TECHNISCHE UNIVERSITÄT MÜNCHEN

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München
Lehrstuhl für Brau- und Getränketechnologie

Temperature induced gene expression in self-cloning industrial yeast

Susann Fischer

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat)

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. H.-Chr. Langowski
Prüfer der Dissertation:	Prof. Dr. T. Becker
	Prof. Dr. A. Schnieke

Die Dissertation wurde am 06.06.2018 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18.01.2019 angenommen.

Why does this magnificent applied science, which saves work and makes life easier, bring us so little happiness?
The simple answer runs:
Because we have not yet learned to make sensible use of it.

Albert Einstein (1879-1955)

Acknowledgment

The world is full of willing people, some willing to work, the rest willing to let them.
Robert Frost (1874 – 1963)

This dissertation was not created solely by my will, but also with the participation of many supporters, colleagues and friends whom I would like to thank. My thanks go to Prof. Dr.-Ing. T. Becker for his tireless support, the freedom of my research and of course for the joint development of the topic of this dissertation. I would also like to thank Prof. Dr. A. Schnieke and Prof. Dr. H.-Chr. Langowski for the review of the dissertation and acting as chief examiner.

My students Daniel Dröge, Carina Engstler, Kai Rupert Büchner, Laure-Elise Pilett, Joaquín A. Steinhäuser, Irina Beer, Tobias Kretzner, Moritz Kärcher, Nadine Thiermann, Lydia Reisinger, Lisa Bachmaier, Rabea Selzer, Saskia Weiß and Yovcho Byalkov share my joy and suffering in a scientific sense and accompanied me on this journey; thank you all.

A special thanks goes to all members of the Institute of Brewing and Beverage Technology, in particular to all my colleagues of the Research Group Beverage and Cereal Biotechnology for their support and the wonderful time. Many Thanks to Dr. Reiner Springer for proofreading this thesis. Roland Kerpes, I thank you for the countless funny and serious moments at the chair and in our private lives.

The biggest thanks go out to those who often had to do without me when my yeast needed a little more attention; the constructive words when things did not go that way again; the joyful dances you shared with me, when the yeasts did what I wanted; the patience you have brought me cannot be grasped by words.

Thank you Mag. Christoph Vierbauch, thank you Zoe and you little Xaver, thank you too!

Preface and peer reviewed publications

The results and publication of this thesis were produced at the Technische Universität München, Institute of Brewing and Beverage Technology, Workgroup Beverage and Cereal Biotechnology from 2012 to 2017.

The following peer reviewed publication were generated in the period of this work and were part of the thesis:

Fischer, S., S. Procopio and T. Becker (2013). "Self-cloning brewing yeast: a new dimension in beverage production." European Food Research and Technology **237**(6): 851-863.

Fischer, S., C. Engstler, S. Procopio and T. Becker (2016). "EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system." LWT - Food Science and Technology **68**: 556-562.

Fischer, S., C. Engstler, S. Procopio and T. Becker (2016). "Induced gene expression in industrial *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70: evaluation of temperature and ethanol inducible native promoters." FEMS Yeast Res **16**(3): 1-10.

Fischer, S. Büchner, K. R., Becker, T. (2018). ”Induced expression of the alcohol acetyltransferase gene ATF1 in industrial yeast *Saccharomyces pastorianus* TUM 34/70.” Yeast doi: 10.1002/yea.3319

Contents

Abbreviations

°P	degree plato
AATase	alcohol acetyltransferase
BC	before Christ
Bp	base pairs
CTA	C-terminal transcriptional activation
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
CWI	cell wall integrity
EBC	European brewery convention
EFFCA	European Food and Feed Cultures Association
EFSA	European Food Safety Authority
EGFP	enhanced green fluorescence protein
ESR	environmental stress response
EU	European union
GMO	genetically modified organism
H_2O_2	hydrogen peroxide
HOG	high osmolarity glycerol
HSR	heat shock response
HSE	Heat shock element
HTS	high throughput system
MAPK	mitogen-activated protein kinase
O_2	Oxygen
O_2^-	Superoxide
O_3	Ozone
OH	Hydroxyl
PCR	polymerase chain reaction
PKA	cAMP-protein kinase A
ROS	reactive oxygen species
SM	sulfometuron methyl
STRE	stress response element
TF	transcription factor
YRE	Yap1p recognition element

