unwanted mutations in the cell culture, similar to what is seen in chemostats (Ferenci, 2007; Gresham & Hong, 2015; Harder et al., 1977; Helling et al., 1987; Novick & Szilard, 1950). Importantly, it has been shown using whole-genome DNA sequencing that no mutations arose during a regular-length changestat study of cell physiology in glucose-limited \( E. \) coli A-stat cultures if appropriate acceleration is used (Nahku et al., 2011).

Generally, the rate of change of an environmental parameter supporting the characterization of physiological states in a changestat experiment comparable with a chemostat depends on \( \mu_{\text{max}} \) of the organism under study: the higher it is, the faster the conditions can be changed. For an A-stat experiment, appropriate acceleration can be estimated based on \( \mu_{\text{max}} \) of the organism to be in the range of 0.01–0.04 \( \times \mu_{\text{max}} \) (Kasemets et al., 2003). This is supported by several studies where \( \mu_{\text{max}} \) was reached for different micro-organisms in A-stats (Adamberg et al., 2009; Barbosa et al., 2003; Kask et al., 1999; Paalme et al., 1995, 1997a, b; Valgepea et al., 2010). A thorough study of the effect of acceleration on the growth of \( L. \) lactis showed that \( \mu_{\text{max}} \) of 0.59 h\(^{-1}\) was reached in an A-stat whilst maintaining the quasi steady state using accelerations \(<0.005\) h\(^{-2}\) (Adamberg et al., 2009). Another study concluded that an acceleration of 0.001 h\(^{-2}\) is the fastest that can be used for approaching steady-state culture characteristics during A-stats with yeasts (van der Sluis et al., 2001). Generally, A-stat data collected from experiments using accelerations in the range of 0.007–0.050 \( \times \mu_{\text{max}} \) are comparable with chemostat data at various levels for many different organisms (Albergaria et al., 2000, 2003; Girbal et al., 2000; Lahtvee et al., 2011; Nahku, 2012; Valgepea et al., 2010, 2011; van der Sluis et al., 2001; van Dijken et al., 2000). That being said, one is still advised to determine the exact rate of change for each case where detailed characterization of a novel organism is required (e.g. the last step in optimization of growth conditions).

Next, we will highlight the literature applying changestats for fundamental research of metabolism, bioprocess optimization and other purposes (summarized in Table 1).

**Accelerostat (A-stat)**

The first changest – the A-stat – was developed in the early 1990s at the National Institute of Chemical Physics and Biophysics in Tallinn, Estonia by the research group of Professor Raivo Vilu whilst studying the growth of \( E. \) coli (Paalme et al., 1995). The A-stat has been the most used method among changestats (Table 1) mainly because it enables the study of the effects and dynamics of \( \mu \), one of the most important physiological parameters, on cell metabolism. The A-stat is based on the stepwise chemostat technique, but instead of increasing \( D \) stepwise after achieving the initial steady state in the chemostat, a continuous increase of \( D \) at constant acceleration is started in the A-stat according to the equation: \( D = D_0 + a_D \times t \), where \( D_0 \) is the initial \( D \), \( a_D \) is acceleration of \( D \) and \( t \) is the time after start of acceleration of \( D \). Identical to the
**Table 1. Literature of changestats of special interest with main results**

