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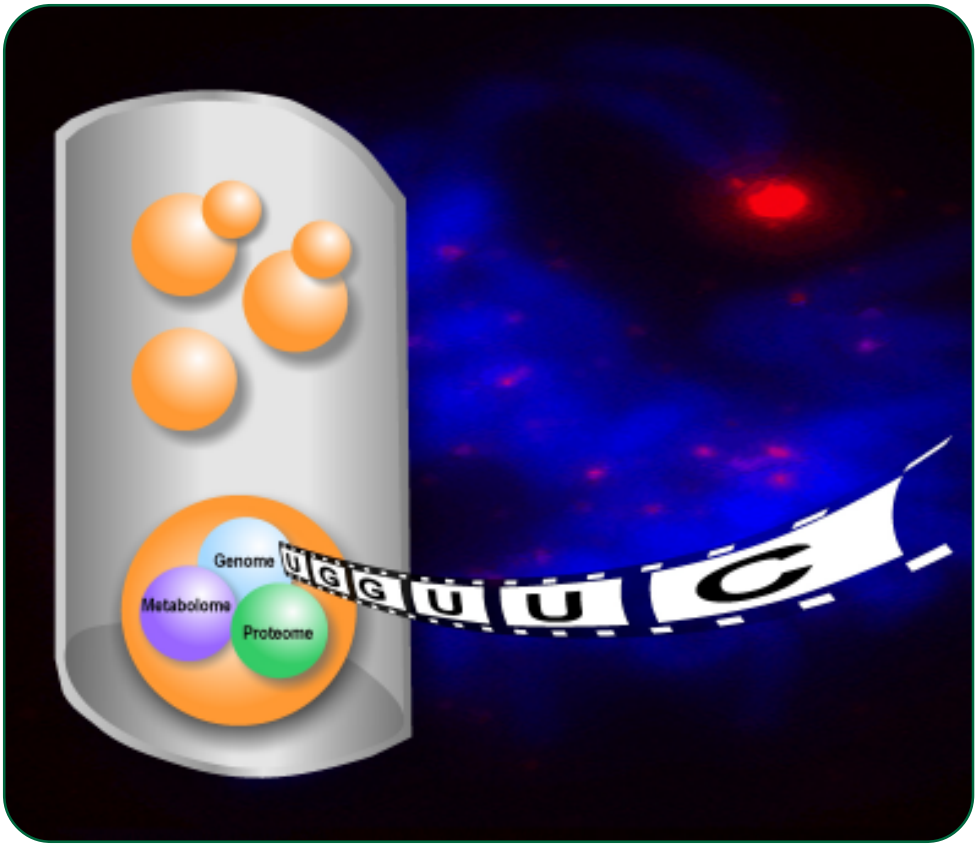

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Development of rapid gene expression analysis and its application to bioprocess monitoring

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Keywords gene expression, RNA analysis, functional genomics, bioprocess monitoring, yeast, filamentous fungi, protein production, brewing

Abstract

Cultivation of a microorganism in a bioreactor offers an ideal environment for optimized production of industrial compounds and for studying biological phenomena under reproducible conditions. In order to be able better to understand and control biological systems both for industrial and scientific purposes, development of methods that generate more detailed information about the biosystems is required. The focus in the development of tools for monitoring and control of bioreactor cultivations is on analyses that report on the physiological status of the production organism. Genetic expression is an important and growing aspect of cellular physiology, because the genomic sequences are becoming available for an increasing number of organisms. Technologies enabling studies of whole genome-wide expression analysis have provided large quantities of gene expression data under various conditions. One consequence of this has been the discovery of smaller sets of genes that provide the essential information about the biological system of interest. This has increased the need for technologies enabling rapid and cost-effective detection of specific gene transcripts.

The aim of the present study was to develop methods suitable for expression analysis of defined gene sets in bioprocess conditions, and to apply the methods for monitoring microbial cultures. The environmental conditions in bioreactor cultivations set certain challenges for the methodology. The environmental surroundings are typically in constant change during bioprocesses, requiring frequent analysis. In addition, the conditions are affected by various factors, such as decreasing nutrient and oxygen levels and increasing levels of secreted proteins or ethanol. Thus the number of relevant genes to be monitored in a process is dozens to hundreds rather than a few. For control purposes the response time of the method should be short.

The solution (sandwich) hybridization principle was applied in the development of two mRNA analysis methods: 1. a sandwich hybridization assay with alkaline phosphatase-based signal amplification and 2. a solution hybridization method called TRAC (Transcript analysis with the aid of affinity capture) using a pool of oligonucleotide probes separable and quantifiable by capillary electrophoresis. The basic sandwich hybridization assay detects one target per sample, whereas TRAC was capable of more than 20-plex RNA target detection. Both methods are performed in 96-well format with crude cell lysates as sample material. The developed methods have many advantages that make them suitable for monitoring microbial cultures. The analysis is simple (RNA extraction and cDNA conversions are avoided), the protocol time is short and for large numbers of samples the methods could be semi-automated by using magnetic bead processors. Multiplex target detection by the TRAC method makes it suitable for high-throughput gene expression analysis.

The TRAC method was applied for monitoring protein production processes and chemostat cultures of the filamentous fungus *Trichoderma reesei*, used widely in industrial enzyme production. In addition conventional beer fermentations by brewer's lager yeast (*Saccharomyces pastorianus*) were monitored by frequent analysis of gene expression facilitated by TRAC. Altogether about 30 *T. reesei* and 70 *S. pastorianus* genes were identified with presumed relevance to the respective processes and were subsequently tested in process conditions. Many of the marker gene expression profiles showed to have value in the prediction of consecutive physiological effects and of process performance both in the filamentous fungus and in yeast. Marker gene expression measured by TRAC could be used *e.g.* in evaluation of growth and of the production potential of secreted proteins, as well as in evaluation of nutrient and oxygen availability. In addition TRAC was used in the evaluation of gene expression stability during steady state conditions during *T. reesei* chemostat cultures as well as during transient oxygen deprivations. These data were applicable in the evaluation of steady state quality, which was useful when selecting samples for further systems-level analyses. The data obtained by TRAC confirmed the value of focused and frequent analysis of gene expression in monitoring biotechnical processes, providing a powerful tool for process optimization purposes.

Rautio, Jari. Development of rapid gene expression analysis and its application to bioprocess monitoring. [Nopean geenien ilmentymisanalyysin kehittäminen ja soveltaminen bioprosessien seurantaan]. Espoo 2007. VTT Publications 661. 123 s. + liitt. 83 s.

Avainsanat gene expression, RNA analysis, functional genomics, bioprocess monitoring, yeast, filamentous fungi, protein production, brewing

Tiivistelmä

Bioprosesseja käytetään teolliseen biologisten komponenttien tuottamiseen ja biologisten ilmiöiden tutkimiseen toistettavissa olosuhteissa. Jotta biologisia systeemejä voitaisiin sekä ymmärtää että kontrolloida paremmin, tarvitaan sellaisten menetelmien kehitystä, jotka tuottavat yksityiskohtaista informaatiota organismien fysiologisesta tilasta. Geenien ilmentyminen on tärkeä osa solujen fysiologiaa. Genominlaajuiset ilmentymisanalyysit ovat mahdollistaneet pienempien geeniryhmien identifioinnin, jonka avulla voidaan selvittää olennainen informaatio kiinnostuksen kohteena olevasta biologisesta systeemistä. Tämä puolestaan on lisännyt tarvetta tekniikoille, jotka mahdollistavat nopean ja edullisen geenien transkriptien mittauksen.

Tässä tutkimuksessa oli tavoitteena kehittää menetelmiä, jotka soveltuvat tiettyjen geeniryhmien ilmentymisen mittaukseen bioprosesseissa. Liuoshybridisaatioperiaatetta sovellettiin kehitettäessä sandwich-hybridisaatiomenetelmää sekä menetelmää nimeltä TRAC (Transcript analysis with the aid of affinity capture), jossa käytetään sellaisten oligonukleotidikoettimien joukkoja, jotka voidaan erotella ja kvantifioida kapillaarielektroforeesilla. Sandwich-hybridisaatiomenetelmää voidaan käyttää havainnoimaan yksittäisiä kohteita kustakin näytteestä, kun taas TRAC-menetelmä mahdollistaa yli 20 RNA-kohteen yhtäaikaisen mittauksen kustakin näytteestä, mikä tekee siitä tehokkaan ilmentymisanalyysin. Kehitettyjen menetelmien edut bioprosessien seurannassa ovat helppokäyttöisyys ja nopea mittaus suoraan solulysaateista. Näytteenkäsittely on osittain automatisoitu suurien näytemäärien yhtäaikaiseen käsittelyyn.

TRAC-menetelmää sovellettiin rihmasieni *Trichoderma reeseillä* suoritettujen proteiinituottoprosessien sekä lager-hiivalla (*Saccharomyces pastorianus*) suori-

tettujen käymisten seurantaan. Yhteensä identifioitiin 30 *T. reesei* ja 70 *S. pastoriasnus* -prosessien kannalta tärkeiksi oletettua merkkigeeniä. Useiden identifioitujen rihmasienen ja hiivan merkkigeenien ilmentymisprofiilien osoitettiin ennustavan sekä fysiologisia vaikutuksia että prosessien etenemistä. Lisäksi TRAC-menetelmää käytettiin geenien ilmentymistasojen stabiilisuuden mittaukseen *T. reesei* -kemostaattikasvatuksissa. Tätä dataa käytettiin arvioimaan kasvatusten laatua valittaessa näytteitä jatkoanalyyseihin. TRAC-menetelmällä kerätty data osoitti fokusoidun ja tiheään tapahtuvan geenien ilmentymismittauksen hyödyn bioteknisten prosessien seurannassa ja tarjosi täten tehokkaan työkalun prosessien optimointiin.

Preface

This study was conducted between 2001 and 2006 in the Protein Production Team at VTT Technical Research Centre of Finland and in the Bioprocess Engineering Laboratory at the University of Oulu. Financial support from Tekes – Finnish Funding Agency for Technology and Innovation, the Academy of Finland (Finnish Centre of Excellence Program, 2000–2005, Project no. 64330 and SUNARE program, Project no. 52796), the Finnish malting and brewing industry (Panimolaboratorio Oy), the Graduate School in Chemical Engineering, Tekniikan Edistämissäätiö, and the Finnish Cultural Foundation is gratefully acknowledged.

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I wish to thank all of my co-authors – Kari Kataja, Reetta Satokari, Merja Penttilä, Hans Söderlund, Markku Saloheimo, Bart Smit, Marilyn Wiebe,

Michael Bailey, Teemu Kivioja, Anne Huuskonen, Virve Vidgren, Heikki Vuokko, John Londesborough, Kim Barken, Juhani Lahdenperä, Antje Breitenstein, Soren Molin, and Peter Neubaer – for their valuable assistance in generating and presenting these results. Special thanks go to my colleagues Kari Kataja and Reetta Satokari for the pioneer work they have done in developing the TRAC method. Teemu Kivioja and Mikko Arvas are gratefully acknowledged for setting up all of the bioinformatic tools that saved a couple of years of my time. Bart Smit and Michael Bailey earn special acknowledgements for carrying out all the time-consuming bioreactor cultivations. The contribution of Marilyn Wiebe to this work by supporting me and Bart with all the cultivations as well as by providing excellent revisions of the manuscripts is highly appreciated. I also thank John Londesborough for his supervision in experimental design and for editing the manuscript of the yeast work. Science was also done during the nighttime with the yeast work. Thanks Anne, Virve, and Heikki for help with the unpleasant shifts.

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I thank my mother Tuula Rautio and my deceased father Mauno Rautio for their care and support over the years. I am sorry father, it seems there's going to be a third Ph.D. in the family, but I think we have been doing all right nevertheless.

My deepest gratitude goes to my wife Annastiina and our son Jarkko. Thanks for your patience while I was working on the thesis and even greater thanks for making me forget it while I was not working on it. You are the most precious things in my life.

Berkeley, CA, 21st of October 2007

Jari Rautio

List of publications

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals (Publications I–V).

I Rautio, J., Barken, K., Lahdenperä, J., Breitenstein, A., Molin, S. and Neubauer, P. 2003. Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates. Microbial Cell Factories. Vol. 2, No. 4.

II Rautio, J. J., Kataja, K., Satokari, R., Penttilä, M., Söderlund, H. and Saloheimo, M. 2006. Rapid and multiplexed transcript analysis of microbial cultures using capillary electrophoresis-detectable oligonucleotide probe pools. Journal of Microbiological Methods. Vol. 65, pp. 404–416.

III Rautio, J. J., Smit, B., Wiebe, M., Penttilä, M. and Saloheimo, M. 2006. Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*. BMC Genomics. Vol. 7, No. 247.

IV Rautio, J. J., Bailey, M., Kivioja, T., Söderlund, H., Penttilä, M. and Saloheimo, M. 2007. Physiological evaluation of the filamentous fungus *Trichoderma reesei* in production processes by marker gene expression analysis. BMC Biotechnology. Vol. 7, No. 28.

V Rautio, J. J., Huuskonen, A., Vuokko, H., Vidgren, V. and Londesborough, J. 2007. Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression. Yeast. Vol. 24, pp. 741–760.

Contents

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List of abbreviations

AP	Alkaline phosphatase
ATP	Adenoside triphosphate
BADGE	Beads array for the detection of gene expression
CE	Capillary electrophoresis
CHO	Chinese hamster ovary
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
DS	Dielectric spectroscopy
DTT	Dithiothreitol
FIA	Flow injection analysis
FPLC	Fast protein liquid chromatography
GC	Gas chromatography
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
HTB	High-throughput bioprocessing
IRS	Infrared spectroscopy
LOV	Lab-on-valve

MPSS	Massive parallel signature sequencing
mRNA	Messenger RNA
NIR	Near infrared
NIRS	Near infrared spectroscopy
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PCR	Polynuclease chain reaction
pO_2	Partial pressure of dissolved oxygen (also DO or DOT)
ppm	Parts per million
PyMS	Pyrolysis mass spectroscopy
RESS	Repression under secretion stress
RNA	Ribonucleic acid
SAGE	Serial analysis of gene expression
siRNA	Short interfering RNA
TRAC	Transcript analysis with the aid of affinity capture
TRF	Time-resolved fluorescence
UPR	Unfolded protein respose
VHG	Very high gravity

1. Introduction

The development of modern biotechnology has increased requirements for methods generating more detailed information about biosystems, so that they can be better understood, developed and controlled. Microbial cultivations in bioreactors offer a controlled environment not only for production of a wide range of biological products that are present in our everyday lives, but also for studying various biological phenomena. The development of tools for monitoring and control of bioreactor cultivations is focused towards analyses that are based on the physiological status of an organism. Genetic expression is one important aspect of cellular physiology that is currently widely studied.

For an increasing number of microorganims of scientific and industrial interest, all the genes in the genome have been sequenced, which in turn has enabled genome-wide expression studies. Global level transcriptomic analysis has generated large volumes of gene expression data from various conditions. One of the key aspects of this research is that in many cases the analysis of thousands of genes leads to the discovery of significantly smaller sets of genes, from a few to a few hundred, that provide the essential information about the given biological system. As a consequence, there is a need for technologies that allow rapid, cost-effective and quantitative detection of specific gene transcripts (RNA molecules). An increasing number of these techniques are becoming available, which are mainly developed for biomedical research but are also finding their way into industrial biotechnology.

1.1 Overview of biotechnical processes

In biotechnical processes (bioprocesses), living cells or cell components are utilized for production of biological products. The exploitation of microbial or animal/plant cell cultures for production of biological products involves growth of the cells in so-called bioreactors and is often referred to as a fermentation process. Today fermentation processes are used in the production of a wide variety of products, including whole cells, enzymes and other proteins, primary and secondary metabolites, DNA and other polymers. These products are used as medicines (*e.g.* insulin, erythropoetin) and vaccines, fuels (*e.g.* ethanol) and

beverages (beer, wine) or in the production of textiles, food and paper (see Table 1). Pharmaceutical products have the highest unit value, but amino acids, ethanol and industrial enzymes are produced in the largest quantities.

Table 1. Industrial fermentation product categories with examples of products, organisms used and application fields.

Category of Product	Products	Organism	Application field
Whole cells	Baker´s yeast	*Saccharomyces cerevisiae*	Beverage and bakery industry
	Lactic acid bacteria	Lactic acid bacteria	Food production
Beverage, food products	Beer, wine	*Saccharomyces cerevisiae, Saccharomyces pastorianus*	Beverage production
Primary metabolites	Ethanol	*Saccharomyces cerevisiae*	Biofuel
	Lactic acid	Lactic acid bacteria	Food additive, Ringer´s solution
	Glutamate	*Corynebacterium glutamicum*	Spice compound
	Riboflavin	*Bacillus subtilis*	Vitamin, Feed additive
Secondary metabolites	Penicillins	*Penicillium chrysogenum*	Medicine
	Statins	*Aspergillus terreus*	Medicine
Recombinant proteins	Insulin	*Saccharomyces cerevisiae*	Medicine
	tPA	Chinese Hamster Ovary cells	Medicine
	Erythropoetin	Chinese Hamster Ovary cells	Medicine
	Vaccines	Bacteria and yeast	Medicine
Enzymes	Cellulases	*Trichoderma reesei*	Detergents, starch and food industries
	Hemicellulases	*Trichoderma reesei*	
	Proteases	*Bacillus subtilis*	
Polymers	Xantan gum	*Xanthomonas capestris*	Food additive
	Polyhydroxyalkanoates	*Alcaligenes erytrophus*	Biodegradable orthopaedic aids
DNA	DNA	*Esherichia coli*	Vaccine, Gene therapy

Recombinant DNA technology, *i.e.* directed manipulation of the genome of a cell, has fundamentally expanded the potential for biological systems during recent decades, and has enabled the production of several of the products from the categories listed above or made their production economically more feasible. Thus genetic and metabolic engineering can be used as a means for constructing production strains for production of novel biotechnical products or for improvement of existing industrial organisms and fermentation processes. (Ryu & Nam, 2000; van der Werf, 2005)

1.1.1 Bioprocesses as tools for systems-wide studies

Microorganisms can be cultivated in bioreactors in carefully controlled environmental conditions. Different factors such as temperature, pH, nutrient and gas concentrations can be monitored and controlled. This makes bioreactor cultivations a preferred environment not only for optimised industrial production

of various compounds, but also for studying the biological behaviour and cellular physiology of a microorganism under reproducible conditions.

The genomes of a large number of microorganisms have been sequenced (www.genome.ad.jp). The number of genes in microbial genomes, depending on the complexity of the cells, ranges from the few hundred genes of a bacterium (*Mycoplasma genitalium* – 500 genes) (Fraser *et al.*, 1995) to the approximately 16600 genes of the filamentous fungus *Stagonospora nodorum* (www.broad.mit.edu). The genome represents all the functions which a cell can express (genotype). The actual functions expressed (phenotype) depend on which genes are transcribed to messenger RNAs (transcriptome) and further translated to active proteins (proteome) that carry out the actual cellular processes *e.g.* by interconverting metabolites (metabolome). A cell may express several different phenotypes depending on the environmental conditions to which it is exposed. The phenotype is controlled at many levels, including mRNA transcription and degradation, protein translation and (in)activation and interaction between proteins, DNA and metabolites (interactome) (see Figure 1). A major effort following the genome sequencing is to assign functions to all genes in the genomes. This field of research is known as functional genomics. Studying the biological interactions within cellular systems by methods including complex mathematical models is generally referred to as systems biology.

Advanced tools enabling systems-wide analysis at the level of the different -omes (genome, transcriptome, proteome, metabolome), in combination with efficient bioinformatic tools, have demonstrated the complexity of biological systems. Functions of living cells are not only defined by the information (genes) contained in the genome, but also by regulatory systems which control the expression of the genes, synthesis and modification of proteins and changes of metabolite profiles allowing cells to respond and adapt to fluctuations in their environment (Castrillo & Oliver, 2004, and references therein).

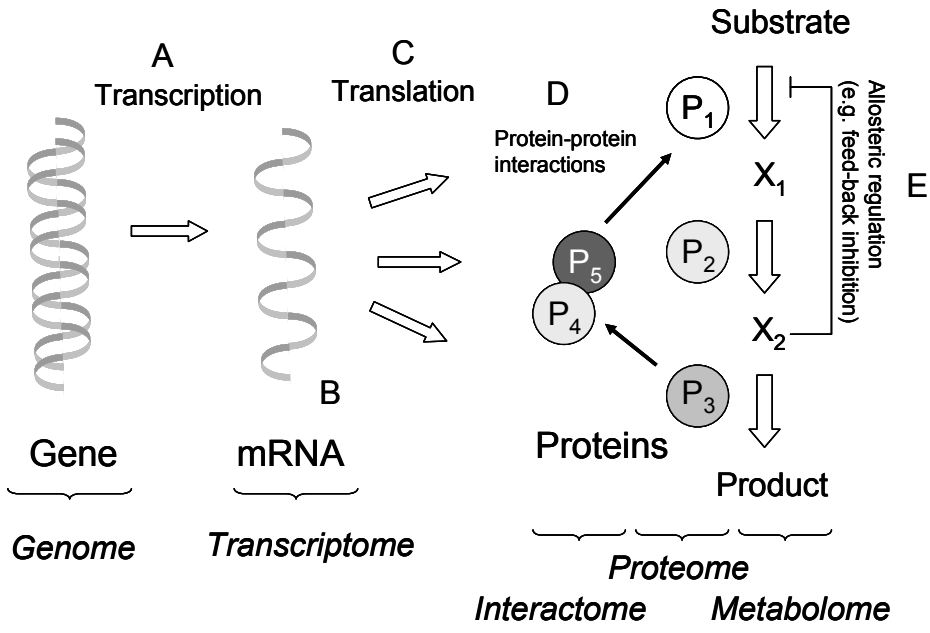

Figure 1. Overview of the process for expression of a specific phenotype in a cell. In transcription genes made of DNA (the genome is composed of all the DNA of the cell) are transcribed into messenger RNAs (the transcriptome consists of all transcibed mRNAs), which are further translated into proteins (the proteome consists of all translated proteins). Many proteins are enzymes (P_1–P_3) that catalyze biochemical reactions, such as catabolism of substrates to products. Other proteins have structural or mechanical functions, or are involved e.g. in cell signaling, adhesion and regulation (P_4–P_5). The phenotype is controlled at many levels: A) Transcriptional control, B) mRNA degradation, C) Translation control, D) Protein activation/inactivation and E) Allosteric regulation of enzymes (regulation by other metabolites). The metabolome represents all the metabolites of the cell. The interactome refers to the whole set of molecular interactions in the cell.

Studying this biological complexity using the -omics technologies is addressed using model organisms that are analysed under controlled conditions. Baker's yeast *S. cerevisiae* is the most widely used eukaryotic organism in these types of studies. This yeast has several features that make it a useful research tool: it is easy to cultivate, it is not pathogenic and powerful techniques for its genetic manipulation are available (Mnaimneh *et al.*, 2004). Furthermore *S. cerevisiae*

was the first eukaryotic organism for which the genome sequence was completed (Goffeau *et al.*, 1996), and thus the use of -omics technologies has been possible with *S. cerevisiae* for a longer period of time than for other eukaryotes.

Besides increasing the understanding of the complexity of cellular functions, the main practical purposes of studying the different -omes in biotechnically relevant microorganisms is to use the understanding gained to improve process conditions and in rational development of the organisms. Identification of rate-limiting steps in the production of desired products can lead to solutions that help in overcoming these bottlenecks by strain engineering, for example in metabolic engineering by overexpressing the gene(s) responsible for the rate-limiting step(s) or by inactivating those pathways contributing to by-product formation as well as deleting futile cycles (Vemuri & Aristidou, 2005). However, metabolic engineering strategies based on omics data have enjoyed only limited success. We are still far from understanding the regulatory mechanisms at the global level. Although these -omics technologies produce extremely useful information, the data which they provide represent snapshots of information transfer from gene to function, while missing the continuity of dynamic changes (Vemuri & Aristidou, 2005).

1.1.2 Modes of bioprocess operation

Bioprocesses are not only used for industrial production of compounds of interest, but also for creating controllable surroundings to study various biological phenomena. Modes of biotechnical process operation used for these purposes are batch, fed-batch and continuous type cultures. A short overview of these is given in the following.

In a **batch** process all substrate components are available from the beginning of the fermentation. Only gaseous compounds such as oxygen and pH- and foam -controlling agents are added during a batch process. The biomass concentration in a batch process increases until some limiting factor, such as low substrate concentration, reduces the growth rate or a side product accumulates to toxic levels (Figure 2a).

In a **fed-batch** process one or more substrate components are added in such a way that its concentration is reaction rate limiting, which enables control of reaction rate by the feed rate. The volume of the medium increases in the bioreactor and the biomass concentration increases at a rate that is proportional to the feed rate (Figure 2b).

In a **continuous** process complete medium is fed to the bioreactor and the content of the reactor is continuously withdrawn at the same rate to keep the volume constant. Continuous processes can be controlled in alternative ways. In the chemostat culture one component is selected to be reaction rate limiting and the feed rate is constant (Figure 2c). In other control modes (auxostats) some variable, such as optical density, pH or substrate concentration, is used to control the medium flow rate (Enfors & Häggström, 2000; Nielsen *et al.*, 2002).

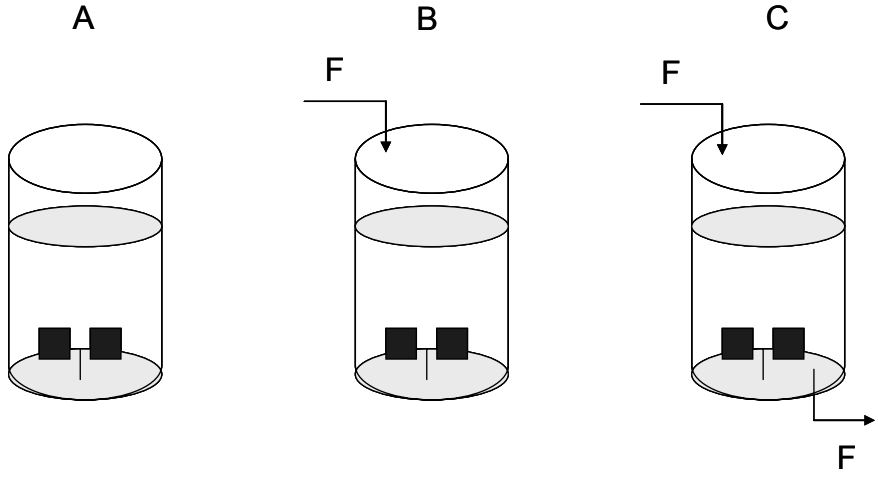

Figure 2. Different modes of fermentation processes. A. Batch, B. Fed-Batch, and C. Continuous (Chemostat) cultures (F = Flow rate).

In industrial production of biological compounds batch and particularly fed-batch type cultures are preferred modes of operation. For large scale production batch and fed-batch cultures are simpler to set up and operate than continuous flow cultures, and evolution of the biological system is avoided (Wiebe *et al.*, 1994). Chemostat and other continuous flow cultures are however increasingly preferred for production of biomass for global omics studies. In this mode of process operation the cell population grows at a constant rate in a constant

environment, providing biomass in a physiological steady state. Thus reproducible physiological studies can be carried out in conditions where a single chosen parameter may be varied while all others are kept constant. By contrast, experiments in batch cultures, with constantly changing environmental conditions, produce data sets that are difficult to interpret (Hayes *et al.*, 2002; Hoskisson & Hobbs, 2005).

1.2 Filamentous fungi and yeasts as industrial production organisms

Bacteria, yeasts, filamentous fungi and animal and plant cells are all employed by the biotechnology industry (Table 1). Selection of a strain for production a compound of interest depends both on the strain characteristics (*e.g.* growth, capability for secretion and post-translational modifications) and on the properties and application of the product (pharma/food product). The two industrial production strains investigated in the present study, the filamentous fungus *Trichoderma reesei* used widely in enzyme production and the yeast *Saccharomyces pastorianus* (*S. carlsbergensis*) used in beer production, are discussed here in more detail.

1.2.1 The filamentous fungus *Trichoderma reesei*

Filamentous fungi have been widely used for industrial scale production of homologous and heterologous proteins, due to their natural ability to secrete large amounts of proteins, especially hydrolytic enzymes. The simple nutritional demands of filamentous fungi are a further advantage. T. reesei is the best studied cellulase-producing fungus, mainly because it is an important organism in industrial cellulose and hemicellulase production. Many super-secreting strains of T. reesei have been made by random mutagenesis and selection, by recombinant DNA techniques and by the combined use of these approaches. As a result, production of native enzymes by *T. reesei* can exceed even 100 g/l (Cherry & Fidantsef, 2003). Many applications for enzymes produced in T. reesei have been developed for textile treatment, animal feed processing and the pulp and paper industry (Buchert et al., 1998; Galante et al., 1998a; Galante et al., 1998b).

T. reesei possesses strong cellulase promoters and can carry out post-translational protein modifications in a manner resembling mammalian cells (glycolysation, cleavage, disulfide bond formation), making it a preferred host for production of heterologous proteins. Especially gene products originating from other fungi can be produced heterologously in *T. reesei* up to gram per litre levels, such as *Aspergillus* phytase (Paloheimo *et al.*, 1993) and *Melanocarpus albomyces* laccase (Kiiskinen *et al.*, 2004).

1.2.1.1 Protein secretion by *T. reesei*

The major part of the proteins secreted by *T. reesei* are cellulases and hemicellulases, that enable the fungus to utilise cellulose and other complex plant polysaccharides for growth. The cellulase activity produced by *T. reesei* is composed of synergistically acting cellobiohydrolases (CBH, exoglucanase), endoglucanase (EG) and β-glucosidases, which hydrolyse cellobiose to glucose. The regulation of the expression of these enzymes in *T. reesei* is complex and only partially understood. Transcription of the major cellulase genes, such as *cbh1* and *egl1* encoding cellobiohydrolase 1 and endoglucanase 1 respectively, is induced by cellulose, oligosaccharides and also by a veriety of disaccharides including lactose, sophorose and cellobiose. Presence of the preferred carbon sources, glucose and fructose, represses these genes (Ilmen *et al.*, 1997; Nogawa *et al.*, 2001). Microarray studies of the partially sequenced genome have shown that transcription of the known biomass-degrading enzymes is highly co-regulated (Foreman *et al.*, 2003).

In filamentous fungi the majority of secreted proteins, including heterologous gene products, have been suggested to be secreted through the growing hyphal tip (Wösten *et al.*, 1991). There is, however, evidence that secretion can also take place in older parts of the mycelium (Nykänen, 2002). Proteins designated for secretion first enter the endoplasmic reticulum (ER), where folding and core glycosylation takes place. Proteins are transported from the ER to the Golgi complex in vesicles, where further modifications are completed. Finally, these proteins are packed into secretory vesicles, which fuse with the plasma membrane resulting in externalization of the proteins to the surrounding medium through the fungal cell wall (Conesa *et al.*, 2001).

One essential function of the ER is protein quality control and subsequent degradation of misfolded proteins. In a response to accumulation of unfolded proteins within the ER a large set of genes is up-regulated to increase the protein folding capacity and transport. This phenomenon is known as the unfolded protein response (UPR). The UPR can be induced by various factors such as dithriothreitol (DTT), a folding inhibitor, mutations in the secretory pathway genes, overloading of the secretory pathway and expression of heterologous proteins (Saloheimo *et al.*, 1999; Saloheimo *et al.*, 2004). A transmembrane serine/threonine kinase encoded by *ire1* functions as the most upstream component of the UPR pathway by sensing unfolded proteins in the ER and transferring the signal to the transcription factor Hac1p. Active Hac1p in turn facilitates transcriptional up-regulation of ER chaperons and foldases. Among the best studied proteins under UPR control are the molecular chaperon BiP and protein disulfide isomerase PDI, which is involved in the formation of disulfide bridges. Expression analyses in *S. cerevisiae* (Travers *et al.*, 2000) and *T. reesei* (Arvas *et al.*, 2006) have shown, however, that many genes are linked to the UPR and have functions that are not limited to protein folding but cover all stages of the secretory pathway, including *e.g.* glycosylation, lipid biosynthesis and vacuolar sorting.

Induction of cellulase production by adding lactose to glucose-grown *T. reesei* culture has been shown to cause coordinate induction of cellulase genes and genes involved in UPR related functions (*pdi1*, *bip1* and *hac1*), indicating an increased requirement for protein folding machinery under these conditions (Collen *et al.*, 2005). However, cellulase induction has been shown not to cause any major induction of genes involved in protein processing and secretion (Foreman *et al.*, 2003). Growth rate has also been shown to affect the rate at which proteins are synthesized and secreted in *T. reesei*. Secreted enzymes were produced most efficiently at low growth rate (0.031 h^{-1}). However, at low growth rates the secretion/synthesis rate ratio was lower and the expression of UPR-related genes higher than at high specific growth rates, indicating limitations in the secretion capacity at low growth rates (Pakula *et al.*, 2005).

Simultaneously with up-regulation of UPR-related genes, a second regulatory response mechanism results in down-regulation of genes encoding the major secreted proteins in fungi. This effect, known as repression under secretion stress

(RESS) (Pakula *et al.*, 2003), was recently identified in *T. reesei* treated with the protein folding inhibitors DDT and brefeldin A and has also been observed in *Aspergillus niger* (Al-Sheikh *et al.*, 2004). Genes subjected to RESS in *T. reesei* included the cellulase genes *cbh1*, *cbh2*, *egl1*, *egl2* and the hemicellulase gene xyn1, which together form a major part of the secreted proteins naturally produced by *T. reesei*. The constant expression of the gene *bgl2* encoding for the intracellular hydrolytic enzyme, β-glucosidase, during treatment with DTT indicates that RESS only affects the genes encoding secreted enzymes.

The genome of *T. reesei* has recently become publically available (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Combination of the genome data with microarray and proteomic analysis enables a more systems-wide approach to study these protein secretion-related phenomena in this fungus. Genomes of several other filamentous fungi, such as various *Aspergillus* species (*A. nidulas, A. fumigatus, A. oryzae*) and *Neurospora crassa*, have also recently been sequenced and annotated. The gradual accumulation of functional genomics data from different filamentous fungi can be thus be expected in the near future, as has happened with *S. cerevisiae* since its genome sequence became available.

1.2.2 Fermentation of wort to beer by *Saccharomyces pastorianus*

The fermentation of wort to beer is one of the most ancient forms of biotechnology, but the complexity of this process from the biological perspective is still being explored. During the brewing process yeast must adapt to sequential and simultaneous environmental changes. During the initial part of fermentation yeast is exposed to high osmolarity caused by high sugar concentrations. As fermentation proceeds, the yeast must adapt to various other stress factors, such as gradual depletion of essential nutrients, anaerobiosis, increasing ethanol concentration and acidification of the medium. Initial osmotic and late high ethanol stresses become more severe when (very) high gravity worts, containing high concentrations of fermentable sugars, are fermented (Casey *et al.*, 1984). Yeast responds to the dynamic surroundings by altering its genetic expression, protein synthesis and/or interaction, accumulating stress-protectants and activation of repair functions. The stress factors affect the viability and vitality

of the yeast (Gasch & Werner-Washburne, 2002), and thus poor adaptation can result in defective fermentations. Various individual stress and environmental factors have been widely studied in laboratory yeasts (Causton *et al.*, 2001; Gasch *et al.*, 2000), but still only a rather limited number of systems-level approaches are available to gain better understanding of the adaptation mechanisms during actual process conditions.

There are two main types of brewer's yeasts. The ale yeasts are *S. cerevisiae* strains that are related to *S. cerevisiae* laboratory strains. Lager type brewer's yeasts, *S. pastorianus* (syn. *S. carlsbergensis*) are, however, hybrids between *S. cerevisiae* and another *Saccharomyces* species, most commonly *S. bayanus* (Casaregola *et al.*, 2001; Naumova *et al.*, 2005). Some lager strains can may also have portions of the *S. uvarum* genome or be hybrids between *S. bayanus* and *S. uvarum* (Rainieri *et al.*, 2006). Two dimensional (2-D) electrophoresis maps of lager yeast proteins were found to consist of two elementary patterns of proteins, presumably encoded by the two different sets of chromosomes in lager yeast (Joubert *et al.*, 2001). This poses a challenge for genome-wide profiling by microarray techniques, since mRNAs originating from the non-cerevisiae part of the transcriptome will either be undetectable or it will be measured as part of the expression from the genes originating from *S. cerevisiae*. An additional challenge to genome-wide data analysis of brewing fermentations arises from the multitude of gradually altering growth conditions, which makes the interpretation of such data patterns complicated.

Despite these hindrances, both the transcriptomic and proteomic analyses of brewer's yeast have provided valuable insights into many aspects of the yeast's responses to the altering surroundings in brewing conditions. In lag and early exponential growth phases, during the initial hours of the fermentations, yeast increases the expression of genes and/or proteins involved in ergosterol biosynthesis, protein synthesis and translocation, amino acid biosynthesis, glycerol metabolism and oxidative stress (Brejning *et al.*, 2003; Higgins *et al.*, 2003a). The yeast membrane component ergosterol has been shown to function in restoring the fermentative capacity after storage (Higgins *et al.*, 2003a). Since ergosterol biosynthesis requires oxygen, wort is generally aerated prior to fermentation in order to ensure membrane synthesis. Ergosterol may also be essential to protect yeast against ethanol stess (Alexandre *et al.*, 2001). During

wine fermentation, in which the ethanol concentration increases to over 10% (vol/vol), oxygen is sometimes added to stimulate sluggish fermentations (Sablayrolles *et al.*, 1996).

When yeast cells enter the stationary phase either in beer or wine fermentations, a variety of heat shock proteins such as Hsp104 and Hsp30 responding to a variety of stress factors, is induced in a strain-dependent manner (Brosnan *et al.*, 2000; Riou *et al.*, 1997). Genome-wide analyses during wine fermentation have shown that onset of the stationary phase triggered major transcriptional reprogramming, resulting in increased expression of starvation and ethanol stress responses (Rossignol *et al.*, 2003). However, many of the stress responses are repressed as the fermentation proceeds, indicating that the major stress responses are transient (Gasch *et al.*, 2000)

Ethanol inhibits yeast growth and key glycolytic enzymes, decreases viability and affects the plasma membrane and transport of amino acids and sugars (Alexandre & Charpentier, 1998). Thus ethanol can be considered as the main stress factor during alcoholic fermentations. *Saccharomyces* species have evolved adaptation mechanisms towards ethanol, such as up-regulation of heat shock proteins, activation of plasma membrane ATPase and regulation of membrane lipid composition (Fernandes & Sa-Correia, 2003; Piper, 1995; Swan & Watson, 1999). Transcriptome analysis has revealed altered expression of more than 6% of the genome during short term ethanol exposure. Ethanol-induced genes were involved in ionic homeostasis, heat protection, trehalose synthesis and antioxidant defence (Alexandre *et al.*, 2001). Many of these genes are regulated by the general stress transcription factors Msn2p and Msn4p, but a transcription factor Asr1p responding specifically to ethanol stress has also been identified (Betz *et al.*, 2004).

Potential practical applications of the gradually increasing knowledge of physiological responses and adaptation of yeast in actual brewing conditions could be *e.g.* development of means for optimisation and control of growth (Brejning *et al.*, 2003; Higgins *et al.*, 2003a; James *et al.*, 2003), creating stress tolerant strains (Hirasawa *et al.*, 2006) and finding strain-dependent differences to be used in further selection of better adapted strains for standard and high-gravity conditions (Brosnan *et al.*, 2000; Devantier *et al.*, 2005).

1.3 Monitoring the physiological state of microbial cultures

As discussed above an increasing number of genome sequences has become available, and consequently technologies enabling -omics studies of microorganisms in controlled conditions have been developed. Furthermore, recent progress in genetic and metabolic engineering has enabled the rapid engineering of new microbial strains, bringing an increased requirement for tools to analyse the physiological consequences of the changes at the cellular level. The biotechnical industry has a growing demand for process monitoring tools allowing better process modelling, control and optimization (Process Analytical Technology iniative, PAT by FDA, 2004). Thus a key issue in the design of novel bioprocesses and in the optimisation of existing ones is a quantitative analysis of the cellular functions that are important for the desired performance of the production organisms and how they respond to the dynamic environment in bioreactor cultures.

Process control aims at keeping a biotechnical process in the desired state. Some variables conventionally analysed from bioprocesses, such as dissolved oxygen, pH and temperature are measured on-line outside the cell, providing only indirect information on the intracellular physiological status. New analytical tools measuring intracellular variables such as proteins, RNA and metabolites, allow more direct evaluation of the physiological status, but their applicability to on-line monitoring is limited (Schuster, 2000). The potential benefits to industry of such monitoring systems include better understanding of processes, increased batch-to-batch reproducibility, increased operation efficiency and cycle time reduction (Schuster, 2000; Schweder & Hecker, 2004). In the following an overview is given of the measurement technologies used in the monitoring of bioprocesses, with emphasis on the methods directly assessing different aspects of the cellular physiology. A summary of the process monitoring methods is given in Table 2.

1.3.1 Sampling technologies

Different process monitoring methods require different sample processing procedures prior to measurement, and this can be also used in the classification

of these methods. The following definitions for the measurement strategies are used in this thesis:

- On-line: The sample is diverted from the production process for analysis with the possibility of returning the sample to the process.
- At-line: The sample is removed manually from the production process and analysed in close proximity to the process.
- In-line: No sample is removed from the manufacturing process, but analysis is by an invasive or non-invasive measurement.
- Off-line: The sample is removed manually from the manufacturing process and transported to laboratory for analysis.

Many analyses in the bioprocess industry are still routinely performed off-line, *i.e.* part of the growth medium is manually withdrawn from the bioreactor through a sterilizable valve and the sample is transported to the laboratory for analysis. Development of measurement strategies has also led to development of *in situ* sampling methods, *i.e.* the analysis sensor is inside the fermentor or *in situ* sampling is used and the sensor is outside the bioreactor (*ex situ*).

1.3.1.1 Flow Injection Analysis

Flow Injection Analysis (FIA) is a sampling technique for instant sampling using injection of small amounts of samples into the carrier stream. FIA is an *ex situ* rather than an *in situ* method, but analysis times are commonly only a few minutes. FIA systems have been connected with various detection systems for on-line monitoring of medium components, such as pH, CO_2, sodium, glucose, ethanol, ammonium, as well as for monitoring production of proteins and antibodies (Harms *et al.*, 2002; Schugerl, 2001, and references therein). Short response time, high flexibility and inexpensive components have made FIA systems widely used in on-line process monitoring. (See Figure 3.)

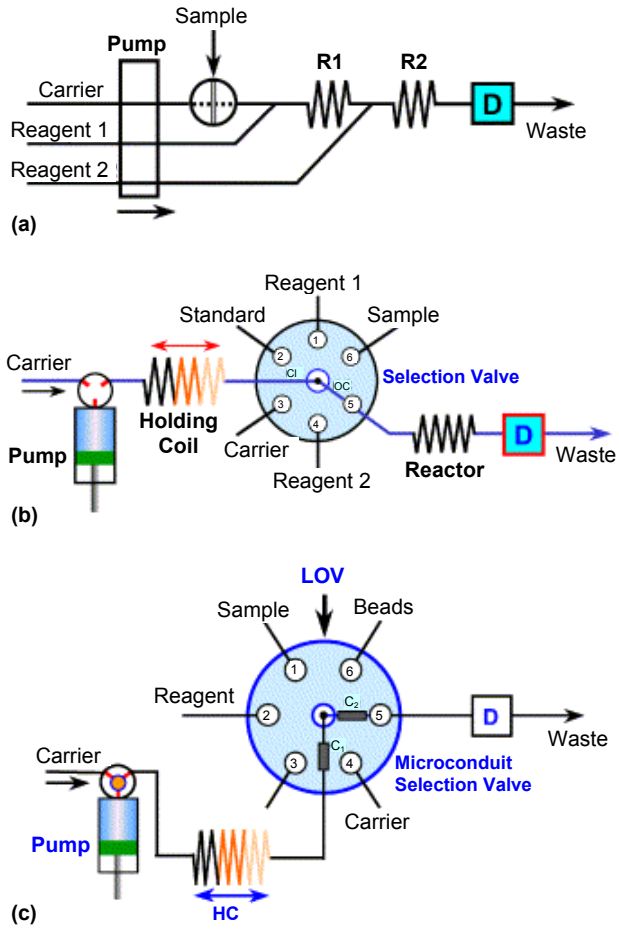

Figure 3. The three generations of FIA: (a) A typical FIA-manifold, where a defined volume of sample is injected into a continuously flowing carrier stream, which is subsequently merged with two reagent streams. Reactions are taking place in reactors 1 and 2 (R1 and R2). A suitable detector (D) is used for sample monitoring. (b) Typical sequential injection analysis SIA, based on the use of a selection valve and a bi-directional syringe pump. (c) A schematic drawing of a lab-on-valve (LOV) system, the concept of which is a microconduit placed atop a selection valve. The microconduit should ideally contain all means for executing sample manipulations and chemistries required, as well as detector, i.e. act as a small laboratory. However, with large instrumental detector devices, it is necessary to employ external detection (D) as shown in the figure. Besides aspirating liquids, it is also possible to handle small beads (furnished with active functional groups) (Hansen, 2004).

A more sophisticated version of FIA is Sequential Injection Analysis (SIA) that uses computer controlled and programmable bi-directional discontinuous flow. SIA enables exact use of very small volumes (< 1 µl), making sample and reagent consumption substantially smaller than in traditional FIA (Hansen, 2004). Yet another generation of injection analysis is the so called lab-on-valve (LOV), which contains integrated analysis equipment, designed to perform all the necessary operations for a given assay. LOV may contain mixing points for analyte and reagents, packed column reactors or beads with active groups (*e.g.* immobilized enzymes or antibodies) as well as detection instruments (Hansen, 2004), such as a capillary electrophoresis device (Wu *et al.*, 2003).

1.3.2 Culture morphology by microscopy

Observing morphology is one of the oldest methods used for monitoring the physiological status of a cell culture. This can give valuable information about the state of an organism, especially in the case of fungal cultures. Both the medium (nutrient concentrations, pH, *etc.*) and physical environment (temperature, fermenter geometry, agitation) affect the morphology of fungal cultures. Particular morphological forms achieve maximum performance, but due to the multiple environmental factors that influence morphology, determining the relationship between process variables, product formation and fungal morphology is rather complex (Papagianni, 2004). Yeasts and bacteria also display distinct morphological changes that can be related to physiological events. For example, oxidative stress has been shown to affect *S. cerevisae* morphology (Belo *et al.*, 2005) and the shift of metabolic pathways between solvent and acid production by *Clostridia* is related to morphology changes (Jones & Woods, 1986).

The subjectivity of microscopic observation makes quantification of the data difficult, and morphological information has often been neglected. Use of novel automated computer-based image analysis makes collection of quantitative information more reliable. Such information can be used, for example, in characterization of complex mycelial morphologies and physiological states and their interactions to build up structured models that have predictive value for process performance (Papagianni, 2004). However, the application of such models for monitoring of industrial productions is limited. Sample preparation

and analysis by microscopy are still too time-consuming for routine process monitoring. Calculation of cell density, and estimations of size distribution and degree of cell aggregation have been performed by *in situ* microscopy, involving a CCD-camera and digital image processing software (Joeris *et al.*, 2002). Such machine vision systems could potentially also be useful in morphology monitoring.

1.3.3 Monitoring based on chemical separation

In addition to microscopy, analytical techniques based on chemical separation (chromatography and electrophoresis) have already been used for decades for monitoring of microbial cultures.

Chromatographic methods including gas chromatography (GC), high performance liquid chromatography (HPLC), fast protein liquid chromatographry (FPLC) and membrane chromatography are mainly used in off-line analysis of bioprocesses and down-stream processes. The long analysis time, need for protein purification from cultivation media prior to analysis and low reproducibility are factors that limit the applicability of these methods for on-line monitoring and process control. As off-line culture analysis they are used *e.g.* for analysis of lipid or fatty acid patterns and protein and carbohydrate concentrations. (Schugerl, 2001; Schuster, 2000)

Protein patterns of whole cells can be analysed by simple one dimensional polyacrylamide gel electrophoresis (PAGE). Such analysis resolves 20 to 50 bands of protein groups according to their molecular mass. For some organisms, changes in the protein composition as a response to physiological events are strong enough to be detected by PAGE (Terracciano *et al.*, 1988) and can be used as semi-quantitative markers for the physiological status. More detailed analysis of cellular proteins can be performed by two-dimensional electrophoresis, with separation based on molecular mass in one direction and isoelectric focusing in the other yielding some hundreds of individual protein spots. Both of these analyses require long analysis times, are rather laborious and require staining, and are thus used as off-line analyses.

Capillary electrophoresis (CE) combines electrophoretic separation with direct and automatic quantitative analysis. It is a rapid analysis method, uses high voltage gradients and does not necessarily require staining. CE is suitable for analysis of proteins, amino acids, plasmids and nucleic acids. Due to the short response time CE-based analyses have potential for on-line monitoring. (Schugerl, 2001)

1.3.4 Flow cytometry

In flow cytometry large numbers of single cells or particles pass through a laser beam in a directed fluid stream. When the laser beam impacts a cell, the excitation light is scattered in forward and sideways directions. The forward scattered light provides information about the the size of the cells, whereas the sideways scattered light is affected by several paramenters, including granularity, cell size and morphology. Intracellular components that possess an intrinsic fluorescence (*e.g.* NAD(P)H), or can be stained with fluorescence dyes allow selective assays of certain cell components. The combination of flow cytometers with sorting units offers the possibility to separate sub-populations. The possibility of analysis of a wide variety of cell characteristics (size, shape, viability) and cell components (DNA, RNA, antigens) and their distribution in the populations has made flow cytometry analysis attractive for microbial culture analysis (Rieseberg *et al.*, 2001).

1.3.4.1 Targets and biotechnological applications

Flow cytometry offers alternative ways to assess viability for evaluation of bioprocesses. The most common way is to measure membrane integrity. Defective membranes leak intracellular components such as proteins and other fluorescent products. Exclusion dyes bind to nucleic acids in the dead non-viable cells (with defective membranes), whereas retention dyes are degraded by intracellular enzymes (present only in living cells) to yield fluorescent products that are trapped in the cell. Alternatively, membrane potential can also be used as an indicator of cell damage, and fluorescent annexin-V conjugates can be used to assay externalisation of phosphatidyl serine (Rieseberg *et al.*, 2001), which is one of the earliest indicators of apoptosis. Cellular growth rates can

also be measured by flow cytometry using bromodeoxyuridine coupled with propidium iodide staining, which allows calculation of the doubling time (Wilson, 1994).

The cytosolic pH regulates various cellular processes and functions, providing an indicator of the physiological state and metabolic activity of the cell culture. A variety of fluorogenic esterase substrates, diacetate derivatives and other pH-sensitive dyes have been developed for this purpose (Boyer & Hedley, 1994). Cytosolic calcium concentration plays an important role in several cellular functions including enzyme activity, and bioprocesses based on a high rate of cell growth are influenced by the concentrations of calcium and magnesium and their interactions (Walker, 1999). Flow cytometry has been used to analyse the effects of different stress factors on calcium concentrations in Chinese hamster ovary (CHO) and insect cell cultures (Aloi & Cherry, 1994; Kaneko *et al.*, 2000).

The DNA content of cells fluctuates depending on the phase of the cell cycle. Thus the nucleic acid-binding fluorescent dyes are commonly used for viability testing and cell cycle analysis. The cell cycle and growth behaviour of *S. cerevisiae* have been studied by flow cytometry in synchronised and unsynchronised cultures for example to correlate with other fermentation parameters for process optimisation (Rieseberg *et al.*, 2001), and to evaluate propagation and fermenting yeast for brewing processes (Hutter & Lange, 2001; Muro *et al.*, 2006).

In addition, flow cytometry has been applied to monitor total intracellular protein content in yeast cells, efficiency of intracellular protein production in bacterial and yeast cultures and antibody production in mammalian cell cultivations, and to study mixed populations of microorganisms using *in situ* hybridization with fluorescently labelled rRNA-targeted oligonucleotide probes (Rieseberg *et al.*, 2001).