Summary

Based on short generation time and ease of use, *Saccharomyces* yeasts are a substantial eukaryotic model organism for molecular biology and pharmacological research. The beverage-processing *Saccharomyces* yeast are adopted on manifold stressors, such as differences in temperature, osmotic pressure, oxygen and nutrient starvation, which induces the genetic regulation of stress response genes and the related metabolic answer. Furthermore, the metabolic processes of the yeast during industrial fermentation have been comprehensively investigated scientifically. In the present study, process technological variations were used to induce the gene expression consciously over stress situations. First, in order to evaluate native promoters of the industrial yeast *Saccharomyces cerevisiae* TUM 68 under industrial brewing conditions, a high throughput method was established. The fluorescence green protein (EGFP) as reporter gene allows a rapid and non-invasive detection of the promoter activity. This also enables a comparative evaluation of ten different stress-related native promoters of the hybrid yeast *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70. Beside the effects of different cold shock scenarios and ethanol concentrations on the induction conditions, the induction triggered by fermentation without targeted induction was determined. The three promoters $_{P}$SSA3, $_{P}$HSP104 and $_{P}$UBI4 were evaluated as the most efficient for temperature induction in industrial fermentation processes. In the final part, the gene ATF1 – which has product-optimising properties against the biosynthesis of ethyl acetate and isoamyl acetate – was brought under the control of these three promoters. Under industry-orientated conditions, the self-cloning yeast with the regulated promoter $_{P}$HSP104 and $_{P}$UBI4 shows significant higher amounts of ethyl acetate after the temperature shift from 12°C to 4°C, which indicates a targeted gene induction.

Zusammenfassung

Die *Saccharomyces* Hefen stellen aufgrund ihrer kurzen Generationsphase und einfachen Handhabung den bedeutendsten eukaryotischen Modellorganismus für die molekularbiologische und pharmakologische Forschung dar. Ebenso sind *Saccharomyces* Hefen an mannigfachen Prozessen in der Lebensmittelindustrie beteiligt. Diese Hefen sind an die im Herstellungsprozess von Getränken und Lebensmitteln auftretenden Umwelteinflüsse adaptiert und deren Stoffwechselvorgänge in den jeweiligen Herstellungsprozessen zudem umfassend untersucht. Die an der Produktion hefefermentierter Getränke beteiligten *Saccharomyces* Hefen unterliegen verschiedenen Stressoren wie Temperatur, osmotischer Druck, Sauerstoff- und Nährstoffmangel, die eine starke Regulation der am Metabolismus beteiligten Enzyme bzw. Gene mit sich führen können. In der vorliegenden Arbeit wurde diese Induktion der Genexpression gezielt über prozesstechnische Variationen durch bewusst herbeigeführte Stressinduktionen hervorgerufen. Dazu wurde im ersten Teil ein Hochdurchsatzverfahren zur Evaluierung der Induktionskonditionen von nativen Promotoren der Hefe *Saccharomyces cerevisiae* Stamm TUM 68 unter industriellen Bedingungen entwickelt. Mittels des grün fluoreszierenden Proteins EGFP und dessen Fluoreszenzdetektion war es möglich, die Promotoraktivität zeitnah und nicht invasiv zu detektieren. Im Weiteren wurden zehn native Promotoren verschiedener stressinduzierter Gene der untergärigen Hefe *Saccharomyces pastorianus* Stamm TUM 34/70 mit der validierten Methode evaluiert. Dabei wurde neben verschiedener Kälteschock-Szenarien und Ethanolkonzentrationen auch die Induktion während der Fermentation ohne gezielte Induktion untersucht. Die drei Promotoren $_{P}$SSA3, $_{P}$HSP104 und $_{P}$UBI4 zeigten dabei die effektivsten Induktionseigenschaften. Das Gen ATF1, welches produktoptimierende Eigenschaften bezüglich der Biosynthese der Aromastoffe Ethylacetat und Isoamylacetat besitzt, wurden anschließend unter die Kontrolle der evaluierten Promotoren gebracht. Unter industrienahen Bedingungen zeigten die selbstklonierten Hefen mit den regulierten Promotoren $_{P}$UBI4 und $_{P}$HSP104 einen signifikant höhere Konzentration an Ethylacetat nach dem Temperatursprung von 12 °C auf 4 °C, was eine gezielte Induktion durch die prozesstechnische Variation der Temperatur belegt.