<table>
<thead>
<tr>
<th>Main results</th>
<th>Reference</th>
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</table>
| **Accelerostat (A-stat)**  
*Escherichia coli*  
- Saturation of respiration capacity proposed to trigger acetate overflow at $\mu = 0.38$ h$^{-1}$  
- Disruption of acetate recycling in the phosphotransacetylase–acetyl-CoA synthetase (PTA–ACS) node proposed to trigger acetate overflow at $\mu = 0.27$ h$^{-1}$; A-stat and chemostat comparable at transcriptome level; $\mu$-dependent metabolome, transcriptome and proteome  
- Acetate overflow postponed and reduced fourfold in a mutant strain with coordinated activation of PTA–ACS and tricarboxylic acid cycles  
- Cells achieve faster growth by increasing catalytic and translation rates of proteins  
*Saccharomyces cerevisiae*  
- Inter-laboratory study determined $\mu_{\text{crit}}$ values for various different laboratory yeasts to select a reference *S. cerevisiae* strain  
- Mutant strain with derepressed glucose control displays 5% higher $\mu_{\text{crit}}$  
- Supplementing mineral medium with oleic acid increases $\mu_{\text{crit}}$ by 8%  
*Hanseniaspora guilliermondii*  
- Two-phase switch from fully respiratory to respiro-fermentative growth with acetate excretion preceding ethanol; optimal range of $\mu$ for high biomass yield  
*Zygosaccharomyces rouxii*  
- Crabtree effect detected under aerobic conditions; effect of acceleration on growth characteristics also a function of the rate of change in environmental substrate concentrations  
*Lactococcus lactis*  
- Continuous shift from mixed acid fermentation to homolactic fermentation with faster growth; $\mu$-dependent metabolome, transcriptome and proteome  
- Cells achieve faster growth by increasing catalytic and translation rates of proteins  
*Corynebacterium glutamicum*  
- Direct coupling between $\mu$ and demeton-S-methyl biodegradation in co-metabolism with fructose; A-stat comparable with De-stat  
*Dunaliella tertiolecta*  
- Optimized kinetic parameters for photobioreactor design and bioprocess conditions for the production of vitamins and carotenoids  
|  |  |
| **Deceleration-stat (De-stat)**  
*Escherichia coli*  
- Determination of maintenance energy requirements near zero-growth conditions  
- Stress response less apparent during a smooth change in nutrient availability compared with nutrient shifts under fed-batch conditions  
*Rhodobacter capsulatus*  
- Optimization of photosynthetic efficiency for the production of hydrogen from acetate and light energy by the determination of important components of the light energy balance  
*Saccharomyces cerevisiae*  
- Stronger stress response toward rapid changes in substrate concentration and $D$ in the chemostat compared with gradual changes in the De-stat  
*Thalassiosira pseudonana* and *Phaeodactylum tricornutum*  
- Up to 94% of time can be saved compared with chemostats using 10 times faster decelerations than required for maintaining the quasi steady state whilst losing only 5% in accuracy to estimate maximal biomass productivity rate  |  
| **Dilution rate stat (D-stat)**  
*Zygosaccharomyces rouxii*  
- Decreasing the threonine/methionine ratio in the ingoing medium proved that methionol synthesis occurs only in the Ehrlich pathway  
*Saccharomyces uvarum* and *Saccharomyces cerevisiae*  
- Dynamic effects of temperature on $\mu$, biomass yield and byproduct profiles; metabolic events accompanying the transition of metabolism from carbon to nitrogen limitation  
|  |  |
Table 1. cont.

<table>
<thead>
<tr>
<th>Main results</th>
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<tr>
<td><strong>Lactic acid bacteria</strong></td>
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<tr>
<td>- Byproduct patterns of fermentative metabolism for various lactic acid bacteria depend</td>
<td>Adamberg et al. (2006)</td>
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<td>on the galactose/arginine ratio in the cultivation medium</td>
<td></td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>- Maximal acetate co-utilization with glucose maintained up to ( D = 0.2 ) h(^{-1}), but totally</td>
<td>Valgepea et al. (2010)</td>
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<td>lost between ( D = 0.45 ) and ( 0.5 ) h(^{-1})</td>
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<tr>
<td><strong>Yarrowia lipolytica</strong></td>
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<tr>
<td>- Best nitrogen/carbon ratio in the cultivation medium for lipid production without carbon loss</td>
<td>Ochoa-Estopier &amp; Guillouet (2014)</td>
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<td>into citric acid between 0.021 and 0.085 N-mol/C-mol</td>
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<tr>
<td><strong>Auxo-accelerostats</strong></td>
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<tr>
<td><strong>Lactic acid bacteria</strong></td>
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<tr>
<td>- Water activity is the most important environmental parameter affecting growth in</td>
<td>Laht et al. (2002)</td>
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<td>cheese-like conditions</td>
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<tr>
<td>- Determination of pH and temperature optima for achieving fastest growth of different</td>
<td>Adamberg et al. (2003)</td>
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<td>lactic acid bacteria</td>
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<td><strong>Saccharomyces cerevisiae</strong></td>
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<tr>
<td>- Dose–effect curves ([IC(_{50})] five times higher when the inhibitory aliphatic monocarboxylic</td>
<td>Kasemets et al. (2006)</td>
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<td>acid is added smoothly to the growth environment compared with when added rapidly</td>
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<tr>
<td>- Similar threshold levels for stress responses toward increased temperature, ethanol, salt</td>
<td>Kasemets et al. (2007);</td>
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<td>or organic acid concentration causing the decrease of ( \mu_{\text{max}} ) for a recombinant and</td>
<td>Nisamedtinov et al. (2008)</td>
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<td>laboratory strain</td>
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<td><strong>Adaptastat</strong></td>
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<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
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<tr>
<td>- Enables the study of cell metabolism near ( \mu_{\text{max}} ) under substrate limitation and the</td>
<td>Tomson et al. (2006)</td>
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<td>first to produce nutrient-limited growth on two substrates</td>
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</table>

chemostat, the experimenter can control \( \mu \) of the cells in the A-stat through \( D \) under steady-state representative conditions. The A-stat produces higher resolution data, and is much more time- and resource-efficient compared with chemostat approaches (Fig. 2).