1.3.4.2 Limitations and applicability for on-line control

The extensive use of flow cytometry for biotechnology applications still has certain limitations. Flow cytometry analysis is suitable only for single cell suspensions. Cell tissues or adherent cells must be mechanically or enzymatically treated prior to measurement. Most applications of flow cytometry are based on

fluorescence monitoring. Studying specific cell components with fluorescent dyes requires staining procedures (including fixation, staining, washing steps) and often also permeabilisation of the cells before the analysis can be performed. These are time-consuming steps, especially considering on-line applications of these techniques. Furthermore, the required instruments are costly and need well trained personnel for their operation. (Rieseberg *et al.*, 2001)

Despite the hindrances, flow cytometers have been coupled with flow-injection analysis (FIA) systems for on-line process monitoring. The bioreactor sample is pumped to a reaction loop containing fluorescent dye solution and is transferred further to the cytometer. Such a system can be used for rapid determination of cell number, viability and size distribution (Rieseberg *et al.*, 2001).

1.3.5 Spectroscopic monitoring of bioprocesses

Many biological compounds interact with radiation (*e.g.* electromagnetic radiation or light) and are thus detectable by spectroscopic methods. Optical sensors are used increasingly for bioprocess monitoring, since they can be used inside the fermenter (*in situ*), with living cells (*in vivo*), providing real-time data that allows effective process control. Spectroscopic methods are also used for analysis of intracellular compounds off-line, at-line or on-line. Optical sensors also offer interesting possibilities for microbioreactor-based high-throughput bioprocessing (Betts & Baganz, 2006), since these systems function with small volumes (1 ml or less) (Ulber *et al.*, 2003). The main spectroscopic methods used in monitoring of cellular cultures are presented in the following.

Infrared spectroscopy (IRS) has great potential to provide off-line and on-line information in different bioprocesses. IRS is at least a semi-quantitative method to follow changes in all major biomass components (protein, carbohydrate, lipid and nucleic acids) from dried microbial samples. The spectra are analysed as multi-component mixtures, *i.e.* characteristic bands in the spectra are identified, the extinction coefficients for each component at each band are determined and the concentrations are calculated by linear equations (Schuster, 2000). As compared to chemical analysis of each component in separate assays, the IRS measures all components simultaneously and relatively quickly. IRS has also

been applied for on-line monitoring of *e.g.* glucose, fructose, proline, ammonia and ethanol in growth media (Harms *et al.*, 2002; Scarff *et al.*, 2006). The high cost, difficulty of calibration and challenges in application of the technique to processes with complex media or filamentous organisms has hitherto prevented widespread use of this method.

Near infrared spectroscopy (NIRS) also has great potential for quantitative chemical analysis in bioprocesses, since many biologically important chemical bonds (aliphatic C-H, aromatic or alkene C-H, amine N-H and O-H) absorb in the NIR range (Marose *et al.*, 1999). NIRS has been widely applied for measurement of biomass, substrate and product concentrations in industrial processes. These applications include NIRS measurement of biomass, glycerol, ammonium and acetate concentrations in recombinant *E. coli* fed-batch culture (Macaloney *et al.*, 1997) and monitoring of key analytes (glucose, lactate, glutamine and ammonia) in animal cell (CHO) cultures (Arnold *et al.*, 2003).

Raman spectroscopy is based on the phenomenon of the shifted wavelength of light scattered from molecules excited with monochromatic light due to non-elastic collision of photons with the molecule. It has been applied for monitoring of the glucose-ethanol transformation by yeast (Shaw *et al.*, 1999) and giberrelic acid 3 produced by *Giberella fujikoroi* (McGovern *et al.*, 2002). Analysis of chemical patterns at the single cell level can also be performed by Raman spectroscopy. Laser beams in the visible or near infrared range are used as excitation light sources and are focused *e.g.* through the optical set-up of a microscope (confocal technique). By this technique single living cells of higher organisms have been studied and strains of bacteria have been identified directly from colonies on solid culture media. (Maquelin *et al.*, 2000; Puppels *et al.*, 1990)

Dielectric spectroscopy (DS) (also known as capacitance monitoring) offers a means for on-line, non-invasive monitoring of the biomass. The capacitance signal of the solution measured by DS increases in direct proportion to the total enclosed biovolume, *i.e.* the volume completely enclosed by the cytoplasmic membranes of all biological material within the electrical field. Thus, objects without an intact membrane do not contribute to the capacitance (Davey *et al.*, 1993). DS has also been used to estimate the radius of hybridoma, yeast and bacterial cells (Hauttmann & Müller, 2001), but with filamentous organisms the interpretation of DS signals is difficult.

Pyrolysis mass spectroscopy (PyMS) can be used for classification and identification of organisms (Goodacre & Kell, 1996). In addition PyMS has been used in monitoring growth stage and morphological differentiation in cultures of *Streptomyces albidoflavus* (Nakajima *et al.*, 1998), and for detection of intracellular substances such as indole or recombinant proteins during cultivation. Although PyMS is powerful in terms of selectivity and sensitivity, the spectra are very complex. The data evaluation requires sophisticated mathematical methods such as supervised learning or neural networks (Schuster, 2000).

High resolution UV spectrophotometers have also been shown to be applicable for monitoring physiological events during bioprocesses. New UV spectrophotometers are capable of measuring continuous spectra, allowing several analytes to be monitored simultaneously (Noui *et al.*, 2002). For instance, Habib *et al.* (2000) demonstrated the usefulness of UV-spectral analysis for real-time monitoring of levels of RNA, protein and cell debris in order to control the flocculation process of *S. cerevisiae*.

Use of **fluorescence sensors** in bioprocess monitoring has mainly concentrated on measurement of NAD(P)H in the cultures. With two-dimensional fluorescence spectroscopy all fluorophores, such as proteins, coenzymes and vitamins, can be simultaneously detected qualitatively and quantitatively in intra- or extracellular samples. This method is suitable for on-line, *in situ* measurement and has been used in monitoring growth and metabolic changes *e.g.* in the shift from aerobic to anaerobic conditions (Marose *et al.*, 1998).

The green fluorescent protein (GFP) has been applied for many purposes in bioprocesses, such as determination of protein concentrations, and localization and trafficking of GFP fusion partners (Li *et al.*, 2002a). GFP-fusion proteins have also been used as model products in studying the effects of process parameters on product formation (Wang *et al.*, 2003). By connecting GFP to particular stress- or environment-sensitive gene promoters, changes in the GFP levels have been studied under changing oxygen, temperature and nutrient levels (March *et al.*, 2003; Reischer *et al.*, 2004). However, measurement of GFP from fermentation broths can be difficult, due to the strong fluorescent background from various medium components.

Affinity-based optical **biosensors** are used to measure biomolecular interactions. These instruments employ antibodies, receptors or enzymes as ligands that are immobilised on a sensor surface and detect reactants in solution. The detector converts the interaction into an electronic signal. For protein analysis during bioprocesses and down-stream processing, different types of affinity sensors have been developed including surface plasmon resonance (SPR) and reflectometric interference spectroscopy (RIF). Since these techniques enable rapid on-line measurements of extracellular proteins in complex environments, they have become attractive alternatives to conventional ELISA assays (Baker *et al.*, 2002). SPR has also been applied to quantification of intracellular recombinant protein production in sonicated *E. coli* samples collected from fermentations (Ivansson *et al.*, 2002).

1.3.6 Marker molecules in detection of physiological events

When gradual or sudden changes in the prevailing environment of the culture occur, the cells exhibit a pattern of responses (temporary) that will result in adaptation (long term) of the culture to the new surroundings. The dynamic responses affect the complex biological network, consisting of gene expression, protein expression and interaction and flow of metabolites. Some of these responses are common to a wide range of environmental factors, whereas others occur in a more specific manner. Those biological molecules, of which the levels or activities behave in a predictable way, can be used as markers for physiological events of interest.

Environmental conditions that can affect the culture performance and could potentially be detected by molecular markers include *e.g.* suitable substrate concentration (to support production but avoid overflow metabolism), limitations or oversupply of other nutrients (phosphate, nitrogen), oxygen, high or low pH. Additionally, physiological markers could be useful in scale-up of bioprocesses, when gradients in the bioreactor often affect the internal physiology of the cells, as well as in detection of cellular response during overproduction of recombinant proteins (Schweder & Hecker, 2004).

1.3.6.1 Enzyme markers

The activities of intracellular enzymes indicate the activity of certain metabolic pathways. Enzymes are assayed by performing their specific reactions under standard conditions *in vitro* using their natural or artificial substrates and often some additional detection reaction. Prior to the analysis, samples are harvested from the cultures and cell extracts are prepared. A relatively large set of glycolytic enzyme activities have been used as markers, for example, to study the fermentative capacity of *S. cerevisiae* after cold storage (Nilsson *et al.*, 2001) or in chemostat cultures under various nutrient limitations (van Hoek *et al.*, 2000).

However, the use of enzymes as physiological markers has a number of disadvantages. The general problem is comparing activities measured *in vitro* with the *in vivo* conditions. Different enzymes can have varying stabilities during sample preparation and storage, which needs to be tested and optimised for individual enzymes. In addition, each enzyme requires a specific assay. Thus, measuring activities of a large set of enzymes at regular intervals is not suitable for the monitoring of bioprocesses (Schuster, 2000). Continuous on-line monitoring of single intracellular enzymes has been performed, however, by cell permeabilisation and optical detection of suitable enzymatic reactions in FIA systems (Steube & Spohn, 1994).

1.3.6.2 Protein markers

A large number of proteins have been identified as markers for both general stress or for specific stress conditions such as temperature, oxidative stress, nutrient starvation *etc*. This work has been carried out by 2D-electrophoresis, which allows investigations of protein patterns of cells under specified conditions. *E. coli* and *B. subtilis* have been the main targets of this stress-related protein identification (Schuster, 2000), but also proteomic profiles of *S. cerevisiae* have also been extensively studied both in defined stress conditions (Boy-Marcotte *et al.*, 1999; Hu *et al.*, 2003) and in actual complex bioprocess conditions (Hansen *et al.*, 2006; Kobi *et al.*, 2004). As mentioned above, 2D-PAGE is not suitable for routine bioprocess analysis, but is a powerful tool for marker identification and in basic physiological studies. The actual monitoring of a few protein stress markers can be more conveniently performed *e.g.* by appropriate antibody reactions.

1.3.6.3 Gene markers

The use of gene expression levels as markers to detect physiological events in microbial cultures has a number of advantages over the use of proteins or enzymes. The assays measuring multiple targets simultaneously are more easily set up for RNA molecules than for enzymatic or antibody reactions. The physiological responses occur faster at the gene expression level than at the protein expression level. The mRNA targets can be measured from one sample preparation, *i.e.* a cell lysate or RNA extract, which is not always the case with proteins and enzymes. RNA is also more stable in the prepared samples than enzymes, if chemicals inhibiting RNAses are used. On the other hand, responses at the level of gene expression are transient and may be detected only during relatively short time periods in a process. The mRNA level describes the potential for translation of a protein, but does not necessarily correlate with the actual protein levels reached. However, increase in mRNA *e.g.* for a stress protein may be a good marker for stress response, even if the cells do not manage to produce the corresponding protein.

Genome-wide DNA microarrays (see below) can be used to identify gene markers*, i.e.* genes responding significantly and specifically to defined conditions. This technology allows, in principle, a semi-quantitative analysis of all mRNA levels of a microbial genome simultaneously. In this respect, genome-wide screening by DNA arrays can give a more comprehensive view of potential specific physiological markers than 2D-electrophoresis of the proteome. 2D-PAGE analysis has severe restrictions concerning the percentage of the total protein pool that can be detected. For example, hydrophobic proteins cannot be separated during iso-electric focusing and very small or extremely basic proteins as well as proteins at very low concentrations are not easily visualised by 2D-PAGE. On the other hand, mRNA analysis does not reveal anything about translation efficiency, protein targeting or post-translational modifications. The advanced approach in identifying suitable markers for the physiological state of an organism results from combination of both proteomics and transcriptomics.

The identification and use of gene markers in the field of microbial bioprocessing is still emerging, but has been extensively studied and applied in the field of clinical diagnostics. Genome-wide expression screening has been

performed to identify potential marker genes for detection of *e.g.* different types of cancers (Cuperlovic-Culf *et al.*, 2005; Wang, 2005), epilepsies (Leppik, 2003) and post-traumatic stress disorders (Zhang *et al.*, 2006). The first commercial applications coming to the market are based on detection of cancer-specific gene markers, such as the APTIMA PCA3 test by Gen-Probe, that measures expression of the prostate cancer-specific gene PCA3 (Groskopf *et al.*, 2006).

Genome-wide expression analysis has also been widely applied for organisms relevant to the bioprocess industry. The focus is to identify genes that are important under industrial bioprocess conditions or function as markers for specific physiological conditions. With yeasts, most published genome-wide screens of industrial fermentations concern beer (James *et al.*, 2003; Olesen *et al.*, 2002), wine (Rossignol *et al.*, 2003) or bioethanol (Devantier *et al.*, 2005) production. However, to date only one study (Higgins *et al.*, 2003b) is available with the specific aim of finding molecular markers for process monitoring, in this case concerning the detection of zinc depletion during brewing fermentations. As discussed above, a vast amount of gene expression data that has been generated by exposing yeast to various stress conditions is available for *S. cerevisiae*. These data can be used to identify genes that are specific for conditions occurring in industrial yeast-based processes, such as lack or presence of oxygen (ter Linde *et al.*, 1999), osmotic stress (Gasch *et al.*, 2000) or starvation (Natarajan *et al.*, 2001). A large number of potential gene markers describing various aspects of *E. coli* and *B. subtilis* processes have been identified. These genes are involved in responses to various stress conditions such as heat/cold shock, protein aggregation, and starvation of nitrogen, glucose, oxygen and phosphate. (Jürgen *et al.*, 2005b; Schweder & Hecker, 2004)

Table 2. Measurement technologies used for monitoring of biotechnical processes.

Measurement technique	Target / Information	
Chromatography		Potential for on-line analysis
Gas	Gas production	
HPLC, FPLC	Proteins, carbohydates	
Flow cytometry	Morphology, Viability, Intracellular pH, DNA, RNA, protein(total)*	
Molecular markers	Specific proteins, enzymes and genes	
Spectroscopy		In routine on-line analysis
Dielectric spectroscopy	biomass, morphology	
2D fluorescence spectroscopy	NAD(P)H, vitamin, protein, coenzyme	
UV- spectroscopy	RNA and protein pattern, cell depris	
Affinity sensors	Biomolecular interactions, protein production	
Infrared spectroscopy	Protein, lipid, nucleic acids, carbohydrates	
Pyrolysis mass spectrometry	growth, morphology	
Raman spectroscopy	Chemical patterns, single cell analysis	
Electrophoresis		Commonly off-line analysis
PAGE, CE	Protein pattern, amino acids, Nucleic acids	
Microscopy	Morphology	

1.4 Overview of gene expression analysis methods

Limited knowledge of the physiology of industrial microorganisms is one evident limitation to the wider use of gene or other molecular markers for monitoring culture performance. Accumulation of transcriptomic and other systems-level data in connection with novel bioinformatic tools is constantly making the prediction of process-relevant responses and their marker genes more reliable. However, another obvious obstacle is the shortage of suitable tools for efficient monitoring of the selected genes. Although data collected by microarray technology is essential for this work, it is not suitable for frequent analysis of microbial cultivations. Measuring expression levels of a set of gene markers for either clinical or biotechnical diagnostics requires methods that can be performed rapidly and preferably with a large number of samples. Furthermore for research purposes it is rational to use alternative methods for more in-depth targeted studies of interesting subsets of genes discovered by microarrays. An overview of both genome-wide transcriptional analysis and non-array methods for focused expression analysis is given in the following sections. A summary of these methods is presented in Table 3.

1.4.1 Nucleic acid hybridization

A common factor between gene expression analysis methods is that they are all based on nucleic acid hybridization reactions. During hybridization, single-stranded nucleic acids are subjected to conditions of temperature and ionic strength that favour base pairing between complementary sequences. Under these conditions, nucleic acids can re-associate with their complementary sequences even in presence of vast amounts of unrelated sequences. There are several factors affecting rate of duplex formation. Rate of hybridization can be increased by increasing temperature to a point, which is related to the hybrid melting temperature (T_m). Also, high salt and nucleic acid concentrations can accelerate the hybridization rate (Anderson, 1998). Inert, high-molecular weight polymers such as polyethylene glycol and dextran sulphate can be used to concentrate the nucleic acids in solution to increase reaction rate (Wetmur, 1975). Formamide destabilises nucleic acids and is used to decrease the T_m (Wetmur, 1991). Stringency, a term often used in connection to hybridization, refers to combination of temperature, salt, and formamide concentration that determines the specificity of the reaction. Stringent conditions allow only well-matched hybrids to form.

1.4.2 Genome-wide expression analysis

Microarray technology has transformed molecular biology in the last decade more than any other methodological approach. Global studies of cellular activities instead of studies of individual biological functions of a few genes, proteins or at best pathways has been enabled by DNA microarrays (Shalon *et al.*, 1996). The principle of this optical DNA-chip technology is straightforward: 1. Pieces of DNA sequences are immobilised or synthesised on defined locations of the chip surface. 2. mRNAs in the samples are converted to cDNA and labelled usually with a fluorescence label. 3. Labelled cDNAs are applied on the chip, where they hybridize to their complementary sequences. 4. Fluorescence label intensity on the chip enables quantification of the mRNAs in the studied sample. (Figure 4.)

The DNA-chips are produced by two fundamentally different techniques: A. By robotic spotting or inkjet printing of DNA (oligonucleotides or full length gene sequences) on the chips or B. (more commonly in industrial chip production) by *in situ* synthesising oligonucleotide arrays by photolithography (Fodor *et al.*, 1991). In method B, UV light is used to trigger stepwise synthesis of DNA-oligonucleotides by deprotection of the oligonucleotides at the appropriate position on the chip, which enables binding of the subsequently added activated nucleotide. After removing uncoupled oligonucleotides the next synthesis cycle follows. This synthesis requires a large number of photolithography masks for each chip in order to direct the UV-light to appropriate positions. Alternatively the synthesis can be performed with a maskless array synthesizer (MAS), that utilises computer-controlled individually addressable micromirrors to reflect the desired pattern of UV-light (Singh-Gasson *et al.*, 1999). This MAS technology allows synthesis of arrays with *e.g.* > 780 000 different 25-mer oligonucleotides in about one hour (Stengele *et al.*, 2005). Oligonucleotide arrays are designed so that approximately 20 oligonucleotides represent one gene. Thus one chip can be used to analyse the expression of approximately 40 000 genes or more.

Although transcriptional analyses represent the main application for DNA microarrays, other applications for this technology are also available. These include comparative genomic hybridization (CGH), identification of microorganisms by RNA/DNA sequences, finding gene regulatory elements and monitoring expression of translation modifiers such as microRNAs. Additionally, microarray-based analyses have been developed for other molecules in addition to nucleic acids. These applications include, for example, combination of chromatin immunoprecipitation with DNA microarrays (ChIP-on-chip) to study protein-DNA interaction or GlycoChip to study protein-carbohydrate interactions (Hoheisel, 2006).

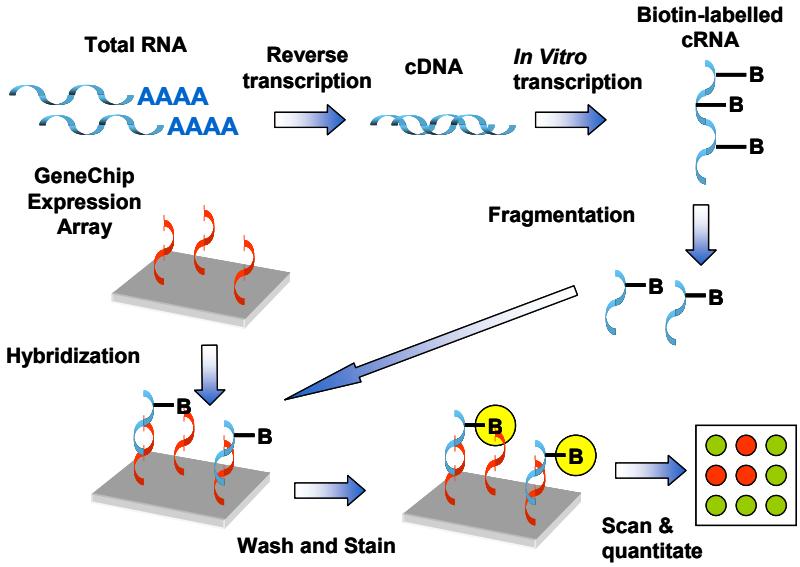

Figure 4. Overview of the different steps required to perform Affymetrix GeneChip experiment.

Serial analysis of gene expression (SAGE) is an alternative method to microarrays for studying the expression of thousands of transcripts. The benefit of SAGE analysis is the possibility to detect previously unknown transcripts. As in microarrays, each mRNA is first converted to cDNA that carries a biotin label at the 3´ end. The cDNA is digested and the product is captured using the biotin. A BsmFI restriction site is ligated to these fragments. The restriction enzyme cleaves DNA approximately 14 base pairs from its recognition site. This results in 14 base long SAGE tags corresponding to each target in the original sample. These tags are ligated together to form concatamers, which are cloned into a plasmid vector and sequenced. Each clone can contain tags from up to 60 mRNA transcripts (Velculescu *et al.*, 1995). In dubbed LongSAGE, tags with a length of 21 bases are used, which makes discrimination between genes and identification of new genes easier. One of the main bottlenecks in terms of time and costs for SAGE analysis and especially in dubbed LongSAGE analysis is the sequencing step (Bonetta, 2006). SAGE has rarely been used in expression analysis of microorganisms under bioprocess conditions. One of the few examples is a SAGE study by Varela *et al.* (2005) of gene expression profiles in an industrial *S. cerevisiae* strain in mid-exponential phase and in the early and late stationary phases of wine fermentation.

Massive parallel signature sequencing (MPSS) is one of the latest tools that have become available for genome-wide sequencing (Brenner *et al.*, 2000). Counting of mRNAs by MPSS is performed by generating a 17-base long sequence for all mRNAs at a specific site upstream from polyA tail. This 17-base sequence functions as an identification signature. MPSS signatures for transcripts are generated by sequencing double stranded cDNA fragments that are prepared from polyA RNA and cloned onto microbeads. This process results in a library of microbeads, where each mRNA is prepresented by one bead and each bead contains approximately 100 000 identical cDNA fragments. In the sequencing process one million of these beads are loaded into a flow-cell, which is connected to a computer-controlled microfluidistic network that provides the required reagents for the sequencing reaction (details of the technology are described in Brenner *et al.* [2000] and Reinartz *et al.* [2002]). A high-resolution CCD (charge-coupled device) camera is used to capture fluorescent images at specific stages of the sequencing reactions.

MPSS is a very powerful technology enabling accurate quantification of genes expressed at the level of a few molecules per cell. No prior knowledge of a gene´s sequence is required, unlike in the case of microarrays, making MPSS suitable for any organism. In addition, in many cases MPSS enables differentiation of even highly homologous genes. However, only a very limited number of samples can be analysed by the process, and thus MPSS is seen more as an complementary method for microarrays in genome-wide gene expression analysis (Reinartz *et al.*, 2002). Solexa and 454 Life Sciences are MPSS-based technologies that have recently become commercially available for genome-wide sequencing and expression analysis.

1.4.3 Focused expression analysis

Although microarray technology is a powerful tool in gene expression analysis, it is not always the optimal tool for a given application, such as frequent expression monitoring of a subset of genes. Production of DNA-chips and the analysis protocol are expensive to perform. The whole assay protocol including conversion of mRNA to cDNA and the slow solid-phase hybridization can take up to 24 h. Alternative techniques can provide more accurate measurements of

subsets of genes in shorter times and more cost-effectively than the microarray chip-techniques, and therefore have higher potential for marker gene expression analysis. Alternative non-array based strategies are presented here.

1.4.3.1 Traditional RNA quantification techniques

The Northern blot hybridization is a conventional method for mRNA analysis of a very limited number of genes (Alwine *et al.*, 1977). In this method RNA molecules are separated by size in an agarose gel, followed by transfer of the mRNA onto a membrane and detection by labelled target sequence-specific probes. The benefit of this technique is that besides the target quantity it can give information about the target size and degradation products. When the size and potential degradation are irrelevant, the RNA targets can be directly blotted to a membrane, in RNA slot-blot analysis, which shortens the detection time significantly. Compared to more modern RNA analysis techniques, these methods are laborious to perform and often require the use of radioactive labels to achieve maximum sensitivity. Even for a small set of genes, the Northern blot analysis can take days to perform. However, since these methods are well established and the facilities to perform them are available in most molecular biology laboratories, they are still widely used, also for monitoring the expression of selected genes in fermentation conditions (Brejning *et al.*, 2003; Higgins *et al.*, 2003a; Perez-Torrado *et al.*, 2005).

1.4.3.2 Quantitative and multiplexed PCR

Real-time PCR offers an alternative for rapid and quantitative analysis of mRNA levels. This technique also involves initial conversion of mRNA to cDNA by reverse transcription. Using the cDNA as a template, thermostable DNA polymerase extends pairs of short single-stranded, gene-specific primers during repeated cycles of heat denaturation, primer annealing and primer extension. The amount of template cDNA is doubled in each cycle, until it reaches a plateau. In real-time PCR, the amplified product is detected in each reaction cycle, in real-time, by the thermocycler instrument. The detection can be realised by using two alternative detection chemistries – the fluorescent dyes can be incorporated in the gene-specific oligonucleotide probes or dyes that become fluorescent after binding to double stranded DNA can be used, such as SYBR Green I (Morrison *et al.*, 1998).

Fluorescence by SYBR Green I steadily increases as the amount of double stranded PCR product increases. This technique is generic for each template to be assayed, but is less specific than probe-based detection methods, since dimeric primers can also generate a signal. In addition, SYBR Green I is not suitable for multiplex assays. TaqMan probes are most widely used for labelled probe-based detection in real time PCR. TaqMan probes have a fluorescent dye at their 5′ end and a quencher moiety at the 3′ end. When a TaqMan probe binds to the amplified template, it is digested by the exonuclease activity of the Taq DNA polymerase. As a result the fluorescent dye is released from the quencher and the fluorescence signal increases. (Heid *et al.*, 1996)

Various real-time PCR instruments are becoming available that can detect up to four or five different fluorophores. Thus more than one PCR product can be detected in a single sample by using gene-specific probes with different fluorescence dyes, thus reducing reagent costs and increasing throughput. The two main challenges in multiplex real-time PCR are that amplification of templates with high abundance in the sample can lead to depletion of reaction components before lower-abundance targets reach the detection limit, and the interactions between probes. PCR buffers that promote 'molecular crowding', bringing probes and templates into closer proximity, have been used to increase specificity and efficiency in multiplex-PCR (Ballantyne *et al.*, 2006). Both end-point and real-time PCR with multiplex target detection are being applied for high-throughput screening applications (Scheurer *et al.*, 2007; Stedtfeld *et al.*, 2007).

Real-time PCR has been used for fermentation monitoring, for example in quantification of lactic acid bacteria in wine fermentations (Neeley *et al.*, 2005) and to quantify the expression of 17 *Clostridium thermocellum* genes during ethanol production from cellulose or cellobiose (Stevenson & Weimer, 2005). These applications mainly use SYBR Green I detection due to the lower expenses and simpler assay set-up compared to the use of gene-specific probes. Current disadvantages of this technology for wider use in bioprocess monitoring are the high contamination risk and the need for extensive template preparation to obtain high quality RNA (Bustin, 2002; Mifflin *et al.*, 2000). However, rapid real-time PCR protocols that can be performed within about 2 hours have been developed. In addition, there are reports showing the possibility of using samples without RNA extraction (Kumamoto *et al.*, 2005). Real-time PCR-based

methods and instruments are under continuous development and are becoming faster and more sophisticated. The combination of multiplex detection with a fast assay protocol including sample preparation could make real time PCR a potential method for rapid diagnostics and at-line bioprocess analysis.

1.4.3.3 *In situ* hybridization

In situ hybridization provides information about the temporal and spatial expression of transcripts in a cell. This technique applies labelled nucleic acid probes that bind to specific targets in cells. The detection is performed by various fluorescence- or enzyme-based protocols. Recent developments in probe design, fluorescent dyes and confocal microscopes are enabling multiplex fluorescence *in situ* hybridizations (FISH; Bonetta, 2006). FISH analysis has also become a method of choice for direct detection of micro-organisms. In the bioprocess industry this technology has been used *e.g.* for rapid detection of lactic acid bacteria (Blasco *et al.*, 2003) and for analysis of yeast population dynamics during wine fermentations (Xufre *et al.*, 2006).

1.4.3.4 Solution hybridization

Solution hybridization-based techniques for transcript analysis include methods developed more than 20 years ago and more recently developed ones with improved throughput. A common way to perform RNA quantification in solution is the so-called sandwich hybridization (Dunn & Hassell, 1977; Ranki *et al.*, 1983). In this method RNA targets are hybridised with a pair of probes. One of the probes is used to immobilise the target on a solid support (the capture probe) and the other one carries a label enabling detection and quantification of the target (the detection probe). There are many alternative ways to carry out the hybridization and final detection. The capture probe can be immobilised prior to or after hybridization (Albretsen *et al.*, 1990; Tenhunen *et al.*, 1990), coated magnetic beads or microwell plates can be used as solid support for the target capture (Fisher *et al.*, 1997; Ishii & Ghosh, 1993) and final detection can be based e.g. on time-resolved fluorescent dyes (Hakala & Lonnberg, 1997), radioactive labels (Palva *et al.*, 1988) or enzymatic reactions (Ishii & Ghosh, 1993). The main commercial applications of solution/sandwich hybridization techniques have been in clinical diagnostics of various viral infections (Nicholls & Malcolm, 1989).

More recent developments of solution hybridization for nucleic acid detection include the use of xMAP technology developed by Luminex (Vignali, 2000). This technique makes use of polystyrene microspheres that carry two spectrally distinct fluorochromes. The use of precise amounts of each of these fluorochromes produces an array of up to 100 different microsphere sets with unique spectral profiles, which can be distinguished. Each microsphere is coupled to a unique probe that recognises a specific target when the beads are mixed with a sample. A third fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction at the microsphere surface. Microspheres are studied individually in a rapidly flowing stream as they pass by two separate lasers in the Luminex flow cytometer instrument. One of the lasers excites the two fluorochromes within the microsphere beads, and the other one excites the reporter fluorochrome. High-speed digital signal processing classifies the microspheres according to their spectral profiles and quantifies the reaction on the surface. Thousands of microspheres and up to 100 different targets per sample can be analysed in just a few seconds. (Dunbar, 2006)

Beads Array for the Detection of Gene Expression (BADGE) is an application of the xMAP technology for gene expression (Yang et al., 2001). In this method the capture oligonucleotide probes (25 bases) are immobilised on the color coded microspheres, and are hybridized with the labelled cRNA sample, followed by direct measurement by flow cytometry. The BADGE method has been used for simultaneous analysis of 20 *Arabidopsis thaliana* pathogenesis-related genes in single samples collected from benzothiadiazole-treated cultures. Hybridization for 1 h was followed by fast read-out of the 20 transcript levels in 96-well format by the Luminex instrument, which shows the high potential of the BADGE method for high-throughput applications. The sensitivity of target detection in BADGE assays is low (1 fmol, 6 x 10^8 molecules), allowing monitoring of only moderately or abundantly expressed genes. Variation between hybridization signals by this assay was around 35%, which is rather high but still comparable to microarray analysis.

To improve the sensitivity of xMAP-based transcript analysis, branched DNA technology (bDNA) (Urdea *et al.*, 1991) has been used for signal amplification in connection with xMAP technology. This technology is commercially available as QuantiGene assay (Zhang *et al.*, 2005). The RNA transcripts are hybridised with a set of specific probes, followed by capture to the respective microsphere

beads. The signal on each bead is amplified by hybridising with branched DNA and biotinylated probes which bind to a fluorescent dye analysed by the Luminex instrument. Improved sensitivity allows the hybridization to be performed directly using cell lysates as samples. However, the analysis requires a rather complex set of probes (capture probes, capture extenders, label extenters, blocking probes, biotinylated probes), amplifier DNA, and alkaline phosphatase as signal amplifier (Figure 5). This increases the complexity of the system and overnight hybridizations are required.

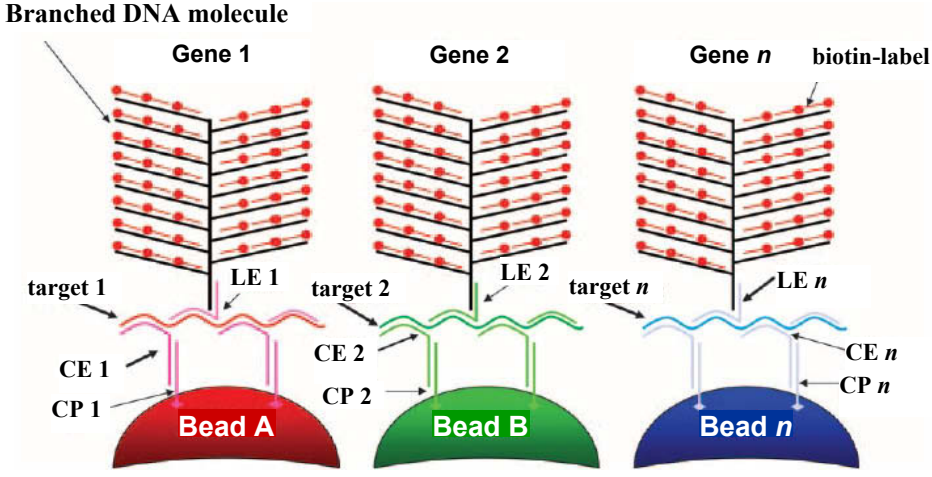

Figure 5. Principle of multiplex branchedDNA (bDNA) assay using Luminex beads. Capture extenders (CE) specific to 1 bead participate in the capture of a specific mRNA. Capture probes (CP) and label extenders (LE) provide a binding site for the amplifier molecule (bDNA) which in turn hybridizes to biotinylated label probes. Streptavidin-conjugated phycoerythrin binding to label probes provides a fluorescent readout that is proportional to the amount of mRNA attached to the bead. (Zhang et al., 2005)

Yet another technology based on solution hybridization is the eTag Multiplex mRNA assay (Tian *et al.*, 2004). In this method two-target-specific oligonucleotide probes (a signal probe with eTag molecule and an Invader oligonucleotide) form an invasive structure by hybridizing in tandem to the mRNA target. Stacker oligonucleotide is used to increase the melting temperature. Cleavase, a thermostable 5´ nuclease, recognizes the invasive structure and cleaves the 5´-base along with the eTag molecule. Released eTag functions as a reporter. Multiplexing

is achieved by allocating distinct eTag molecules to multiple target-specific signal probes, and the migration time in capillary electrophoresis can be used to identify the mRNA targets (Figure 6a and b). The signal amplification by Cleavase enzyme enables detection of 1000–5000 copies per assay. Multiplex detection (44-plex) with the possibility of performing the assay in a 96-well format is useful for high-throughput screening of marker gene expression levels.

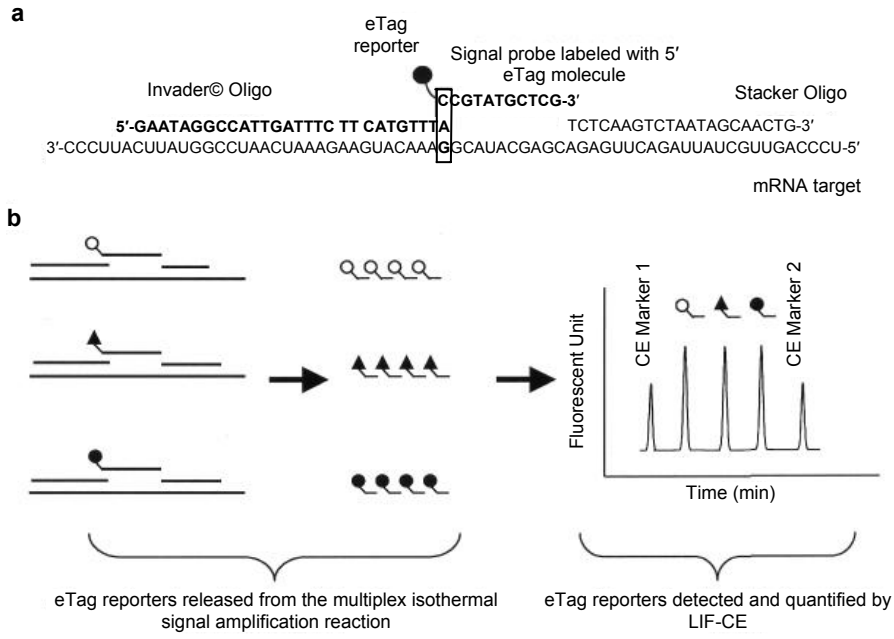

*Figure 6. Schematic representation of eTag multiplex mRNA assay. (**a**) A probe set includes invader oligonucleotide, signal probe with distinct eTag molecule and stacker oligonucleotide specifically designed to anneal to the RNA target. The 3′ terminus of the invader oligonucleotide invades one base (non-complementary to the template) into the DNA–RNA duplex between the signal probe and target, forming an overlapped DNA–RNA triplex structure shown in the box. The Cleavase enzyme, possessing 5′ nuclease activity, recognizes and cleaves this specific structure, generating an eTag reporter. Consequently signal probes, in large excess, rapidly undergo association with the mRNA target replacing cleaved signal probes. Multiple signal probes are cleaved per RNA target, resulting in target-specific accumulation of eTag reporters. (**b**) Multiplex assay is achieved by allocating unique eTag molecules to specific probe sets. Multiple cleaved eTag reporters are separated by CE (Tian et al., 2004).*

Electric DNA-chips based on hybridization in solution have also been developed specifically for rapid monitoring of selected transcript levels in bioprocesses (Jürgen *et al.*, 2005a). This method combines a redox electrode on a silicon chip (Hintsche *et al.*, 1997) and sandwich hybridization. The detection probe is labelled with an alkaline phosphatase conjugate, which hydrolyses the substrate electode inactive para-aminophenyl-phosphate into the electrode active product para-aminophenol. The product is measured with the electric DNA-chip by the redox recycling reaction. The current generated can be measured at the electrode and it corresponds to the level of hybridised mRNA. This method was used to study expression of three *B. subtilis* genes in glucose-limited cultures. The advantages of this kind of electrical chips are rapid detection of RNA hybridizations and lower equipment costs compared to methods based on *e.g.* PCR or flow cytometry. On the other hand, the multiplex detection is not an option with electric detection and RNA extraction is required. When only one to two mRNA targets are to be monitored in bioprocesses, this technique has potential to be used in continuous at-line monitoring.

Table 3. A summary of the most common methods used for gene expression analysis.

Method	Advantages	Disadvantages
Genome-wide analysis		
Microarray	40 000 genes per assay, applications beyond gene expression	Laborious, expensive
SAGE	No prior sequence information required	Sequencing increases cost and analysis time
MPSS	Highly sensitive, No prior sequence information required, Differentiation of homologous sequences	Low throughput, expensive
Focused analysis		
Northern blot	Gives information of target size and degradation products	Labour intensive, low throughput and sensitivity, radioactive labels
qPCR	Sensitive	High contamination risk, good quality RNA required, assessment of small changes
In situ hybridization	Gives information of gene expression inside the cell	Suitable for limited number of genes
Sanwich hybridization	Fast, no RNA purification or cDNA conversion required	Moderate sensitivity
BADGE	Fast, multiplex, high-throughput	Low sensitivity
QuantiGene	Multiplex, no RNA purification or cDNA conversion required	Complex probe sets required, requires manufacturing of gene specific beads
eTag	Multiplex, robust, sensitive	Complex probe sets required

Performing the hybridization in solution has certain benefits as compared to solid-phase hybridization (microarray, Nothern blot) or PCR-based methods, making it an attractive alternative for applications in which rapid quantification of a set of genes is required. Hybridization reactions in solution reach their plateau in minutes rather than hours (Thompson & Gillespie, 1987). Most methods employing hybridization in solution avoid conversion of RNA to cDNA and target amplification, thus avoiding quantification inaccuracies associated with the amplification and resulting in a shorter protocol time. Furthermore, in many cases RNA extraction can be avoided.

1.5 Aims of the present study

The first aim of this study was to develop methods suitable for expression analysis of sets of marker genes in biotechnical production processes. The dynamic environmental conditions in such processes, combined with transient genetic responses, necessitate frequent sampling. Due to the multitude of environmental factors affecting the cellular physiology during the processes, the number of genes to be assayed is potentially high. In order to apply gene expression analysis for process control the response time of the method should be short. Thus the preferred characteristics of the gene expression analysis method to be developed were short protocol time and reliable quantification of a few to a few hundred transcript levels from a large number of samples.

The other aim of the study was to apply the developed method to gain process-relevant gene expression data from microbial cultures. The target organisms in this study were the filamentous fungus, *Trichoderma reesei*, used widely in industrial enzyme production, and the lager type brewer's yeast, *Saccharomyces pastorianus* (see Section 1.2). Work completed to reach this aim included optimisation of sample treatment protocols, identification of genes of which the expression patterns predict aspects of cellular physiology relevant for the production processes, and an evaluation of the developed methods and expression analysis of the chosen genes in actual process conditions.

2. Materials and methods

The methods used in this thesis are summarised in Table 4 and are described in detail in the original articles (Publications I–V). The principle of the solution hybridization method TRAC that was developed (Publication II) and applied (Publications III–V) in these studies is presented in Figure 7.

Table 4. The methods used in this study.

Method	Description	Publication
Cell lysis	Enzymatic	I
	Non-enzymatic	II–V
Synthesis of contol RNA	In vitro transcription with T7-mRNA polymerase	I, II, V
Oligonucleotide probe design and synthesis	Computer-aided design and selection	I–V
	Binding efficiency evaluation	IV
	custom made / 3´end labelling with digoxigenin	I–V
RNA extraction and analysis	Extraction	I, II
	Slot Blot / Northern blot	I–II
	Real-Time PCR	I
	Total RNA quantification	II–V
	polyA RNA quantification	III–V
	Sandwich hybridisation: AP, TRF detection	I, II
	TRAC	II–V
Steady state expression stability	Calculation of gene expression variation during chemostat cultures	III
Enzymatic activity measurements	Invertase	I
	Cellulase	II–IV
	Laccase	IV
Dry weight	Biomass quantification	III–V
Total protein quantification	Biorad test kit	III
Ammonium quantification	Roche test kit	III
Sugar analysis	HPLC	V
Microbial cultures	*T. reesei* batch, fed-batch, continuous	IV
	T. reesei chemostat (lactose limited)	III
	T. reesei shake flask	II
	S. cerevisiae shake flask	I
	S. pastorianus brewing fermentations	V

2.1 Probe binding efficiency evaluations

Binding efficiencies between probe-target pairs can vary. This was investigated in experiments that are not described in Publications I–V and therefore the methods used for probe binding efficiency evaluation are described here in detail. To evaluate binding efficiencies of alternative oligonucletide probes to

corresponding mRNA targets in conditions resembling the TRAC protocol, 4–5 probes (23–33 nt) labelled with digoxigenin (DIG oligonucleotide Tailing Kit, Roche), were hybridized (see Publication I, Methods) with 7 *in vitro* transcribed (Mega script, Ambion) biotinylated mRNA targets (32 probes in total) in the presence or absence of 20% formamide. The signal intensities of alternative probes were detected by anti-DIG-alkaline phosphatase FAB fragments (Roche) and AttoPhos substrate (Promega, Leiden, the Netherlands) according to the Sandwich hybridization protocol (Publication I). The alternative probes were designed using the Primer3 program (Rozen & Skaletsky, 2000), with melting temperature (T_m) limits of 50–80 °C, GC% limits of 45–65, and excluding probes with potential hairpin structures. The thermodynamic properties that were computed and compared to relative signal intensities were the free energy change in the hybridization ΔG_H (computed with the program Melting [Le Novere, 2001]), the melting temperature of the probe target duplex (Melting [Le Novere, 2001]), and the free energy changes of the probe (Mfold [Zuker, 2003]) and the target secondary structures (ViennaRNA [Hofacker, 2003]). The global secondary structure of the target outside the probe binding site was assumed to be maintained upon oligonucleotide probe hybridization and the free energy change was estimated using the measure A_c of Luebke *et al.* (Kivioja, 2004; Luebke *et al.*, 2003). A_c is calculated by substracting the free energy of the most stable predicted secondary structure of the analyte transcript from the free energy of the secondary structure where the probe binding site is maintained.

2.2 Target selection

To summaries the target selection basis in the work presented in Publications II–V, it can be stated that two approaches were applied. Potentially useful marker genes were selected on the basis of their responses in conditions of interest as reported in publically available transcriptional analysis data for various species of filamentous fungi or *S. cerevisiae*. In addition, many of the target genes were selected based on their role in pathways considered to be relevant for the studied *T. reesei* and *S. pastorianus* processes. The genes selected for transcriptional analysis, with their corresponding target specific probes, are listed in Table 1 in Publications I–IV and in Tables 1 and 2 in Publication V.

3. Results

3.1 Development and comparison of gene expression analyses (Publications I, II, IV)

As presented in the introduction, alternative technological principles, based on hybridization between target and probe/primer sequences, are available for expression analysis of focused sets of genes. When hybridization between probes and transcripts takes place in solution, there are a number of benefits compared to solid phase or amplification based methods, such as enhanced reaction kinetics and lowered requirement for purified RNA extract. Thus solution hybridization was selected as the most promising basis for developing gene expression analysis methods for robust monitoring of microbial cell cultures.

The solution (sandwich) hybridization principle was applied in the development of two mRNA analysis methods used in monitoring of fungal cultures (see Figure 7): 1. A sandwich hybridization assay with alkaline phosphatase-based signal amplification (Publication I), which was used in expression analysis of single genes per sample and 2. a multiplex solution hybridization method called TRAC (transcript analysis with the aid of affinity capture) using pools of probes separable and quantifiable in capillary electrophoresis (Publication II). Both of these methods are performed in 96-well format with crude cell lysates as sample material. Simultaneously to these studies the TRAC method was also being developed for analysis of bacterial populations based on ribosomal RNA (Satokari *et al.*, 2005) and for sensitive detection of transcripts by amplifiable DNA probes and PCR (Kataja *et al.*, 2006).

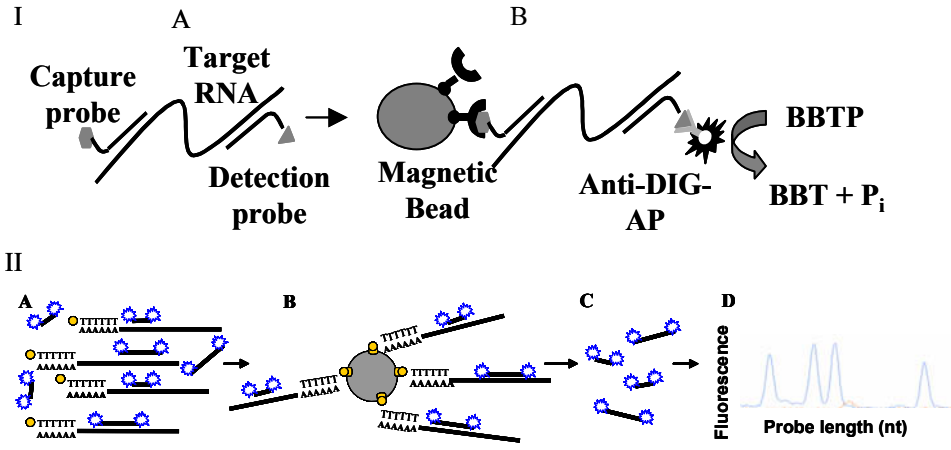

Figure 7. The principles of the solution hybridization methods. ***I)*** *In the sandwich hybridization the biotin labeled capture probe and the digoxigenin labeled detection probe are first hybridized with target RNA (A) followed by hybrid immobilization on magnetic beads and detection with anti-digoxigenin (DIG) – alkaline phosphatase FAB fragments (B). Alkaline phosphatase cleaves BBTP (2´-[2-benzothiazoyl]-6´-hydroxybenzothiazole phosphate) to inorganic phosphate (Pi) and BBT (2´-[2-benzothiazoyl]-6´-hydroxybenzothiazole).* ***II)*** *In the TRAC method the target RNAs are hybridized with a probe pool consisting of differently sized oligonucleotides with double fluorophore labels and a biotinylated oligo(dT) probe (A). Hybridized targets are immobilized by affinity capture to streptavidin-coated magnetic beads (B). Unbound material is washed off and the probes are eluted from the beads (C). The probes are identified and quantified by capillary electrophoresis (D).*

3.1.1 Use of cell lysates as sample material (Publications I, II)

It has been shown that hybridizations performed in solution allow the use of crude cell lysates of various sample types including neuroblastoma cells (Tenhunen *et al.*, 1990) and insect cells (Chandler *et al.*, 1993). In order to investigate whether this is also possible with yeast and filamentous fungi, crude lysate preparations were tested and optimised. A lysate for solution hybridization could be made simply by disrupting the cells with glass beads in a homogenizer in a lysis buffer. The lysis buffer contained either lyticase enzyme (Publication I) or sodium dodecyl sulphate (SDS, Publication II) to aid the mechanical

disruption. SDS was chosen as a standard ingredient in the lysis buffer since there is no requirement for a separate incubation step that can affect the transcript levels in enzymatic treatment of the cells. In addition, SDS is an efficient RNase inhibitor (Tenhunen, 1989). Guanidine thiocyanate (GuSCN) was also tested in the lysis buffer instead of SDS. However, GuSCN inhibits the hybridization reaction (Thompson & Gillespie, 1987) and sample dilution was therefore required prior to hybridization, which lowered the sensitivity of the measurement (data not shown). 1 to 2% SDS was found to be sufficient for lysis of *T. reesei,* whereas for lysis of *S. cerevisiae* up to 4% SDS was required in order to obtain sufficient concentrations of polyA RNA for direct use in hybridization (data not shown). Possibly the filamentous structure of *T. reesei* is more vulnerable to lysis by glass beads than the unicellular *S. cerevisiae*, and this could lower the requirement for agents aiding the lysis of the cells.

The use of crude *T. reesei* cell lysates in the TRAC assay showed faster hybridization kinetics as compared to total RNA extracts. Using a pool of 7 oligonucleotide probes, with cell lysates and total RNA as sample material, the hybridization reactions reached equilibrium in 30 min and 120 min respectively (data not shown). Faster kinetics of lysate hybridization is one probable explanation why up to 60% higher signal levels of 18S rRNA were detected when yeast cells as crude lysate were added to the hybridization solution as compared to the signal measured from total RNA extracted from the same amount of cells (Publication I, Figure 2a). Biological compounds present in the hybridization reactions when lysates are used as sample material can potentially function as 'molecular crowding' reagents, bringing the RNA and probes into closer proximity, which results in faster reaction kinetics.

High concentration of biological material has a negative effect on the hybridization (Tenhunen *et al.*, 1990). This effect was studied by adding known amounts of *in vitro* transcribed RNA (*SUC2*, *cbh1*) to solutions containing various amounts of crude lysates of *S. cerevisiae* and *T. reesei*. The yeast was cultured in conditions in which the target *SUC2* mRNA expression was insignificant, and the *T. reesei* strain used was a *cbh1* disruptant. For yeast cell lysates no inhibition of hybridization was observed when 10^6 or less lysed cells were added to the hybridization (Publication I, Figure 4). However, addition of 10^7 to 10^9 lysed cells to the hybridization solution decreased the signal of *SUC2*

mRNA by 40–55%. Using RNA extracted from the same number of cells, the corresponding decrease in *SUC2* mRNA signal was 15–40%. With *T. reesei,* inhibition of the RNA quantification was negligible when less than 1 µg/µl (wet weight of the lysed mycelium) of the mycelial lysate was applied to the hybridization (Publication II, Figure 3). 10 µg/µl lysate concentration reduced the signal by about 50%. This effect was independent of the growth phase of the culture. The effect of lysate was taken into account in signal quantification when high cell concentrations were used (Publication I), or the amount of biomass was kept low in the hybridizations so that this effect could be neglected in signal quantification (Publications II–V).

