Development of scalable high throughput fermentation approaches for physiological characterisation of yeast and filamentous fungi

Knudsen, Peter Boldsen; Workman, Mhairi; Nielsen, Kristian Fog; Thykær, Jette

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Peter Boldsen Knudsen
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Development of scalable high throughput fermentation approaches for physiological characterisation of yeast and filamentous fungi

Ph.D. Thesis
Peter Boldsen Knudsen
Department of Systems Biology
Technical University of Denmark

Supervisors
Assoc. Prof. Mhairi Workman
Assoc. Prof. Kristian Fog Nielsen
Dr. Jette Thykær
Summary

The pursuit of identifying efficient cell factory candidates for production of pharmaceutically relevant products and commodity chemicals relies heavily on the successful selection of process suitable microorganisms. Hence the selection of efficient cell factories is paramount for successful scale-up to economically viable industrial processes. Accurate quantitative assessment of cellular performance is required for the evaluation of the overall suitability of a microorganism as an industrial cell factory, ensuring that not only product, but also process parameters are optimised. With the increasing number of strains generated through genetic engineering programmes, the traditionally applied methods for strain characterisation, which are typically labour intensive and time consuming, have become somewhat limited due to throughput capacity. Unfortunately, most high throughput methods only provide low levels of information compared to larger scale cultivations, explaining why these systems have not been broadly implemented.

The overall aim of the thesis was, therefore, to shift this paradigm towards higher throughput systems for assessment of cellular performance with a higher level of information. This was pursued through development and validation of small, scalable microtiter based systems, for cultivating yeast and filamentous fungi, validated by comparable results from bioreactors. The experimental work was performed using Saccharomyces cerevisiae (yeast) and Aspergillus nidulans (filamentous fungus) strains producing the heterologous model polyketide, 6-methylsalicylic acid (6-MSA).

An automated methodology for high throughput screening focusing on growth rates, together with a fully automated method for quantitative physiological characterisation in microtiter plates, was established for yeast. Full scalability was demonstrated through comparative physiological characterisation of yeasts, cultivated in both microtiter plates and bioreactors, revealing that the growth rate and yield coefficients of all non-volatile products including biomass could be correlated. The highly correlated results were taken as
an indication of comparable growth physiology in both microtiter plates and bioreactors, which was substantiated by metabolic flux analysis resulting in identical flux distributions over micro-scale and lab scale cultivations.

The thesis further presents a novel automated high throughput method for cultivating filamentous fungi in microtiter plates, without compromise to morphology and with product yields and growth rates identical to bioreactors. This was made possible by the dispersive effect on morphology of the anionic polymer carboxypolymethylene, enabling the application of optical density measurements as a means to evaluate growth rates. Again full scalability was demonstrated for a heterologous 6-MSA producing A. nidulans strain, cultivated in both microtiter plates and in bioreactors, displaying identical growth rates and 6-MSA yields on glucose. This versatile and robust method was shown to be applicable for a wide range of different filamentous fungi and conditions, with growth rates comparable with those reported in the literature.

A final comparative study of cell factory potential, involving the heterologous 6-MSA producing S. cerevisiae and A. nidulans strains, applied throughout the thesis, demonstrated superior yields and productivities for an A. nidulans strain where two copies of the 6-MSA gene had been chromosomally integrated. Implementation of a chemostat cultivation strategy for A. nidulans proved successful for conditional comparison, while metabolic oscillations and low productivities in continuous cultivations of S. cerevisiae rendered it less favorable for production of 6-MSA.

Overall the methods developed and validated during the course of this study, have contributed to a progression towards higher throughput, together with an improved level of detail in physiological characterisation of cultivations at micro-scale. This paves the way for further advances in detailed quantitative physiology based on omics analyses, which would further increase the possibilities for advanced quantitative physiology at different scales.
**Resumé**

I denne afhandling præsenteres tre validerede “high throughput” metoder til fysiologisk karakterisering af mikroorganismer under bioreaktor sammenlignelige forhold.

Rent eksperimenterligt blev dette gennemført via anvendelse af bl.a. genmodificerede *Saccharomyces cerevisiae* (gær) og *Aspergillus nidulans* (skimmel svamp) stammer, designet til at producere det heterologe model polyketid 6-metylsalicyl syre (6-MSA).

I studiet blev der etableret en automatiseret “high throughput” screeningsmetode til gær med primær fokus på vækst hastigheder. Videreudvikling af denne metode resulterede i en fuld automatiseret metode til kvantitativ fysiologisk karakterisering i mikrotiter bakker.

Fuld skalerbarhed blev demonstreret gennem komparativ fysiologisk karakterisering af gær dyrket i både mikrotiter bakker og bioreaktorer, hvor både væksthastigheder og udbytte koefficienter blev vist sammenlignelige. Disse resultater blev yderligere understøttet ved hjælp af metabolsk flux analyse resulterende i til alle formål identiske flux distributionerne.

I afhandlingen præsenteres endvidere en nyskabende automatiseret “high throughput” metode til kultivering af skimmelsvampe i mikrotiter bakker. Dette blev muliggjort ved hjælp af den anioniske polymer carboxypolymetylen, der sikrede en fordelagtig morfologi, hvilket muliggjorde estimering af væksthastigheder via optisk densitets målinger. I lighed med det tidligere studie på gær, blev der også her demonstreret fuld skalerbarhed, med ens væksthastigheder og 6-MSA udbytte koefficienter for *A. nidulans* dyrket i både mikrotiter bakker og i bioreaktorer. Denne alsidige og robuste metode viste sig yderligere, anvendelig for en bred vifte af forskellige skimmelsvampe, resulterende i litteratur sammenlignelige væksthastigheder.
Afslutningsvis udførtes et komparativt studie til afklaring af cellefabrik potential af de, i afhandlingen, anvendte 6-MSA producerende gær og skimmelsvampe. Højest produktivitet og udbytte blev identificeret for en *A. nidulans* stamme, hvor 2 kopier af 6-MSA genet var blevet kromosomalt integreret.

I dette studie endvidere udviklet en *A. nidulans* kemostat kultiveringsmetode til anvendelse i komparative studier. Tilsvarende *S. cerevisae* kultiveringer blev kompromitteret af metaboliske oscilleringer under bestemte forhold, hvilket besværliggjorde sammenligning. Den oscilleringe fænotype blev stabiliseret ved tilsætning af en mindre mængde ethanol til fødemediet, hvilket muliggjorde sammenligning af de to forskellige microorganismer. På baggrund af disse forsøg kunne det konkluderes, at *A. nidulans* er væsentlig overlegen, som celle fabrik til produktion af 6-MSA i forhold til *S. cerevisiae*.

Samlet set har de i afhandlingen udviklede metoder bidraget til en progression imod højere evalueringskapacitet med et væsentlig højere detaljeringsniveau i relation til fysiologisk karakterisering af mikro-skala fermenteringer. Med disse studier er der banet vej for yderligere fremskridt i detaljeret kvantitativ fysiologi baseret på omics analyser, hvilket yderligere vil øge mulighederne for anvendelse af avanceret kvantitativ fysiologi i en bredere vifte af fermenteringssystemer.
Preface

The work presented in this thesis was performed between March 2011 and March 2015 at Section for Eukaryotic Biotechnology, Institute for Systems Biology, Technical University of Denmark (DTU). The PhD study was part of the former Centre for Microbial Biotechnology’s (CMB) IVC (Ingeniør-Videnskabelige Centre) supported by the Danish Research Council for Technology and Production. The work was supervised by Dr. Jette Thykær, Associated Professor Kristian Fog Nielsen and Associated Professor Mhairi Workman.

I wish to express my gratitude to my original main supervisor Jette Thykær and Kristian Fog Nielsen for putting your trust in me and offering me the opportunity to pursue a PhD here at DTU. I am grateful for all of your guidance, supervision and for keeping your doors open for questions, ideas and problems even when you were not at work.

A special thanks to Jette for your unbeatable optimism and for keeping me from despair, even when nothing worked and everything seemed hopeless.

My deepest gratitude goes to Mhairi Workman that generously took over main supervision in the last crucial period after Jette. Thank you for taking on this responsibility, for all of our both valuable and fun discussions and finally for all your help allowing me to finish my PhD.

None of this would have been possible if not for the tireless efforts of Tina Johansen, Martin Nielsen and Elisabeth Krøger. Thank you for your technical assistance and for being able to solve even those problems that seems unsolvable.

To all of my colleagues at Systems Biology, I would like to thank you all for making it a fun and inspiring place to work. I would especially like to thank the following: Subir Kumar Nandy for valuable discussions about flux analysis, fermentation and for the great collaboration that lead to a joint manuscript, my former office mate Dorte Koefoed Holm for interesting and fun discussions that lead to several joint manuscripts.
together with Lene Petersen, and for constructing several of the strains that this thesis is built on, Zofia Jarczynska for helping with Dortes strains, providing the basis for a joint manuscript, Olivera Magdenoska for great collaboration forming the basis for two manuscripts. Thanks also to Tomas Strucko, Jakob Blæsbjerg, Andreas Klitgaard, Daniel Svenssen, Ana Ley, Gustav Hansen Paiman Khorsand-Jamal, Stig Rattleff, Phillipe Holt, Kasper Rasmussen, Anne Lund, Martin Schälen, Diana Anyaogu, Anna-Lena Heins for improving the daily life at the section.

Last but not least, a special place is reserved for my girlfriend Louise Dauggard for being endlessly patient with me and for understanding the difficulties and complications that inherently is associated with fermentation “steeling” more than its fair share of my time.

Peter Boldsen Knudsen
Publications

The results presented in this thesis have formed the basis for the following articles and manuscripts:


Furthermore minor contributions have been given to the following articles and manuscripts:


- Klitgaard A, Frandsen RJN, Holm DK, **Knudsen PB**, Frisvad, JC and Nielsen KF. Combining UHPLC-high resolution MS and feeding of stable isotope labeled polyketide intermediates for linking precursors to end products. Journal of Natural Products 2015. (Accepted, pending revisions).


- Frandsen RJN, Rasmussen SA, **Knudsen PB**, Uhlig S, Petersen D, Lysøe E, Gotfredsen CH, Giese H and Larsen TO. Perithecial pigmentation in *Gibberella* species is to the accumulation of 5-deoxybostrycoidin-based melanin. Chemistry and Biology (Submitted).

## Contents

**Summary** .................................................................................................................. I  
**Resumé (dansk)** ......................................................................................................... III  
**Preface** ....................................................................................................................... V  
**Publications** ............................................................................................................... VII  
**Chapter 1** .................................................................................................................. 3  
  Introduction and outline ................................................................................................. 3  
  Outline of the thesis ...................................................................................................... 12  
  References ..................................................................................................................... 18  
**Chapter 2** .................................................................................................................. 23  
  A high throughput method for quantifying metabolically active yeast cells ............ 23  
  Abstract ....................................................................................................................... 24  
  Introduction .................................................................................................................. 25  
  Materials and Methods ............................................................................................... 27  
  Results and Discussion .............................................................................................. 30  
  Conclusions ................................................................................................................. 37  
  References .................................................................................................................... 39  
**Chapter 3** .................................................................................................................. 41  
  A scalable high throughput fermentation approach for exploring cell factory potential 41  
  Abstract ....................................................................................................................... 42  
  Introduction .................................................................................................................. 43  
  Materials and methods ............................................................................................... 46  
  Results and discussion ............................................................................................... 57  
  Conclusion ................................................................................................................... 76  
  References .................................................................................................................... 77  
**Chapter 4** .................................................................................................................. 81  
  A high throughput microtiter plate based screening approach for physiological characterisation of filamentous fungi ........................................................................... 81  
  Abstract ....................................................................................................................... 82  
  Introduction .................................................................................................................. 84  
  Materials and Methods ............................................................................................... 87
Chapter 1

Introduction and outline
Introduction

In recent years there has been a dramatic increase in the number of fungal stains generated with potential applications in industrial biology. The revolution in genome sequencing has made rational design and metabolic engineering strategies available, and this has been coupled with a simultaneous advancement of increasingly efficient genetic engineering tools [1]. These developments have been amplified by the advent of new high throughput molecular biology techniques such as USER cloning/fusion and EasyClone [2]–[6] and recently the genome editing system CRISPR/Cas9 has been demonstrated for filamentous fungi [2]–[7]. In addition there has been an increasing interest in the design of cell factories capable of utilising non-conventional substrates such as glycerol, a by-product of from biodiesel production, where both molecular biology techniques and evolutionary engineering are employed in the design process [8]–[11]. Consequently, the bottleneck has shifted from strain construction to characterisation that with the increasing strain numbers makes it imperative to develop and employ high throughput systems for quantitative physiological characterisation.

Quantitative physiology

The selection of an efficient cell factory from screening experiments is paramount for successful scale-up to economically viable industrial biotechnology processes. While the criteria for a successful candidate is highly dependent on the product of interest, all potential cell factories must perform well with respect to growth rate, ability to efficiently convert substrates to the desired products, and be manageable both in large scale bioreactors and downstream processes for product recovery. Thus, for successful identification of the best performing cell factory candidate, accurate quantitative assessment of cellular performance is required for the evaluation of the overall suitability for scale-up, ensuring that not only product, but also process development is optimised [12], [13].
Dependent on the stage of advancement in the identification process, different quantitative characterisation approaches may be chosen, aiming at evaluating growth rates with end point titers or full scale characterisation of growth rates, yield coefficients and productivities (figure 1) [14].

Figure 1. Schematic summary of available systems for quantitative physiological characterisation of cell factory potential. With increasing information levels, throughput is compromised making it imperative to advance the high throughput systems to accommodate the increasing number of strains which are available.

The choice of system for identification and characterisation of a potential cell factory is highly variable with the number of candidate strains to be evaluated, where the number often determines the system chosen. It is therefore crucial that the quality of the data generated in high throughput systems mirror the reality in larger volume bioreactors, ensuring that the chosen candidate strain will perform equally well when the process is scaled up to production levels [14].
Amongst the applied cultivation systems, Erlenmeyer flasks have traditionally been first choice for strain selection, medium optimisation and, to a moderate extent, physiological characterisation. Though this approach presents reasonable throughput of individual experiments manageable for a single individual, the sheer number of strains made available, with the introduction of new molecular biology techniques, challenges this practice [15], [16]. The increasing screening load has, to some extent, been alleviated by the use of microtiter plates (MTP) allowing for a high degree of parallelisation with a massive increase in number of single experiments manageable for one person [12]. Unfortunately, these methodologies, mostly provide yes/no results, in the form of end point titers and to some extent growth rates, under conditions not validated against fully instrumented bioreactor systems [14]. Consequentially, there has been a substantial interest in downscaling bench top bioreactors to micro- and mini-bioreactor scale, increasing throughput by parallelisation and reducing the labour cost invested [12], [17], [18]. However, even for these systems, current throughput is limited to a maximum of 48 parallel cultivations in 8-12 mL scale with the BioReactor 48 system by 2mag AG [12], [18].

A similar development in MTP based cultivation systems has enabled even higher throughput of 48 to several hundred parallel cultivations with variable levels of control presented by e.g. the BioLector and Hamilton STAR systems [12], [19]–[21]. With these systems a high degree of automatisation and process control allows for a significant improvement in screening throughput, with control levels for the BioLector systems approaching instrumented bioreactors, e.g. pH and dissolved oxygen (DO) control, and recently also the option for feeding and actively modulating the culture pH [22], [23]. Application of such systems is well established for a range of unicellular microorganisms, in particularly bacteria. With an increasing number of studies validating MTP based physiological parameters, with those of the instrumented cultivation systems, the selection of the best performing strain may be done with increasingly higher confidence [1], [14], [23]–[25]. Thus MTP based systems, together with micro- and mini-bioreactors, can to some extent substitute the more laborious shake flask and bench top bioreactor systems for the basic
physiological characterisation of unicellular microorganisms, enabling confident selection of best performing strains, thereby significantly reducing the number of strains for further in-depth characterisation [14].

Unfortunately, the more complex morphology of most filamentous organisms precludes the use of these high throughput systems, where at best, end point titers comparable with shake flasks may be obtained [26]. To further advance eukaryotic cell factories and have the possibility for expediting the process time from strain discovery to bioprocess application, it would be highly desirable to define and validate the conditions required for controllable, micro-scale cultivations with these organisms.

**Advanced quantitative physiology through Omics approaches**

Though an increasing level of information may be extracted from high throughput systems the need for high quality biomass in quantities relevant for omics based characterisation poses a challenge with regards to the microscale systems (MTP and micro-bioreactors), necessitating the use of larger instrumented bioreactors. A few examples exist describing methods for fluxomics [27], metabolomics [28], [29] and proteomics [30] in scaled down MTP based cultivations, however the need for quality biomass produced under highly controlled conditions (tailor-made biomass) more or less precludes use of small scale systems for most types of omics analysis and is especially problematic with filamentous microorganisms [1].

To produce the quality biomass needed for omics analyses, not only volume, but also highly defined conditions are needed [13]. To meet these requirements mini-bioreactors and standard bench scale bioreactors are preferred. These ensure sufficient control, allowing the researcher to monitor and modify a number of different parameters such as pH, dissolved oxygen concentration and temperature, as well as measure biomass, substrate and product concentrations in order to calculate growth rates and yield coefficients [13], [31]. By exerting control over the growth rate by means of continuous medium supply in chemostat cultures, detailed physiological studies of cellular performance under highly controlled and similar conditions enables more reliable comparison of strains displaying different kinetic behaviour [32].
Together with batch cultivations where individual kinetics may be assessed, the chemostat, and to some extent fed-batch cultivations, provides high quality, and especially for comparative studies, tailor-made biomass for a range of more advanced systems biology omics analysis approaches (figure 2) [13], [32], [33].

Figure 2. Schematic overview of the relation between classical physiological characterisation techniques, generating information for basic assessment of cellular performance, and the required “tailor-made” biomass for advanced omics analysis (systems biology).

Cellular performance may be assessed either by a simple overall model termed the “black box model” or by application of structured models utilising advanced omics analysis. The former enables direct analysis of cellular performance taking advantage of a simple mathematical representation of cellular growth, lumping all reactions into one overall cellular reaction, assuming adherence to a simple kinetic model such as the Monod model. Here no information on the pathways by which the various products and biomass are
produced is known, and the principle requirement states that all substrates must be retrievable as products on elementary balances [32], [34]–[36]. While this model is vastly underdetermined, considering only the major metabolic products produced with no information on the pathways applied it has been widely used and accepted for cellular performance assessment [32], [37]. More information may be generated from a systems biology approach were biomass generated under highly controlled conditions in batch, fed-batch and chemostat, cultivations is analysed by application of a range of omics methods [11], [32], [33], [38]. Application of these methods enables in-depth analysis of the metabolic pathways by which the aforementioned products are generated, providing a deeper, holistic understanding of the cellular performance. Thus by application of the vast array of omics methods, detailed information applicable for strain optimisation programs may be generated, resulting in an iterative process where cellular performance analysis is applied for the further optimisation of the cell factory strain in question, as illustrated in figure 3 [39], [40].
Figure 3. Illustration of the iterative process of physiological strain characterisation producing quality biomass for omics based analysis, both generating information's applicable for further strain optimisation. Figure based on [13].

The term omics covers a wide range of different approaches for investigating cellular function with each focusing on particular elements [39]. These techniques are increasingly applied for identifying targets for genetic engineering and thus strain optimisation, especially accelerated by the increasing number of available genomes [32], [40]. This has sparked a revolution in genomics and thus genome scale modelling, enabling the identification of gene targets for deletion or overexpression, thereby increasing the strain construction success rate [41]. This revolution has driven a rise in the application of transcriptomics studies, as a means to evaluate the dynamic transcription of genes under a given set of conditions. This information is highly applicable for identification of particular beneficial cultivation conditions and the global response to insertion of heterologous genes [39], [42]. Though transcriptomics enables in-depth analysis of which genes are actively transcribed, the subsequent translation into proteins does not reveal the extent of post-
translational modifications nor the protein quantities, leading to a different type of omics termed proteomics.

Since proteins, in interplay with other effectors, are responsible for most of the biological functions in the cells, the application of proteomics becomes an important tool for investigating the regulatory networks of the microorganism [39], [42], [43]. Where proteomics and transcriptomics outline the metabolic network capacity of the microorganism, metabolomics summaries an immense array of intracellular metabolite pools. The analyse of the metabolome is therefore the measurement of, not only the driving forces of cells, such as energy and redox metabolites, but also building blocks and signal molecules pools. Diligent analysis of these intracellular metabolite pools may yield vital information on potential bottlenecks in precursor supply, applicable for identification of potential targets for genetic engineering [33], [39]. Where the other omics techniques represent various levels of the cellular network, fluxomics represents the final endpoint of all network events describing the intracellular in vivo reaction rates of molecular fluxes. By determining the metabolic fluxes of the central carbon metabolism the activity of the essential pathways is examined, enabling in-depth analysis of the metabolic response to a change in the biological system, yielding valuable information concerning potential bottlenecks applicable for rational metabolic engineering [32], [39], [44].

While micro-scale fermentations are becoming increasingly applicable for classical quantitative physiological characterisation of growth rates, yield coefficient and overall microorganism kinetics, bioreactor based cultivation remains almost exclusively the source for the high quality biomass needed for generating omics data [13]. Thus, the ability to perform controlled, robust and reproducible submerged cultivations is the essential step towards advanced physiological characterisation of microorganisms.
Outline of the thesis

The cell factory concept includes a wide range of organisms from microbes to higher eukaryotes including those of insect, plant and mammalian origin, amongst which the most commonly applied are bacteria (e.g. *Escherichia coli*), mammalian cells (e.g. CHO cells), and fungi (including both yeasts and filamentous fungi) [45], [46].

In this study the experimental work is confined to yeast (*Saccharomyces cerevisiae*) and filamentous fungi, focusing on development of scalable methodologies for cultivating these important industrial cell factories.

The industrial application of these vastly diverse microorganisms covers a wide range of different products ranging from pharmaceutically relevant proteins, antibiotics and immunosuppressants to commodity products like citric acid, ethanol and enzymes [46], [48]. Though yeasts and filamentous fungi are both eukaryotic, belonging to the same phylogenetic kingdom, they differ on a range of vital levels making yeast significantly easier to cultivate, whereas the more complex filamentous fungi presents a wider range of products. The principal cell factory related properties are listed in table 1.

### Table 1. Comparison of selected cell factory characteristics of yeast and filamentous fungi cell factories.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Yeast</th>
<th>Filamentous fungi</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rates</td>
<td>High</td>
<td>Low</td>
<td>[47]</td>
</tr>
<tr>
<td>Substrate range</td>
<td>Narrow</td>
<td>Wide</td>
<td>[13], [47], [49], [50]</td>
</tr>
<tr>
<td>Native product range</td>
<td>Low</td>
<td>High</td>
<td>[42], [51], [52]</td>
</tr>
<tr>
<td>Productivity</td>
<td>Medium</td>
<td>High</td>
<td>[49], [52]</td>
</tr>
<tr>
<td>Toxic by-products</td>
<td>None</td>
<td>Several, conditional production</td>
<td>[53], [54]</td>
</tr>
<tr>
<td>Morphology</td>
<td>Unicellular</td>
<td>Heterogeneous, filamentous</td>
<td>[49], [51], [53], [55]</td>
</tr>
<tr>
<td>Stability</td>
<td>Oscillating metabolism</td>
<td>Oscillating morphology</td>
<td>[51], [53], [56]</td>
</tr>
<tr>
<td>Metabolic models</td>
<td>Established</td>
<td>Emerging</td>
<td>[13], [48]</td>
</tr>
<tr>
<td>Amenable to genetic engineering</td>
<td>Established</td>
<td>Few complex methods</td>
<td>[47]</td>
</tr>
<tr>
<td>Omics</td>
<td>Established</td>
<td>Emerging</td>
<td>[13], [47]</td>
</tr>
</tbody>
</table>
The main focus of the thesis is the development and validation of scalable systems for cultivating yeast and filamentous fungi, ensuring comparable results with instrumented bioreactors. The goal was to shift the paradigm outlined in figure 1, towards higher throughput systems for assessment of cellular performance with a higher level of information.

Although, several microorganisms were employed in this study, the focal point was a set of *Saccharomyces cerevisiae* and *Aspergillus nidulans* strains producing a heterologous model polyketide 6-methylsalicylic acid (6-MSA).

This polyketide presents one of the structurally simplest of the class \((C_8H_8O_3)\), produced naturally by *Penicillium patulum* as a biosynthetic intermediate for the toxin patulin [57]–[59]. The polyketide synthase (PKS) gene responsible for the production of 6-MSA (6-MSAS) was the first fungal PKS to be cloned and characterised, hence this polyketide has served as model for both investigation of biosynthetic pathways [60], [61] as well as a for characterisation of potential industrially relevant heterologous cell factories [62]–[65].

Polyketides comprise a structurally diverse class of secondary metabolites isolated from a broad range of different bacteria, filamentous fungi and plants with a wide range of bioactivities from pigments for protection against UV-radiation, signalling and chemical warfare against competitors [66]. Amongst these are several mycotoxins with harmful effects to human health, such as fumonisins and the carcinogenic aflatoxins [67], [68]. While certain polyketides and derived compounds display detrimental effects toward humans, others exhibit pharmacological effects, which have been harnessed for a range of different medical purposes amongst which are antibiotics, anticancer and cholesterol lowering agents [69], [70]. This is well illustrated by the fact that 20 % of the top-selling drugs are polyketide or derived products, as surveyed in 2005 [71]. Thus, there is an economically driven motivation for isolation and characterisation of polyketide based compounds and, importantly, developing systems for the production of polyketides [65].
For all of the 6-MSA producing strains studied here, the 6-MSAS gene(s) were heterologously expressed on the chromosome of the respective strains under constitutive promoters, and expression was thus growth related. In order to advance detailed physiological characterisation of fungal cell factories and validate robust and reproducible methodologies for cultivation, the PhD study comprised several approaches towards the overall goal.

The thesis core comprises three independent chapters each outlining the development of downscaled methodologies for cultivation in microtiter plates with a strong focus on validating quantitative physiological parameters to fully instrumented bioreactors. In support to these chapters a method (see Appendix I) entitled “A robust validated GC-MS methodology for $^{13}$C-measurements of proteinogenic amino acids with application in metabolic flux analysis” was developed. The purpose of this work was to develop and validate a protocol for hydrolysis, sample purification using solid phase cartridges and revising the already established derivatisation and analysis protocol, thus reducing the biomass requirement. The reduction of biomass quantities was crucial for the successful application of metabolic flux analysis in chapter 3.

In chapter 2 entitled “A high throughput method for quantifying metabolically active yeast cells” an already established methylene blue reduction test for bacteria was revised and adapted for automated high throughput analyses of the metabolic activity of yeast cells in batch cultures. A high throughput cultivation approach applying 48 well microtiter plates with automated liquid handling and atline spectrophotometric optical density measurements was devised, ensuring full autonomy of the entire process. Growth rates extrapolated from optical density measurements of both microtiter plate wells and fully instrumented bioreactors were highly correlated, which was taken as evidence for comparable growth physiology. This observation was supported by means of the adapted methylene blue reduction test, displaying a markedly
high correlation for both bioreactors and microtiter plates. The methylene blue reduction test was furthermore shown to be applicable for growth rate determination and it was suggested that this approach might be superior to traditional gravimetric and optical methods for estimating growth rates, due to the discriminatory nature of the method, ensuring that only metabolically active cells are quantified. The field of application for this method includes characterisation of the death phase of stationary phase cultures and drug screens with pathogenic fungi.

In chapter 3 entitled “A scalable high throughput fermentation approach for exploring yeast cell factory potential” an automated methodology for high throughput screening focusing on growth rates, together with a fully automated method for quantitative physiological characterisation, in 48 well microtiter plates were established. For screening purposes, part of the outlined methodology from chapter 2 was revised, employing semipermeable membranes ensuring low evaporation thereby enabling diauxic growth characterisation. Full scalability was demonstrated through a comparative physiological characterisation of the reference S. cerevisiae, CEN.PK113-7D, and a heterologous 6-MSA producing S. cerevisiae strain cultivated in both MTPs and bioreactors, revealing that the growth rate and yield coefficients of all non-volatile products including biomass could be correlated. This was made possible by diligent analysis, displaying no statistically significant differences between the individual growth rates of the microtiter wells, supported by only minor relative standard deviations for the quantified extracellular metabolites. The highly correlated results were taken as an indication of a comparable growth physiology in both microtiter plates and bioreactors. This notion was substantiated by metabolic flux analysis (partly discussed in Appendix I) of the CEN.PK113-7D strain cultivated in 24-well microtiter plates, shake flasks and bioreactor exhibiting identical flux distributions, proving that the difference in cultivation mode had no measurable effect on metabolism. The field of application for this method includes automated physiological
characterisation of yeasts together with micro-scale metabolic flux analysis, reducing the price and labour investments, significantly.

In chapter 4 entitled “A high through-put microtiter plate based screening approach for physiological characterisation of filamentous fungi” a novel automated high throughput method for cultivating filamentous fungi in microtiter plates, without compromise to morphology and with product yields and growth rates identical to bioreactors, is presented. This was made possible by the dispersive effect on morphology of the anionic polymer carboxypolymethylene, enabling the application of optical density measurements as a means to evaluate growth rates. Full scalability was demonstrated for a heterologous 6-MSA producing A. nidulans strain cultivated in both 48 well microtiter plates and in bioreactors, displaying identical growth rates and 6-MSA yields on glucose. Since the 6-MSAS gene was chromosomally integrated under a constitutive promotor, the metabolite was expressed as a function of growth, and was shown to mirror the growth rate precisely, enabling validation of the optical density based growth rate estimations. Statistical analysis, comparable to those applied in chapter 3, of both growth rates and quantified extracellular metabolites, displayed no significant differences for growth rates with only minor relative standard deviations for the quantified extracellular metabolites.