**Acetate overflow metabolism in E. coli**

As the A-stat enables the precise determination of \( \mu \)-dependent metabolic switch-points and follows the metabolic events around the switch-point with high resolution, there has been a lot of interest in applying the A-stat for studying acetate overflow metabolism in *E. coli* (i.e. excretion of acetate above a certain \( \mu \) when growing on glucose).

Initially, saturation of respiratory chain capacity was proposed to cause acetate accumulation in cultivation broth at high \( \mu \) (Paalme et al., 1995, 1997b). Later, the first transcriptome study in an A-stat suggested that gene expression patterns of the main acetate-producing ([pta, ackA, poxB] and \(-\)consuming enzymes (acs)) might play a role in the regulation of acetate overflow (Nahku et al., 2010). Indeed, a systems biology study proposed that acetate overflow is triggered by downregulation of the enzyme acetyl-CoA synthetase (ACS). This phenomenon can be explained by disruption of acetate recycling in the phosphotransacetylase–acetyl-CoA synthetase (PTA–ACS) node above \( \mu = 0.27 \) h\(^{-1}\) [corresponding to a specific glucose uptake rate \( q_S = 4.2 \) mmol (g dry cell weight)\(^{-1}\) h\(^{-1}\)], leading to a decreasing capability of acetate reassimilation (Valgepea et al., 2010). This hypothesis was recently proven as coordinated activation of the PTA–ACS and tricarboxylic acid (TCA) cycles postponed the start of acetate overflow up to \( q_S = 6.0 \) mmol (g dry cell weight)\(^{-1}\) h\(^{-1}\) and reduced carbon flow to acetate fourfold at \( \mu_{\text{max}} \) (Peebo et al., 2014). Importantly, this overflow-reduced *E. coli* strain does not accumulate any other detrimental byproduct besides acetate and showed identical \( \mu_{\text{max}} \) compared with the wild-type. Application of an A-stat approach was instrumental in these studies for proposing a novel hypothesis and engineering more efficient strains as it enabled the precise detection of the switch-point of acetate overflow and monitoring of metabolic events at high resolution.

**Crabtree effect in yeast**

The A-stat approach has been widely used due to its ability to study \( \mu \)-dependent metabolic switch-points with high resolution for detailed characterization of the Crabtree effect in yeast (De Deken, 1966) where cells switch from fully respiratory to respiro-fermentative growth above a certain \( \mu \) (Crabtree-positive yeasts), termed the critical \( \mu \) (\( \mu_{\text{crit}} \)).

One significant inter-laboratory study determined \( \mu_{\text{crit}} \) values using A-stats for various different laboratory yeasts to select a reference *Saccharomyces cerevisiae* strain amenable to
experimental techniques used in genetic, physiological and biochemical engineering research (van Dijken et al., 2000). The A-stat approach has also been used for $\mu_{\text{crit}}$ screening in a study of an enological yeast strain, *Saccharomyces uvarum*, used in wine production, which led to a purely oxidative fed-batch process (Albergaria et al., 2000) and in a study of *S. cerevisiae* for the development of a mathematical model enabling the quantitative description of transient changes in metabolism (Herwig et al., 2001). Two studies have investigated the effects of cultivation medium composition on $\mu_{\text{crit}}$ of *S. cerevisiae* whilst one study concluded that supplementing glucose-based mineral medium with ethanol had no effect on $\mu_{\text{crit}}$ (Paalme et al., 1997a), the other study showed an 8 % increase of $\mu_{\text{crit}}$ on medium supplemented with oleic acid (Marc et al., 2013). In addition, a *S. cerevisiae* double-knockout strain engineered for derepression of glucose control displayed a 5 % higher $\mu_{\text{crit}}$ compared with the wild-type, possibly explained by elevated respiratory capacity (Klein et al., 1999). Interestingly, detailed $\mu$-dependent characterization of the non-*Saccharomyces* yeast *Hanseniaspora guilliermondii* (Albergaria et al., 2003) and *S. cerevisiae* (Kasemets et al., 2003) revealed a two-phase switch from fully respiratory to respiro-fermentative growth with the excretion of acetic acid preceding that of ethanol. Notably, the same two-phase switch was seen in a series of 20 chemostats of *S. cerevisiae* (Postma et al., 1989).