3.1.2 Sensitivity, linear range and reproducibility (Publications I, II)

The sensitivity and linear range of the solution hybridization assays were evaluated by using *in vitro* transcribed RNAs as target molecules. Using the sandwich hybridization assay with alkaline phosphatase (AP) signal amplification it was possible to detect 2 fmol (1.2×10^9 molecules) target molecules and there was a 1×10^3 -fold linear range when tested with *in vitro* transcribed 18S rRNA as target (Publication I, Figure 2). The same target was used for comparison in slot blot hybridization and in real-time PCR assays (see Section 1.4.2). The slot blot hybridization assay with AP-based chemiluminescence signal amplification had a similar level of sensitivity to that of the sandwich hybridization assay but the linear range of detection was only about 25-fold. The detection limit of the real-time PCR method was approximately 0.6 amol and the linear range of detection was greater than 1×10^4 (Table 5 and Publication I, Figure 2).

The corresponding characteristics of the TRAC assay were studied by performing measurements with a mixture containing $6 \times 10^6 - 1.2 \times 10^{10}$ – molecules (10 amol – 20 fmol) of *in vitro* transcribed RNAs from the *Trichoderma reesei* major cellulase (*cbh1*) and UPR transcription factor (*hac1*) genes. The detection probes were labeled with FAM (6-carboxy fluorescein) fluorescent labels at their 3´-and 5´-ends and detected by capillary electrophoresis (CE). The lowest detectable amounts of mRNA for *cbh1* and *hac1* were 30 and 50 amol respectively. Detection was linear up to 10 fmol of *cbh1* mRNA, and peak saturation was reached at 20 fmol. Thus, TRAC with capillary electrophoresis-based detection

allowed RNA quantification over a range of approximately 300-fold (Publication II, Figures 2a, b). The sensitivity of the TRAC assay has later been improved by 3 to 5 -fold (limit of detection 5–15 amol) by using the ABI PRISM 3100 instrument instead of the ABI PRISM 310 for capillary electrophoresis (data not shown).

Capillary electrophoresis as a detection method was compared with fluorescence signal amplification by AP and time-resolved fluorescence (TRF) -based detection with Europium (Eu^{3+}) labeled oligonucleotide. Lanthanide ions, such as Eu^{3+}, emit a long-lived fluorescence, which can be detected after a time delay, leading to decay of background fluorescence and increased measurement sensitivity (Soini & Lövgren, 1987). Compared to the sandwich hybridization assay described in Publication I, here the oligo dT was used in target capture instead of a specific probe and the detection probe was labeled with a DIG -tail instead of single 3´-end label to increase the sensitivity. Analysis of variable amounts (10 amol – 1 pmol) of *in vitro* transcribed *T. reesei cbh1* RNA was performed with the three detection methods, using similar hybridization and washing conditions for each method. In addition, the same amounts of the *in vitro* transcribed target RNAs were applied on agarose gel and analyzed by Northern hybridization with the full length *cbh1* cDNA probe.

TRAC RNA quantification with capillary electrophoresis detection was about 5-fold more sensitive than Northern blot analysis with 1–3 day exposure on a phosphor storage screen, and about 8-fold more sensitive than alkaline phosphatase amplification and time-resolved fluorescence. However, the latter two detection methods had wider linear ranges of detection ($1–2 \times 10^3$) than TRAC (3×10^2) and Northern blot (1×10^2). The relative performance of these different methods is summarised in Table 5.

Table 5. Comparison of alternative detection methods for in vitro transcribed RNA.

Analysis method	Detection method	Separation method	Limit of detection		Linear range	Protocol time	Sample material	Targets / sample	Ref
			amol	pg					
Solution hybridisation:									
TRAC	Fluorescence label	Capillary electrophoresis	5	4	3×10^2	2-3 h	Lysate/RNA	20-30	II
Sandwich hybridisation	Fluoresence amplification by single/multiple AP label	Fluorometry	250	150	1×10^3	2-3 h	Lysate/RNA	1	I / II
	Time resolved fluorescence	TRF-Fluorometry	250	150	2×10^3	2-3 h	Lysate/RNA	3	II
Real-time PCR	SYBR-Green		0.6	0.5	1×10^4	2-3 h	RNA	3-4	I
Membrane hybridisation:									
Northern blot	^{32}P label	Phosphoimager	150	90	1×10^2	2-5 days	RNA	1-2	II
Slot blot	Chemiluminescence amplification by AP label	Densitometry	2000	1200	25	1-2 day(s)	RNA	1	I

The reproducibility of the TRAC assay was determined by quantifying the variation between duplicate samples in assays performed with a 10-probe set (Publication II, Table 1, Pool II). 550 randomly selected expression levels analyzed in duplicate samples by TRAC were plotted to an x-y axis (Publication II, Figure 2c). A linear function of y = 1.006 and correlation coefficient of $R^2 = 0.987$ showed the high reproducibility of sample-to-sample analysis. Variation between duplicate assays was 9% on average and 90% of the replicate analyses showed less than 20% variation. Variation was also independent of the signal strength. The expression profiles measured from the samples with TRAC and conventional Northern hybridization were in excellent agreement (Publication II, Figures 5 and 6), which also demonstrates the good reliability of the TRAC method.

3.1.3 Multiplex hybridization (Publication II)

Separation of fluorescent probes by CE in the TRAC analysis allows multiplex target detection in one electrophoresis run due to the possibility of probe sizing as well as the possibility of using probes with different fluorescent dyes. The probe pools were designed based on varying molecular weights of the probes, using 2 nt length differences. Varying length, GC content and melting temperature of the probes used in the same pools requires the use of average reaction conditions concerning e.g. hybridization temperature and formamide concentration.

In order to optimize the conditions for probe pool hybridizations, different formamide concentrations and hybridization temperatures were tested in the TRAC analysis. Five different probes specific for the *T. reesei* genes *act1, cbh1, egl1, hac1* and *pdi1* (melting temperatures 63.7, 58.7, 61.4, 75.2 and 66.7 °C, respectively) were used for the detection of the respective mRNAs in *T. reesei* total RNA. The use of formamide in the solutions generally resulted in lower signals in hybridizations with oligonucleotide probes. However, low amounts of formamide (5–7%) resulted in somewhat higher signals with probes having T_m of 64 °C or above, but a signal decrease of the same level was observed for probes with T_m lower than 64 °C (data not shown). The oligonucleotide probes mentioned above (in a pool with T_m range 58.7–75.2 °C, 23 nt – 33 nt) each reached a maximum signal in the hybridization temperature range 58–62 °C. At temperatures below this range, the relative decreases in signal strengths were increased with the T_m value of the probe. Use of hybridization temperatures 0–3 °C above the calculated T_m resulted in 20–45% decrease in signals compared to the optimal temperature (Publication II, Figure 4). A hybridization temperature of 60 °C without added formamide was chosen as standard conditions for all probe pools.

The use of oligo dT for polyA-tailed RNA capture instead of mRNA target-specific biotinylated oligonucleotide capture probe resulted in 3–5 -fold increases in the measured signals in the TRAC analysis (data not shown), probably due to faster binding kinetics and higher binding efficiency of oligo dT in comparison to sequence specific probes. Secondary structures of the target mRNAs might also affect the hybridization efficiency at the polyA tail less than at a probe binding site located in the middle of the structure. Use of oligo dT simplifies the probe design and is more cost effective than the use of custom designed probes. Because the biotinylated oligo dT captures all mRNA species it can be used at high enough concentrations to do this without exceeding the capture capacity of the streptavidin coated beads. Oligo dT also enables quantification of total mRNA levels (see Section 3.2.1).

3.1.4 Probe design and selection (Publications I, II, IV)

When oligonucleotide hybridizations are used, the probe design has high importance. To accelerate and improve the oligonucleotide probe selection for TRAC analysis, algorithms presented in Kivioja *et al.* (2002); Kivioja (2004) were used in automated design of the probes. The probe sequences were selected by these algorithms using various probe quality criteria, such as Tm and GC% range, specificity criteria (maximal repeat size and similarity, taking into account the whole genome of the organism) and were finally organised into a minimal number of pools (see *e.g.* Publication IV, Methods). One 25–45 nt oligonucleotide probe for each marker gene was selected for the expression analysis. In order to study the binding efficiency of oligonucleotide probes to corresponding targets, alternative probe sequences were hybridised with the *in vitro* transcribed mRNA and the relative signal intensities were correlated with different thermodynamic properties (see Materials and Methods). The free energy change of the target upon probe binding (A_c) showed relatively high correlation with the relative signal intensity both in the presence (r=0.517) and absence (r=0.712) of 20% formamide (Figure 8). For all the 7 mRNA targets A_c predicted one of the 2 most efficiently binding probes. The prediction was slightly improved by including the free energy change in hybridization ΔG_H (Le Novere, 2001) and the T_m values of the probes. Probes that fulfilled all the set quality criteria were prioritised according to prediction based on the linear squares fitting of A_c, ΔG_H and T_m. This mathematical procedure was used to find the best-fitting curve to a given set of points by minimizing the sum of the squares of the offsets of the points from the curve. The function of such prediction is not to find the most efficiently binding probe sequence, but to eliminate the ones that bind poorly.

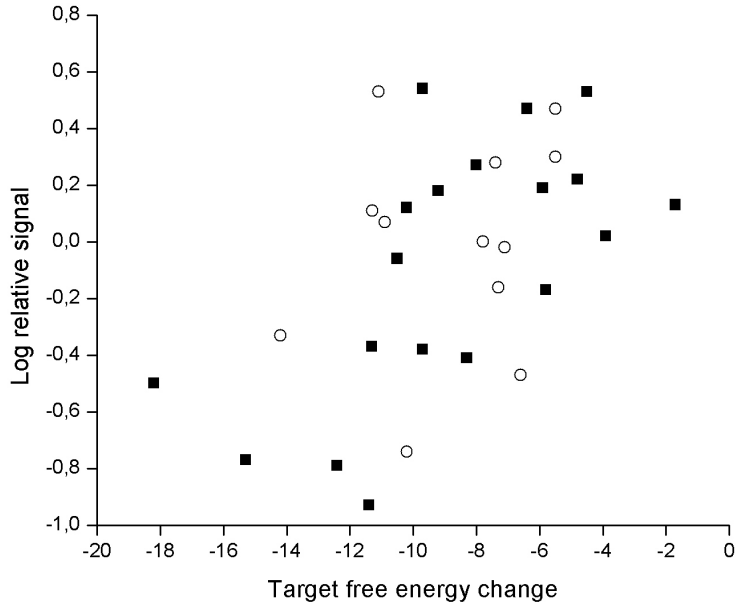

Figure 8. The effect of the target RNA secondary structure on the hybridization signal. The natural logarithm of a relative hybridization signal of the probe is plotted as a function of the predicted target energy change in hybridization (kcal/mol). The relative hybridization signal is defined as the signal of the probe divided by the average signal of all probes to the same target. 19 probes for 4 mRNAs were tested in 0% formamide (■) and 13 probes for 3 targets were tested in 20% formamide (○).

The sequence lengths of the probes assigned by the computer programme to the same pool differed from each other by at least 2 nt. The migration rate of the oligonucleotide sequences in CE is not, however, directly related to the molecular weight (Cordier *et al.*, 1994; Guttman *et al.*, 1992), and thus some manual reorganization of the pools was also required in order to avoid signal overlapping and to optimize the number of probes per pool. Up to 16 probes with one type of fluorescence label (FAM, 6-carboxy fluorescein) have been fitted into one pool. Using another type of fluoresencent label NED (2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxy-fluorescein) along with FAM in the same pool, the multiplexity of the TRAC assay has been further increased (22-plex, used in Publication V) and with additional label types multiplexity can potentially be increased up to 60. Signal leakage between different label types in CE analysis is the main factor limiting the introduction of new labels to the TRAC analysis.

The specificity of some designed *T. reesei* probe sequences was evaluated by using them in hybridization reaction with 1 µg of *S. cerevisiae* total RNA as target. The measured signals using yeast RNA were compared to the corresponding signals measured from an equal amount of *T. reesei* RNA. As expected, the signals measured from yeast RNA were dependent on the stringency of the washing conditions. In standard TRAC washing conditions (*e.g.* Publication II, Materials and Methods) the signals measured using yeast total RNA were 0%, 0%, 7%, 5%, 3% of the signal obtained with *T. reesei* RNA for *cbh1, egl1, hac1, pdi1*, and *act1*, respectively. The cross-hybridization between *T. reesei* specific probes and *S. cerevisiae* RNA was thus below the average deviation between duplicate measurements and the oligo(dT) sustains well the stringency used in the TRAC protocol and does not significantly lower the specificity of the assay.

3.2 Gene expression monitoring during microbial cultures (Publications III–V)

The developed TRAC assay was applied in transcriptional monitoring of various types of cultures performed with *T. reesei* and *S. pastorianus*. The *T. reesei* strain used in these studies expressed *Melanocarpus albomyces* laccase (Kiiskinen *et al.*, 2002) along with the full repertoire of native cellulases and was grown in chemostat, continuous, batch and fed-batch cultures. *S. pastorianus* was used for beer production in high gravity conditions. Approximately 30 *T. reesei* and 70 *S. cerevisiae* marker genes from process-relevant pathways were selected and used in evaluation of the physiology of these organisms in standard and altered process conditions with TRAC (see Publication IV, Table 1 and Publication V, Table 1).

3.2.1 Normalization of gene expression data

In production processes the conditions in the fermentor may be in constant change, causing variation in physiological parameters. This makes conventional gene expression data normalization methods, such as the use of so called house-keeping genes, unreliable. The TRAC gene expression analysis is performed directly from cell lysates, offering the possibility of directly relating the

expression of specific genes to the biomass content in the hybridization. Alternatively, the expression levels can be related to the total polyA RNA content of the lysate, which can be measured using oligo dT-based target capture in the TRAC protocol (see Publication IV, Methods). The ratio of marker gene mRNA to total polyA mRNA shows the change in the expression of a specific gene relative to overall gene expression, whereas the ratio of marker gene mRNA to biomass shows the changing level of a transcript relative to the total mass of viable and non-viable cells.

In order to study the variation between the biomass- and polyA mRNA-based normalizations, they were compared in normalization of the major cellulase *cbh1* expression in a fed-batch culture (Publication IV, Figure 4b). An expected difference between the *cbh1* to biomass and *cbh1* to polyA RNA ratio profiles was observed at the end of the culture. The *cbh1* to biomass ratio showed a faster decreasing trend after about 70 h than *cbh1* expression relative to polyA, since an increasing proportion of the biomass became metabolically inactive and polyA RNA was produced only by the metabolically active part of the culture. It should be noted, however, that *e.g.* increase in a gene mRNA to polyA RNA ratio is not necessarily a sign of specific up-regulation, but can also be a result of slower down-regulation than for the majority of genes. However, in either case an increase or decrease in this ratio indicates the growing or decreasing physiological importance of the gene product in the particular conditions.

The polyA RNA content was routinely determined in the *S. pastorianus* and *T. reesei* cultures. The polyA RNA level in the lysed biomass was culture phase dependent (Figure 9) (Publication IV, Figure 2; Publication V, Figure 1). In aerated, industrially cropped yeast culture the polyA RNA level increased rapidly after transfer to wort, reaching a maximal level 20 h after pitching. After a 70 h fermentation period, when yeast started to sediment, the polyA RNA was at the level of the pitching yeast (Figure 9). The average expression value of all ORFs determined from daily samples from lager fermentation in studies by Olesen *et al.* (2002) showed a similar profile to the total mRNA levels measured here. In a *T. reesei* batch culture that was freshly grown for a batch production of recombinant laccase, the polyA RNA level remained at a rather constant level until lactose was consumed and growth stopped (Figure 9). In addition, it was also observed that a process disturbance or batch-to-batch variation affecting the

T. reesei culture performance (growth and protein production) was also evident at the overall gene expression level (Publication IV, Figure 2). Thus overall gene expression is not only connected to growth phase/rate in fermentations, but is also affected by possible environmental perturbations.

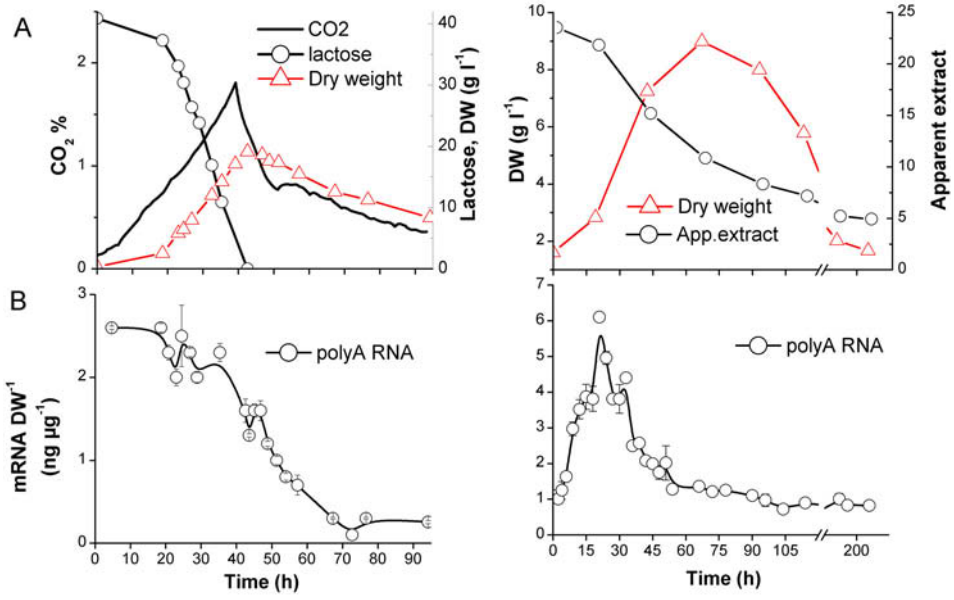

Figure 9. PolyA RNA profiles in T. reesei batch fermentation and in brewer's yeast (S. pastorianus) during beer fermentation. Apparent extract is the specific gravity of wort.

3.2.2 Monitoring of steady state of *T. reesei* by TRAC (Publication III)

Chemostat cultures are commonly used in production of biomass for systems-wide biological studies, since they provide more reproducible conditions than biologically non-steady batch cultures (see Section 1.1.2). However, the establishment of chemostat cultures for filamentous fungi faces particular challenges, because of their multi-nuclear, polar growth form, which introduces an inherent heterogeneity to the system. In addition, conidia production, pellet formation and growth on solid surfaces, such as the walls of the bioreactors (Larsen *et al.*, 2004), may further increase culture heterogenity. The quality of a

chemostat steady state is generally assessed from the measurement of various process parameters such as biomass concentration, CO_2 evolution and alkali consumption rates. However, short term changes in environmental conditions, such as sampling or poor mixing, can have an impact on cellular physiology (Enfors *et al.*, 2001), but will not necessarily result in measurable changes in biomass-related parameters. Culture perturbations may however affect the transcriptome, since cells can rapidly adapt to changing conditions by transcriptional regulation (Gasch *et al.*, 2000; Gasch & Werner-Washburne, 2002). The degree of gene expression stability in fungal or any other type of continuous flow cultures has not hitherto been reported. The TRAC method was used in this work to monitor transcriptional steadiness of approximately 30 *T. reesei* genes and to identify potential disturbances in a large number of chemostat cultures of a *T. reesei* transformant producing the laccase of *M. albomyces* and its parental strain. In addition, TRAC was used to assess transcriptional responses to intentional steady state disturbances caused by transient oxygen deprivations.

In chemostat cultures with steady states, stabilisation of gene expression occurred quickly after the start of continuous medium feeding (*i.e.* at 0 residence times (R), Publication III, Figure 1). The analysed gene set showed on average less than 20% variation between sequential samples after approximately 0.5 R (R = 33.3 h), and this degree of stability remained for at least 5 or 6 R. The average gene expression levels varied significantly between the batch and the continuous phase. In the batch phase, the analysed gene set showed on average a 1.3–2.0-fold change relative to the expression phase defined as most stable during the continuous feeding, and the average deviation in the fold change between the genes was large. Stabilisation of average gene expression occurred within approximately the same time period as stabilisation of CO_2 and dry weight production, but stabilization of secreted protein production required one to two R longer (Publication III, Figure 1).

Many of the *T. reesei* chemostats showed some level of instability during the (pseudo-) steady state, although the external parameters were carefully controlled. Disturbances in the steady state and the consequent physiological instability were most sensitively detected at the transcription level. Instabilities in chemostat cultures were observed due to technical disturbances, biological

factors and by unknown effectors. Instabilities in the steady states were observed either as changes in the transcript levels and in parameters related to biomass, or only as changes in transcript levels (Publication III, Figure 2).

Intentional perturbation of the steady state by exposure of the culture to anaerobic phases caused strong responses at the transcriptional level. More than 1.5-fold change in expression level was observed for approximately 50% of the marker genes as a response to anaerobic conditions. The longer the anaerobic phase was sustained, the stronger the effect was for the analysed set of genes (Publication III, Figure 3). However, the expression levels recovered to the initial levels rapidly after the restart of culture aeration. No more than 3 to 5 h after aerobic conditions were restored, the transcript levels of the gene set showed less than 1.3-fold variation, on average, from the initial steady state levels and the gene expression levels also remained constant for the next 20 to 30 h.

Both intentional and unintentional process perturbations showed that several of the genes involved in growth, protein production and secretion are sensitive markers for process disturbances. Disturbances, such as high concentration of polypropylene glycol used as antifoam agent, lack of air or high cell density stress, were manifested in particular by the down-regulation of genes encoding native hydrolytic enzymes (*cbh1*, *egl1*, *bgl2*, *bga1*) (Publication III, Figures 2 and 4), which are also the major products of *T. reesei* in industrial production. The *cbh1* promoter is also widely used for the expression of foreign proteins.

An additional aim of the chemostat-related studies was to investigate the physiological effects of foreign protein production on *T. reesei*. Transcriptional stability was used as the ultimate criterion in selection of the most stable cultures among those assessed as being in steady state based on conventional process analyses for focused and genome-wide expression comparison. Only 3 genes showed more than 1.5-fold difference in expression between the parental and laccase-producing strain, out of approximately 30 marker genes studied by TRAC. *Hsp70* coding for a cytoplasmic protein chaperon was the only gene up-regulated in the recombinant strain in the analysed gene set. Genes coding for native secreted cellulases *cbh1* and *egl1* were down-regulated in the recombinant strain by 1.9 and 2.6-fold respectively, which corresponded with the decreased activities of CBHI and EG (Publication III, Figure 5). These TRAC results were

later confirmed by genome-wide microarray analysis. Only 32 genes showed more than 1.5-fold difference between the compared strains, most of which encoded secreted enzymes, which were down-regulated in the recombinant strain (data not shown). The RESS effect is one potential explanation for this observation, *i.e.* secrection stress as a result of production of the foreign protein caused down-regulation of native secreted enzyme. However, UPR was not detected in these conditions (see Section 1.2.1). Another possibility is that the secreted enzymes were down-regulated due to titration of their activating transcription factors as described in *Aspergillus niger* (Verdoes *et al.*, 1994), although the laccase expression construct was present in the analysed strain as a single copy (data not shown).

3.2.3 Physiological state evaluation of *T. reesei* in protein production processes (Publication IV)

Close to 80% of the chosen *T. reesei* genes (33 in total) were regulated during the different protein production processes in a way that could have predictive value in evaluation of different aspects of the culture physiology, such as growth, productivity and nutrient availability.

3.2.3.1 Ribosomal proteins and growth

In yeast, expression of ribosomal protein mRNAs is dependent on the growth rate (Ju & Warner, 1994). Consistently with this, changes in the amounts of two ribosomal protein mRNAs relative to fungal biomass showed the most similar trends to specific growth rate (μ) out of the set of analysed genes in batch and continuous cultures (Publication IV, Figures 2 and 6). A similar correspondence was also observed earlier for *S. cerevisiae* 18S rRNA level and μ (Publication I, Figure 5), but in anaerobic brewing conditions ribosomal protein mRNA levels showed a clear decrease prior to the exponential growth phase (Publication V, Figure 3). Lower expression or slower induction of ribosomal protein mRNAs in *T. reesei* batch cultures (Publication IV, Figure 1) or batches of brewer's yeast fermentations (Publication V, Figure 3 and supplementary data) also preceeded the corresponding effects on growth rates of the cultures. In addition, a consistent, approximately 2-fold, increase in the two ribosomal protein mRNAs was observed in *T. reesei* batch cultures 3 to 5 h before exhaustion of carbon source and the end of growth.

3.2.3.2 Marker genes reflecting oxygen level

Expression levels of the *T. reesei* heat shock protein gene *hsp70* and the heme biosynthesis gene *hem6,* most consistently responded to the availability of oxygen during the transient anaerobic phases in the chemostat cultures (Publication III, Figure 4) and between cultures run with different oxygen levels (Publication IV, Figure 3). These genes were up-regulated rapidly under anaerobiosis, followed by rapid down-regulation after the restart of aeration (Publication III, Figure 4). In aerated batch cultures and batch phases of fed-batch cultures, the expression of these genes increased about 2-fold between 18 to 45 hours, while the DO decreased from 90 to 30% and biomass increased from 3 to 20 g l^{-1}. A 20–35% decrease in the mRNA levels relative to total polyA RNA was observed for these genes after maximal expression was reached (Publication IV, Figure 3). In batch and fed-batch cultures (16–20 g l^{-1} DW, 30% DO), the maximal expression levels of the *hsp70* and *hem6* genes were approximately 3-fold lower than in anaerobic conditions, but 2 to 3 -fold higher than in chemostat cultures with high DO% (80%) and low cell density (4 g l^{-1}) (Publication IV, Figure 3). A level of 30% DO is apparently sufficient to support aerobic culture conditions, growth and production, whereas at 10% DO increased levels of these oxygen sensitive gene transcripts and decreased levels of cellulase gene transcripts were observed (data not shown).

3.2.3.3 mRNA expression and enzymatic activity of secreted proteins

The *T. reesei* transformant strains used in this study produced *M. albomyces* laccase under the *cbh1* (cellobiohydrolase) promoter, as well as all native cellulases, including cellobiohydrolyase I (CBHI). Gene expression levels for *cbh1* and *lac1* (laccase) and two folding factors (*pdi1, bip1*) were followed in the different growth phases of *T. reesei* batch and fed-batch cultures and compared with the specific extracellular production rates of laccase and CBHI (Publication IV, Figure 4). All these genes showed increasing expression during the first 40 h of growth in batch cultures. Starting the lactose feed into the fed-batch culture caused 1.8 and 2.6 -fold increases in the mRNAs for *lac1* and *cbh1*, respectively. There were corresponding increases in the specific extracellular production rates of laccase (3.8-fold) and CBHI (4.3-fold) between batch and fed-batch phases. The specific production rates of both CBHI and laccase followed trends similar

to the corresponding *cbh1* and *lac1* gene expression profiles. However, a delay was observed between the gene expression and the corresponding extracellular enzyme production rate. The specific extracellular laccase production rate, which was more frequently measured than CBHI, was highest 6 to 10 h after the highest gene expression level was measured.

The highest amounts of *lac1*, *cbh1, pdi1* and *bip1* mRNAs were measured during a short time interval between 7 and 12 h after the start of the lactose feed, when μ was 0.05–0.03 h^{-1} and conditions had become growth limiting. During most of the feeding phase, μ was relatively constant at a value of 0.02 h^{-1} and constantly decreasing transcript levels of *lac* and *cbh1* genes were measured (Publication IV, Figure 4). In batch cultures, the highest gene expression levels of the carbohydrate degrading enzymes (*lac1*, *cbh1*, *egl1*, *bgl2*) were measured in the same range of μ. By the time of complete lactose starvation, transcript levels of these genes had decreased by 1.5-fold (Publication IV, Figure 4).

3.2.3.4 Starvation-induced responses

To study the responses of the marker genes during starvation, samples were collected frequently around the time when lactose was expected to be consumed in two batch cultures. Decrease in the expression of genes encoding carbohydrate-degrading enzymes (*cbh1*, *egl1*, *lac1* and *bgl2*) was observed 4 to 6 h prior to the point where lactose concentration was undetectable and base consumption had stopped. Altogether 9 genes in the analysed gene set showed increasing expression levels under starvation or slow growth, indicating their importance in these conditions. These genes coded for the heat shock proteins *hsp105* and *hsp30*, metacaspase (*mca1*), involved in apoptosis, neutral trehalase (*nth1*), membrane fusion factor (*nsf1*), copper transporter (*ctaA*) and two conidiation-related proteins (*ccg9* and *con6*) (Figure 10). In addition, the acetyl-CoA-synthase (*acs1*) gene was up-regulated at low lactose concentration earlier than the above genes (Publication IV, Figure 2).

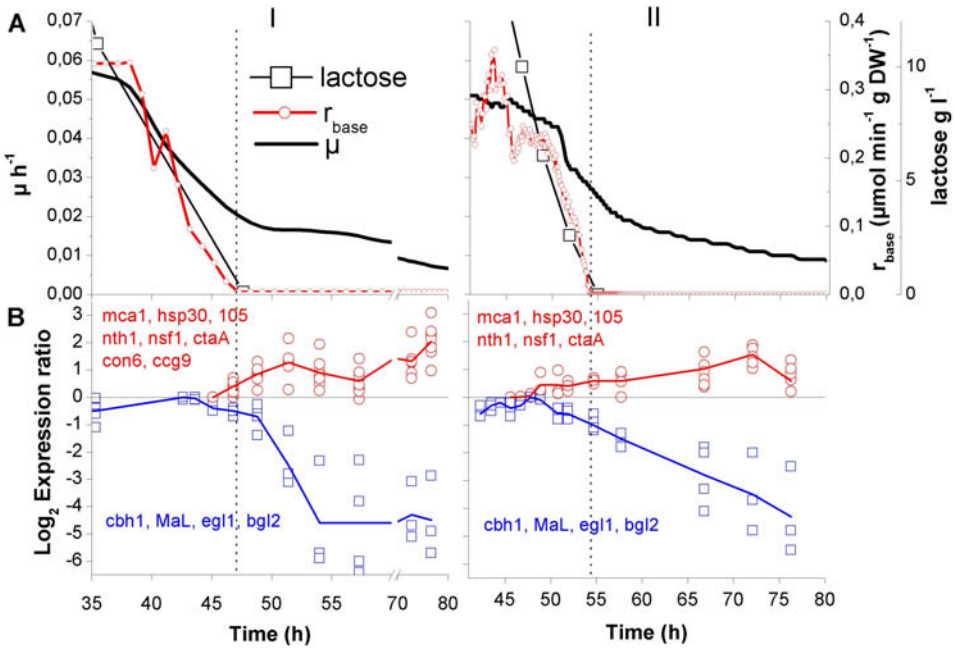

Figure 10. Responses of genes to starvation in two T. reesei batch cultures. A. Specific growth rate μ, lactose concentrations, specific base consumption rate r_{base} (μmol min^{-1} g^{-1}). B. Expression of cellulase and laccase genes as log$_2$ ratio to maximal expression measured in the cultures (blue symbols). Expression of genes showing > 2-fold increasing mRNA level relative to polyA RNA after exhaustion of lactose as log$_2$ ratio to the expression level in a sample taken before the observed increase (red symbols).

3.2.3.5 Production optimisation in continuous culture

A continuous culture was used to study futher the effect of growth conditions on the productivity of secreted proteins and to optimise their production. The medium feed was controlled by the DELTABAS algorithm, which is based on the rate of base consumption and aims at maintaining the optimal production phase by limiting the growth of the culture but avoiding total carbon source exhaustion (Bailey & Tähtiharju, 2003). Different growth conditions were applied, *i.e.* the medium feed rate and composition as well as the process temperature were altered during this process, which was run for 1000 h (Publication IV, Figure 6).

As could be expected on the basis of the batch and fed-batch cultures, similar trends were observed between expression of *cbh1* and the extracellular protein production rate (CBHI being the major component of the secreted proteins), both being highest at a growth rate of approximately 0.03 h^{-1}. Decrease of dilution/growth rate resulted in lower expression of *cbh1* and lower production of the extracellular proteins. In addition, starvation-related induction of the *acs1* gene was observed at low growth rates and the growth rate-dependent ribosomal protein mRNA *rpl16b* was expressed at a low level (Publication IV, Figure 6), indicating slow growth. As in chemostat cultures (Publication III), secreted enzymes, as well as growth-related genes, were sensitive markers for process disturbances, which in this case were caused by malfunctioning of the vessel weight controller and oscillation in the feed control mechanism.

3.2.4 Brewer's yeast physiology in very high gravity fermentations (Publication V)

TRAC was also applied to study the physiology of brewer's yeast in very high gravity (VHG) fermentations. The expression of > 70 selected yeast genes was measure at high frequency (up to 10 times a day) through VHG (25 °P) wort fermentations lasting 9 to 10 days. The analysed genes code for proteins involved in various pathways relevant to beer fermentations, such as maltose, glycerol and lipid metabolism, glucose fermentation, amino acid biosynthesis, aroma formation and flocculation. Two independent VHG fermentations were performed using industrially cropped yeast that was aerated in the laboratory prior to pitching.

Most of the oligonucleotide probes for the target genes of interest were designed using the *S. cerevisiae* genome database. However, the lager brewing yeast, *S. pastorianus*, is a hybrid between *S. cerevisiae* and *S. bayanus* genomes (see Section 1.2.2). In order to evaluate the specificity of the measured signals, we aligned each designed *S. cerevisiae* probe sequence against each predicted ORF or contique sequence in the *S. bayanus* genome using the Needlman-Wunsch algorithm (Needleman & Wunsch, 1970). The number of mismatches was determined for the sequence differences between the best alignments. Out of all the 70 *S. cerevisiae* probes, 11 had ≤ 3 mismatches to the most similar sequence in *S. bayanus* (Publication V, Table 1). In these cases, the *S. bayanus* version of the target transcript may have contributed to the observed signal.

3.2.4.1 Maltose metabolism and glucose fermentation

Maltose typically comprises about 60% of the fermentable sugar in brewer's wort. Glucose causes repression of many genes including those involved in utilisation of alternative carbon sources, such as maltose. The expression of genes involved in malto(trio)se metabolism, glucose phosphorylation and metabolism of acetaldehyde and ethanol were compared with glucose, maltose and maltotriose concentrations during VHG fermentations (Publication V, Figure 2).

Unexpectedly, strong and co-ordinate increases in expression of genes for maltase (*MALx2*), maltose transporter (*MALx1*) and the maltotriose-preferring transporter (*MTT1*), were observed only 6 h after pitching, when the glucose had hardly decreased. The highest expression of these three genes was observed during a short period between 20 and 33 h, while glucose was still present at 4 to 15 g l^{-1} and consumption of both maltose and maltotriose was slow (Publication V, Figure 2). The transcript levels of these genes began to decrease while glucose was still present and maltose and maltotriose had just started to decrease. When approximately 50% of the fermentable sugars had been consumed, expression levels of *MALx1*, *MALx2* and *MTT1* were below 25% of their maximum levels.

Genes involved in glucose phosphorylation are differently regulated with respect to the availability of glucose. *HXK2* codes for the predominant hexokinase isoenzyme during growth on glucose and is an important mediator of glucose repression (Entian, 1980). Hxp2p mediates repression of *HXK1* and *GLK1* (glucokinase) (Rodriguez *et al.*, 2001). Consistently with this, *HXK2* expression increased rapidly during the intial hours of fermentation, while expression of *HXK1* and *GLK1* strongly decreased. The *HXK2* transcript level started to decrease after about 25 h while glucose was still above 10 g l^{-1} (Publication V, Figure 2). Expression of *HXK1* and *GLK1* started to increase strongly after 21 h, when the level of glucose was below 15 g l^{-1}. The data indicate that Hxk1p and Glk1p are probably the major enzymes phosphorylating glucose during the second half of the fermentation.

3.2.4.2 Growth and flocculation

Flocculation of lager yeast towards the end of brewery fermentations provides sedimented yeast that can be conveniently cropped and used for the next fermentation. In addition, flocculation provides an effective way to initiate clarification of the beer. Premature flocculation, however, is a serious problem for brewers, resulting in slow and incomplete fermentations. (Verstrepen *et al.*, 2003) Expression of three flocculation-related genes (*FLO1*, *FLO8* and *FLO11*) was followed in yeast collected from the suspension (*i.e.* cells not yet flocculated and sedimented) (Publication V, Figure 3).

Expression of the cell surface glycoprotein gene *FLO11* followed the profile of the yeast dry mass curve. *FLO11* gene expression level increased sharply after approximately 20 h simultaneously with exponential growth, whereas decreasing *FLO11* mRNA level was observed when the yeast started to sediment. *FLO8* (a transcription factor required for flocculation) and *FLO1* (a mannose-binding cell wall protein) were expressed at similar and rather constant levels through the fermentations (Publication V, Figure 3).

Unlike in the case of aerobic *T. reesei* batch cultures, the ribosomal protein mRNAs measured in brewing conditions (*RPL5* and *RPL22*) showed decreasing levels before the yeast started to grow and did not thus correlated with μ. The highest expression of ribosomal protein mRNAs was measured during the 6 to 9 h after pitching. By 45 h (when the yeast was still growing), expression of *RPL5* and *RPL22* had fallen to 5% and 20%, respectively, of their maximum levels (Publication V, Figure 3c).

Expression of cell cycle-dependent genes was used to evaluate the degree of synchrony of cell division. Five genes (*ELO1, CLN2, HTB2, PMA1, ASH1*) with strongly cell cycle-dependent expression (Spellman *et al.*, 1998) showed sharp expression peaks during the first 15 h after pitching (Publication V, Figure 3d). *ELO1* and *CLN2* genes (induced in G1-phase) peaked 4 h after pitching. The histone gene, *HTB2*, (induced in S-phase) peaked at 6 h. Peaks in the expression of *PMA1* and *ASH1* at 9 and 12 h indicated that most cells reached M and late M/G1 phases at these times. Smaller, second peaks in the expression of these genes were observed 6 to 9 h after the first peaks, suggesting that a second round

of synchronised cell division involved a smaller sub-population of the cells. The degree of synchronization, however, varied between cultures (Publication V, Figure 3 and supplementary material). Furthermore, a lower degree of synchronisation was observed in the expression of these genes during the first day of fermentation, when freshly grown yeast was pitched to wort instead of aerated cropped yeast (data not shown).

3.2.4.3 Sterol synthesis and oxygen-sensitive genes

Ergosterol as the predominant sterol in yeast is an essential component in maintaining integrity of the plasma membrane and has been shown to be involved in restoring the fermentative capacity of cells after storage (Bloch, 1983; Higgins *et al.*, 2003a). Expression of 4 genes from the ergosterol biosynthesis pathway, *ERG3*, *ERG10*, *ERG13* and *HMG1*, and the sterol biosynthesis and transport gene *PDR16,* weas analysed by TRAC (Publication V, Figure 6). After pitching, the mRNA levels increased for *ERG3*, *ERG13* and *PDR16* by approximately 1.2 to 2.5 fold during the first 6 h, but were immediately down-regulated for *ERG10* and *HMG1* by more than 3-fold. 10 h after pitching the average expression of these genes was below 50% of the levels measured during the initial hours of fermentation.

Repression of genes involved in aerobic metabolism (*ROX1*, *COX5a*), and increased expression of the anaerobically induced *HEM13*, *COX5b* and *OLE1* genes (ter Linde & Steensma, 2002) during the first 15 to 30 hours of fermentation, indicated that the oxygen in wort and aerated yeast slurry was consumed during this initial period. Unexpectedly, these “aerobic” genes were up-regulated along with ergosterol biosynthesis and *PDR16* genes during the end phase of the fermentation, when fermentable sugars had been consumed and the yeast had stopped fermenting (Publication V, Figures 2 and 6). In addition, two oxidative stress-related genes, *YAP1* and *AAD6,* which are considered to be up-regulated in response to accumulation of reactive oxygen species (ROS) (Delneri *et al.*, 1999; Kuge *et al.*, 1997), showed increased expression towards the end of the fermentations, despite the anerobic conditions. Although genes involved in ergosterol biosynthesis showed increasing transcript levels in the end phase of fermentations, ergosterols were not synthesized, but squalene accumulation was observed (Publication V, Figure 1).

3.2.4.4 Comparison of *S. cerevisiae* and *S. bayanus* genes

In order to compare the expression profiles of *S. cerevisiae* and *S. bayanus* type genes, the *S. bayanus* sequences corresponding to five *S. cerevisiae* genes (*ADH1*, *ATF1*, *ERG3*, *ILV5* and *MALx1*) were identified from the genome and probes specifically recognising these sequences were designed (Publication V, Table 2). For *MALx1, ATF1* and *ILV5*, expression profiles of the *S. cerevisiae* and *S. bayanus* components were very similar (Publication V, Figure 7). In the case of *ILV5*, the *S. cerevisiae* probe may also recognise *S. bayanus* mRNA because there are only two mismatches (Publication V, Table 1), but the *S. bayanus* probe is expected to be specific (8 mismatches; Publication V, Table 2). However, for *ADH1* and *ERG3*, the *S. cerevisiae* and *S. bayanus* genes were regulated differently. Following a peak in expression at 25 h for both components, expression of the *S. bayanus ADH1* fell to a very low level for the remainder of the fermentation, whereas the *S. cerevisae ADH1* remained at about 40% of its maximum value until the very end of the fermentation (225 h). The observed decrease of *S. cerevisiae ADH1* mRNA may be an artefact, because the probe probably also recognises mRNA of the strongly down-regulated *S. bayanus ADH1* gene (1 mismatch; Table 1). For *ERG3,* the situation was reversed: the *S. cerevisiae* gene showed an early peak and then low expression until the second peak at 220 h, whereas expression of the *S. bayanus* gene from 50 h to the end of the fermentation remained rather constant at about 60% of its maximal level.

4. Discussion

4.1 Solution hybridization methods in expression monitoring

Two solution hybridization-based methods for transcript analysis with single- and multiplex detection of target RNAs using specific oligonucleotide probes were developed and applied in this study. Solution hybridization has a number of advantages over solid phase hybridization for gene expression analysis applications that require short protocol time and are used for large numbers of samples. The reaction equilibrium in solution is reached faster than on a solid surface (Thompson & Gillespie, 1987), variations arising from membrane or array quality are avoided and crude cell lysates can be used directly as sample material without purification of their RNA. Additionally, use of cell lysates provides the possibility to quantify the signals directly in relation to the cell amount used in the analysis. Avoiding extraction of RNA simplifies substantially the analysis protocol, and increases the feasibility and decreases the costs of the assays. We found up to 60% higher signal intensities per hybridization with crude lysates of less than 3×10^7 lysed yeast cells as compared to RNA extracted from corresponding amounts of cells (Publication I, Figure 4). This was probably due to two factors, *i.e.* avoidance of loss of target RNA during the extraction procedures, which can be up to 30% with affinity resin-based extraction (Carlsson *et al.*, 2000), and the "molecular crowding" effect, which brings the target and probe sequences into closer proximity in the reaction conditions (Sanders *et al.*, 1994).

With alkaline phosphatase-based fluorescence signal amplification (Publication I), the sensitivity reached was sufficient only for detection of RNA targets with high abundance. However, the sensitivity of this assay was further increased by digoxigenin tail labelling (Publication II, Leskelä *et al.*, 2005; Soini *et al.*, 2005). In the TRAC analysis, with fluorescent probe detection by capillary electrophoresis, a sensitivity level of 30 amol (20 pg) was reached (Publication II). Using a more advanced capillary electrophoresis device, the detection limit has been lowered by 5 to 10 -fold (data not shown). This is comparable to published solution hybridization methods using either radioactively labeled probes (Ishii & Ghosh, 1993; Palva *et al.*, 1988) or signal amplification by an enzyme (Albretsen *et al.*,

1990; Wicks *et al.*, 1998; Zammatteo *et al.*, 1997). With capillary electrophoresis-based detection, it is possible to reach a sensitivity level of hundreds (Swerdlow *et al.*, 1991) to even single molecules (Castro *et al.*, 1993) of DNA. In practice the background signal, caused by fluorescent probes unspecifically bound to nucleic acids or to magnetic beads, is the limiting factor of the sensitivity.

Solution hybridization methods using affinity capture to magnetic beads have not previously received much attention for high-throughput analyses due to the lack of automation possibilities. Equipment now available enabling automated magnetic particle processing even in 96-well format, makes this type of analysis more attractive. The TRAC analysis presented here is automated from affinity capture to probe elution, but we aim to increase the degree of automation of the analysis to increase the sample processing capacity. The simplicity of the sample preparation and the assay protocol permit fully automated analysis without complicated robotics, in contrast to microarray and real-time RT-PCR methods (Mifflin *et al.*, 2000).

The possibility of separating the oligonucleotide probes by size or by type of fluorophore label enables multiplex detection of mRNA targets. Probes with a relatively wide Tm range (59.7–75.2 °C) showed similar binding kinetics and efficiency in the hybridization conditions used (Publication II). In the studies presented here, TRAC was performed maximally as a 22-plex analysis (Publication V), but by using fluorescent labels with different emission spectra and using the available resolution of probe sizing even up to 60-plex TRAC assays could be achieved with oligonucleotide probes. Detection of multiple RNA sequences in a single sample reduces time, labor and costs as compared to singleplex detection methods. Performing the TRAC assay as even only 20-plex analysis for 96 samples yields 1920 expression levels, showing its potential for high-throughput expression analysis.

Deviation between 550 replicate TRAC measurements was 9% on average (Publication II, Figure 2) and intra (sample-to-sample) and interassay (day-to-day) coefficients of variation have been determined in other studies to be 2–5% and 3–6% respectively, depending on the probe-target pair (data not shown). The reproducibility of TRAC analyses is excellent as compared to microarray or RT-PCR based methods (Chuaqui *et al.*, 2002; Zhang *et al.*, 1997). The high

reproducibility of the results is partly due to automated sample processing. In addition, the conversion of RNA to cDNA as well as the target amplification required in methods such as RT-PCR can be expected to increase assay variability.

The TRAC assay is easy to set up for new targets. The probe design and pooling were automated (Kivioja, 2004; Kivioja *et al.*, 2002) and all probes used in these studies fulfilled defined specificity and sensitivity criteria. Based on the probe sequence, some thermodynamic properties such as T_m can be estimated rather reliably (SantaLucia, 1998), but the binding efficiency of oligonucleotide probes to the corresponding mRNA targets is less predictable. In oligonucleotide array analysis, it has been shown that only 10% of the randomly selected oligonucleotide sequences give a signal that is more than 5 to 15% of the highest signal (Luebke *et al.*, 2003). The effect of disrupting the secondary structure of the target RNA molecule has been especially difficult to observe in practice (Luebke *et al.*, 2003; Matveeva *et al.*, 2003), and is therefore often ignored in probe selection even though it is thought to be an important factor (Southern *et al.*, 1999). With a relatively small probe test data set, we saw a clear correlation between relative signal efficiencies and a target secondary structure measure which estimates the free energy difference between the target and target-probe pair, although in microarray hybridizations no correlation between these two factors has been observed (Luebke *et al.*, 2003). The basic difference between solution and solid phase hybridization, *i.e.* free interaction between probe and target *vs*. probe or target bound on a solid surface, probably causes differences in the thermodynamic properties of the probe-target binding. However, the data set used here is too small to draw any definite conclusions. It is not possible always to find the most efficiently binding probe sequence by predictive calculations of RNA secondary structures, but these calculations can be useful to eliminate poorly binding sequences. In our experiments, about 85% of the designed and tested probes have given a signal/noise ratio of more than 2 under conditions in which the genes were expected to be expressed, showing the good success rate of our probe selection.

Taken together, the solution hybridization methods developed and applied here have many key advantages that make them suitable for their intended applications in bioprocess monitoring. Analysis is simple and protocol time is short, RNA extractions and manipulations (cDNA conversion and amplification) are avoided and the protocols can be made fully automatic by robotic instruments,

allowing simultaneous handling of large sample numbers. In addition, with TRAC the possibility of performing the assay in a highly multiplexed manner makes it potent for high-throughput screening applications. Compared to other multiplex expression analysis methods xMAP (Zhang *et al.*, 2005) and eTag (Tian *et al.*, 2004), the main advantage of TRAC is the simplicity of the protocol and high flexibility in selection of the targets to be measured. In both xMAP and eTag, rather complex probe settings are required to perform the analysis (see Figures 5 and 6). Furthermore, both these methods require an enzymatic amplification step at some point of the protocol.

The selection of a method for analysis of gene expression levels is highly dependent on the intended application, the number of samples or target genes and the organism under study. For example, the single-plex sandwich hybridization assay is effective in routine quantification of 1 to 5 target genes from some dozens of samples. However, as the number of target RNAs and samples increases, the advantages of the TRAC analysis become more evident. The potency of TRAC is probably most evidently demonstrated in the work presented in Publication V, where yeast samples from two brewing fermentations were collected at frequencies of up to 2 hours and the expression of more than 70 marker genes were analysed in all the samples, yielding altogether more than 10 000 expression levels. With conventional Northern blot analysis and also with single-plex qPCR, this work would have taken months to complete, whereas with TRAC it was performed within one week.

4.2 Signal normalization and quantification

The unsteady environmental conditions in production processes cause variability in practically all physiological parameters, making most commonly used expression normalization methods unreliable. In these studies, the expression levels of specific genes were mainly related to the amount of biomass or the total polyA RNA content used in the hybridization reaction. Relating marker gene expression to biomass can be more useful in predictions of culture performance such as specific growth and production rates (Publication IV, Figures 1 and 5), whereas normalization to overall mRNA expression (polyA RNA) predicts more accurately the physiological responses in metabolically active cells (Publication IV, Figure 5).

The most common way of normalizing non-biological variation with focused gene expression methods is to use so called house-keeping genes, the expression of which with respect to overall gene expression is assumed to be constant. *Act1* coding for actin protein and *gpd1* (glyceraldehyde-3-P-dehydrogenase) are two examples of genes widely used in data normalization (Foreman *et al.*, 2003; Higgins *et al.*, 2003a; Pakula *et al.*, 2003). However, we have not hitherto been able to identify any genes for which the expression relative to polyA RNA was constant independently of the conditions. With genome-wide microarray expression analysis the average of the signal levels of all genes is used in data normalization, which is similar to the polyA RNA-based normalization used here with TRAC. However, for normalization of genome-wide transcription data, the distributions of the expression signal intensities between samples are equalised, *i.e.* it is assumed that global shifts in the mRNA population do not occur, in other words the ratio of mRNA to total RNA is assumed to be constant. However, measurement of both polyA RNA to biomass and polyA RNA to total RNA ratios showed that neither of these ratios are constant and that they can also be affected by batch-to-batch variations (Publication IV, Figure 1). Disregarding global changes in mRNA levels during non-steady conditions (*e.g.* batch and fed-batch cultures) can result in masking of true gene expression changes between compared samples (van de Peppel *et al.*, 2003). Using a quantitatively measured factor to normalise expression levels between samples from different phases of a cultivation should thus be preferred over normalization based on on the assumption that a chosen factor is constant.

Relative quantification of the gene expression levels in microbial cultures is in the majority of cases sufficient to provide the required information. For example, the fold-change in transcript levels between the compared conditions describes the physiological responses of the culture. For modelling purposes and to compare transcript levels of different genes, absolute target quantification (*e.g.* copy number per cell) would be preferable. For a limited number of targets this can be achieved by using *in vitro* transcribed RNA as a standard, as was done for quantification of *SUC2* and 18S rRNA in yeast cultures (Publication I, Figures 5 and 6). In order to present the expression levels as absolute target amounts in the TRAC assay, the probe signals must be corrected by two factors, *i.e.* probe fluorescence intensity (fluorescence unit per mole of probe) and probe hybridization efficiency (percent of target captured by a probe). Since the

hybridization efficiency varies between probe-target pairs, both these approaches become impractical for large sets of genes.

4.3 Marker gene expression analysis

For the measurement of focused gene sets in the changing environmental conditions of biotechnical processes, the two major issues to be considered are 1) which genes respond in a manner that has predictive value for the outcome of the process and 2) how frequently should they be measured so that the potential responses are not missed.

Changes in gene expression levels measure physiological responses, which in turn lead to long-term physiological adaptation to prevailing surroundings. Responses of gene expression caused by some environmental factors are transient and differ in their kinetics. With the eukaryotic organisms *T. reesei* and *S. pastorianus* used in these studies, the impact of the experimental conditions studied appeared to affect the expression levels of responding genes at least for some hours. Maximal responses of certain genes due to addition of DTT causing the unfolded protein response (UPR) (Publication II, Figure 6) or to sudden anaerobic pulses (Publication III, Figure 4) were observed 1 to 2 hours after the shift in conditions, followed by dilution of the responses. Similar response times of stress related genes have been observed in studies with *S. cerevisiae* subjected to various stress conditions (see *e.g.* Gasch *et al.*, 2000). In prokaryotic organisms, the responses at the transcript level appear to occur in significantly shorter time periods. High concentrations of glucose given to *Escherichia coli* in transient pulses caused significant induction of stress-related genes within less than 1 minute, and 10 minutes was sufficient to relax the response (Enfors *et al.*, 2001). Similar response kinetics have been observed for *E. coli* heat shock genes following temperature increase (Soini *et al.*, 2005).