This versatile and robust method was shown to be applicable for a wide range of different filamentous fungi and conditions, with growth rates comparable to those reported in literature. The field of application for the novel method includes: high throughput basic physiological characterisation of strain libraries providing a basis for guiding cultivation strategies and selection of best candidate strains based not only on growth rates, but also certain yield coefficients.
In chapter 5 entitled “Comparative physiological characterisation of Saccharomyces cerevisiae and Aspergillus nidulans cell factory potential for heterologous polyketide production” a more in-depth quantitative physiological characterisation of the heterologous 6-MSA producing S. cerevisiae and A. nidulans strains, applied in chapter 3 and 4, is outlined. From this study it was concluded that an A. nidulans strain, with 2 copies of the 6-MSAS gene integrated into the chromosome in insertion site (IS) 1 and 53, presents the best candidate for polyketide production. Comparative analysis of both 1 copy A. nidulans strains, where the 6-MSA gene had been integrated in respectively IS1 and IS53, revealed a 40 % higher expression level for IS53. In comparison the 9-copy (6-MSAS) S. cerevisiae strain produced 100 fold lower yields on glucose than the 2 copy A. nidulans strain, emphasising the superiority of the filamentous host. Chemostat cultivation strategies were successfully devised for both the S. cerevisiae and A. nidulans strains. While S. cerevisiae was significantly easier to manage in continuous culture systems, the 9-copy strain exhibited a strong oscillatory behavior, which was only attenuated by the application of ethanol to the feed medium. This behavior was speculated to be connected with a redox imbalance introduced by the expression of 6-MSA, which could be controlled by the continuous presence of ethanol, as also observed for continuous cultures performed above the critical dilution rate for ethanol production. The successful implementation of a chemostat cultivation strategy for A. nidulans will be highly applicable for conditional comparative analysis allowing for omics based studies, potentially identifying bottlenecks relevant for rational metabolic engineering.

In the final chapter, conclusions and perspectives based on the outlined results and developed methods are presented.
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Chapter 2

A high throughput method for quantifying metabolically active yeast cells

Subir Kumar Nandy1*, Peter Boldsen Knudsen1#, Alexander Rosenkjaer1, Anna Eliasson Lantz2, Jette Thykaer1, Mhairi Workman1*

1Department of Systems Biology, Technical University of Denmark, Building 223, Søltofts Plads, DK – 2800 Kgs. Lyngby, Denmark.

#These authors contributed equally to the work

*Corresponding Author: Mhairi Workman, email: mwo@bio.dtu.dk, Tel.: +45 4525 2700

2Current address: Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 229, Søltofts Plads, 2800 Kgs. Lyngby, Denmark

Keywords: Saccharomyces cerevisiae, metabolically active cells, quantification, high throughput, methylene blue.
Abstract

By redesigning the established methylene blue reduction test for bacteria and yeast we present a cheap and efficient methodology for quantitative physiology of eukaryotic cells applicable for high throughput systems. Validation of the method in fermenters and high throughput system proved equivalent, displaying reduction curves that interrelated directly with CFU counts. For growth rate estimation the Methylene Blue Reduction Test (MBRT) proved superior, since the discriminatory nature of the method allowed only for the quantification of metabolically active cells; excluding dead cells. The drop in metabolic activity associated with the diauxic shift in yeast proved more pronounced for the MBRT derived curve compared with OD curves, consistent with a dramatic shift in the ratio between live and dead cells at this metabolic event. This method provides a tool with numerous applications e.g. characterising the death phase of stationary phase cultures, or in drug screens with pathogenic yeasts.
Introduction

Several standard microbiological procedures have been established to differentiate between viable and non-viable cells. Classical microbiology equates viability with culturability and researchers typically differentiate viable and non-viable cells as metabolically active or inactive, respectively [1], [2]. The determination of metabolically active or culturable viable cells is an essential analytical measurement in physiological evaluation of cell cultures. Such data can be used for process optimisation or understanding cell death mechanisms as a means of controlling pathogenic organisms.

One of the most commonly applied methods for estimating viability in microbial cultures is to count colony forming units (CFU) on plates. These methods however, have several disadvantages; particularly time, labor and inaccuracy [3], [4]. A widely applied method to evaluate microbial load is optical density measurement, where both live and dead cells are pooled. In this case the disadvantage is that there is no distinction between dead/live microorganisms and moreover the sensitivity of OD is very limited.

Live and dead cell ratios may also be assessed using commercially available dead/live staining kits (such as those available from Molecular Probes Inc.) or enzymatic assays. A major disadvantage of these methods, besides being expensive and time consuming, is the need for dedicated instruments such as fluorescence microscopes and flow cytometers [5]–[8].

The methylene blue dye reduction test (MBRT) is widely used in the dairy industry to determine the microbial load in milk. This method relies on sample decoloration rate as a prognostic indicator for the microbial load in the sample. Though the mechanism by which methylene blue is reduced is not entirely elucidated, it is suggested to be a combination of oxygen consumption in conjunction with production of redox equivalents and reduction directly by membrane bound reductases [7], [9], [10]. The MBRT technique has previously been described for bacterial and, more recently, yeast cell cultures, focusing on quantification of metabolically active bacteria and evaluation of overall yeast vitality, respectively [11], [12].
Though, the method has been described for yeast, previous work focused solely on evaluation of culture vitality as a means to identify when a culture is at its metabolic apex and therefore optimal as an inoculum for a new fermentation [12]. In a recent study, an absorption and desorption assay based on methylene blue was devised proving a linear correlation between methylene blue absorption/desorption and dry weight [13]. This method, however, necessitates microorganism specific correlation coefficients. In this study we expand the MBRT method to a high through-put quantitative tool for physiological characterisation of yeast with full scalability from 1 L bioreactors to high through-put microtiter scale. By discriminating between metabolically active and inactive cells, this method allows for establishment of growth rates, representing only the relevant cultivable fraction of the cultures not attainable with standard methods. This discriminatory nature of the method further enables for the detection of the viability drop at the diauxic shift and for death phase characterisation with further applications in studies where cell viability and death measurements are required e.g. anti-microbial screening [14]. Finally, the presented method offers an attractive alternative for CFU estimation compared with the traditional spread plate technique.
Materials and Methods

Microorganism

*Saccharomyces cerevisiae* CEN.PK113-7D (MATα, MAL2-8c SUC2) was applied as the model organism for this study. The strain was maintained on Yeast extract-Peptone-Glucose agar plates. A single colony from a plate was sub cultured before each test in a shake flask with 100 mL of sterile minimal medium and grown for 14-16 h at 30 °C at 150 rpm. Bioreactors and microtiter plates were inoculated to an OD<sub>600</sub> of 0.01 and the entire submerged cultivation process carried out over ~30-40 h.

Media

Yeast extract, peptone and glucose medium (YPG) was used throughout the experiments to grow the organism. YPG agar plates was used for culture maintenance on plates and for viable counts using the spread plate method. The minimal medium composition used for cultivation in the bioreactors and 48 well plates was as follows: 20 g·L<sup>-1</sup> glucose, 5 g·L<sup>-1</sup> ammonium sulphate, 3 g·L<sup>-1</sup> monopotassium phosphate, 0.5 g·L<sup>-1</sup> magnesium sulphate, 1 mL trace metal solution, 1 mL vitamin solution, 50 µL antifoam 204 (Sigma-Aldrich, St. Louis, MO/USA). The trace metal solution consisted of (per liter): 15 g EDTA (sodium salt), 0.45 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1 g MnCl<sub>2</sub>, 2H<sub>2</sub>O, 0.3 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.3 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.4 g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.45 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.3 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g H<sub>3</sub>BO<sub>3</sub>, 0.1 g KI. The vitamin solution contained (per liter): 0.05 g biotin, 0.2 g p-amino benzoic acid, 1 g nicotinic acid, 1 g Ca-pantothenate, 1 g pyridoxine-HCl, 1 g thiamine-HCl and 25 g myo-inositol. For high throughput experiments the medium was supplemented with 100 mM 4-morpholineethanosulfonic acid hemisodium salt (MES) buffer (Sigma-Aldrich, St. Louis, MO/USA) and adjusted to pH 5. 1 % Methylene Blue dye (Sigma-Aldrich, St. Louis, MO/USA) was used throughout the experiments. The chemicals for YPG and minimal medium were obtained from Merck Millipore (Darmstadt, Germany) or Sigma-Aldrich Co. (St. Louis, MO/USA).
**Standard calibration curve and sensitivity analysis of MBRT**

Correlation curves for optical density, viable cells and methylene blue decoloration rates were based on yeast shake flask cultures incubated at 30 °C under constant shaking at 150 rpm. The whole culture (about 100 mL) was aseptically harvested after 16 h, while still in the exponential growth phase, and centrifuged at 17,000 g, for 10 min. at 4 °C after which serial dilutions were prepared in phosphate saline buffer [11]. The sensitivity analysis, previously described by Bapat *et al*, 2006 [11], was modified for the automated high throughput setup in 96 well plates and performed in triplicates.

In brief, serial diluted yeast samples were dispensed in aliquots of 100 µL into a 96 well plate (Greiner Bio-One, Frickenhausen, Germany) and the optical density (OD) measured at 600 nm. After OD measurement, 2 µL methylene blue dye was automatically dispensed into the wells and the plate subsequently shaken for 5 s before color reduction time was recorded by kinetic measurements at 650 nm. For validation, 3-4 diluted yeast samples were applied for CFU spread plate counts and incubated at 30 °C for 36 h prior to counting.

**S. cerevisiae growth experiment in bioreactors**

The reference cultivations of *S. cerevisiae* were carried out in batch bioreactors with a working volume of 1 L (Applikon Biotechnology, Schiedam, The Netherlands). Temperature was maintained at 30 °C, agitation at 600 rpm and aeration was set to 1 volume of air per volume of liquid per minute (vvm). The pH was maintained at 5 by automatic addition of 2N NaOH. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer’s standard procedures. The bioreactors were equipped with a condenser to avoid evaporation from the medium. The effluent gas from the fermentation was monitored using an acoustic gas analyser (1311, Innova Air Tech Instrument A/S. Nærøm, Denmark). Samples were taken at regular intervals for optical density measurement, viable plate count and MBRT. The decoloration slope for each sample was evaluated spectrophotometrically over a 200 s period, following the procedure outlined in Bapat *et al*, 2006 [11]. Experiments were conducted as three true replicates with the average
reported. The linear interval obtained from the sensitivity analysis was correlated with the MBRT to yield CFU estimates, which was validated by independent CFU counts.

**Standard calibration curve and sensitivity analysis of MBRT**

For high throughput fermentations, the inoculum was prepared from overnight cultures to a final OD of 0.01. The inoculum was automatically seeded into 48 well microtiter (Greiner Bio-One, Frickenhausen, Germany) plates in aliquots of 400 µL using a Hamilton STAR liquid handling workstation (Hamilton, Bonaduz, Switzerland) with an integrated Thermo Cytomat 2 C450 shaking incubator (Thermo-Fischer Scientific, Waltham, MA/USA) and Biotek Synergy 2 microplate reader equipped with a dual reagent dispenser (Biotek, Winooski, Vermont/USA). The outer wells were filled with 400 µL water to reduce the effects of evaporation on the microcultures and four inner wells were filled with 400 µL medium serving as negative reference. Plates were incubated at 30 °C in the integrated incubator under constant shaking at 1200 rpm with an amplitude of 3 mm. Humidity was kept constant by a heated water bath integrated into the shaking incubator. The handling workstation was programed to extract individual plates from the incubator at user defined time intervals (~ 2 h) for measurements and liquid handling. As a precaution random samples were taken and investigated under microscope for cross-contamination.

Briefly, plates were extracted and transported by the handling system to the plate reader for OD$_{600}$ measurements after which samples of 100 µL from three individual wells were automatically harvested for the MBRT method. The 48 well cultivation plate was then returned to the incubator. Finally, the 96 well plates containing said samples was transferred to the plate reader where 2 µL of methylene blue dye was dispensed into the wells using the inbuilt dual reagent dispenser. The methylene blue was mixed in by continuous shaking and reduction time was recorded using kinetic measurements at 650 nm over 2 min. with 7 s intervals.
Results and Discussion

The principal aim of this study was to introduce an efficient and inexpensive tool for quantifying metabolically active yeasts and relating this to actual OD_{600} measurements. The scalability was thoroughly challenged in bioreactor settings and microtiter plates before introduction of an automated high-throughput setup. Through these studies a new method was developed, broadening the range of physiological parameters that may be evaluated in automated high-throughput settings. This new tool is well suited for automated growth curve analysis and will be highly applicable for automated high-throughput antimicrobial drug screening, such as described in [14]. Towards the aim of implementing MBRT for high-throughput setup for \textit{S. cerevisiae}, we focused on instrumented bioreactors as reference for the high-throughput system.

Comparing OD and CFU with MBRT

The sensitivity analysis using \textit{S. cerevisiae} demonstrated that the MBRT derived decoloration slope can be used to correlate the OD_{600} of metabolically active cell cultures with CFU for an order of magnitude greater than 1000 cells. By adding thus step in, the MBRT method time were reduced from several hours to ~40-60 seconds. The correlation of MBRT slope to OD_{600} and CFU (as shown in Figure 1a and 1b, respectively) was established to be linear and sensitive. Linearity of the MBRT slope was documented up to an OD_{600} of 10 resulting in a range of $10^2$ – $10^7$ cells for the viable count.
Figure 1. MBRT sensitivity and validation: (a) Correlation between OD$_{600}$ and MBRT slope. Open circle symbol represents experimental points. Solid line represents best fit with $R^2 = 1$. Best-fit equation is given by $Y = 0.0008 X$. (b) correlation between CFU and metabolically active cells calculated from MBRT slope. In this case, Log (MBRT slope) and Log (CFU) were fitted as a straight line. Solid line represents best fit with $R^2 = 0.99$. Best-fit equation is given by $Y = -0.5848 X + 6.0032$, (c) inhibition experimental data from ethanol concentration in $S. cerevisiae$, where open square and open circles describe the CFU from MBRT and from agar plates, respectively and (d) Methylene blue decoloration rates (slopes) recorded as a kinetic measurement at 650 nm over 2 min. with 7 s intervals. MBRT was determined from the slope in the linear phase.

Verifying the MBRT in the robotic setup

To validate the correlations observed from the sensitivity analysis and to establish the applicability of the MBRT, samples from an exponential growing culture were challenged with a range of different concentrations of ethanol (10-50 %) with the purpose of inhibiting/killing portions of the cells. These
samples were divided into two fractions; one that was used for a spread plate CFU experiment and the other were applied for the MBRT. By utilizing the previously established relationship between MBRT slopes and metabolically active cells (based on CFU·mL⁻¹), it was possible to quantify the concentration of metabolically active cells in the samples. Finally, these calculated concentrations were shown to be well correlated with the results generated on the spread plates, validating the methodology. All experiments were carried out in duplicate to establish the robustness of the method through evaluation of the standard deviations, as illustrated in Figure 1c. Figure 1d shows the time profile of the methylene blue decoloration at 650 nm as an output of the kinetic measurements (see materials and methods).

**Assessment of metabolically active cell number by MBRT**

To evaluate the applicability of the method as a tool for physiological characterisation, it was tested in a standard physiological characterisation of a batch cultivation of *S. cerevisiae* CEN.PK113-7D. Figure 2a displays the actual OD₆₀₀ measurements for the full batch characterisation (open triangles) plotted with the calculated OD₆₀₀ based on the MBRT (open circles).
Figure 2. Physiology of *S. cerevisiae* CEN.PK113-7D correlated to MBRT slope: (a) Correlation between actual OD\textsubscript{600} (open triangle) and calculated OD\textsubscript{600} from MBRT slope (open circle), (b) Change of MBRT slope with time (open triangle), (c) Correlation between actual CFU from plate method (open square) with the metabolically active cell count from MBRT slope (open triangle).

From this figure it is evident that there was a high degree of correlation between the OD\textsubscript{600} values for the entire exponential growth phase and it was only as the culture entered the stationary phase that substantial deviations started to occur. These deviations were representative of the drop in metabolic activity observed as the culture progressed into the stationary phase. Since OD measurements are non-discriminating with regards to the metabolic state of the cells, the calculated OD\textsubscript{600} based on the MBRT measurements are actually a better physiological tool for characterising microbial growth phases. This was further evident from Figure 2b in which the decoloration rates are plotted against time, yielding a growth curve entirely based on only live, metabolically active and dividing cells. Closer investigation of MBRT based growth curve in Figure 2b further reveals a sharp drop in the metabolic activity at 23 hours consistent with the diauxic shift, which is less pronounced in case of the non-discriminatory optical density curve of Figure...
2a. Extrapolation of the growth rate from both this curve and the corresponding curve based on OD<sub>600</sub> measurements were in good agreement with the OD<sub>600</sub> based $\mu_{\text{max}} = 0.372\pm0.01 \text{ h}^{-1}$ and the MBRT based resulting in $\mu_{\text{max}} = 0.365\pm0.03 \text{ h}^{-1}$, both correlating well with literature [15]. Though easily within reasonable standard deviation, the slightly lower growth rate based on the MBRT may be explained by the fact that this measurement does not take the increasing fraction of non-metabolic active cells into account. In Figure 2c the calculated concentration of metabolically active cells is plotted against CFU spread plate results, proving the method highly accurate with only minor standard deviations attached to each individual measurement.

**High throughput scalability study**

The high throughput microtiter based cultivations were evaluated on two levels to ascertain the scalability of the MBRT methodology and to evaluate the applicability of the high throughput method for growth rate characterisation.

Growth rates were calculated by measuring the optical density of the individual wells of the microtiter plates over time thereby treating each well of the microtiter plates as separate biological replicates (n=49) (data not shown). In addition to this individually based calculation strategy, the results on a plate to plate level (n=3) were summarised and plotted as a single growth curve by taking the average optical density of up to 20 well replicates and plotting them for the individual time points that the plates were measured. This strategy of evaluating the growth rate both as a total of all individual wells, but also summarising all plate results as coherent measurements, resulting in a single growth curve, makes it possible to ascertain the robustness of the methodology. This was evaluated using an one-way ANOVA test on for the individual growth rates, evaluated as the three different populations represented by the separate microtiter plates.

With a population variance in the range of $\sigma^2 = 2.89 \times 5.88 \times 10^{-5}$ and the test displaying no statistical significant difference ($F(2,48) = 0.439, \ p = 0.347$) the methodology was considered robust. The individual well assessment resulted in a maximal growth rate of $\mu_{\text{max}} = 0.366\pm0.007 \text{ h}^{-1}$ (figure 3a) with a total sample variance of $\sigma^2 = 4.38 \times 10^{-5}$. The low variance and the fact that the individual growth rates displayed no
significant differences indicates that the growth phase of each individual well follows an almost identical course, which opens up for the possibility for treating individual wells as representative for single batch cultivation.

Figure 3. High throughput physiology data: (a) Overall OD$_{600}$ summarised on plate to plate basis (n=3) with the OD$_{600}$ of up to 20 individual wells logged with their respective standard deviation on a time point basis, (b) OD @ 600 nm (open diamond) demonstrate the same specific growth rate that was measured from MBRT slope (open triangle) and it is similar to the maximum specific growth rate from batch cultivation of S. cerevisiae CEN.PK113-7D, (c) Correlation between actual OD$_{600}$ (open triangle) and calculated OD$_{600}$ from MBRT slope (open rectangle).

To test the downscaled MBRT methodology, triplicate samples were automatically harvested at user designated time points and treated with methylene blue, in accordance with the described method, before the decoloration rates were recorded. In Figure 3b the decoloration rate vs sample time point are plotted together with the OD$_{600}$ at the sample time point. Extrapolation of the growth rates from both the MBRT and OD$_{600}$ curve are in good agreement with the OD$_{600}$ based $\mu_{max} = 0.367 \pm 0.009$ h$^{-1}$ and the MBRT based,
resulting in $\mu_{\text{max}} = 0.371 \pm 0.007 \, \text{h}^{-1}$ (Figure 3b). Though in overall good correlation with both literature and bioreactor data a higher degree of evaporation must be expected, which may affect the growth physiology. With evaporation not accounting for more than approximately 5 % in the presented case, the results were not substantially affected and evaporation was disregarded. However, for slower growing microorganisms including more in-depth physiological analysis, evaporation becomes a factor that should be taken into account.
Conclusions

This study was an extension of previous work on prokaryotes and in part yeast using methylene blue reduction to gauge metabolic activity in cells in order to correlate CFU with OD. In this work we have proven that the method can be efficiently extended to also include eukaryotes and that it with few modifications is adaptable for high throughput systems. This modified method allows for fast and automatic assessment of the physiological state of the culture and due to the discrimination between metabolic active and inactive cells a superior method for estimating bioprocess relevant growth rates. In addition the accurate quantification of metabolic active cells in a culture makes it a good alternative to the more laborious and time requiring method of colony counting. The method could further be adapted for antimicrobial screens that are commonly carried out in microtiter plates. Here this method would offer a significantly cheaper alternative to many of the staining procedures applied with regards to time, manpower and running costs.
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Author Contributions

SKN performed all experiments and drafted the manuscript, PBK designed the methods applied for the high throughput setup and co-wrote the manuscript, AR programmed the methods for high throughput process, AEL and JT discussed the design of experiments, JT and MW supervised the work and assisted in the manuscript writing.

Competing interests statement

The authors declare no competing interests.
References


Chapter 3

A scalable high throughput fermentation approach for exploring cell factory potential
Abstract

This study presents an automated high throughput methodology for cultivating yeast in microtiter plates (MTPs) with application for both high throughput strain library screening focusing on growth rates and for automated quantitative physiological characterisation. While several other studies have investigated these subjects, this is the first study to correlate quantitative physiological characterisation and metabolic flux distributions of both MTPs and bioreactors. For the screening methodology, the growth rates of a selection of well characterised yeast strains cultivated in membrane sealed MTPs were correlated with reported values in the literature. The application of membranes ensured a high degree of vapor retention and made characterisation of the diauxic growth on ethanol a possibility. From comparative physiological characterisation of the reference Saccharomyces cerevisiae strain, CEN.PK113-7D, and a heterologous 6-methylsalicylic acid (6-MSA) producing strain cultivated in both MTPs and bioreactors, it was concluded that the growth rate and yield coefficients of all non-volatile products including biomass could be correlated, indicating that the systems were highly comparable. Statistical analysis did not reveal any significant difference between the individual well growth rates, and with only minor relative standard deviations (RSD) for the quantified products. The scale-down method was proven highly robust. From a comparative metabolic flux distribution analysis of the reference strain in bioreactor, shake flasks and MTPs it was concluded that neither fluctuations in oxygen transfer rates nor pH drifts posed any measurable influence on the central carbon metabolism.
Introduction

Selection of efficient cell factories is paramount for economically viable industrial biotechnology processes. Strains selected as possible candidates for the production of valuable compounds must perform to a high standard with respect to growth rate, ability to efficiently convert substrates to the desired products, and their ease of handling both in large scale bioreactors and in downstream processes for product recovery. The precise parameters depend on the process under development, but in all cases a quantitative assessment of cellular performance is required in order to accurately evaluate and compare the numerous strains that may be generated in strain development programmes.

Shaken cultivation systems have a long history of application within strain selection and medium optimisation, since these systems have few requirements with regards to instrumentation and labour. Traditionally, Erlenmeyer flasks have been used extensively for these tasks, due to the low cost and reasonably high number of individual experiments that may be managed by a single individual [1, 2]. This practice has been challenged by the development of high throughput molecular biology techniques such a USER cloning/fusion, increasing the volume of strains to be characterised, dramatically [3, 4].

To process the increasing volume of strains, dedicated equipment has been developed allowing for automated cultivation increasing the throughput of strain characterisation and reducing the time and labour invested. Several systems have been developed focusing on either micro-bioreactors with variable levels of control [5, 6] or microtiter plate (MTP) based cultivation systems such as the BioLector systems from m2p-labs [7, 8] and the STAR system by Hamilton [9, 10]. Amongst these, the MTP based systems offer the highest throughput, albeit differing in a number of ways from bench top bioreactors and micro-bioreactors, which can hamper direct comparison and hence accurate conclusions [2, 11].

One challenge of selecting the most suitable candidate strains is obtaining uninhibited growth, and consequentially unrestricted product formation, in MTPs. There are, for example, several factors that may limit the extend of aerobic growth rates such as pH control, evaporation and most importantly volumetric
oxygen transfer rates (kLa) [2]. In comparison to bench top bioreactors and micro-bioreactors, MTP systems rely solely on shaking as a means of mixing and oxygen supply, since neither stirring nor active aeration is possible [2]. Substantial resources have therefore been invested in the estimation of the volumetric oxygen transfer rates (kLa) at different volumes, MTP well size and shape, shaking speed and amplitude to establish conditions with bioreactor comparable kLa’s [12–17]. Amongst these a study by Kensy et al [7] reported kLa values of up to 1600 h\(^{-1}\) for 48 well MTPs, well in the range of industrial bioreactors; making MTPs an excellent alternative to shake flask screening, increasing the throughput substantially [12].

While pH stability constitutes a problem for several systems, diligent application of buffers may, to a certain extent, solve these issues [18]. Recently, a BioLector based system was introduced, combining an established fiber-optic online monitoring device relying on fluorescence, pH and dissolved oxygen sensor spots (optodes), with microfluidic control by means of micro-channels [8, 13]. With this new system, fed-batch fermentation with active pH control becomes an option in MTP format and the data produced is now approaching a complexity, previously solely offered by fully instrumented bench scale bioreactors [13, 19–21]. Though process control in certain MTP based systems can approach bench-top bioreactor standards, the degree of complexity introduced into e.g. the BioLector systems limits high throughput experimentation, since only one plate may be handled [22]. Higher throughput systems, such as the Hamilton STAR, offer the opportunity for multiple plate handling combined with automated liquid handling, however compromises are made with regard to process control, since measurements and liquid handling are made at-line [10, 23, 24].

One further reported drawback with MTP applications is evaporation, where several authors describe procedures to reduce evaporation by humidifying the circulated air or sealing the plates with breathable membranes, with reported evaporation losses have been reported to be in the range of 5-12 % [10, 20, 24, 25].
Several studies report on the scalability of MTP based systems with overall good results primarily focusing on growth rate and kLa correlation [11, 13, 26, 27]. Furthermore, a few studies report similar product and biomass concentrations compared with bioreactors, however none of these studies include a more detailed comparative physiological characterisation of strains in both MTPs and instrumented bioreactors [13, 27, 28].

The aim of this study was to establish a scalable high throughput microtiter based screening approach for quantitative physiological characterisation of yeasts in MTPs applying OD_{600} measurements for monitoring growth rates. This design was modified and expanded aiming at establishing an automated MTP based cultivation platform designed for fast autonomous cultivation, with measurements and sampling based on user pre-specifications for the sample time regime. The scalability of the method was challenged in a comparative quantitative physiological study where *Saccharomyces cerevisiae* CEN.PK113-7D was cultivated and characterised in both 24 and 48 well microtiter plates and in 0.5, 1 and 5 L fully instrumented and controlled bioreactors. Scalability was further challenged in a comparative flux distribution analysis for both MTP, shake flask and bioreactor cultivations with potential application for micro-scale metabolic flux experimentation.
Materials and methods

Strains

*Saccharomyces cerevisiae* CEN.PK113-7D (MATα, MAL2-8c SUC2) was applied as the “proof-of-concept” organism for this study. All other applied strains are listed in table 1. The strains were maintained on Yeast extract-Peptone-Dextrose agar plates. Single colonies from plates were sub-cultured before each test in shake flasks with 100 mL of sterile minimal medium and grown for 14-16 h at 30 °C at 150 rpm. Bioreactors and microtiter plates were inoculated to an OD600 of 0.01 and the entire submerged cultivation process carried out over ~ 30-40 h.

Table 1 Strains applied for the study. a) Diploid 6-MSA multicopy yeast strain based on the two annotated haploid strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>CEN.PK113-7D</td>
<td>MATα, MAL2-8c SUC2</td>
</tr>
<tr>
<td>CEN.PK448</td>
<td>gdh1Δ</td>
</tr>
<tr>
<td>CEN.MS1-10C T1</td>
<td>gdh1(209,1308): :loxP gdh2: :PGKp-GDH2-KanMX3</td>
</tr>
<tr>
<td>GAX10-1C-LM1</td>
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</tr>
<tr>
<td>x</td>
<td></td>
</tr>
<tr>
<td>CEN.PK113-7B</td>
<td>MATα MAL2-8C SUC2 leu2-3_112 trp1-289 ura3-52</td>
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</table>

Media

Yeast extract, peptone and dextrose medium (YPD) was used throughout the experiments to grow the organism. The minimal medium composition [29] used for either cultivation in the bioreactors or in 24 and 48 well plates was as follows: 20 g·L⁻¹ D-glucose or [1⁻¹³C]glucose (for shake flasks 5 and 20 g·L⁻¹), 5 g·L⁻¹ ammonium sulphate, 3 g·L⁻¹ monopotassium phosphate, 0.5 g·L⁻¹ magnesium sulphate, 1 mL trace metal solution, 1 mL vitamin solution, 50 µL antifoam 204 (Sigma-Aldrich, St. Louis, MO/USA). The trace metal solution consisted of: 15 g EDTA (sodium salt), 0.45 g·L⁻¹ ZnSO₄. 7H₂O, 1 g·L⁻¹ MnCl₂. 2H₂O, 0.3 g·L⁻¹ CoCl₂.
6H₂O, 0.3 g·L⁻¹ CuSO₄·5H₂O, 0.4 g·L⁻¹ Na₂MoO₄·2H₂O, 0.45 g·L⁻¹ CaCl₂·2H₂O, 0.3 g·L⁻¹ FeSO₄·7H₂O, 0.1 g·L⁻¹ H₃BO₃, 0.1 g·L⁻¹ KI. The vitamin solution contained: 0.05 g biotin, 0.2 g·L⁻¹ p-amino benzoic acid, 1 g·L⁻¹ nicotinic acid, 1 g·L⁻¹ Ca-pantothenate, 1 g·L⁻¹ pyridoxine-HCl, 1 g·L⁻¹ thiamine-HCl and 25 g·L⁻¹ myo-inositol. For MTP experiments the medium was supplemented with 100 mM 4-morpholineethanosulfonic acid hemisodium salt (MES) buffer (Sigma-Aldrich, St. Louis, MO/USA) and adjusted to pH 5. The chemicals for YPD and minimal medium were obtained from Merck Millipore (Darmstadt, Germany) or Sigma-Aldrich Co. (St. Louis, MO/USA).

**Shake flask cultivations**

All shake flask cultivations were carried out in 500 mL baffled flasks with a working volume of 100 mL and incubated at 30 °C under constant shaking at 150 rpm. As part of a validation of the high throughput setup suitability for metabolic flux analysis, shake flasks containing medium with 5 g·L⁻¹ [¹³C]glucose were inoculated from a pre-culture of the reference strain (CEN.PK113-7D) at a start OD₆₀₀ of 0.15, in accordance with the procedure outlined by Gombert et al (2001) [30]. As reference, shake flasks containing medium with 20 g·L⁻¹ [¹³C]glucose were inoculated to OD 0.01. All cultivations were carried out in duplicates.