1. Introduction

Yeasts have been used for centuries by humankind for the production of food and beverages, even prior to the knowledge of their existence. In ancient Egypt between 4000 and 1000 BC, sour dough was prepared through a mixture of wheat flour and fermented beer. The earliest evidence of wine-making was dated as early as the seventh millennium BC in China (P. E. McGovern et al., 2004). The wine was made from rice, honey grapes and hawthorn. In the first century BC, Germanic and Celtic tribes established the knowledge of beer brewing in Europe (Corran, 1975). Initially, only monasteries were allowed to brew for their own consumption. During Lent, beer was the daily diet for the friars (Reinheitsgebot, 2016). The historic evolution of beer is indisputable, which was inextricably connected to bread. The phenomena of fermented beverage production leads to the ethanol-combined analgesic, disinfectant and mind-altering effects (Vallee, 1998). Besides these profound effects, fermentation preserves and enhances the natural value of foods and beverages. This is provoked to pharmacological, nutritional and sensory benefits of fermented beverages. Furthermore, fermentation plays a key role in developing human culture and technology and contributing to the advancement and intensification of horticulture, agriculture and finally food-processing techniques (Patrick E McGovern, 2013; Underhill, 2002).

Many industrial fermentation processes are performed by the yeast *Saccharomyces cerevisiae,* such as the production of alcoholic beverages (wine, sake and beer) as well as for bread dough fermentation. Additionally, the yeast is used in the bioethanol industry and as the best-known eukaryotic microorganism for the molecular biology. This yeast was the first eukaryotic organism to be fully sequenced in 1996 (Goffeau et al., 1996). Besides knowledge of the sequence, two-thirds of the ≈6,000 genes have been characterised (Kumar & Snyder, 2001; Pena-Castillo & Hughes, 2007). Furthermore, due to the short generation time, modesty on the media and simple genetic modification, the yeast *Saccharomyces* has gained usage in the molecular biology and biotechnology. The scientific effort is based on the characterisation of coding genes and metabolic engineering of complete pathways (Ostergaard, Olsson, & Nielsen, 2000). Therefore, it became possible to produce heterologous compounds such as hepatitis and human papillomavirus vaccines and human insulin (Hou, Tyo, Liu, Petranovic, & Nielsen, 2012).

This industrialisation of *Saccharomyces* yeast in the context of metabolic engineering and usage in food and beverage industry is the focus of the following chapter.