Other studies of cell metabolism

The A-stat approach has also been used for $\mu$-dependent characterization of several other metabolic processes in various organisms, e.g. energy and amino acid metabolism, and regulation of gene expression. A-stat studies of these and other metabolic processes are discussed next.

One of the first studies using A-stats determined several key $\mu$-dependent physiological characteristics of *E. coli* growing on several carbon sources (e.g. glucose, acetate, succinate and Casamino acids) (Paalme et al., 1997b). Interestingly, although the maximum specific respiration rates ($q_{O_2}$) were very similar for all substrates, $\mu_{\max}$ values varied from ~0.2 to ~0.8 h$^{-1}$. The authors concluded that $\mu_{\max}$ is dependent on growth yield, and respiratory and glycolytic capacity of the strain. In addition, analysis of A-stat data with a stoichiometric flux model indicated that ATP production in *E. coli* exceeds the energy (theoretically) needed for biomass synthesis two fold, consistent with earlier calculations (Stouthamer, 1973). Notably, the initial calculations by Paalme et al. (1997b) were advanced by including the futile PTA–ACS cycle detected in Valgepea et al. (2010) to the model network, which revealed that energy spilling in *E. coli* is dependent on $\mu$ and strongly declines after the start of acetate overflow (Valgepea et al., 2011). The apparent discrepancy between reduced ATP spilling with rising $\mu$ and a constant biomass yield could be explained by the concomitant increase of carbon flux (wasting) to byproducts, mainly acetate and pyrimidine synthesis pathway intermediates (Valgepea et al., 2011). The high-resolution data of the A-stat was instrumental for detecting the dynamics of metabolism in these studies.

Two studies of lactic acid bacteria show that the A-stat approach is suitable for describing $\mu$-dependent dynamics of amino acid metabolism. Firstly, metabolism of *L. lactis* shifts from mixed-acid fermentation, with extensive consumption of arginine, serine, asparagine and alanine, to homolactic fermentation, with balanced amino acid consumption, gradually with the increase of $\mu$ from 0.1 to 0.6 h$^{-1}$ (Lahtvee et al., 2011). Similarly, a continuous decrease of amino acid consumption and increase of lactic acid production with faster growth is seen in A-stat cultures of *Lactobacillus plantarum* (Kask et al., 1999). Both of these studies support fine-tuning of growth media for lactic acid bacteria, thus lowering costs of expensive defined rich media used in bioprocesses and enabling more accurate studies of amino acid metabolism through their lower residual concentrations.

Integration of high-resolution steady-state cultures with -omics measurements and metabolic modelling has strong potential for leading to a more accurate and quantitative understanding of metabolism at the whole-cell level. Such a systems biology approach was recently applied for *E. coli* (Valgepea et al., 2010) and *L. lactis* (Lahtvee et al., 2011) by coupling A-stat cultivation with transcriptome, proteome and metabolome analyses, and metabolic flux analysis. Both studies represent a unique dataset of global regulation of metabolism with rising $\mu$. For instance, shifts in substrate utilization patterns (e.g. respiratory and fermentative) could be linked with gene expression dynamics (see above). Also, the high mRNA/protein correlations ($R$ up to 0.8) observed in A-stats imply that the state of the culture for analysis (steady state versus non-steady state) could be an important factor for mRNA/protein correlation determination. Further computational analysis of these data combined with absolute quantitative proteome analysis (Arike et al., 2012) reveals that both micro-organisms with very different metabolism, i.e. *E. coli* and *L. lactis*, use the same principles in the regulation of gene expression levels (mRNA, protein and metabolic fluxes). Furthermore, combining high-resolution A-stats with high-throughput -omics analyses led to novel biological discoveries. In more detail, it was shown using absolute quantitative transcriptomics and proteomics that cells achieve faster growth through post-transcriptional control of protein abundances (changes in protein levels are not strictly determined by changes in mRNA levels). Secondly, and even more importantly, higher flux throughput supporting faster growth is achieved through the increase of apparent *in vivo* catalytic rates of enzymes (Adamberg et al., 2012; Valgepea et al., 2013).