**Bioreactor based batch cultivations**

All bioreactor based cultivations were carried out using Sartorious 0.5 L (¹³C experiments), 1 L and 5 L bioreactors (Satorious, Stedim Biotech, Goettingen, Germany) with equivalent working volumes and equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer’s standard procedures. The bioreactor was sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5 % (v/v) CO₂, 0.04 % (v/v) ethanol and methanol, 1 % (v/v) argon, 5 % (v/v) and 15 % (v/v) oxygen all with nitrogen as carrier gas (Linde Gas, AGA, Enköping, Sweden). Temperature was maintained at 30 °C throughout the cultivation and pH controlled by automatic addition of 2 M NaOH and
H₂SO₄. Culture conditions were pH: 5, stirring rate: 800 rpm and air flow: 1 volume of air per volume of liquid per minute (vvm). The strains were cultivated until carbon depletion and the culture had entered stationary phase as monitored by off-gas CO₂ concentration. HPLC, OD₆₀₀ and dry weight (DW) samples were taken across the exponential growth phases for metabolite profiling.

**High throughput MTP batch cultivations**

**Multi-strain growth rate screening**

For high throughput multi-strain screening the inoculum suspensions were prepared to an OD₆₀₀ of 0.2 and dispensed into 96 well U-bottomed microtiter master-plates (Greiner Bio-One, Frickenhausen, Germany). All liquid handling was carried out using a Hamilton Microlab STAR liquid handling workstation (Hamilton, Bonaduz, Switzerland) with an integrated Thermo Cytomat 2 C450 shaking incubator (Thermo-Fischer Scientific, Waltham, MA/USA) and Biotek Synergy 2 microplate reader (Biotek, Winooski, Vermont/USA). Medium was automatically dispensed into the inner wells of 48 well MTPs (Greiner Bio-One, Frickenhausen, Germany) in aliquots of 380 and 400 µL for cultivations and negative reference (two wells), respectively. The medium aliquots of cultivation were subsequently inoculated with 20 µL of the inoculum from the master-plate and automatically mixed by pipetting (final OD₆₀₀ 0.01). Medium and water were stored in sterilised liquid reservoirs mounted in the handling workstation.

For initial screening tests a semipermeable membrane (Mepore, Mölnlycke Healthcare, Göthenborg, Sweden) were manually fixed to the lidless plates to reduce both evaporation of water and volatile metabolites.

The plates were placed in the integrated incubator under constant shaking at 1200 rpm with an amplitude of 3 mm. Temperature and humidity (≥ 95 % rH) were kept constant at 30 °C, by a heated water bath integrated in the shaking incubator, reducing evaporation. The handling workstation was programed to
extract individual plates from the incubator at user defined time intervals (~ 1-3 h) for measurements and/or liquid handling.

Briefly, plates were extracted and transported by the handling system to the plate reader for measurements. The biomass evolution of each individual well was monitored by logging the average of a nine point matrix $OD_{600}$ measurement.

**High throughput MTP batch cultivation**

For quantitative physiological characterisation and metabolic flux analysis, an inoculum was prepared from overnight cultures to a final $OD_{600}$ of 0.01. The inoculated medium, sterile medium and water were stored in sterilised liquid reservoirs mounted in the handling workstation. The inoculum was automatically seeded into 48 and 24 well ($^{13}$C experiments) microtiter plates in aliquots of 400 and 800 µL, respectively.

The outer wells were filled with 400 µL water to reduce the effects of evaporation on the micro-cultures. In addition two inner wells were filled with 400 µL water for evaporation rate evaluation. Medium and water were stored in sterilised liquid reservoirs mounted in the handling workstation. Incubation parameters and handling parameters for the 48 well MTPs followed the procedure described earlier, except the lid were automatically removed before measurements. For the 24 well plates shaking was lowered to 800 rpm to avoid spill out.

To compensate for water evaporation, path length reduction rate was measured as the difference between baseline absorbance at 900 nm and the absorbance peak at 977 nm divided with the difference in a standard 1 cm cuvette as specified in McGown et al, 1998 [31]. Measurement of path length reductions were carried out concurrently with $OD_{600}$ allowing for the calculation of a time dependent evaporation coefficient (figure 1) enabling reconciliation of non-volatile metabolite concentrations.
Figure 1. Correlation curve describing water evaporation as a function of path length reduction over time in 48 well microtiter plates with a working volume of 400 µL.

As part of the design, OD$_{600}$ measurements always preceded sampling (harvesting of well contents) ensuring individual measurements of the biomass accumulation for all wells including those to be sampled. At the designated time point, a sequential assembly of the vacuum manifold was initiated by the repositioning of three elements to the vacuum station: a 96 well U-bottomed microtiter plate (Greiner Bio-One, Frickenhausen, Germany) placed on the vacuum station followed by the positioning of the manifold house on top and finalised by placement of a 96 well 0.45 µm filter plates (Merck Millipore, Cork, Ireland) on top of the manifold house.

Triplicate samples of 200 µL were automatically harvested from individual wells after the OD measurement and dispensed into the filterplate after which the cultivation plate was returned to the shaking incubator, while the samples were filtered through the filter plate by the activation of the vacuum pump for 5 s. After filtration, the vacuum manifold was automatically dismantled and the U-bottom 96 well plate was transported back to the holding position. Samples were then transferred into a 96 well flat bottomed microtiter plate (Greiner Bio-One, Frickenhausen, Germany) placed on the inbuilt cooling plates and kept at 2 °C thereby ensuring sample stability.
As a precaution random samples were taken and investigated off-line under a microscope for cross-contamination.

**Cell mass determination**

Throughout all cultivations cell mass concentration was monitored by measurement of $\text{OD}_{600}$ in standard 1 cm cuvettes for bioreactors and as direct measurements in 48 well plate wells. For physiological characterisation in bioreactors dry cell mass was further determined gravimetrically. Shortly, a known weight of cell culture was filtered through pre-dried 0.45 µm polyether sulfone filters (Frisenette, Knebel, Denmark), and the biomass washed with saline (0.9 % NaCl). The filter was the folded to lock the biomass within the filter and finally dried in a microwave oven at 150 W for 20 min. Mass gain or biomass dry weight (DW) was ascertained after a cooling period of at least 30 min in a desiccator.

By correlating optical density in 1 cm cuvettes with direct measurements in 48 well microtiter plates, a correlation coefficient was calculated allowing for conversion of microtiter based optical densities to standard 1 cm cuvette measurements, see figure 2.

\[
y = 5.5202x - 0.1552 \\
R^2 = 0.99
\]

![Correlation curve](image)

**Figure 2** Correlation curve describing the relation between the optical density in a 1 cm cuvette and in a 48 well microtiter plate with a working volume of 400 µL. Standard deviations describe both variations in cuvette (x-axis) and MTP based $\text{OD}_{600}$ (y-axis).
In a similar manner, a correlation coefficient was calculated by relating OD$_{600}$ based in standard 1 cm cuvettes with gravimetical measurements [32], allowing for conversion of OD$_{600}$ measurements to dry weights, as exemplified in figure 3.

![OD$_{600}$ (cuvette) to biomass dry weight correlation](image)

Figure 3. Correlation curve describing the relation between the optical density in a 1 cm cuvette and dry weight in g/L.

With these two correlation coefficients, OD$_{600}$ measurements in microtiter plates could be converted into dry weights allowing for calculation and comparison of biomass yield coefficients.

**Quantification of extracellular metabolites by HPLC**

For quantification of extracellular metabolites, culture samples from bioreactors were taken and filtered through a 0.45 µm cellulose acetate filter (Frisenette, Knebel, Denmark). For the MTP cultures, samples were automatically sampled and filtered in accordance with previous descriptions. The samples were frozen and kept at -20 °C until analysis. Glucose, glycerol, pyruvate, succinate, acetate and ethanol were detected and quantified on an Agilent 1100 HPLC system equipped with a refractive index and Diode array detector (Agilent Technologies, Waldbronn, Germany) and equipped with an Aminex HPX-87H cation-exchange column (BioRad, Hercules, Ca, USA). Compounds were separated by isocratic elution at 60 °C,
with 5 mM H$_2$SO$_4$ at a flow rate of 0.6 mL·min$^{-1}$. Quantification was performed using a six-level external calibration curve with pyruvate detected at a wavelength of 210 nm and glucose, succinate, glycerol, acetate and ethanol by refractive index measurements.

6-MSA was quantified using a Dionex Ultimate 3000 UHPLC coupled with an ultimate 3000 RS diode array detector (Dionex, Germering, Germany) equipped with a Poroshell 120 phenyl hexyl 2.1 x 100 mm, 2.7 µm (Agilent, Santa Clara, CA/USA) column by gradient elution at 60 °C and a flow rate of 0.8 mL·min$^{-1}$ starting at 15 % acetonitrile and MiliQ water and ramped up to 100 % in 7 min. before returned to the starting point for an additional 5 min.. Quantification was performed using an eight-level external calibration curve with concentrations from 1-300 mg·L$^{-1}$ at a wavelength of 210 nm.

**Quantitative physiology analysis**

All bioreactor experiments were carried out in triplicates and all microtiter based experiments in duplicates with three biological replicates for each time point. Maximum specific growth rates were estimated from exponential fits of biomass concentrations either determined by OD$_{600}$ or gravimetrically as a function of time. The robustness of the microtiter based strategy was assessed by a one-way ANOVA test of the individual growth rates, evaluated as independent populations represented by the individual microtiter plates of the given experiment supported by total population variance analysis. Yield coefficients were estimated through linear regression of product accumulation as a function of substrate consumption with a coefficient of determination above 0.95. Standard biomass composition was assumed to be CH$_{1.8}$O$_{0.5}$N$_{0.2}$ corresponding to 24.6 g DW C·mol$^{-1}$ for all cultivated yeast strains [33].

Yield of CO$_2$ on glucose was estimated by calculating firstly the off-gas CO$_2$ concentrations (g·L$^{-1}$), using equation (1):

$$\text{Accumulated CO}_2 \text{ at time } t_n = \sum_{i=1}^{n} \left( \frac{(Q_i + Q_{i-1}) \cdot (t_i - t_{i-1})}{2 \cdot V_R} \right) \cdot M_{CO_2} \ (g \cdot L^{-1})$$ (1)
where $t_n$ is the sample time, is time given in hours, $V_r$ is the bioreactor volume (L), $M_{CO_2}$ is the molecular mass of CO$_2$ (44.0095 g·mol$^{-1}$) and $Q_i$ is defined as mol CO$_2$ produced per hour at the given time point. $Q_i$ is calculated using equation (2):

$$Q_i = \frac{F_{\text{air},i} \cdot (\%CO_2,t_i - \%CO_2,t_0)}{V_m \cdot 100} \ (\text{mol} \cdot \text{h}^{-1}) \quad (2)$$

Where, $F_{\text{air},i}$ is the flow of air (L·h$^{-1}$) at the given time point, $\%CO_2,t_i$ is the CO$_2$ percentage in the off-gas air to the given time point and $CO_2,t_0$ is the background concentration of CO$_2$ in the supplied gas. The molar volume $V_m$ is derived from the ideal gas law, equation (3).

$$V_m = \frac{V}{n} = \frac{R \cdot T}{P} \ (L \cdot mol^{-1}) \quad (3)$$

Where, $R$ is Reynolds number (0.08314 L·bar·K$^{-1}$), $T$ is the temperature in the off-gas analysator (298.15 K) and $P$ is the ambient pressure (0.9885 bar).

The ethanol concentration in the gas phase were calculated by similar fashion, substituting the molecular mass of CO$_2$ ($M_{CO_2}$) with the molecular mass of ethanol ($M_{\text{ethanol}} = 46.06844$ g·mol$^{-1}$) and the CO$_2$ percentage ($\%CO_2,t_0$) with the ethanol percentage ($\%$EtOH$_{t,i}$) and at the given time point.

The specific glucose uptake rate was estimated from the yield of biomass on glucose ($Y_{sx}$) and the specific growth rate ($\mu$) using equation (4).

$$r_{glc} = Y_{sx} \cdot \mu \ (mmol \cdot g \text{ DW}^{-1} \cdot h^{-1}) \quad (4)$$
Amino acid derivatisation and GC-MS analysis

For metabolic flux analysis culture broth was harvested from shake flasks, bioreactor and 24 well plate cultivations in mid- to late-exponential growth phase, while glucose were still present, as gauged by glucose test strips (Machery-Nagel, Germany). The broth was immediately centrifuged for 5 min, at 12,000 \( \cdot \) g at 4 °C, washed twice with cold saline (0.9 % NaCl) and lyophilised until complete dryness. For bioreactor derived biomass, aliquots of approximately 5 mg and for 24 well MTP based cultivations aliquots of approximately 1.6 mg dry weight were resuspended in 400 µL 6 M HCl and hydrolysed for 16 h at 105 °C. Purification, derivatisation and GC-MS (HP-G1723A, Hewlett-Packard, Palo Alto, CA/USA) analysis of labelling patterns of intracellular metabolites (glucose-6-phosphate and proteinogenic amino acids) were done in accordance with the procedure outlined in Appendix I “A robust validated GC-MS methodology for \(^{13}\)C-measurements of proteinogenic amino acids employed in Metabolic Flux Analysis” also partly outlined by Christensen and Nielsen (1999) and (2000) [34, 35].

The gaseous derivates were bombarded with free electrons in the ion source released from a filament, resulting in the formation of positive ion fragments, which in turn, when analysed, resulted in fragment mass distributions measurements [35]. As a result of the fragmentation several ion clusters of the individual metabolites were observed in the mass spectrum each corresponding to the abundancy of the mass isotopomers of the respective metabolite fragment. By relating the measured mass peaks from the mass spectrum summed fractional labelling (SFL) of the various derivates fragments could be calculated applying equation (5) with \( m \), indicating the abundancy of the \((m+i)\) mass isotopomers and \( n \) the total number of carbon atoms in the given fragment. SFL is defined as the sum of fractional labelling of the individual carbon atom positions of the fragment, with labelling referring to \(^{13}\)C.

\[
SFL = \frac{\sum_{i=0}^{n} i \cdot m_i}{\sum_{i=0}^{n} m_i} 
\]

(5)
With a natural occurrence of isotopes the SFLs were corrected, accordingly, to reduce the noise from other atoms in accordance with the method described by [36, 37].

Mathematical modelling

Through combination of metabolite and isotope balancing, quantification of metabolic fluxes was achieved without the need for assumptions about cofactor requirements among others [34, 38]. Metabolic fluxes were estimated applying the mathematical framework described in [34] using the SFLs as input for mathematical modeling of the central carbon metabolism, slightly modified from a model, previously described by [30] and [39]. Other input to the model includes the specific glucose uptake and biomass production rate and the drain for biomass synthesis, the latter adopted from the model described by [30] and recalculated in accordance with the observed growth rate and biomass yield coefficient. All calculations were performed using an in-house-developed C^{13} Matlab program [40–42], based on the metabolic network analysis framework of [43].
Results and discussion

This study presents a fully automated high-throughput method for quantitative physiological characterisation and fast screening of yeast in microtiter plates. With this method either high throughput basal growth rate determination with optional endpoint product titer determination or in-depth quantitative physiological characterisation supported with metabolic flux analysis may be achieved.

Method design

To optimise and maximise the information obtained from the MTP based fermentation methodology two approaches were developed aiming at a) obtaining quality information on growth rate for comparative analysis of strain performance and b) obtaining data for quantitative physiological characterisation of not only growth rates, but also yield rates and metabolic flux analysis.

For either of the approaches to be successful several differences between instrumented bioreactors and MTP were addressed; principal amongst these were evaporation, oxygen transfer rate and pH drift.

Where instrumented bioreactors are commonly fitted with condensers ensuring minimal evaporation of water, but also volatile metabolites such as acetate and ethanol, MTP cultivations suffered substantial liquid loss of up to 12 % (v/v) over 30 hours when only lids were fitted. To counter evaporation gas permeable membranes were applied in the basic screening approach, reducing not only the loss of water, but also the loss of ethanol and acetate to evaporation. Based on a study by Zimmerman et al [48] a transparent MölnLycke Mepore membrane was chosen that, unlike most gas permeable membranes, exhibited a high degree of liquid retention while ensuring moderate oxygen transfer rates [25].

Though gas permeable membranes are routinely applied for both shake flasks and MTP’s providing adequate oxygen transfer rates for screening purposes such as comparative growth rate analysis [7], [44], [5], the absence of active aeration of the MTP wells represents a significant difference to instrumented bioreactors. In comparison to actively aerated bioreactors exhibiting volumetric oxygen transfer
coefficients, $k_{La}$, between 370-600 h$^{-1}$ [21], the values for shaken cultures are markedly lower often in the range of 30 to 60 h$^{-1}$, dependent on whether baffles are applied or not [45]. Comparable volumetric oxygen transfer rates have been demonstrated in a study by Kensy et al. [7] where $k_{La}$ of up to 550 h$^{-1}$ have been achieved for unsealed 48 well plates with a fill volume of 400 µL, by shaking at 1200 rpm at an amplitude of 3 mm [12]. Thus by adopting these parameters bioreactor comparable oxygen transfer rates could be achieved for the shaken MTP. Eliminating the use of gas permeable membranes was crucial in the design of an automated sampling regime, since the elasticity of such seals inhibits automated sampling. Though resulting in higher $k_{La}$ and enabling automatisation, omitting the use of membranes increases evaporation significantly, resulting in up-concentration of metabolites and loss of volatiles [46]. While this effect has no apparent effect on yield coefficient calculations of non-volatiles, since the concentration of both the carbon source and the product increases with the same rate, final concentrations and biomass yields may not be ascertained unless corrections are made.

By monitoring the absorbance of water at 977 nm, a time dependent evaporation coefficient was calculated allowing for reconciliation of nutrient and metabolite concentrations. It was ascertained that the linear water evaporation accounted for approximately 12 % loss of volume in 30 h.

Non-uniform evaporation across the different wells is reported as a potential concern when comparing growth in MTP’s [11]. As a countermeasure, the outer wells, which were most exposed to non-uniform evaporation, were filled with 400 µL sterile water. By comparing the average evaporation of the outer wells with the inner wells it was concluded that the outer wells were exposed to ~20-30 % higher evaporation. No such correlation was observed for any of the inner wells, where the well position could not be linked to higher or lower evaporation (data not shown). As a precaution evaporation was always calculated as an average of two wells differently located with respect to the edge of the plates.

Due to growth associated acid production and the release of protons upon uptake of ammonium ions [47] considerable acidic drifts can be encountered. To counter this problem, the medium for MTP cultivations
was supplemented with MES buffer, however pH stability was not achieved and the pH dropped to an endpoint level of ~ 3.5.

**Establishing a MTP based screening platform**

The suitability of the high throughput screening strategy for comparative growth rate analysis was evaluated by cultivation of four well described yeast strains, each displaying a different growth rate [48]: CEN.PK113-7D, CEN.MS5-3A, CEN.MS1-10CT1 and CEN.PK448. The strains were cultivated in membrane sealed 48 well MTPs for approximately 40 h with the maximal specific growth rates determined by at-line optical density measurements of individual wells over time. Three independent cultivations, with 30-36 individual well replicates each, were carried out for all strains, as illustrated with the cultivation plots in figure 4.
Figure 4. Comparison of the basic growth physiology of the four different strains in 48 well MTPs. A total of between 30-36 separate well replicates were summarised and plotted with their respective standard deviations. A clear distinction of the diauxic growth physiology was observed for all four strains indicating a high retention of produced ethanol.

From figure 4 it was evident that ethanol is well retained allowing characterisation of diauxic growth with potential application for the experimental design of detailed bioreactor based physiological characterisations.

Maximum specific growth rates were calculated from OD$_{600}$ measurements of individual wells over time treating each well as an independent replicate (99-108 replicates) (data not shown). By calculating individual growth rates for each independent set of cultivations in-depth assessment of the method
robustness was performed by one-way ANOVA tests. No statistically significant differences were identified for any of the four strains with p values ranging from 0.16-0.65, as specified in table 2.

Table 2. Comparative growth rate analysis of 48 well MTP based cultivations (b) with literature data (a) [48]. Growth rates were ascertained by OD600 measurements over time. No standard deviations were provided for CEN.PK448 in the literature.

<table>
<thead>
<tr>
<th>Strains</th>
<th>( \mu_{max}(h^{-1})^a )</th>
<th>MTP replicates</th>
<th>( \mu_{max}(h^{-1})^b )</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK113-7D</td>
<td>0.36±0.02</td>
<td>104</td>
<td>0.38±0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>CEN.PK448</td>
<td>0.28</td>
<td>108</td>
<td>0.23±0.01</td>
<td>0.65</td>
</tr>
<tr>
<td>CEN.MS1-10C T1</td>
<td>0.35±0.03</td>
<td>99</td>
<td>0.38±0.01</td>
<td>0.28</td>
</tr>
<tr>
<td>CEN.MS5-3A</td>
<td>0.34±0.06</td>
<td>102</td>
<td>0.34±0.01</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The MTP cultivation based growth rates were overall in good correlation with literature data displaying significantly lower standard deviations, with one notable difference observed for CEN.PK448 where MTP based growth rates were \( \sim 20\% \) lower than reported in [48]. While this difference cannot be attributed to standard deviations and might indicate an incoherence in the MTP based screening methodology it was noted that the standard deviations for the literature values, were based on duplicates suggesting that the ascribed growth rate was based on a single cultivation. Since the values for the other three strains were in good correlation and furthermore displayed higher average growth rates than CEN.PK448, it seems reasonable to conclude that the lower growth rate was not caused by inferior oxygen transfer rates. More likely the substantial deviations ascribed the other reported growth rates; indicate that the CEN.PK448 growth rate might be overestimated.

The absence of significant differences in the extrapolated growth rates together with low sample variation indicated that the growth phase of each individual well followed an almost identical course, which allowed for treating individual wells as representative for a single batch cultivation [23].

While growth rates were overall comparable with literature values, the application of gas permeable membranes does reduced the volumetric oxygen transfer rates [25] with potential unpredictable metabolic effects [11] resulting in dissimilar yield rates. Though this makes the method unsuitable for more
comprehensive analysis, the reduction in kLa is not necessarily vital for comparative analysis of growth rates, making the method applicable for high throughput screening of strain libraries.

**Validation of MTP based cultivations through comparative physiological characterisation with bioreactors**

To benchmark the proposed high throughput method, the scalability was challenged in a set of scale-up experiments with the reference strain, CEN.PK113-7D, ranging from 400 µL in MTPs to bioreactors with working volumes of 1 and 5 L. The MTP method was validated by comparative quantitative physiological characterisations performed for all scales.

For the proposed method, complete automatisation was implemented with full autonomy with regards to all liquid handling, sampling, filtration and optic measurements, with the setup program only relying on pre-specified user inputs. All cultivations were carried out in MTPs with non-fixed lids. While this ensured bioreactor comparable kLa’s and enabled automated sampling, quantitative analysis of ethanol and acetate yields were compromised due to unpredictable losses to evaporation. Thus, while ethanol and acetate yields were quantified, correlation with bioreactor yields was not possible.

Calculation for MTP biomass yields were only possible by reconciling substrate concentrations, eliminating the effects of evaporation occurring during the cultivation period (figure 1). Since biomass accumulation was assessed by OD$_{600}$ directly in the MTP’s, all measurements were converted to OD$_{600}$ in a 1 cm standard cuvette (figure 2) and in turn to gram dry weight using a correlation factor (figure 3). Thus application of the described correlations enabled re-calculation of OD$_{600}$ measurements to gravimetrical units and hence biomass yield coefficient. With these re-calculations, comparison of bioreactor and MTP cultivation profiles were possible as can be seen in figure 5 where reproducible, quantitative data is plotted for each cultivation type.
As evident from figure 5, substrate consumption, biomass and product formation follow similar trends. The exception is the formation of the volatile products that, due to evaporation, were measured in lower concentrations. The substantial losses observed for the volatile metabolites further explains the reduction in the second phase of the diauxic growth, explaining why this growth phase was not characterised.

Figure 5. Cultivation profiles for a bioreactor cultivation (top) and MTP based cultivation (bottom). Standard deviations have been included for all plotted MTP values based on triplicate samples.
For the comparative physiological characterisation three independent MTP cultivations were compared with triplicate cultivations in 1 and 5 L bioreactors, respectively (Table 3). As prerequisite for any comparison, the individual wells of the MTPs must be representative of a single batch cultivation. This was ascertained by one-way ANOVA tests of the individual growth rates, evaluated as independent populations on microtiter plate to plate basis. For the three independent MTP cultivations no statistically significant difference was observed, with values between $p = 0.09-0.73$ and variance from $\sigma^2 = 1.11\times10^{-4}-9.41\times10^{-6}$, indicating that the growth phase followed an almost identical course for the individual experiments. This was further supported by triplicate samples for each time point enabling basic statistic evaluation for each set of samples applied for calculation of yield coefficients. For the time intervals where yield coefficients (non-volatiles) were calculated the maximal relative standard deviations (RSD) were lower than 6 %, excepting only pyruvate where the deviations were between 1.3 - 9.6 %. The relatively high deviation observed for pyruvate may likely be attributed to the low abundancy of the metabolite, thus reducing the quantification accuracy, which also explains the relative high variation observed in both 1 and 5 L bioreactors. Though evaporation hampered any correlation of the volatile metabolites it could overall be concluded that the loss due to evaporation amounted to approximately 50 %, assuming that the overall physiology was comparable. For all of the non-volatile products a high degree of correlation was observed across all scales providing validation that the systems are comparable (Table 3). The comparability of the systems was further supported by the growth rate of the three independent MTP cultivations ($n = 88$) exhibiting a high degree of coherence with bioreactor derived values (Table 3).

To evaluate the method applicability for exploring cell factory potential, a heterologous 6-MSA producing $S.\cerevisiae$ strain was characterised in both 1 L bioreactors and MTPs with regards to the non-volatile metabolites. Since the 6-MSA genes were chromosomally integrated between essential genes, the expression and accumulation of the compound should in principle mirror the growth rate of the yeast, as previously shown for $Aspergillus\ nidulans$ [24].
Table 3. Results from quantitative physiological characterisation of *S. cerevisiae* CEN.PK113-7D in MTP’s, 1 L and 5 L bioreactors. All yield coefficients are given as C-mol product per C-mol glucose. Though the volatile products of the cultivations indicated by a), b) and c) have been quantified for the MTP cultivations with exception of c) no comparison could be made due to evaporation. Bioreactor ethanol yields (d) have been given as a total of the ethanol measured in samples and in the off-gas analysis.

<table>
<thead>
<tr>
<th>Cultivation vessel</th>
<th>Growth rate (h⁻¹)</th>
<th>Yield coefficients on glucose (C-mol·C⁻¹-mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD600</td>
<td>Ysx</td>
</tr>
<tr>
<td>MTP</td>
<td>0.37±0.01</td>
<td>0.17±0.00</td>
</tr>
<tr>
<td>1 L bioreactor</td>
<td>0.38±0.01</td>
<td>0.17±0.00</td>
</tr>
<tr>
<td>5 L bioreactor</td>
<td>0.39±0.02</td>
<td>0.17±0.01</td>
</tr>
</tbody>
</table>

For benchmarking, three independent cultivations were carried out in both bioreactor and MTP. As with the reference strain, growth rates based on OD₆₀₀ were comparable across scale (*n* = 87), but also growth rates based on 6-MSA accumulation were highly correlated with both OD₆₀₀ and across scale, further validating the comparability of the two systems (Table 4). This was supported with one-way ANOVA analysis of growth rates displaying no statistically significant difference for any of the MTP cultivations (data not shown).

Table 4. Growth rates for the heterologous 6-MSA producing yeast strain based on both time dependent optical density measurements and 6-MSA accumulation.

<table>
<thead>
<tr>
<th>Cultivation vessel</th>
<th>Growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD₆₀₀</td>
</tr>
<tr>
<td>MTP</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td>1 L bioreactor</td>
<td>0.37±0.01</td>
</tr>
</tbody>
</table>

Quantitative physiological analysis of the strain in both bioreactors and MTPs displayed good correlation with the previous analysis of the reference strain exhibiting comparable yields for all of the non-volatile products (Table 5). In the time intervals where yield coefficients (non-volatiles) were extrapolated, the
maximal RSDs were ≤ 6.4 %, in good correlation with previous findings. Both pyruvate and 6-MSA displayed a RSD of ≤ 10.3 %, likely owing to the relatively low abundancy of both compounds.

Table 5. Results of the quantitative physiological characterisation of the heterologous 6-MSA producing yeast strain in both MTPs and 1 L bioreactors. All yield coefficients are given as C-mol product per C-mol glucose. Though the volatile products of the cultivations indicated by a), b) and c) have been quantified for the MTP cultivations with exception of c) no comparison could be made due to evaporation. Bioreactor ethanol yields (d) have been given as a total of the ethanol measured in samples and in the off-gas analysis.

<table>
<thead>
<tr>
<th>Cultivation vessel</th>
<th>Yield coefficients on glucose (C-mol·C-mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ysx</td>
</tr>
<tr>
<td>MTP</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>1 L bioreactor</td>
<td>0.17±0.01</td>
</tr>
</tbody>
</table>

Despite the poorly controlled culture pH, and the short periods during handling where oxygen transfer rates were diminished, no measurable differences were identified in the comparative physiological characterisation of neither the reference nor 6-MSA producing strain. This indicated that while certain process conditions were difficult to control, the design could accommodate bioreactor comparable growth conditions. This high degree of robustness with regards to interrupted oxygen transfer was also supported by Zimmermann and Degussa (2006) [49]. Though the pH drop was substantial, similar growth rates and production rates for both biomass and glycerol were reported in literature in the pH range from 4 to 6 with a minor drop for pH at 3.5 [50]. In this study, the pH drop to 3.5 was not observed before the end of the cultivation, thus the majority of the cultivation remained at a pH level where according to literature no effect should be measurable [50].
Validation through metabolic flux analysis

Although no measurable differences were identified in the comparative physiological characterisation the pH drift and the fluctuations in oxygen transfer rates, may have triggered intermediary changes from aerobic to anaerobic growth causing unpredictable metabolic reactions, as suggested in previous work [11].

To investigate whether pH or intermediary changes in oxygen transfer rates affected the intracellular flux distribution, metabolic flux analysis was performed based on $^{13}$C labeling. If the oxygen transfer rate was not meeting the requirements for full respirative-fermentative growth, a shift towards fermentative growth was expected, which in principle would cause a change in the metabolic flux distribution [51].