1.1 Industrialisation of yeast

> *…alcoholic fermentation is a process correlated with the life and organisation of yeast cells, not with the dead or putrefaction of the cells. Nor is it a phenomenon of contact, for in that case the transformation of the sugar would occur in the presence of the ferment without giving anything to it or taking anything from it.* PASTEUR, 1860

Pioneering studies from a scientific and technological perspective of yeasts start with PASTEUR and HANSEN in the mid-1800s with the observation of beer and wine fermentations (Barnett, 2000; Guilliermond, 2003). In 1859, PASTEUR refuted the theory of "spontaneous generation" throughout an experimental conclusion (Wyman, 1862). Furthermore, HANSEN separated yeast from bacteria and isolated different yeasts from mixed beer fermentations to describe the different types of yeast for a targeted production (Guilliermond, 2003). However, before this isolation of such pure cultures, the foodstuffs were prepared by using endogenous microflora. At present, some 100 yeast genera represent over 1,400 yeast species (Kurtzman, Fell, & Boekhout, 2011). However, this number does not indicate the expected wealth of yeast biodiversity, given that the majority of yeast genera has simply not been isolated or described. As an example, the unknown parental strain of the bottom fermented yeast *Saccharomyces pastorianus* var. *carlsbergensis* was found in Tibet and published in 2014, named as *S. eubayanus* (Bing, Han, Liu, Wang, & Bai, 2014). The habitat of this strain is not a coincidence, based on the strong hypothesis that Far East Asia is the origin centre of the *Saccharomyces* yeast (Bing et al., 2014; Q. M. Wang, Liu, Liti, Wang, & Bai, 2012).

At present, microbial food cultures are defined by the EFFCA (European Food and Feed Cultures Association) as *"live bacteria, yeasts or molds used in food production*" (Bourdichon et al., 2012). For food-based use, microbial food additives have to be authorised and many of these microorganisms are included in the "Qualified Presumption of Safety" (QPS) list of EFSA (Barlow et al., 2007). Microbial food additives with a long history of safe use are listed in the International Dairy Federation review of microorganisms with technological beneficial use (Bourdichon et al., 2012).

The domestication of yeast and its later industrialisation has adopted the yeast to specific production steps and led to a genetic appropriateness. The yeast strains used in industrial processes today have little in common with the antecedent yeast that was used thousands of years ago to transform sugar into alcohol inside human-made containers (Bauer & Pretorius, 2000; Querol & Bond, 2009).

According to the latest findings, current industrial yeast can be grouped into five subsets, depending on the genotypic and phenotypic differences compared with wild yeast. Furthermore, industrial yeast originates from only a few common ancestors and further mutating through domestication and regional differences (Verstrepen, 2017). In Table 1, different yeasts for the various industrial relevant applications in food and beverage production as well as foreign proteins, bioethanol and metabolites are listed.

Table 1: Application of industrial relevant yeast (modified to (Attfield & Bell, 2003))

Genius	**Species**	**Application**
Saccharomyces	*cerevisiae*	Bread, wine beer, sake, distilling, fuel alcohol and foreign proteins (e.g. Insulin, invertase, ergosterol),
Saccharomyces	various species	Beer, sake, wine, amylase, melibiase
Candida	*utilis*	Food yeast and nucletides
Candida	*paraffinica*	Food yeast
Candida	*lipolytica*	Citric acid
Rhodotorula	species	Carotene, itaconic acid
Pichia	*angusta*	Foreign proteins
Pichia	species	Food yeast, steroid precursors, riboflavin, α-galactosidase
Kluyveromyces	*fragilis*	Invertase, food yeast
Kluyveromyces	*marxianus*	Chees
Kluyveromyces	*lactis*	Food yeast, β-galactosidase
Hansenula	species	Steroid precursor
Torulopsis	species	Food yeast, gluconic acid
Zygosaccharomyces	*rouxii*	Soy sauce, miso

Compared to natural conditions, in industrial processes stressors appear in a higher intensity and – due to the process – also sequentially and simultaneously. What is stress and how does a unicellular organism adapt to stress? A theoretical definition of stress that is universal, transferable to living organisms and succinctly summaries all

previous definitions was established by the psychologist STRICKLAND in 2001: "*The physiological and psychological responses to situations or events that disturb the equilibrium of an organism*" (Strickland, 2001).