High-resolution $\mu$-dependent characterization of *Zygosaccharomyces rouxii* (a yeast used in soy sauce production) metabolism shows that this yeast species also displays the Crabtree effect under aerobic conditions and the effects of acceleration on culture characteristics at specific $D$ values are not only a function of the metabolic adaptation rate of the yeast, but also of the rate of change in environmental substrate concentrations during the A-stat (van der Sluis et al.,...
Another study investigating the evolution of physiological growth parameters of the yeast *H. guilliermondii* in an A-stat detected an optimal range of $\mu$ for achieving the highest biomass yield and further revealed that this non-*Saccharomyces* yeast continues to increase its oxygen uptake even after switching from fully respiratory to respiro-fermentative metabolism (Albergaria *et al.*, 2003).

Lastly, Lindley and colleagues used A-stats for investigating the high efficiency of demeton-S-methyl (a pesticide and a chemical warfare agent analogue) biodegradation by *Corynebacterium glutamicum* in co-metabolism with fructose and its dependence on $\mu$ (Girbal *et al.*, 2000). They discovered a direct coupling between the specific demeton-S-methyl consumption rate and $\mu$ in the range of 0.1–0.4 h$^{-1}$. Following high-cell-density cultures demonstrated the potential of using the latter bacterium for degradation of demeton-S-methyl in industrial processes.

All the latter studies demonstrate the benefits of applying high-resolution A-stats for determining $\mu$-dependent switches and dynamics in metabolism. These examples are also useful for bioprocess development due to the industrial relevance of these micro-organisms. Next, direct application of A-stat for bioprocess optimization is reviewed.

### Bioprocess development

The Dutch group led by Professor R. H. Wijffels has used the A-stat approach for the optimization of kinetic parameters of photobioreactors for microalgae cultivation (Barbosa *et al.*, 2003) and bioprocess conditions for vitamin and carotenoid synthesis by *Dunalieilla tertiolecta* (Barbosa *et al.*, 2005). Notably, the A-stat yields comparable data with chemostats even in such systems where the limiting nutrient is not evenly distributed within the bioreactor, and is thus suitable as a fast and accurate tool for the determination of kinetic parameters and optimization of cultivation conditions (e.g. for maximal biomass volumetric productivity) in specific photobioreactors (Barbosa *et al.*, 2003, 2005). Notably, the benefits of the A-stat approach were evident in optimization of production yields of vitamins C and E, and carotenoids lutein and $\beta$-carotene in microalgae *D. tertiolecta*, as optimal yields were achieved at different light intensities for each product (Barbosa *et al.*, 2005).

### Deceleration-stat (De-stat)

The changestat method deceleration-stat (termed De-stat in this review) is in principle an A-stat with the only difference that $D$ is decreased at a constant rate (opposite to the A-stat). Although the first study to implement De-stat was Paalme *et al.* (1997b) for the determination of maintenance energy requirements in *E. coli*, the De-stat approach has been most extensively used by Professor R. H. Wijffels’ group. An important advantage of the De-stat compared with the A-stat is the time saved because of the shorter time period needed to attain the initial steady state at higher $\mu$ and the use of a faster rate of change of $D$ (Hoekema *et al.*, 2014). Notably, a De-stat has been shown to yield comparable results with A-stats in the study of demeton-S-methyl biodegradation capability by *C. glutamicum* (Girbal *et al.*, 2000).

Wijffels and colleagues first used the De-stat approach for optimizing the photosynthetic efficiency of the purple non-sulphur bacterium *Rhodobacter capsulatus* for the production of hydrogen from acetate and light energy by determining important components of the light energy balance: biomass growth and maintenance, generation of hydrogen, and photosynthetic heat dissipation (Hoekema *et al.*, 2006). They saw that culture characteristics were strongly dependent on $\mu$. In another De-stat study, the maximum photosynthetic yield of green microalgae was investigated, revealing that photobioreactor designs should provide relatively high specific light supply rates as biomass yield at high biomass densities decreases at low light supply rates due to high demands for biomass maintenance (Zijffers *et al.*, 2010). Additionally, Professor R. H. Wijffels’ group has developed a simulation for estimating maximal productivity of algal biomass based on the De-stat data, including cases where deceleration exceeded the rate at which the quasi steady state could be maintained (Hoekema *et al.*, 2014). They showed that up to 94% of the time can be saved compared with chemostats using 10 times faster decelerations than required for maintaining the quasi steady state whilst losing only 5% in accuracy to estimate the maximal biomass productivity rate.