For comparison, the parameters and procedure outlined by Gombert et al (2001) [30] was adopted and carried out in shake flasks, MTPs and ½ L bioreactor. Due to the relatively small working volume of the MTP wells (800 µL), the starting $[1$-$^{13}$C]glucose concentration was increased to 20 g·L$^{-1}$ compared with only 5 g·L$^{-1}$ applied by Gombert et al (2001) [30]. By increasing the substrate concentration it was possible to extract approximately 1.6 mg DW biomass from each well at mid to late exponential growth (OD$_{600}$ of 0.6, as measured in the well) (data not shown).

For comparative flux analysis between the various cultivation setups and literature values, duplicate cultivations in shake flasks were carried out with 5 g·L$^{-1}$ (as specified by Gombert et al (2001)) and 20 g·L$^{-1}$ labelled glucose, respectively, supported by cultivation in a 0.5 L instrumented and controlled bioreactor with 20 g·L$^{-1}$labelled glucose.

To ensure comparability, the precursor drain for biomass formation were adopted from [30] and re-calculated and normalised to the glucose uptake rate (100). This re-calculation took into account the higher biomass yield on glucose and the slightly higher growth rate observed for this study, thereby normalising the adopted data. Since biomass yield coefficients were reported to be significantly lower than were...
estimated in the present study, the glucose uptake rate was calculated to be 14.76 mmol·g(DW)⁻¹·h⁻¹, where 15.6 mmol·g(DW)⁻¹·h⁻¹ was reported in [30].

While these values corresponded well, scrutiny revealed, through re-calculation of the uptake rate from the reported growth rate and biomass yield coefficient, that the uptake rate was not 15.9 mmol·g (DW)⁻¹·h⁻¹, but rather 19.6 mmol·g (DW)⁻¹·h⁻¹ corresponding well with similar values obtained previously [52].

Coupling the labelling results, re-calculated to SFLs (table 6), with the biomass drain and the extracellular fluxes (where available), fluxes were estimated using the model outlined in Gombert et al (2001) [30], as illustrated in figure 6.
Table 6. Summed fractional labelling (measured and calculated) of derivatised fragments for the reference strain CEN.PK113-7D, cultivated in shake flasks with 5 g·L\(^{-1}\) [\(^{1}\)C]glucose in accordance with the procedure outlined by Gombert et al (2001) [30], and in shake flasks and bioreactor with 20 g·L\(^{-1}\) [\(^{13}\)C]glucose inoculated to an OD\(_{600}\) of 0.01 (15 fold lower). The SFLs have been calculated as the mean from duplicate measurements with standard deviations less than 5 %.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>C atoms in fragment</th>
<th>Biosynthetic origin</th>
<th>SFL measured</th>
<th>SFL calculated</th>
<th>SFL measured</th>
<th>SFL calculated</th>
<th>SFL measured</th>
<th>SFL calculated</th>
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<th>SFL calculated</th>
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<th>SFL calculated</th>
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<td>104.2</td>
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<td>44.4</td>
<td>48.2</td>
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<td>PYR(2, 3)</td>
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<td>8.1</td>
<td>9.8</td>
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<td>OAA(2)</td>
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<td>OAA(1, 2, 3, 4)</td>
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<tr>
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<td>2, 3, 4, 5, 6</td>
<td>+ OAA(2, 3, 4)</td>
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<td>98.5</td>
<td>100.2</td>
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<tr>
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<td>2-KG(1, 2)</td>
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<td>48.0</td>
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<td>2-KG(2, 3, 4, 5)</td>
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<td>93.0</td>
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<td>+ AKG(2)</td>
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<td>PE(1, 2)</td>
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<td>Phe</td>
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<td>2, 3, 4, 5, 6, 7, 8, 9</td>
<td>+ PE(1, 2, 3, 4)</td>
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<td>2.9</td>
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</table>
Figure 6. Central metabolism flux distribution of CEN.PK113-7D batch cultivations in a) fully instrumented bioreactor (20 g·L⁻¹ [1-¹³C]glucose) with all extracellular fluxes included, b) fully instrumented bioreactor, c) shake flask with 5 g·L⁻¹ and d) shake flask with 20 g·L⁻¹ glucose all including the extracellular flux of glycerol. All fluxes are net fluxes and have been normalised to the glucose uptake rate of 14.76 mmol.gDW⁻¹·h⁻¹. Abbreviations: Glc, Glucose; G6P, Glucose-6-Phosphate; PSP, Pentose-5Phosphate; S7P, Sedoheptulose-7-Phosphate; G3P, Glycerol-3-Phosphate; F6P, Fructose-6-Phosphate; E4P, Erythrose-4-Phosphate; PEP, Phosphoenolpyrovate; ACA, Acetaldehyde; ACE, Acetate; AcCoA, Acetyl-CoA; PYR, Pyruvate; OAA, Oxaloacetate; ICIT, Iso-Citrate; AKG, Alpha-ketoglutarate; FUM, Fumarate; THR, Threonine; Gly, Glycine; SER, Serine.
Since evaporation compromised thorough quantitative analysis of ethanol and acetate, no extracellular fluxes for these could be included in the simulation for neither shake flasks nor MTPs. With especially ethanol comprising a significant carbon flux during respirative-fermentative growth, a compromise was made, to stabilise the model, by eliminating the flux of acetate. While eliminating this flux does have an impact on the system dynamics, comparative analysis with the bioreactor based cultivation, where all extracellular fluxes were included, indicated only minor deviations as a consequence, as illustrated in figure 6.

To ascertain the robustness of the model after this elimination, a simulation of the metabolic fluxes was made for the bioreactor derived data, excepting the extracellular fluxes to ethanol and acetate. While eliminating these fluxes had an effect, the overall flux distribution remained highly correlated, as evident from both table 6 and figure 6. By comparison of the measured and calculated SFLs from all experiments, it was evident that the model minimisation routine was capable of fitting the majority of all measurements within the standard deviation interval outlined, suggesting that the model was correct and applicable for flux estimation.

Notably, a substantial difference was observed for the metabolic fluxes and SFLs dependent on the glucose concentration applied. Though the reproduced experiments were in in overall good correlation with the metabolic fluxes estimated by Gombert et al (2001) [30], both the SFLs and metabolic fluxes differed substantially from the corresponding values estimated from the bioreactor and shake flask cultivations (20 g·L⁻¹ [1-¹³C]glucose). The metabolic flux though the pentose phosphate pathway was estimated to be 16.9 % for the shake flask cultivations with 5 g·L⁻¹ [1-¹³C]glucose, corresponding well with the 16.2 % reported by Gombert et al (2001) [30]. In comparison, the corresponding flux for all cultivations, applying low inocula and 20 g·L⁻¹ [1-¹³C]glucose, were estimated to approximately 9 %, indicating an incoherence. This difference suggested that though one of the main topics of the aforementioned publication was the establishment of a well-defined interval with isotopic steady state, relevant for metabolic flux analysis, that the authors did
not achieve this for their metabolic flux estimations. The high inoculum applied together with low substrate concentration likely explains why an isotopic steady state was not achieved, arguing for the use of higher substrate concentrations with reduced inoculum.

The estimations of Gombert et al (2001) [30] were also disputed by Fiaux et al (2003) [53], estimating the key flux between glycolysis and pentose phosphate pathway to be >4 %.

While they argued that the model and global minimisation function, applied by Gombert et al (2001) [30] and in this study, were the primary causes, the more likely explanation was that extracellular fluxes were not included in their estimations.

To evaluate the effect of leaving out extracellular fluxes, metabolic flux analysis was performed applying the SFLs derived from the bioreactor cultivation. The resulting metabolic fluxes revealed a pentose phosphate flux of 2.5 % corresponding well with the findings of Fiaux et al (2003) [53]. Based on these tests it was concluded that the model could not estimate the metabolic fluxes accurately and with sufficiently high confidence unless extracellular fluxes were applied together with high [1-^{13}C]glucose concentrations and low start inoculum.

Since the MTP based cultivation strategy is autonomous, allowing for continuous OD monitoring, a low inoculum was applied together with a 20 g·L^{-1} [1-^{13}C]glucose ensuring sufficient biomass for metabolic flux analysis. By comparison, the measured SFLs from the MTP derived biomass correlated well with both the calculated SFLs and the corresponding values for the bioreactor, underlining the applicability of the model for flux analysis (Table 7). From the comparative metabolic flux analysis it was concluded that the flux distribution of the MTP based cultivation (figure 7), closely resembled the bioreactor distribution, suggesting that neither acid drift nor the intermediary changes in oxygen transfer had any measurable effects on the metabolic flux of the strain.
Table 7. Summed fractional labelling (measured and calculated) of derivatised fragments for the reference strain CEN.PK113-7D, cultivated in MTP and bioreactor with 20 g·L⁻¹ [1-¹³C]glucose inoculated to an OD₆₀₀ of 0.01. The SFLs have been calculated as the mean from duplicate measurements for the bioreactor and quadruplicates for the 24 well MTP with standard deviations less than 5 %.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>C atoms in fragment</th>
<th>Biosynthetic origin</th>
<th>Bioreactor SFL measured</th>
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<td>2, 3, 4</td>
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<td>+ OAA(2, 3, 4)</td>
<td>100.5</td>
<td>99.9</td>
<td>100.2</td>
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<td>Glu</td>
<td>143</td>
<td>1, 2</td>
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<td>46.1</td>
<td>46.6</td>
<td>48</td>
<td>48.28</td>
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<td>Phe</td>
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<td>2, 3, 4, 5, 6, 7, 8, 9</td>
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<td>5.2</td>
<td>4.0</td>
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<td>3.233</td>
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Figure 7. Central metabolism flux distribution of CEN.PK113-7D batch cultivations in a) fully instrumented bioreactor (20 g·L⁻¹ [1-\(^{13}\)C]glucose) with all extracellular fluxes included and b) 24 well microtiter plate with 20 g·L⁻¹ glucose, including the extracellular flux of glycerol. All fluxes are net fluxes and have been normalised to the glucose uptake rate of 14.76 mmol·gDW⁻¹·h⁻¹. For abbreviations see figure 2.
Though a modified model had to be applied for stabilising the model structure, the flux distributions were remarkably closely correlated, even for the ethanol and CO₂ flux, which were not included as extracellular fluxes. This, not only suggests that the physiology of the MTP cultivations were comparable with the bioreactor cultivation, but also expands the utility of MTP cultivations as a platform for high throughput metabolic flux analysis.
Conclusion

Despite the dissatisfactory level of pH control and even though the method design required short periods where plates could not be shaken, diminishing the oxygen transfer rates, the comparative quantitative physiological analysis in MTPs and bioreactors revealed robust correlations. Quantitative characterisation was not an option for volatile metabolites in MTPs constraining the general suitability to characterisation of only non-volatile metabolites, making the method ideal for microorganisms not producing volatile metabolites (CO₂ not included). In-depth metabolic flux analysis revealed that though the model could not be constrained by inclusion of volatile extracellular metabolite fluxes, omitting the acetate flux from the mathematical model enabled micro-scale flux analysis. From the comparative analysis with the metabolic fluxes calculated for fully instrumented and controlled bioreactors it was concluded that the metabolic fluxes in MTP cultures resembled those of bioreactors. Based on the highly similar growth rates, yield coefficients and the correlating metabolic fluxes it was concluded that the outlined method may be used to generate high quality quantitative data comparable to those of bioreactors. Thus by applying this methodology, data generated in MTPs can be used to make informed decisions concerning further strain optimisation and for selection of best performing strains with the confidence that the same conclusions would have been made in instrumented bioreactors, excepting only information’s regarding volatiles.
References


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Chapter 4

A high throughput microtiter plate based screening approach for physiological characterisation of filamentous fungi

Peter Boldsen Knudsen, Alexander Rosenkjaer, Zofia Dorota Jarczynska, Kristian Fog Nielsen, Jette Thykaer, Mhairi Workman*

Department of Systems Biology, Technical University of Denmark, Building 223, Søltofts Plads, DK – 2800 Kgs. Lyngby, Denmark.

PBK: pebok@bio.dtu.dk; AR: alexr@bio.dtu.dk; ZDJ: zofja@nio.dtu.dk; KFN: kfn@bio.dtu.dk; JT: jthykaer@yahoo.com

*Corresponding Author: Mhairi Workman, email: mwo@bio.dtu.dk, Tel.: +45 4525 2700
Abstract

Background

Efficient cell factories are in constant demand as our need for novel and sustainable bio-products increases. Similarly, advanced genetic engineering systems generate an increasing number of possible variants, which must be evaluated as potential hosts for value added products. Such demands and possibilities, challenge traditionally applied methods for cell factory assessment, being based on time consuming and laborious shake flask or bioreactor cultivations. In this study we present a novel, automated high throughput methodology for cultivating filamentous fungi in microtiter plates (MTPs), without compromise to morphology, and with product yields and growth rates identical to bioreactors. This was made possible by the supplementation with the anionic polymer carboxypolymethylene, ensuring full hyphal dispersion throughout the entire exponential growth phase.

Results

Due to consistent hyphal dispersion, fungal growth rates could be estimated in MTPs through optical density measurements, allowing for miniaturised physiological studies in microtiter plates. A sequence of initiatives aimed at controlling, monitoring and compensating for pH drift and evaporation were implemented and validated. Cultivations were proven equivalent in a comparative scale-down experiment from bioreactors to the high throughput MTP based system, where identical growth rates and product yield coefficients were obtained for an Aspergillus nidulans producing heterologous 6-methylsalicylic acid (6-MSA). Since the 6-MSA gene was integrated chromosomally, the metabolite was expressed as a function of growth and mirrored the growth rate precisely, adding an additional level of confidence to the scale-down experiment. Method versatility and robustness were proven in a screen of 5 filamentous fungi representing industrially applied strains as well as novel species with cell factory potential. The growth rates of the 5 were shown to be highly correlated with literature data.
Conclusions

An automated high through-put methodology was introduced for cultivating filamentous fungi in MTPs taking advantage of the dispersive effect on morphology of the anionic polymer carboxypolymethylene. Cultivation of selected fungi in both bioreactors and MTPs proved that not only growth rates, but also yield coefficients were identical across scale suggesting that this method may be used for fast quantitative physiological studies assessing cell factory potential with full scalability.

Keywords: Microtitre, physiological characterisation, filamentous fungi, carboxypolymethylene
Introduction

The traditional practice of performing strain evaluation and medium optimisation in shake flasks has faced some serious challenges over recent years due to the significant advances made in genetic engineering and construction of mutant libraries with a consequential rise in numbers of strains to be characterised. To deal with these advances a range of methods have been developed to increase the through-put of candidate strain evaluation. The main effort for enhancing throughput has focused on miniaturisation of fermentation processes in order to reduce analysis costs, not only in terms of materials used, but also in time and labour. These efforts have been fairly successful for dealing with both yeast and bacteria, where the application of robotics and microtiter plates (MTPs) have allowed for fast and reliable evaluation of growth and end point product concentrations [1]–[3]. Dedicated instruments have even been developed for automated high throughput experimentation and an increasing number of papers are published on the topic of miniaturised fermentations for physiological characterisation [4]–[6].

Unfortunately, automated systems rely principally on UV/Vis based plate readers for growth evaluation, which constitutes a problem when non-unicellular microbial organisms, such as filamentous fungi and bacteria, are under investigation. Some studies have been published on the topic, but mainly focus on filamentous bacteria such as *Streptomyces coelicolor* that due to a more homogeneous morphology can be evaluated using optical density (OD) measurements [7], [8]. Filamentous fungi however, have a more complex morphology ranging from freely dispersed hyphae, through entangled clumps to loose or compact pellet like structures [9]–[11], making it rather inaccurate to apply plate readers for growth rate determination [7], [8], [12]. A few studies do exist that describe the application of OD measurements for the determination of filamentous fungal growth rates, but they focus on a narrow range of fungi such as *Geotrichum candidum, Verticillium agaricnum* and *Mucor hiemalis* that, similar to the filamentous bacteria, display a homogeneous dispersed morphology in submerged cultures [13]. Attempts on small scale fermentations in 24 well plates have been made and were shown superior to shake flask with regards to
standard deviations, but with no control of the morphology, pellets predominated and growth rates could conversely not be estimated by OD [14]. Though OD measurements are not generally applicable for filamentous fungi, a correlation between dry weight and OD has been proven by comparison of the OD of homogenised biomass with dry weight [15].

Morphology is not only an issue with regards to physiological characterisation, but can also indirectly influence production levels of various metabolites of interest and consequently receives considerable attention from both academia and industry [11], [16]–[19]. It is difficult to predict, which morphology is optimal based on the product alone, as the interplay between process parameters complicates the process of separating cause and effect [11], [19].

While product formation may be enhanced under certain morphological states, the dynamic nature of morphological development makes these organisms difficult to control. Physiological characterisation and process optimisation therefore becomes a bottleneck in many processes involving filamentous microorganisms. In most cases, a trade-off must be accepted between optimal processing with regards to viscosity, mixing and mass transfer, which is to a large extent determined by morphology, and the product yield [20]–[23]. For highly detailed physiological studies based on quantification of cellular performance indicators, a dispersed hyphal morphology is required. This ensures a homogeneous culture and the representative samples required for quantitative analysis [8], [20].

The morphology of filamentous fungi can be influenced by process variables, but many of the methods require active control to change pH, aeration and stirring during germination and the time course of the fermentation in order to maintain a uniform dispersed morphology; parameters that are not easily controlled in MTP [20], [24]. Other effectors which do not require a high level of active process control include increasing the inoculum size [24], [25], changing the process pH [26], [27], altering vessel form to increase oxygen transfer [11] and using additives such as Tween 80 [25], talc powder (aluminum oxide and magnesium silicate) [28], [29], alginate [30] or anionic polymeric additives such as polyacrylic acid and
carboxypolymethylene [30]–[32]. Comparative studies of select effectors have concluded that the most efficient of these were addition of the anionic polymeric additives [11], [33] as agglomeration was reduced for both spores and hyphae thereby inhibiting pellet formation [31]. The reduction in agglomeration is crucial if growth rates are to be calculated from OD₆₀₀ measurements, where culture homogeneity is an absolute requirement.

Since both filamentous bacteria and fungi can be evaluated using OD measurements, provided that they form completely dispersed mycelia in culture; supplementation of anionic polymers should make OD a reliable option for biomass estimation. This has been demonstrated for an A. niger culture supplemented with the anionic polymer Junlon (polyacrylic acid), where a linear correlation between biomass dry weight and culture turbidity were obtained [32]. The method has further been suggested applicable for growth rate estimations of both filamentous fungi and bacteria though it has not been substantiated with exponential growth phase data [32], [34].

The aim of this study was to establish a high throughput microtiter based screening approach for physiological characterisation of filamentous fungi in MTPs applying OD₆₀₀ measurements for monitoring growth rates. As a means to control the morphology polyacrylic acid and carboxypolymethylene were tested. The scalability of the method was challenged in a comparative physiological study where Aspergillus nidulans strains were cultivated in both MTPs and fully automated and instrumented 1 L bioreactors. The universality of the method was tested by growth experiments on nine different filamentous fungi representing five different genera of both well characterised and novel fungal cell factories.
Materials and Methods

Fungal strains

The novel screening study was performed with nine different filamentous fungi: *Aspergillus niger* (ATCC 1015), *Aspergillus oryzae* (A 1560), *Penicillium chrysogenum* (wis54 1255), *Talaromyces atroroseus* (IBT 11181), *Fusarium oxysporum* (IBT 40467) and the in-house constructed heterologous 6-methylsalicylic acid (6-MSA) producing *Aspergillus nidulans* strains IBT 33253, IBT 33254, IBT 33255 and the corresponding reference strain IBT 33252. IBT strains are available from the IBT culture collection (Department of Systems Biology, Technical University of Denmark).

Media

Czapek yeast extract (CYA) medium was used for spore propagation, containing 30 g·L\(^{-1}\) Sucrose, 5 g·L\(^{-1}\) Yeast extract, 3 g·L\(^{-1}\) NaNO\(_3\), 1 g·L\(^{-1}\) K\(_2\)HPO\(_4\), 0.5 g·L\(^{-1}\) MgSO\(_4\)· 7 H\(_2\)O, 0.5 g·L\(^{-1}\) KCl, 0.01 g·L\(^{-1}\) FeSO\(_4\)· 7H\(_2\)O, 15 g·L\(^{-1}\) Agar, 1 mL·L\(^{-1}\) trace element solution containing 0.4 g·L\(^{-1}\) CuSO\(_4\)· 5 H\(_2\)O, 0.04 g·L\(^{-1}\) Na\(_2\)B\(_2\)O\(_7\)· 10 H\(_2\)O, 0.8 g·L\(^{-1}\) FeSO\(_4\)· 7 H\(_2\)O, 0.8 g·L\(^{-1}\) MnSO\(_4\)· H\(_2\)O, 0.8 g·L\(^{-1}\) Na\(_2\)MoO\(_4\)· 2 H\(_2\)O, 8.0 g·L\(^{-1}\) ZnSO\(_4\)· 7 H\(_2\)O. pH was adjusted to 6.2 with NaOH prior to autoclavation. Unless otherwise stated all chemicals applied were procured from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO/USA).

For medium optimisation, microtiter and bioreactor cultures the following defined medium was used: 5 g·L\(^{-1}\) D-glucose, 9.75 g·L\(^{-1}\) NaNO\(_3\) or 7.5 g·L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 1.5 g·L\(^{-1}\) KH\(_2\)PO\(_4\), 1 g·L\(^{-1}\) MgSO\(_4\)· 7 H\(_2\)O, 1.0 g·L\(^{-1}\) NaCl, 0.1 g·L\(^{-1}\) CaCl\(_2\), 0.1 mL Antifoam 204, 1 mL·L\(^{-1}\) trace element solution. Trace element solution: 0.4 g·L\(^{-1}\) CuSO\(_4\)· 5 H\(_2\)O, 0.04 g·L\(^{-1}\) Na\(_2\)B\(_2\)O\(_7\)· 10 H\(_2\)O, 0.8 g·L\(^{-1}\) FeSO\(_4\)· 7 H\(_2\)O, 0.8 g·L\(^{-1}\) MnSO\(_4\)· H\(_2\)O, 0.8 g·L\(^{-1}\) Na\(_2\)MoO\(_4\)· 2 H\(_2\)O, 8.0 g·L\(^{-1}\) ZnSO\(_4\)· 7 H\(_2\)O. For optimisation the medium was applied with or without supplementation of polyacrylic acid (Mw – 100,000) or carboxypolymethylene (Carbopol-934) (Kindly provided by Lubrizol, Wickliffe, OH/USA) and either without pH regulation or buffered with 100 mM 4-Morpholineethanesulfonic acid hemisodium salt (MES) buffer.
Inoculum preparation

Spores were propagated on Czapek Yeast extract Agar (CYA) plates for 10-21 days, dependent on the individual strain, and subsequently harvested with 10 mL cold 0.9 % NaCl followed by filtration through mira cloth (Merck Millipore, Darmstadt, Germany) and spun down at 8000 x g. The spores were then washed twice with cold 0.9 % NaCl prior use. The cultivations were initiated by inoculation of the relevant spore suspension to a final concentration of 5·10^9 spores·L^-1 as determined by hemocytometer.

Media design

Optimal medium composition was identified by shake flask experiments with Aspergillus nidulans IBT 33253. Duplicate cultivations were carried out in MES buffered or non-buffered media with either sodium nitrate or ammonium sulfate as nitrogen source with or without supplementation of polyacrylic acid or carboxypolymethylene. Glucose concentration was assessed after 36 h of cultivation, by glucose test strips (Machery-Nagel, Düren, Germany) and the degree of dispersion was assessed by qualitative visual inspection with microscopy.

Bioreactor based batch cultivations

All bioreactor based cultivations were carried out using Sartorious 1 L bioreactors (Satorious, Stedim Biotech, Goettingen, Germany) with a working volume of 1 L and equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer’s standard procedures. The bioreactor was sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5 % (v/v) CO2, 0.04 % (v/v) ethanol and methanol, 1 % (v/v) argon, 5 % (v/v) and 15 % (v/v) oxygen all with nitrogen as carrier gas (Linde Gas, AGA, Enköping, Sweeden). Temperature was maintained at 30 °C throughout the cultivation and pH controlled by automatic addition of 2 M NaOH and H_2SO_4. Start culture conditions were pH: 5, stir rate: 100 rpm and air flow: 0.1 volume of air per volume of liquid per minute (vvm). Following inoculation these
conditions were changed in a linear fashion over a period of 720 min., stirring rate: 800 rpm and air flow: 1 vvm. The strains were cultivated until glucose was depleted and the culture had entered stationary phase as monitored by off-gas CO₂ concentration. Samples were taken across the exponential growth phase for metabolite profiling.

**High throughput MTP batch cultivations**

For all high through-put fermentations the spore suspensions were prepared to a concentration of $1 \cdot 10^8$ spores·mL⁻¹ and dispensed into a 96 well U-bottomed microtiter master-plate (Greiner Bio-One, Frickenhausen, Germany). All liquid handling was carried out using a Hamilton Microlab STAR liquid handling workstation (Hamilton, Bonaduz, Switzerland) with an integrated Thermo Cytomat 2 C450 shaking incubator (Thermo-Fischer Scientific, Waltham, MA/USA) and Biotek Synergy 2 microplate reader (Biotek, Winooski, Vermont/USA). Medium was automatically dispensed into the inner wells of 48 well MTPs (Greiner Bio-One, Frickenhausen, Germany) in aliquots of 380 and 400 µL for cultivations and negative reference, respectively. The medium aliquots of cultivation were subsequently inoculated with 20 µL spore suspension from the master-plate and automatically mixed by pipetting. The outer wells were filled with 400 µL water to reduce the effects of evaporation on the micro-cultures. In addition four inner wells were filled with 400 µL water for evaporation rate evaluation.

The plates were incubated in the integrated incubator under constant shaking with an amplitude of 3 mm starting at 500 rpm for the first 6 h then ramped up to 800 rpm in a stepwise fashion of 100 rpm per 45 min. Temperature and humidity (≥95 % rH) were kept constant at either 25 or 30 °C, dependent on the strain specific requirements, by a heated water bath integrated into the shaking incubator. The handling workstation was programed to extract individual plates from the incubator at user defined time intervals for measurements and liquid handling. Briefly, plates were extracted and transported by the handling system to the plate reader for measurements. The biomass evolution of each individual well was monitored by logging the average of a nine point matrix measurement at 600 nm. Water evaporation or path length
reduction rate was measured as the difference between baseline absorbance at 900 nm and the absorbance peak at 977 nm divided with the difference in a standard 1 cm cuvette as specified in McGown et al, 1998 [46].

**Quantification of extracellular metabolites**

For quantification of extracellular metabolites, culture samples were taken and immediately spun down at 10,000 x g to sediment the polymer and biomass solids and then filtered through a 0.45 µm cellulose acetate filter (Frisenette, Knebel, Denmark). For the MTP cultures, samples were taken manually immediately after the automated OD<sub>600</sub> measurements. The samples were frozen and kept at -20 °C until analysis. Glucose, glycerol, pyruvate, succinate, acetate and ethanol were detected and quantified on an Agilent 1100 HPLC system equipped with a refractive index and diode array detector (Agilent Technologies, Waldbronn, Germany) and equipped with an Aminex HPX-87H cation-exchange column (BioRad, Hercules, Ca, USA). Compounds were separated by isocratic elution at 60 °C, with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL·min<sup>-1</sup>. Quantification was performed using a six-level external calibration curve with pyruvate detected at a wavelength of 210 nm and glucose, succinate, glycerol, acetate and ethanol by refractive index measurements.

6-MSA was quantified using a Dionex Ultimate 3000 UHPLC coupled with an ultimate 3000 RS diode array detector (Dionex, Germering, Germany) equipped with a Poroshell 120 phenyl hexyl 2.1 x 100 mm, 2.7 µm (Agilent, Santa Clara, CA/USA) column by gradient elution at 60 °C and a flow rate of 0.8 mL·min<sup>-1</sup> starting at 15 % acetonitrile and MiliQ water and ramped up to 100 % in 7 min. before returned to the starting point in and additional 5 min. Quantification was performed using an eight-level external calibration curve with concentrations from 1 - 300 mg·L<sup>-1</sup> at a wavelength of 210 nm.
Results and Discussion

The principal aim of this study was to introduce a fast and reliable method for physiological characterisation of filamentous fungi applying an automated microtiter based screening strategy. The design of this method allows for basic high throughput estimation of growth rates and in-depth characterisation of potential cell factories through metabolite profiling.

Media design

In order to investigate the effect of two anionic polymers (polyacrylic acid and carboxypolymethylene) and screen for optimal media compositions, a series of shake flask experiments was carried out, optimising for superior growth/morphology and pH stability (data not shown). The issue of pH stability constituted one of the major problems due to the production of organic acids and the concomitant release of protons upon the uptake of ammonium ions causing acidic drift [35]. As a countermeasure, supplementation with MES buffer was tested, but was found ineffective. A more effective measure was to substitute ammonium sulfate with sodium nitrate, since the uptake of nitrate occurred simultaneously with the uptake of a proton [35], which ensured only minor pH drifts of ± 0.4.

Carboxypolymethylene and polyacrylic acids were both applied in concentrations of 0.3 % (w/v) based on several studies concluding that this concentration provided the optimal morphology and product titers [31], [33]. In comparison carboxypolymethylene supplementation resulted in a higher degree of dispersion compared with polyacrylic acid, where clumps were also observed. Carboxypolymethylene was therefore selected for further work, and nitrate for all fermentations with the exception of those where supportive literature data prescribed use of ammonium sulfate.

Though the application of these anionic polymers has been extensively studied the exact effect is not entirely elucidated. It has been suggested that the increasing medium viscosity caused by the addition of the polymer results in the more dispersed morphology observed [34]. This theory has been somewhat
contradicted in a study on *Rhizopus arrhizus*, where a pH dependent 400 fold decrease in the medium viscosity did not change dispersion while the polymer was present, indicating that viscosity was not the main effector [30]. Another hypothesis suggested that the polymer inhibited pellet formation by sequestering sites on spores and hyphae thereby preventing salt bridging caused by binding of divalent cations from the medium [30]. However this hypothesis was later disproven, since leaching the media of divalent cations by dialysis did not change morphology [36]. More likely, the dispersion is caused by absorption of the negatively charged polymer onto the spore and hyphal surfaces, resulting in electrostatic repulsion preventing interaction and thereby agglomeration [30], [31], [37]–[39].

To ensure that carboxypolymethylene did not constitute an alternative carbon source and thereby complicate any quantitative analysis, all fungi were cultivated in substrate deficient medium containing carboxypolymethylene for five days. While 4 out of the 9 of the tested fungi germinated, none were capable of any further growth, corresponding well with literature data on both filamentous fungi and bacteria [31], [34].