Stress is provoked by exogenous factors that disturb the organism through changes in the optimal situation or equilibrium. As a result, the organism responds with the stress syndrome. This syndrome could be caused by an arbitrary number of stress factors. The following reaction of the organism is the stress resistance, which is characterised by stress tolerance and stress adaption (Schopfer & Brennicke, 2011). The best-characterised response against stress by cells is the immediate synthesis of a limited number of proteins, the so-called *stress proteins* (Estruch, 2000). Therefore, global signalling pathways triggered by environmental stress lead to defined gene expression and physiological response in cellular adaption to environmental changes and repairing possible damages (Gasch et al., 2000; Hohmann & Mager, 2003). The response to stress is classified in three phases: i) immediate cellular changes occur as a direct consequence of stress exposure and damage; ii) defence process are triggered; and iii) the resumption of proliferation. These phases can be distinguished more or less depending on the type of stress (Hohmann & Mager, 2003). Especially unicellular organisms such as yeast are confronted with large variations of their natural or industrial environment in a direct way in comparison to complex metazoans (Bauer & Pretorius, 2000; Hohmann & Mager, 2003).

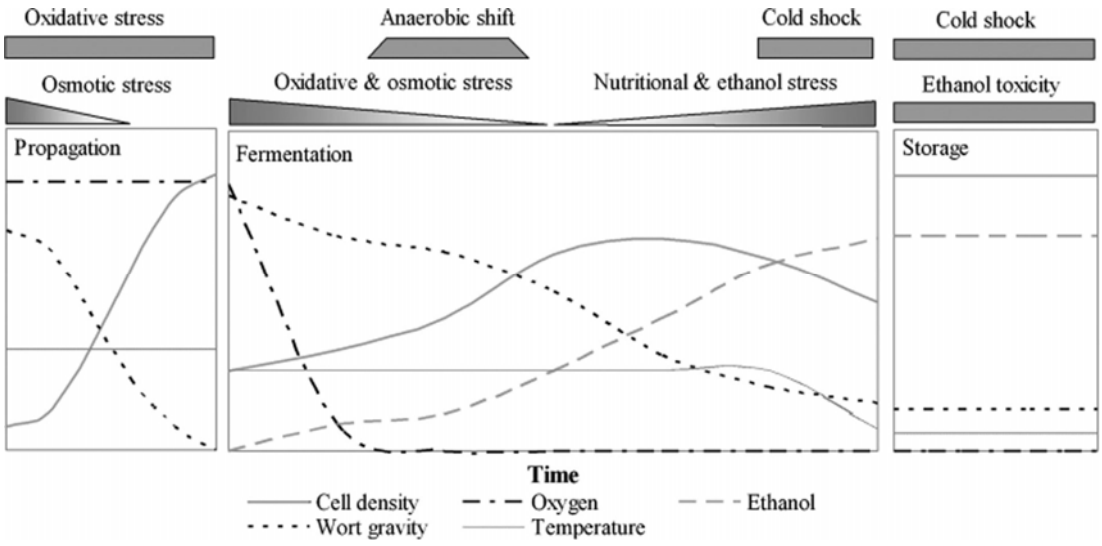

Figure 1: Types of stress during beer fermentation procedure, propagation and storage of the yeast cells (B. R. Gibson, Lawrence, Leclaire, Powell, & Smart, 2007).