In addition to the transience of genetic responses, it appears that the regulatory profiles between different groups of genes vary. This was evident especially from the frequently monitored brewing fermentations (Publication V). Expression levels of some mRNAs, such as those involved in maltose metabolism and glucose fermentation, appeared consistently to follow smooth and simple

profiles related to the sugar concentrations. In contrast, for mRNAs involved *e.g.* in flocculation, amino acid metabolism or in cell cycle-related functions, fluctuating levels were measured through the fermentations. The transcriptional control mechanism is potentially more complex with these "fluctuating" genes. An important practical consequence of the transience of some genetic responses is that the "snapshots" of genome-wide expression profiles taken at isolated timepoints during complex industrial processes give incomplete and in some cases even misleading data of the genetic responses. This is particularly evident when the cell cycle-regulated genes are considered. Approximately 13% of the *S. cerevisiae* genome is under cell cycle-dependent regulation. Based on the expression of five yeast genes induced during G1, S, M or M/G1 phases of the cell cycle during the first 20 h of wort fermentation (Publication V, Figure 3), it is obvious that the result of genome-wide expression analysis would vary significantly depending on the exact sampling time during the first 20 h of fermentation. This problem does not arise in the constant conditions of chemostat cultures, which are therefore preferable for "theoretical" systems-wide analyses (Hayes *et al.*, 2002; Hoskisson & Hobbs, 2005; ter Linde *et al.*, 1999). However, the problem must be faced for practical studies of real industrial processes, necessitating frequent analyses of the kind facilitated by TRAC methodology.

In these studies some of the genes for TRAC analysis were selected on the basis of their responses in conditions of interest as reported in publically available gene expression data for various species of filamentous fungi or *Saccharomyces cerevisiae* (see Publication IV, Table 1 and Publication V, Tables 1 and 2). This data has mainly been produced by experiments in which wild-type cells have been shifted from standard growth conditions to stressful environments, such as elevated temperatures, acidic or alkalic surroundings, medium containing high concentrations of salt, sorbitol or drugs that damage cell structures or cellular redox potential (*e.g.* Causton *et al.*, 2001; Gasch *et al.*, 2000). In most cases these environmental shifts change the expression of hundreds or thousands of genes. Comparison of expression programs generated by different conditions reveals that some expression changes are specific to a particular environment, whereas others occur in all environments tested (Gasch & Werner-Washburne, 2002). Consequently, this markedly limits the number of potential target genes that could be used in prediction of a certain physiological state. On the other

hand identification of such marker genes requires large data sets with sufficient number of repeated experiments and sophisticated bioinformatic tools. One such tool in the identification of relevant genes could be the COSA algorithm (Friedman & Meulman, 2004), which can be used to organise genes into clusters according to their expression and score the genes within the cluster according to their importance. There is still only a limited amount of published data available for prediction of different aspects of bioprocess performance by gene markers, and in particular there are generally far too few replicate measurements for reliable identification of gene markers. In addition, there is a lack of tools for convenient monitoring of the identified markers in industrial environments. These limitations can be expected to change as systems-wide data accumulate and new high-throughput analysis tools are developed.

4.4 Use of TRAC in off-line analysis of fungal cultures

In these studies, TRAC was used as an off-line analysis method, *i.e.* samples withdrawn from the fugal cultures were analysed after the processes were finished. The TRAC results presented here demonstrated the usefulness of TRAC not only as a tool for function genomics but also as potential aid in optimisation of protein production processes and in quality control of brewing fermentations.

4.4.1 Optimisation of protein production

The changing specific extracellular production rates of the secreted enzymes, CBHI and laccase, were consistent with the expression profiles of the corresponding genes *cbh1* and *lac1* in (fed)-batch and continuous cultures (Publication IV, Figures 4 and 6). The expression of these genes was found to be highest in the specific growth rate range of 0.03 to 0.05 h^{-1}, which was in accordance with the optimal growth rate (0.031 h^{-1}) for production of extracellular proteins in T. reesei determined from chemostat cultures (Pakula et al., 2005). In addition to growth or substrate feeding rates, medium composition (Publication IV, Figure 6) and O_2 level (Publication III, Figure 4) also affected the transcript levels of these secreted enzymes. Furthermore, it was observed that genes coding for hydrolytic enzymes (*cbh1*, *egl1*, *bgl2*, *bga1*) responded sensitively to various process disturbances such

as high concentrations of antifoam reagent (polypropylenen glycol) or to high cell density stress (Publication III, Figure 2). Thus, it can be concluded that these enzymes are expressed optimally in a narrow physiological window and that their intensive monitoring is useful in defining the optimal production conditions.

In order to identify optimal process conditions and to detect process disturbances, several other potentially useful gene markers were defined besides the genes of the hydrolytic enzymes. Altogether 9 studied genes were induced close to exhaustion of carbon source and during starvation (Publication IV, Figure 5). Enzymes coded by these genes were involved in physiological events that are connected to nutrient limitation and slow growth, such as mobilisation of storage carbohydrates, stress protection, acquisition of nutrients from the medium and apoptosis. Induced expression of these genes can thus indicate conditions in which carbon source or another nutrient concentration is too low for optimal product formation. Consistently with this, in the fed-batch and continuous cultures performed, some of these genes were induced when the growth rate was below the optimal range (*e.g. acs1* in Publication IV, Figure 6). These genes form a potential group of markers to be used in optimisation of feeding strategies. They could be particularly useful in combination with the DELTABAS feed control system (Bailey & Tähtiharju, 2003) in order to maintain conditions that favour maximal secretion of proteins but avoid exhaustion of the carbon source and subsequent cell death.

The heme biosynthesis gene *hem6* and heat shock protein *hsp70* were shown to be the most sensitive markers for anaerobic conditions among the *T. reesei* genes assessed here (Publication III, Figure 4a; Publication IV, Figure 3). The *S. cerevisiae* homologue of *hem6* (HEM13), along with several other heme biosynthesis genes, has been shown to be induced under anaerobic conditions, even though heme cannot be synthesised without oxygen (ter Linde & Steensma, 2002). The Ssa type Hsp70 proteins have been shown to be part of a complex that regulates transcription of genes expressed in aerobic conditions (Hon *et al.*, 2001). Based on the expression of these genes during batch, fed-batch and continuous processes, 30% dissolved oxygen (DO) concentration is apparently sufficient to maintain aerobic culture conditions even at relatively high cell density (DW ~20 g l^{-1}). Strong induction of these anaerobic marker genes and decreased levels of the mRNAs for cellulase genes were observed only when

DO was below 10% (data not shown). In large-scale industrial bioprocesses with slurries of filamentous fungi, inhomogeneity of the system can cause local limitations in oxygen supply (Li *et al.*, 2002b). Thus, monitoring the expression of these markers in such processes could be useful in designing conditions that would better maintain sufficient overall oxygenation and high productivity.

Taken together, many of the selected *T. reesei* genes appeared to have value for prediction of physiological events and process performance, such as growth rates, enzyme production rates and nutrient and oxygen limitations. TRAC-assisted optimisation of such factors could be particularly useful for novel processes aiming at production of new protein products, where prior knowledge of preferable conditions is limited. Transcriptional analysis can provide a more complete picture of the physiological state of an organism than is obtained from the external parameters conventionally measured during production processes, and thus established processes may also benefit from the increased knowledge of culture physiology that TRAC offers. The future accumulation of microarray data from *T. reesei* and other industrially relevant organisms grown under various conditions can be expected to identify further marker genes that are valuable for prediction of culture performance.

4.4.2 Steady state control

Chemostat or other continous flow cultures provide a reproducible environment that enables production of cell populations that are in a physiological steady state. The quality of a large number of *T. reesei* chemostat cultures was addressed in these studies by comparison of conventional process parameters and transcriptional stability as assessed by TRAC. To reach a physiological steady state in chemostat cultures with filamentous fungi is difficult, because various factors can cause inhomogeneity in the culture (wall growth, viscosity, presence of metabolically active and inactive biomass *etc.*). Consistently with this, only in some of the *T. reesei* chemostat cultures performed in this work were good physiological steady states observed, based on the stability of all measured parameters (conventional and mRNA). In these cultures, steady state gene expression levels were reached within less than 1 generation from the onset of the continuous medium flow. Expression levels remained stable for 5 to 6 generations.

Instabilities were always evident at the level of transcription in those cultures where steady states were not reached, but were not always detectable in the conventional process analyses. The instability at the transcriptional level indicated that some undetected perturbation in the environmental conditions had occurred. Microorganisms are able rapidly to adjust their internal physiology to variable conditions (Gasch & Werner-Washburne, 2002), and adjustment of genetic expression is expected to be among the earliest detected changes in cellular physiology in response to environmental perturbations. By choice of appropriate marker genes, the TRAC data can also indicate the source of the perturbations.

After intentional perturbation of the steady states of the fungal cultures by exposing them to anaerobic conditions for 0.3–3 h, the recovery of transcript levels of affected genes to their initial level was rapid after restart of culture aeration. Expression of the genes reached the previous steady state levels within 5 h once aeration was restarted. *T. reesei* sustained metabolic activity surprisingly well without oxygen, although it is not able to grow in anaerobic conditions. Short-term deprivation of oxygen triggered physiological responses, but *T. reesei* recovered relatively fast even from 3 h of total anaerobiosis (Publication III, Figure 4) and with no signs of cell lysis (data not shown). Bonaccorsi *et al.* (2006) observed a similar rate of recovery of gene expression in another *T. reesei* strain (QM9414). Within 2 h following a 2 h period of anaerobic conditions at $D = 0.01\ h^{-1}$ with glucose as the carbon source, the genes affected were expressed at their previous level. It is worth noting that *S. cerevisiae* appears to require more time to adjust to a change in conditions than does *T. reesei*. Four to five *S. cerevisiae* generations are required to attain a transcriptional steady state following a change to anaerobic conditions (Wiebe *et al.*, 2007), whereas a shift from growth on galactose to growth on glucose required more than 10 generations (Braun & Brenner, 2004).

This study demonstrated that transcriptional monitoring of chemostat cultures is beneficial prior to costly and laborious systems-wide analyses in order to increase their reliability by ensuring that samples from only reproducible cultures are analysed. TRAC was successfully applied for two purposes, *i.e.* as a functional genomics tool for studying transient responses of selected genes and as a quality control tool to ensure steady state prior to selection of cultures for global level biological studies.

4.4.3 Quality control of alcoholic fermentation

From the gene expression point of view, the most active phase of brewing fermentations was the first two days. Total mRNA levels peaked at about 20 h, when yeast had finalised the first round of cell division (Publication V, Figure 3). 90% of the genes selected for TRAC analysis showed their highest expression during the first 75 h, when the yeast concentration in suspension was still increasing and about 50% of fermenatable sugars had been consumed. These observations agreed with the microarray studies of Olesen *et al.* (2002), who found expression of all *S. cerevisiae* ORFs to be at their highest on day 2 in industrial lager fermentation.

4.4.3.1 Novel findings of brewer's yeast gene regulation

An unexpectedly strong and coordinate increase in expression of the malto(trio)se metabolism genes, *MALx1*, *MALx2* and *MTT1,* was observed only 6 h after pitching when glucose was still above 20 gl^{-1} (Publication V, Figure 2). This showed that glucose did not repress maltose metabolism in the strains used under these conditions (wort maltose/glucose = 4.8). Maltose transport activity measured in another industrial strain (A63015) also increased during the initial phase of fermentation, suggesting that catabolite inactivation of maltose transport was also ineffective. In our earlier work (Rautio & Londesborough, 2003) maltose transport of both lager and ale strains decreased strongly during the first 20 h of fermentation in high glucose wort (maltose/glucose = 1.8). The maltose/ glucose ratio of wort has a strong influence on yeast physiology and this ratio varies widely in industrial worts.

The high expression of maltose metabolism genes decreased to 20% of the maximum already by 50 h, when concentrations of maltose and maltotriose were still high (90 and 35 g l^{-1}, respectively). One possible mechanism for turning off the maltose metabolism genes could be the COMPASS histone methylation complex that represses expression of telomerically located maltose utilisation genes during late stages of fermentations starting at high maltose concentrations (Houghton-Larsen & Brandt, 2006). Whatever turns off the expression of *MALx1*, *MALx2* and *MTT1*, it means that maltose metabolism through most of the fermentation is dependent on the proteins synthesised during the first 50 h.

Another set of yeast genes for which the regulation during wort fermentations was unforeseen, were genes involved in oxygen-regulated pathways and oxygen-requiring ergosterol synthesis. High expression of ergosterol genes as well as the aerobic genes, *ROX1* and *COX5a*, was measured during the first 6 to 10 h of the fermentations. This was expected, since both the yeast and wort were aerated and yeast synthesizes ergosterols during the very first hours of fermentation and the corresponding genes are up-regulated during this period (Higgins *et al.*, 2003a). However, between 100 and 170 h, when most sugars had been consumed, there was a second up-regulation of these genes, including *ROX1*, *COX5a*, *ERG3*, *ERG10*, *ERG13* and *HMG1* (Publication V, Figure 6). This up-regulation was observed in a replicate fermentation despite extra precautions to exclude air from the fermentations. Markedly stronger expression of genes in the ergosterol pathway as well as of *ROX1* and *COX5a* was also observed by James *et al.* (2003) in lager fermentations, when microarray data from Day 8 was compared to Day 1, although they dismissed these data from discussion.

There are at least three possible explanations for the observed activation of aerobic genes in anaerobic conditions. In the final stages of the fermentations some of the yeast cells are dying, and it is possible that the heme released from the dying cells could be taken up by other cells and activate the *HAP1-ROX1* pathway. However, viabilities in some of the cultures where these effects were observed were still rather high in the end of fermentations (up to 93%). The present day brewer's strains are evolutionary products of repeated recycling through anaerobic brewer's fermentations with a few hours' exposure to oxygen in each cycle. Thus, the observed response could be a sign of evolutionary adaptation of the brewer's yeast to these conditions. Yeast would gain a significant growth advantage by synthesising squalene (the end point of the anaerobic part of the ergosterol synthesis pathway) before repitching into fresh aerated wort, where squalene must be synthesised in a few hours. Another possibility is that the observed inductions are a sign of a general stress response to the environment with insufficiency of nutrients and high concentration of ethanol. Induced levels of ROX1 have also been observed in yeast damaged by a mildly toxic dose of a monofunctional SN2 alkylating agent, methyl methanesulfonate (MMS) (Jelinsky *et al.*, 2000).

Monitoring of the expression of several cell cycle-dependent genes at 2 to 3 hour intervals during the first 54 h of wort fermentation revealed an ordered series of sharp expression peaks. Yeast entered G1-, S-, M-phases and the M/G1 boundary at about 4, 6, 9 and 12 hours, respectively. This suggests that the first round of cell division was strongly synchronised, which has previously been reported for wort fermentations (Hutter, 2002). Smaller and broader peaks of these cell cycle-associated genes were detected 6–9 h after the first peaks, indicating a decreasing degree of synchrony for the second round of division.

Comparison of expression profiles of genes originating from the *S. cerevisiae* and *S. bayanus* parts of the lager strain's genome revealed that profiles of *MALx1, ILV5* and *ATF1* were very similar for the two components, but the expression profiles of *ADH1* and *ERG3* were markedly different (Publication V, Figure 7). Thus, the use of microarrays of *S. cerevisiae* genes alone gives incomplete and possibly misleading information about the physiological behaviour of the hybrid lager strains. Better knowledge of their physiological adaptation through the wort fermentations requires that expression levels of both sets of genes are measured.

4.4.3.2 Potential applications of TRAC for brewing

The frequent sampling allowed by TRAC showed a very dynamic pattern of gene expression especially during the first two days of wort fermentations. By providing an effectively continuous time profile of the expression of chosen genes, TRAC reveals how changes in gene expression predict their physiological consequences at least several hours in advance. This offers the possibility of using this kind of data to monitor and control fermentations. For industrial brewers TRAC could be a potential tool for predicting when the fermentation is going to be finished and the tank can be used again. With gene markers predicting beer quality (*e.g.* flavour profile), brewers could decide whether to sell the beer as it is or blend it with beer from better fermentations. Thus TRAC could be used in planning very large scale operations, where mistakes are expensive.

TRAC may also have applications in assisting brewers to design and optimize novel processes. TRAC could be used as a tool *e.g.* in testing and optimisation of wort composition. In addition TRAC may be useful with a proper set of gene

markers, for testing the suitability of yeast batches for subsequent fermentations. Our unpublished data indicate that different batches of cropped yeast respond differently to stimulation, such as aeration, in a way that may predict the subsequent fermentation performance. This idea requires testing with statistically significant numbers of replicates under brewery conditions.

Yet another application of TRAC that could be useful for brewing science is screening of strains that have qualities advantageous for wort fermentations, such as the ability to activate maltose metabolism genes in the presence of glucose.

4.5 Future perspectives

4.5.1 The potential of TRAC for on/at-line analysis

For studies involving process optimisation, quality control or functional genomics measurement of transcript levels off-line is in general sufficient. However, in order to control bioprocesses by TRAC, at-line or even on-line instrumentations would be required. Further development of, particularly, the sample processing and detection technology are needed for such on-line measurements. TRAC analysis in its present form can be performed in 2–3 h for 96 samples. For a lower number of samples with an optimised protocol this time can be shortened. An analysis response time of 1–2 h would be sufficient in long lasting processes, such as those performed in these studies with brewer's yeast and filamentous fungus. One potential solution for applying the TRAC method for on-line measurement is the use of miniaturised silicon chips that could be used for rapid analysis of small sample amounts. Such a TRAC chip solution is currently being developed, the installation of which at bioprocess sites would be more convenient compared to present day TRAC instrumentation. Alternatively, TRAC assays could potentially be performed as on-line analysis using lab-on-valve (LOV) systems (see Section 1.3.1.1). These LOV systems enable handling of small beads with active functional groups as required by TRAC. In addition, CE analysers have been used as detectors in connection to LOV (Wu *et al.*, 2003). As well as technical development, biological research is also required to identify marker genes, the monitoring of which would be useful in prediction of the culture performance. Data from large sets of bioprocesses (including

industrial scale) should be correlated with gene expression profiles to verify statistical significance. Combination of TRAC with other measurement technologies used for evaluation of the different physiological aspects of the cell, such as different spectroscopic measurements as well as flow cytometry analysis (see Section 1.3), would be most beneficial in gaining comprehensive data sets.

The cost-to-benefit ratio of development of such on-line analysis systems should also be low enough that investments would be reasonable. With bioprocesses producing high cost products, such as pharmaceuticals (*e.g.* erythropoetin), the process optimisation and control possibilities gained by on-line gene expression analysis could be highly beneficial. In the case of medicine production, legislation is becoming stricter, requiring comprehensive monitoring of the productions (FDA, 2004).

There are a number of benefits that could be gained by on-line TRAC analysis. Because the responses at gene expression levels predict the physiological outcomes, suitable marker genes in combination with rapid expression analysis could function as early warning signs of process failure. By placing sampling sites at different locations of an industrial scale fermentor, culture gradients such as local limitations of oxygen or nutrients could be detected. Gene markers providing a signal for optimal process termination time could also be useful in avoiding problems in down-stream processing due to cell lysis.

4.5.2 Novel applications for TRAC

The TRAC technique has a wide application field. In addition to the *T. reesei* and *S. pastorianus* processes studied in this work, the TRAC method has been applied to transcriptional analysis of other eukaryotic microorganisms, including *S. cerevisiae* (Kataja *et al.*, 2006; Wiebe *et al.*, 2007), *Pichia pastoris* (Gasser *et al.*, 2007), *Fusarium graminearum* (Rautio *et al.*, unpublished data), for quantification of bacterial populations (Maukonen *et al.*, 2006; Satokari *et al.*, 2005) and for oncogene expression analysis in colon cancer cell lines (Rautio, unpublished data).

The use of TRAC is most advantageous in applications involving focused expression analysis in large numbers of samples, which could make this method powerful in connection to high-throughput bioprocessing (HTB). HTB techniques are used in small volumes for screening optimal production conditions and strains for a given production process (Betts & Baganz, 2006). Transcriptional analysis, and particularly analysis of focused gene sets having predictive value for the performance of mini-scale bioprocesses, could also be an effective way for screening of preferred process or strain qualities. The TRAC assay developed in this study offers a potential tool for this purpose.

Monitoring of *e.g. S. pastorianus* fermentation by TRAC was performed from less than 300 µl of VHG fermentation volume. This amount of liquid could easily be fitted in 96-well format, opening interesting possibilities for screening applications. Such mini-scale cell culture TRAC analysis is already being performed with human cell lines, with the purpose of studying the effects of various chemicals or drug candidates on the expression of disease-releated gene sets. However, this application would require the development of robotic solutions to increase the sample processing capacity at least to some thousands of samples per day instead of the current 200 to 300 samples per day. With 30 to 60-plex target detection such high-throughput solutions would yield 50 000 to 200 000 expression levels daily.

There are also a number of other applications where the TRAC method could be applied or already has been applied. Short interfering RNA (siRNA) molecules are used in silencing of mRNAs of selected genes (Leung & Whittaker, 2005) and are currently widely applied in screening the effects of loss of gene functions (Bjorklund *et al.*, 2006). TRAC has been used in connection to siRNA screening in verification of gene silencing by the RNA interference process, and a further application could be to follow the expression of the identified genes and the pathways in which these genes are involved.

We have recently developed a new application for TRAC that enables identification of single nucleotides in expressed RNA (Söderlund & Rautio, 2007). This enables analysis of single nucleotide polymorphism (SNP) in expressed RNA and expression analysis of splice variants of a given transcript. This application has a great potential *e.g.* in studies of expression of genes causing inherited diseases.

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PUBLICATION I

Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates

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Research

Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates

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Abstract

Background: A rapid microtiter plate based sandwich hybridization assay was developed for detection and quantification of single RNA species using magnetic beads. Following solution hybridization target RNA molecules were collected by biotin-streptavidin affinity binding and detected by fluorescence signal generated by alkaline phosphatase. The 18S rRNA and SUC2 mRNA of *Saccharomyces cerevisiae* were used as model RNA target molecules.

Results: The sensitivity of the assay was approximately 1.2×10^9 (2 fmol) molecules of target RNA. The developed method was feasible with crude cell lysates of *S. cerevisiae carlsbergensis* and was evaluated by measuring the levels of 18S rRNA during cell growth and SUC2 mRNA under repressive and inductive conditions. The 18S rRNA expression level followed the changes in the specific growth rate. SUC2 mRNA levels were in good correlation with the measured invertase enzyme activities.

Conclusions: The here presented sandwich hybridisation method was succefully applied for monitoring the amounts of ribosomal RNA and mRNA with high expression level in shake flask cultivation conditions. Sandwich hybridisation method offers a fast and convenient tool for following single key RNA species of interest in the production conditions.

Background

Development of novel methods for fast, sensitive and reliable RNA quantification has recently got increasing attention. Conventional methods for RNA analysis, like Northern and slot blot hybridization are in general time consuming, laborious and allow only relative quantification within a narrow concentration range. More novel methods for RNA analysis e.g. RT-PCR and real time RT-PCR are highly sensitive, but also slightly susceptible to experimental interferences, like template inhibition due to insufficient purification [2] and lack accuracy for quantification due to biases connected to PCR and reverse transcription reactions, which in general are accepted errors connected to these methods.

The sandwich hybridization method is a suitable alternative for RNA quantification. The method is based on the detection of hybridization events between two specific

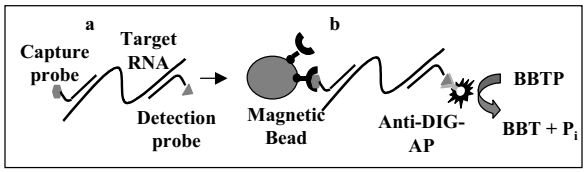

Figure 1
Principle of the sandwich hybridization assay. In solution hybridization the biotin labeled capture probe and the digoxigenin labeled detection probe are first hybridized with target RNA (a) followed by hybrid immobilization on magnetic beads and detection with anti-DIG – alkaline phosphatase FAB fragments (b). Alkaline phosphatase cleaves BBTP (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate, Atto-Phos®) to inorganic phosphate (Pi) and BBT (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole)

oligonucleotide probes and the target nucleic acids. The capture probe is used to immobilize the target sequence on a solid support and the detection probe is labeled with a detectable marker (see Fig. 1). Sandwich hybridization is relatively sensitive and can be performed with crude biological samples [20].

Sandwich hybridization assays from crude cell samples or in connection to PCR have been extensively used in clinical diagnostics for detection of nucleic acids from bacteria [4,6,7,13,15,21,23], viruses [1,3,9,11,14,17,25] of gene mutations [5] and for cell typing [19]. The aim in our laboratory is to apply this method for monitoring of bioprocesses. The sandwich hybridization method would be ideal for measuring the levels of specific mRNAs in yeast and bacterial cells during the fermentation processes.

Here we describe a sandwich hybridization method for quantification of RNA to be applied for measuring levels of specific RNAs in yeast cells as an informative tool for the control and state analysis of bioprocesses. The developed method is evaluated by measuring the levels of *Saccharomyces cerevisae carlsbergensis* 18S rRNA and SUC2 mRNA in shake flask experiments.

Results

The aim of the present study was the development of a fast and reliable method for quantification of single RNA species in solution using a sandwich hybridization assay applicable for analysis of bioprocesses. The influence of different parameters like probe concentrations, concentrations of the components of the hybridization solution, reaction times, amount of beads and hybridization temperatures were investigated to optimize the hybridization protocol and the sensitivity level. The 18S rRNA and SUC2 mRNA encoding invertase enzyme of *S. cerevisiae* were used as model RNA target molecules within this study.

Detection limit and linear range of the developed sandwich hybridization assay

The sensitivity level and the linear range of the assay were determined with the solution based sandwich hybridization system using 3.1×10^6 – 7.2×10^{11} (5 amol – 1.2 pmol) *in vitro* transcribed 18S rRNA molecules of *S. cerevisiae* as target. The biotin labeled oligonucleotide probe 18S rRNA-400 was used as capture probe and the digoxigenin labeled oligonucleotide probe 18S rRNA-1302 was used for detection. The signals of the sandwich hybridization method were compared to those of the slot blot hybridization.

1.2×10^9 (2 fmol) target molecules in the solution hybridization gave a signal that was 4-fold above the noise level. The reaction was linear up to 5.9×10^{11} (980 fmol) target molecules (Fig 2.)

The same amounts of 18S rRNA target molecules were immobilized onto a filter membrane in slot blot hybridization assay. Digoxigenin labeled 18S rRNA-1302 probe was used for chemiluminescence detection of the target molecules. Approximately the same amount of target molecules as in sandwich hybridization assay gave a signal that was 4-fold above the noise level. With the used detection system the linear range of the slot blot method was between 1.2×10^9 and 3×10^{10} (2 – 50 fmol) target molecules.

Real-time PCR experiments were performed with 18S rRNA ranging from 2×10^4 – 6×10^9 target molecules (0.04 amol – 10 fmol). Detection limit was estimated to be 4×10^5 molecules (0.6 amol) and the linear range was greater than 4 orders of magnitude, making this method most sensitive with widest linear range.

Comparison of hybridization with crude cell lysates and extracted RNA

To create a fast analytical system it was investigated, whether a separate total RNA extraction step is necessary or whether crude cell lysates could be used as sample material. The assay was performed using either crude lysates of 7×10^4 – 1.2×10^9 exponentially grown *S. cerevisiae carlsbergenesis* cells or total RNA extracted from the same amount of cells. 18S rRNA was used as target for method comparison since it is abundant in the cell.

Fig. 3 shows that when less than 3×10^7 cells as crude lysate were added to the hybridization solution the signal of the respective target molecules was up to 60% higher

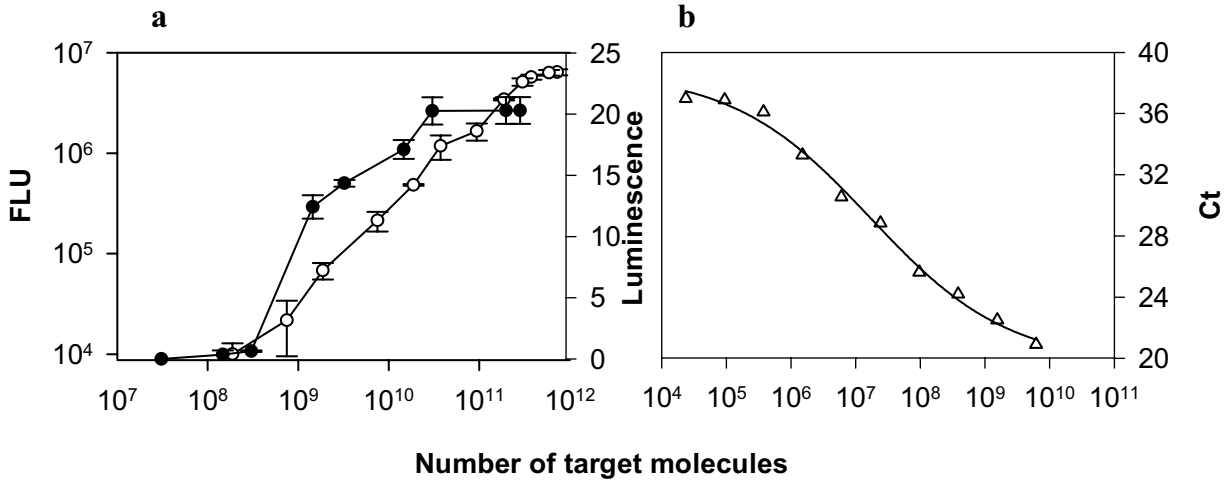

Figure 2
Comparison of sandwich solution hybridization with slot blot filter hybridization assay and real-time PCR. In **a)** 3.1 × 10^7 – 7.2 × 10^{11} 18S rRNA target molecules were added to the sandwich hybridization solution (○) or to the membrane for slot blot hybridization (●). Fluorescence (sandwich assay) and chemiluminescence (slot blot assay) signals were both generated by anti-DIG-alkaline phosphatase-FAB fragments. In **b)** 2 × 10^5 – 6 × 10^{10} 18S rRNA target molecules were amplified by the real-time PCR reaction, using SYBR-green as detection dye (Δ). Ct is number of cycles needed to reach threshold fluorescence signal level. The error bars show the ± SD of three parallel samples.

when compared to signal measured from total RNA extracted from the same amount of cells. When the amount of crude cell lysate was further increased the signal started to decrease probably due to unspecific reactions of the crude cell material. With total RNA the same inhibition effect was observed when more than 2.5 × 10^8 yeast cells were used for extraction.

The effect of biological material

The effect of biological material on the signal of the solution hybridization was studied by incubating various amounts (2 × 10^5 – 1 × 10^9) of lysed *S. cerevisiae* BY4737 cells or total RNA extract of the cells together with 1 × 10^{11} *in vitro* transcribed SUC2 mRNA target molecules. BY4743 yeast cells contained undetectable levels of SUC2 mRNA when grown on glucose (data not shown). No inhibition was observed when 10^6 or less lysed yeast cells or their RNA content were added to the hybridization (Fig. 4). 40 – 55% decrease in the signal of SUC2 mRNA was observed when 10^7 to 10^9 lysed cells were added to the hybridization solution. With extracted RNA the decrease was respectively 15 – 40%.

The inhibition effect of cell lysate on the hybridization was taken in to consideration, when the signal of target RNA in sandwich hybridization was quantified in growth experiments (see Fig. 5 and 6).

Monitoring of 18S rRNA and SUC2 mRNA in growth experiments with S. cerevisiae carlsbergensis

To evaluate the developed sandwich hybridization assay with respect to its application in monitoring of different RNA species in bioprocesses, 18S rRNA and SUC2 mRNA were analyzed at different growth phases during shake flask cultivations of *S. cerevisiae carlsbergensis*. 18S rRNA was used as target for method evaluation due to its high cellular amount and stability. mRNA molecules represent only approximately 5% of total RNA in yeast [24] and thus are suitable targets for sensitivity evaluation. Expression of SUC2 mRNA expression can additionally be easily controlled by glucose repression [10].

Fig. 5 shows the level of 18S rRNA detected by the solution based sandwich hybridization assay in relation to the optical density and the specific growth rate of the yeast. The amount of 18S rRNA molecules per cell was calculated on the basis of a standard curve created with *in vitro* transcribed 18S rRNA. The hybridisation efficiencies of probes to native 18S rRNA and *in vitro* transcribed 18S rRNA were assumed to be the same. The assay was performed with a low number of 1 × 10^6 – 3 × 10^6 lysed yeast

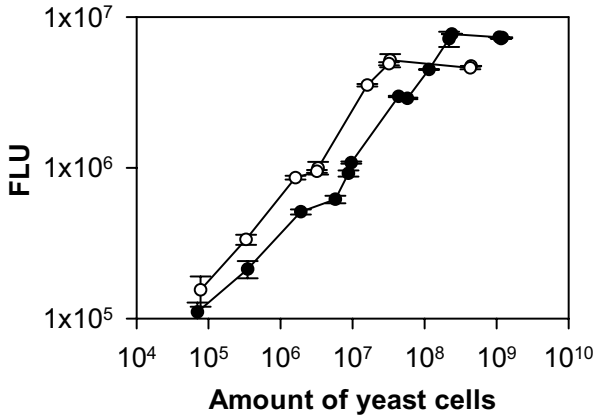

Figure 3
Comparison of extracted total RNA and crude cell lysate as sample material in the sandwich hybridization assay. 18S rRNA was used as target molecule for method evaluation. 5 ml of OD_{600} = 2 yeast culture was lysed (❍) or used for extraction of total RNA (●). Different dilutions of lysed yeast cells or total RNA extracted from the same number of cells were added to the sandwich hybridization solution. Fluorescence (FLU) measured with 18S rRNA probes is presented in relation to the amount yeast cells used for lysis or extraction of RNA. The error bars show the ± SD of three parallel experiments.

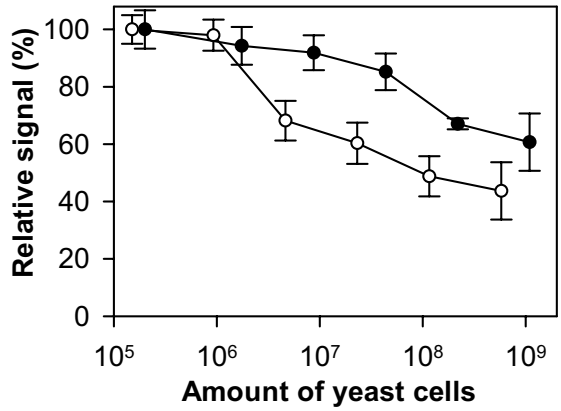

Figure 4
Effect of cell lysate and total RNA on the signal of the solution hybridization. 1.5 × 10^{11} SUC2 *in vitro* transcribed RNA molecules were added to the hybridization solution containing crude cell lysate (❍) or RNA extract (●) from 1 × 10^6 – 1 × 10^9 *S. cerevisiae* cells grown on glucose. The results are expressed as a percentage value of the signal compared to that obtained with the same amount of pure *in vitro* transcribed target SUC2 mRNA. Approximately 1.4 μg total RNA was extracted from 1 × 10^8 cells. The error bars show the ± SD of three parallel samples.

cells. Therefore the inhibitory effect of the cell extract on the assay was negligible (see Fig. 4).

During the first 5 hours the 18S rRNA level was slightly increasing and after that a nearly constant decrease was observed until 60 hours of growth. These results are in good correlation with the determined growth rate μ at the examined sampling points. Due to the fact that the preculture used for inoculation was in exponential growth phase, the increase of the 18S rRNA during the initial lag phase is relatively low.

Invertase activity is known to be under glucose repression and the corresponding SUC2 gene expression is induced by sucrose [10].

In Fig. 6 the level of SUC2 mRNA and the invertase enzyme activity were detected in cultures of *S. cerevisiae carlsbergensis* grown in shake flask cultures. In the two experiments the cultivation medium contained either sucrose or glucose as carbon source. For the calculation of the level of SUC2 mRNA the hybridization assay was performed with a cell extract containing 7 × 10^7 – 1.2 × 10^8 lysed cells. In this case the inhibition effect of the

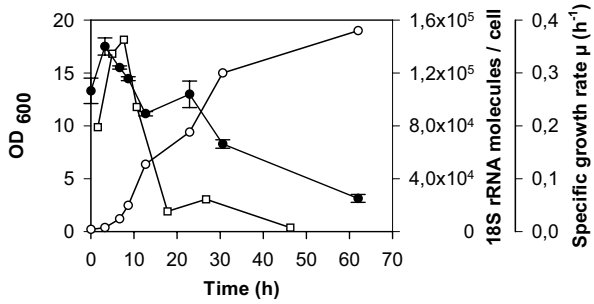

Figure 5
Level of 18S rRNA of *S. cerevisiae carlsbergensis* determined by sandwich hybridization as molecules per cell (●) in different growth states. The growth was followed by measuring the optical density at 600 nm (❍) and the specific growth rate μ (□) was determined. The yeast was cultured in medium containing 1% yeast extract, 2% Bacto peptone solution and 2% glucose as carbon source. 1 × 10^6 – 3 × 10^6 yeast cells as crude lysate was added to sandwich hybridization for 18S rRNA analysis. OD_{600} = 1 corresponds to 5.5 × 10^8yeast cells ml^{-1}. The error bars of show the ± SD of three parallel samples.

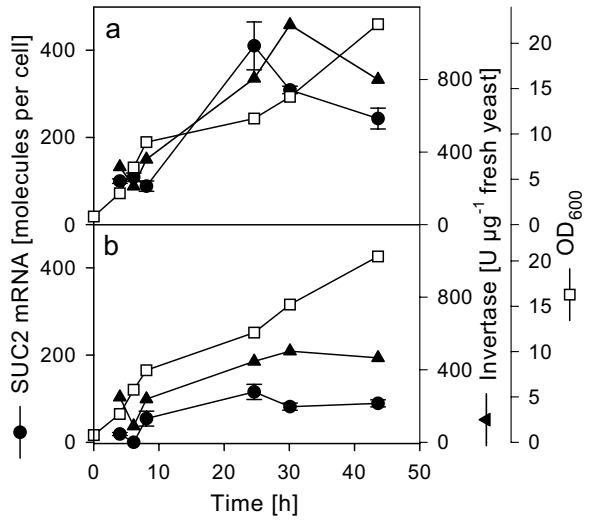

Figure 6
Level of SUC2 mRNA of *S. cerevisiae carlsbergensis* given as molecular amount per cell determined by sandwich hybridization (●) and the activity of invertase (▲) during exponential growth in medium containing 1% yeast extract, 2% Bacto peptone solution and either **a)** 2% sucrose or **b)** 2% glucose as carbon source. Growth was followed by optical density at 600 nm (□). $7 \times 10^7 - 1.2 \times 10^8$ yeast cells as crude lysate were added to the sandwich hybridization for 18S rRNA analysis. The error bars show the ± SD of three parallel samples.

biological material on the assay had to be considered according to Fig. 4 and the signals were accordingly corrected by 50 – 55% in dependence on the specific amounts of extract used.

During the initial growth phase the invertase activity was about 200 to 300 U μg^{-1} fresh cells in sucrose grown culture but slightly lower (100 – 200 U μg^{-1}) in glucose grown culture. In contrast the level of SUC2 mRNA was 5 – 10 times higher in the sucrose culture than in the glucose containing culture. After 24 h of cultivation the total activity of invertase was significantly higher in the sucrose grown culture than in the glucose grown culture (800 to 1100 U μg^{-1} versus 450 U μg^{-1}). Correspondingly there was an approximately four times higher amount of SUC2 mRNA detected in the sucrose grown culture than in the glucose containing culture (Fig. 6).

Discussion

The developed assay is based on hybridization of RNA target molecules with a biotin labeled capture probe and a digoxigenin labeled detection probe. Alkaline phosphatase enzyme is attached to the detection probe after hybridization, which amplifies a fluorescence signal used for quantification. The indirect enzyme labeling system was able to detect 10^9 RNA target molecules. This is comparable to the sensitivities reached by others with similar detection systems used for PCR product quantification [16,23]. With direct probe labeling by thermostable alkaline phosphatase 100-fold higher sensitivities have been reached in other studies [12,26]. Although the direct labeling is more sensitive, the indirect labeling method of the probes is beneficial if the aim is to measure a large number of different RNA targets in one microwell plate. Each detection probe does not have to be labeled separately with an amplifying enzyme, which decreases the storage time of the probes.

The aim here was to apply the developed method for RNA expression monitoring of fermentation processes. Recently, this was performed by Jürgen *et al.* [13] and Schweder *et al.* [17] by slot blot analysis, which is in general time consuming and laborious. Here we present a simpler and faster method, with the possibility to use crude cell lysates instead of extracted RNA as a major improvement. We found up to 60% higher signal intensities with crude lysates when less than 3×10^7 lysed yeast cells were used per hybridization, possibly because a loss of target RNA during extraction procedure can be avoided. Carlson *et al.* [3] have reported approximately 30% loss of RNA with affinity resin based extractions.

Avoiding this by use of lysates provides a great benefit compared to RT-PCR and array based methods, which always require a separate RNA purification step. Use of high concentrations of either cell lysate or total RNA caused a decrease of the fluorescence signal possibly due to unspecific binding. With low cellular levels of target RNA more cell material is required in the assay, and correspondingly, then inhibition has to be considered in signal quantification [20]. Thus the amount of cell lysate used in hybridization assay should be optimized for routine analysis.

The sensitivity of the used sandwich hybridization assay was determined to be approximately 2 fmol, which was similar to the membrane based slot blot hybridization assay. Nevertheless, the linear range of the solution hybridization was approximately 20-fold higher compared to membrane hybridization. The time required for the sandwich assay from sampling to detection was about three hours, which could be further shortened. In contrast, the membrane based hybridization and detection could not be completed within less than 12 hours. The main difference of the two methods is the time it takes for the hybridization to reach equilibrium. Real-time PCR takes about 5 hours to complete and was in our investigation the most sensitive method (0.6 amol), but this

method suffers from the demand of a very pure RNA preparation in order to avoid inhibition of the reverse transcriptase and DNA polymerase.

The described method was evaluated in shake flask experiments, where 18S rRNA and SUC2 mRNA levels were measured during different growth phases. rRNA can be used as an indicator of cellular activity or growth state [9]. Results showed that the changes in 18S rRNA expression level were simultaneous with the changes in the specific growth rate μ during different growth phases of the yeast *S. cerevisiae carlsbergensis*.

SUC2 mRNA levels were in good correlation with the measured invertase enzyme activities. Cultivation of *S. cerevisiae carlsbergensis* in either glucose or sucrose containing medium resulted in different levels of SUC mRNA and invertase activity. Our experiments confirm the knowledge that brewery strains are less sensitive to glucose repression than laboratory strains and are thus often more adapted to disaccharide utilization [8]. Whereas we found expression of SUC2 mRNA in *S. cerevisiae carlsbergensis* glucose-grown cultures, no expression of SUC2 mRNA was found in corresponding cultures of the laboratory strain *S. cerevisiae* BY4737 (data not shown).

The molecular amount of RNA per cell was calculated using *in vitro* transcribed 18S rRNA or SUC2 mRNA as standards. We determined 25 000 – 140 000 18S rRNA molecules per cell in dependence on the different growth phases. These values are in good agreement with results presented by Warner, who calculated about 200 000 ribosomes in exponentially growing yeast cells [24]. For highly expressed genes 200 – 400 mRNA copies per cell have been determined with SAGE analysis [22]. In our current study the SUC2 mRNA level has been calculated in the same range. The number of mRNA molecules varied between 100 – 400 molecules per cell when invertase formation was induced. Quantification of the *in vitro* standard RNA, sample mRNA stability, and the effect of the biological material on the hybridization efficiency can cause inaccuracies to the quantification of the target mRNA species, but our method seems to provide a fair estimation of the molecular levels of RNA.

The sensitivity of the developed method was found to be sufficient for detection of intracellular rRNA molecules and highly expressed mRNA species. For RNA molecules with a low expression level a higher sensitivity would be desirable if crude cell lysates are used as sample material for the analysis, to avoid the use of cell concentrations which inhibit the analysis. Although for physiological studies the number of target RNA molecules of low expressed genes can be increased by increasing the amount of cell extract, signal inhibition has then to be included into the calculations.

Automation of the sandwich hybridization and technical improvements e.g. detection of targets with electric biochips [21] are making the sandwich hybridization method interesting for many application areas especially for clinical diagnostics, food industry use and even for the detection of bioweapons [18]. One of the challenging applications is the state analysis of bioprocesses based on transcriptional profiling of key genes that reflect the physiological state of the producing organism. The here presented method in view of its automation capabilities offers a valuable tool in this direction.

Conclusions

Alternative and novel methods for RNA analysis are being developed for clinical and industial diagnostics as well as for studing the physiology of living cells. The aim of the present study was the development of a fast and reliable method for quantification of single RNA species in solution using a sandwich hybridization based assay applicable for physiology analysis of organisms used in bioprocesses. The presented method allows the use of crude cell lysates as sample material and offers a sensitivity level suffiecient for measurement of ribosomal RNAs and messenger RNAs with relatively high expression levels. The developed method was evaluated by measuring the levels of *Saccharomyces cerevisae carlsbergensis* 18S rRNA and SUC2 mRNA in shake flask experiments. The measured 18S rRNA level was consistent with the specific growth rate and thus could be used as an indicator of growth state of a cell. SUC2 mRNA was in accordance with measured invertase activities under glucose repression and sucrose induction.

Methods

Strains and cultivation

S. cerevisiae strain BY4743 and the industrial *S. cerevisiae carlsbergensis* strain LK01 were used for cultivation. Cells were grown in medium containing 1% yeast extract (Difco), 2% Bacto Peptone (Difco) with 2% glucose or sucrose as carbon source at 30°C and 200 rpm in baffled shake flasks. For the culture used in analysis 20 ml of a 24 h grown preculture was inoculated in a 1 l flask containing 200 ml fresh medium. Growth was followed spectrophotometrically by measuring the optical density at 600 nm. The culture was grown for 60 h, during which yeast samples were taken for analysis. For RNA analysis 5–10 ml samples were taken depending on the growth state. The cell number in relation to OD was deterimed by Thoma cell counting. Cells were centrifuged at 20800 × g for 10 min at 4°C, washed with cold DEPC water and frozen at -70°C before analysis.

Table 1: Sequences of the oligonucleotide probes used in sandwich and slot blot hybridizations. All probes are designed for *S. cerevisiae*.

Target name	Probe name	Probe sequence 5'->3'	Location of probe
	Capture probes		
YIL162W	SUC2-1400	UUGUAGUCUUGUGAUUUGGCAGC- (AAGAG)*	1400–1422
RDN18-2	18S rRNA-400	CUGCUGCCUUCCUUGGAUGUGGUAGCCGUU- (GAGAG)	400–429
	Detection probes		
YIL162W	SUC2-581	GACUUGAAUGGUUGGUUGUUGAC- (GUGAC)	581–603
RDN18-2	18S rRNA-1302	UCUCGUUCGUUAUCGCAAUUAAGCAGACAA- (GCAGC)	1302–1331

* 5nt long spacer arms in brack

Yeast cell lysis and RNA extraction

Frozen yeast cells were suspended to 250 – 500 mg fresh yeast ml^{-1} with lysis buffer containing 550 U ml^{-1} Lyticase (Sigma), 1 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 0.2% RNA guard RNase inhibitor (Amersham Pharmacia). After addition of 100 µl of acid-washed beads (diameter 425–600 µm, Sigma) cells were disrupted with a FastPrep cell homogenizer (ThermoSavant) using 6 m sec^{-1} for 45 sec. Lysed cells and beads were rinsed with 100 µl DEPC-treated water. The cell lysate was directly used for RNA analysis.

Total RNA from freshly grown yeast cells was extracted using the TOTAL RNA kit (A&A Biotechnology, Poland) following the protocol provided with the kit. A maximum of 50 mg of cells was used per extraction to not exceed the binding capacity of the silica columns. Extracted RNA was quantified by absorbance measurement at A_{260} (DNA Quant, Pharmacia).

Synthesis of external control RNA

RNA used as an external control for the sandwich hybridization was synthesized in vitro by T7-RNA polymerase from the respective PCR products containing a T7 promoter sequence using the DIG RNA labeling Kit (Roche Diagnostics) as recommended by the manufacturer but with unlabeled nucleotides. The following primers were used for synthesis of the appropriate PCR product:

5'CCACATCTCCATCGTTGAAG and 5'CTAATACGACTCACTATAGGGAGATTCC-TTTTCCTTTTGGCTGG for SUC2

5'TCCTCTAAATGACCAAGTTTG and 5'CTAATACGACTCACTATAGGGAGATGGA-AGAGATGTATTTATTAGAT for 18S rRNA

Synthesized RNA was quantified by RiboGreen RNA Quantification Kit (Molecular Probes) as recommended by the manufacturer.

Synthesis of oligonucleotide probes

3' end biotin labeled capture probes, were custom-synthesized by Sigma Genosys. Detection probes were labeled with digoxigenin with oligonucleotide 3' end labeling Kit (Roche Diagnostics) (Table 1) as recommended by the manufacturer.

Sandwich hybridization assay

The total RNA extract, crude cell lysates or *in vitro* synthesized control RNA were diluted in buffer containing H_2O, 20 × SSC and deionized formamide in a ratio of 5:3:2 and denaturated at 65°C for 3–5 min before addition to hybridization reaction with 3 × 10^{12} molecules of biotin labeled capture probe and 2.4 × 10^{12} molecules of DIG-labeled detection probe. The hybridization reactions were carried out in 96-well plates (Greiner) at 50°C for 30 min at 600 rpm shaking (Thermomixer Comfort, Eppendorf) in a total volume of 100 µl containing 5 × SSC, 0.5% SDS, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 20% deionized formamide, 4% dextran sulfate.

The hybrid collection was performed by addition of 20 µg of streptavidin coated magnetic beads (Promega) to the hybridization reaction followed by incubation at 37°C for 30 min at 600 rpm. The beads were washed three times with 1 × SSC buffer and afterwards 50 µl of conjugation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.3% Tween 20, 1% BSA) containing 35 mU anti-DIG-alkaline phosphatase FAB fragments (Roche Diagnostics) were added to the beads and incubated for 30 min at room temperature. After six washing steps with washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.3% Tween 20), 150 µl of Atto-Phos substrate (Promega) were added to the beads and incubated for 20 min at 37°C. The magnetic beads were separated between the washing steps with a MagnaBot 96 Magnetic Separation Device (Promega). The clear supernatants were transferred to a black 96-well plate (Greiner) for fluorescence measurement performed with Victor 2 fluorescence reader (Wallac) at exitation wavelength 430 nm and emission wavelegth 560 nm.

Slot Blot hybridization

For detection of yeast 18S rRNA by slot blot hybridization the 3' end DIG-labeled probe 18SrRNA-1302 (table 1) was used. After denaturation in buffer containing H_2O, 20 × SSC and formaldehyde in the ratio of 5:3:2 at 65°C for 10 min dilution series of *in vitro* transcribed 18S rRNA were blotted onto positively charged nylon membranes (Amersham Pharmacia) using Bio Dot vacuum (BioRad). 1 h prehybridization and over night hybridization were carried out at 50°C using High SDS hybridization buffer (7% SDS, 50% formamide, 5 × SSC, 2% Blocking Reagent (Roche Diagnostics), 50 mM sodium phosphate, 0.1% N-lauroylsarcosine, pH 7.0). Signal detection was performed by chemiluminescence using CDP Star (Roche Diagnostics) as a substrate as recommended by the manufacturer and exposure to chemiluminescence films (Hyperfilm ECL, Pharmacia) for 60 min at room temperature. The film sheets were scanned by use of a flatbed scanner (Epson) and relative amounts of detected 18S rRNA were calculated by volume densitometric analysis using the Quantity One® Quantitation Software from BioRad.