Two studies have utilized the De-stat approach to specifically study stress responses. Firstly, Neubauer and colleagues studied the stringent and general stress response during entry of *E. coli* into glucose starvation (Teich *et al.*, 1999). Whilst they observed clear responses of corresponding response regulators (ppGpp and $\sigma^S$ components) to nutrient shifts under fed-batch conditions, the concerted reaction of ppGpp and $\sigma^S$ was less apparent during a smooth change in nutrient availability in De-stats. Secondly, the response of *S. cerevisiae* to a sudden or gradual decrease in glucose concentration and $D$ has been followed based on the expression of the general stress response protein Hsp12p (Nisamedtinov *et al.*, 2008). Similar to Teich *et al.* (1999), Nisamedtinov *et al.* (2008) detected a stronger stress response, through protein Hsp12p expression, toward rapid changes in substrate concentration and $D$ in a chemostat compared with gradual changes in De-stat. Application of the De-stat approach in both studies led to the suggestion that stress response mechanisms in cells have adapted to rapid changes in environmental conditions.

### Dilution rate stat (D-stat)

The D-stat approach is the most powerful if it is planned to study the impact of an environmental parameter (including cultivation medium composition) on cell physiology at constant $\mu$. A range of values at high resolution can be screened within one experiment, thus more potentially detecting optimal growth conditions (Fig. 1). Although the D-stat was first used to study the Ehrlich pathway in the yeast *Z. rouxii* (van der Sluis *et al.*, 2002), D-stat characteristics were first
thoroughly formulated (Kasemets et al., 2003) and later most extensively practiced by the groups of Professor R. Vilu and Professor T. Paalme in Tallinn, Estonia. The concept of the D-stat is simple: one environmental parameter (e.g. temperature, pH or cultivation medium composition) is smoothly changed whilst D and other parameters are kept constant according to the equation: \( N = N_0 + a \times t \), where \( N \) is the parameter being changed, \( N_0 \) is the initial value of the parameter being changed, \( a \) is the rate of change of parameter \( N \) and \( t \) is time (Kasemets et al., 2003).

The D-stat approach is suitable for determining critical values and limits of both environmental parameters (e.g. temperature, pH) and composition of cultivation medium (e.g. relative nitrogen or vitamin concentration) that affect \( \mu \). For instance, D-stats with a smooth increase in temperature detected the dynamic effects of temperature on \( \mu \), biomass yield and byproduct profiles of \( S. uvarum \) (Kasemets et al., 2003). The same study also utilized a D-stat to determine the metabolic events accompanying the transition of metabolism from carbon to nitrogen limitation in \( S. cerevisiae \) by smoothly decreasing the nitrogen/carbon ratio in the ingoing medium. Similarly, analysis of the effect(s) of pH and/or temperature on \( L. lactis \) growth physiology showed dependence of both biomass yield and lactate production on pH and temperature (Lahtvee et al., 2009). Additionally, substrate consumption and product formation patterns, and gene expression showed no difference between increasing or decreasing pH within the range of 5.4–6.3. In addition to using a De-stat approach (see above), Paalme and colleagues also applied a D-stat to study the gradual stress response of \( S. cerevisiae \) to salt, ethanol or high temperature through protein Hsp12p expression (Nisamedtinov et al., 2008). High-resolution D-stats at \( D = 0.09 \text{ h}^{-1} \) exactly detected the threshold values for the stress response for each treatment: expression of Hsp12p increased above temperature 34°C, salt concentration 185 mM or ethanol concentration 0.54 mM.

The D-stat approach is possibly the most useful, especially regarding bioprocess development, for studying the consumption patterns of different substrates. In such cases, after steady state in a chemostat has been achieved on one medium, feeding of another medium with a different composition [added, removed or modified concentration of component(s)] is started and increased in time whilst simultaneously decreasing the feeding rate of the initial medium to keep \( D \) constant. These D-stats have yielded the following main results: (i) decreasing the threonine/methionine ratio in the ingoing medium of \( Z. rouxii \) cultivations revealed that methionol synthesis occurs in the Ehrlich pathway (van der Sluis et al., 2002); (ii) byproduct patterns of fermentative metabolism for various lactic acid bacteria depend on the galactose/arginine ratio in the cultivation medium (Adamberg et al., 2006); (iii) maximal acetate consumption of \( \sim 35 \text{ mmol (g dry cell weight)}^{-1} \) by \( E. coli \) under glucose limitation is maintained up to \( D = 0.2 \text{ h}^{-1} \), whilst co-utilization is totally lost between \( D = 0.45 \) and \( 0.5 \text{ h}^{-1} \) (Valgepa et al., 2010); and (iv) the best ratio nitrogen/carbon ratio in the cultivation medium for producing lipids without carbon loss into citric acid by \( Yarrowia lipolytica \) is between 0.021 and 0.085 N-mol/C-mol (Ochoa-Estopier & Guillouet, 2014).