**Method design**

There are several major differences between instrumented bioreactors and shaken microtiter cultures, impeding comparison of quantitative physiological data. Amongst these are the small volumes of the microtiter wells, which are subject to significant water evaporation resulting in up-concentration of biomass, metabolites and nutrients. With the proposed method relying on plate readers for logging biomass growth, the substrate concentrations had to be kept low to ensure that the OD$_{600}$ remained within the linear range of the plate reader. By keeping a low substrate concentration the rheological issues [24] associated with biomass concentration, fungal cell morphology and in this case the supplementation of carboxypolymethylene, were attenuated improving hydrodynamics and aeration.

Compared to stirred bioreactors with volumetric oxygen transfer coefficients, kLa, in the range of 370-600 h$^{-1}$ [40], shaken cultures only exhibit kLa’s in the range of 30-60 h$^{-1}$ [41] due to the lack of active aeration. A
shaking ramp was therefore designed for the MTPs going from 400 to 800 rpm that, while ensuring that spores were not lost during the germination phase due to vigorous shaking, also accommodated a higher kLa during the exponential growth phase. In accordance with a study by Kensy et al (2005), increasing the shaking to 800 rpm allowed for an elevation of the volumetric transfer rate to approximately 100 h\(^{-1}\) [42]. While higher shaking frequencies have been shown to further increase the kLa, depositing of biomass on the sides of the wells were observed for frequencies higher than 800 rpm leading to incorrect OD\(_{600}\) measurements. In addition, the dispersive effect of carboxypolymethylene has been shown to alleviate some of the mass transfer issues associated with filamentous cultures as well as reducing the surface tension of the liquid and improving the hydrodynamics of the broth [31], [34].

Though an attractive feature of microtiter cultivations, the small volumes are subject to a degree of water evaporation resulting in up-concentration of metabolites and nutrients. While this effect has no apparent effect on yield coefficient calculations, since the concentration of both the carbon source and the product increases with the same rate, final concentrations may not be ascertained unless corrections are made. By monitoring the absorbance of water at 977 nm, a time dependent evaporation coefficient was calculated allowing for reconciliation of nutrient and metabolite concentrations to their true values. It was ascertained that the linear water evaporation accounted for approximately 12 % loss of volume in 30 hours.

**Comparative analysis of bioreactor and microtiter based cultivations**

Method suitability for growth rate determination was evaluated by duplicate cultivations of *Aspergillus nidulans* (IBT 33252) in carboxypolymethylene supplemented media in fully instrumented bioreactors. Since it was difficult to separate biomass from the polymer additive, no dry weight measurements were obtained, and growth rate determination was therefore based on accumulated CO\(_2\), as illustrated in Figure 1B and 1D.
Figure 1. Comparative analysis of bioreactor and microtiter based cultivations of *Aspergillus nidulans* (IBT 33252). (A) Microtiter based optical density growth curve with standard deviations, (B) Bioreactor based accumulated CO$_2$ growth curve, (C) Exponential growth fit of 42 individual wells from four independent cultivations with their respective standard deviations, (D) Exponential bioreactor growth fit based on accumulated CO$_2$.

For the microtiter based cultivations, maximum specific growth rates were calculated from OD$_{600}$ measurements of the individual wells over time, treating each well of the plate as a separate replicate (n = 48) (data not shown). In addition to this individually based calculation strategy, summarised results on a plate to plate level (n = 4) were plotted as a single growth curve by taking the average OD$_{600}$ of up to 15 well replicates and plotting them for the individual time points that the plates were measured, as illustrated in Figure 1A and 1C. In the comparative analysis the maximum specific growth rates were proven equivalent to the instrumented bioreactors, indicating that microtiter plate based cultivations provides a reliable alternative for growth rate determination, as illustrated and noted in Figure 1 and Table 1.
Table 1. Quantitative physiological results from microtiter and bioreactor based cultivations of all *Aspergillus nidulans* strains.

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<th>6-MSA yield</th>
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<td>mg(<em>{6-\text{MSA}}/g</em>{\text{glc}})</td>
<td>CO(_2) 6-MSA</td>
<td>mg(<em>{6-\text{MSA}}/g</em>{\text{glc}})</td>
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<td>32.9±0.07</td>
<td>0.27±0.02 0.27±0.02</td>
<td>30.1±0.49</td>
</tr>
</tbody>
</table>
Applying a strategy of evaluating the growth rate both as a total of all individual wells, but also summarising all plate results as coherent measurements, resulted in a single growth curve, permitting in-depth assessment of the methodology robustness. This was evaluated by a one-way ANOVA test of the individual growth rates, evaluated as four independent populations represented by the separate microtiter cultivations. The total population variance was determined to be within the range of $\sigma^2 = 15.9 - 6.80 \cdot 10^{-5}$ and with the test displaying no statistical significant difference ($F(3,44) = 0.87, p = 0.47$), the method was considered robust. The summarised growth rate based in the individual well assessment resulted in a maximal growth rate of $\mu_{max} = 0.268 \pm 0.007 \text{ h}^{-1}$ with a total sample variation of $\sigma^2 = 5.02 \cdot 10^{-5}$. The absence of significant differences in the observed growth rates together with low sample variation indicated that the growth phase of each individual well followed an almost identical course, which allowed for treating individual wells as representative for a single batch cultivation.

**Comparative physiological characterisation**

To test the downscaled microtiter cultivation methodology, a comprehensive physiological characterisation of the heterologous 6-MSA producer *Aspergillus nidulans* (IBT 33253) was carried out in MTPs and instrumented bioreactors. Since the 6-MSA gene was chromosomally integrated in-between essential genes the compound was expressed as a function of growth and the 6-MSA accumulation should, in principle, mirror the growth rate of the fungi.

To benchmark the physiology of the strain a traditional physiological characterisation was carried out in bioreactors with growth rate evaluation based on accumulated CO$_2$ and 6-MSA quantified over time, as illustrated in Figure 2b and 2d. A comparison of accumulated CO$_2$ and 6-MSA production revealed a high degree of similarity proving that 6-MSA production may be applied as a secondary gauge for growth rate evaluation.
Figure 2. Comparative physiological characterisation of *Aspergillus nidulans* (IBT 33255) in bioreactors and MTPs. (A) Duplicate microtiter based optical density growth fits of 40 individual wells with their respective standard deviations, (B) Duplicate bioreactor based accumulated CO$_2$ growth fits, (C) Duplicate microtiter based growth fits on accumulated 6-MSA based on triplicate samples with respective standard deviations, (D) Duplicate bioreactor growth fits based on accumulated 6-MSA.

For bioreactor and microtiter based cultivations to be comparative, each individual well of the MTP must be representative of a single batch cultivation. In Figure 2a and 2c, OD$_{600}$ and 6-MSA concentrations from triplicate samples harvested from individual wells are plotted. Extrapolation of the growth rate from both OD$_{600}$ and 6-MSA concentrations over time were in good correlation with the growth rates derived from accumulated CO$_2$ and 6-MSA in bioreactors indicating that the microtiter based cultivations were comparable with instrumented bioreactors. This was further substantiated with a one-way ANOVA test (data not shown) displaying no statistically significant difference with variances comparable to those previously shown.
An additional two 6-MSA producing strains were characterised using the microtiter based methodology outlined above, for further validation (Figure 3). A parallel development in 6-MSA production was observed for both strains mirroring the development in the OD_{600}. Neither for these were any statistical significant differences observed for the growth rates extrapolated validating the previous observations (data not shown). Although no parallel physiological experiments were carried out in bioreactors for these strains, the 6-MSA yield coefficients (Table 1) were comparable with unpublished results from bioreactor cultivations.

Figure 3. Supportive physiological characterisation of *Aspergillus nidulans* (IBT 33253 and 33254) in MTPs. In support to the conclusions that 6-MSA may be applied as a secondary gauge for growth rate estimations, the two 6-MSA producing strains *Aspergillus nidulans* IBT 33253 (A and C) and 33254 (B and D) were cultivated in MTPs and the optical density along with 6-MSA accumulation logged. It is evident from this comparison that the two methods for estimating growth rates are alike.
Analysis of the 6-MSA concentrations for all the above experiments revealed a high degree of culture homogeneity with relative 6-MSA standard deviation ranging from 0.20 - 6.93 %, corresponding well with the 0.50 – 5.50 % observed for the OD$_{600}$ at the time of sampling, indicating that the individual wells of a MTP may tentatively be considered representative for a single batch cultivation. This claim is further substantiated by the similar 6-MSA yield coefficient on glucose in both bioreactor and MTPs, as indicated in Table I.

With no active pH control in the microtiter based cultivations, the pH of each individual sample taken was measured revealing a maximal final pH of approximately 5.4. Though there were no indications that this change in pH affected the overall growth physiology of the culture it may have had an effect on metabolism, which would require further investigation, such as by metabolic flux analysis, if the method were to be expanded for more in-depth characterisation.

**Method robustness and versatility**

To evaluate the versatility of the proposed methodology, 5 different filamentous fungi were selected for characterisation. This selection was made to cover both industrially applied fungi and strains not well characterised in bioreactors.

Though the optimal medium composition for maintaining a stable pH level and securing dispersed growth relied on sodium nitrate, the vast majority of quantitative studies of the chosen fungi are based on ammonium sulfate as nitrogen source. Ammonium sulfate was consequentially applied for all cultivations where literature data stipulated the use. As part of the evaluation of the method versatility, fungi with different pH optima were selected including citric acid producer *Aspergillus niger* (ATCC 1015) [43] at pH 2 and β-lactam producer *Penicillium chrysogenum* (wis54 1255) [44][45] at pH 6.5. Amongst other industrially applied fungi, the α-amylase producing *Aspergillus oryzae* (A1560) [26] was tested along with a previously uncharacterised strain of *Fusarium oxysporum* (IBT 40467). To test the effect of water evaporation over extended cultivation times, the slow growing fungus *Talaromyces atroroseus* (IBT 11181) was included.
(growth rate of 0.069±0.004 h\(^{-1}\); exponential growth phase approximately 65 h). While evaporation was significant (~24 %) the maximal specific growth rate did not seem affected in comparison with bioreactor based growth rates, indicating that even lengthy cultivations can be accurately analysed using the outlined setup. In addition neither the choice of nitrogen source nor the slight variation in pH showed detrimental effects on the growth rates of the tested fungi, all of which displayed exponential growth (Figure 4) with growth rates in good correlation with literature (Table 2).
Figure 4. Method versatility test of a range of different filamentous fungi. A selection of five different strains were chosen for basic physiological characterisation as an evaluation of the versatility of the proposed methodology. The chosen fungi: (A) *Talaromyces artoroseus* (IBT 11181), (B) *Penicillium chrysogenum* (wis54-1255), (C) *Fusarium oxysporum* (IBT 40467), (D) *Aspergillus niger* (ATCC 1015) and (E) *Aspergillus oryzae* (A1560) all displayed exponential growth that compared with literature were shown directly comparable.
Table 2. Versatility test results noted with the corresponding reference values.

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture code</th>
<th>OD(_{600})</th>
<th>Reference value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>ATCC1015</td>
<td>0.23±0.01</td>
<td>0.23±0.02</td>
<td>Poulsen et al (2012)</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>A1560</td>
<td>0.25±0.01</td>
<td>0.25</td>
<td>Carlsen et al (1996)*</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>WisS4-1255</td>
<td>0.14±0.01</td>
<td>0.14</td>
<td>Bajaj et al (2014)* and Thykaer et al (2008)*</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>IBT 40467</td>
<td>0.21±0.00</td>
<td>-</td>
<td>None reported</td>
</tr>
<tr>
<td><em>Talaromyces atroroseus</em></td>
<td>IBT 11181</td>
<td>0.069±0.02</td>
<td>0.069±0.004</td>
<td>Unpublished results</td>
</tr>
</tbody>
</table>

*No standard deviation is provided.*
Conclusion

In this study an automated high throughput methodology was introduced for cultivating filamentous fungi in MTPs taking advantage of the dispersive effect on morphology of the anionic polymer carboxypolymethylene. Cultivation of selected fungi in both bioreactors and MTPs proved that not only growth rates, but also yield coefficients were identical across scale suggesting that this method may be used for fast quantitative physiological studies assessing cell factory potential with full scalability. The overall homology of the individual wells in the MTPs was remarkably high, which is a prerequisite for reliable quantitative studies based on microtiter plates. The method was furthermore proven highly versatile, with 5 different fungal species displaying similar growth rates with those reported in literature, making the methodology applicable for broad-spectrum analysis of filamentous fungi. Though the presented method does not constitute a complete alternative to stirred bioreactors, since no volatile products may be analysed, it does provide an adequate basis for guiding cultivation strategies and selection of best candidate strains based not only on growth rates, but also certain yield coefficients. The method might further be adapted for metabolic flux experiments, provided that the similar metabolic fluxes can be generated in both MTPs and bioreactors. This would not only confirm the comparability of the two systems, but also provide a significantly cheaper alternative to standard bioreactor cultivations and making metabolic flux analysis more widely available.
Competing interests

The authors declare no competing interests.

Author contributions

PBK performed the experimental work, data analysis and interpretation, and wrote the main draft of the manuscript. AR contributed to the experimental design and assisted in running the MTP cultivations and the subsequent data handling and analysis. ZDJ constructed the 6-MSA producing A. nidulans strains. KFN, JT and MW supervised the work and contributed to the experimental design, data analysis and interpretation, writing and critical assessment of the manuscript.

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We would like to thank Lubrizol for kindly providing a sample of Carboxypolymethylene (Carbopol-934) for our study and Gerit Nymschefsky for the unpublished growth rates on Taleromyces atroroseus (IBT 11181).

Finally, the authors acknowledge the Technical University of Denmark Fermentation Platform at the Department of Systems Biology for providing access to both the Hamilton Microlab STAR liquid handling workstation and bioreactor equipment.
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44. Bajaj I, Veiga T, Dissel D van: Functional characterization of a Penicillium chrysogenum mutanase gene induced upon co-cultivation with Bacillus subtilis. BMC ... 2014.

Chapter 5

Comparative physiological characterisation of *Saccharomyces cerevisiae* and *Aspergillus nidulans* cell factory potential for heterologous polyketide production
Abstract

In this study a comparative analysis of heterologous 6-MSA producing *Aspergillus nidulans* and *Saccharomyces cerevisiae* strains, including their respective reference strains, is presented. A thorough quantitative physiological characterisation of all strains was performed in batch bioreactors identifying an *A. nidulans* strain with 2 copies of the 6-MSA synthase gene integrated into the chromosome as the top candidate for polyketide production. This strain displayed 100 fold higher 6-MSA yields on glucose (0.14±0.02 C·mol\(_{6\text{-MSA}}\)·C\(_{\text{glucose}}\)\(^{-1}\)) and 17.2 fold higher productivities (51.6±8.0 \(\text{mg}_{6\text{-MSA}}\)·g(DW)\(^{-1}\)·h\(^{-1}\)) compared with the 6-MSA producing *S. cerevisiae*. For the 6-MSA producing *S. cerevisiae* strain, a 6 fold higher 6-MSA yield was observed during growth on ethanol compared to glucose, likely due to increased flux towards the 6-MSA precursor acetyl-CoA. When cultivated in a chemostat below the critical growth rate for the onset of the Crabtree effect, the 6-MSA producing *S. cerevisiae* exhibited strong oscillations, which were speculated to be connected with a redox imbalance induced by the production of 6-MSA. This phenomenon was attenuated by addition of 0.3 g·L\(^{-1}\) ethanol ensuring a stable steady state applicable for in-depth analysis. No such phenomenon was observed when the strain was cultivated at a dilution rate of 0.36 h\(^{-1}\), where respiro-fermentative metabolism ensured steady ethanol production, which was speculated to stabilise the metabolism of the yeast. Finally, a procedure for cultivating *A. nidulans* under chemostat conditions was established and shown applicable for conditional comparative analysis of two of the 6-MSA producing *A. nidulans* strains.
Introduction

Polyketides comprise a structurally diverse group of secondary metabolites isolated from a broad range of different bacteria, filamentous fungi and plants. This diverse group of compounds displays a wide range of bioactivities from pigments for protection against UV-radiation, signalling and microbial warfare against competitors [1]. Amongst these are several mycotoxins with harmful effects to human health such as fumonisins and the carcinogenic aflatoxins [2], [3]. While certain polyketides and derived compounds display detrimental effects toward humans, others exhibit pharmacological effects, which have been harnessed for a range of different medical purposes amongst which are antibiotics, anticancer and cholesterol lowering agents [4], [5]. This is well illustrated by the fact that 20 % of the top-selling drugs are polyketide or derived products, as surveyed in 2005 [6]. Thus, there is an economically driven motivation for isolation and characterisation of polyketide based compounds and, importantly, developing systems for the production of these polyketides [7].

Producing relevant polyketides in the native hosts often constitutes a problem due to several factors, such as poor growth characteristics, low yields and productivities including lack of genetic background information and tools for genetic manipulation of the native strain [7]–[9]. These drawbacks may be alleviated by transferring the relevant biosynthetic gene clusters to well described cell factory hosts, enabling easier genetic manipulation in bioreactor amenable strains [9], [10].

Several studies describe the production of polyketides in heterologous hosts, principally *Escherichia coli*, and *Aspergilli*. Though there are many studies describing production, genetic modification and assignment of function for different polyketides, only few studies characterising potential cell factories for industrial polyketide production, are available [11]. These studies mainly focus on the polyketide 6-methyalsalicylic acid (6-MSA), which is structurally one of the simplest polyketides (C₈H₈O₃), produced naturally by *Penicillium patulum* as a biosynthetic intermediate for the toxin patulin [12]–[14]. The polyketide synthase (PKS) gene responsible for the production of 6-MSA was the first fungal PKS to be cloned and characterised,
hence this polyketide has served as model for both investigation of biosynthetic pathways [15], [16] as well as a for characterisation of potential industrially relevant heterologous cell factories [7], [8], [11], [17].

While 6-MSA is only encoded by one gene, co-expression of 4’-phosphopantetheine transferase gene is required for the conversion of the inactive apo-enzyme into its active holo-form in both *E. coli* and *Saccharomyces cerevisiae* hosts as demonstrated by Kealey *et al* [7]. In this study, final titers for *E. coli* were demonstrated to be 75 mg·L⁻¹ and up to 1700 mg·L⁻¹ for *S. cerevisiae*, with the latter 2 fold higher than the natural host [7], [18]. Unfortunately, in this study no details concerning the quantitative physiology of the cultivations were given making comparison with other studies difficult.

Applying a different metabolic engineering strategy Wattanachaisaereeekul *et al* [8] obtained a final titer of approximately 200 mg·L⁻¹ 6-MSA for *S. cerevisiae*, cultivated on galactose. In a later study Wattanachaisaereeekul *et al* [19] demonstrated final titers of 554 mg·L⁻¹ 6-MSA, applying metabolic engineering for optimising both the precursor supply and substrate range, ensuring also production of 6-MSA on glucose. In both studies a substantially higher yield coefficient for 6-MSA on ethanol (12 fold), compared with glucose or galactose, was observed. This observation was consistent with the bioconversion of ethanol into acetaldehyde then to acetate, which in turn can be converted into, the 6-MSA precursor, acetyl-CoA with the concomitant oxidation of NADP⁺ to NADPH also required for the production of 6-MSA [8], [20]–[22]. Recently, a 3 fold increase in 6-MSA titers to a final concentration of 343 mg·L⁻¹ was achieved by engineering the acetyl-CoA carboxylase ensuring constitutive activation thereby increasing the precursor malonyl-CoA levels in a *S. cerevisiae* strain [23].

Motivated in part by the close relation between the model organism *A. nidulans* and the industrially important cell factories *A. niger* and *oryzae*, Panagiotou *et al* [17] engineered *A. nidulans* for heterologous expression of the 6-MSAS (6-MSA Synthase) gene. Quantitative physiological characterisations of the engineered strains were conducted applying a range of different substrates including, xylose, ethanol,
glycerol and glucose. While ethanol was theoretically expected to result in the highest yield of 6-MSA it was concluded that glucose was the most efficient carbon source with a final titer on 455 mg·L⁻¹ 6-MSA [17].

In this study, a *S. cerevisiae* strain expressing 9 copies of the heterologous 6-MSAS gene chromosomally integrated was compared with two *A. nidulans* strains expressing one of the heterologous 6-MSAS gene integrated each at a different site in the chromosome and a two copy strain with the gene inserted at both sites. The immediate physiological effects of the introduction of several copies of a non-native to yeast polyketide gene were assessed by batch and continuous cultivation. By cultivating the producing *S. cerevisiae* both above and below the critical growth rate for the onset of the Crabtree effect, the influence of overflow metabolism on 6-MSA production was investigated. To increase the potential for advanced omic’s based physiological characterisation, chemostat cultivation approaches were investigated, principally focusing on the establishment of steady state cultures of the morphologically challenging *A. nidulans* strains. The stability of the *A. nidulans* cultures were benchmarked to the *S. cerevisiae* steady states to assess the applicability of fungal continuous cultures for generating quality tailor-made biomass for supporting detailed physiological characterisation through omic’s studies.
Materials and Methods

Strains

For the study two diploid *S. cerevisiae* strains were applied: one heterologous 6-MSA producing strain with 9 copies of the 6-MSAS gene and the ‘4-phosphopantetheinyl transferase (PPTase) gene chromosomally inserted and its respective reference strain, also diploid. These two strains will from this point henceforth be referred to as *S. cerevisiae* reference and *S. cerevisiae* 9-copy 6-MSA strain, respectively. Four *A. nidulans*: 33252, a non-producing reference strain; 33253, a strain with 2 copies of the 6-MSA gene chromosomally inserted in insertion site (IS) IS1 and IS53, respectively; 33254, a strain with 1 copy of the gene chromosomally inserted at IS1 and finally 33255, a strain with the gene 1 copy of the gene chromosomally inserted at IS53. All applied strains are listed in table 1.

Table 1. *S. cerevisiae* and *A. nidulans* strains applied for the study. The *S. cerevisiae* strains applied are diploid and the genotypes of the two haploid parents are given in the table.

<table>
<thead>
<tr>
<th>Strains</th>
<th>IBT/ID code</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em></td>
<td>33252</td>
<td>veA1, nkuΔ</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>33253</td>
<td>argB2, pyrG89, veA1, nkuΔ, IS1::44965::argB, IS53::44965::pyrG</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>33254</td>
<td>argB2, pyrG89, veA1, nkuΔ, IS53::44965::pyrG, IS1::argB</td>
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<tr>
<td><em>A. nidulans</em></td>
<td>33255</td>
<td>argB2, pyrG89, veA1, nkuΔ, IS1::44965::argB, IS53::pyrG</td>
</tr>
<tr>
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<td>CEN.PK113-6A</td>
<td>MATa MAL2-8C SUC2 leu2-3_112 trp1-289 ura3-52</td>
</tr>
<tr>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CEN.PK113-7B</td>
<td>MATa MAL2-8C SUC2 leu2-3_112 trp1-289 ura3-52</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>GAX10-1C-LM1</td>
<td>GAX10-1C+LM1, trans 1, pop, unamplified MATa MAL2-8C SUC2 his3Δ trp1-289 ura3-52, (PTEF1-6MSA+PPGK1-npga) in site X-2, X-3, X-4, XI-1, XI-2, XI-4a, XII-1, XII-2, XII-3, XII-4</td>
</tr>
<tr>
<td></td>
<td>x</td>
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<td></td>
<td>CEN.PK113-7B</td>
<td>MATa MAL2-8C SUC2 leu2-3_112 trp1-289 ura3-52</td>
</tr>
</tbody>
</table>
I

Occulum preparation

Preparation of \textit{S. cerevisiae} inoculum

The \textit{S. cerevisiae} strains were maintained on Yeast extract-Peptone-Dextrose (YPD) medium, consisting of: 20 g·L\(^{-1}\) dextrose, 10 g·L\(^{-1}\) yeast extract, 20 g·L\(^{-1}\) peptone and 15 g·L\(^{-1}\) agar. Single colonies from plates were sub cultured before each test in shake flasks with 100 mL of sterile minimal medium and grown for 14-16 h at 30 °C at 150 rpm. Bioreactors for both batch and chemostat were inoculated to an OD\(_{600}\) of 0.01.

Preparation of \textit{A. nidulans} spore inoculum

Spores were propagated on CYA media plates for 10 days at 30 °C and subsequently harvested with 10 mL 0.9 % NaCl followed by filtration through mira cloth. The spores were finally washed twice with 0.9 % NaCl prior application. The batch fermentation was initiated by inoculation of spore suspension to a final concentration of 5·10\(^9\) spores·L\(^{-1}\).

Media

\textit{S. cerevisiae} cultivations

The \textit{S. cerevisiae} strains were maintained throughout the experiment on Yeast extract-Peptone-Dextrose (YPD) medium consisting of: 20 g·L\(^{-1}\) dextrose, 10 g·L\(^{-1}\) yeast extract, 20 g·L\(^{-1}\) peptone and 15 g·L\(^{-1}\) agar.

The minimal medium composition [24] used for cultivation in the bioreactors was as follows: 20 g·L\(^{-1}\) (batch) and 10 g·L\(^{-1}\) D-glucose (chemostats), 5 g·L\(^{-1}\) ammonium sulphate, 3 g·L\(^{-1}\) monopotassium phosphate, 0.5 g·L\(^{-1}\) magnesium sulphate, 1 mL trace metal solution, 1 mL vitamin solution, 50 µL antifoam 204 (Sigma-Aldrich, St. Louis, MO/USA). The trace metal solution consisted of: 15 g EDTA (sodium salt), 0.45 g·L\(^{-1}\) ZnSO\(_4\). 7H\(_2\)O, 1 g·L\(^{-1}\) MnCl\(_2\). 2H\(_2\)O, 0.3 g·L\(^{-1}\) CoCl\(_2\). 6H\(_2\)O, 0.3 g·L\(^{-1}\) CuSO\(_4\). 5H\(_2\)O, 0.4 g·L\(^{-1}\) Na\(_2\)MoO\(_4\). 2H\(_2\)O, 0.45 g·L\(^{-1}\) CaCl\(_2\). 2H\(_2\)O, 0.3 g·L\(^{-1}\) FeSO\(_4\). 7H\(_2\)O, 0.1 g·L\(^{-1}\) H\(_3\)BO\(_4\) 0.1 g·L\(^{-1}\) KI. The vitamin solution contained: 0.05 g biotin, 0.2 g·L\(^{-1}\) p-amino benzoic acid, 1 g·L\(^{-1}\) nicotinic acid, 1 g·L\(^{-1}\) Ca-pantothenate, 1 g·L\(^{-1}\) pyridoxine-HCl, 1 g·L\(^{-1}\) thiamine-HCl and 25 g·L\(^{-1}\) myo-inositol. pH was adjusted to 5 with 2 M NaOH. For the 9 copy 6-MSA
producing strain, the medium composition was altered after the semi-steady state cultivation to include both 10 g·L⁻¹ D-glucose and 0.3 g·L⁻¹ 99.9 % ethanol as a means to obtain a steady state. The chemicals for YPD and minimal medium were obtained from Merck Millipore (Darmstadt, Germany) or Sigma-Aldrich Co. (St. Louis, MO/USA).

**A. nidulans cultivations**

Czapek yeast extract (CYA) medium was used for spore propagation, containing 30 g·L⁻¹ Sucrose, 5 g·L⁻¹ Yeast extract, 3 g·L⁻¹ NaNO₃, 1 g·L⁻¹ K₂HPO₄, 0.5 g·L⁻¹ MgSO₄ · 7 H₂O, 0.5 g·L⁻¹ KCl, 0.01 g·L⁻¹ FeSO₄ · 7H₂O, 15 g·L⁻¹ Agar, 1 mL·L⁻¹ trace element solution containing 0.4 g·L⁻¹ CuSO₄ · 5 H₂O, 0.04 g·L⁻¹ Na₂B₂O₇ · 10 H₂O, 0.8 g·L⁻¹ FeSO₄ · 7 H₂O, 0.8 g·L⁻¹ MnSO₄ · H₂O, 0.8 g·L⁻¹ Na₂MoO₄ · 2 H₂O, 8.0 g·L⁻¹ ZnSO₄ · 7 H₂O. pH was adjusted to 6.2 with NaOH prior to autoclavation. Unless otherwise stated all chemicals applied were procured from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO/USA).

For batch and chemostat cultivation the following media composition was applied: 13.5 g·L⁻¹ (batch) and 5 g·L⁻¹ (chemostat) D-glucose, 7.5 g·L⁻¹ (NH₄)₂SO₄, 1.5 g·L⁻¹ KH₂PO₄, 1.0 g·L⁻¹ MgSO₄ · 7 H₂O, 1.0 g·L⁻¹ NaCl, 0.1 g·L⁻¹ CaCl₂, 0.1 mL Antifoam 204 (sigma), 1 mL·L⁻¹ trace element solution. Trace element solution: 0.4 g·L⁻¹ CuSO₄ · 5 H₂O, 0.04 g·L⁻¹ Na₂B₂O₇ · 10 H₂O, 0.8 g·L⁻¹ FeSO₄ · 7 H₂O, 0.8 g·L⁻¹ MnSO₄ · H₂O, 0.8 g·L⁻¹ Na₂MoO₄ · 2 H₂O, 8.0 g·L⁻¹ ZnSO₄ · 7 H₂O.

**Bioreactor based batch cultivations**

All bioreactor batch cultivations were carried out in Sartorious 1 L bioreactors (Satorious, Stedim Biotech, Goettingen, Germany) with an equivalent volume, equipped with baffles (yeast only) and 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer’s standard procedures. Bioreactors were sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5 % (v/v) CO₂, 0.04 % (v/v) ethanol and methanol, 1 % (v/v) argon, 5 % (v/v) and 15 % (v/v) oxygen all with nitrogen as carrier gas.
Temperature was maintained at 30 °C throughout the cultivation and pH controlled by automatic addition of 2 M NaOH and H$_2$SO$_4$.

**S. cerevisiae**

Culture conditions were pH: 5, stir rate: 800 rpm and air flow: 1 volume of air per volume of liquid per minute (vvm). The strains were cultivated until carbon depletion and the culture had entered stationary phase as monitored by off-gas CO$_2$ concentration. HPLC, OD$_{600}$ and dry weight (DW) samples were taken across the exponential growth phases for metabolite profiling.