Real-time PCR

Real-time PCR was carried out on an ABI Prism 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA), using SYBR-green as detection dye. cDNA synthesis of *in vitro* transcribed 18S rRNA was performed with Enhanced Avian RT first Strand Synthesis kit (Sigma), using the antisense specific primer: 18S-741R; 5'CTTGGCAAATGCTTTCGCAG. The PCR reaction was done with SYBR Green JumpStart Taq ReadyMix (Sigma), using the primers: 18S-523F; 5'CCTTGTGGCTCTTGGCGAAC and 18S-741R.

Invertase activity measurement

Invertase activity was measured in cell lysates in a two-phase reaction. In the first phase invertase hydrolyses sucrose to glucose and fructose. In the second phase glucose and ATP form glucose-6-phosphate and ADP in a reaction catalyzed by hexokinase. Glucose-6-phosphate and NADP form 6-phosphogluconate and NADPH in a glucose-6-phosphate dehydrogenase catalyzed reaction. NADPH is measured at 340 nm.

Reaction was started by adding lysed cells to a concentration of 0.6 mg ml^{-1} in 50 mM sodium acetate (pH 4.5) and 20 mM sucrose at 30°C with a final volume of 1 ml. Reaction was stopped after 10 min by boiling. 50 µl of the reaction solution were added to 950 µl of 50 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 3 mM ATP and 1 mM NADP. A_{340} was measured spectrophotometrically (Ultrospec 2100 pro, Amersham Pharmacia) before addition of 4.5 U hexokinase (Sigma) and 1.2 U glucose-6-phosphate dehydrogenase (Sigma) and again after 30 min reaction at room temperature.

Author's contribution

JR designed the experiments and carried them out for the method development and growth experiments. KBB performed the experiments with real-time PCR. KBB and JL participated in the method development and optimisation work. SM contributed with ideas and discussions in the writing of the manuscript. AB and PN participated in the design and supervision of the work. All authors read and approved the final manuscript.

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PUBLICATION III

Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*

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Research article

Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*

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Abstract

Background: Chemostat cultures are commonly used in production of cellular material for systems-wide biological studies. We have used the novel TRAC (transcript analysis with aid of affinity capture) method to study expression stability of approximately 30 process relevant marker genes in chemostat cultures of the filamentous fungus *Trichoderma reesei* and its transformant expressing laccase from *Melanocarpus albomyces*. Transcriptional responses caused by transient oxygen deprivations and production of foreign protein were also studied in *T. reesei* by TRAC.

Results: In cultures with good steady states, the expression of the marker genes varied less than 20% on average between sequential samples for at least 5 or 6 residence times. However, in a number of *T. reesei* cultures continuous flow did not result in a good steady state. Perturbations to the steady state were always evident at the transcriptional level, even when they were not measurable as changes in biomass or product concentrations. Both unintentional and intentional perturbations of the steady state demonstrated that a number of genes involved in growth, protein production and secretion are sensitive markers for culture disturbances. Exposure to anaerobic conditions caused strong responses at the level of gene expression, but surprisingly the cultures could regain their previous steady state quickly, even after 3 h O_2 depletion. The main effect of producing *M. albomyces* laccase was down-regulation of the native cellulases compared with the host strain.

Conclusion: This study demonstrates the usefulness of transcriptional analysis by TRAC in ensuring the quality of chemostat cultures prior to costly and laborious genome-wide analysis. In addition TRAC was shown to be an efficient tool in studying gene expression dynamics in transient conditions.

Background

Systems-wide methods have become an important part of physiological research in industrial biotechnology, with the aim of improving industrially relevant production strains and processes, for example by identifying physiological reactions that limit metabolism or the production of proteins. Transcriptional profiling can be particularly useful, since it can reveal previously unknown, but relevant pathways [1].

Chemostat and other continuous flow cultures are the technique of choice for producing biomass for global studies, such as transcriptomic, proteomic and metabolomic profiles, since parameters such as growth rate, dissolved oxygen and nutrient concentrations can be kept constant, providing a reproducible environment and populations of cells in physiological steady state [2]. Thus reproducible physiological studies can be carried out and single parameters can be varied while others are kept constant, increasing the reliability of systems-wide datasets. Batch culture systems are also used to obtain systems-wide datasets, but because environmental conditions and growth rate are constantly changing, the interpretation of these datasets and comparison with other results is complicated [2-4].

Filamentous fungi form a notable group of cell factories that are widely exploited in the production of industrial enzymes because of their ability to produce large amounts of extracellular proteins. Production of native enzymes by the fungus *Trichoderma reesei* can exceed 100 g l^{-1} [5]. Such levels of secretion, however, have not been reached for non-fungal recombinant proteins [6]. Use of global level 'omics' technologies, coupled with continuous chemostat cultivations, as a strategy to improve productivity is thus also emerging in the fungal research community [7,8] as the genomes of these organisms, including the one of *T. reesei* [9], become available.

Establishing chemostat cultures for filamentous fungi, however, faces particular challenges, because of their multi-nuclear, polar growth form, which introduces an inherent heterogeneity to the system. Differentiation, particularly for conidia production, may further increase culture heterogeneity for some species. In addition, fungi have a greater ability than unicellular organisms to adhere to each other, forming pellets, or to grow on solid surfaces, such as the walls of the bioreactor [10]. The filamentous growth form increases the viscosity of the culture, causing mass transfer limitations [11,12]. As with other organisms culture evolution as a result of mutations and selection occurs [13].

The quality of a chemostat steady state is generally assessed from the measurement of various process parameters such as biomass and product concentrations, CO_2 evolution and alkali consumption rates. However, short term changes in environmental conditions, for example as a result of sample removal, and poor mixing, resulting in nutrient gradients, will have an impact on cellular physiology [14], but would not necessarily result in measurable changes in biomass related parameters. Since cells can rapidly adapt to changing conditions by transcriptional regulation [15,16], these perturbations may be affecting the transcriptome. However, the degree of stability of gene expression in continuous flow cultures has not been reported.

In these studies we have applied a novel transcriptional analysis method called TRAC (Transcript analysis with aid of affinity capture) [17] to study expression levels of a set of 30 marker genes, relative to polyA RNA content, in chemostat cultures of a *T. reesei* strain producing the laccase of *Melanocarbus albomyces* [18] and its parental strain. The TRAC method was used to monitor transcriptional steadiness and to identify disturbances in the steady state. In addition, TRAC was used to assess transcriptional responses during transient periods of oxygen deprivation and subsequent recovery and during recombinant protein production.

Results

Gene expression stability during steady state

The stability of several physiological parameters were monitored before and after the onset of continuous medium feeding to evaluate the steady state of chemostat cultures. Standard on-line (e.g. base consumption, dissolved O_2 concentration and off gas concentrations for CO_2, O_2, N_2) and off-line (e.g. dry weight, NH_3 concentration, cellulase activity) measurements were used and are referred to as conventional process analyses. In addition to these conventional process analyses a novel method for rapid transcriptional profiling called TRAC [17] was used to monitor the expression of appr. 30 marker genes.

Stabilisation of gene expression occurred rapidly after the start of continuous medium feeding (i.e. at 0 R) in chemostats which subsequently showed good steady states (Figure 1). The genes analysed showed, on average, less than 20% variation from the most stable expression phase after approximately 0.5 R (R = 33.3 h), even though F_{SS} (see Materials & Methods) showed that the average gene expression levels varied significantly between the batch and the continuous phase. In the batch phase, the genes analysed here showed on average a fold-change of 1.3 – 2.0 relative to the most stable expression phase during the chemostat culture and the average deviation in the fold change between the genes was large. The expression of secreted protein (*cbh1*, *egl1*) and folding factor genes was strongly up-regulated when the culture was in transition

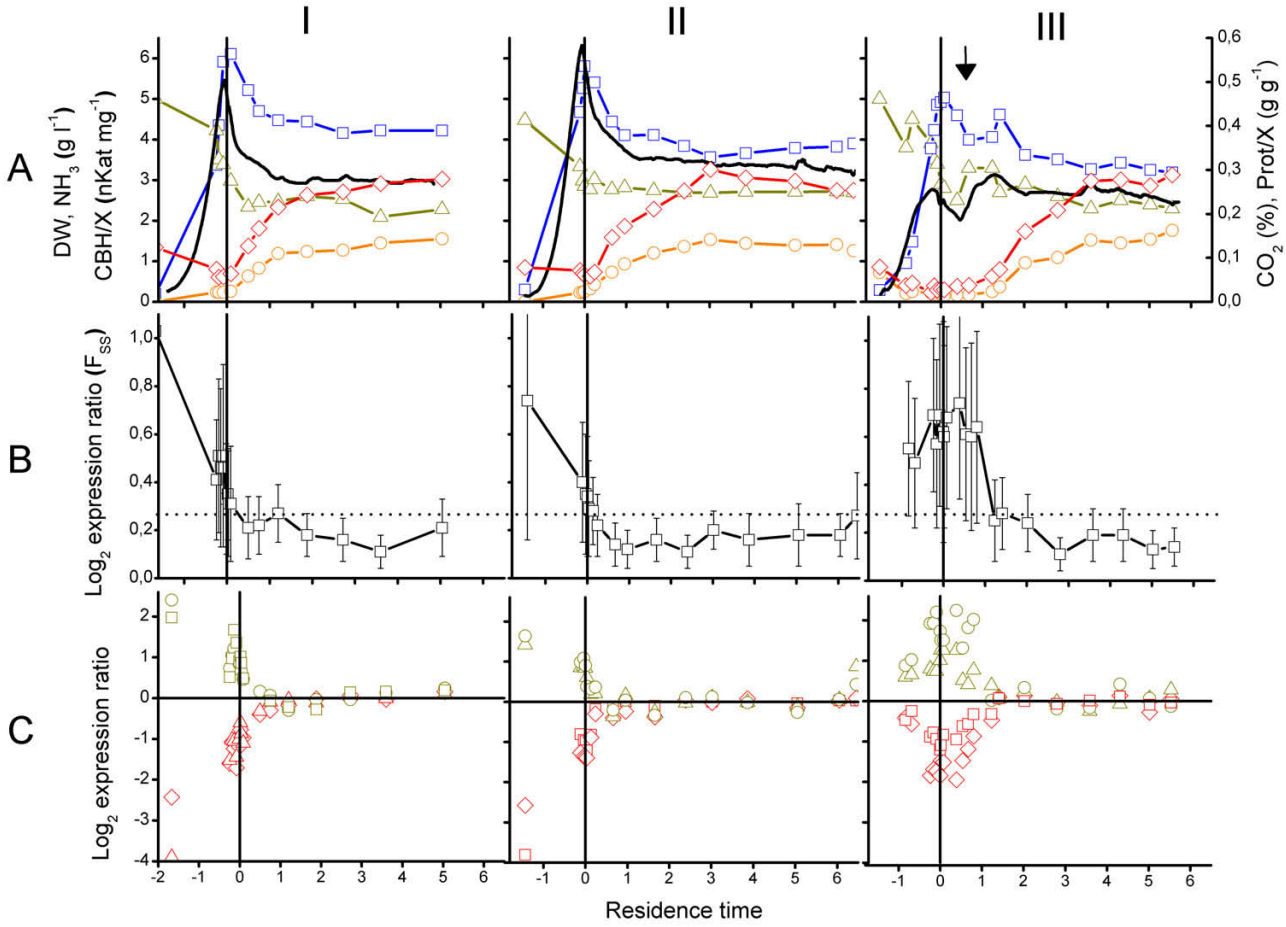

Figure 1

Evaluation of marker gene expression stability during chemostat cultures with *Trihoderma reesei* RutC30. A. Dry weight (blue squares), off gas CO_2 concentration (black line), protein concentration (g g^{-1} DW) (red diamonds) and specific cellobiohydrolase activity (nkat g^{-1} DW) (orange circles) and ammonium concentration (green triangles) in chemostat cultures I – III (D = 0.03 h^{-1}). In culture III arrow indicates the addition of trace mineral solution. B. Average fold-change of expression from predicted steady state of 30 marker genes (F_{SS}). Error bars indicate the average deviation in *Fss* between the different marker genes. C. Expression ratios of genes showing the highest variation between batch and continuous phase (increasing expression, red symbols *cbh1*, *egl1*) and (decreasing expression, green symbols *gpd1*, *hsp70*). Residence time 0 indicates the start of the continuous growth phase. Expression levels as $\log_2$ expression ratios from the F_{ma} predicted steady state expression level.

from batch to chemostat phase, whereas, for example, glycolysis gene *gpd1* and heat shock protein *hsp70* gene were strongly down-regulated (Figure 1 C).

Most chemostat cultures were lactose limited. In one batch culture, however, limitation of trace minerals ($FeSO_4$, $MnSO_4$, $ZnSO_4$, $CoCl_2$) was applied (Figure 1 III) and the transient phase between batch and chemostat growth was further disturbed by increasing the concentrations of the trace minerals to those used in other cultures, to shift the limitation from trace minerals to lactose at 0.5 R after the start of continuous medium feeding. The gene expression levels reached the same level of stability as was observed in lactose limited cultures in less than 1 R after lactose became limiting. In all cultures, the average expression levels remained constant for the next 4.5 – 5 R, with a small average deviation in F_{SS} (Figure 1 I–III).

In stable chemostat cultures all measured parameters had become constant within 3 R, although different parameters required slightly different lengths of time to become constant. Stabilisation of average gene expression

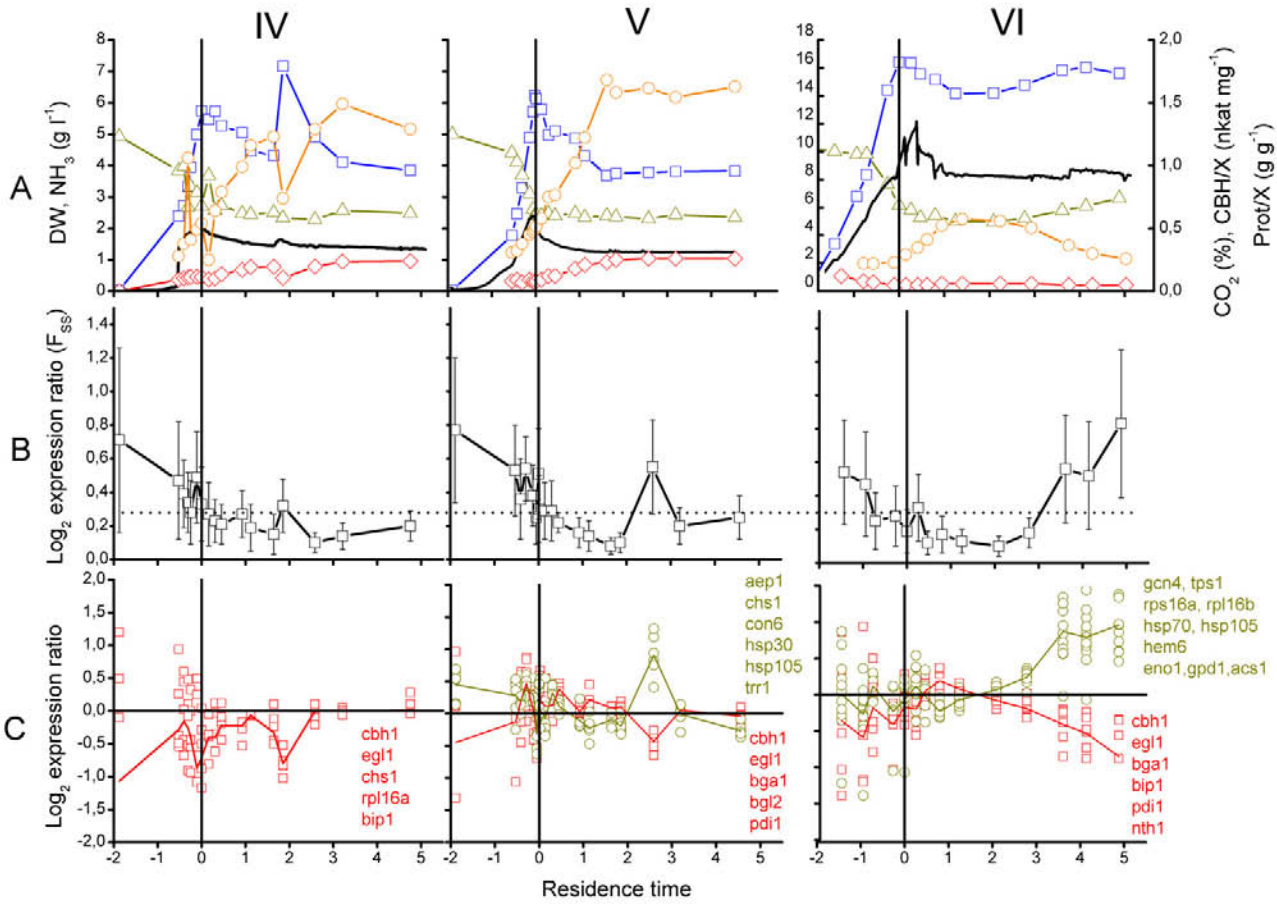

Figure 2

Monitoring of disturbances in gene expression stability in chemostat cultures. A. Dry weight (blue squares), off gas CO_2 concentration (black line), protein concentration (g g^{-1} DW) (red diamonds) and cellobiohydrolase activity (nkat g^{-1} DW) (orange circles) and ammonium concentration (green triangles) in chemostat cultures IV–VI (D = 0.03 h^{-1}). B. Average fold-change of expression from predicted steady state of 30 marker genes (F_{SS}). Error bars indicate the average deviation in *Fss* between the different marker genes. C. Marker genes show up (green circles) or down (red squares)-regulation during the continuous phase of the cultures. Expression levels as $\log_2$ expression ratios from the F_{ma} predicted steady state expression level

occurred within a similar time frame as stabilisation of CO_2 and dry weight production, but one to two R earlier than required for secreted protein and enzyme concentrations (Figure 1).

Some marker genes showed consistent variation during the steady state phase. Expression of cellulases (*cbh1*, *egl1*) and folding factor (*pdi1*) was very constant in the F_{ma} predicted steady state phase, with sequential measurements generally varying less than 10 % from their mean. However, variation in the expression levels for the stress related genes *gcn4*, *hsp30* and *hsp105*, the conidiation genes *con6* and *ccg9* and vacuolar protease *vpa1* was 15–20%. This level of variation was detected by TRAC with high reliability [17] and was not dependent on the fluorescence signal strength in the TRAC assay.

Monitoring of disturbances in the steady state by TRAC

Several fungal chemostats showed some instability during the (pseudo-)steady state, in spite of the carefully controlled external parameters. Potential disturbances in the steady state and the resulting physiological instability were most sensitively detected at the level of gene expression. Figure 2 illustrates three representative chemostat cultures (IV – VI), in which instabilities occurred due to a technical disturbance (IV), an unknown effector (V) or a biological factor (VI). Instabilities were detected either as a change in gene expression and in conventionally moni-

tored parameters (IV and VI) or as a change in gene expression only (V).

In culture IV (Figure 2) a disturbance occurred between 1.6 and 2.6 R as a result of a failure in the antifoam control. The concentration of polypropylene glycol increased 9-fold above the normal concentration causing an apparent increase in biomass concentration and a decrease in extracellular protein and enzyme concentrations. At the transcriptional level the disturbance was observed as a small increase in the average fold-change of expression of the gene set used (F_{SS}), compared to the F_{MA} predicted steady state. The deviation from the steady state was primarily caused by down-regulation of two main cellulases (*cbh1*, *egl1*), protein chaperon (*bip1*), ribosomal protein mRNA (*rpl16a*) and chitin synthase (*chs1*) (Figure 2 IV C).

In another culture (Figure 2 V), a number of genes showed a significant response to an unknown effector, while other physiological parameters were constant. Genes coding for extracellular enzymes were down-regulated together with a protein folding factor (*pdi1*), while up-regulation was observed for some stress related markers (*hsp30*, *hsp105*, *trr1*), conidation genes (*con6*, *chs1*) and alkaline extracellular protease (*aep1*).

In high density chemostat cultures, with cell dry weight 4-fold higher than in the standard culture conditions, steady states were achieved in a similar time frame to the lower biomass cultures, based on all the measured parameters. However, in cultures with high biomass a large change in expression occurred for several of the gene markers after 3 to 4 R (Figure 2 VI). The disturbance in the steady state was less evident in the conventional process measurements, although a small increase in biomass concentration and a 50% decrease in the cellobiohydrolase concentration did occur (Figure 2 VI A). The gene markers which showed down regulation after 3 R in the high biomass culture (Figure 2 VI) were the same as in other cultures which showed disturbances (Figure 2 IV, V) i.e. cellulases and folding factors. In high biomass cultures, up-regulation was consistently observed for stress related genes (*gcn4*, *tps1*, *hsp70*, *hsp105*), central carbohydrate metabolism genes (*gpd1*, *eno1*, *acs1*) and ribosomal protein genes.

Monitoring transcriptional responses to anaerobic phases

We studied by marker gene expression analysis the physiological responses to fluctuation in oxygen availability and recovery of the cultures from such perturbations by exposing *T. reesei* cultures to anaerobic conditions. In several fungal chemostat cultures the steady state was disturbed after approximately 5 R by making the conditions anaerobic for 20 to 180 min. Replacing the air supply with N_2 caused an immediate drop of dissolved and off-gas O_2 levels to 0% (data not shown).

Approximately 50 % of the marker genes showed greater than 1.5-fold change in expression level in response to anaerobic conditions. The longer the anaerobic phase, the stronger the effect for the set of genes analysed (Figure 3). However, recovery of the initial expression levels was quick after aeration of the culture was restarted. In 3 to 5 h after restoring aerobic conditions the expression of these genes showed less than 1.3-fold variation on average, from the initial steady state expression level and the expression levels remained constant for the next 20 to 30 h.

Four different expression patterns were observed for gene markers that showed a response to sudden changes in availability of O_2 (Figure 4 A–D). The most sensitive markers to lack of oxygen availability were *hsp70* and *hem6* (Figure 4 A). Both of these genes were strongly induced during anaerobiosis, and immediately repressed after aerobic conditions were restored, returning the previous, aerobic expression levels within less than 1 h.

The amino acid permease gene *gap1* showed little or no response to the anaerobic conditions, but showed 5–8-fold induction 15 min after restoring aeration (Figure 4 B). The genes *gpd1* and *acs1*, involved in central carbohydrate metabolism, also showed induced expression during the transition from anaerobic to aerobic conditions (data not shown). These genes only returned to the previous steady state after 5–6 h. When cultures were starved of carbon for 60 min, *gap1* was induced. It was then down-regulated after medium feed was restarted (Figure 4 B ii).

Ribosomal protein and chitin synthase gene (*chs1*) expression levels decreased during anaerobiosis and also during lactose starvation. The average mRNA half life ($T_{1/2}$) for these genes was shorter during lactose starvation (33 min) than anaerobic conditions (41 min) with ribosomal protein mRNAs having 15–40% shorter $T_{1/2}$ than *chs1*. Up-regulation to previous steady state expression level occurred after the restoration of aeration (Figure 4 C).

The genes for secreted cellulases, *cbh1* and *egl1*, and the protein folding factor gene, *pdi1*, which had been observed to be down-regulated in various process disturbances (Figure 2), were also down-regulated (by appr. 2-fold) by anaerobiosis, along with the trehalose-6-phosphate synthase (*tps1*) gene (Figure 4 D). These genes slowly recovered their previous expression levels after restoration of aerobic conditions. The specific activity of CBH and EG (nkat g^{-1} dry weight) decreased 15 to 30% in anaerobic conditions, but increased to the previous activities once aerobic conditions were restored (data not

shown). In contrast, during 60 min lactose starvation expression of these genes did not change and they were up-regulated after medium feed was restarted. (Figure 4 D iv).

To determine whether the responses described above were specific for anaerobiosis and subsequent restoration of aeration or due to the accumulation of substrate or other metabolites during the anaerobiosis, chemostat cultures were also carried out in which the medium feed was stopped with or without simultaneous termination of air feed (see Materials & Methods). The effect of anaerobic conditions was the same, regardless of whether the culture was starved of lactose or had excess of lactose (Figure 4 A i – D iv). The transcriptional response to oxygen starvation was different than the response to lactose starvation for all genes except the ribosomal protein and chitin synthase genes (Figure 4 C iii), and thus for the other genes observed response was specific for lack of oxygen.

Comparison of marker gene expression between a recombinant and the parental strain

One of the aims in our studies was to address the physiological effects of foreign protein production to the cell. For focused and genome-wide expression comparison, using transcriptional stability (<20% variation from F_{MA} predicted steady state) as a decisive criterion, the most stable cultures among those cultures assessed as being in steady state based on conventional process analyses were selected. Figure 5 gives a comparison of marker gene expression between *T. reesei* RutC-30 and the *M. albomyces* laccase producing strain during steady state in lactose limited chemostat cultures at D = 0.03 h^{-1}. Results of the genome-wide analysis will be published elsewhere.

Expression of each marker gene was averaged over 5 samples during the F_{MA}-predicted steady state from triplicate cultures. Out of approximately 30 marker genes, only 3 showed significant difference in expression (>1.5-fold difference) between the two strains (Figure 5 A). Gene expression of native secreted cellulases *cbh1* and *egl1* were down-regulated in the recombinant strain by 1.9 and 2.6-fold respectively. Another cellulase, intracellular β-glucosidase 2 (*bgl2*) showed a smaller 1.3-fold down regulation in the recombinant laccase producing strain, compared to the host strain. The reduced expression levels corresponded with the observed 2.1- and 3.3-fold lower specific extracellular activities for CBH and EG, respectively (Figure 5 B). The unfolded protein response (UPR) (*pdi1*, *hac1*) and trafficking related (*nsf1*) genes were moderately down-regulated in the recombinant strain.

Only one gene in the gene set analysed, coding for protein chaperon *hsp70*, was up-regulated in the recombinant strain. The expression level of this gene showed considerable variation between the triplicate cultures of the same strain, and the observed variation may therefore be partly culture dependent.

Discussion

In this study we addressed the quality of a large number of chemostat cultures performed with *Trichoderma reesei*. This was done by conventional process analysis and by a novel transcriptional analysis method TRAC that allows measurement of multiple mRNA levels in a large number of samples.

To achieve a physiological steady state in chemostat cultures with filamentous fungi can be considered as challenging due to various factors causing inhomogeneity to the culture (wall growth, viscosity, presence of metabolically active and inactive biomass *etc.*). Consistent with this, only in some of the *T. reesei* chemostat cultures performed in this work good physiological steady states were observed, based on the stability of all measured parameters (conventional and mRNA). In these cultures, monitoring of marker genes showed that most transcripts had reached their steady state expression level (<20% deviation from the predicted steady state) within less than 1 generation of the onset of continuous medium flow and that expression levels remained constant for the following 5 to 6 generations or until the culture was terminated or deliberately perturbed. Cultures, chosen by TRAC for comparison of the two strains, showed a high degree of reproducibility during the steady state (Figure 5). The average variation between parallel TRAC measurements has been show to be approximately 9% [17].

Steady states were not obtained in all cultures, nor were the reasons for this always apparent. Instabilities were always evident at the level of transcription, but were not always detectable in the conventional process analyses. The changes observed at the transcriptional level indicated that some undetected perturbation in the environmental conditions had occurred. Microorganisms are able to rapidly adjust their internal physiology to variable conditions [15], and adjustment of genetic expression is expected to be among the earliest detected changes in cellular physiology in response to environmental perturbations. Monitoring of relevant marker genes will also give an indication as to the source of perturbation.

In chemostat cultures with *T. reesei*, the most sensitive markers for process disturbances were the genes coding for hydrolytic enzymes (*cbh1*, *egl1*, *bgl2*, *bga1*), which were consistently down-regulated in response to various factors like high concentrations of polypropylene glycol, lack of aeration or high cell density stress. This observation has special importance, since these enzymes are the major products of *T. reesei* in industrial production, and the *cbh1*

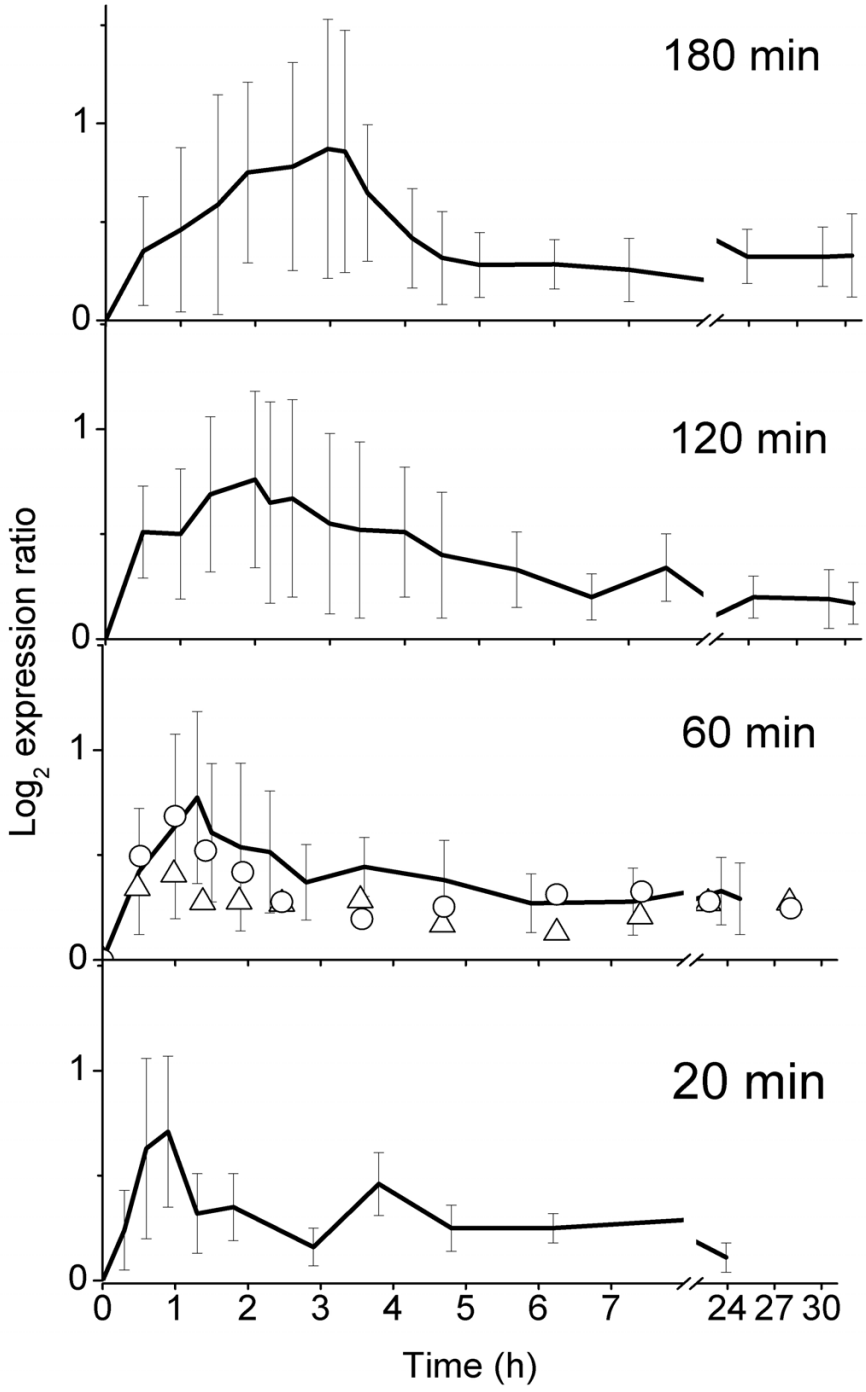

Figure 3
Average responses of 30 marker genes to anaerobic phases. In steady state phase of a chemostat culture (D = 0.03 h^{-1}, pH 5) the air supply was replaced with N_2 for 20, 60, 120 and 180 min. Curves show the average variation of the entire marker gene set (Table 1) in each sample point from the 0 h sample (initial steady state). In the figure for 60 min, circles show the corresponding average response for 60 min without either air or medium feed and triangles show the average response to 60 min without medium feed, but with air. The average variation in the responses between different marker genes is indicated by the bars.

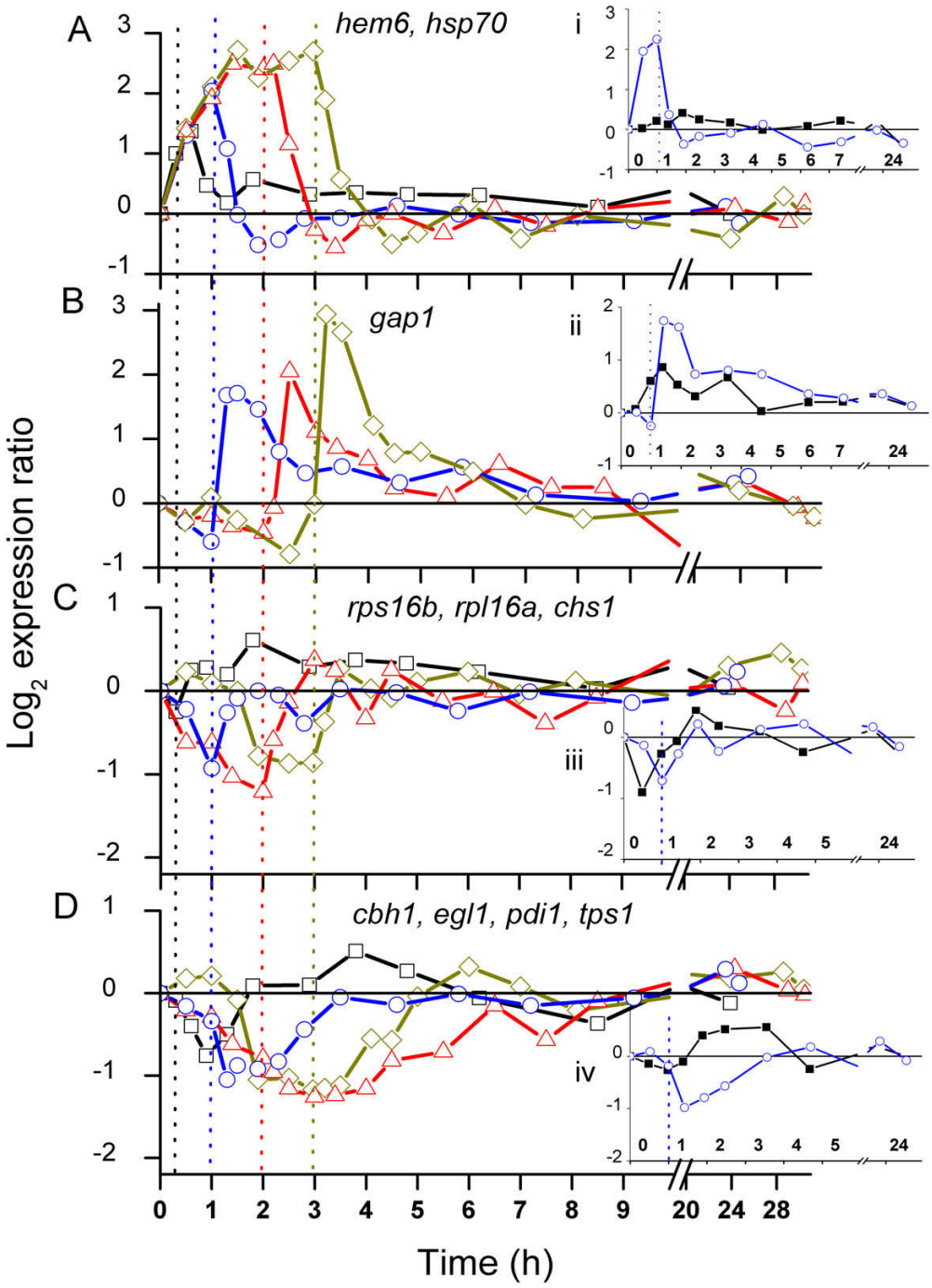

Figure 4

Expression of specific marker genes in *T. reesei* during transient anaerobic phases. Duration of anaerobic phases were 20 (black squares), 60 (blue circles), 120 (red triangles) and 180 (green diamonds) min. 0 min indicates the start of the anaerobic phase and the coloured dotted line the end of an anaerobic phase. A-D shows the average $\log_2$ expression ratios from the 0 min for different genes that showed similar responses during the experiment, subfigures i-iv show the average responses of the corresponding genes during 60 min without either medium or air feed (blue circles) or without medium feed (black squares).

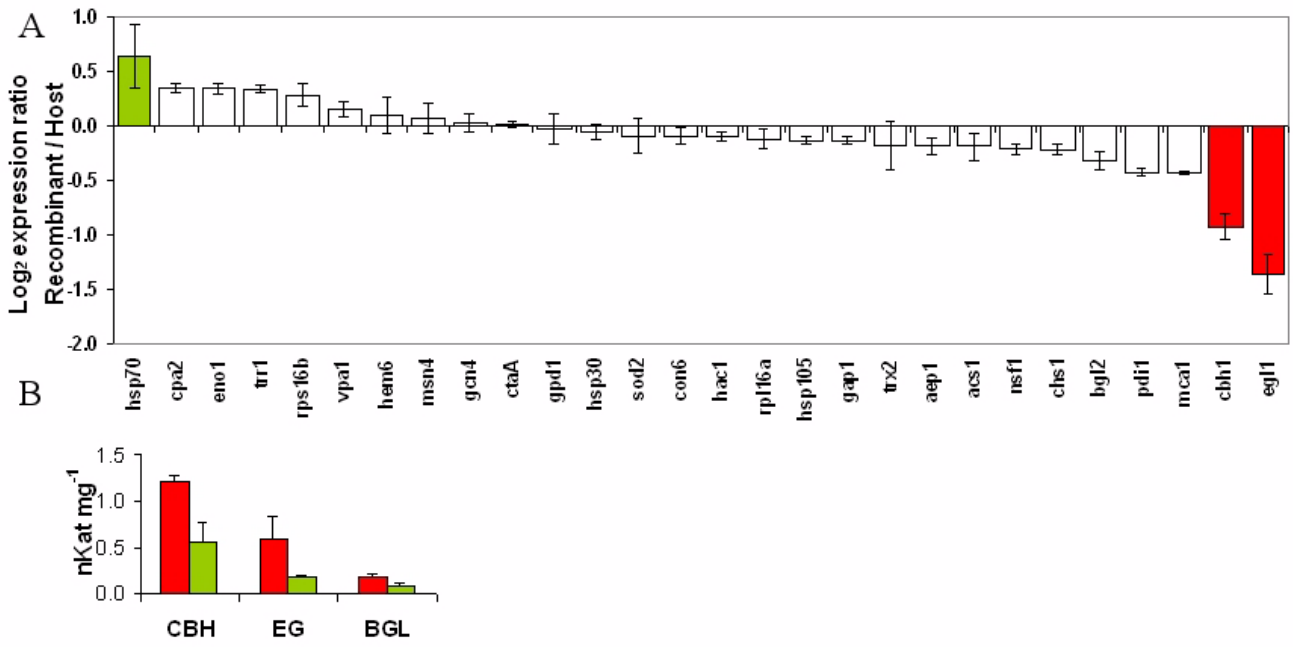

Figure 5

Comparison of marker gene expression and cellulose production between recombinant laccase producing and parental strains. A. Log_2 ratios of gene expression between recombinant and parental strains in chemostat cultures (D = 0.03 h^{-1}). Error bars indicate the standard deviation between triplicate cultures. Genes showing higher expression ratios than 1.5-fold are marked as green (higher in recombinant strain) or red (higher in parental strain) B. Comparison of specific production levels (nKat mg^{-1} DW) of three secreted hydrolases (CBH, EG and BGL) between parental (red) and recombinant (green) strains.

promoter is widely used to drive expression of foreign proteins. The down-regulation of secreted enzyme genes was accompanied by down-regulation of UPR-related folding factors (*pdi1*, *bip1*). The co-regulation of these two gene groups has been observed earlier [19]. Down-regulation of *cbh1* and *egl1* was also reflected in subsequent reduction in the corresponding enzyme activity (Figures 2 and 5). The *T. reesei* cultures clearly respond to several stress conditions at the expense of protein production and secretion.

Expression of genes coding for conidiation related (*con6*, *chs1*) and ribosomal proteins (*rps16a*, *rpl16b*) was also sensitive to process disturbances. Reduced transcription of ribosomal protein mRNAs has also been observed in yeast under various stress conditions, such as nutrient starvation, protein secretion stress and osmotic stress [16]. Since ribosomal RNA genes make up a considerable fraction of the entire genome, the reduced synthesis of rRNA and ribosomal protein transcripts and of the ribosomes may help to save energy while the cells adapt to changing conditions [20]. In rapidly growing cells the expression of ribosomal protein mRNA is, by contrast, strongly induced [21].

Con6 encodes a short protein that is homologous to *Neurospora crassa* conidiation specific gene 6. *N. crassa con6* is expressed during the formation of asexual conidia (spores), but is not expressed in mycelium [22]. Chitin synthase (*chs1*) has a role in cell wall biogenesis and is required for formation of conidia in *N. crassa* [23]. These conidiation related genes showed relatively high instability during non-disturbed as well as disturbed chemostat cultures. High levels of conidiation were observed in several of the cultures that did not attain steady state (data not shown) and variation in the expression of these genes during steady states could reflect a need to continually adjust the balance between filamentous growth and conidium formation, in order to maintain constant biomass in the chemostat.

In high cell density *T. reesei* chemostat cultures after 3 to 4 R several of the marker genes showed a significant fold-change, relative to their expression levels during the most stable expression phase (Figure 2 VI). The up-regulation of the trehalose-6-phosphate synthase gene *tps1* and repression of the trehalase gene *nth1* suggested that trehalose synthesis occurred in the high biomass *T. reesei* cultures, as has been observed in *S. cerevisiae* when grown in high

substrate concentrations [24]. Up-regulation of the carhohydrate metabolism genes (*acs1*, *gpd1*, *eno1*) in *T. reesei* indicated elevated glycolytic flux and higher energy demand [24,25]. *Hem6* and/or *hsp70* genes were induced after 3 R in the high cell density cultures (Figure 2 VI), possibly indicating limitation in oxygen availability, since regulation of these genes was also observed to be oxygen dependent (Figure 5 A). The increase in transcription factor *gcn4* mRNA could suggest an amino acid starvation response [26]. Unexpected increase in biomass concentration was observed, which could be connected to up-regulation of ribosomal protein mRNAs (Figure 2 VI).

When the steady states of the fungal cultures were deliberately disturbed by exposing them to anaerobic conditions for 0.3 – 3 h, the return of transcript levels of affected genes to their previous level was surprisingly rapid once aeration of the culture was restarted. Within 5 h of restarting aeration the expression of the genes were at the previous steady state levels. Although *T. reesei* is not able to grow in anaerobic conditions, it sustained metabolic activity surprisingly well without oxygen. Short term deprivation of oxygen level to the culture did trigger physiological responses, but *T. reesei* recovered in relatively short time from even 3 h of total anaerobiosis (Figure 4) and no signs of cell lysis were observed (data not shown). Bonaccorsi *et al*. [27] observed a similar restoration of gene expression in *T. reesei* QM9414 within 2 h following a 2 h period in anaerobic conditions at D = 0.01 h^{-1} with glucose as the carbon source. Bonaccorsi *et al*. [27] monitored 2000 genes, 19.6 % of which were affected by oxygen concentration, but did not confirm steady state expression levels, as was possible here using TRAC with a smaller number of genes. Along with medium composition, the exchange of gasses is a critical parameter in industrial fermentation processes [11,28]. In large scale fermentations with a thick filamentous slurry, inhomogeneity of the system leads to local limitations in oxygen supply [12]. As with other process disturbances, the cellulase genes were repressed during oxygen deficiency (Figure 4 D), as has previously been observed for *cbh1* and *egl1* [29]. This is a crucial factor considering the productivity of the *T. reesei* production process.

Genes coding for coproporphyrinogen III oxidase (*hem6*) and heat shock protein 70 (*hsp70*) were the most sensitive markers among the genes assessed here for availability of O_2. Coproporphyrinogen III oxidase is involved in heme biosynthesis. Heme functions in yeast as an oxygen sensor by regulating the activity of transcription factor Hap1. Heme permits Hap1 to be released from a high-molecular-weight complex (HMC) in the presence of oxygen, and Hap1 subsequently activates transcription of aerobic genes and represses expression of anaerobic genes by repressing Rox1 activity [30-32]. The absence of oxygen induces the expression of half of the heme biosynthesis genes in *S. cerevisiae*, with the *hem6* homologue HEM13 showing the strongest up-regulation, even though heme cannot be synthesised in anaerobic conditions [33]. In *S. cerevisiae* Ssa type Hsp70 proteins have been shown to be a part of the HMC [34] and to be involved in activation of anaerobic genes via the binding of Hap1p in the absence of heme. The O_2 dependent regulation of the *T. reesei* Ssa homologue *hsp70* shown here, may thus reflect its role in the regulation of HAP1 activity.

Inhibition of growth by either lack of lactose or of oxygen resulted in down-regulation of ribosomal protein and chitin synthase genes as expected. Rapid up-regulation to previous expression level was observed after restoring previous steady state conditions. Yeast ribosomal protein mRNAs decline in mild heat shock with $T_{1/2}$ of 5–7 min [35], which was much faster than the turn-over times observed in this work for two *T. reesei* ribosomal protein mRNAs under lactose starvation ($T_{1/2}$ = 28 min) or anaerobiosis ($T_{1/2}$ = 37 min). The rapid increase (doubling in 60 min) in *chs1* and ribosomal protein mRNA levels when culture again became aerobic was consistent with the observation that cell density had not decreased during the short term anaerobiosis. The rapid and strong up-regulation of aminoacid permease may also be part of the quick adaptation of the culture to growth conditions (Figure 4 B).

TRAC analysis demonstrated that the expression levels of the marker genes were very similar for RutC-30 and the strain producing recombinant laccase, with only few exceptions (Figure 5). Down-regulation of native endo- and exoglucanases (*cbh1*, *egl1*) was observed in the recombinant strain, corresponding to the lower specific production of these enzymes (Figure 5 B). This could be due to repression of the genes by the RESS mechanism (repression under secretion stress, [8]), even though no unfolded protein response (UPR, [36]) was observed. Another possible explanation could be that the cellulase genes were down-regulated due to titration of their activating transcription factors [37]. This idea, however, is questioned by the fact that the laccase expression construct is present in the analysed strain as a single copy (data not shown).

Transcriptional monitoring of chemostat cultures is beneficial prior to costly and laborious systems-wide analyses in order to increase their reliability by ensuring that samples from only reproducible cultures are analysed. In addition to offering a high degree of reproducibility, TRAC also has the advantage of allowing simultaneous analysis of a large number of samples, in a cost effective, time efficient manner. Therefore, in addition to monitoring of transcriptional stability, TRAC is well suited for analysis of

gene expression dynamics in experiments involving transients.

Conclusion

We have analysed the expression stability of approximately 30 marker genes in a large number of chemostat cultures of the filamentous fungus *Trichoderma reesei*, using the novel transcriptional analysis method TRAC. We have demonstrated that gene expression levels were stable in those chemostat cultures showing good physiological steady states. Instabilities in the chemostat cultures were always detected at the level of transcription, but were not always detectable by conventional process analyses. Various disturbances, as well as expression of a recombinant laccase, were particularly manifest by the down-regulation of genes coding for the hydrolytic enzymes, which are the major industrial products of *T. reesei*. Deliberate exposure of the *T. reesei* chemostat cultures to transient anaerobic phases, revealed that the fungus was able regain previous steady state expression levels rapidly once aeration was restarted. This study demonstrates the usefulness of TRAC in transcriptional analysis of transient conditions, as well as ensuring the quality of chemostat cultures prior to systems-wide analysis.

Methods

Strain and medium

Trichoderma reesei Rut-C30 [38] and its transformant pLLK13/295 producing *Melanocarpus albomyces* laccase [18] were used in these studies.

The medium contained: KH_2PO_4 15 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.6 g l^{-1}, $MgSO_4 \cdot 6H_2O$ 0.6 g l^{-1}, $CuSO_4 \cdot 5H_2O$ 30 mg l^{-1}, $FeSO_4 \cdot 5H_2O$ 5 mg l^{-1}, $MnSO_4 \cdot H_2O$ 1.6 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 1.4 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 3.7 mg l^{-1} and lactose 10, 20 or 40 g L^{-1}. Salt concentrations in cultures containing 40 g lactose l^{-1} were increased to: KH_2PO_4 15 g l^{-1}, $(NH_4)_2SO_4$ 12.5 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 1.5 g l^{-1}, $MgSO_4 \cdot 6H_2O$ 1.5 g l^{-1}, $CuSO_4 \cdot 5H_2O$ 30 mg l^{-1}, $FeSO_4 \cdot 5H_2O$ 12.5 mg l^{-1}, $MnSO_4 \cdot H_2O$ 4.0 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 5.6 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 14.8 mg l^{-1}

Cultivation conditions

Inoculum was prepared by transferring 1×10^8 spores into a 1.5 l conical flask containing 500 ml growth medium (lactose 20 g l^{-1}). The culture was grown for approximately 72 h at 28°C with shaking at 200 rpm and then 200 or 500 ml were transferred to a bioreactor (Braun Biostat CT2-DCU3 or CT5-2, B. Braun Biotech International GmbH, Meisungen, Germany) along with sufficient medium to give total final volumes of 2 or 5 l respectively. Cultivations in the bioreactors were carried out at 28°C, with aeration of 0.5 vvm (volumes of air per volume of liquid per minute) and stirring at 800 rpm. The pH was maintained at 4.8 ± 0.1 using 1 M NaOH. Approximately 0.2% v/v (final concentration) polypropylene glycol (mixed molecular weights; [39]) was used as an antifoam agent. Nitrogen, oxygen and carbon dioxide were monitored online by measuring peak areas at M/z = 24, 32 and 44 with an OmniStar mass specrometer (Pfeiffer Vacuum, Germany).

For lactose limited chemostat culture, the feed was started immediately after CO_2 production stopped at the end of the batch phase, approximately 50 h after inoculation. The feed was supplied at constant rate to maintain a dilution rate of 0.03 h^{-1} for 5 to 6 residence times (R).

To study the effect of anaerobic conditions, air supply to the fermentor was replaced by N_2 for 20, 60 (in duplicate), 120 or 180 min in chemostat cultures after 5 R when the cultures were in steady state. It was assumed that during anaerobic conditions the fungus would not be able to metabolise all the fed lactose and thus substrate would accumulate. To study which of the transcriptional responses observed were specific for the transition from anaerobic to aerobic conditions and which resulted from the increased concentration of substrate or other metabolites, the supply of growth medium was stopped for a 60 min period in one culture and in another culture medium supply was stopped simultaneously with replacing the air flow by N_2, also for a 60 min period.

Samples (20 – 50 ml) were withdrawn from the fermentor via a sterilisable port. Two times five mL aliquots were immediately filtered using glass-fibre filter disks (Whatman GF/B 47 mm ∅, Kent, UK). The biomass was immediately washed with 20 ml sterile, demineralised water, after which the biomass was removed to liquid nitrogen and stored at -80°C for further analysis. The filtrate was collected for protein, sugar and enzyme analyses. These samples were also frozen in liquid nitrogen and stored at -80°C until analysed. Samples were collected most frequently some hours before and after the transient from batch to continuous phase and 2–3 times per day during the continuous phase. Samples for transcriptional analysis during and after the anaerobic conditions were collected every 15 to 60 min for the first 8 h and every 2 to 6 h until 30 h.

Analyses

Dry weight

For determination of dry weight, two sample aliquots were weighed and collected on pre-weighed filter disks (Whatman GF/B 25 mm ∅). The biomass was washed with approximately 50 ml demineralised water and dried overnight in an oven at 110°C, before the disks were re-weighed.

Enzyme activities

The activities of the cellulolytic enzymes were determined using a Cobas-mira (Roche, Switzerland) autoanalyser. For measurement of total cellulolytic (i.e. cellobiohydrolase (CBH), endoglucanase (EG) and β-glucosidase (BGL)) activity 10 μl sample was added to 100 μl reagent containing 100 mM sodiumcitrate buffer pH = 5.0 and 6.0 mM p-Nitrophenyl-β-D-lactopyranoside (Sigma, St. Louis, MO). After 16 min incubation at 37°C, 50 μl 1.5 M sodium carbonate was added to raise the pH above 10 and the absorbance was read at 405 nm. Activity was determined from the amount of p-Nitrophenol released, as determinded from a calibration curve made under the same reaction conditions, and is reported as nkat mg^{-1} biomass. Individual activities of CBH, EG and BGL were estimated by adding 10 mM cellobiose (inhibits mainly CBH) or 100 mM glucose (inhibits BGL) to the assay.