**Auxo-accelerostats**

The A-stat and D-stat approaches are suitable for high-resolution study of steady-state cell physiology, but only under nutrient limitation. To study the effects of environmental parameters and medium components on the metabolic switch-points and optimal growth conditions of cells at maximal specific growth rates, the auxo-accelerostat approach is the most suitable (Fig. 1). The classical method for studying cells in the steady state under substrate excess conditions is the turbidostat (Bryson & Szybalski, 1952) where cells are forced to grow with \( \mu_{\text{max}} \) by adjusting \( D \) based on biomass concentration (with optical density as the indicator) using the following simple logic: if the optical density is below or above the set-point value, \( D \) is decreased or increased, respectively. In addition to culture turbidity, cells can be forced to grow with \( \mu_{\text{max}} \) through feedback from pH (pH-auxostat; Martin & Hempfling, 1976), \( \text{CO}_2 \) (\( \text{CO}_2 \)-auxostat; Watson, 1969), electrical capacitance of the culture (permittistat; Markx et al., 1991) or other parameters. However, turbidostat and auxostats are not suitable for high-resolution studies of steady-state growth space under nutrient excess as, similar to the chemostat, the culture has to be stabilized after every shift in environmental conditions. Therefore, auxo-accelerostats were developed (Kasemets et al., 2003) for achieving higher resolution through smoothly changing one environmental parameter under nutrient excess conditions. Auxo-accelerostat culture is controlled online by the experimenter through a parameter that is either directly (e.g. optical density) or indirectly [e.g. pH, percentage of dissolved oxygen concentration in bioreactor (pO2%), percentage of \( \text{CO}_2 \) in the off-gas] related to cell growth. The equation for operating an auxo-accelerostat is the same as for the D-stat (see above).

Several studies have demonstrated the effectiveness of auxo-accelerostats for screening out optimal or inhibitory conditions for the growth of yeasts and lactic acid bacteria. Applying various algorithms of the auxo-accelerostat for studying the effects of pH, temperature, salt concentration and water activity on the growth of the cheese bacterium \( Lactobacillus paracasei \) revealed that water activity is the most important environmental parameter affecting the growth of cells under cheese-like conditions (Laht et al., 2002). Interestingly, the response curve of slowly growing \( L. paracasei \) (\( \mu_{\text{max}} = 0.3 \text{ h}^{-1} \)) to pH and temperature was rather flat, whilst fast-growing \( Streptococcus thermophilus \) (\( \mu_{\text{max}} = 2.2 \text{ h}^{-1} \)) showed a narrow range of temperature (44°C) and pH (6.6) for growth at \( \mu_{\text{max}} \) (Adamberg et al., 2003).

Auxo-accelerostats have also been used to study stress responses of yeast in detail. Characterizing the toxic effects of aliphatic monocarboxylic acids (e.g. acetic, formic and propionic acids) on \( S. cerevisiae \) growth using \( \text{CO}_2 \)-auxo-accelerostats revealed an immediate decline of \( \mu \) and growth yield with slowly increasing acid concentrations (Kasemets et al., 2006). Furthermore, they showed that the dose-effect
curves (IC_{50}, acid concentration causing a 50 % decrease in either μ or growth yield) are five times higher when the inhibitory acid is added smoothly to the growth environment (as in the auxo-accelerostat) compared with when added rapidly (by pulse). Two studies using auxo-accelerostats and metabolic modelling for studying the effect of smoothly changing stress conditions (increasing temperature, ethanol, salt or organic acid concentration) on a laboratory and a recombinant S. cerevisiae strain determined similar threshold levels of stress responses causing the decrease of μmax (Kasemets et al., 2007; Nisamedtinov et al., 2008). Interestingly, these threshold values determined under nutrient excess and μmax were very similar to those of glucose-limited D-stats at D=0.09 h⁻¹ (Nisamedtinov et al., 2008). In conclusion, the latter studies highlight that high-resolution auxo-accelerostats can successfully be used for determination of the quantitative effects of environmental conditions on growth characteristics.