**A. nidulans**

Temperature was maintained at 30 °C throughout the cultivation and pH controlled by automatic addition of 2 M NaOH and H$_2$SO$_4$. Start culture conditions were pH: 5, stir rate: 100 rpm and air flow: 0.1 volume of air per volume of liquid per minute (vvm). Following inoculation these conditions were changed in a linear fashion over a period of 720 min., stirring rate: 800 rpm and air flow: 1 vvm. The strains were cultivated until glucose was depleted and the culture had entered stationary phase as monitored by off-gas CO$_2$ concentration. Samples were taken across the exponential growth phase for metabolite profiling.

**Bioreactor based chemostat cultivations**

**S. cerevisiae**

All chemostat cultivations were carried out in Sartorious 0.5 L and 1 L bioreactors (Satorious, Stedim Biotech, Goettingen, Germany), equipped with baffles and 2 Rushton six-blade disc turbines. The ½ L bioreactors were solely used for *S. cerevisiae* continuous cultivations at $D = 0.36$ h$^{-1}$ with a working volume of 0.4 L. 1 L bioreactors were used for *S. cerevisiae* continuous cultivations at $D = 0.1$ h$^{-1}$ with a working volume of 0.6 L. Continuous cultivations were initiated and cultivated as batch until late exponential phase where feed medium supplementation was initiated. The feed medium was identical to the batch medium with the exception of the glucose concentration which was 10 g·L$^{-1}$ and 10 g·L$^{-1}$ supplemented with 0.3 g·L$^{-1}$ ethanol dependent on experiment and strain. Feed supply was controlled by a gravimetrically controlled
peristaltic pump and bioreactor volume was kept constant applying a level probe ensuring continuous removal of excess volume. Continuous cultivations at a dilution rate of 0.1 h\(^{-1}\) were carried out in 1 L bioreactors with a working volume of 0.6 L (60 mL·h\(^{-1}\)). For the continuous cultivations with a dilution rate of 0.36 h\(^{-1}\), 0.5 L bioreactors were applied with a working volume of 0.4 L (144 mL·h\(^{-1}\)). Automatic addition of feed was initiated at late exponential growth and feed initiation the culture was left for at least 5 residence times before samples were taken, ensuring steady state conditions.

\textit{A. nidulans}

All bioreactor batch cultivations were carried out in Sartorious 1 L bioreactors (Satorious, Stedim Biotech, Goettingen, Germany) with an equivalent volume, equipped with 2 Rushton six-blade disc turbines situated respectively, 0.5 and 3 cm from the end of the shaft. Chemostat cultivations were initiated and cultivated as batch until late exponential phase feed medium supplementation was initiated. The feed medium was identical to the batch medium with the exception of the glucose concentration, which was 5 g·L\(^{-1}\). Feed supply and bioreactor outflow were controlled by a gravimetrically controlled peristaltic pumps ensuring constant bioreactor volume with a dilution rate of 0.05 h\(^{-1}\) (50 mL·h\(^{-1}\)). Automatic addition of feed was initiated at late exponential growth and the culture was left for at least 5 residence times before samples were taken ensuring steady state conditions.

\textbf{Cell mass determination}

For physiological characterisation in bioreactors dry cell mass concentrations (DW) were determined gravimetrically. Shortly, a known weight of cell culture was filtered through pre-dried 0.45 µm polyether sulfone filters (Frisenette, Knebel, Denmark), and the biomass washed with saline (0.9 % NaCl). The filter was the folded to lock the biomass within the folded filter and finally dried in a microwave oven at 150 W for 20 min. Mass gain or biomass dry weight (DW) was ascertained after a cooling period of approximately 30 min. in a desiccator.
Quantification of extracellular metabolites by HPLC

For quantification of extracellular metabolites, culture samples were taken and filtered through a 0.45 µm cellulose acetate filter (Frisenette, Knebel, Denmark). The samples were frozen and kept at -20 °C until analysis. Glucose, glycerol, pyruvate, succinate, acetate and ethanol were detected and quantified using an Agilent 1100 HPLC system equipped with a refractive index and Diode array detector (Agilent Technologies, Waldbronn, Germany) and with an Aminex HPX-87H cation-exchange column (BioRad, Hercules, Ca, USA). Compounds were separated by isocratic elution at 60 °C, with 5 mM H₂SO₄ at a flow rate of 0.6 mL·min⁻¹. Quantification was performed using a six-level external calibration curve with pyruvate detected at a wavelength of 210 nm and glucose, succinate, glycerol, acetate and ethanol by refractive index measurements.

6-MSA was quantified using a Dionex Ultimate 3000 UHPLC coupled with an ultimate 3000 RS diode array detector (Dionex, Germering, Germany) equipped with a Poroshell 120 phenyl hexyl 2.1 x 100 mm, 2.7 µm (Agilent, Santa Clara, CA/USA) column by gradient elution at 60 °C and a flow rate of 0.8 mL·min⁻¹ starting at 15 % acetonitrile and MiliQ water and ramped up to 100 % in 7 min. before returned to the starting point in an additional 5 min.. Quantification was performed using an eight-level external calibration curve with concentrations from 1 - 300 mg·L⁻¹ at a wavelength of 210 nm.

Quantitative physiological analysis

Batch bioreactor experiments were carried out in triplicates and chemostat based experiments in triplicates for S. cerevisiae and duplicates for A. nidulans. Maximum specific growth rates were estimated from exponential fits of biomass concentrations either determined by OD or gravimetrically as a function of time. Yield coefficients for batch cultivations were estimated through linear regression of product and biomass accumulation as a function of substrate consumption with a coefficient of determination above 0.95. For A. nidulans cultivations the yield coefficient for 6-MSA on glucose was estimated as an overall value for the
cultivation. For chemostat cultivations yield coefficients were estimated as a total value at the sample time point applying equation (1) assuming Monod kinetics without maintaince:

$$Y_{si} = \frac{i}{(c_s^f - c_s)}$$

(1)

Where $Y_{si}$ is the yield coefficient of the $i$'th product on substrate, $c_s^f$ is the feed concentration of the substrate and $c_s$ is the immediate concentration of substrate in the bioreactor. Standard biomass composition was assumed to be CH$_{1.8}$O$_{0.3}$N$_{0.2}$ (24.6 g DW C-\text{mol}$^{-1}$) for both 	extit{S. cerevisiae} strains and CH$_{1.72}$O$_{0.55}$N$_{0.17}$ (24.9 g DW C-\text{mol}$^{-1}$) for all four 	extit{A. nidulans} strains [25].

For batch cultivations the specific 6-MSA production rate were estimated from the overall yield of 6-MSA on biomass ($Y_{xp}$) and the maximal specific growth rate ($\mu_{max}$) using equation (2):

$$r_p = Y_{xp} \cdot \mu_{max}$$

(2)

For chemostat cultivations the specific 6-MSA production rate (g 6-MSA g (DW).h$^{-1}$) were estimated from the volumetric production of 6-MSA (g·L·h$^{-1}$) and the average biomass concentration (g·L$^{-1}$) using equation (3):

$$r_p = \frac{q_p}{\Delta x} (g \cdot g \text{DW}^{-1} \cdot h^{-1})$$

(3)

Where $\Delta x$ is the average biomass concentration (g·L$^{-1}$) in the analysed interval and $q_p$ is the volumetric production rate of the product (6-MSA) in the given time interval calculated using equation (4):

$$q_p = \Delta c_p \cdot D (g \cdot L^{-1} \cdot h^{-1})$$

(4)

Where $\Delta c_p$ is the average product concentration (g·L$^{-1}$) in the analysed interval and D is the dilution rate (h$^{-1}$).
**Batch cultivations**

Yield of CO$_2$ on glucose was estimated for batch cultivations by calculating firstly the off-gas CO$_2$ concentrations (g·L$^{-1}$), using equation (5):

$$\text{Accumulated CO}_2 \text{ at time } t_n = \sum_{i=1}^{n} \left( \frac{(Q_i + Q_{i-1}) \cdot (t_i - t_{i-1})}{2 \cdot V_R} \right) \cdot M_{\text{CO}_2} \quad (g \cdot L^{-1})$$  \hfill (5)

Where $t_n$ is the sample time (hours), $V_R$ is the bioreactor volume (L), $M_{\text{CO}_2}$ is the molecular mass of CO$_2$ (44.0095 g·mol$^{-1}$) and $Q_i$ is defined as mol CO$_2$ produced per hour at the given time point. $Q_i$ is calculated as an estimate using equation (6):

$$Q_i = \frac{F_{\text{air},i} \cdot (\%\text{CO}_2_{i,t} - \%\text{CO}_2_{t,0})}{V_m \cdot 100} \quad (mol \cdot h^{-1})$$  \hfill (6)

Where, $F_{\text{air},i}$ is the flow of air (L·h$^{-1}$) at the given time point, $\%\text{CO}_2_{i,t}$ is the CO$_2$ percentage in the of-gas air to the given time point and $\text{CO}_2_{t,0}$ is the background concentration of CO$_2$ in the supplied gas. The molar volume $V_m$ is derived from the ideal gas law, equation (7).

$$V_m = \frac{V}{n} = \frac{R \cdot T}{P} \quad (L \cdot mol^{-1})$$  \hfill (7)

Where, $R$ is Reynolds number (0.08314 L.bar.K$^{-1}$), $T$ is the temperature in the off-gas analysator (298.15 K) and $P$ is the ambient pressure (0.9885 bar).

The ethanol concentration (yeast only) in the gas phase were calculated by similar fashion, substituting the molecular mass of CO$_2$ ($M_{\text{CO}_2}$) with the molecular mass of ethanol ($M_{\text{Ethanol}} = 23.03422$ g·mol$^{-1}$) and the CO$_2$ percentage ($\%\text{CO}_2_{i,t}$) with the ethanol percentage ($\%\text{EtOH}_{i,t}$) and at the given time point.
Chemostat cultivations

Overall yield of CO$_2$ on glucose was estimated for chemostat cultivations by calculating firstly the off-gas CO$_2$ concentrations (g·L$^{-1}$), using equation (8):

$$
CO_2 \text{ at time } t_n = \sum_{i=1}^{n} \left( \frac{Q_i \cdot D}{V_R} \right) \cdot M_{CO_2} \ (g \cdot L^{-1}) \quad (8)
$$

where D is the dilution rate (h$^{-1}$), V$_R$ is the bioreactor volume (L), M$_{CO_2}$ is the molecular mass of CO$_2$ (44.0095 g·mol$^{-1}$) and Q$_i$ is defined as mol CO$_2$ produced per hour at the given time point. Q$_i$ is calculated using equation (6).

Where, F$_{air,i}$ is the flow of air (L·h$^{-1}$) at the given time point, %CO$_{2,i}$ is the CO$_2$ percentage in the of-gas air to the given time point and CO$_{2,0}$ is the background concentration of CO$_2$ in the supplied gas. The molar volume V$_m$ is derived from the ideal gas law, equation (7).

The ethanol concentration (Yeast only, D = 0.36 h$^{-1}$) in the gas phase were calculated by similar fashion, substituting the molecular mass of CO$_2$ (M$_{CO_2}$) with the molecular mass of ethanol (M$_{Ethanol} = 23.03422$ g·mol$^{-1}$) and the CO$_2$ percentage (%CO$_{2,i}$) with the ethanol percentage (%EtOH$_{i}$) at the given time point.
Results and Discussion

Characterisation of heterologous 6-MSA producing S. cerevisiae and A. nidulans in batch

A quantitative physiological characterisation of triplicate cultivations of the four A. nidulans and two S. cerevisiae strains were performed in 1 L batch bioreactors. The cultivations were executed as to assess the overall aptitude of S. cerevisiae and A. nidulans for the production of the model polyketide 6-MSA by estimating kinetic parameters and overall yield coefficients on glucose.

Due to distinct morphological differences between the yeast and filamentous microorganisms a marked reduction in dissolved oxygen was expected for the A. nidulans strains at higher biomass concentrations. To control this, different initial glucose concentrations (figure 1) were chosen as to ensure uninhibited growth, thus maximising the potential for 6-MSA production.

For the S. cerevisiae cultivations, 6-MSA was characterised in relation to diauxic growth to ascertain the effect of growth on glucose and ethanol, respectively (figure 1). Despite the antifungal properties of 6-MSA, no effects on the growth rate (0.38±0.01 h⁻¹) were observed compared with the reference strain (0.37±0.01 h⁻¹), likely due to the relatively low final concentration of 6-MSA; approximately 64 mg·L⁻¹ (data not shown). Consistent with the observations made by Wattanachaiaereeukul et al [8], [19] a higher yield was observed for the diauxic growth on ethanol where yields were ~ 6 fold higher than for growth on glucose (table 2). This significant increase in 6-MSA yield indicated a bottleneck in the precursor supply for this non optimised strain, in good agreement with the studies by both Wattanachaiaereeukul et al [19] and Choi et al [23], in which significant increases in the yields were achieved by increasing precursor levels by metabolic engineering. The 50 % drop in specific 6-MSA productivity (r_p) (table 2) likely reflected the significantly lower growth rate on ethanol (0.12±0.01 h⁻¹) correlating well with the observations made by Wattanachaiaereeukul et al [8], [19].
Figure 1. Representative batch profiles of the two *S. cerevisiae* and the four *A. nidulans* strains carried out as triplicate cultivations for each strain.

Three 6-MSA producing *A. nidulans* strains were characterised in this study with respectively one copy of the 6-MSAS gene in insertion site (IS) 1 and 53, and a strain with the 6-MSAS gene inserted at both sites.
(data not shown). Interspecies comparison revealed no influence of the 6-MSA expression on growth rates with only a minor reduction in overall growth rated observed for the 1 copy strain (IS1) (33255) as illustrated and noted in figure 1 and table 2. While this reduction in growth rates of (33255) cannot be explained without thorough analysis by application of advanced omics’ techniques, the comparable growth rates for the 2 copy strain (33253) with the reference strain (33252) suggests no effects of the 6-MSAS expression.

For all three 6-MSA producing A. nidulans strains, high yields were observed ranging between 0.06-0.14 C-mol$_{6\text{-MSA}}$·C-mol$_{\text{glucose}}$⁻¹. Most notably, the 1 copy strain (33254), where the 6-MSAS gene was integrated into site 53, displayed significantly higher yield coefficients (0.10±0.01 C-mol$_{6\text{-MSA}}$·C-mol$_{\text{glucose}}$⁻¹) compared with strain (33255), where the gene was integrated into site 1 (0.06±0.01 C-mol$_{6\text{-MSA}}$·C-mol$_{\text{glucose}}$⁻¹), indicating an approximately 40 % higher expression level for site 53 (table 2).

Estimation of the 6-MSA yield coefficient from the 2 copy strain (33253) resulted in 0.14±0.02 C-mol$_{6\text{-MSA}}$·C-mol$_{\text{glucose}}$⁻¹, indicating an accumulative effect of the double integration. An accumulative effect was expected, due to the constitutive expression of the genes as a function of growth, which was not affected by the increase in 6-MSA expression, indicating a potential metabolic capacity for even higher yields, provided further metabolic engineering were to be pursued.

With the relatively high growth rates (0.21-0.25 h⁻¹), also considerable specific 6-MSA production rates ($r_p$) were achieved, with the 2-copy strain displaying a maximum with $r_p = 51.6\pm8.0$ mg.g(DW)⁻¹.h⁻¹ (table 2). In comparison with the studies by Panagiotou et al [26] and Wattanachaisaereekul et al [19] this rate is 4 and 10 fold higher, respectively.

Unfortunately, since no information is given in Kealey et al [7] concerning substrate concentrations, comparison with this study was not possible. However in comparison with Wattanachaisaereekul et al [19] and Panagiotou et al [26] reporting, respectively, 554 and 435 mg·L⁻¹ for S. cerevisiae and A. nidulans on 20 g·L⁻¹ glucose, we achieved an average of 1771 mg·L⁻¹ normalised to the reported substrate concentration.
applied in the corresponding studies (data not shown). The 3 fold increase in 6-MSA concentrations with up to 10 fold higher productivity emphasises the overall cell factory potential of A. nidulans for polyketide production.
Table 2. Comparative physiological characterisation of the two *S. cerevisiae* and four *A. nidulans* strains with maximal specific growth rates, yield coefficients and specific 6-MSA production rates annotated for all strains cultivated as triplicates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>IBT/ ID code</th>
<th>( \mu_{\text{max}} ) (h(^{-1}))</th>
<th>Yield coefficients on glucose (Cmol·Cmol(^{-1}))</th>
<th>( r_{6-\text{MSA}} ) (glucose)</th>
<th>( r_{6-\text{MSA}} ) (ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td>Reference</td>
<td>( \begin{array}{c} 0.37 \ 0.38 \end{array} ) (0.00) (0.01)</td>
<td>( \begin{array}{c} 0.19 \ 0.19 \end{array} \ 0.04 \ 0.04 ) (0.01) (0.01)</td>
<td>( \begin{array}{c} 0.004 \ 0.049 \ 0.001 \end{array} \ 0.53 \ 0.01 ) (0.00) (0.01)</td>
<td>( \begin{array}{c} 0.25 \ 0.22 \ 0.004 \end{array} \ 0.0015 ) (0.000) (0.001)</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td>9-copy, 6-MSA</td>
<td>( \begin{array}{c} 0.24 \ 0.25 \end{array} \ 0.21 ) (0.00) (0.01) (0.00)</td>
<td>( \begin{array}{c} 0.67 \ 0.62 \ 0.62 \end{array} \ 0.03 \ 0.02 ) (0.01) (0.03)</td>
<td>( \begin{array}{c} 0.03 \ 0.04 \end{array} \ - ) (0.02) (0.01)</td>
<td>( \begin{array}{c} 0.30 \ 0.35 \ 0.30 \end{array} \ 0.14 ) (0.01) (0.01)</td>
</tr>
<tr>
<td><strong>A. nidulans</strong></td>
<td>(reference)</td>
<td>( \begin{array}{c} 33252 \ 33253 \ 33254 \ 33255 \end{array} \ 0.24 \ 0.24 \ 0.25 \ 0.21 ) (0.00) (0.00) (0.01) (0.00)</td>
<td>( \begin{array}{c} 0.67 \ 0.50 \ 0.62 \ 0.62 \end{array} \ 0.03 \ 0.02 \ 0.02 ) (0.01) (0.03)</td>
<td>( \begin{array}{c} 0.03 \ 0.02 \ 0.004 \end{array} \ - ) (0.001) (0.001)</td>
<td>( \begin{array}{c} 0.30 \ 0.29 \ 0.30 \end{array} \ 0.10 ) (0.01) (0.01)</td>
</tr>
<tr>
<td><strong>A. nidulans</strong></td>
<td>(2-copy, IS1 &amp; SS3)</td>
<td>( \begin{array}{c} 33252 \ 33253 \ 33254 \ 33255 \end{array} \ 0.24 \ 0.24 \ 0.25 \ 0.21 ) (0.00) (0.00) (0.01) (0.00)</td>
<td>( \begin{array}{c} 0.67 \ 0.50 \ 0.62 \ 0.62 \end{array} \ 0.03 \ 0.02 \ 0.02 ) (0.01) (0.03)</td>
<td>( \begin{array}{c} 0.03 \ 0.02 \ 0.004 \end{array} \ - ) (0.001) (0.001)</td>
<td>( \begin{array}{c} 0.30 \ 0.29 \ 0.30 \end{array} \ 0.10 ) (0.01) (0.01)</td>
</tr>
<tr>
<td><strong>A. nidulans</strong></td>
<td>(1-copy, IS53)</td>
<td>( \begin{array}{c} 33252 \ 33253 \ 33254 \ 33255 \end{array} \ 0.24 \ 0.24 \ 0.25 \ 0.21 ) (0.00) (0.00) (0.01) (0.00)</td>
<td>( \begin{array}{c} 0.67 \ 0.50 \ 0.62 \ 0.62 \end{array} \ 0.03 \ 0.02 \ 0.02 ) (0.01) (0.03)</td>
<td>( \begin{array}{c} 0.03 \ 0.02 \ 0.004 \end{array} \ - ) (0.001) (0.001)</td>
<td>( \begin{array}{c} 0.30 \ 0.29 \ 0.30 \end{array} \ 0.10 ) (0.01) (0.01)</td>
</tr>
<tr>
<td><strong>A. nidulans</strong></td>
<td>(1-copy, IS1)</td>
<td>( \begin{array}{c} 33252 \ 33253 \ 33254 \ 33255 \end{array} \ 0.24 \ 0.24 \ 0.25 \ 0.21 ) (0.00) (0.00) (0.01) (0.00)</td>
<td>( \begin{array}{c} 0.67 \ 0.50 \ 0.62 \ 0.62 \end{array} \ 0.03 \ 0.02 \ 0.02 ) (0.01) (0.03)</td>
<td>( \begin{array}{c} 0.03 \ 0.02 \ 0.004 \end{array} \ - ) (0.001) (0.001)</td>
<td>( \begin{array}{c} 0.30 \ 0.29 \ 0.30 \end{array} \ 0.10 ) (0.01) (0.01)</td>
</tr>
</tbody>
</table>
Characterisation of heterologous 6-MSA producing *S. cerevisiae* in chemostats

Since the production of ethanol during batch cultivations drains a considerable part of the acetaldehyde pool, as illustrated in chapter 3 “A scalable high throughput fermentation approach for exploring cell factory potential”, a substantial part of the flux towards acetate and consequentially acetyl-CoA is diverted. Conversely, the production of ethanol might increase the flux towards acetyl-CoA by increasing the drain on the central carbon metabolism pyruvate node. To investigate the effect of ethanol production, the 9 copy 6-MSA producing *S. cerevisiae* strain was cultivated as continuous cultures (triplicates) below and above the onset of the Crabtree effect at a dilution rate of 0.1 and 0.36 h\(^{-1}\), respectively (figure 2).

As evident from figure 2, steady state was achieved at both dilution rates for the reference strain and was kept in steady state for between 4 and 7 residence times. Notably, off-gas ethanol concentrations were applicable for assessment of steady state conditions; however steady state concentration was achieved at a significantly later time point compared with off-gas CO\(_2\) values. Whereas steady state was achieved at both dilution rates for the reference strain, a steady state was only achieved for the 9-copy strain at a dilution rate of 0.36 h\(^{-1}\). In comparison, steady state values for 6-MSA and biomass were achieved at the dilution rate of 0.1 h\(^{-1}\), however a strong and self-sustained oscillatory behaviour was observed for the CO\(_2\) values, which consequentially only reached a pseudo-steady state.

Metabolic oscillation in yeast is a relatively well known phenomenon that while not completely understood has been extensively studied. The literature offers several explanations for the phenomenon, which occurs primarily for *S. cerevisiae* continuous cultures at growth rates below 0.25 h\(^{-1}\) [27], [28]. The principal explanation given, describes a disproportional relationship between the generation time of parent and daughter cells resulting in synchronisation of the cell population [27], [29]. The disproportionality is explained by the observation that the cell cycle for smaller daughter cells is twice the length of the parent cell population resulting in a synchronisation of cell division, which may be observed as oscillations. The relationship results in periodic oscillations associated directly to the dilution rate, which has been shown to
correlate with an oscillation frequency of ~4-5 h at a dilution rate of 0.1 h\(^{-1}\) in good correlation with the average frequency of 4.3 h observed in this study [21], [30]. This phenomenon, which in some studies is termed energy-metabolism oscillations or metabolic cycles, has further been linked with a redox imbalance causing periodic change between reductive and oxidative states in the NAD(P)\(^{+}\)/NAD(P)H ratio [21], [31], [32].

The oscillations manifests in a periodic accumulation of ethanol, even at sub-Crabtree dilution rates, which is strictly coupled to the mobilisation of internal carbohydrate reserves, e.g. trehalose and glycogen, allowing for brief periods of unrestricted growth resulting in the accumulation of ethanol and acetate in the medium [21], [29]. As a result of this cellular event, causing production of ethanol, the critical cell size for budding decreases, while the critical size for cell division increases [30], [33]. This was consistent with the detection of ethanol (below the limit of quantification) in samples harvested exclusively at time points immediately after a CO\(_2\) spike (figure 2) connected with the mobilisation of carbohydrate reserves (data not shown).

Thus, the observed oscillations may be a consequence of a NAD(P)\(^{+}\)/NAD(P)H ratio imbalance induced by the NADPH dependent production of 6-MSA occurring as a function of growth. While further investigations are needed, this may explain why no oscillations were observed for the reference strain.

Oscillations were not observed for any of the cultivations performed at dilution rate 0.36 h\(^{-1}\), where respiro-fermentative metabolism ensured a constant presence of ethanol, which has been shown to disturb the mechanism maintaining the partially synchronous culture [30]. This is further supported by the observation of larger mother cells and smaller daughter cells for low dilution rates, whereas at high dilution rates a more proportional relationship in mother/daughter cell size is observed [30].

Despite the CO\(_2\) oscillations of the 9-copy 6-MSA strain (D = 0.1 h\(^{-1}\)), both biomass and 6-MSA concentrations proved steady for more than 7 residence times. A comparison of kinetic parameters and yield coefficients were therefore possible, excepting only CO\(_2\) yields.
Figure 2. Representative continuous culture profiles for the reference and 9-copy *S. cerevisiae* strain cultivated as triplicates at a dilution rate of $D = 0.1 \text{ h}^{-1}$ and $D = 0.36 \text{ h}^{-1}$.

Estimation of yield coefficients (table 2) revealed a 39 % lower biomass yield on glucose for the 9-copy strain compared with the reference strain, with a concomitant 2 fold higher ethanol yield, indicating different critical growth rates for the onset of the Crabtree effect. 6-MSA yield on glucose was shown to be 3 fold lower for the chemostats performed at a dilution rate of 0.36 h$^{-1}$ (0.003 C-mol$_{\text{6-MSA}}$/C-mol$_{\text{glucose}}$) compared with the chemostats at 0.1 h$^{-1}$ (0.009 C-mol$_{\text{6-MSA}}$/C-mol$_{\text{glucose}}$) in agreement with the substantial ethanol drain on the central carbon metabolism (0.23±0.02 C-mol$_{\text{Ethanol}}$/C-mol$_{\text{glucose}}$).

The estimated specific 6-MSA production rate was significantly higher (81 %) for cultivations at $D = 0.36 \text{ h}^{-1}$, indicating that the increased flux from the pyruvate node to acetaldehyde might promote the overall flux towards acetyl-CoA, thereby increasing the 6-MSA precursor pool. This might be disclosed by metabolomics.
to reveal whether the size of the acetyl-CoA pools changes for cultivations performed above and below the onset of the Crabtree effect.

**Modulating the oscillatory behaviour of the 6-MSA producing *S. cerevisiae* strain**

Since no true steady state was obtained for the 9-copy strain at $D = 0.1 \text{ h}^{-1}$ due to oscillating metabolism, it was difficult to evaluate cellular performance by means of continuous cultivations and impossible to generate high quality biomass for omics analysis. To control the phenomenon several studies have investigated possibilities for attenuating the oscillatory behaviour by e.g. increasing or lowering dissolved oxygen concentrations and by addition of acetaldehyde and ethanol to the medium [28], [34].

As a means to generate a steady state culture, oscillating cultures (triplicates) of the 9-copy *S. cerevisiae* strains were supplemented with 0.3 g·L$^{-1}$ ethanol in the feed in accordance with the method of [34]. The addition of ethanol to the feed medium caused an immediate attenuation of the oscillation resulting in the establishment of steady state cultivations, as illustrated in figure 3.
Figure 3. Representative continuous culture profile for the 6-MSA producing (9-copy) *S. cerevisiae* strain. The strain exhibits strong oscillatory behaviour (CO$_2$) during growth on glucose as the sole carbon source. After addition of 0.3 g·L$^{-1}$ ethanol (intersecting line) to the feed medium the oscillations was attenuated resulting in stable parameters.

Supplementation with ethanol did not affect the biomass yield on the combined substrate, and since the oscillation was attenuated, a true yield coefficient for CO$_2$ on the substrate was estimated to 0.32 C-mol$_{CO2}$·C-mol$_{substrate}$$^{-1}$. Though easily within standard deviation, a minor enhancement in 6-MSA yield on substrate and specific production rate were observed for cultivations of the 9-copy *S. cerevisiae* strain on the combined substrate. This observation would be consistent with the bioconversion of ethanol to acetyl-CoA with the concomitant generation of NADPH both substrates for 6-MSA production.

While the outlined approach was limited by the need for ethanol supplementation for this otherwise oscillating strain, it offered the possibility for generating the stable conditions required for generating the tailor-made biomass needed for advanced omics analysis as previously shown in [34].
Table 3. Physiological coefficients from all chemostat/continuous cultivations. Triplicate cultivations were carried out for all *S. cerevisiae*. For *S. cerevisiae* cultivated on glucose and ethanol together, the yield coefficients were calculated as a total output of both substrates. For the *A. nidulans* strains duplicate cultivations were carried out for 33252 and 33253 where only one successful cultivation was achieved for 33254 (B).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>IBT/ID code</th>
<th>D (h⁻¹)</th>
<th>Ysx</th>
<th>Ysg</th>
<th>Yse</th>
<th>Ysa</th>
<th>Ysc</th>
<th>YsMSA</th>
<th>r₆-MSA (mg·g(DW)⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Glc</td>
<td>Reference</td>
<td>0.10</td>
<td>0.64</td>
<td>0.0004</td>
<td>-</td>
<td>-</td>
<td>0.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Glc</td>
<td>Reference</td>
<td>0.37</td>
<td>0.61</td>
<td>0.11</td>
<td>0.005</td>
<td>0.34</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Glc</td>
<td>9-copy, 6-MSA</td>
<td>0.10</td>
<td>0.63</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>0.46</td>
<td>0.009</td>
<td>1.04</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Glc + EtOH⁴</td>
<td>9-copy, 6-MSA</td>
<td>0.10</td>
<td>0.64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.32</td>
<td>0.011</td>
<td>1.36</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Glc</td>
<td>9-copy, 6-MSA</td>
<td>0.36</td>
<td>0.44</td>
<td>0.23</td>
<td>0.002</td>
<td>0.30</td>
<td>0.003</td>
<td>0.000</td>
<td>1.88</td>
</tr>
<tr>
<td><em>A. nidulans</em> (reference)</td>
<td>Glc</td>
<td>33252</td>
<td>0.05</td>
<td>0.57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.41</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. nidulans</em> (2-copy, IS1 &amp; IS53)</td>
<td>Glc</td>
<td>33253</td>
<td>0.05</td>
<td>0.42</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.42</td>
<td>0.18</td>
<td>16.71</td>
</tr>
<tr>
<td><em>A. nidulans</em> ⁸ (1-copy, IS53)</td>
<td>Glc</td>
<td>33254</td>
<td>0.05</td>
<td>0.47</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.39</td>
<td>0.13</td>
<td>10.57</td>
</tr>
</tbody>
</table>
Establishing a methodology for chemostat cultivation of *A. nidulans*

The substantial 6-MSA yields and high specific productivities estimated for the *A. nidulans* strains makes these strains promising candidates for industrial polyketide production. To gain information concerning potential metabolic bottlenecks, a method for cultivating *A. nidulans* in chemostats was established.