Protein and ammonium

Protein was assayed by mixing 10 μl sample with 200 μl reaction mixture (Biorad, Hercules, CA) in a microtiter plate. After 15 minutes incubation at 20°C, absorbance was measured at 595 nm. Samples were measured in duplicate and protein concentration was determined from a Bovine Serum Albumin (Biorad) calibration curve.

The ammonium concentration was determined using the Roche ammoniak test kit (Basel, Switzerland), according to the instructions of the manufacturer, adapted for automated analysis with the Cobas-mira (Roche).

Transcriptional analysis by TRAC

Transcriptional analysis was performed with the TRAC assay as described in Rautio et al. [17]. 0.3 – 1 mg (wet weight) of lysed mycelium, containing 50 – 200 ng of polyA mRNA, was added to each hybridisation reaction with 4 pmol biotinylated oligo(dT) capture probe and 1 pmol of each 6-FAM-labeled detection probe. The hybridizations were carried out in 96-well PCR plates (ABgene, Epsom, UK) at 60°C for 30–40 min with shaking at 600 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany) in a total volume of 100 μl, containing 5 × SSC, 0.2% SDS, 1 × Denhardt solution (0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl pyrrolidone, 0.02% (w/v) BSA) and 3% (w/v) dextran sulfate.

The steps following hybridization, including affinity capture, washing and elution, were automated with a magnetic bead particle processor KingFisher 96 (Thermo Electron, Vantaa, Finland) in 96-well plates, as follows: 1) affinity capture of hybridized RNA targets to 50 μg of streptavidin-coated MyOne DynaBeads (Dynal, Oslo, Norway) at room temperature for 30 min, 2) washing of the beads two times for 1.5 min in 150 μl of 1 × SSC, 0.1% (w/v) SDS at room temperature, 3) washing twice for 1.5 min in 150 μl of 0.5 × SSC, 0.1% (w/v) SDS at RT, 4) washing once for 1.5 min in 150 μl of 0.1 × SSC, 0.1% (w/v) SDS at RT and 5) elution of probes to 10 μl deionised formamide (Sigma) for 20 min at 37°C.

The eluents were analyzed by capillary electrophoresis with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). In order to compare individual samples and to calibrate the separation of the detection probes by size, GeneScan-120LIZ size standard (Applied Biosystems) was added to each sample. The identity of the probes was determined by the migration and the quantity by the peak area.

Total polyA RNA quantification from prepared lysates was performed with the above TRAC protocol without addition of detection probes. The final elution of polyA mRNA was performed in 50 μl DMPC treated water. RNA concentration in the eluent was quantified with a RiboGreen RNA quantitation kit (Molecular Probes, Leiden, the Netherlands).

Probe pools

The detection probe oligonucleotides, labelled at the 3' and 5' ends with 6-carboxy fluorescein (6-FAM), were synthesized by Thermo Electron (Ulm, Germany). The biotinylated Oligo(dT) capture probe was from Promega. The HPLC-purified oligonucleotide detection probes were organised into three pools (Table 1). Oligonucleotide probes were designed using mathematical algorithms presented in Kivioja *et al*. [40]. Criteria used in probe selection were the following: melting temperature, T_m, limits 60 – 70°C, GC% limits 38 – 62, maximum repeat size 15 nt, maximum similarity e = 0.8, maximum free energy change in hybridisation [41] > -15 kcal/mol and minimum target energy change, A_c, [41] < -10 kcal/mol. Tms were calculated with the nearest neighbouring method according to le Novére [42] using 10 nM nucleic acid and 750 mM salt concentrations.

The marker genes used are involved in various cellular functions, including carbohydrate metabolism, protein production and degradation, transport, growth, conidiation and stress protection against various factors such as unfolded proteins and reactive oxygen species (Table 1).

Calculation of gene expression stability in chemostat cultures

The fluorescence signal intensity measured for gene specific probes in capillary electrophoresis is normalised using the total polyA mRNA amount in each hybridisation reaction. To estimate the stability of the expression of the set of gene markers in the different phases of the chemostat cultures we have calculated the moving average of fold change (F_{ma})(equation 1). F_{ma} predicts the time inter-

Table 1: Marker genes used in the TRAC analysis.

Gene name	Length nt	ORF no.	Function	Sequence
Pool1				
cbh1	25	22421	cellobiohydrolyase	CATTCTGGACATAGTATCGGTTGAT
egl1	27	42363	endoglucanase	CGGACTTTGTACACTTGTAGGTTGTCA
sod2	29	42675	Superoxide dismutase	TTGATGACGTCCCAGATGGCGCTGAAGTA
pdi1	33	45146	protein disulfide isomerase	GGTCAAAGGGGAACTTGAGGTTCTTCTCAATGT
gap1	33	43090	general amino acid transporter	TGATACTTCCAGGCATTGCGGAATCGGATGTGG
axp1	33	21826	acidic extracellular protease	AAGTTGAAGGTGGCATCCTTGATGTTTGCTTTG
trx2	39	9865	thioredoxin protein	CAAACTTGACAAAGTGGACCTTGTCCTTGAACTCTGCGT
ccg9	39	43571	Clock controlled gene, cell wall biogenensis	AAACTTTGACTTCGAACCCTTCATACGTCGACAGTTGAA
bgl2	41	42449	β-glucosidase	CGTTATAGTACTTGACCCTGAAGTCATCTTCGAGAATCTTC
bga1	43	45191	β-galactosidase	TTTGGAATGCCAATGATCTCGAGCGGCGTTGTGACGTTGAAAT
Pool2				
mca1	25	20144	metacaspase, cysteine protease, apoptosis	AATACCCTGCGTGGAGTAGATGTAC
bip1	27	42955	protein chaperon	AGGGGGTTGACGTCCATGAGAACAATG
aep1	25	46483	alkaline extracellular protease	CATGGAGGTGCCGCTAATCGTGTTT
vpa1	27	44744	vacuolar protease A	GTGATGTCGGGGAGGGAATCACGCTTG
hsp30	31	29985	heat shock protein	GTACTTTGCGTTGTCGGTAGGCTTGTTGCTG
con6	31	35051	ligth induced conidiation gene	TGCTTAGCGTTTTCCTTTGCTTCCTCCGACA
trr1	31	45052	thioredoxin reductase	AATGACGAAGAGGGGCTTGTTGCGGAAGATG
gcn4	37	37844	transcription factor	TGAAGAAGACGATCGGTACATGGGCTCTGATTCCAAA
chs1	37	43213	chitin synthase	GAAAGAAGCGATAAAGTAGAGGCCGTAAATGGTAATC
nsf1	41	42584	general membrane fusion factor	CAACAGGGCATCGTCAATCATGTCTTTTCGATTCGTCATTC
nth1	43	44650	neutral trehalase	AACGTAACTGGCATTGACCCATCCAAATCCTTCTTTCGCAACG
ctaA	41	21112	ATPase copper transport	ACGAGTGATTGTGCCGGTTTTGTCCAAGACGACTTTGGTAA
Pool3				
eno	25	43415	enolase	TTACGGAAGTTGGTGCCAGCGTAGA
rpl16a	27	43406	ribosomal protein L13A, 60S subunit	CAACCTTCTTGCGCTCGTAGTAGGCAG
gpd1	29	20456	glyceraldehyde-3-phosphate dehydrogenase	ACGAAGTTGGGGTTCAGGGAGATACCAGC
hem6	29	13475	Coproporphyrinogen III oxidase, heme biosynthesis	ACTTCTTGAACCGAGGGTAGTACGTCTTG
hsp70	33	27833	heat shock protein	TTGGTGATGACAATCTTGTTGGACTTACCAGTG
hsp105	37	42578	ER chaperon	CGGGCTTATCCTCAGTGTCAACTTGTTGATAGAATAA
rps16b	35	29002	ribosomal protein S16A, 40S subunit	TGACACGGACGCGGATGTCGACGTTGGCGAACTTG
acs1	39	20403	acetyl CoA synthase	TTGTGCTTCTCAATAATGTCCCAGTACCTTGAGAAGTTG
tps1	41	21151	trehalose-6-phosphate synthase	AACTTGCGGATGAACTTGGTGATCCACGACTGGACATTCTG

Oligonucleotide probes binding to respective mRNA targets are organised into 3 pools according to their migration in capillary electrophoresis.

val in which the culture was in steady state based on the average deviation in the expression of the marker genes from their mean expression level between 5 sequential measurements being at its lowest. F_{ma} is calculated over all groups of 5 sequential measurements throughout the experiment.

$$F_{ma} = \frac{1}{5N}\sum_{k=1}^{N}\sum_{i=0}^{4}\left|\log_2\left(\frac{5x_k(t+i)}{\sum_{j=0}^{4}x_k(t+j)}\right)\right| \qquad 1)$$

where,

$x_k(t)$ = Expression value of gene k (k = 1, ..., N) at time point t

t = starting time point of 5 sequential measurements

Average relative deviation of the marker gene set from the F_{ma} predicted steady state at any given time point is given by F_{SS} (Average fold change of expression from steady state) (equation 2).

$$F_{ss} = \frac{1}{N}\sum_{k=1}^{N}\left|\log_2\left(\frac{5x_k(t)}{\sum_{j=0}^{4}x_k(t_s+j)}\right)\right| \qquad 2)$$

where,

t_s = starting time point of F_{ma} predicted steady state

Authors' contributions

JJR carried out the TRAC analysis, all data analysis and drafted the manuscript. BAS designed and carried out all bioreactor cultivations and growth and protein produc-

tion analyses and helped to draft the manuscript. MW participated in the design of the study and helped to draft the manuscript. MP participated in the conception, design and coordination of the study. MS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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PUBLICATION IV

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Research article

Physiological evaluation of the filamentous fungus *Trichoderma reesei* in production processes by marker gene expression analysis

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Abstract

Background: Biologically relevant molecular markers can be used in evaluation of the physiological state of an organism in biotechnical processes. We monitored at high frequency the expression of 34 marker genes in batch, fed-batch and continuous cultures of the filamentous fungus *Trichoderma reesei* by the transcriptional analysis method TRAC (TRanscript analysis with the aid of Affinity Capture). Expression of specific genes was normalised either with respect to biomass or to overall polyA RNA concentration. Expressional variation of the genes involved in various process relevant cellular functions, such as protein production, growth and stress responses, was related to process parameters such as specific growth and production rates and substrate and dissolved oxygen concentrations.

Results: Gene expression of secreted cellulases and recombinant *Melanocarpus albomyces* laccase predicted the trends in the corresponding extracellular enzyme production rates and was highest in a narrow "physiological window" in the specific growth rate (μ) range of 0.03 – 0.05 h^{-1}. Expression of ribosomal protein mRNAs was consistent with the changes in μ. Nine starvation-related genes were found as potential markers for detection of insufficient substrate feed for maintaining optimal protein production. For two genes induced in anaerobic conditions, increasing transcript levels were measured as dissolved oxygen decreased.

Conclusion: The data obtained by TRAC supported the usefulness of focused and intensive transcriptional analysis in monitoring of biotechnical processes providing thus tools for process optimisation purposes.

Background

Microorganisms used in various types of biotechnical processes encounter constantly changing environmental conditions, to which they adapt by changing their cellular physiology. The performance of the microorganism has a major impact on the performance of the process and as a consequence, bioprocess monitoring and control strategies based on the physiological status of the culture have become more popular [1,2]. In bioprocesses the physiological status of the culture is generally measured indirectly by analysis of extracellular variables such as enzyme activities, pH and exhaust gas concentrations. Recent

developments in analytical methods allow direct evaluation of cellular physiology by analysis of intracellular variables such as proteins, RNAs or metabolites, either in focused or systems-wide manner [1,3].

By combination of transcriptomic, proteomic and/or metabolic profiles with advanced bioinformatic analysis it is possible to predict from the massive amount of data biologically meaningful analytes, *i.e.* biomarkers which can predict certain physiological events. In biotechnical processes such biomarkers have been used *e.g.* for detection of osmotic and oxidative stress conditions or trace element deficiencies in yeasts [2,4] and bacteria [3]. Simple and robust assays are required in order to use these biomarkers in monitoring or control of bioprocesses. Transcriptional analysis of selected marker genes provides one potential way for robust monitoring of physiological events, since adaptation to changes in the environment takes place rapidly at the level of gene expression [5,6]. In addition it is possible to measure multiple targets in a single assay with RNA which is more difficult to achieve with enzymes or metabolites.

Few microarray studies have been performed specifically to identify gene markers to be used in monitoring of industrial fermentations [4,7]. Conventional Northern blot analysis is still abundantly used for the actual monitoring of the selected markers in process conditions [2,4,7]. New tools for more robust RNA analysis are emerging, such as quantitative RT-PCR [8], and combination of sandwich hybridisation with fluorescent [9] or electric detection [10,11]. Depending on the organism and length of the process these methods may be feasible for at-line mRNA expression monitoring, but would generally be applicable for only a limited number of targets and/or samples.

We have recently developed a method for transcriptional analysis method called TRAC (Transcript analysis with aid of affinity capture) [12,13]. The TRAC method is suitable for rapid simultaneous analysis of 96 samples with multiplex target detection, allowing high-throughput, focused expression analysis [12] or alternatively it can be performed with amplifiable DNA probes and PCR [13] to reach high sensitivity. TRAC has been applied for gene expression analysis of microbial cultures [12], multiplex quantification of bacterial populations [14,15] and to monitor gene expression stability in continuous cultures [16]. In this study we have used TRAC for expression monitoring of more than 30 marker genes in different types of protein production processes performed with the industrially important filamentous fungus *Trichoderma reesei*. Expression levels of the marker genes, which are involved in different process relevant pathways, were compared with extracellular process parameters from the cultures, such as specific growth rates, production rates of secreted enzymes and availability of nutrients and oxygen. Identification of relationships between gene expression fingerprints and process performance would offer possibilities for process optimisation and control.

Results

polyA RNA- and biomass-based normalisation of gene expression data

Expression of a set of 34 marker genes, representing various physiological states, was monitored in different phases of two batch and fed-batch cultures and one continuous culture of an *M. albomyces* laccase producing strain of the filamentous fungus *Trichoderma reesei*. Potentially useful molecular marker genes were selected on the basis of their responses in conditions of interest as reported in publicly available transcriptional analysis data for various species of filamentous fungi or *Saccharomyces cerevisiae*. The genes used in this study are listed in Table 1 with references to available data on these genes or corresponding homologues in other organisms.

The TRAC expression analysis for the selected genes was performed directly from cell lysates, and the gene expression levels were related to the amount of biomass used in the hybridisation or to overall polyA RNA concentration of the lysate (see Methods). The ratio of marker gene mRNA to total polyA RNA shows the change in the expression of a specific gene relative to overall gene expression, whereas the ratio of marker gene mRNA to biomass shows the changing level of a transcript relative to the overall culture (viable/non-viable).

PolyA RNA concentration in the lysed biomass in different growth phases of the two batch cultures, which differed considerably in growth and substrate consumption rate are presented in Figure 1. In batch I the polyA RNA concentration varied between 2 and 2.5 mg g^{-1} dry weight (DW) for the first 35 h, while μ was above 0.06 h^{-1} and lactose concentration above 10 g l^{-1}. After 35 h, when the culture conditions had become growth limiting, the polyA RNA content relative to biomass decreased to approx. 0.4 mg g^{-1} dry weight. However in batch culture II the specific growth rate and uptake of lactose were slower than in batch I after 20 h and the polyA RNA per g biomass decreased at this time already, although the polyA RNA content in the biomass subsequently increased between 30 and 45 h. After 45 h the cultures were similar. To verify that the decrease in the polyA RNA content after 20 h was not a result of experimental error (*e.g.* variation in cell lysis efficiency), total RNA was extracted from the samples of batch culture II and the polyA RNA to total RNA ratio was determined. This confirmed that a decrease in overall gene expression had occurred after 20 h (Figure 1B).

Table 1: *T.reesei* genes used in the TRAC analysis grouped into functional categories.

Gene name	Function	Probe Length (nt)	Probe sequence 5'-3'	Location of the probe in CDS	Reference data
Carbohydrate degradation/oxidation					
bgl2	β- glucosidase	41	CGTTATAGTACTTGACCCTGAAGTCATCTTCGAGAATCTTC	1143–1183	51
cbh1	Cellobiohydrolyase	25	CATTCTGGACATAGTATCGGTTGAT	889–913	19
egl1	Endoglucanase	27	CGGACTTTGTACACTTGTAGGTTGTCA	107–133	19
lac1	*Melanocarpus albomyces* laccase	35	GACCAACGCATGTCGAAAGTGAACAACAAGTAACC	1952–1986	24
Stress					
bip1	Protein chaperon	27	AGGGGGTTGACGTCCATGAGAACAATG	1008–1034	58
gcn4	Transcription factor	37	TGAAGAAGACGATCGGTACATGGGCTCTGATTCCAAA	347–410	52
hac1	Transcription factor	31	AGAGAGTGATGCTGTCCTGGAGAGAGTCGAG	562–592	58
hsp105	ER chaperon	37	CGGGCTTATCCTCAGTGTCAACTTGTTGATAGAATAA	1522–1558	37
hsp30	Heat shock protein 30	31	GTACTTTGCGTTGTCGGTAGGCTTGTTGCTG	750–780	36
msn4	Transcription factor	35	CGAGAGAACTTCTTGCCGCACTCGTTGCACTCAAA	1327–1361	5
nsf1	General membrane fusion factor	41	CAACAGGGCATCGTCAATCATGTCTTTTCGATTCGTCATTC	1388–1428	59
nth1	Neutral trehalase	43	AACGTAACTGGCATTGACCCATCCAAATCCTTCTTTCGCAACG	2061–2103	30
pdi1	Protein disulfide isomerase	33	GGTCAAAGGGGAACTTGAGGTTCTTCTCAATGT	926–958	58
sod2	Superoxide dismutase	29	TTGATGACGTCCCAGATGGCGCTGAAGTA	637–665	5
tps1	Trehalose-6-P-phosphate synthase	41	AACTTGCGGATGAACTTGGTGATCCACGACTGGACATTCTG	1612–1652	53
trr1	Thioredoxin reductase	31	AATGACGAAGAGGGGCTTGTTGCGGAAGATG	444–474	18
trx2	Thioredoxin protein	39	CAAACTTGACAAAGTGGACCTTGTCCTTGAACTCTGCGT	230–268	18
Central carbohydrate metabolism					
acs1	Acetyl-CoA-synthethase	39	TTGTGCTTCTCAATAATGTCCCAGTACCTTGAGAAGTTG	1263–1301	17
eno1	Enolase	25	TTACGGAAGTTGGTGCCAGCGTAGA	1277–1301	17
gpd1	Glyceraldehyde-3-P-dehydrogenase	29	ACGAAGTTGGGGTTCAGGGAGATACCAGC	895–923	17
Growth and conidiation					
ccg9	Trehalose synthase	39	AAACTTTGACTTCGAACCCTTCATACGTCGACAGTTGAA	902–940	34
chs1	Chitin synthase	37	GAAAGAAGCGATAAAGTAGAGGCCGTAAATGGTAATC	2133–2169	54
con6	Conidiation related gene	31	TGCTTAGCGTTTTCCTTTGCTTCCTCCGACA	278–308	35
rpl16a	Ribosomal protein L13A, 60S subunit	27	CAACCTTCTTGCGCTCGTAGTAGGCAG	1612–1652	55
rps16b	Ribosomal protein S16A, 40S subunit	35	TGACACGGACGCGGATGTCGACGTTGGCGAACTTG	174–208	55
Proteases					
aep1	Extracellular protease	25	CATGGAGGTGCCGCTAATCGTGTTT	1038–1062	56
axp1	Extracellular protease	33	AAGTTGAAGGTGGCATCCTTGATGTTTGCTTTG	933–965	56
mca1	Metacaspase, cysteine protease	25	AATACCCTGCGTGGAGTAGATGTAC	861–885	33
vpa1	Intracellular aspartic protease	27	GTGATGTCGGGGAGGGAATCACGCTTG	957–983	57
O_2 regulated					
hem6	Coproporphyrinogen III oxidase	29	ACTTCTTGAACCGAGGGTAGTACGTCTTG	798–826	39
hsp70	Heat shock protein 70	33	TTGGTGATGACAATCTTGTTGGACTTACCAGTG	1545–1577	40
Transport					
ctaA	ATPase copper transport	41	ACGAGTGATTGTGCCGGTTTTGTCCAAGACGACTTTGGTAA	2309–2349	31
gap1	Amino acid transporter	33	TGATACTTCCAGGCATTGCGGAATCGGATGTGG	1335–1367	52

The specific growth rates (μ) were compared to the expression of two ribosomal protein mRNAs (*rpl16a*, *rps16b*) as growth-related marker genes in these batch cultures. The changes in the transcription of these genes relative to biomass were similar to μ (Figure 1C). The relatively low specific growth rate between 20 and 30 h for batch II (25% lower than in batch I), corresponded to a 1.7-fold lower expression of the two ribosomal mRNAs during this period. However, in both fermentations, an approximately 2-fold increase in the ribosomal protein mRNA to biomass ratio was observed 3 to 5 h before the decrease in specific growth rate which occurred after all lactose had been consumed.

Monitoring carbohydrate metabolism markers

A number of genes coding for enzymes involved in central carbohydrate metabolism (glycolysis, TCA cycle) are known to be transcriptionally regulated according to the availability of glucose in *T. reesei* [17]. We measured the relative expression of three of these genes involved in central carbohydrate metabolism in batch and fed-batch fermentations with lactose as carbon source (Figure 2). Lack of glucose has been shown to have an increasing (acetyl-CoA-synthethase, *acs1*), decreasing (enolase, *eno1*) or indifferent effect (glyceraldehyde-3-P-dehydrogenase, *gpd1*) on their expression [17]. Both *gpd1* and *acs1* increased from 25 to 45 h in lactose-rich conditions (>10

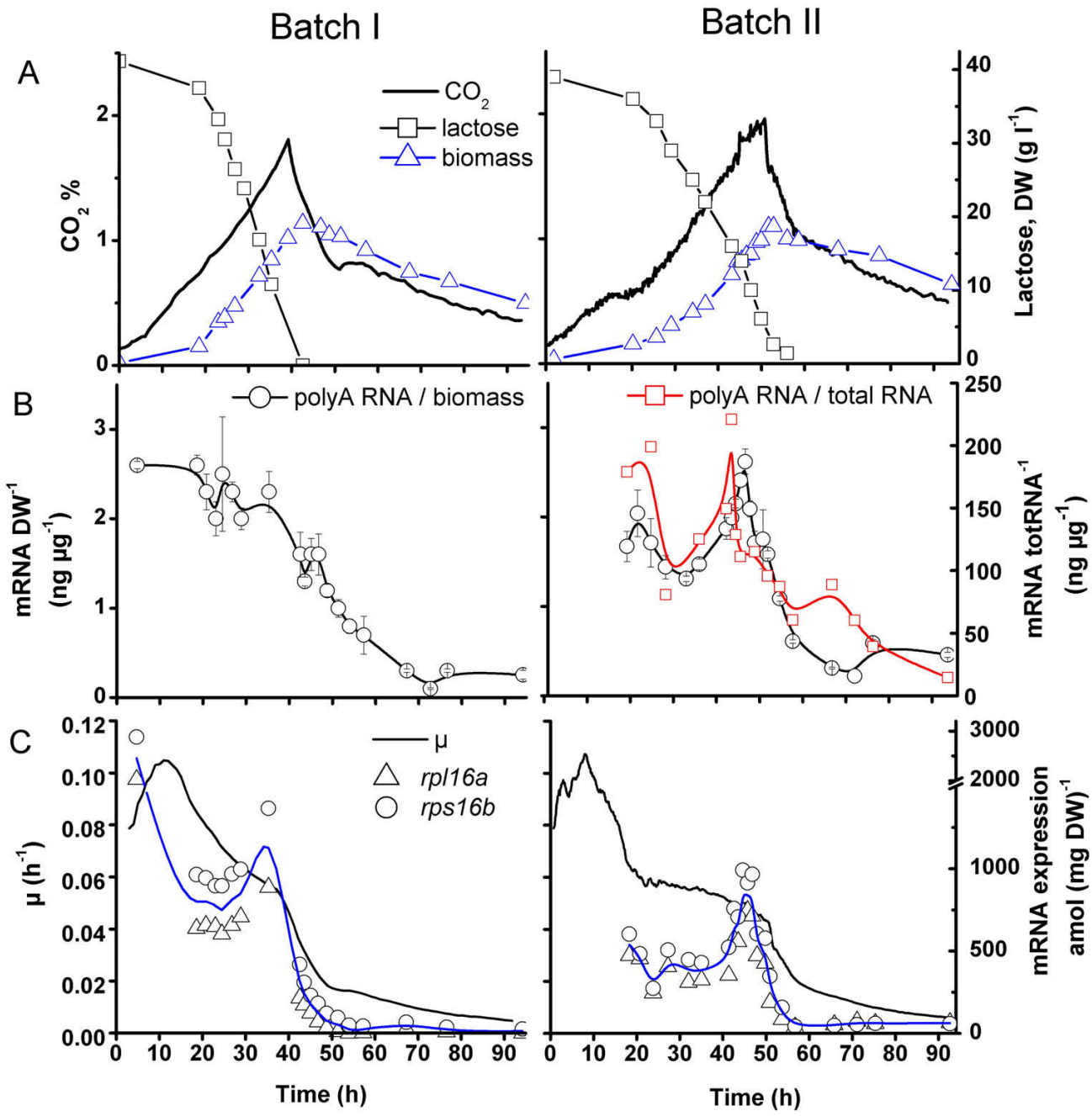

Figure 1
PolyA RNA and ribosomal protein mRNA levels in twobatch cultures of *T. reesei*. A. Lactose, biomass and CO_2 concentrations in batch cultures I and II. B. PolyA RNA concentration relative to biomass in cultures I and II and polyA RNA concentration relative to total RNA in culture II. C. Specific growth rate (µ) and expression of two ribosomal protein mRNAs (*rpl16a*, *rps16b*) relative to biomass. Cultures were maintained at 28°C and pH 5.5 – 6.

g l^{-1}) in batch cultures (Figure 2A). After the lactose concentration decreased below 10 to 6 g l^{-1}, the abundance of *gpd1* mRNA relative to polyA RNA decreased 30 – 65% compared to its maximal expression around 50 h, whereas *acs1* mRNA continued to increase at low lactose concentration (below 5 g l^{-1}) and during starvation. About 20 h after complete exhaustion of lactose, *acs1* was expressed at a 2.5 to 3 -fold higher level than in high lactose concentra-

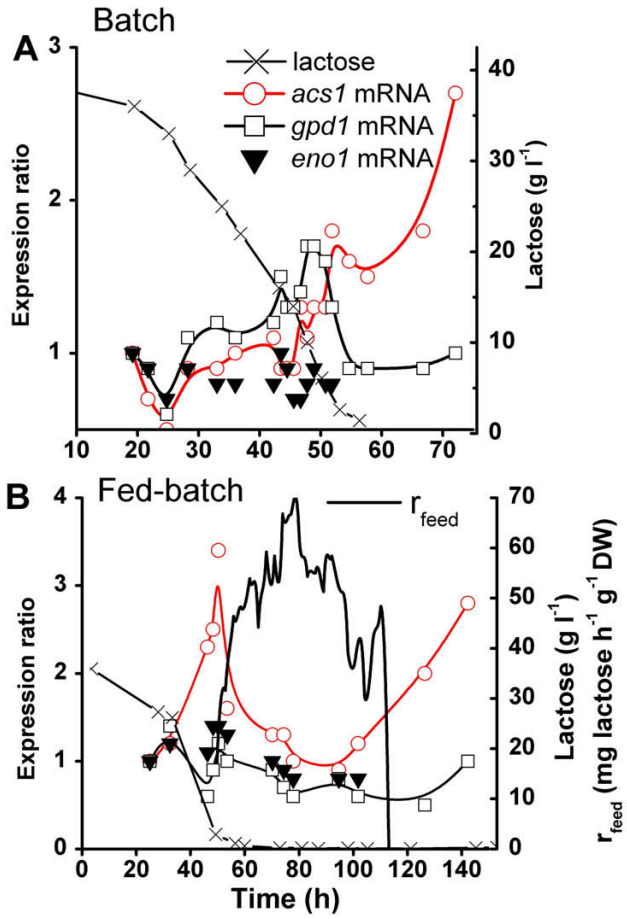

Figure 2
Expression of carbohydrate metabolism marker genes in a batch culture (A) and a fed-batch culture (B) of *T. reesei*. Lactose concentration, Feed rate of lactose r_{feed} (mg lactose h^{-1} g^{-1} DW), mRNA expression levels of *acs1*, *gpd1* and *eno1* relative to expression level measured in the first sample (19.2 h). Expression levels were normalised using polyA RNA.

tions. The *eno1* mRNA to polyA RNA ratio was constant when lactose concentration was above 5 g l^{-1}, whereas during starvation *eno1* mRNA was undetectable.

In the fed-batch fermentations a 2 to 3 -fold increase of *acs1* mRNA level occurred when lactose concentration decreased from 40 to below 5 g l^{-1}, before the start of the lactose feed (Figure 2B) as in the deceleration to stationary phases of the batch cultures. Lactose was fed to the fermentor after 45 h at a rate that maintained the rate of base (NH_4OH) consumption at around 0.012–0.014 ml l^{-1} min^{1}. As long as the feed rate of lactose was below 30 mg h^{-1} g^{-1} DW, *acs1* mRNA level continued to increase, but it decreased rapidly after the feed rate increased above this value (Figure 2B), even though the residual lactose concentration in the medium remained zero. Termination of the lactose feed caused another increase in *acs1* expression. *gpd1* and *eno1* mRNA levels showed only minor changes during the different phases of the fed-batch cultures, and *eno1* was again undetectable under starvation conditions.

Monitoring marker genes responding to oxygen

Expression of the heat shock protein gene *hsp70* and the heme biosynthesis gene *hem6* has been shown to be regulated by oxygen availability in *T. reesei* [16]. The *trx2* gene encoding thioredoxin was chosen as a potential marker for oxidative stress based on *S. cereviciae* transcriptional data [18]. In batch cultures (Figure 3) and in the batch phase of fed-batch cultures the expression of these three genes increased 1.6 to 2-fold between 18 to 45 h, while the biomass increased from approximately 3 to 20 g l^{-1} and pO_2 decreased from 90 to 30 %. A 20–35% decrease in the expression level relative to polyA RNA was observed for these 3 genes after the maximal expression was measured (Figure 3A).

The maximal expression levels of these genes in batch cultures were compared to the corresponding levels measured in steady state of chemostat cultures with high and low cell density, and in anaerobic conditions (TRAC data of chemostat and anaerobic cultures for this comparison from Rautio *et al.* [16]) as well as in shake flask precultures (Figure 3B). In anaerobic conditions the expression levels of *hsp70* and *hem6* were 3.5 and 2.8 -fold higher than the maximal expression levels measured in batch or fed-batch cultures. Comparable *hsp70* and *hem6* expression levels were measured between high cell density (16–20 g l^{-1}) batch or fed-batch cultures and chemostat cultures with 30% pO_2, whereas at low cell density (4 g l^{-1} and a pO_2 of 80%) the expression level of *hsp70* was 2.7-fold lower and that of *hem6* was appr. 2-fold lower than in the high cell density aerated cultures. In the shake flask cultures (~2 g l^{-1}) *hem6* expression levels were comparable to those observed in high density cultures, whereas the *hsp70* transcript level was 2-fold lower. This indicates that other stress factors besides oxygen limitation resulted in up-regulation of the *hsp70* mRNA level in the high density cultures. *Trx2* expression was similar in all aerobic bioreactor cultures, but was on average 1.5-fold higher in aerobic than in anaerobic or shake flask culture conditions.

Comparison of secreted enzyme production rate and mRNA expression

The transformant strain LLK13/295 used in these studies produced *M. albomyces* laccase under the cellobiohydrolase (*cbh1*) promoter, as well as all the native cellulases, including cellobiohydrolase I (CBHI). *Cbh1* promoter is induced by cellulose and by oligosaccharides and disaccharides derived from cellulose, such as cellubiose or

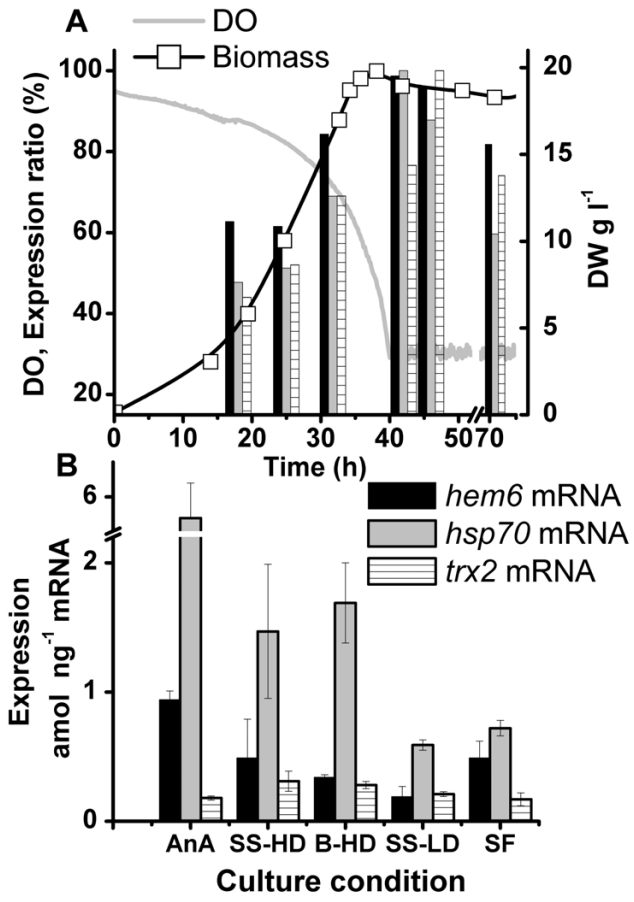

Figure 3
Expression of oxygen sensitive *hem6, hsp70* and *trx2* genes in different cultures of *T. reesei*. Expression of *hem6*, *hsp70* and *trx2* genes (A) during batch culture of *T. reesei* compared with DO and biomass concentration and (B) maximal expression levels of *hem6*, *hsp70* and *trx2* measured in an anaerobic culture (AnA, DW 4 g l^{-1}, pO_2~0%), steady state with high cell density (SS-HD, DW 16 g l^{-1}, pO_2 ~30%) and with low cell density (SS-LD, DW 4 g l^{-1}, pO_2 ~80%), in batch cultures with high cell density (B-HD, DW 20 g l^{-1}, pO_2 ~30%) and in shake flask precultures (SF, DW ~2 g l^{-1}). Error bars show the standard deviation between triplicate cultures.

sophorose. Also several other disaccharides such as lactose induce *cbh1* expression [19]. Transcript levels of the genes (*cbh1*, *lac1*) expressing these secreted enzymes and two folding factors (protein disulfide isomerase *pdi1* and protein chaperon *bip1*) were monitored during batch and fed-batch cultures and were compared with specific extracellular production rates of laccase and CBHI (Figure 4). Both biomass (*lac1*, *cbh1*) and polyA RNA (*bip1*, *pdi1*, *cbh1*) concentrations were used to normalise gene expression so that *lac1* and *cbh1* could be readily compared with biomass specific production rates and to allow physiological interpretation of the data.

Expression of *cbh1*, *lac1*, *pdi1*, and *bip1* increased during the first 40 h of growth in batch cultures. In the fed-batch culture there was a 1.8 and 2.6 -fold increase in mRNAs for *lac1* and *cbh1* respectively, after the lactose feeding was started, whereas their expression decreased in the batch cultures. There were corresponding increase in the specific extracellular production rates of laccase (3.8 -fold) and CBHI (4.3 -fold), after starting of the lactose feed in the fed-batch culture. The highest amounts of *lac1*, *cbh1*, *pdi1* and *bip1* mRNAs were measured between 7 and 12 h after the start of the lactose feed. 36 h after the lactose feed had been started both the gene expression (mol/biomass) and the specific enzymes production rates (nkat h^{-1} g^{-1} DW) had decreased to levels similar to those observed during the batch phase.

To study the variation between the biomass and polyA mRNA based normalisation they were compared in the normalisation of the major cellulase *cbh1* expression in the fed-batch culture (Figure 4B). An expected difference between the profiles of *cbh1* to biomass and *cbh1* to polyA RNA ratios was observed at the end of the culture. *Cbh1* to biomass ratio showed a faster decreasing trend after about 70 h than *cbh1* expression relative to polyA RNA, since increasing proportion of the biomass became metabolically inactive and polyA was produced only by the metabolically active part of the culture. It should be noted, however, that *e.g.* increase in a gene mRNA to polyA RNA ratio is not necessarily a sign of specific up-regulation, but can also indicate slower down-regulation than for the majority of genes. However increase or decrease in this ratio indicates the growing or decreasing physiological importance of the gene product in the particular conditions.

The specific production rate of both CBHI and laccase followed trends similar to the *cbh1* and *lac1* gene expression profiles, although a delay was observed between the gene expression and the corresponding extracellular enzyme production rate. For example the specific extracellular laccase production rate, which was more frequently measured than CBHI, was highest 6 to 10 h after the highest gene expression level was measured. An increase in laccase production rate was consistently observed at the end of the fed-batch cultures after lactose feed was stopped (Figure 4). This was not observed for CBHI.

Monitoring responses caused by starvation

The highest expression levels for genes coding the cellulases, laccase and folding factors were detected at growth rate 0.05 – 0.03 h^{-1}, at low lactose concentration in batch and lactose limiting in fed-batch cultures (Figure 4). To

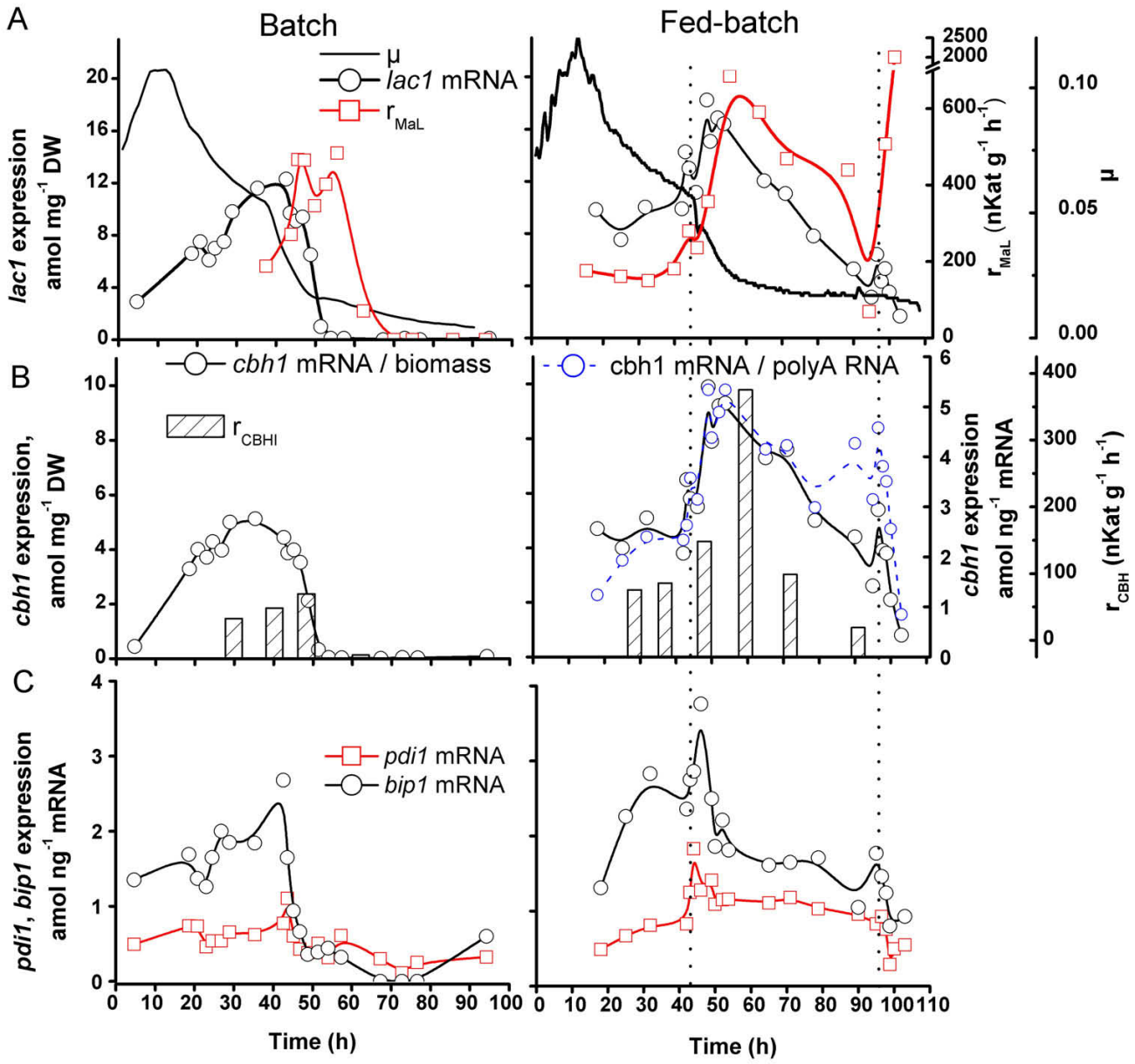

Figure 4

Expression of the recombinant *M. albomyces* laccase gene *lac1*, the cellulase gene *cbh1* and protein folding factor genes *pdi1* and *bip1* in batch and fed-batch cultures of *T. reesei*. A. *lac1* mRNA level relative to biomass, specific extracellular production rate of laccase r_{MaL}, specific growth rate μ. B. *cbh1* mRNA relative to biomass (batch and fed-batch) and relative to polyA RNA (fed-batch) and specific extracellular production rate of CBHI. C. *pdi* and *bip1* mRNA relative to polyA RNA. Dotted, vertical lines in the fed-batch culture represent the start and end of lactose feed.

determine the responses of the marker genes during the transition from deceleration to stationary phase, samples were taken frequently around the time when lactose was expected to be exhausted in batch cultures (Figure 5). Gene expression levels are presented relative to overall gene expression (polyA RNA), because the responses of metabolically active cells during starvation was of interest. The time when the base consumption rate had decreased to zero and the lactose was undetectable was considered to be the time at which starvation began. The expression of genes coding for carbohydrate degrading enzymes was at this time on average 1.5 -fold lower than the maximal expression level measured during these cultures. The time of maximal expression of *cbh1*, *lac1*, *egl1* (endoglucanase)

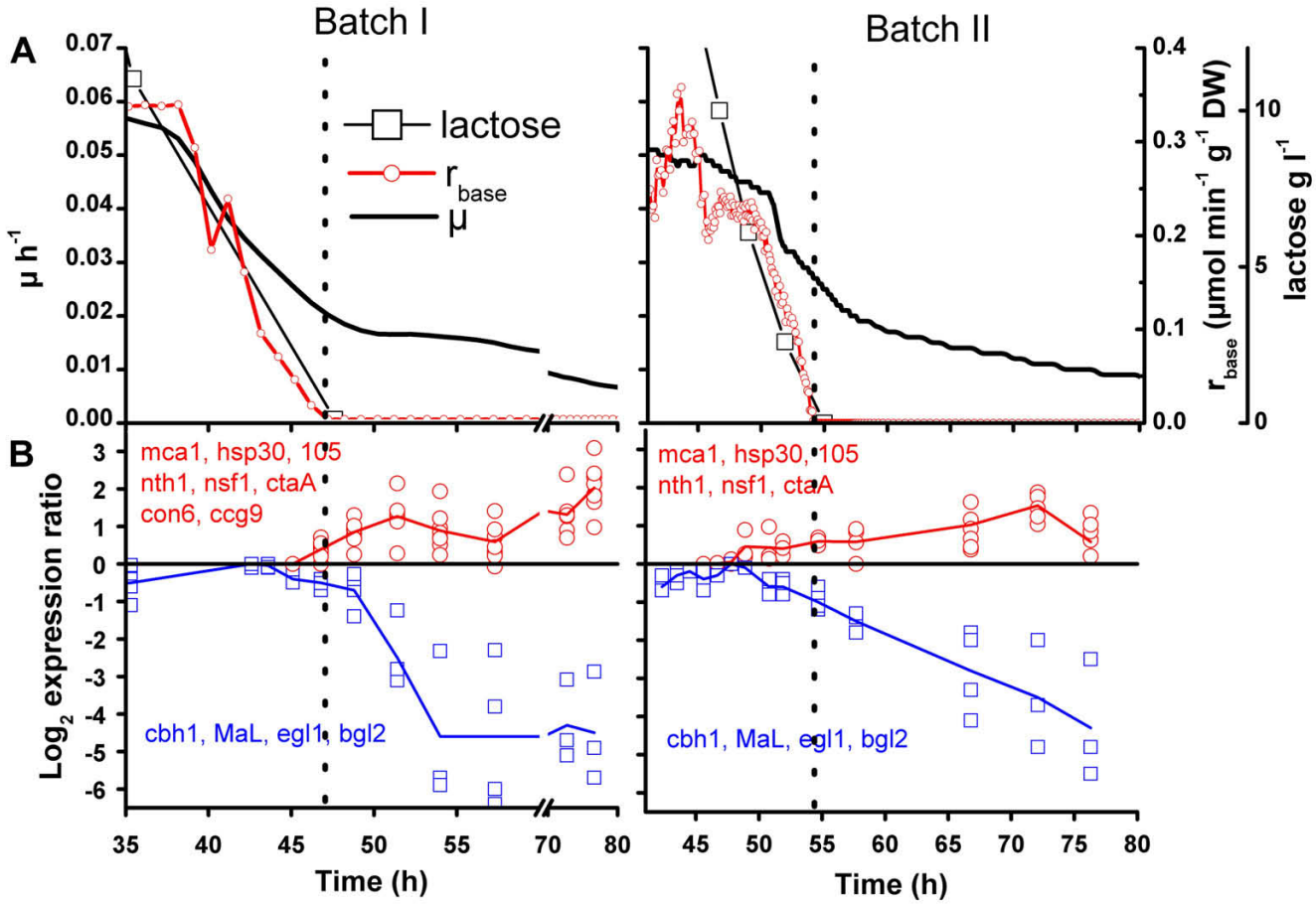

Figure 5

Responses of marker genes to starvation in two batch cultures (I and II) of *T. reesei*. A. Specific growth rate μ, lactose concentrations, specific base consumption rate r_{base}. B. Expression of cellulase and laccase genes as log_2 ratio to maximal expression measured in the cultures (blue symbols). Expression of genes showing >2-fold increasing mRNA level relative to polyA RNA after exhaustion of lactose as log_2 ratio to the expression level in a sample taken before the observed increase (red symbols).

and *bgl2* (β-glucosidase) was 3.6 to 6.4 hours prior to the start of starvation, when μ was 0.03 – 0.045 h^{-1}, the rate of base consumption was between 0.1 and 0.2 μmol min^{-1} g^{-1} DW and the lactose concentration was below 6 g l^{-1}. Along with cellulases and laccases the majority of the genes analysed showed down-regulation during starvation, including the folding factors (*pdi1*, *bip1*), oxidative stress genes (thioredoxin reductase *trr1* and thioredoxin protein *trx2*) and glycolysis genes (*eno1*, *gpd1*) (data not shown).

However, six to eight marker genes out of the 34, in addition to *acs1* (Figure 2), showed more than 2 -fold increased mRNA levels during starvation (Figure 5). The trend of increasing expression of these genes started 0.5 to 5 hours before base consumption stopped. These genes coded for heat shock proteins *hsp105* and *hsp30*, metacaspase (*mca1*) involved in apoptosis, neutral trehalase (*nth1*), membrane fusion factor (*nsf1*), copper transporter (*ctaA*) and two conidiation related proteins (*ccg9* and *con6*, measured only in batch I).

Monitoring of a continuous culture

Continuous culture was used to study further the effect of growth conditions on the productivity of extracellular proteins and to optimise their production. Different growth conditions were applied during the culture, *i.e.* altering the medium feed rate and composition and process temperature. In addition, unexpected disturbances occurred during the process, which was maintained for 1000 h. The TRAC assay was used to monitor expression 20 genes in a single pool, including markers that showed predictive value for cellulase productivity (*cbh1*), specific growth rate (*rpl16a*), and starvation (*acs1*) (Figure 6). A

more detailed description of the process parameters in the continuous culture will be published elsewhere (M. Bailey, unpublished results).

The feed rate of the culture was controlled by the rate of base consumption (see Methods). The medium feed to the fermentor started at 41 h (Figure 6I) when the rate of base consumption decreased below the DELTABAS criterion value (0.02 ml (5% NH_4OH) l^{-1} min^{-1}). This criterion kept the dilution rate (D) at approximately 0.04 h^{-1} and the specific base consumption rate, r_{base}, was 0.165 μmol min^{-1} g^{-1} DW. The criterion for base consumption was decreased in two steps: at 309 h by 25% (Figure 6II) and at 407 h by additional 20% (Figure 6III). The average r_{base} value decreased to 0.14 and 0.12 μmol min^{-1} g^{-1} dry weight as a result of these two changes and the average dilution rate decreased to 0.036 and 0.026 h^{-1}. The *cbh1* mRNA level increased 1.6-fold (Figure 6B) and the specific extracellular protein production rate (r_{prot}) increased 1.2 – 1.5 -fold (Figure 6A) after the first step (II). After the second reduction in base consumption rate (III) there was no immediate effect on *cbh1* mRNA or extracellular protein production rate, but after 48–57 h (1.2–1.5 residence times) these values decreased to the level measured prior to the changes in the base consumption rate (before 309 h).

At 550 h the organic nitrogen concentration was halved in the medium (Figure 6IV), which decreased D to an average value of 0.017 while the r_{base} increased to an average level of 0.134 μmol min^{-1} g^{-1} DW. The lower level of nitrogen in the medium will have been partly compensated for by the increased base (NH_4OH) consumption. The specific production rate of extracellular proteins was unaffected by this change and the only marker gene which responded to the lowered N concentration was the amino acid permease *gap1*, the mRNA level of which was increased 1.8-fold 65 h after change in the medium (data not shown).

The expression profile of the ribosomal protein mRNA (*rpl16a*) followed the changes in the dilution (=growth) rate throughout the culture, consistent with earlier observations from batch cultures (Figure 1). The gene coding for acetyl-CoA-synthase (*acs1*), which showed its highest expression at a low lactose concentration (Figure 2), was somewhat induced when the dilution rate was reduced (Figure 6C). Two other genes (*hsp30*, *nsf1*) showing increased mRNA level under starvation conditions (Figure 5) were also transitorily induced after 309 h when the rate of medium feed decreased (data not shown).

Finally at 861 h the temperature of the culture was reduced from 28 to 24 °C over a 50 h period (Figure 6V). A number of marker genes responded by increasing their expression levels. Some of these genes (*cbh1*, *egl1*, *lac1* and vacuolar protease *vpa1*) returned to the expression levels measured before the temperature change in less than 45 h, whereas others (*hsp30*, *nsf1*, *bgl2*, *gpd1*) remained at the higher expression level, even after the temperature was increased to 28°C again for the remainder of the culture. However, it can not be concluded that these responses were specifically related to the temperature change, since a temporary increase in the dilution rate occurred simultaneously because of a process control problem when growth and base consumption were reduced at the lower temperature (see Figure 6V).

Two technical disturbances occurred during the process. The first occurred at 68 h when the vessel weight controller malfunctioned and the culture volume decreased to about 8 litres. The volume was increased to 10 l by medium addition, which caused increased expression of *e.g. cbh1* and ribosomal protein mRNA (*rpl16a*) genes. Both reached stable expression levels within 70–90 hours (2.8 – 3.8 residence times) of the disturbance (Figure 6*). At 504 h, oscillation in the feed control mechanism was observed. Samples were collected at 1 h interval after this disturbance and temporarily increased expression in many of the marker genes (e.g. *cbh1*, *rpl16b*, *pdi1*, *gpd1*) was observed (Figure 6O).

Discussion

In this work we monitored the expression of 34 marker genes during changing conditions in batch, fed-batch and continuous fermentations of the filamentous fungus *T. reesei* using the transcriptional analysis method TRAC. In production processes the environmental conditions in the fermentor may be variable, causing corresponding variability in physiological parameters. This makes conventional gene expression data normalisation methods, such as the use of house-keeping genes, unreliable. In this study we related the expression of specific genes either to the amount of biomass or to the total polyA RNA content used in the hybridisation. Relating marker gene expression to biomass can be more useful in prediction of culture performance parameters such as specific growth and production rates, whereas normalisation to polyA RNA predicts the physiological responses in metabolically active cells more accurately.