Adaptastat
To further complement the array of changestat methods, the adaptastat was developed for studying cells near μmax under substrate limitation in aerobic cultures a decade ago in the National Institute of Chemical Physics and Biophysics in Tallinn, Estonia by Dr Kalju Vanatalu. In an adaptastat, μ of cells is raised stepwise until near μmax through increasing D according to activation of oxygen consumption by the micro-organism (Tomson et al., 2006). More specifically, D is controlled through a feedback loop as follows: after attaining steady state in a chemostat, feeding (ΔD) is abruptly increased followed by stop (or reduction) of nutrient inflow. Next, the time needed to exhaust the residual substrate is calculated by measuring the rise of the dissolved oxygen concentration. The shorter the time, the faster the growth. Subsequently, ΔD is increased or decreased depending on the ratio of the feeding pulse and substrate exhaustion durations. The adaptastat is an attractive method as it enables the conversion of a chemostat culture to near μmax conditions whilst maintaining substrate limitation. The adaptastat is also the first method to produce nutrient-limited growth on two substrates. The method should be efficient for isotopic labelling of bacterial cultures and their components produced abundantly at fast growth (e.g. ribosomes and RNA).

Conclusion
Currently, the number of recombinant cells designed in silico exceeds the throughput of comprehensive experimental characterization of their metabolism, which is vital for validating the in silico predictions. More complete description and in silico reconstruction of their phenotypes has great potential for accelerating systems microbiology-based bioprocess optimization. With this review, we hope to have presented that a more accurate and quantitative understanding of metabolism could be achieved through steady-state GSA utilizing high-resolution changestat cultivation methods.

The changestat methods have several advantages over the widely used batch and chemostat methods, and provide unique knowledge about shifts in metabolism and optimal growth conditions. Thus, we propose that systematic high-resolution characterization of the steady-state growth space of cells using changestats should not only be used for fundamental studies of metabolism, but also incorporated into the systems microbiology-based metabolic engineering pipeline (Fig. 3) (Van Dien, 2013). Comprehensive systems microbiology through coupling advanced continuous cultivation methods, -omics technologies and metabolic modelling could lead to more efficient cell designs. Notably, remarkable interest in continuous cultivations has recently emerged in the pharmaceutical industry due to sustained operation with consistent product quality, reduced equipment size, high volumetric productivity, streamlined process flow, low process cycle times, and reduced capital and operating costs (Konstantinov & Cooney, 2015).

In the future, more automated control and higher throughput of changestats is required to achieve their wider acceptance and use. One approach to accomplish this is the use of iterative control algorithms during a changestat experiment through continuous modification of the rate of change of the environmental parameter according to adaptation of cells. This approach would enable the change of the direction of growth space scanning and change of several environmental parameters simultaneously. There already exists an approach to increase the throughput of changestats: a method enabling the multiplication of the steady state by a sequential parallel cultivation system, termed the MD (‘mother–daughter’) system (Erm et al., 2014). Higher throughput in the MD

![Fig. 3. Systems microbiology-based pipeline of metabolic engineering of superior cell factories.](image-url)
system is achieved by distributing the steady-state culture from the ‘mother’ vessel to several ‘daughter’ vessels without disturbing the physiological state of cells, thus allowing changes to take place in each ‘daughter’ vessel after the transfer. Hopefully, other successful developments will follow.

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References


van der Sluis, C., Westerink, B. H., Dijkstal, M. M., Castelein, S. J.,
van Boxtel, A. J., Giuseppin, M. L., Tramper, J. & Wijffels, R. H.
(2001). Estimation of steady-state culture characteristics during
van der Sluis, C., Rahardjo, Y. S. P., Smit, B. A., Kroon, P. J., Hartmans, S.,
extracellular accumulation of alpha-keto acids and higher alcohols by
Van Dien, S. (2013). From the first drop to the first truckload:
commercialization of microbial processes for renewable chemicals.
Curr Opin Biotechnol 24, 1061–1068.
van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M.,
authors (2000). An interlaboratory comparison of physiological and
genetic properties of four Saccharomyces cerevisiae strains. Enzyme
Microb Technol 26, 706–714.
Watson, T. G. (1969). Steady state operation of a continuous culture
at maximum growth rate by control of carbon dioxide production.
J Gen Microbiol 59, 83–89.
Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt,
J., Khandurina, J., Trawick, J. D., Osterhout, R. E. & other authors
(2011). Metabolic engineering of Escherichia coli for direct
& Wijffels, R. H. (2010). Maximum photosynthetic yield of green

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