Since it can be vastly complicated to obtain and maintain filamentous fungal steady state, due to the complex morphology of the cells [35], [36], several initiatives were taken. To ensure complete mycelial dispersion, and thereby homogeneous biomass, high spore concentrations of $5 \times 10^9$ spores·L$^{-1}$ [37] where ubiquitously applied for all batch cultivations, including those applied for chemostat cultivations. This initiative was supported by rigid control of both pH and aeration ensuring low initial spore agglomeration during the germination phase by keeping a pH of 3 [38], [39], while low initial aeration (0.1 vvm) ensured that the hydrophobic spores were not expelled from the medium. The start conditions were slowly changed during the germination phase to reach final process conditions only after full germination had occurred, as observed by microscopy (data not shown). By reducing the glucose concentration to 5 g·L$^{-1}$ in the feed medium, low biomass concentration was achieved supporting mycelial dispersion.

Chemostat cultivations at a dilution rate of 0.05 h$^{-1}$ were performed for the reference strain and the two high yielding strains (1 and 2 copy strains) both with the 6-MSAS gene integrated into IS53. As evident from figure 4, steady state was achieved for all three strains and was maintained stable for between 5 and 8 residence times. Though feed was initiated in the late part of the exponential growth phase, a significant drop in CO$_2$ concentrations of 20 to 100 hours was observed, prior to a secondary “semi-batch” phase after which a carbon limited steady state culture was established. This phenomenon might indicate an adaptation phase, where the microorganism metabolism stabilised to the new physio-chemical conditions.
Figure 4. Representative chemostat profiles for the three *A. nidulans* strains: 33252, 33253 and 33254 cultivated as duplicates, excepting 33254 (only one cultivation), at a dilution rate of $D = 0.05 \text{ h}^{-1}$. 
Duplicate cultivations of all three strains were performed; however only a single cultivation of the 1-copy (33254) strain was successful, explaining the lack of deviations for the specific 6-MSA production rate in table 3.

In comparison to *S. cerevisiae* steady state cultivations the *A. nidulans* chemostat cultures displayed considerably more variability in the CO₂ evolution curve. However both biomass and 6-MSA yields exhibited remarkably low standard deviations corresponding to those of the *S. cerevisiae* strains emphasising the robustness of the outlined method.

A markedly lower biomass yield on glucose was observed for the 6-MSA producing strains corresponding to the increase in 6-MSA yield, fitting well with the constitutive expression of the polyketide (Table 3). For the batch cultivations a 26 % increase in the relative 6-MSA production rate was observed for the 2-copy strain compared with the 1-copy (IS53). The corresponding difference during chemostat cultivations was estimated to be 60 %, emphasising the advantage of chemostat cultivations for conditional comparative studies. The presented method may furthermore be applied to gain information concerning potential bottlenecks applicable for rational metabolic engineering [36], [40].
Conclusion

From the comparative analysis of the 6-MSA producing strains, it was concluded that the *A. nidulans* presented by far the most attractive alternative for producing polyketides with 4 fold higher production rates than for any other heterologous host reported. With a final 6-MSA titer, normalised to the substrate concentrations applied in the literature reference studies, reaching approximately 1.8 g·L$^{-1}$, a conversion rate of almost 10 % of the substrate to product was achieved outperforming, to the author’s knowledge, all reported values. Though *S. cerevisiae* presented the best alternative in respect to the ease of cultivation, strong oscillation rendered glucose limited chemostats with the 6-MSA producing strain at the low dilution rate impossible, likely due to a redox imbalance. An approach for stabilising the oscillatory behaviour was established by supplementing the feed medium with 0.3 g·L$^{-1}$ ethanol, thereby allowing for conditional comparative studies and generation of tailor-made biomass for omics analysis.

By tightly controlling external parameters an approach for cultivating *A. nidulans* in chemostats was devised enabling not only conditional physiological studies with this morphologically challenging strain, but also generation of tailor-made biomass for omics analysis. Through such studies, it will likely be possible to identify potential bottlenecks applicable for metabolic engineering of the strains potentially increasing polyketide yields and productivities of this promising cell factory candidate.
References


138

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Chapter 6

Conclusions and Perspectives
The principle goal of this PhD thesis was to shift the paradigm outlined in figure 1, chapter 1, towards higher throughput systems for the assessment of cellular performance, improving the degree of information both in terms of quantity and quality. To reach this goal three dedicated studies (chapters) on the development of high throughput techniques applicable for microtiter based cultivations have been presented. Diligent attention was given to all aspects during the design of these methodologies, including ascertainment of evaporation rates and parameters with influence on oxygen transfer rates, pH control and morphology. All of the presented methods were thoroughly validated in instrumented bioreactors and, while not all parameters could be controlled, adequate documentation and analysis of the potential ramifications of these parameters were taken into account.

In terms of advancing towards higher quantity with improved quality, the method, presented in chapter 2 did not only provide a high throughput method for the assessment of metabolically active yeast cells, but also evidence suggestive of the scalability of the system in terms of basic physiology. This evidence was achieved through the comparative quantification of metabolically active cells, from microtiter plate based cultivations, with automated growth rate assessment, and bioreactor based cultivations where a high correlation was observed.

Taking advantage of these observations, a high throughput method for exploring yeast cell factory potential was developed and validated in a scale-down study, from 5 L and 1 L bioreactors to 48 well microtiter plate wells. In this comparative study, growth rates and yield coefficients were shown comparable with bioreactor based values, excepting volatile metabolites, which could unfortunately not be assessed due to unpredictable losses to evaporation.

Parallel to this study a scaled down methodology for advanced physiological characterisation by metabolic flux analysis in microtiter plates was developed. This objective was achieved by optimisation of several sample preparation parameters and instrumental conditions, thus ensuring that the biomass quantities, available in microtiter well concentrations, could be confidently analysed.
This method was implemented for a thorough analysis of the metabolic flux distributions of *S. cerevisiae* cultivated with [1-13C]glucose as carbon source in both microtiter plates, shake flasks and in a bioreactor. Flux distributions were shown comparable for bioreactor, microtiter plates and shake flasks, though a few modifications to the model was necessary, since volatile metabolites could not be assessed. Further analysis revealed that these modifications did not change the final distributions significantly and the model was therefore considered robust and applicable for microtiter cultivation based metabolic flux analysis. From these two studies it was concluded, that the overall physiology of microtiter based yeast cultivations, were comparable with bioreactor cultivations and that both growth rates, yield coefficients and metabolic flux distributions of the cultivated yeast could be directly correlated. These results will be highly applicable for confident identification of best candidate strains in large scale screening efforts with the option of fast and automated physiological characterisation that, while not making bioreactors obsolete, are applicable for quick assessment of cell factory potential and for thorough design of bioreactor experiments.

Extending the outlined observations and analysis above, a similar approach for cultivating filamentous fungi in microtiter plates was developed, taking advantage of the dispersive effect of carboxypolymethylene, enabling biomass evolution monitoring by optical density measurements. By implementing several initiatives a method was devised and validated against bioreactor experiments. The constitutive expression of 6-MSA as a function of growth, provided an additional method for growth rate assessment, corresponding well with growth rates estimated from optical density, validating the methodology. Basic physiological characterisation resulted in identical 6-MSA yields on glucose in both bioreactors and microtiter plates, which was taken as evidence for the scalability of the method. A broad-spectrum, basic characterisation of a range of different filamentous fungi, some of which are industrially applied, revealed a high degree of correlation for all strains and conditions, proving the versatility and robustness of the method.
While this novel method in its own right provides relevant information, further developments should aim at characterising different additives for obtaining the dispersed morphology needed, such that samples may be filtrated, thereby enabling full implementation of the degree of automatisation outlined for the yeast based method in chapter 3. Clarification of the metabolic effects of the polymer additions should be pursued and compared with cultures without supplementation. Dependent on the conclusions from such experiments, the method may be extended for metabolic flux analysis, in which case the only volatile metabolite not accounted for would be CO$_2$, usually not implemented into the model.

Finally an in-depth analysis of all of the aforementioned 6-MSA producing strains was conducted to evaluate the overall cell factory potential for polyketide production in yeast (S. cerevisiae) and filamentous fungi (A. nidulans). From this study it was concluded, that the 2-copy 6-MSA expressing A. nidulans strain was the best cell factory candidate for polyketide production with yields outcompeting all other strains by at least 40 %, exceeding, to the authors knowledge, all reported results in literature. Analysis of chromosomal specific integration sites (IS) revealed a 40 % higher expression level from IS53 compared with IS1. The results are suggestive of the possibility of identifying sites with equal, or higher, expression levels, with the potential for increasing yield and specific production rates even further.

Hypothetically, due to redox imbalance, it was not possible to obtain glucose limited steady state cultures for the 6-MSA producing S. cerevisiae, suggesting the presence of a major metabolic bottleneck in this strain. The resulting oscillatory behaviour was effectively attenuated by ethanol supplementation, paving the way for obtaining steady state cultures for, amongst others, the identification of said bottleneck. Finally, a procedure for cultivating the morphologically challenging A. nidulans in chemostat cultures was developed by the diligent implementation of several initiatives. While not pursued in this study, the option for obtaining steady state cultures, as demonstrated here with A. nidulans might have great ramifications for omics driven optimisation of not only the high yielding A. nidulans strain identified in this study, but also other studies of this important model organism.
To conclude, several methods have been developed and validated for high throughput characterisation of both yeast and filamentous fungi in microtiter based systems, extending the application of these systems to include quantitative physiological characterisation. It was furthermore demonstrated that metabolic flux analysis could confidently be carried out based on microtiter cultures and validated by comparison to bioreactor cultivations. In perspective, future developments in microtiter based cultivation designs, together with downscaled procedures for omics analysis, might eventually allow for rational omics driven cell factory design based on micro-scale studies.
Appendix I

A robust validated GC-MS methodology for 13C-measurements of proteinogenic amino acids with applications in Metabolic Flux Analysis
Abstract

This chapter presents a robust gas chromatography-mass spectrometry (GC-MS) methodology for measuring $^{13}$C-labelling patterns of derivatised proteinogenic amino acids. The resulting labelling data represents the foundation for quantification of fluxes in metabolic networks. The method was validated on a range of microorganisms (Saccharomyces cerevisiae, Streptomyces coelicolor, and Aspergillus nidulans) cultivated on a range of differently $^{13}$C-labelled glucose sources, ([1-$^{13}$C] glucose, [U-$^{13}$C$_6$]glucose and D-glucose), and the harvested biomass was hydrolysed to cleave amino acids from the proteins. Compared to other flux methods, the sensitivity and robustness were increased by cation-exchange solid-phase-extraction purification of amino acids prior to derivatisation. Two types of volatile derivatives were produced and analysed by GC-MS. Fragment ions from both derivatives provided redundant labelling information and was used as a quality control of the data. The required amount of biomass was determined to be 5 mg dry weight based on extensive testing of biomass derived from different microorganisms, however, by reducing the MS collision energy from 70 eV to 50 eV and further concentrating the samples, 1 mg dry weight for yeast biomass provided a full dataset, making it fully compatible with microtiter based cultivations.
Introduction

Metabolic flux analysis (MFA) has been used extensively to describe network topology and the distribution of metabolic fluxes within microbial cells [1], [2]. There are many applications of MFA within biotechnology processes, such as in connection with strain improvement for amino acid production by Corynebacterium glutamicum and Escherichia coli [3]. MFA combines $^{13}$C-labelling feeding, of e.g. $[^{1-13}]$C-glucose with metabolite balancing, enabling a quantitative as well as a qualitative study of metabolic pathways, providing an integrated characterisation of microbial cell metabolism. The analytical method applied for extracting $^{13}$C-labelling information from the labelled biomass is of key importance in MFA and has thus been subject to extensive scrutiny by researchers.

Though isotopomeric distributions may be measured by either mass spectrometry (MS) or Nuclear magnetic resonance (NMR) methods, MS offers a significant advantage over NMR, with superior sensitivity allowing for measurements of metabolites down to $10^{-15}$ g, more than $10^{9}$ times lower than NMR. Thus, MS has become the state of the art technique for high throughput $^{13}$C amino acid analyses, especially when limited biomass supplies from growth experiments in micro-bioreactor and microtiter plates are applied [4], [5].

For a successful identification of each sample analyte and reduction of the background noise, a chromatographic step must be included prior to MS analysis. Though superior in many aspects, liquid chromatography (HPLC, CE) has some limitations, as it can only be combined with atmospheric ionisation techniques (ESI, APCI, APPI, SSI etc.), where C-C bond cleavages are difficult to induce. It is vital to ensure C-C bond cleavage to achieve information of labelling positions, which requires a much harder ionisation, such as electron-impact-ionisation (EI’), necessitating the application of gas chromatography (GC) [6], [7]. By measuring the resulting fragments by MS, the labelling pattern may be deduced ensuring vital data for the MFA.
GC analysis of volatile amino acid derivatives is an old technique dating back to the 1950’s [8] and was already interfaced to mass spectrometers (GC-MS) in the 1960’s [9]. However, for MFA the isotopomer distribution of fragments, providing labelling position, are needed for calculation of the $^{13}$C-labelling degree of the measured metabolites [10]. For a comprehensive review on the application of GC-MS in MFA, refer to the review by Witmann et al (2007) [2].

A GC-MS approach has been employed extensively in our labs and by associated partners for the study of flux distributions in bacteria [11]–[14], yeast [2], [15]–[17] and filamentous fungi [18]–[22]. The method applied was designed for detection of proteinogenic amino acids as two different derivatives, N-ethoxycarbonyl-amino ethyl-esters (ECF) and N-dimethyl-amino-methylene-methyl-esters (DMFDMA). A similar approach has been described employing derivatisation by tert-Butyldimethylsilyl [24]. Compared with the method by Zamboni et al (2009) [24], the introduction of the SPE purification step in the presented method makes it better suited for $^{13}$C isotopomer analysis of large sequences of samples.

In this current study, we present a highly robust and thoroughly validated GC-MS methodology for generating $^{13}$C-labelling data. Based on the work of Christensen et al. [19], [23], a superior method, implementing several initiatives in the MS method along with the sample preparation and purification by solid phase extraction (SPE) purification, has been developed. The presented GC-MS method has moreover been validated with GC-MS results, performed on cell biomass generated by growing microbial cultures on mixtures of naturally labelled and $^{13}$C uniformly labelled glucose, proving a high degree of accuracy.

An overview of the developed method from $^{13}$C-labelling experiment to GC-MS analysis is given in Figure 1.
Figure 1. Schematic overview of the GC-MS method from biomass harvest to GC-MS analysis of the derivatised metabolites.
Materials and methods

Growth experiments

Microorganisms

Saccharomyces cerevisiae CEN.PK113-7D (MATα, MAL2-8c SUC2), Streptomyces coelicolor A3(2) and Aspergillus nidulans were applied as the “proof-of-concept” organisms for this study. S. cerevisiae was maintained on Yeast Extract-Peptone-Dextrose agar plates. Single colonies from plates were sub cultured before each test in shake flasks with 100 mL of sterile minimal medium and grown for 14-16 h at 30 °C at 150 rpm. Bioreactors and microtiter plates were inoculated to an OD₆₀₀ of 0.01 and the entire submerged cultivation process carried out over ~ 30-40 h. S. coelicolor was maintained and cultivated as described in Borodina et al. (2008) [14], and A. nidulans was maintained and cultivated as described in David et al. (2005) [21].

The yeast minimal medium composition used for either cultivation in the bioreactors or in 48 well plates was as follows: 20 g·L⁻¹ D-glucose/[1⁻¹³C]glucose (Sigma-Aldrich, St. Louis, MO/USA)/[U⁻¹³C₆]glucose (Cambridge Isotope Laboratories, Andover, MA/USA) or mixtures hereof, 5 g·L⁻¹ ammonium sulphate, 3 g·L⁻¹ monopotassium phosphate, 0.5 g·L⁻¹ magnesium sulphate, 1 mL trace metal solution, 1 mL vitamin solution, 50 µL antifoam 204 (Sigma-Aldrich, St. Louis, MO/USA). The trace metal solution consisted of: 15 g EDTA (sodium salt), 0.45 g·L⁻¹ ZnSO₄, 7H₂O, 1 g·L⁻¹ MnCl₂, 2H₂O, 0.3 g·L⁻¹ CoCl₂· 6H₂O, 0.3 g·L⁻¹ CuSO₄· 5H₂O, 0.4 g·L⁻¹ Na₂MoO₄· 2H₂O, 0.45 g·L⁻¹ CaCl₂· 2H₂O, 0.3 g·L⁻¹ FeSO₄· 7H₂O, 0.1 g·L⁻¹ H₃BO₃, 0.1 g·L⁻¹ KI. The vitamin solution contained: 0.05 g biotin, 0.2 g·L⁻¹ p-amino benzoic acid, 1 g·L⁻¹ nicotinic acid, 1 g·L⁻¹ Ca-pantothenate, 1 g·L⁻¹ pyridoxine-HCl, 1 g·L⁻¹ thiamine-HCl and 25 g·L⁻¹ myo-inositol. For high throughput experiments the medium was supplemented with 100 mM 4-morpholineethanosulfonic acid hemisodium salt (MES) buffer (Sigma-Aldrich, St. Louis, MO/USA) and adjusted to pH 5.
Unless otherwise stated all chemicals applied for this study were procured from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO/USA).

**Cultivations**

Cultivations with the microorganisms were carried out using D-glucose, [1-\(^{13}\)C]glucose and with a mixture of naturally labelled and [U-\(^{13}\)C\(_6\)]glucose. When applying \(^{13}\)C labelled glucose, preferably 100 % should be used, but acceptable results could also be obtained with 50-80 % labelled glucose, the remainder non-labelled D-glucose.

The required amount of culture volume, corresponding to minimum 5 mg dry weight for *S. coelicolor* and *A. nidulans* and ~ 1 mg dry weight for *S. cerevisiae*, was withdrawn from the culture. The culture broth was then centrifuged for 5 min at 12,000 x g at 4 °C. The supernatant was discarded and the biomass kept at -20 °C until use.

**Biomass hydrolysis for amino acid analysis**

The biomass samples were thawed (~ 15 min.) and washed with 1 mL water. Aliquots corresponding to a least 5 mg dry weight (~ 1 mg for *S. cerevisiae*) were distributed in 2 mL Eppendorf tubes, and centrifuged at >12,000 x g at 10 min., the supernatant was discarded, ~ 10 % of the re-dissolved biomass was saved for glucose analysis. One quality control (QC) sample with 1 mg BSA was included. For hydrolysis 400 µL 6 M hydrochloric acid was added, the vial sealed with crimp caps, vortexed for 30 s, and then hydrolysed at 105 °C for 16 h. After hydrolysis the vials were cooled to room temperature and de-capped. The contents of the vials were, transferred to 1.5 mL Eppendorf tubes, centrifuged at 15,000 x g for 2 min. and supernatant discarded. The hydrolysed biomass was then split into two glass vials or in case of low quantity biomass samples, one vial. The samples were the evaporated to dryness for 3 h under a stream of nitrogen, and one stored as backup sample.
**Solid phase extraction of amino acids from biomass hydrolysate**

The samples were then redissolved in 200 µL water, vortexed vigorously for 30 s, after which a further 800 µL water was added along with 100 µL 2.5 mM 4-Chloro-DL-phenylalanine (internal standard). The Strata SCX columns (100 mg, 1 cc, Phenomenex, Torrance, CA, USA) were placed in a SPE manifold and conditioned with 2 mL methanol and subsequently 2 mL water. Air was passed through the columns for ~ 2-4 s to empty it (not drying the phase). Samples were then loaded onto the columns and allowed to percolate through, after which 1 mL of 50 % ethanol in water was added to wash out contaminants.

Vials (2 mL) were then placed under the columns and 0.2 mL 1 M NaOH was loaded onto the columns. The reagent was allowed to percolate through, after which the samples were completely eluted with a mixture of (9:5:1) 1 % (w/v) NaOH in saline (0.9 % NaCl):Ethanol:Pyridine. The content was divided into 2 parts (500 µL each) in 2 mL glass vials (for ECF derivatisation) and (for DMFDMA derivatisation).

**Preparation of ECF-amino acids derivatives**

To all samples (including a blank and a quality control sample) 50 µL of ECF was gently mixed in by pipetting (yellow colour, which fades gradually with the reaction effervescence). The lid was closed and the mixture was vortexed gently for 5-10 s intervals between which pressure generated by gas released from the reaction was released. After 1 min a further 5 µL ECF was added and the contents mixed gently by pipetting upon which the samples were vortexed gently for 30 s.

The derivatised amino acids were then extracted by addition of 200 µL propyl acetate. The mixture was vortexed vigorously for 30 s, and the vial unscrewed to release pressure. Residual pyridine in the propyl acetate phase were shifted into the water phase by mixing in 50 µL 1 M HCl and vortexing the mixture vigorously for 30 s after which the vial was carefully opened to release pressure. 200 µL of the upper organic layer was then transferred to a clean 2 mL vial containing approximately 100 mg anhydrous MgSO₄. To a new 2 mL vial 100 µL was transferred with a 200 µL tip insert (concentrated sample), and 15 µL for a 2 mL vial along with 1485 µL propyl acetate (100 X diluted sample) and the vials sealed and GC-MS analysis.
could then be performed.

If only a small sample of biomass was available the 200 µL extract were dried under a stream of nitrogen and re-dissolved in 40 µL propyl acetate (5 X concentration).

**Preparation of DMFDMA-amino acids derivatives**

One extra QC of 50 µL amino acid mix solution with 50 µL internal standard was included. 200 µL 1 M HCl was mixed into all samples (including a blank and the QC) and subsequently dried in an oven for 2 to 4 h at 105 °C without caps. The vials were then removed and cooled in a desiccator (for 30 min) after which crystal formation was observed.

Derivatisation was carried out by mixing in 200 µL DMFDMA and 200 µL acetonitrile upon which the vials were sealed with crimp caps and the liquid thoroughly mixed by 30 s of vortexing. Vials were then kept for derivatisation at 100 °C for 15 to 20 min. The vials were then removed and cooled at -20 °C for 10 min, where after the vials were decapped and 200 µL of the content transferred into glass vials with inserts (concentrated sample) and 15 µL for a 2 mL vial along with 1485 µL reagent acetonitrile (100 X diluted sample). The vials were sealed prior to GC-MS analysis.

If only a small sample of biomass was available the 200 µL extract were dried under a stream of nitrogen and re-dissolved in 40 µL propyl acetate (5 X concentration).

**Glucose incorporation from complex carbohydrates**

Biomass samples were thawed and washed with 1 mL water and centrifuged at >12,000 x g for 3 min after which the supernatant was discarded. For hydrolysis the biomass was mixed with 400 µL 4 M trifluoroacetic acid, transferred to a 2 mL vial and sealed with crimp caps.

The vials were kept for hydrolysis at 105 °C for 2.5-4 h. After hydrolysis the vials were decapped and samples evaporated to dryness under a continuous nitrogen flow at 60 °C. 300 µL water was then added to each vial and the samples vortexed for 30 s.
**SPE purification of glucose from biomass hydrolysate**

One extra QC with 300 µL 100 µM glucose was included. The Strata X-C SPE columns, placed in the vacuum manifold, were conditioned with 2 mL methanol and subsequently 2 mL water. Air was passed through the columns for approximately 2-4 s to empty it, but not totally drying the phase after which 2 mL vials placed under columns. Samples were loaded onto columns and allowed to percolate slowly through the columns. To ensure full elution a further 700 µL water was loaded onto the columns and the whole 1 mL sample collected. The eluate was then evaporated to dryness under a continuous nitrogen flow.

**Derivatisation of glucose**

The reagent mixture was freshly prepared: pyridine: acetic acid anhydride (1:1 v/v) and 200 µL added into the dry vial, and mixed in gently by pitting. The mixture was left to react for approximately 8-12 min. after which 600 µL 5 % NaHCO₃ was mixed in, followed by 400 µL propyl acetate. After careful mixing, the upper clear phase was transferred into a new vial containing ~ 100-200 mg anhydrous MgSO₄ thereby removing all residual water. The clear liquid was transferred to a 2 mL vial with insert, and the vial capped before GC-MS analysis. If only a small sample of biomass was available, the 200 µL extract were dried under a stream of nitrogen and re-dissolved in 40 µL propyl acetate (5 X concentration).

**GC-MS analysis**

For all the above methods, samples of 1 µL were injected in the splitless (30 s, split 1:20) mode. MS scan range m/z 40-400. The MS was setup with impact ionisation (EI⁺) using an electron energy of 50 eV for the ECF derivates and 70 eV for the DMFDMA and glucose penta-acetate derivates. For the analytical sensitivity analysis, electron energies from 10 eV to 80 eV were tested in intervals of 10 eV.

The GC was equipped with a 4.0 mm i.d. Siltek gooseneck splitless deactivated liner (Restek, Bellefonte, PA, USA), and a Supelco (Bellefonte, PA, US) Equity®-1701 (15 m, 0.25 mm i.d., 0.25 µm film) column. Helium was used as carrier gas at a constant linear gas velocity of 38 cm/s. Transfer line temperature was 280 °C,
quadrupole temperature 150 °C and MS source 230 °C. The GC-MS system was controlled from Agilent MSD Chemstation v. D.01.02.16, and auto-tuned prior to every sequence.

**Specific details for amino acid-ECF derivatives**

Injection temperature was 220 °C, and oven temperature initially held at 75 °C for 1 min. Hereafter the temperature was raised 40 °C · min⁻¹ until 165 °C, then 4 °C · min⁻¹ until 190 °C and then 40 °C · min⁻¹ to 240 °C. At the end, temperature was increased to 260 °C at 4 °C · min⁻¹ and held constant for 4 min.

**Specific details for amino acid-DMFDMA derivatives**

Injection temperature was 230 °C, oven temperature was initially held at 60 °C for 1 min. Hereafter the temperature was raised at 20 °C · min⁻¹ up until 130 °C, then 4 °C · min⁻¹ until 150 °C and 40 °C · min⁻¹ to 260 °C and held constant for 4.25 min.

**Specific details for acetylated-glucose**

Injection temperature 260 °C, starting with an oven temperature of 75 °C for 1 min, 40 °C · min⁻¹ to 190 °C, and finally 50 °C · min⁻¹ to 280 °C keeping this for 3 min.

**Data extraction**

Isotope ratios were obtained by integrating the extracted ion chromatogram peaks (-0.3 amu to +0.7 amu) of the fragments of interest and their X+1, X+2,…, X+n isotopomers using auto quantification module of the Chemstation program, in a time window of ± 0.3 min for all AA’s except leucine and isoleucine where a time window ± 0.1 min was used. This approach should be possible to perform in all MS manufacturers’ software. Before data extraction, pre and post-sequence validation samples (pure AA mix and BSA samples) were analysed to validate consistent retention times. Ions used for data extraction are listed in supplementary table 1.
Results and discussion

Cultivation, hydrolysis and sample purification

In recent years, significant effort has been made towards improving quenching, derivatisation, data-extraction and data analysis for metabolome GC-MS data [2], [25]–[28], however little attention has been paid to sample-purification especially by solid phase extraction (SPE). Sample purification is of key importance, since hydrolysed biomass samples contains a multitude of pollutants as a result of the hydrolysis, complicating routine analysis [23] with a consequent loss of sensitivity after 5-20 samples, resulting in replacements of column and change of the liner.

After washing non-basic impurities off the column, the amino acids were eluted by the combined action of: i) Na\(^+\) ions; ii) increasing pH; and iii) pyridine, which displaced some amino acids on the resin as well as acting as catalyst for the ECF reaction in the subsequent step. Ethanol was added to the eluting solvent mixture to ensure complete elution of the aromatic amino acids otherwise retained by reversed phase interactions. To maintain high recoveries of the amino acids it was vital to ensure complete evaporation of the HCl solvent applied in the previous step. This SPE purification procedure not only ensured a significant reduction in background noise, but also allowed for up-concentration making the amino acids the major peaks in the chromatograms. With the reduction in background noise, further up-concentration by evaporation was possible, reducing the critical sample sizes and making small scale cultivation available for metabolic flux analysis experimentation.

The purification and subsequent up-concentration allowed for the detection of ornithine, cysteine and methionine, previously not detected (data not shown). This suggested that the SPE step may render them available for derivatisation as well as reducing the amount of interfering material that could react or interfere with their derivatisation. This was important as the yields of e.g. methionine was poor during the harsh hydrolysis step [2].
For cultivations with $^{13}$C-labelled glucose the first branch point after uptake is glucose-6-phosphate at which the carbon flux branches into the pentose phosphate (PP) pathway and the Embden-Meyerhof-Parnas (EMP) pathway. Measurement of the $^{13}$C-labelling degree of this particular metabolite was therefore of key importance for ensuring accurate flux quantification. Unfortunately, the old protocol [19] for hydrolysing polysaccharides, of which glucose-6-phosphate is a precursor, was quite destructive for both the MS and the column.

This was solved via the following adaptations: i) a milder hydrolysis (4 M TFA rather than 6 M HCl); ii) the reversed-phase (RP) purification on the very polar Strata-X-C phase (retaining fats, peptides and other medium to very apolar compounds) [29]; and iii) capture of amino acids and glucose-amine from the chitin as well as other basic compounds by the cat-ion-exchange moieties in the Strata-X-C. The resulting chromatograms revealed only acetylated sugars. A time scale study (30 min to 6 h in steps of 30 min) of the hydrolysis of biomass and pure glucose showed that the glucose concentration peaked after ca. 2-3 h depending on the growth conditions and organism (S. cerevisiae, Streptomyces coelicolor, and A. nidulans) (data not shown).

Separation of derivates by gas chromatography

In this new and improved GC protocol, dichloromethane was replaced with the less toxic propyl acetate, allowing for splitless injection at 75 °C instead of 45 °C [30], reducing the oven cooling time to a third. Other tested alternatives included butyl acetate and propyl propionate, which unfortunately were shown to co-elute with the early eluting ECF derivatised amino acids and ethyl acetate that did not result in the required phase separation (due to ethanol and pyridine).

Injection temperature optimisation resulted in a reasonable compromise of 220 °C, at which sufficient quantities of the later eluting (and thus higher boiling) ECF derivatised amino acids were achieved, while avoiding decomposition of the sulphur and alcohol containing ECF-amino acid derivatives. With a temperature programmable injector, this could likely be significantly improved by introducing a
temperature gradient from 180 to 250 °C in approximately 20-30 s. Since the DMFDMA derivatisation procedure required the analyte to be in the salt form, acidification of the eluate prior to drying was absolutely crucial.