Both polyA RNA to biomass and polyA RNA to total RNA ratios were shown to be growth phase dependent, and batch-to-batch variation in growth and protein production was also evident at the level of overall mRNA expression (Figure 1). Quantitative determination of total RNA per g biomass from mycelial biomass is not reliable, however comparing the polyA RNA per g biomass with polyA RNA per total RNA from the same culture (Figure 1) indicated that the total RNA to biomass ratio was not constant

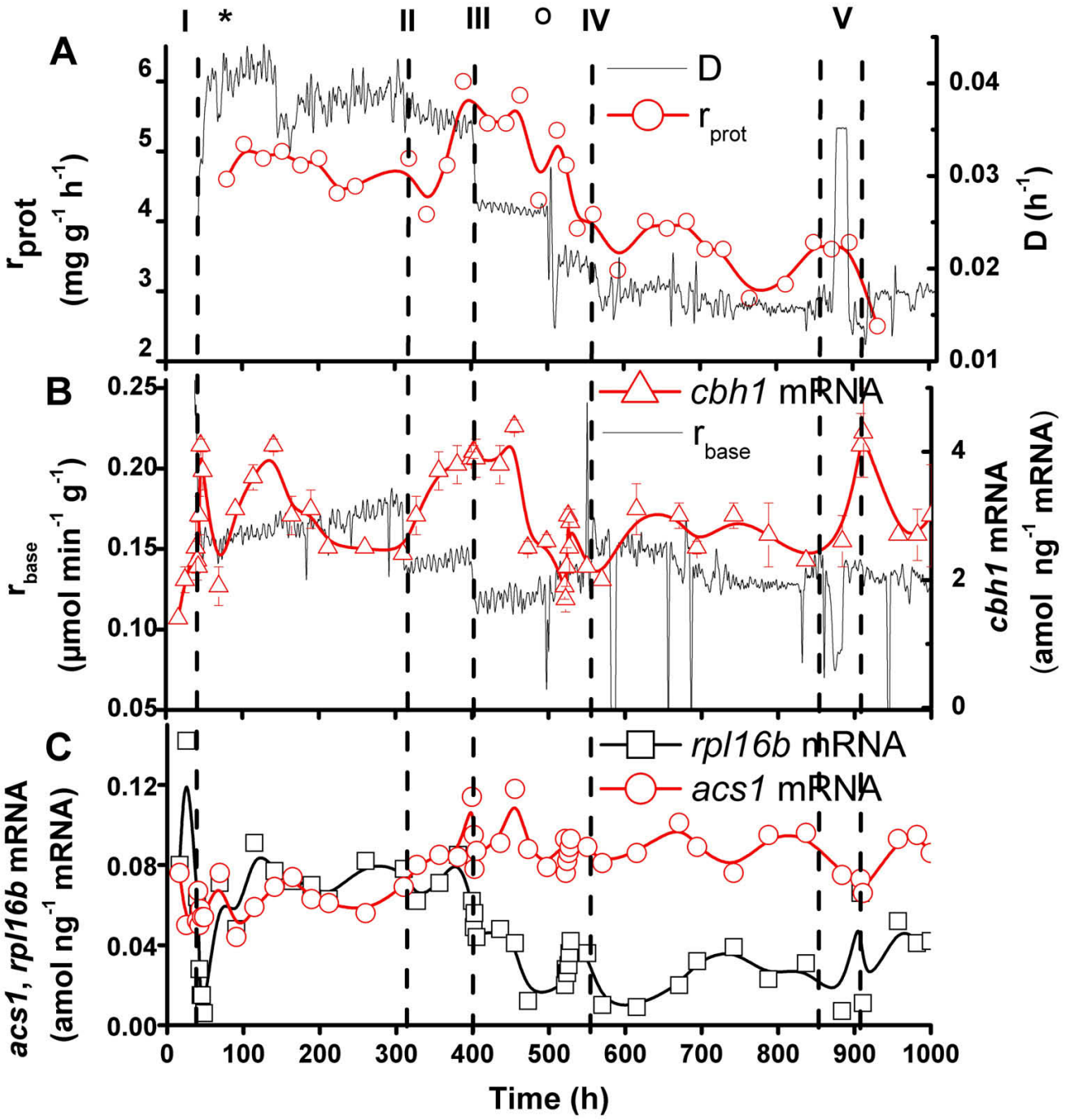

Figure 6

Expression of *cbh1*, ribosomal protein mRNA and *acs1* in a continuous culture of *T. reesei* run for 1000 h. A. Culture dilution rate D, specific productivity of extracellular proteins r_{prot}(mg h^{-1} g^{-1} DW). B. Specific base consumption rate r_{base}(µmol min^{-1} g^{-1} DW), *cbh1* mRNA expression relative to polyA RNA. C. *acs1* and *rpl16b* mRNA expression relative to polyA RNA. I Start of continuous medium feed. II, III Changes of base consumption rate criterion (DELTABAS). IV Reduction in nitrogen provision. V Temperature gradient from 28°C to 24°C. Process disturbances (*) and (O).

in changing environmental conditions of the batch cultures. It is thus beneficial to use a quantitatively measured factor to normalise expression levels between samples collected from different phases of a cultivation, rather than basing the normalisation on the assumption that a chosen factor is constant.

The normalisation based on polyA RNA is similar to the use of the expression levels of all genes for a normalisation in microarray experiments. However, for normalisation of genome-wide transcription data the distributions of the expression signal intensities between samples are equalised, *i.e.* it is assumed that global shifts in the mRNA population do not occur. This is a reasonable assumption *e.g.* in steady state conditions that can be achieved in continuous cultures, however disregarding global changes in mRNA levels during non-steady conditions (*e.g.* batch and fed-batch cultures) can result in the masking of true gene expression differences between compared samples [20]. On the other hand, variability in oligo dT-based mRNA capture can be caused by decreasing polyA tail length, that has been observed in stationary phase yeast cells [21]. However, since deadenylation leads to degradation of the mRNA body [22], and the polyA tail is required for translation initiation [23], the physiological relevance of mRNA species with short or no polyA tail is presumably insignificant, when the mRNA synthesis rate is low.

Expression of the native cellulase gene *cbh1* and the recombinant laccase gene *lac1* showed correlation with the specific extracellular production rates of the corresponding enzymes. There was a delay of 6 to 10 h between increased level of *lac1* transcripts and laccase production (Figure 4). *M. albomyces* laccase is processed at its C-terminus and is presumably activated by the processing [24], and a delay in this processing might contribute to the difference between the *lac1* expression and laccase production profiles. C-terminal processing of laccase could also explain the strong increase of its activity in the fed-batch cultures after termination of the substrate feed, which was not observed for CBHI (Figure 4). Gene expression of the secreted enzymes was highest when the growth rate was decreasing from 0.05 to 0.03 h^{-1} in batch and fed-batch cultures (Figures 4 and 5) or was constant between 0.026 and 0.036 h^{-1} in the continuous culture (Figure 6). This is in accordance with the optimal growth rate (0.031 h^{-1}) for production of extracellular proteins observed in chemostat cultures of *T.reesei* [25]. When the growth rate was below 0.03 h^{-1} decreased gene expression of cellulases and recombinant laccase was observed in addition to the responses associated with starvation (Figure 5). This data indicates that the "physiological window" in which the productivity of these extracellular proteins is optimal is narrow. In general the relation between specific product formation rate of a secreted protein and specific growth rate seems to be more dependent on the protein and its transcriptional promoter than on the organism. The specific product formation rate of a secreted protein can increase consistently with increasing growth rate, as shown *e.g.* for Fab fragment production in *Pichia pastoris* [26] and β-galactosidase production in *Escherichia coli* [27]. Whereas for other secreted proteins, such as α-galactosidase of *P. pastoris*, the specific product formation rate is at highest at low growth rates [28].

The DELTABAS feed control system, uses the rate of base consumption to try to maintain a culture in the optimal production phase by limiting the growth of the culture but avoiding total carbon source depletion [29]. During the optimal production phase the base (NH_4OH) was consumed at a rate of 0.1 – 0.2 μmol min^{-1} g^{-1} DW (Figures 5 and 6). However, in the fed-batch and continuous cultures in which this feeding strategy was applied, the optimal production was maintained for rather short periods of time. In the continuous culture most of the time the growth (and base consumption) rates were either too high or too low (Figure 6). During the feeding phase in the fed-batch cultures the growth rate was mainly below 0.025 h^{-1} (Figure 4), being too low for optimal production. Induced expression level of some of the starvation markers during the lactose (fed-batch, data not shown) or medium feed (*acs1*, continuous culture, Figure 6C) further indicated that too little substrate was available for the culture at this low dilution rate. However, at the end of the fed-batch cultures it was observed that the metabolically active part of the culture was still expressing cellulase genes (*cbh1*, Figure 4B) at a relatively high level, suggesting that improved productivity would be attained if culture viability could be maintained for a longer time.

Altogether 9 out of 34 marker genes (Figures 3 and 6) were observed to have more than 2-fold increased mRNA level under starvation and slow growth conditions, indicating their increasing importance under these conditions. Of the induced genes, *nth1* codes for neutral trehalase, which is involved in degradation of storage carbohydrates [30]. *CtaA* codes for a copper transporter [31]. and in these fermentations the copper requirement may have been increased because of production of the copper-containing laccase [32]. The cysteine protease encoded by *mca1* [33] is presumably involved in programmed cell death. In yeast the corresponding Mca1p is under post-translational regulation [33], but in *T. reesei* it would appear also to be subjected to transcriptional regulation. In *Neurospora crassa* the expression of the clock controlled gene *ccg9* has been shown to be induced during glucose deprivation [34]. The increased level of *con6* mRNA may be a response to nitrogen starvation, as shown by Bailey-Shrode and Ebbole [35]. High gene expression of *hsp30 S. cerevisiae* homologue (HSP30) has been observed in late stationary

phase yeast cells [36] and *hsp105* is homologous to the mammalian glucose-regulated protein GRP170, for which up-regulation has been shown when cellular UPD-glucose is reduced [37]. *S. cerevisiae* SEC18 (a homologue of *T. reesei nsf1*) is involved in starvation-induced degradation of tryptophan permease in yeast [38]. The *acs1* gene has been shown to be strongly induced in glucose starvation in *T. reesei* [17]. The induction of these genes elucidates some of the physiological events during starvation and slow growth: mobilisation of storage carbohydrates, acquisition of nutrients from the medium, stress responses, onset of conidia formation and triggering of apoptosis. The consistent induction of these genes preceding the complete exhaustion of the carbon source can make them useful markers in optimisation of substrate feeding strategies, when their expression is monitored continuously.

The heme biosynthesis gene HEM13 of *S. cerevisiae* has been shown to be induced under anaerobic conditions [39] and the Ssa type Hsp70 proteins have been shown to be part of a complex that regulates transcription of aerobic genes [40]. The homologues of these genes are induced by anaerobic conditions in *T. reesei* (Figure 3). Based on the expression of these genes during the batch, fed-batch and continuous processes, 30% dissolved oxygen (DO) concentration even at relatively high cell density (DW ~ 20 g l^{-1}) is apparently sufficient to maintain aerobic culture conditions, although at lower density (4 g l^{-1}) and higher DO (80%) the expression of these genes was more than 2-fold lower. Strong induction of these anaerobic marker genes and decreased levels of the cellulase gene mRNAs were observed only when DO was lower than 10% (data not shown). In large scale industrial fermentations with thick filamentous slurry, inhomogeneity of the system can cause local limitations in oxygen supply [41]. Thus monitoring of the expression of these markers in such fermentations could be useful in devising intervention strategies which would maintain better aeration and productivity. Simultaneously with the increase in *hsp70* and *hem6* expression, moderately increased expression of the thioredoxin gene (*trx2*) was observed (Figure 3), which indicated a response to accumulation of reactive oxygen species presumably because of culture ageing [42].

The TRAC method was shown to be an efficient tool for monitoring sets of marker genes in different bioprocess conditions. The possibility to analyse simultaneously in 96-well format large number of samples, allows frequent sampling during the cultivations. Crude lysed cell material can be directly used in the hybridisations, thus RNA extraction and cDNA conversions are avoided. Detection of multiple mRNA targets from a single sample and low hands-on time of the protocol makes the assay cost-effective. For off-line monitoring, the advantages of the system are most clearly demonstrated in the analysis of the continuous culture. The protocol starting from the lysis of all the 48 mycelial samples to analysis of expression of 18 genes in duplicate (1728 expression levels) was carried out in 7 h with 1 to 2 h hands-on time. For at line monitoring (less than 10 samples at once) the entire protocol (from sampling to results) can be carried out in approximately 2 h. Increasing the degree of automation by combining some or all of the 4 steps (cell lysis, hybridisation, sample treatment, CE analysis) would make the overall protocol time even shorter and frequent at-line monitoring more practical. Such an analysis system would have a wide application potential.

We have demonstrated here that by marker genes analysis it is possible to evaluate various physiological factors of the culture, such as nutrient and oxygen limitation, growth and extracellular protein production rates in the changing environmental conditions. Transcriptional analysis can provide a more complete picture of the physiological state of an organism than can be achieved by the external parameters that are measured from production processes. In addition to increasing the knowledge of gene regulation, gene expression data collected at high frequency may suggest strategies for optimising process parameters, such as medium composition and feeding strategy. Accumulation of microarray data from *T. reesei* and other industrially relevant microorganisms grown under various conditions will further help in the selection process of marker genes, the expression of which has a predictive value in the evaluation of the physiological state and performance of the cultures.

Conclusion

We have monitored by the TRAC method the expression of a set of 34 marker genes, involved in different process relevant pathways, in batch, fed-batch cultures and continuous cultures of a filamentous fungus *T. reesei* transformant strain producing *M. albomyces* laccase. Many of the marker gene expression levels measured at frequent intervals showed to have value in prediction of consecutive physiological effects and process performance. mRNA levels of genes coding for industrially relevant secreted cellulases and recombinant laccase followed the trends in the corresponding extracellular enzyme production rates and was at highest in narrow specific growth rate range of 0.03 – 0.05 h^{-1}. The specific growth rate of the fungal cultures was possible to evaluate on the basis of ribosomal protein mRNA expression. Increasing expression of altogether nine starvation related genes preceded the complete exhaustion of carbon source at least by some hours, indicating their usefulness in prediction of insufficient substrate feed for optimal production. Deficiency in oxygen supply was manifested by increased level of two oxygen sensitive genes *hem6* and *hsp70*. The TRAC method was shown to be an effective tool in focused transcrip-

tional monitoring of biotechnical processes and the data produced by this method supported the usefulness of intensive gene expression analysis for process optimisation work.

Methods

Strain, medium and cultivation conditions

Trichoderma reesei Rut-C30 transformant pLLK13/295 producing *Melanocarbus albomyces* laccase [43] was used in these studies. The inoculum was cultivated 3 × 200 ml flasks at 28°C, 200 rpm, for 2 days, starting from a stock spore suspension maintained in 15% glycerol at -80°C. The buffered inoculum medium contained 20 g lactose l^{-1} and other components described previously [44].

The fermentor medium contained (g l^{-1}): lactose 40, peptone 4, yeast extract 1, KH_2PO_4 4, $(NH_4)_2SO_4$ 2.8, $MgSO_4 \times 7H_2O$ 0.6, $CaCl_2 \times 2H_2O$ 0.8, $CuSO_4 \times 5H_2O$ 0.025 and 2 ml 2 × trace element solution l^{-1} (Mandels & Weber, 1969). pH was adjusted to 5.5 – 6 with NH_4OH and H_2PO_4 and the cultivation temperature was 28°C. Dissolved oxygen level was maintained above 30% with agitation at 600 r.p.m., aeration at 0.5 vvm (volumes of air per volume of liquid per minute) and 0–20% O_2-enrichment of incoming air. Foaming was controlled by automatic addition of Struktol J633 polyoleate antifoam agent (Schill & Seilacher, Germany, Hamburg) or polypropylene glycol (mixed molecular weights; [45]). Feeding of 24% (w/v) lactose solution into fed-batch fermentations and of complete culture medium in the continuous fermentation was controlled by the rate of base consumption in the culture using the algorithm described by Bailey and Tähtiharju [29]. The value of the DELTBAS variable [29] was calculated as the amount of base consumed per litre (kg) of medium or fermentor volume within 5 min intervals. Samples were taken frequently for dry weight, lactose, total protein, laccase, β-1,4-endoglucanase and cellobiohydrolyase I (CBHI) activity and gene expression measurements. Both batch and fed-batch cultures were performed as independent duplicates and the continuous culture was performed as a single culture maintained for 1000 h.

Analyses of fermentations

Dry weights were measured by filtering and drying mycelium samples at 105°C to constant weight. Residual lactose in the culture filtrate was measured enzymatically (Lactose kit, Roche, Basel, Switzerland). Soluble protein concentration was measured using the Bio-RAD Protein assay (Hercules, CA). Laccase activity was calculated by measuring oxidation of 5 mM ABTS in 25 mM succinate buffer (pH 4.5) at 436 nm, using the absorption coefficient (ε) of 29 300 M^{-1} cm^{-1} [46]. Cellobiohydrolyase I (CBHI) activity was measured according to Bailey & Tähtiharju [29].

Transcriptional analysis by TRAC

Biomass was harvested from fermentations for transcriptional analysis by anaerobically withdrawing medium containing 50–150 mg fresh biomass. Biomass was separated from medium by quick filtration with glass-fibre filter disks (Whatman GF/B 47 mm ∅, Kent, UK). The biomass was immediately washed with RNAse-free (dimethyl pyrocarbonite (DMPC)-treated) water, after which the biomass was transferred in tarred screw-cap tubes to liquid nitrogen and stored at -80°C. This sampling procedure took <5 min.

Transcriptional analysis was performed with the TRAC assay from crude cell lysates as described in Rautio *et al*. [12]. Frozen *T. reesei* mycelia were suspended (100 – 400 mg wet weight ml^{-1}) in buffer containing 5 × SSC (750 mM sodium chloride, 75 mM sodium citrate), 2% (w/v) SDS and 66 U ml^{-1} RNA guard RNase inhibitor (Amersham Biosciences, Buckinghamshire, UK). Mycelia were disrupted with a FastPrep cell homogenizer (6.5 m s^{-1}, 45 s) (ThermoSavant, Dreiech, Germany) using 500 µl acid-washed glass beads (Sigma). 0.3 – 1 mg (wet weight) of lysed mycelium, containing 50 – 200 ng polyA RNA, was added to the hybridisation reaction with 4 pmol biotinylated oligo(dT) capture probe and 1 pmol of each 6-carboxy fluorescein (6-FAM) labeled detection probe. The hybridizations were carried out in 96-well PCR plates (ABgene, Epsom, UK) at 60°C for 30–40 min with shaking at 600 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany) in 100 µl total volume, containing 5 × SSC, 0.2% (w/v) SDS, 1 × Denhardt solution (0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl pyrrolidone, 0.02% (w/v) BSA) and 3% (w/v) dextran sulfate.

The steps following hybridization, including affinity capture, washing and elution, were automated with a KingFisher 96 magnetic bead particle processor (Thermo Electron, Vantaa, Finland) in 96-well plates as follows: 1) affinity capture of hybridized RNA targets to 50 µg of streptavidin-coated MyOne DynaBeads (Dynal, Oslo, Norway) at room temperature (RT) for 30 min, 2) washing of the beads twice for 1.5 min in 150 µl of 1 × SSC, 0.1% (w/v) SDS at RT, 3) washing twice for 1.5 min in 150 µl of 0.5× SSC, 0.1% (w/v) SDS at RT, 4) washing once for 1.5 min in 150 µl of 0.1 × SSC, 0.1% (w/v) SDS at RT and 5) elution of probes to 10 µl of deionised formamide (Sigma) for 20 min at 37°C.

The eluents were analyzed by capillary electrophoresis with ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). In order to compare individual samples and to calibrate the separation of the detection probes by size, GeneScan-120LIZ size standard (Applied Biosystems) was added to each sample. The identity of the probes was determined by the migration

and the quantity by the peak area. In order to convert the measured peak area to the molar amount of a probe, the fluorescence signal intensity relative to molar amount was determined for each 6-FAM labelled probe.

Total polyA RNA quantification from prepared lysates was performed with the above TRAC protocol without addition of detection probes. The final elution of polyA RNA was performed in 50 µl DMPC treated water. RNA concentration in the eluent was quantified with a RiboGreen RNA quantitation kit (Molecular Probes, Leiden, the Netherlands).

The expression levels of the marker genes during the cultivations are presented here either as changes relative to a control sample or as molar amounts of the target specific probe detected. The expression levels presented as molar amounts can be compared between conditions for a given gene, but not between different genes, since the hybridisation efficiency between probe-target pairs can vary.

Probe selection

The detection probe oligonucleotides, labelled at the 3' and 5' ends with 6-FAM, were synthesized by Thermo Electron (Ulm, Germany). The biotinylated Oligo(dT) capture probe was from Promega. The HPLC-purified oligonucleotide detection probes (Table 1) were mixed in three pools, according to their migration in capillary electrophoresis. Oligonucleotides were designed using the algorithms presented in Kivioja *et al*. [47,48]. The quality criteria used in probe selection were the following: melting temperature (T_m) limits 60 – 70°C, GC% limits 38 – 62, maximum free energy change in hybridisation ΔG_H [49] -15 kcal/mol and minimum target energy change A_c [50] -10 kcal/mol. A maximum repeat size of 15 nt and a maximum similarity of 80% were used as probe specificity criteria. T_m values were calculated with the nearest-neighbour method [49] using 10 nM nucleic acid and 750 mM salt concentrations.

Authors' contributions

JJR carried out the TRAC analysis, all data analysis and drafted the manuscript. MB designed and carried out most of the bioreactor cultivations and growth and protein production analyses and helped to draft the manuscript. TK participated in the computer based design of the TRAC probes and helped to draft the manuscript. HS participated in the design of the TRAC analysis and helped to draft the manuscript. MP participated in the conception, design and coordination of the study. MS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression

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Research Article

Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression

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Abstract

Brewer's yeast experiences constantly changing environmental conditions during wort fermentation. Cells can rapidly adapt to changing surroundings by transcriptional regulation. Changes in genomic expression can indicate the physiological condition of yeast in the brewing process. We monitored, using the transcript analysis with aid of affinity capture (TRAC) method, the expression of some 70 selected genes relevant to wort fermentation at high frequency through 9–10 day fermentations of very high gravity wort (25 °P) by an industrial lager strain. Rapid changes in expression occurred during the first hours of fermentations for several genes, e.g. genes involved in maltose metabolism, glycolysis and ergosterol synthesis were strongly upregulated 2–6 h after pitching. By the time yeast growth had stopped (72 h) and total sugars had dropped by about 50%, most selected genes had passed their highest expression levels and total mRNA was less than half the levels during growth. There was an unexpected upregulation of some genes of oxygen-requiring pathways during the final fermentation stages. For five genes, expression of both the *Saccharomyces cerevisiae* and *S. bayanus* components of the hybrid lager strain were determined. Expression profiles were either markedly different (*ADH1, ERG3*) or very similar (*MALx1, ILV5, ATF1*) between these two components. By frequent analysis of a chosen set of genes, TRAC provided a detailed and dynamic picture of the physiological state of the fermenting yeast. This approach offers a possible way to monitor and optimize the performance of yeast in a complex process environment. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: yeast physiology; brewer's yeast; gene expression profiling; RNA; TRAC; expression regulation; fermentation monitoring

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Introduction

Brewer's wort is made from malted barley with or without the addition of cheaper carbohydrate adjuncts. Its main fermentable sugars are maltose, maltotriose and glucose and it contains many amino acids and di- and tripeptides. The conventional fermentation of this wort to beer is a biologically complex process, during which yeast must adapt to constantly changing environmental conditions, such as changing carbon and nitrogen sources, deprivation of nutrients, including oxygen, and a variety of stresses, such as hyperosmotic stress, high ethanol concentration, anaerobiosis and temperature changes. In very high-gravity (VHG) fermentations, yeast is exposed to greater osmotic stress at the start of fermentation and to greater ethanol stress at the end than in conventional

processes, because of the high concentrations of fermentable sugars in VHG worts (Casey *et al*., 1984). Failure to adapt to various dynamic environmental challenges can result in decreased fermentative capacity and viability of the yeast (Attfield, 1997; Powell *et al*., 2000), which also decreases its performance in the following fermentations when repitching is used (i.e. when yeast cropped from one fermentation is used to ferment the next batch of wort, which is the usual industrial practice).

Yeast cells can rapidly adapt their physiology to prevailing surroundings by reorganizing their genomic expression (Gasch and Werner-Washburne, 2002) and so changing the patterns of cellular proteins and metabolites. Since *Saccharomyces cerevisiae* has been a model organism in functional genomics, numerous studies have been performed to identify individual genes or groups of genes (clusters) that have altered expression patterns in given conditions (Boer *et al*., 2003; Gasch *et al*., 2000; ter Linde *et al*., 1999). Alternatively, the expression levels of genes with known responses to environmental changes can be used to evaluate the physiological condition of yeast and identify significant environmental features.

Genome-wide analyses of batch cultures provide datasets that are complicated to interpret, because of the multitude of changing growth conditions (Hayes *et al*., 2002). Thus, continuous culture systems, where single parameters may be varied while others are kept constant, have been preferred to assess specific genome-scale responses (Hoskisson and Hobbs, 2005). However, most industrial fermentations are intrinsically complicated batch processes. Genome-wide expression profiling has previously been applied to assess the physiological changes that yeast goes through during both beer (Higgins *et al*., 2003a; James *et al*., 2003; Olesen *et al*., 2002) and wine (Backhus *et al*., 2001; Novo *et al*., 2006; Rossignol *et al*., 2003) fermentations and to search for molecular markers of process events, such as zinc depletion (Higgins *et al*., 2003b). Data from beer fermentations have been collected at isolated time points (Higgins *et al*., 2003a; James *et al*., 2003) or once a day (Olesen *et al*., 2002) during fermentations that last a week or longer. However, this frequency of sampling gives too few data points to describe the details of gene regulation (Olesen *et al*., 2002). Transcriptional analysis techniques for the frequent analysis of such genes are emerging that are economically and practically more suitable than microarray technology (Bonetta, 2006). However, conventional Northern blot analysis is still widely used to follow the expression of selected genes during fermentations (Brejning *et al*., 2003; Higgins *et al*., 2003b; Perez-Torrado *et al*., 2005).

We here apply to VHG wort fermentations the transcriptional analysis method called transcript analysis with aid of affinity capture (TRAC) (Kataja *et al*., 2006; Rautio *et al*., 2006a, 2006b; Satokari *et al*., 2005) for frequent analysis of a focused gene set. TRAC enables analysis of multiple mRNA species from a large number of samples in a cost- and time-efficient manner. We followed at frequencies up to 10 times/day the expression of a set of genes involved in various pathways relevant to beer fermentations, such as maltose, glycerol and lipid metabolism, glucose fermentation, amino acid biosynthesis, aroma formation and flocculation. The expression profiles were compared to fermentation parameters, such as sugar concentrations and yeast growth and sedimentation. Our main aims were: (a) to determine how rapidly expression levels change in a complex batch fermentation and whether some genes show coordinated changes under these conditions; (b) to test whether expression changes in a brewer's yeast fermenting brewer's wort can be understood in terms of the changes described for genetically simpler laboratory yeast under laboratory conditions; (c) to compare the expression of the *S. cerevisiae* and corresponding *S. bayanus* genes present in (hybrid) lager yeasts; and (d) to examine whether the TRAC technique offers possibilities to monitor and predict the course of industrial fermentations.

Materials and methods

Yeast and cultivation conditions

Two samples of the same production lager yeast were obtained, 7 months apart, from a local brewery as slurries containing about 600 g fresh yeast mass/kg. The samples were taken from the yeast storage tank about 10 h after recropping from a production fermentation of high-gravity wort by

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yeast recycled several times in normal brewery operations. The samples were chilled to 0 °C, transported to the laboratory, diluted with water to 200 g/kg and aerated at 15 °C for 5–6 h in a stirred fermentor before pitching (Huuskonen *et al*, in preparation). Laboratory grown yeast of another production lager strain, A63015 from VTT's collection, was used in one experiment reported below.

Wort and fermentations

Brewers report wort strengths in degrees Plato (°P). Wort of x °P has the same specific gravity as an aqueous solution containing x g sucrose in 100 g solution, but the solids ('extract') in wort mainly comprise a mixture of fermentable sugars and non-fermentable oligomeric carbohydrates. A 25 °P wort was made at VTT from malt with a high-maltose syrup as adjunct (40%) and contained (g/l): maltose, 120; glucose, 25; maltotriose, 40; fructose, 5; and sucrose, 2; and (mg/l): free amino nitrogen (FAN), 420; Mg, 170; Ca, 60; and Zn, 0.17. An additional 0.1 mg/l Zn was added as $ZnSO_4$ before use. The fermentations were carried out essentially as described previously (Rautio and Londesborough, 2003) in 100 × 6 cm stainless steel tall tubes (designed to imitate on a small scale the cylindroconical unstirred vessels used in most breweries) with conical bottoms and anaerobic sampling ports 23 cm above the cones. Well-mixed yeast slurries were pitched at 10 °C by mass into 2 l oxygenated 25 °P wort (ca. 12 p.p.m. O_2) to 8.0 g (fermentation I) or 8.6 g (fermentation II) centrifuged yeast mass/l. After pitching, the tubes were warmed over 10 h to 14 °C. At 24 h (fermentation I) or 23 h (fermentation II) the temperature was further raised to 18 °C.

The course of fermentation and yeast growth were followed by anaerobically withdrawing samples and separating the yeast and wort by centrifugation. The specific gravity of the wort was determined with an Anton Paar DM58 instrument and used to calculate the apparent extract, which is essentially a measure of the conversion of carbohydrate into ethanol, from which, with certain assumptions, both remaining carbohydrate and ethanol can be calculated (EBC, 2004). The yeast was washed with distilled water and dried to constant weight at 104 °C.

Ergosterol and squalene analysis

Samples (5–30 ml) from yeast slurries and fermenting wort were centrifuged for 5 min at 10 000 × g. The pellets were washed twice with ice-cold distilled water and then resuspended in ice-cold 0.9% NaCl (4.0 ml/g centrifuged yeast mass). Duplicate 750 µl portions were transferred to 12 ml screw-cap tubes (Kimax) and centrifuged for 10 min at 5000 × g. The supernatants were removed by Pasteur pipette and the pellets (150 mg yeast) stored under N_2 at −80 °C and then freeze-dried. The pellets were hydrolysed under N_2 with 20% KOH in ethanol and sterols extracted into pentane. The pentane was evaporated at room temperature under a stream of N_2 and the sample was silylated at room temperature by adding 180 µl of a 2 : 1 mixture of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide : trimethylchlorosilane and analysed in an HP 6890 gas chromatograph equipped with FID and on-column injector. The column was DB-1 (15 m × 0.53 mm i.d., 0.15 µm film thickness). After 1.5 min at 100 °C the temperature was raised at 12 °C/min to 330 °C and held for 1.0 min.

Maltose transport

Yeast samples for maltose transport were collected and assayed as previously described in Rautio and Londesborough (2003).

Transcriptional analysis

Yeast in suspension was harvested from tall tubes for transcriptional analysis by anaerobically withdrawing wort containing 50–150 mg fresh mass of yeast via the sampling port, 23 cm above the cone (see above). Yeast was separated from wort by quick filtration with glass-fibre filter disks (GF/B, 47 mm diameter; Whatman, Kent, UK). The biomass was immediately washed with RNAse-free [dimethyl pyrocarbonate (DMPC)-treated] water, after which the biomass was transfered in tared screw-cap tubes to liquid nitrogen and stored at −80 °C. This sampling procedure took <5 min. An additional 2 ml anaerobically withdrawn wort was centrifuged for 1 min at 20 800 × g and sugars in the supernatant were analysed by HPLC (Waters, Milford, MA).

Transcriptional analysis was performed using the TRAC assay, essentially as described previously (Rautio *et al*., 2006a). Sample tubes were weighed

to give the fresh yeast mass. Frozen yeast samples were suspended (50–200 mg fresh weight/ml) in buffer containing 5× SSC (750 mM sodium chloride, 75 mM sodium citrate), 4% sodium dodecylsulphate (SDS) and 66 U/ml RNA guard RNase inhibitor (Amersham Biosciences, Buckinghamshire, UK). The yeast cells were disrupted with 500 µl acid-washed glass beads (Sigma), twice, in a FastPrep cell homogenizer (ThermoSavant, Dreiech, Germany), using 6 m/s for 45 s.

Hybridization reaction mixtures contained in 100 µl, yeast lysate (50–300 µg biomass; 100–150 ng polyA RNA), 4 pmol biotinylated oligo(dT) capture probe (Promega), 1 pmol each of 6-carboxy fluorescein (6-FAM) or 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxy-fluorescein (NED)-labelled detection probe, 5× SSC, 0.2% SDS, 1× Denhardt solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA), 3% dextran sulphate and 1.5 fmol *in vitro*-transcribed *Escherichia coli* traT mRNA as internal standard (see below). The hybridizations were carried out in 96-well PCR plates (ABgene, Epsom, UK) at 60 °C for 30–40 min with shaking at 650 r.p.m. (Thermomixer Comfort, Eppendorf, Hamburg, Germany).

The steps following hybridization, including affinity capture, washing and elution, were automated with a magnetic bead particle processor KingFisher 96 (Thermo Electron, Vantaa, Finland) in 96-well plates at room temperature as follows:

1. Affinity capture of hybridized RNA targets to 50 µg of streptavidin-coated MyOne DynaBeads (Dynal, Oslo, Norway) for 30 min.
2. Washing of the beads twice for 1.5 min in 150 µl 1× SSC, 0.1% SDS.
3. Washing twice for 1.5 min in 150 µl 0.5× SSC, 0.1% SDS.
4. Washing once for 1.5 min in 150 µl 0.1× SSC, 0.1% SDS.
5. Elution of probes with 10 µl formamide (Applied Biosystems) for 20 min at 37 °C.

The eluates were analysed by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). To calibrate the separation of the detection probes by size, GeneScan-120LIZ size standard (Applied Biosystems) was added to each sample. The identity of the probes was determined by the migration speed and the quantity by the peak area.

Quantification of total polyA RNA in lysates was performed with the above TRAC protocol without addition of detection probes. The final elution of polyA RNA was performed with 50 µl DMPC-treated water. The RNA concentration in the eluate was quantified with a RiboGreen RNA quantitation kit (Promega).

To minimize non-biological variation in the TRAC assay, the signal intensities measured for the target genes were normalized between samples, using the signal measured for the internal standard *E. coli* traT RNA. The resulting signal intensities were related to the total polyA RNA level in the respective samples. The relative expression level of each gene is presented as a percentage of the maximum level observed for that gene during the fermentation. Recovery of probes through the whole hybridization and affinity capture procedure is probe–target pair-dependent and absolute calibration was not made for most the probes used here. Therefore, absolute signal levels between different genes cannot be exactly compared quantitatively. However, signal strength is strongly correlated to the absolute level of each mRNA, so we assigned the genes into three classes, high-, moderate- and low-expression, based on the maximum signal strength observed for each gene. We arbitrarily set the fluorescence signal limits as: <900, low expression; 900–15 000, moderate expression; and >15 000, high expression. This classification is presented in the Supplementary Material.

Synthesis of internal standard mRNA

E. coli traT RNA was used as an internal standard in the hybridizations. The following PCR primers (Metabion, Martinsried, Germany) were used to synthesize, from *E*. coli DNA, a template containing the T7 promoter sequence and a 25 nt long T tail: 5′ CTAATACGACTCACTATAGGGAGA-ATGAAAAAATTGATGATGGT and 3′ TTTTTT-TTTTTTTTTTTTTTTTTT-CAGAGTGCGATT-GATTTGGC. The traT RNA was transcribed *in vitro* by T7-RNA polymerase from this template, using the MEGAscript transcription kit (Ambion, Austin, TX, USA) as recommended by the manufacturer. The synthesized traT RNA was quantified by Agilent Bioanalyser and RiboGreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands) as recommended by the manufacturer.

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Oligonucleotide probes

The biotinylated Oligo(dT) capture probe was from Promega. The detection probe oligonucleotides, labelled at the 3′- and 5′-ends with 6-FAM or at the 5′-end with NED, were synthesized by Thermo Electron (Ulm, Germany), Metabion or Applied Biosystems (Table 1). The HPLC-purified oligonucleotide detection probes were organized into pools according to their migration in capillary electrophoresis. Oligonucleotide probes were designed using mathematical algorithms presented previously (Kivioja, 2004; Kivioja *et al*., 2002). The criteria used in probe selection were the following: melting temperature (T_m), limits 60–70 °C; GC% limits, 38–62; maximum free energy change in hybridization (ΔG_H), −15 kcal/mol (Le Novere, 2001); and minimum target energy change (Ac), −10 kcal/mol (Luebke *et al*., 2003). A maximum repeat size of 15 nt and a maximum similarity of 80% were used as probe specificity criteria. T_m values were calculated using the nearest-neighbour method according to Le Novére (2001), using 10 nM nucleic acid and 750 mM salt concentrations.

Results

We used the TRAC method frequently to measure the expression of >70 selected yeast genes throughout two VHG fermentations lasting 9–10 days. Most of the oligonucleotide probes binding to the target genes of interest were designed using the *S. cerevisiae* genome database. However, lager brewing yeast, *S. pastorianus* (*S. carlsbergenesis*), is a hybrid of the *S. cerevisiae* and *S. bayanus* genomes (Casaregola *et al*., 2001; Naumova *et al*., 2005). To evaluate the specificity of the measured signals, we aligned each *S. cerevisiae* probe sequence used in these studies against each predicted ORF or contiguous sequence in the *S. bayanus* genome (Kellis *et al*., 2003) according to the Needleman and Wunch (1970) algorithm. The sequence difference between the best alignments was defined as number of mismatches (MM) listed in Table 1. Of 70 *S. cerevisiae* probes, only 11 had <3 mismatches to the most similar sequence in *S. bayanus* (*ADH1, ADH3, ALD6, COX5A, ELO1, GPD1, HEM13, HXK1, ILV5, PMA1* and *RPL5*). In these cases, the *S. bayanus* version of the target transcript may contribute significantly to the observed signal.

Two VHG fermentations (I and II) were run under almost identical conditions, using two independent lots of yeast cropped in a brewery at a 7 month interval and aerated in the laboratory prior to pitching (see Materials and methods). All TRAC analyses were performed in duplicate. On average, the duplicate measurements varied from their mean by 7.5% and more than 70% of the parallel analyses showed <10% variation. The measured expression levels and reproducibility of the measurements are shown in the Supplementary Material for all the genes listed in Tables 1 and 2.

Fermentation II was slower than fermentation I and the peak yeast concentration was smaller (Figure 1; the apparent extract shown in (B) is a measure of the remaining carbohydrate, of which the fermentable sugars are shown in Figure 2A). Presumably the differences reflect variations at the brewery or unintended differences in laboratory handling. Total polyA RNA concentrations in the cell lysates were similar during the two fermentations. Gene expression profiles are shown for only one or the other fermentation. Profiles for the duplicate fermentation are given in the Supplementary Material. Gene expression features described below were reproducibly observed in both fermentations, with the few exceptions mentioned.

Expression of genes involved in maltose metabolism and glucose fermentation

Maltose typically accounts for about 60% of the fermentable sugar in brewers' wort (63% in our worts). Under laboratory conditions, glucose represses many genes, including those involved in the utilization of alternative carbon sources, such as maltose (Entian and Shüller, 1997). The expression of genes involved in maltose metabolism, glucose phosphorylation and the metabolism of acetaldehyde and ethanol were compared with the concentrations of glucose, maltose and maltotriose during fermentation (Figure 2). The *MALx2* and *MALx1* probes used bind, respectively, to the maltase or transporter genes at all *MAL1-4* and *MAL6* loci. Strong, coordinate increases in expression of *MALx2, MALx1* and *MTT1* (a *S. bayanus* gene encoding a maltotriose-preferring α-glucoside transporter, and also known as *MTY1*; Dietvorst *et al*., 2005; Salema-Oom *et al*., 2005)) began by 6 h after pitching, when the glucose concentration had hardly decreased. The expression of *MALx1,*

Table 1. TRAC probes for *S. cerevisiase* genes

Probe name	System. name	Function	Probe length (nt)	Probe sequence	MM[1]	L[2]
Maltose metabolism						
MAL13	YGR288W	Activator at MAL1 locus	39	TTGGTATTTGCCCATTGTTAGCGTTCAATGAAAAGTTGC	6	FAM
MALx1	YBR298C	Maltose transporter	33	GGTTTCTGGTAAATCGACAACAGCCCAAGCTAA	8	FAM
MALx2	YBR299W	Maltase	25	ACCGGGCTTGATCGTGATTCTCGAT	4	FAM
MAL33	YBR297W	Activator at MAL3 locus	35	AAGCTGTTGCTCACGAAATGATAGTGATTCGTATG	9	FAM
Glucose fermentation						
ADH1	YOL086C	Alcohol dehydrogenase	25	GCAAGGTAGACAAGCCGACAACCTT	1	FAM
ADH2	YMR303C	Glucose-repressible alcohol dehydrogenase	27	TGGTAGCCTTAACGACTGCGCTAACAA	4	FAM
ADH3	YMR083W	Mitochondrial alcohol dehydrogenase isozyme III	27	TTACTAGTATCGACGACGTATCTACCC	2	FAM
ADH4	YGL256W	Alcohol dehydrogenase type IV	27	TGTCTTTACCGTCTTTGTATGCAGCGA	6	FAM
ALD6	YPL061W	Cytosolic aldehyde dehydrogenase, acetate biosynthesis	39	GACAGCTTTTACTTCAGTGTATGCATGGTAGACTTCTTC	1	FAM
GLK1	YCL040W	Glucokinase	33	GTTGACAGTTTCTGGTCAATTACGACGTCATAC	5	FAM
HXK1	YFR053C	Hexokinase isoenzyme 1	31	TGGAGATTGTTCGTCGACAGCAACATCGTAC	2	FAM
HXK2	YGL253W	Hexokinase isoenzyme 2	27	TGATTGGGTAGTCGTCTAGTGAGGTTT	6	FAM
YAK1	YJL141C	Serine-threonine protein kinase	35	TTTGTTATGAAGGGGTGTAGCATAGCTTGTTGTGG	5	FAM
Growth & protein production						
RPL22A	YLR061W	Protein component of the large (60S) ribosomal subunit	37	CCAGAGAACTTGGCAGTGGAAACAACAGTAACAACGG	4	FAM
RPL5	YPL131W	Protein component of the large (60S) ribosomal subunit	33	GCCACCAAAGATGTAAGATCTCAATAATTCTGG	2	FAM
SMY2	YBR172C	Unknown function, involved in cytoskeleton biogenesis	43	CTTCCTTTTGAGTTCTCTGTTCCGTTTCTCTTGTTCATCCAAT	10	FAM
RAS1	YOR101W	Ras proto-oncogene homolog, G-protein signaling	31	GGCTCAGCAGACTGTTTACTTCTCGAATTTA	6	FAM
RAS2	YNL098C	GTP-binding protein	37	GGAGTTGTCATTTTCCGTCAAAGTCTTGTTGTACTTG	10	FAM
Flocculation						
FLO1	YAR050W	Lectin-like protein involved in flocculation, cell wall protein	31	CAAGCCATTGGTTCCAGTGACAGTAGACATT	3	FAM
FLO11	YIR019C	GPI-anchored cell surface glycoprotein	37	AATCAAAGTGGTTATGTAAGTGGTTGGGATGGACTTG	7	FAM
FLO8	YER109C	Transcription factor (required for flocculation)	35	TAACTTTACCCTTCGCTTTTGAGGTTGCAGATCTA	8	FAM
Cell cycle-regulated genes						
HTB2	YBL002W	Histone 2 B, peaks in S-phase	39	CTAGCAGAAATAGTGGATTTCTTGTTATAAGCGGCCAAT	7	FAM
PMA1	YGL008C	Plasma membrane H + ATPase, peaks in S-phase	29	GTTGGTACCCAAACCCAATTGTCTACAAG	1	FAM
PMA2	YPL036W	Plasma membrane H + ATPase, peaks in S-phase	39	TAAACCCAGGAAAATCTCCAAATGTAAGGATAGGGCAAT	7	NED
CLN2	YPL256C	G1 cyclin controls cell cycle	27	GAGAGTGAGCTCATGTTTCTCATTGGA	4	FAM
PIR1	YKL164C	Glycoprotein needed for cell wall stability	29	AATTGGAGCTGGAGCTGGAGATGGTATAA	5	NED

Table 1. Continued

Probe name	System. name	Function	Probe length (nt)	Probe sequence	MM[1]	L[2]
Cell cycle-regulated genes						
WHI3	YNL197C	RNA binding protein, peaks in G2	33	CGATGCTGCAGAGGGTAAAAAGAACGAAGAACC	4	FAM
ASH1	YKL185W	Zinc-finger inhibitor of HO transcription	31	TGGTACGATCAAATGATTCCTTAGACGGGGA	5	FAM
Lipid metabolism						
ATF1	YOR377W	Alcohol acetyl transferase I	31	TAAAGTCTATCACCTTTTCGATCGGTTCTGG	5	FAM
ATF2	YGR177C	Alcohol acetyl transferase II	31	AAGAGTTGGCAAGAAGGCTGTCTATATTCTT	8	FAM
ELO1	YJL196C	Elongation medium chain fatty acids	41	CAATACATAAGAACGTGAACAGCTAAGTTTAAGGTGACAGG	9	FAM
ELO2	YCR034W	Elongation C16 and C18 to C24	43	CTTTTTCTTCTCTGTTGAGGTTTTGGTGATGGAGATGGAGTAG	2	NED
ERG10	YPL028W	Acetoacetyl-CoA thiolase	27	CTCGTCCTTCGTGACTTGAGTATCAGG	5	FAM
ERG13	YML126C	HMG-CoA synthase	39	TGTGACCAATTTTACAGGTTGGAACATGGAAAACGTTGTA	5	FAM
ERG3	YLR056W	C-5 sterol desaturase	33	TGCAAAAACCCGTCTACAGGATGGAAAGAATGA	5	FAM
HMG1	YML075C	3-Hydroxy-3-MethylGlutaryl-coenzyme a reductase	43	ACCATATCCTCAATGCGTTGGTAGGACTTAATGGGTTTGTAAT	11	FAM
OLE1	YGL055W	Fatty acid desaturase	35	AGAGATATAACCAGATACGTCGTGAACAATACCAG	6	FAM
PDR16	YNL231C	Phosphatidylinositol transfer protein	45	AACCTCTTAAAATAATGATCTCTTTTTTCACGCGCGGTTTCCACC	9	FAM
Glycerol metabolism						
GPD1	YDL022W	NAD-dependent glycerol-3-phosphate dehydrogenase	41	CTTCATTGGGTAGTTGTTGTAAACGATTTGGTATACGGCTT	2	FAM
GPD2	YOL059W	NAD-dependent glycerol 3-phosphate dehydrogenase	27	CTATCTGGTAGACTGCCTCGAATAATG	3	FAM
GPP2	YER062C	DL-Glycerol-3-phosphatase involved in glycerol biosynthesis	25	GATATCCTAAGCCATTCCTGCCCTT	4	FAM
GUT1	YHL032C	Glycerol kinase	41	AACTCGTTGTCGTACTTTAAAGTGGAGAGGTTCATAAATCC	9	FAM
Stress						
AAD6	YFL056C	Aryl-alcohol dehydrogenase, oxidative stress response	29	CCACCAACGTCGTATTTCTTATAGTCAGT	6	FAM
GPX2	YBR244W	Glutathione peroxidase activity, oxidative stress	33	ACTTAACAGGCTTTGGATTTCTTGGTCCAAGGA	6	FAM
HSP104	YLL026W	Hsp, reactivate denatured proteins, heat, ethanol stress	45	TTCAGATTCTGACAACTTCTTAACAGGGATACCAGTCAATCTTGC	3	FAM
MCA1	YOR197W	Cysteine protease, involved in apoptosis	25	ATGGAAGATCCAACACTGTACCCGA	4	FAM
MSN2	YMR037C	Transcriptional activator in stress conditions	43	ATAGTAGTGGTATTCGGACTTGCATTGGAACTTGAGGTAGAAT	10	FAM
TPS1	YBR126C	Trehalose-6-phosphate synthase	39	TCTTCTTGGCAAGCAATATATTCGTAGGAAACCAAGTTC	5	FAM
YAP1	YML007W	bZip transcription factor required for oxidative stress tolerance	29	ACTCGGATATGAATGGGAGTACATCATCG	4	FAM

Table 1. TRAC probes for *S. cerevisiae* genes

Probe name	System. name	Function	Probe length (nt)	Probe sequence	MM[1]	L[2]
VDK, amino acid metabolism						
ARG1	YOL058W	Arginosuccinate synthetase	35	GGATTCTGTCCGGATCGTACAACTTTTCAGTCTTTG	5	FAM
BAT1	YHR208W	Mitochondrial branched-chain amino acid aminotransferase	41	AGTGATGTTCTTTTCTGGGCCGAACAACCATAGATTTTTGTT	6	FAM
BAT2	YJR148W	Cytosolic branched-chain amino acid aminotransferase	43	TAGTGGAGCAGTAACTAGTTCCTTCTTGCCCGTTTTACTATCT	10	FAM
LEU1	YGL009C	Isopropylmalate isomerase	41	CGCCATTCATAAAACAACCCTTTTTTCAAACCCGTTCTCTT	5	FAM
ILV2	YMR108W	Acetolactate synthase	37	TTGGCAACCTTGGATAGTTTCTTTATAACCGTCTGTG	6	FAM
ILV6	YCL009C	Regulatory subunit of acetolactate synthase	25	GATGTCGACGACACGACCTCCAAAG	3	NED
ILV3	YJR016C	Dihydroxyacid dehydrates	27	GGCTTAATAATCTCTTGTCCTTCAGGT	6	FAM
ILV5	YLR355C	Acetohydroxyacid reductase	39	TGGTTTTCTGGTCTCAACTTTCTGACTTCCTTACCAACC	2	FAM
TKL1	YPR074C	Transketolase, synthesis of aromatic amino acids	29	TGGTAGAGAAACAACACGAGCCTTGATGT	3	FAM
TKL2	YBR117C	Transketolase, synthesis of aromatic amino acids	31	AGTAATTGATTGTCTTTTCAGCCCTTGACGC	5	FAM
PUT1	YLR142W	Proline oxidase, glutamate biosynthesis	39	CTTGATTATGTTTTTAGCGCCATGGTTGGTAATTAGGTC	7	FAM
(An)aerobic metabolism						
CCP1	YKR066C	Cytochrome *c* peroxidase	43	TGAAAAAATGTTCTGACATAGTCAGCGTCTTTATCAGCGTCAG	10	FAM
COX5A	YNL052W	Cytochrome *c* oxidase, aerobic	45	CACCCCAAGGATTAGCATTCTTCGACTTCAAATATTCGTCACTCT	2	FAM
COX5B	YIL111W	Cytochrome *c* oxidase, anaerobic	43	GGATTGGCTAATAGTCTCACTAAACCAAAGAGCCCAAATGAGA	11	FAM
CYC7	YEL039C	Cytochrome *c* isoform 2	37	CTATCCTCATCCCATTTGACGTTCTTGTTGATGTTTG	4	FAM
HEM13	YDR044W	Coproporphyrinogen III oxidase, anaerobic induction	39	CTTCAATATTTCTTGTGGGTCACGTTCATCATAATCGTC	2	FAM
ROX1	YPR065W	Transcription repressor	25	AAGGGTCGTGTGGAGCTGAGTAATA	5	FAM
Sulphur metabolism metabolism						
MET1	YKR069W	Component of sulphite reductase	33	TGAAGTCTCGAAATCTTCCAACTTGATGTCACT	6	FAM
MET16	YPR167C	PAPS reductase (giving sulphite) peaks in S phase	37	GTAAACACAGCACTTATATGTAGCTCTTTGTAGGCAC	6	NED
SAM1	YLR180W	SAM synthase	35	CCAACATGTCTCTTGGGATGACTTTTTCAATGATC	8	FAM
SUL1	YBR294W	High-affinity sulphate transporter	45	AAGAAGGCGTCAGTTAAACAATAAAGTGCTAATAGAACGCAACCG	7	FAM
SUL2	YLR092W	High-affinity sulphate transporter	35	TTCTTTCAAACCGGATGGAACTGAGCCTAGGATGC	3	FAM

[1] MM shows the number of base differences between *S. cerevisiae* probe sequence and the most similar sequence in the *S. bayanus* genome (Kellis *et al.*, 2003).
[2] L shows the fluorescent label, FAM or NED attached to the probe.