Numerous DMFDMA reagent derived peaks, made it difficult to universally method for free amino acids. However, extracting the right ions provided important labelling information of fragments originating from the amino acids glycine, alanine, valine, aspartic acid, glutamic acid and phenylalanine. Thus, by combining the two derivatisation methods, redundant labelling information of the central metabolites important for the method validation was acquired.

**Enhancing analytical sensitivity**

The good sensitivity obtained, relative to the amount of available biomass made it possible to run the quadrupole MS in full scan, which meant that data files could be reprocessed for more information.

Dependent on the microorganism and experimental design, biomass samples may vary in size, resulting in sub-optimal amino acid recovery, complicating metabolic flux analysis. This was in part solved by the sample purification step, resulting in an up-concentration of the amino acids. By getting rid of a significant portion of the pollutants, the background noise was dramatically reduced allowing for further up-concentration prior to analysis increasing concentrations up to 5 fold (data not shown).

Due to the wide applications of GC-MS in various fields such as toxicology and environmental research, standard operation condition for the MS source was early on established to be El⁺ of 70 eV, since this ionisation energy provides the most reproducible MS spectra [31]. As a consequence these conditions have widely been adopted for also metabolic flux analysis [23], [32]–[34] even though 70 eV does not necessarily offer optimal results for the very fragile ECF-amino acids.

As part of an optimisation of the methodology, different ionisation energies from 10 eV to 80 eV were tested, to identify the optimal source conditions for maximising sensitivity. The tests were carried out for
the ECF, DMFDMA and penta-acetate derivates in steps of 10 eV, illustrated in figure 2 by a summarisation of the improvement/reduction in sensitivity for the all amino acid fragments normalised to the standard of 70 eV. Though 40 eV provided the best sensitivity for alanine, valine, proline, leucine and isoleucine, 50 eV yielded superior sensitivity for the more elusive ECF derivates of serine and threonine (data not shown). Based on these tests it was concluded that 50 eV provided the optimal source condition (cannot be changed during analytical run on this instrument) for the ECF derivates with between 37 and 59 % improved sensitivity for all derivatised amino acids. In case of the DMFDMA derivates and the glucose penta-acetates, no significant improvements were observed for any of the energy, and 70 eV was thus maintained.
Figure 2. Enhancing the analytical sensitivity. ECF and DMFDMA derivatised amino acids were analysed by MS with fragmentation at 10 eV to 80 eV in steps of 10 eV. The improvement/reduction in the overall sensitivity was assessed by summarisation of all the informative amino acid fragments normalised to the standard of 70 eV (dotted line). Though 40 eV provided the best sensitivity for alanine, valine, proline, leucine and isoleucine, 50 eV yielded superior sensitivity for the more elusive ECF derivates of serine and threonine (data not shown). Based on these tests it was concluded that 50 eV provided the optimal source condition for both the ECF derivates with between 37 and 59 % improved sensitivity for all derivatised amino acids.

Technically, sensitivity may be improved further by reducing biomass quantities to the µg range. However, due to the wide range of quantities of the different amino acids and variation in the amino acid composition for biomass originating from different types of organisms, it is advisable to inject the samples, as described; 100 fold diluted samples may be included allowing for better separation and peak integration of the major amino acids.

Glucose

The glucose analyses used for validation/determination of the relative $^{13}$C incorporation showed mainly penta-acetyl α, and β anomers almost co-eluting as the only peaks in the chromatogram (no other peaks higher than 10 % in the TIC chromatogram). This was in sharp contrast to previously applied protocols.
where glucose-penta acetate was a minor peak, and the glucose measurements were the most troublesome samples with respect to instrument maintenance.

Data analysis and method validation

In MFA based on GC-MS measurements, summed fractional labelling (SFL) is widely applied for data analysis of the labelling degrees of the analysed metabolites. The SFL of an amino acid, or fragment thereof, is calculated from the abundances of the mass isotopomers (via peak areas of extracted ion chromatograms) [23] and corrected for the occurrence of natural labelling from other atoms [2]. An example of deriving correction matrices required for calculation of the corrected mass isotopomers for metabolic flux analysis has been exemplified for alanine in supplementary table 2. SFL’s are defined as the sum of the fractional labelling (FL) of each carbon position in the fragments, i.e. the fraction of carbon atoms being $^{13}$C. SFL is estimated from the corrected mass isotopomers according to Equation (1).

$$SFL = \frac{\sum_{i=0}^{n} i \cdot m_i}{\sum_{i=0}^{n} m_i}$$  \hspace{1cm} (1)

Effect of biomass amount on CNL

Carbon normalised labelling (CNL) is defined as the average labelling of all the carbon atoms in a given fragment. The advantage of using CNL is that the number of carbon atoms in the fragment is taken into account, which facilitates direct comparisons of labelling degrees in experiments carried out with a uniformly labelled carbon source e.g. [U-$^{13}$C$_6$]glucose.

Thus the sensitivity of the method as function of quantity biomass applied, could be evaluated by employing samples with increasing amounts of biomass from cultures of actinomycetes, yeast and fungi. The substrate was 1.1 % $^{13}$C-labelled glucose (naturally labelled) as the only carbon source, thus CNL’s of 1.1 % were expected for all AA fragments. For each microorganism samples of 1, 2, 5, 8 and 12 mg dry weight
were analysed. An average CNL was estimated for each sample amount based on the measured CNL of all measurable AA’s fragments from the biomass samples from the three different microorganisms (n\text{total} = 69). Accordingly, an overall standard deviation was also estimated based on the 69 measurements. The sensitivity of the method was investigated by plotting the overall CNL and standard deviations (SD) as a function of the amount of biomass used for the analysis, Figure 3.

![Figure 3. Sensitivity analysis of the method. The overall average CNL of all measured fragments (n=69) originating from actinomycete, yeast and fungal biomass is illustrated as function of the quantity of biomass (dark grey area). The light grey area shows the overall standard deviation of the measurements of all the CNL’s. The dashed line indicates the expected naturally labelling of the fragments i.e. 1.1 %. The optimal criteria for the method are a measured average CNL of 1.1 % together with the lowest possible standard deviation.

The method performed well in the range of 1 mg to 5 mg of biomass with respect to measured CNL (dark grey area Figure 3), however, the standard deviation on the measurements was high, but decreasing with increasing biomass amount (light grey area Figure 3). For biomass levels higher than 5 mg dry weight, both the CNL and the standard deviation were stabilised at 1.1 % to 1.2 % and 0.2 % to 0.4 %, respectively. The optimal conditions for the method were a measured average CNL of 1.1 % and the lowest possible standard deviation. Thus, with this method and MS instrument, a minimum of 5 mg of dry biomass is recommended.
for doing metabolic flux analysis independent of the applied microorganism, pending that up-concentration is not pursued.

The high method performance in the range between 1 mg and 5 mg indicated that the method could be made applicable for microtiter cultivations, provided that the standard deviations observed in this interval were reduced. In a recent study on yeast, biomass quantities of 0.3 mg from deep-well 96 well plates were applied to perform MFA [35]. While this method was not validated with CNL, it does suggest that significant down-scaling is possible for yeast cultivations.

Taking advantage of the sample purification, yeast biomass samples of 0.25, 0.50 and 0.75 mg were up-concentrated after ECF derivatisation and the improved MS protocol was applied in order to maximise sensitivity. The CNL ratios for the 0.25 mg sample were in the range of 1.1 to 1.2 % for all but the serine and threonine derivates, which displayed CNL’s between 1.1 and 1.4 %, emphasising the particular difficulties associated with the analysis of these amino acids. For the 0.5 and 0.75 mg samples the CNL’s ratios stabilised between 1.1 and 1.2 % (data not shown). Therefore to obtain the required labelling information for metabolic flux analysis, with the previously described redundancy provided by the DMFDMA, a minimum of ~ 1 mg (yeast only) was needed (for the instrument applied), provided that the samples were up-concentrated.

**Method accuracy**

To test the accuracy of the method, an experimental setup using biomass with 5 different (known) degrees of labelling was designed. The $^{13}$C-labelled biomass was generated by feeding separate cultures of yeast in 48 well microtiter plates (well volume – 0.4 mL ~ 0.5 mg) with a mixture of naturally labelled glucose and $[U-{^{13}}C_6]$glucose, Table 1. By performing these experiments in microtiter plates, not only the accuracy of the method, but also the scalability was challenged allowing for an evaluation of the aptitude for microtiter cultivation.
Table 1. Experimental design for validating the GC-MS method for labelling experiments.

| Exp. No. | Fraction (w/w) C₆H₁₂O₆ | Fraction (w/w) ¹³C₆H₁₂O₆ | CNL₉⁶glc | GC chromatograms | MS-spectra | Presumed CNL₀glc
|---------|-------------------------|---------------------------|----------|------------------|------------|-----------------
| 1       | 100 %                   | 0 %                       | 1.1 %    | ECF              |            | 1.1 %
| 2       | 96.2 %                  | 3.8 %                     | 5.0 %    |                  |            | 5.0 %
| 3       | 91.2 %                  | 8.8 %                     | 10.0 %   | DMFDMA           |            | 10.0 %
| 4       | 76.2 %                  | 23.8 %                    | 25.0 %   |                  |            | 25.0 %
| 5       | 51.2 %                  | 48.8 %                    | 50.0 %   |                  |            | 50.0 %

As indicated in Table 1, the 5 experiments were designed to reach ¹³C-labelling degree of each position of glucose (CNL) of: 1.1, 5, 10, 25 and 50 %, respectively. The fraction of labelled glucose needed to obtain the required Carbon Normalised Labeling (CNL) of glucose was calculated using equation (2):

\[
99\% \cdot \text{Frac}_{lab} \cdot \frac{M_{\text{glc}}}{M_{\text{glc}}} + 1.1\% \cdot (1 - \text{Frac}_{lab}) \cdot \frac{M_{\text{glc}}}{M_{\text{glc}}} = \text{CNL}_{\text{glc}}
\]

In these experiments all positions in glucose were equally labelled with ¹³C and since glucose was the only carbon source in the cultures, all carbon containing compounds in the metabolism including the proteinogenic amino acids should have exactly the same CNL as the original glucose molecules [36] illustrated by the CNL of proline (column 7, Table 1). The SFL was calculated from the extracted mass distribution data from the mass spectra, exemplified by the mass spectra for proline under different degrees of ¹³C-labelling, column 6, Table 1. The accuracy of ¹³C-measurements of the proteinogenic amino
acids was subsequently assessed by correlating the measured CNL of the AA’s with the CNL of the provided glucose, Figure 4.

![Method Accuracy](image)

**Figure 4.** The carbon normalised labeling CNL (average $^{13}\text{C}$ labeling) of each carbon atoms of the amino acid fragments as a function of the carbon normalised labeling of the supplied glucose. The black curve shows the result of the linear regression. The upper and lower dotted curve show the 95 % predicted band. Triplicate samples with 14 different fragments resulting in 210 points were used for the analysis.

<table>
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<tr>
<th>Best-fit values</th>
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<tr>
<td>Slope</td>
<td>0.9934 ± 0.001528</td>
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<tr>
<td>Y-intercept when X=0.0</td>
<td>0.2816 ± 0.03896</td>
</tr>
</tbody>
</table>

**95% Confidence intervals**

| Slope           | 0.9904 to 0.9964 |
| Y-intercept when X=0.0 | 0.2052 to 0.3580 |
| X-intercept when Y=0.0 | -0.3611 to -0.2062 |

**Goodness of Fit**

<table>
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<th>$R^2$</th>
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</table>

**Data**

<table>
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</thead>
<tbody>
<tr>
<td>Maximum number of Y replicates</td>
<td>42</td>
</tr>
<tr>
<td>Total number of values</td>
<td>209</td>
</tr>
</tbody>
</table>

The linear regression on Figure 4 shows a consistent correlation with a correlation constant ($R^2$) of 0.99, with all data points situated along the linear regression curve. Notably, the 95 % prediction bands closely flanks the linear regression curve, illustrating not only the robustness, but also the accuracy of the data.

Statistical evaluation of the entire data set revealed a standard deviation of the $^{13}\text{C}$-labelling degree of individual carbon atoms of 0.45 labelling %. Based on the statistical analysis of these results it was concluded that experiments with CNL of glucose of up to 50 % can be performed with a high degree of accuracy even in microtiter plates. Thus a reliable and sensitive platform for investigating co-metabolism has been established.
Method reproducibility

The reproducibility of the GC-MS method was validated by running the 5 samples with proteinogenic amino acids with known degrees of $^{13}$C-labelling described above. Column 5 in Table 1 shows the GC chromatograms resulting from the two derivatisation approaches, ECF and DMFDMA, respectively. To further illustrate the reproducibility of the method, a zoom of the proline peak in the ECF chromatogram is presented. In summary, the chromatograms clearly demonstrate that the analyses of the derivatised AA’s were reproducible. In addition, running the reproducibility test on samples with different degrees of $^{13}$C labelling validated that the reproducibility of the method was independent of the labelling degree.

Effect of measurement error on flux analysis simulations

The main focus of this presented protocol was to develop a GC-MS method for generating consistent $^{13}$C-labelling data applicable for MFA. The flux calculation procedure will therefore not be discussed here, but for a specific protocol on flux quantification, refer to Zamboni et al (2009) [24].

The final part of the method validation involved an investigation of the effect of measurement errors on the flux estimations. Through constructed data sets with different levels of standard deviations, the sensitivity of the flux estimations was evaluated. As a reference data set, the results of Gombert et al (2001) were applied [15]. Flux calculations were first carried out with the reference data set and subsequently with data sets constructed in the following way: ± 5 % (relative) and ± 1 % (absolute), as seen in Figure 5.
Figure 5. Sensitivity test of the effect of deviations in the $^{13}$C labeling data on selected key fluxes in the central metabolism. As reference literature labeling data, the result of Gombert et al [15] was used. The labeling data was modified with ± 5 % (relative) and ± 1 % (absolute) of the literature values. The fluxes of the EMP, the PP and the TCA pathways were subsequently estimated by using the flux estimation procedure described in Gombert et al. [15]. The bar graphs illustrate the differences between the estimated fluxes and the corresponding reference fluxes.

From these calculations it was evident that the estimations of the fluxes through glycolysis and the pentose phosphate pathway displayed the highest sensitivity towards the labelling measurements, whereas no significant differences were observed in the flux estimations of the TCA cycle. Focussing on the estimations based on the ± 1 % (absolute) data set, corresponding to the statistical frame work of this method development, it was observed that the deviation related to the constructed error in the data corresponds to ~ 3 flux units, which was acceptable considering that the effect was an additive effect caused by the addition/subtraction of 1 % from each data point. To conclude, this sensitivity test proved that the developed method can be applied to generate confident flux estimations.
Conclusion

In this study a validated and robust purification protocol is outlined, taking advantage of solid phase extraction SPE cartridges for capturing the relevant molecules, thereby reducing noise introduced by an array of various pollutants generated during the hydrolysis. Besides reducing the concentration of pollutants, the hydrolysis solvents were also completely removed reducing the wear on the GC-MS. Purification further allowed for sample up-concentration, enabling significant reductions in the biomass quantity needed. The introduction of a new hydrolysis and derivatisation protocol for glucose derivates increased analysis sensitivity substantially, while reducing instrument wear. GC temperature control was optimised, compromising between obtaining reasonable quantities of the late eluting derivates and avoiding decomposition of sulfur and alcohol derivates. Enhanced analytical sensitivity was achieved by optimising MS source conditions reducing the EI’ to 50 eV for ECF derivates, thereby obtaining 37-59 % improved sensitivity. From sensitivity analysis of a range of different biomass concentrations derived from *S. cerevisiae*, *A. nidulans* and *S. coelicolor*, it was concluded that a minimum of 5 mg dry biomass is needed for analysis. By up-concentration and diligent attention to detail, the quantity could be lowered to ~ 1 mg for *S. cerevisiae*, making the methodology highly applicable for analysis of microtiter based cultivations. The presented method was proven highly accurate and reproducible in a study correlating the expected labelling degree of the amino acids with the measured, by application of ratios of naturally and [U-$^{13}$C$_6$]glucose.
References


Supplementary appendix 1

Materials

Reagents

- D-(+)-Glucose-1\(^{13}\)C. (e.g. Sigma-Aldrich 297046).
- D-U\(^{13}\)C\(_6\)-labeled glucose. (e.g. Sigma-Aldrich 310808).
- D-(+)-Glucose monohydrate, \(\geq 98.0\%\) (Fluka Glucose 49160).
- Bovine serum albumin (BSA), lyophilised powder \(\geq 98\%\) (e.g. Sigma-Aldrich A7906).
- Water, 18.2 M\(\Omega\) cm\(^{-1}\) (MilliQ purified, Millipore, Bedford, MA, USA).
- Hydrochloric acid (HCl), 37\% (e.g. Sigma-Aldrich 320331).
- Standard amino acid mix (2.5mM in 0.1N HCl, Fluka, AAS18).
- Sodium chloride (NaCl), \(\geq 99.5\%\) (Sigma-Aldrich S7653).
- Sodium hydroxide (NaOH), \(\geq 98\%\) pellets (e.g. Sigma-Aldrich S8045).
- Magnesium sulphate (MgSO\(_4\)), anhydrous, \(\geq 99.5\%\) (e.g. Sigma-Aldrich M7506).
- Propyl acetate, 99.5\% (e.g. Sigma-Aldrich 537438).
- Pyridine, 99.8\% (e.g. Sigma-Aldrich 270970).
- Ethyl chloroformate (ECF), \(\geq 98.0\%\) (e.g. Fluka 23131).
- 4-Chloro-DL-phenylalanine, internal standard I.S.(Sigma-Aldrich C6506).
- Ethanol, HPLC grade. (e.g. Fluka, 02860).
- Trifluoroacetic acid 99\%, (e.g. Sigma-Aldrich, T62200).
- Acetonitrile, HPLC grade. (e.g. Sigma-Aldrich, 34998).
- N,N-Dimethylformamide dimethyl acetal (DMFDMA), derivatisation grade (e.g. Sigma-Aldrich, 394963).
- Methanol, HPLC grade (e.g. Sigma-Aldrich, 34860).
• Acetic anhydride, reagent grade, ≥ 98 % (e.g. Sigma-Aldrich, A6404).
• Sodium bicarbonate (NaHCO₃), ≥ 99.5 % (e.g. Sigma-Aldrich S6297).
• Acetone, HPLC grade (e.g. Sigma-Aldrich, 34850).
• Dichloromethane, HPLC grade (e.g. Sigma-Aldrich, 439223).
• 2-Propanol, LC-MS grade (e.g. Sigma-Aldrich, 34965).
• Helium 99.999 % carrier gas for GC.

Equipment
• Desiccator, containing dried Silica gel orange (e.g. Sigma-Aldrich, 13767).
• Capper and decapper for 2-mL vials.
• Analytical balance.
• SPE vacuum manifold (e.g. Visiprep™ SPE workstation, Supelco 57250-U).
• Eppendorf tubes 1.5 and 2 mL.
• Autosampler vials 2 mL, clear glass crimp top.
• Autosampler vials 2 mL with 500 µL insert, clear glass crimp top or screw cap.
• Crimp caps PTFE/silicone/PTFE septa for 2 mL vials. ! CAUTION many plastic caps and some septa melts above 90-100 °C, so test such before using for the steps involving high temperatures.
• Variable Volume Pipettes, 1 – 10 µL, 10 – 100 µL, and 100– 1000 µL.
• Motorised dispenser is recommended for pipetting regents.
• Racks for 2 mL vials and Eppendorf tubes.
• Oven capable of at least 105 °C (should be kept in fume hood due to HCl vapors).
• Strata SCX 100 mg 3 cc SPE columns (Phenomenex 8B-S010-EBJ)
• Strata X-C 30 mg 3 cc SPE columns (Phenomenex 8B-S029-TBJ).
- Centrifuge for 1.5 and 2 mL Eppendorf tubes, capable of at least 12,000 x g.
- **GC-MS Agilent 6890 gas chromatograph coupled to an Agilent 5973 quadruple MS.**
- Siltek double gooseneck splitless deactivated liner 4.0 mm i.d. (Restek, Bellefonte, PA, USA).
- Supelco (Bellefonte, PA, US) Equity®-1701 column, 15 m, 0.25 mm i.d., 0.25 µm film.
- CTC Combi PAL with PAL cycle composer software version 1.5. (CTC Analytics), setup with 2 x 50 sample cooled trays for 2 mL vials, 32-sample tray 20 mL vials (extra wash solvents), and four 20 mL syringe wash solvent reservoirs and a Hamilton 701 N 10 µL syringe.
Supplementary appendix 1

GC-MS setup

The MS was setup with impact ionisation (EI’) using an electron energy of 50 eV for the ECF derivates and 70 eV for the DMFDMA and glucose penta-acetate derivates. The GC was equipped with a 4.0 mm i.d. Siltek gooseneck splitless deactivated liner (Restek, Bellefonte, PA, USA), and a Supelco (Bellefonte, PA, US) Equity®-1701 (15 m, 0.25 mm i.d., 0.25 µm film) column. Helium was used as carrier gas at a constant linear gas velocity of 38 cm/s. Transfer line temperature was 280 °C, quadruple temperature 150 °C and MS source 230 °C. The GC-MS system was controlled from Agilent MSD Chemstation v. D.01.02.16, and auto tuned for prior to every sequence.

Other 1701 columns (J&W and Phenomenex) were tested and performed equally well.

To avoid carry-over between samples and especially polymerisation of reagent in the syringe leading to blockage of needle or locking of the plunger, the syringe was cleaned pre-injection using five times 5 µL acetone, then five times 5 µL dichloromethane and finally 3 times rinsing with 3 µL sample. The post injection cleaning protocol was, eight times 5 µL acetone then five times dichloromethane, then five times 5 µL 2-propanol, then five times 5 µL water, and finally three times 5 µL 2-propanol. Vials (20 mL) for 2-propanol and water were positioned in the 32-sample tray along with 2 empty 20 vials for waste (all capped).

The GC-MS instrument used was an old model (11 years), so newer and more sensitive instruments will probably require even less biomass. It is recommended not to use an ion-trap, since even minor ion-to-molecule reactions will obscure isotope ratios.
Supplementary appendix 2

Troubleshooting

Loss of signal during GC-MS sequence

The major problem observed was sample overload, when more biomass than recommended was used. In these cases peak intensities above $10^7$ counts have occasionally been observed, giving rise to a sudden 100-1000 fold decline in instrument sensitivity on the Agilent 5973 quadruple MS. Heating the quadrupole and ion-source overnight could usually solve the problem.

Over producing strains

Analysis of lysine over-producing mutant strains also yielded this problem and here 3 fold dilutions were made to find an area where the other amino acids could still be detected, while lysine did not overload the instrument.

The 100 fold general dilution step for re-analysis of sample was introduced for the ECF and DMFDMA, since separation was sometimes not sufficient for the most abundant amino acids alanine, valine and phenylalanine, while some of the isotope ratios of minor amino acids were not determined accurately enough in the diluted samples.

Blocked syringes

Syringe cleaning gave numerous problems with plunger and needle blocking, and thus an extensive syringe cleaning program were introduced, with the acetone being the best cleaning solvent and the 2-propanol as lubricant.
**Internal standards**

Norvaline was also tested as internal standard, but co-elution with valine was observed for the DMFDMA derivatives (but not ECF), and thus it was not included in the method.
Supplementary appendix 3

Example of the data extraction procedure

To demonstrate the data extraction procedure, an example of the process from mass spectrometry data to summed fractional labelling data is presented below.

For data extraction, the Agilent MDS ChemStation software was applied. A list of retention times and the corresponding target ions (Supplementary Table 1) was constructed in ChemStation and by running the auto quantification software; a CSV-file with information on the areas of the required target ions is generated (data not shown). Similar software is to our knowledge supplied from all GC-MS instrument manufactures. To illustrate the further data analysis, mass isotopomer data from the Alanine 116 fragment will be used as an example. Mass isotopomer data (peak areas) for Ala116, Ala117 and Ala118 were extracted: \( x_0 = 82123412; x_1 = 8412744; x_2 = 2355974 \) (data not shown). These mass isotopomer data were corrected for the contribution of natural labelling from the derivative part of the fragment according to the procedure described in Supplementary Table 2. Thus, the corrected mass isotopomer matrix becomes:

\[
\begin{bmatrix}
  m_0 \\
m_1 \\
m_2 \\
\end{bmatrix} = C^{-1} \begin{bmatrix}
x_0 \\
x_1 \\
x_2 \\
\end{bmatrix} = \begin{bmatrix}
1.0494 & 0 & 0 \\
-0.0415 & 1.0494 & 0 \\
-0.0033 & -0.0415 & 1.0494 \\
\end{bmatrix} \begin{bmatrix}
82123412 \\
8412744 \\
2355974 \\
\end{bmatrix} = \begin{bmatrix}
85757373 \\
5397721 \\
1853727 \\
\end{bmatrix}
\]

The summed fractional labelling SFL for the Alanine (m/z 116) fragment was then calculated using Equation (1):

\[
SFL = \frac{\sum_{i=0}^{n} i \cdot m_i}{\sum_{i=0}^{n} m_i} = \frac{0 \cdot m_0 + 1 \cdot m_1 + 2 \cdot m_2}{m_1 + m_2 + m_3} = \frac{5397721 + 2 \cdot 1853727}{85757373 + 5397721 + 1853727} = 0.098 = 9.8\%
\]
For flux estimations, the SFL data for fragments of amino acids originating from different parts of the central metabolism were inserted into flux estimation software and the distribution was estimated. However, this was beyond the scope of this protocol and was therefore not illustrated in this example. A detailed description of the flux estimation procedure is given in Gombert et al. (2001) [15].

In the sensitivity analysis of the method, the carbon normalised labelling was used as a measure of the consistency between expected and measured labelling. In this example with alanine, the CNL was 4.9 %, since the fragment consists of two carbon atoms.
Supplementary Table 1

Measured m/z ions and retention times for the different derivative fragments

<table>
<thead>
<tr>
<th></th>
<th>ECF-derivatives</th>
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<th>DMFDMA-derivatives</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>m/z ions (X, X+1, ..., X+n)</td>
<td>Ret time (min)</td>
<td>m/z ions (X, X+1, ..., X+n)</td>
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<td>Alanine</td>
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<td>Glycine</td>
<td>175, 176, 177</td>
<td>3.88 ± 0.00</td>
<td>85, 86</td>
<td>4.90 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>127, 128, 129, 130, 131</td>
<td>3.77 ± 0.00</td>
<td>143, 144, 145</td>
<td>5.60 ± 0.00</td>
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</tr>
<tr>
<td></td>
<td>144, 145, 146, 147,148</td>
<td>3.77 ± 0.00</td>
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<tr>
<td>Leucine</td>
<td>158, 159, 160, 161, 162, 163</td>
<td>4.42 ± 0.00</td>
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<tr>
<td>Isoleucine</td>
<td>158, 159, 160, 161, 162, 163</td>
<td>4.50 ± 0.00</td>
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<td></td>
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</tr>
<tr>
<td>Proline</td>
<td>142, 143, 144, 145, 146</td>
<td>5.04 ± 0.00</td>
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</tr>
<tr>
<td>Threonine</td>
<td>146, 147, 148, 149</td>
<td>5.54 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>175, 176, 177</td>
<td>5.54 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>132, 133, 134</td>
<td>5.69 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>175, 176, 177</td>
<td>5.69 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx*</td>
<td>188, 189, 190, 191</td>
<td>7.30 ± 0.00</td>
<td>115, 116</td>
<td>9.60 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Glx*</td>
<td>202, 203, 204, 205, 206</td>
<td>9.51 ± 0.00</td>
<td>143, 144, 145</td>
<td>10.70 ± 0.00</td>
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<tr>
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<td></td>
<td>230, 231, 232, 233, 234, 235</td>
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<td></td>
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<tr>
<td>Phenylalanine</td>
<td>192, 193, 194, ..., 200</td>
<td>10.37 ± 0.00</td>
<td>143, 144, 145</td>
<td>11.20 ± 0.00</td>
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</tr>
<tr>
<td>Lysine</td>
<td>156, 157, 158, 159, 160, 161</td>
<td>15.33 ± 0.00</td>
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</tr>
</tbody>
</table>

* Asx represents both aspartate and asparagine since the D-amino group of asparagine is lost during hydrolysation. Correspondingly, Glx represents both glutamate and glutamine.
Supplementary Table 2

Setting up correction matrices illustrated for the fragment Alanine 116.

<table>
<thead>
<tr>
<th>Metabolite part</th>
<th>2 C-atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Derivative part</td>
<td>3 C-atoms; 2 O-atoms; 10 H-atoms; 1 N-atom</td>
</tr>
</tbody>
</table>

**Correction for $^{13}$C**

$$C_{\text{corr}} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 0 & 1.0000000 & 0 \\ 0 & 0 & 1.0000000 \end{bmatrix}$$

**Correction for $^{17}$O**

$$C_{\text{corr}} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 2.0000000 & 1.0000000 & 0 \\ 0 & 0 & 1.0000000 \end{bmatrix}$$

**Correction for $^{18}$O**

$$C_{\text{corr}} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 0 & 1.0000000 & 0 \\ 2.0000000 & 0.0000000 & 1.0000000 \end{bmatrix}$$

**Correction for $^{15}$N**

$$C_{\text{corr}} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 1.0000000 & 0 & 0 \\ 0 & 0 & 1.0000000 \end{bmatrix}$$

**Correction for $^2$H**

$$C_{\text{corr}} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 0 & 1.0000000 & 0 \\ 0 & 0 & 1.0000000 \end{bmatrix}$$

**Total correction matrix**

$$C_{\text{total}} = C_{\text{corr}} \cdot C_{\text{corr}} \cdot C_{\text{corr}} \cdot C_{\text{corr}} \cdot C_{\text{corr}} \cdot C_{\text{corr}}$$

**The inverted total correction matrix**

$$C_{\text{total}}^{-1} = \begin{bmatrix} 1.0000000 & 0 & 0 \\ 0 & 1.0000000 & 0 \\ 0 & 0 & 1.0000000 \end{bmatrix}$$

**Corrected mass isotopomer matrix**

$$m = C_{\text{total}}^{-1} \cdot \begin{bmatrix} x_0 \\ x_1 \\ x_2 \end{bmatrix}$$


# The X-matrix refers to the mass isotopomer distribution obtained directly from the GC-MS measurement shown in Table 1, column 6.