Table 2. TRAC probes for *S. bayanus* genes

Probe name	Function	Probe length (nt)	Probe sequence	MM[1]	L[2]
MTT1	Maltotriose transporter	39	ACTGTTTGTATAGCCAATCCAAATGCGTAAAGGTCA	10	NED
ADH1	Alcohol dehydrogenase I	27	TTGGCCGGCAGACAAGTTAGCGGAC	6	NED
MALx1	Maltose transporter	31	CAGCGACCACAATATTTCCTATGTTGTAC	7	NED
ERG3	C-5 sterol desaturase	35	GCGCTCAGTTATTTTTCTTGGTGTTTGGATC	8	NED
ILV5	Acetohydroxyacid reductase	37	ATGACGTAGTTACCCTTCTTGATGGCATCTTCGA	8	NED
ATF1	Alcohol acetyl transferase	27	ATGCTTCTTGTCATTTATCGCCCCT	7	NED

[1] MM shows the number of base differences between *S. bayanus* probe sequence and the most similar sequence in the *S. cerevisiae* genome.
[2] L shows the fluorescent label, FAM or NED, attached to the probe.

MALx2 and *MTT1* genes was highest during a short period between 20 and 33 h, while glucose was still present at 4–15 g/l and consumption of maltose and maltotriose was still slow (Figure 2A, B). The mRNA levels of these genes began to decrease when maltose and maltotriose started to decrease. When approximately 50% of the fermentable sugars had been consumed, the expression levels of *MALx1, MALx2* and *MTT1* were below 25% of their maximum levels and remained low to the end of the fermentation. We also measured expression of the *AGT1* gene (for a maltose/maltotriose transporter), which is known to be defective in this and other lager strains (Vidgren *et al*., 2005). *AGT1* absolute signal strengths were very low and within experimental error changed little during the fermentation (data not shown).

The same pattern of *MALx1* expression was also observed (with measurements at less frequent intervals) with another industrial lager strain, A63015, in a similar (25 °P) VHG fermentation (Figure 2Bi). In this case, maltose transport activity was measured in yeast samples taken from the fermentation. Transport activity peaked at about the same time (25 h) as *MALx1* expression, but remained at about 30% of its maximum value through the rest of the fermentation, whereas *MALx1* expression fell to very low levels.

HXK2 encodes the dominant hexokinase isoenzyme during growth on glucose and is a major mediator of glucose repression (Entian, 1980). Hxp2p mediates repression of *HXK1* and *GLK1* (glucokinase) (Rodriguez *et al*., 2001). Consistent with this, *HXK2* expression increased rapidly during the first 4 h, while expression of *HXK1* and *GLK1* strongly decreased. Maximal *HXK2* expression was measured at about 25 h, while glucose was above 10 g/l (Figure 2A, C). Expression of

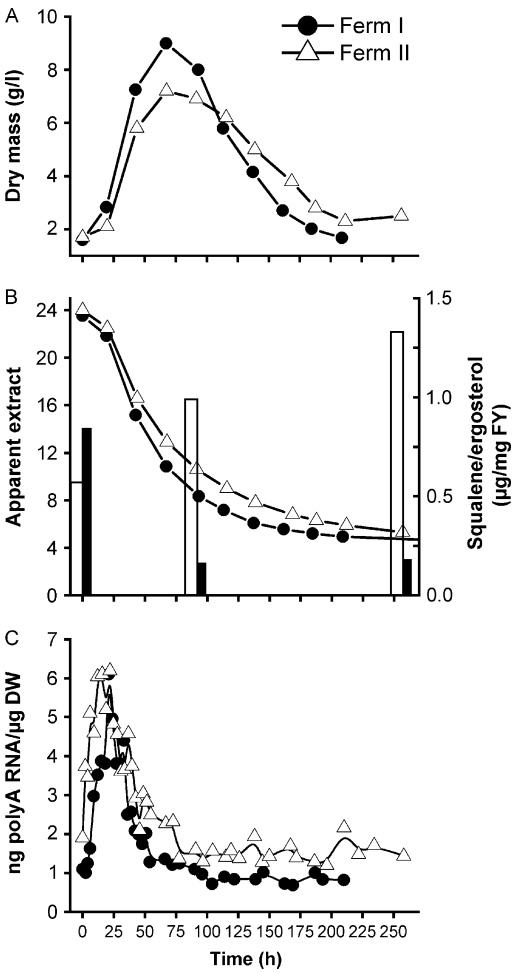

Figure 1. Comparison of dry mass (g/l) (A), apparent attenuation (B) and polyA RNA concentrations in cell lysates (ng/µg) (C) during VHG brewing fermentations I and II. In (B) black bars show the concentration of ergosterol and white bars the concentration of squalene in fresh yeast collected from fermentation II. The smoothed curves in this and other figures were produced by B-spline function

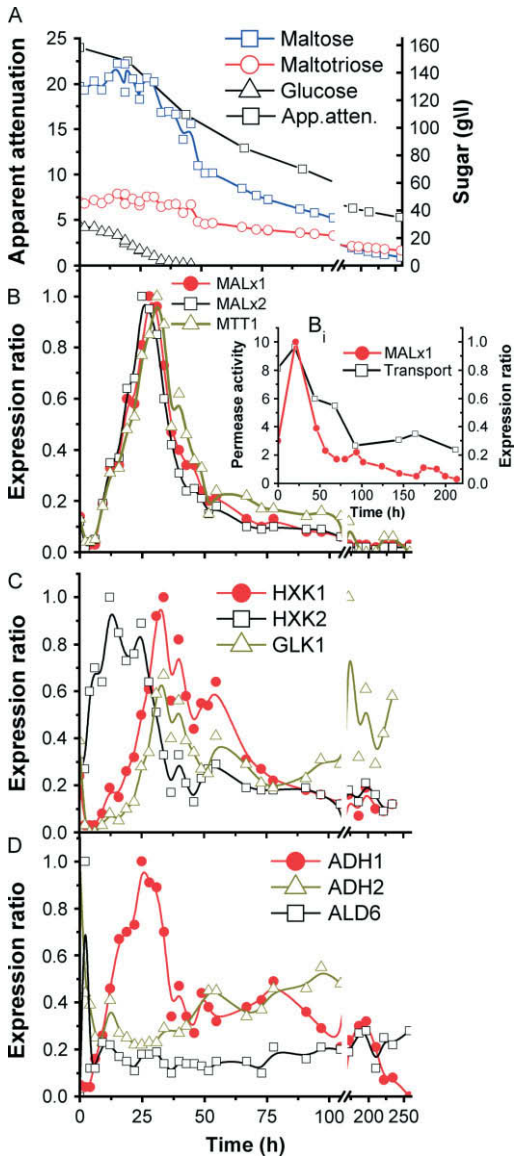

Figure 2. Expression of maltose metabolism- and glucose fermentation-related genes through (A–D) VHG fermentation II and (inset Bi) a similar VHG fermentation using/lager strain A63015. In this and other figures, for each gene the expression ratio shows the measured expression level as a fraction of the maximum expression level found for that gene during the fermentation. (A) Apparent attenuation and concentrations of maltose, glucose and maltotriose. (B) mRNA levels of maltose (*MALx1*) and maltotriose (*MTT1*) transporters and maltase (*MALx2*). (Inset Bi) *MALx1* expression and maltose permease activity (μmol/min/g dry mass) for strain A63015. (C) mRNA levels of glucose phosphorylation genes (*HXK1, HXK2, GLK1*). (D) mRNA levels of alcohol dehydrogenase (*ADH1, ADH2*) and aldehyde dehydrogenase (*ALD6*) genes

HXK1 and *GLK1* started to increase at 9 h (glucose >25 g/l), followed by strong increases after 21 h (glucose ≤15 g/l). As a result, all three glucose phosphorylation genes were strongly expressed at about 30 h and 7 g glucose/l. There was an interesting second upregulation of *GLK1* very late in the fermentation, at about 100 h. Hxk1p and Glk1p are thus probably the major enzymes phosphorylating glucose during the second half of fermentation.

ALD6 mRNA, coding for an aldehyde dehydrogenase that physiologically produces acetate, increased four-fold 2 h after pitching, and abruptly returned to its initial level in the next 2 h. This dramatic spike was reproducibly observed (see Supplementary Figure 1). Simultaneously, the *ADH2* gene coding for the glucose-repressible alcohol dehydrogenase II (involved in ethanol oxidation) was downregulated by four-fold. Expression of the fermentative alcohol dehydrogenase I (*ADH1*) was low in pitching yeast and increased six-fold after 4 h, reaching a peak at 25 h, when *ALD6* and *ADH2* were minimally expressed. *ADH2* mRNA showed a moderate increase (< two-fold) between 25 and 50 h (when glucose was <4 g/l), whereas expression of *ADH1* decreased three-fold in this period. The expression profiles of *ADH3* and *ADH4* resembled that of *ADH1* (data not shown).

Expression of genes related to growth and flocculation

Flocculation of yeast towards the end of brewery fermentations provides the crop of yeast traditionally used for the next fermentation and initiates clarification of the beer. Premature flocculation is a serious problem that leads to slow and incomplete fermentation. (Verstrepen *et al.*, 2003). Three flocculation-related genes (*FLO1, FLO8* and *FLO11*) were followed in cells collected from the suspension (i.e. cells that had not yet flocculated and sedimented). *FLO8* (coding a transcription factor required for flocculation) and *FLO1* (coding a mannose-binding cell wall protein) showed similar and fairly constant expression profiles throughout the fermentations (Figure 3B). Expression of the cell surface glycoprotein gene *FLO11* followed the profile of the yeast dry mass curve; *FLO11* mRNA sharply increased after ca. 20 h, simultaneously with exponential growth, while decreasing *FLO11* expression was observed (in yeast still in suspension) after the yeast started to sediment.

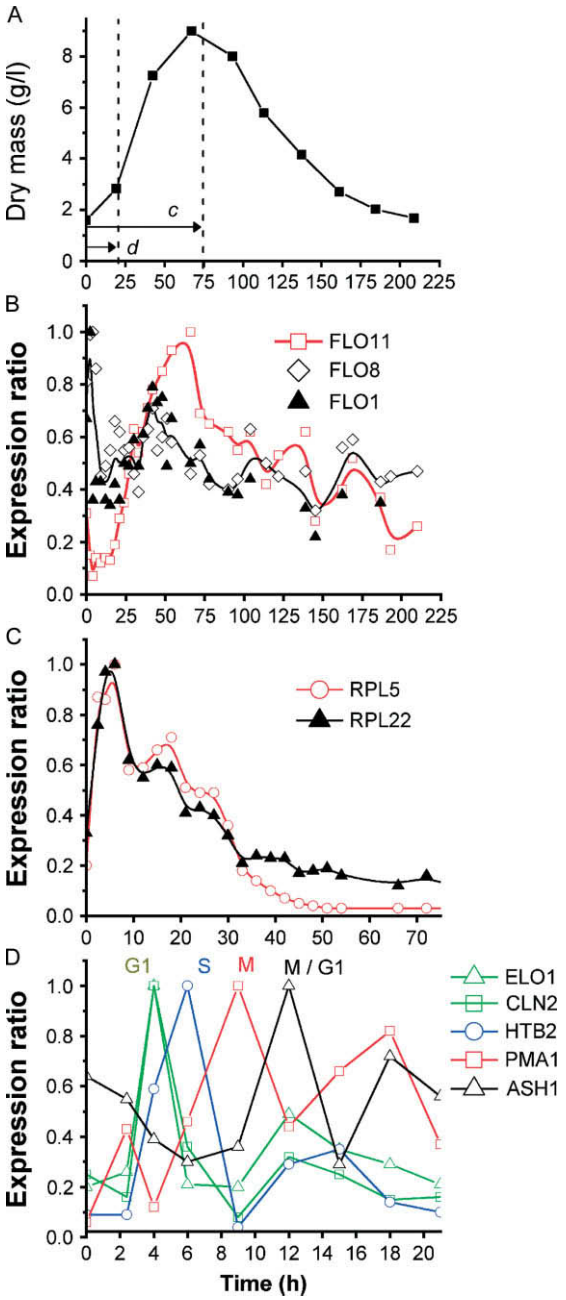

Figure 3. Expression of growth, flocculation and cell-cycle related genes during VHG fermentation I. (A) Dry mass of yeast in suspension. The intervals *c* and *d* correspond to the time spans in (C) and (D). (B) Flocculation-related mRNAs *FLO1, FLO8* (average shown by black line) and *FLO11* thoughout the VHG fermentation. (C) Two ribosomal protein mRNAs, *RPL5* and *RPL22A*, during the first 75 h. (D) Expression of cell cycle-dependent genes *ELO, CLN2* (G_1), *HTB2* (S), *PMA1* (M) and *ASH1* (M–G_1) during the first 22 h of the VHG fermentation

Expression of mRNAs for ribosomal proteins is growth rate-dependent (Ju and Warner, 1994). The two ribosomal protein mRNAs measured here (*RPL5* and *RPL22A*) increased three- to eight-fold during the 6–9 h after pitching, and reached peak levels before the yeast started to grow rapidly. These inductions were followed by rapid decreases and by 45 h (when the yeast was still growing) expression levels of *RPL5* and *RPL22A* had fallen to 5% and 20%, respectively, of their maximum levels (Figure 3C), and remained so for the rest of the fermentation (not shown).

Expression of cell cycle-dependent genes was used to evaluate possible synchrony of cell division. Five genes (*ELO1, CLN2, HTB2, PMA1* and *ASH1*) with strongly cell cycle-dependent expression (Spellman *et al.*, 1998) showed sharp expression peaks during the first 15 h after pitching (Figure 3D). The *ELO1* and *CLN2* genes, which are induced in G_1 phase, peaked 4 h after pitching. The histone gene, *HTB2* (induced in S phase), peaked at 6 h. Peaks in the expression of *PMA1* and *ASH1* at 9 and 12 h indicated that most cells reached M phase and the M–G_1 boundary at these times. Smaller, second peaks in the expression of these genes were observed 6–9 h after the first peaks. Ash1p is a transcriptional activator of *FLO11* (Pan and Heitman, 2000) and the second increase in *ASH1* mRNA (at 18 h) coincided with increasing *FLO11* expression (Figure 3B, D).

Expression of amino acid biosynthesis genes

The parallel pathways for biosynthesis of isoleucine and valine share several enzymes and are important for the development of beer flavour. *ILV2* and *ILV5*, respectively, code the enzymes making and consuming the two α-aceto-α-hydroxy acids from which the vicinal diketones, diacetyl and 2,3-pentadione, are derived by non-enzymatic, oxidative decarboxylation (Inoue *et al.*, 1968). Diacetyl, especially, causes a buttery off-flavour, not desired in lager beers. The α-keto acid precursors of isoleucine and valine can be formed either *de novo* or from the corresponding, wort-derived amino acids, by reactions catalysed by transaminases coded by *BAT1* and *BAT2*. These α-ketoacids are the sources of secondary alcohols and esters with beneficial flavours (Dickinson *et al.*, 1997). We therefore measured the expression of six genes in this isoleucine–valine biosynthesis pathway. *ILV2,*

ILV3, ILV5, BAT1, BAT2 and *LEU1* all showed a similar trend throughout the VHG fermentations (Figure 4). In particular, there was no evidence for a relative change in the expression of *ILV2* and *ILV5* that might promote accumulation of the α-aceto-α-hydroxy acid precursors of VDKs. Expression was highest during the first 15 h, i.e. before the yeast started to grow. By 40 h, when the yeast was growing rapidly, expression had fallen to roughly 30% of the maximal values.

Transketolases coded by *TKL1* and *TKL2* are involved in the production of erythrose 4-phosphate, required for synthesis of aromatic amino acids (Phe, Tyr and Trp) and nicotinamide coenzymes. These aromatic amino acids are precursors of flavour compounds in beer, such as tyrosol and tryptophol. Both genes showed complex variations in expression levels throughout the fermentations and there were some differences in detail between fermentations I and II (cf. Figure 4B and Supplementary Figure 3B). In fermentation I, *TKL1* and *TKL2* both showed sharp peaks at 2–4 h, followed by moderate expression during yeast growth. *TKL1* was then strongly downregulated, while *TKL2* continued to be expressed at about 40% of its maximal level. In fermentation II, the 2–4 h peak was less marked and *TKL1*, as well as *TKL2*, continued to be moderately expressed throughout the fermentation.

Proline is an alternative source of nitrogen for yeast that is utilized when preferred nitrogen sources, such as ammonia, glutamine and asparagine, are no longer available (Perpéte *et al*., 2005). Proline is converted to glutamate by proline oxidase (Put1p) and Δ1-pyrroline-5-carboxylate dehydrogenase (Put2p). *PUT1* was the only amino acid biosynthesis gene in the analysed set that showed increasing transcript level towards the end of the fermentations (Figure 4C), possibly as a response to nitrogen limitation (Boer *et al*., 2003).

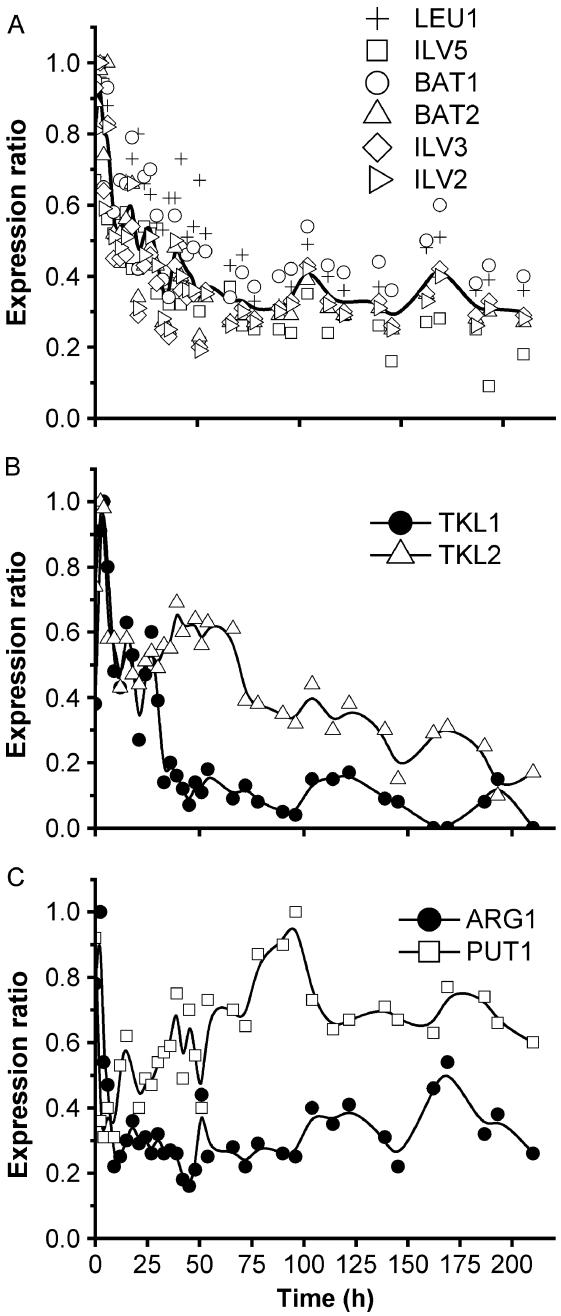

Figure 4. Expression of genes related to amino acid metabolism during VHG fermentation I. (A) Six isoleucine–leucine pathway genes, *BAT1–2, ILV2–3, ILV5* and *LEU1*. (B) Transketolase genes *TKL1–2*. (C) Arginine (*ARG1*) and glutamate (*PUT1*) biosynthesis genes

Expression of genes involved in stress responses

We selected several genes known to be involved in responses to some of the stresses yeast meets in VHG fermentations. Hsp104p and trehalose have a role in acquisition of tolerance to a variety of stresses, such as heat, ethanol, osmotic and oxidative stress (Elbein *et al*., 2003; Sanchez *et al*., 1992). Both *HSP104* and *TPS1* (trehalose 6-phosphate synthase) have stress responsive elements (STRE) in their promoters and are induced by the stress-related transcription factor, Msn2p (Boy-Marcotte *et al*., 1999). These genes showed a similar expression pattern throughout most of

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the fermentation (Figure 5A), only differing at the very start and in very late stages. Immediately after pitching, *TPS1* mRNA was increased by two-fold, while *HSP104* expression was unaffected. The observed increase in *TPS1* mRNA level could be due to osmotic stress. This is supported by the observation that the glycerol biosynthesis genes, *GPD1* and *GPP2*, showed a similar induction as *TPS1* during the first 2–6 h of fermentation (Figure 5B). These three glycerol and trehalose synthesis genes are induced by hyperosmotic stress (Gasch *et al*., 2000). In contrast, *GPD2*, which is not involved in the hyperosmotic response but in anaerobic regeneration of NAD (Ansell *et al*., 1997), was not induced immediately after pitching (data not shown).

About 20–50 h after the start of fermentation, while the glucose concentration fell to 0 g/l (based on fermentation II), *HSP104, TPS1* and *GPD1* mRNA levels increased seven-, three- and two-fold, respectively, and then returned to their inital levels. The expression of these genes, as well as *TKL2*, has been shown to increase in batch cultures on glucose during the diauxic shift, when metabolism changes to aerobic growth on ethanol (DeRisi *et al*., 1997). Possibly the responses of these genes observed here (Figures 4B, 5A) are also related to exhaustion of glucose, but to a shift to utilization of di- and trisaccharides rather than ethanol.

Unexpectedly, two oxidative stress-related genes, *YAP1* and *AAD6*, which are considered to be upregulated in response to accumulation of reactive oxygen species (ROS) (Delneri *et al*., 1999; Kuge *et al*., 1997), showed increased expression towards the end of fermentations (Figure 5C), despite the very anaerobic conditions expected at this stage. *YAP1* showed also double peaks of induction at 24 and 42 h (*AAD6* shared the 24 h peak in fermentation I but not in fermentation II).

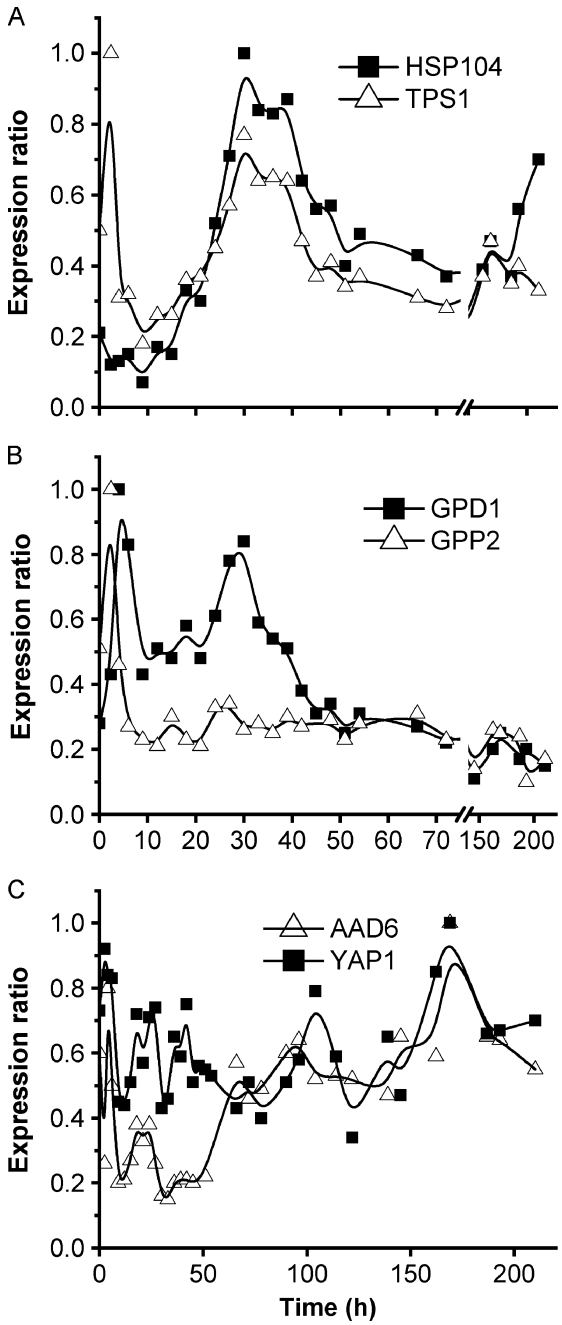

Figure 5. Expression of genes involved in stress responses during VHG fermentation I. (A) *HSP104* and *TPS1* (general stress response). (B) Glycerol biosynthesis genes *GPD1* and *GPP2* (osmotic stress). (C) *AAD6* and *YAP1* (oxidative stress)

Expression of genes sensitive to oxygen

Expression levels of several oxygen-sensitive genes are shown in Figure 6. Expression of *ROX1* is induced by the oxygen-dependent synthesis of haem, which triggers the release of free Hap1, which in turn activates *ROX1*. Rox1p is a repressor of several hypoxic or anaerobic genes (ter Linde and Steensma, 2002). As expected, the level of *ROX1* mRNA fell steeply during the first few hours and this transcript disappeared after 9 h in fermentation 1 (Figure 6) or 5 h in fermentation 2 (Supplementary Figure 5), by which time oxygen

in the wort is expected to be very low. Unexpectedly, *ROX1* mRNA reappeared late in fermentation, and reached maximum levels at 175 h (fermentation 1) or 200 h (fermentation 2), when conditions are expected to be strictly anaerobic. Because fermentable sugars were nearly used up and CO_2 production relatively slow at this time, we considered that small amounts of air might enter the tops of the tall tubes when samples (ca. 30 ml) were taken through the anaerobic sampling ports, and that traces of oxygen might then diffuse through the CO_2 cushion above the wort and dissolve in the wort. Although this seemed unlikely, in fermentation II the tall tubes were protected from the atmosphere by leading the exhaust gas via marprene tubing to water locks, in order to exclude possible entry of traces of air. However, this did not decrease the strong induction of *ROX1* in late fermentation (Supplementary Figure 5).

HMG1 (also activated by Hap1), *ERG10* and *COX5a* (coding the cytochrome oxidase 5A, which is predominantly expressed under aerobic conditions) showed behaviour very similar to *ROX1*. *ERG3, ERG13* and *PDR16* showed peaks about 2 h after pitching into aerated wort, followed by slower decreases than observed for *ROX1* and rises in late fermentation, which were, however, not as pronounced as for *ROX1*. Although tested genes for sterol biosynthesis (*ERG10, ERG13, HMG1* and *ERG3*) were activated in late fermentation, no ergosterol was synthesized between 90 and 250 h (Figure 1B), presumably because oxygen, needed as a reactant to convert squalene into sterols, was not available. Squalene, the last intermediate before the oxygen-requiring steps, increased from about 1.0 to 1.3 mg/g fresh yeast.

Expression of *COX5b*, coding the cytochrome oxidase 5B that predominates under anaerobic conditions, fell sharply after pitching, began to rise strongly after 6 h (when oxygen has probably disappeared), reached its maximal level at around 50 h and remained at about 30% of this maximal level through the second half of the fermentation. *HEM13*, coding the oxygen-utilizing coproporphyrinogen III oxidase, showed somewhat different behaviour in the two fermentations: in fermentation I there was an early peak, a minimum at 9 h and then a second longer peak at 25–40 h, whereas in fermentation II the early peak was not seen (Supplementary Figure 5). In

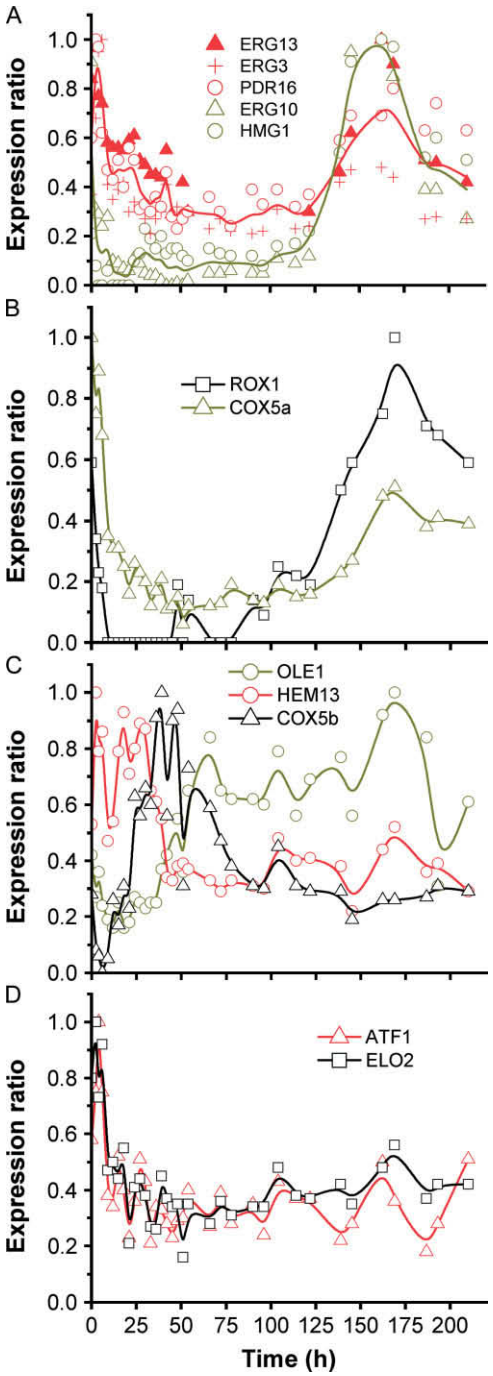

Figure 6. Expression of genes involved in sterol, fatty acid and ester biosyntheses and oxygen responses during VHG fermentation I. (A) Ergosterol biosynthesis genes, *ERG3, ERG10, ERG13* and *HMG1*, and phosphatidylinositol transfer protein *PDR16*. (B) Two oxygen-induced genes, *ROX1* and *COX5a*. (C) *OLE1, HEM13* and *COX5b*. (D) *ATF1* (acetyltransferase) and *ELO2* (fatty acid elongase)

both fermentations, expression of *OLE1*, encoding the oxygen-utilizing enzyme, fatty acid desaturase, decreased during the first few hours, and then gradually rose and maintained its highest levels during the second, anaerobic, half of the fermentation.

We also measured expression of *ELO2* (for a fatty acid elongase) and *ATF1* (for alcohol acetyltransferase), because both of these enzymes are implicated in membrane function and the alcohol acetyltransferases are involved in formation of esters that contribute importantly to beer flavour. *ATF1* is reported to be repressed by oxygen and by unsaturated fatty acids (Fujii *et al*., 1997). Both genes were most strongly expressed in the first few hours, but remained at around half of this maximal level for the rest of the fermentations (Figure 6).

Comparison of *S. cerevisiae* and *S. bayanus* genes

Expression levels discussed above were measured with probes designed to recognize genes from the *S. cerevisiae* part of the lager yeast genome (see above and Table 1) and also the *S. bayanus MTT1* gene. For five *S. cerevisiae* genes (*ADH1, ATF1, ERG3, ILV5* and *MALx1*), the corresponding *S. bayanus* sequences were looked up from the genome and probes specifically recognizing these sequences were designed (Table 2). For *MALx1, ATF1* and *ILV5*, expression profiles of the *S. cerevisiae* and *S. bayanus* components were very similar (Figure 7). For *ILV5*, the *S. cerevisiae* probe may also recognize *S. bayanus* mRNA because there were only two mismatches (Table 1), but the *S. bayanus* probe is expected to be specific (eight mismatches; Table 2). For *MALx1* and *ATF1*, the *S. cerevisiae* and *S. bayanus* probes are expected to be specific (five to eight mismatches; Tables 1 and 2). In contrast, for *ADH1* and *ERG3*, the *S. cerevisiae* and *S. bayanus* components behaved differently. After a marked peak in expression at 25 h for both components, expression of the *S. bayanus ADH1* fell to very low levels for the rest of the fermentation, whereas the *S. cerevisae ADH1* remained at about 40% of its maximum value (with some fluctuations) until the end of the fermentation (225 h). The observed decrease of *S. cerevisiae ADH1* mRNA may be an artifact, because the probe probably also recognizes mRNA of the strongly downregulated *S. bayanus ADH1* gene (one mismatch; Table 1). For *ERG3*, the situation was reversed: the *S. cerevisiae* gene showed an early peak and then low expression until the second (unexpected) peak at 220 h, whereas expression of the *S. bayanus* gene from 50 h to the end of the fermentation remained rather constant at about 60% of its maximal level.

Discussion

We used TRAC to follow the expression levels of a focused set of genes at frequent intervals throughout VHG fermentations. The genes were selected because of their apparent relevance to brewery fermentations. Many of their expression levels changed markedly and frequently, especially during the first 2 days of fermentation. This complex dynamic pattern of expression was, in general, reproducible between two fermentations carried out with industrially cropped yeast at a 7 month interval.

Total mRNA levels peaked (ca. 6 mg/g dry yeast) at about 20 h, when the yeast were just entering the rapid growth phase (although probably they had already completed one round of cell division with little increase in biomass; see below). This seems to agree with Olesen *et al*. (2002), who followed expression levels by microarray at daily intervals through an industrial (14 °P) lager fermentation and found the average expression level of all ORFs to be highest on day 2. Roughly 90% of our selected genes showed their highest expression levels at some point during the first 75 h, i.e. while yeast concentrations in suspension were still increasing. These relative expression levels record the expression of each selected gene as a proportion of the total mRNA from the about 12 000 genes of the hybrid *S. pastorianus* genome.

Several genes related to growth regulation, flocculation and the synthesis of amino acids, proteins and lipids (including *RAS1, RAS2, ALD6, FLO1, TKL1, TKL2, ILV5, RPL5, RPL22A, ERG3, ELO1, ATF1*, etc.) showed a rapid increase in expression immediately after pitching, followed by a return to original levels within 4–10 h. Another group (including genes for maltose metabolism and glucose phosphorylation) reached peak levels at 25–40 h, during the most rapid growth phase. A third group (including *BAT1, BAT2, ILV2, ILV3* and *ILV6*, involved in metabolism of branched chain amino acids and secondary alcohols and

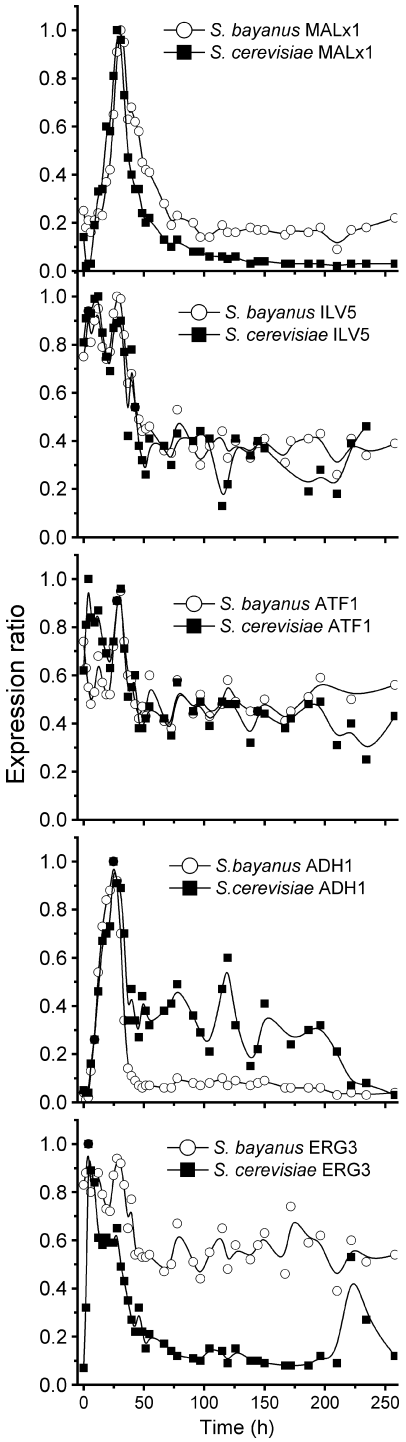

Figure 7. Expression of five *S. cerevisiae* genes and their *S. bayanus* counterparts during VHG fermentation II

vicinal diketones important to beer flavour) were already highly expressed in the pitching yeast and decreased during the growth phase. Only about 10% of genes (*PUT1, ROX1, OLE1, AAD6, GLK1, ERG10, ERG13*) in our selected set reached their highest relative expression level towards the end of the fermentation. Olesen *et al*. (2002) also found that genes for protein and lipid biosynthesis were highly expressed during the start of the fermentation, whereas genes for gluconeogenesis and the glyoxylate cycle (not measured in this study) peaked at day 4 or 5.

As Olesen *et al*. (2002) remarked, sampling at daily intervals does not permit examination of the details of gene expression. We sampled at 2–3 h intervals during the first 54 h. This revealed an ordered series of sharp expression peaks for several genes associated with phases of the cell division cycle, with (in fermentation I; Figure 3D) yeast entering the G_1, S and M phases at about 4, 6 and 9 h, respectively, and reaching the $M–G_1$ boundary at about 12 h. This suggests that the first round of cell division was strongly synchronized, which has been reported previously for wort fermentations (Hutter, 2002; Muro *et al*., 2006). Smaller and broader peaks of these cell cycle-associated genes were detected 6–9 h after the first peaks, indicating that the degree of synchrony was decreasing. In *S. cerevisiae*, expression levels of about 800 genes (13% of the genome) are cell cycle-dependent (Spellman *et al*., 1998). The rapid fluctuations in the levels of these genes cannot be observed without frequent sampling, and they include several genes with functions relevant to brewery fermentations, such as lipid metabolism.

The worts for fermentations I and II contained 120 g maltose/l and 25 g glucose/l (maltose: glucose ratio = 4.8). Strong, coordinate increases in expression of maltose metabolism genes (*MALx1, MALx2* and *MTT1*), starting when glucose was still >20 g/l (Figure 2A, B, Bi) showed that under these conditions glucose did not repress maltose metabolism by the two lager strains used. Maltose transport activity, measured for strain A63015, also increased at this time (Figure 2Bi), suggesting that glucose-triggered catabolite inactivation of maltose transporters was ineffective. This contrasts with earlier work (Rautio and Londesborough, 2003) in which the maltose transport activity of both lager and ale strains sharply decreased during the first 20 h of fermentation in a high-glucose wort (100 g maltose/l and 55 g glucose/l; maltose : glucose ratio

= 1.8). The maltose : glucose ratio is a wort characteristic that strongly influences yeast physiology. This ratio varies widely in industrial worts and can be low in high-gravity worts containing non-malt carbohydrate adjuncts. In the present work, high expression of maltose metabolism genes decreased to 20% of maximum by 50 h, when maltose and maltotriose were still abundant (90 and 35 g/l, respectively) and the cells were still growing rapidly. It has recently been shown that the COMPASS histone methylation complex represses expression of the telomerically located maltose utilization genes during the late stages of fermentations starting at high maltose concentrations (Houghton-Larsen and Brandt, 2006). Whatever may be the mechanism that turns off expression of *MALx1, MALx2* and *MTT1*, it means that maltose metabolism for the rest of the 9–10 day fermentation relies largely upon transporters and maltases synthesized during the first 50 h.

The two ribosomal protein genes assayed, as well as genes in the leucine, isoleucine/valine, arginine and aromatic amino acid synthetic pathways, reached peak expression very early (<10 h) and fell to 5–30% of these levels while the yeast were still growing (Figures 3, 4). As might be expected, frequent transcription analysis revealed strong expression of genes for some physiological functions, in this case amino acid and protein synthesis, before those functions were manifested, in this case by yeast growth. Perhaps less expected was the finding that many genes were already downregulated again by the time the physiological results of their actions were observable.

PUT1 showed increasing transcript levels towards the end of the fermentation (Figure 4), as also observed in wine fermentations (Rossignol *et al*., 2003). *PUT1* encodes proline oxidase, involved in the conversion of proline to glutamate. Proline is the most abundant amino acid in wort, but is barely used by yeast during wort fermentations, because of nitrogen catabolite repression (Perpéte *et al*., 2005). Maximum expression of *PUT1* and *PUT2* genes occurs when proline is the sole nitrogen source (Huang and Brandriss, 2000). The increased levels of *PUT1* mRNA at the end of the fermentations may indicate that preferred nitrogen sources were largely depleted. *PUT1* expression is repressed by anaerobic conditions, but this control is overridden by the presence of proline (Wang and Brandriss, 1987).

We followed the expression of two genes, *TPS1* (trehalose phosphate synthase) and *HSP104* (heat shock protein 104), as examples of genes activated by a wide range of stresses, including oxidative, hyperosmotic, heat, ethanol and starvation stresses (see e.g. Alexandre *et al*., 2001; Gasch *et al*., 2000; Sanchez *et al*., 1992). The expression of these genes coordinately increased (5–15-fold) between 10 and 30 h, possibly in response to increasing ethanol (by 30 h ethanol was about 20 g/l, compared to final values of about 90 g/l), the temperature shift from 14 °C to 18 °C, and to near-exhaustion of some essential nutrients. During fermentation of normal gravity (12 °P) worts, the amount of the stress-protectant trehalose in lager yeasts remains quite low (<1.5% of yeast dry mass; Boulton, 2000), but in 16 °P and 24 °P worts trehalose reaches 5–14% of dry mass in mid-fermentation and remains high to the end (Reinman and Londesborough, 2000). The timing of the activation of *TPS1* is consistent with subsequent production of trehalose 6-phosphate synthase and increasing synthesis of trehalose as the cells enter S phase. Peak expression of *TPS1* and *HSP104* was over before yeast growth stopped (i.e. by 40 h). Decreased expression of *HSP104* has previously been observed towards the end of beer and wine fermentations (Brosnan *et al*., 2000; Riou *et al*., 1997) and Brosnan *et al*. (2000) reported that *HSP104* cannot be induced by heat shock in cells taken from the end of fermentations. The closely coordinated responses of *HSP104* and *TSP1* during most of the fermentation are consistent with the synergism between these protective agents observed during heat shock and ethanol stress (Alexandre *et al*., 2001; Piper, 1995).

Synthesis of sterols requires oxygen as a reactant in several steps between squalene and ergosterol, the major yeast sterol. Ergosterol is an essential component of the plasma membrane. It has been shown to be involved in restoring fermentative capacity of the cells after storage (Bloch, 1983; Higgins *et al*., 2003a) and is required for proper functioning of maltose permease (Guimarães *et al*., 2006). Yeast cropped from industrial wort fermentations has grown into stationary phase after depletion of oxygen, and contains very little sterol. Conversion of squalene into ergosterol and *de novo* synthesis of sterols are vital processes when such yeast is pitched into fresh wort containing

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oxygen at the start of a new fermentation. Higgins *et al*. (2003a) performed microarray analyses of yeast collected 1 and 23 h after pitching into 12 °P wort and found a strong upregulation at 1 h of genes involved in sterol synthesis, as well as several other oxygen-sensitive genes, including *OLE1* and *ROX1*. The initial upregulation of these genes, shown in Figure 6, is in agreement with expectations. However, between about 100 and 170 h, when most sugars had been consumed, there was a second upregulation of several of these genes, including *ROX1, COX5a, ERG3, ERG10, ERG 13* and *HMG1* (Figure 6). This upregulation was not decreased by taking extra precautions (in fermentation II) to exclude oxygen from the fermentation tubes (see Results). Markedly stronger expression of several genes in the ergosterol pathway (e.g. *ERG1, ERG3* and *ERG5*) on day 8 compared to day 1 of a lager fermentation was also observed by James *et al*. (2003) in a microarray study. Higher expression of *ROX1* and *COX5a* on both day 5 and day 8 compared to day 1 was also observed by these authors (other days were not studied; see their data at: **http://www.tcd.ie/Microbiology/Staff/jthrppel/yeast/brewstress.html**). High ethanol concentrations have been shown to increase the expression of genes involved in protection against oxidative stress (Piper, 1995). However, Alexandre *et al*. (2001) found either no response or moderate downregulation of *ROX1, COX5a* and genes involved in sterol synthesis during short-term exposure to ethanol (7% for 30 min), although this did cause upregulation of *GLK1*, which we also observed in late fermentation (Figure 2C). At this stage of the fermentation, some cells are dying, so that possibly haem released from dying cells could be taken up by other cells and activate the *HAP1–ROX1* pathway. Whatever the mechanism behind the upregulation of *ROX1* and sterol synthesis genes in late fermentation, cells showing this response have a significant growth advantage when the cropped yeast is repitched into fresh, aerated wort. Oxygen disappears from this wort in a few hours, so that sterols must be synthesized rapidly, and present-day brewer's strains are the evolutionary product of repeated recycling through anaerobic brewers' fermentations with a few hours' exposure to oxygen in each cycle. It would be interesting to test whether *Saccharomyces* strains with different evolutionary histories also show this response.

Although expression profiles of *MALx1, ILV5* and *ATF1* were, in each case, very similar for the *S. cerevisiae* and *S. bayanus* components of each gene, for *ADH1* and *ERG3* the expression of the *S. cerevisiae* and *S. bayanus* genes followed markedly different profiles (Figure 7). Thus, the use of microarrays of *S. cerevisiae* genes alone does give incomplete and possibly misleading information about the physiological behaviour of the hybrid lager strains. Better knowledge of their physiological adaptation through the wort fermentations requires that expression levels of both sets of genes are measured. This can be done by constructing arrays that recognize both sets of genes. Meanwhile, TRAC can be used to approach this problem with appropriately designed probes.

Although there was broad agreement between the expression profiles of fermentations I and II, some differences were noted. Fermentation II was slower and showed less yeast growth, and also showed no or smaller activations during the first few hours of certain stress-related genes (*TPS1, GPD1, GPP2*; cf. Figure 5 and Supplementary Figure 4) and genes for the synthesis of sterols, unsaturated fatty acids and esters (cf. Figure 6 and Supplementary Figure 5). Data from many more fermentations (including industrial-scale operations) must be correlated to establish the statistical significance of these findings, but they indicate how TRAC might be used to predict the speed of wort fermentations within the first few hours.

In conclusion, the frequent sampling allowed by TRAC showed a surprisingly dynamic pattern of gene expression, especially during the first 2 days of wort fermentation by industrially cropped yeast. Some genes were switched on and off so quickly that their period of active expression could be missed altogether by genome-wide techniques applied only once or twice a day. By providing an effectively continuous time profile of the expression of a limited number of chosen genes, TRAC reveals how changes in gene expression precede their physiological consequences by at least several hours. This offers the possibility of using this kind of data to monitor and control fermentations.

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Development of rapid gene expression analysis and its application to bioprocess monitoring

Abstract

Bioprocesses are used for production of industrial compounds and for studying biological phenomena under reproducible conditions. In order to be able to better understand and control biological systems, development of methods that generate detailed information about the physiological status of the organisms is required. Genetic expression is an important aspect of physiology. Genome-wide expression analyses have led to the discovery of gene sets that provide the essential information about the biological system of interest. This has increased the need for technologies enabling time- and cost-effective detection of specific gene transcripts.

The aim of the present study was to develop methods suitable for expression analysis of defined gene sets in bioprocess conditions. The solution hybridization principle was applied in the development of a sandwich hybridization method and a method called TRAC (Transcript analysis with the aid of affinity capture) using a pool of oligonucleotide probes separable and quantifiable by capillary electrophoresis. The basic sandwich hybridization assay detects one target per sample, whereas TRAC was capable of more than 20-plex RNA target detection, making it suitable for high-throughput expression analysis. The advantages of the developed methods for bioprocess monitoring are simple and rapid analysis directly from cell lysates and semi-automated processing of large sample numbers.

The TRAC method was applied for monitoring of protein production processes by the filamentous fungus *Trichoderma reesei* and of lager yeast fermentations by *Saccharomyces pastorianus*. Altogether about 30 *T. reesei* and 70 *S. pastorianus* genes with presumed process relevance were identified. Many of the marker gene expression profiles both in the filamentous fungus and yeast were shown to have value in the prediction of consecutive physiological effects and of process performance. In addition, TRAC was used in the evaluation of gene expression stability during *T. reesei* chemostat cultures. These data were applicable in the evaluation of steady state quality and were useful when selecting samples for further analyses. The data obtained by TRAC confirmed the value of focused and frequent analysis of gene expression in the monitoring of biotechnical processes, providing a powerful tool for process optimization purposes.

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Nimeke

Nopean geenien ilmentymisanalyysin kehittäminen ja soveltaminen bioprosessien seurantaan

Tiivistelmä

Bioprosesseja käytetään teolliseen biologisten komponenttien tuottamiseen ja biologisten ilmiöiden tutkimiseen toistettavissa olosuhteissa. Jotta biologisia systeemejä voitaisiin sekä ymmärtää että kontrolloida paremmin, tarvitaan sellaisten menetelmien kehitystä, jotka tuottavat yksityiskohtaista informaatiota organismien fysiologisesta tilasta. Geenien ilmentyminen on tärkeä osa solujen fysiologiaa. Genominlaajuiset ilmentymisanalyysit ovat mahdollistaneet pienempien geeniryhmien identifioinnin, jonka avulla voidaan selvittää olennainen informaatio kiinnostuksen kohteena olevasta biologisesta systeemistä. Tämä puolestaan on lisännyt tarvetta tekniikoille, jotka mahdollistavat nopean ja edullisen geenien transkriptien mittauksen.

Tässä tutkimuksessa oli tavoitteena kehittää menetelmiä, jotka soveltuvat tiettyjen geeniryhmien ilmentymisen mittaukseen bioprosesseissa. Liuoshybridisaatioperiaatetta sovellettiin kehitettäessä sandwich-hybridisaatiomenetelmää sekä menetelmää nimeltä TRAC (Transcript analysis with the aid of affinity capture), jossa käytetään sellaisten oligonukleotidikoettimien joukkoja, jotka voidaan erotella ja kvantifioida kapillaarielektroforeesilla. Sandwich-hybridisaatiomenetelmää voidaan käyttää havainnoimaan yksittäisiä kohteita kustakin näytteestä, kun taas TRAC-menetelmä mahdollistaa yli 20 RNA-kohteen yhtäaikaisen mittauksen kustakin näytteestä, mikä tekee siitä tehokkaan ilmentymisanalyysin. Kehitettyjen menetelmien edut bioprosessien seurannassa ovat helppokäyttöisyys ja nopea mittaus suoraan solulysaateista. Näytteenkäsittely on osittain automatisoitu suurien näytemäärien yhtäaikaiseen käsittelyyn.

TRAC-menetelmää sovellettiin rihmasieni *Trichoderma reeseillä* suoritettujen proteiinituottoprosessien sekä lager-hiivalla (*Saccharomyces pastorianus*) suoritettujen käymisten seurantaan. Yhteensä identifioitiin 30 *T. reesei* ja 70 *S. pastoriasnus* -prosessien kannalta tärkeiksi oletettua merkkigeeniä. Useiden identifioitujen rihmasienen ja hiivan merkkigeenien ilmentymisprofiilien osoitettiin ennustavan sekä fysiologisia vaikutuksia että prosessien etenemistä. Lisäksi TRAC-menetelmää käytettiin geenien ilmentymistasojen stabiilisuuden mittaukseen *T. reesei* -kemostaattikasvatuksissa. Tätä dataa käytettiin arvioimaan kasvatusten laatua valittaessa näytteitä jatkoanalyyseihin. TRAC-menetelmällä kerätty data osoitti fokusoidun ja tiheään tapahtuvan geenien ilmentymismittauksen hyödyn bioteknisten prosessien seurannassa ja tarjosi täten tehokkaan työkalun prosessien optimointiin.

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The aim of the present study was to develop methods suitable for transcript analysis of defined gene sets in bioprocess conditions. A sandwich hybridization assay and a multiplex high-throughput gene expression analysis called TRAC (Transcript analysis with the aid of affinity capture) were developed. These methods enable simple and rapid analysis directly from cell lysates and semi-automated processing of large sample numbers. The TRAC method was applied for frequent monitoring of fungal bioprocesses and was shown to have value in physiological evaluation of the organisms and in process optimization.

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