During industrial beer fermentation, yeast is exposed to multiple stress situations in different stages of the fermentation (Figure 1). First, the pitching of the yeast into the fermentation medium exposes the yeast to high concentrations of osmotically-active substances, in particular glucose, fructose, sucrose, maltose and maltotriose (from 10 °P (C. Boulton & Quain, 2013)) and in later phases of fermentation higher concentrations of ethanol (up to 4%(v/v)) (Piper, 1995). Such hypertonic conditions lead to an efflux of water from the cell, reduction of the water availability and diminished turgor pressure (B. R. Gibson et al., 2007; Hohmann, 2002; Markus J. Tamás & Hohmann, 2003). In order to overcome this situation, cell wall modifications and an increase of the osmolyte glycerol are the direct response (Bauer & Pretorius, 2000). After the adaption of the cells to the media, further stressors occur during the fermentation process itself. Nutrient limitations – which could result in a reduction of fermentation efficiency or starvation – occur at different stages of the fermentation (see Figure 1), with the highest stress phenomena at the end of fermentation, provoked by the simultaneous appearance of ethanol toxicity (R. B. Boulton, Singleton, Bisson, & Kunkee, 2013). The response against malnutrition includes the synthesis of intracellular trehalose and the accumulation of heat shock proteins (Werner-Washburne, Stone, & Craig, 1987). During the fermentation, higher amounts of ethanol are produced. Ethanol has a toxicity against organism at low concentrations, low as 2% (v/v) and influences membrane fluidity by changing the levels of hexadecanoic, octadecanoic and palmitoleic acids to enhance the membrane fluidity and reduce the intracellular water activity (Kajiwara, Suga, Sone, & Nakamura, 2000). This affect all compartments of the cell; for instance, the membrane structure, enzyme activity and protein folding. Equally, in response against starvation, the accumulation of trehalose and heat shock proteins occur, as well as the enhancement of the membrane rigidity (Arneborg, Hoy, & Jorgensen, 1995). Temperature shifts occur at the beginning (pitching) and the end of fermentation through the switch from fermentation to maturation. All changes of temperature are recognised as a stress by the yeast cell (Piper, OrtizCalderon, Holyoak, Coote, & Cole, 1997). Throughout the temperature shift (for heat and cold shock equally), membrane fluidity decreases and triggers the stress response. A temperature shock results in an accumulation of heat shock proteins. Additionally, an increment of intracellular trehalose and glycogen occur due to a cold shock situation (Murata et al., 2006; Panadero, Pallotti, Rodriguez-Vargas, Randez-Gil, & Prieto, 2006).

Oxidative stress is controversially debated. An incomplete oxygen metabolism could cause a reduction of oxygen to reductive oxygen species (ROS), which are derivate forms of O_2 such as H_2O_2, OH•, O_2^-, O_3 and $^1O_2$. ROS causes damage via the peroxidation of lipids, oxidation of proteins and DNA (B. R. Gibson et al., 2007; Morano, Grant, & Moye-Rowley, 2012). The stress response of the yeast cell is the synthesis of antioxidants to the catalytic reduction of ROS through electronic transfer; for instance, superoxide dismutase, catalases, glutathione and thioredoxin (B. R. Gibson et al., 2007; Toledano, Delaunay, Biteau, Spector, & Azevedo, 2003). During propagation, for the production of biomass with a high value of vitality and viability, higher concentrations of catalase and an accumulation of glycogen and trehalose are detectable (Bleoanca & Bahrim, 2013). Further studies have shown the influence of growth-limiting conditions during fermentation – and therefore the depletion of glucose in the wort – on the increment of antioxidants in the absence of oxygen (Gibson et al., 2008). The genetic regulation for an effective stress response is the objective of the following section.

1.2 Genetic regulation affected by stress during fermentation

As mentioned above, stress is a sudden change of environmental factors. Therefore, only in the stress situation is a response necessary to outlast this unfavourable situation. Furthermore, to avoid metabolic burden – namely an increased energy demand or dilution of molecular factors required for transcription and translation (Nevoigt, 2008) – a “fine tuning” of gene expression is indispensable. Consequently, the expression of genes that are involved in regulatory or rate-limiting steps of the metabolic process are altered in comparison to the entire pathway (Gasch, 2003). Therefore, a rapid return to a “non-stress” situation in gene expression is possible and reduces the effort and energy requirement (Belanger, Larson, Kahn, Tkachev, & Ay, 2016; Gasch, 2003; Richter, Haslbeck, & Buchner, 2010).

The trigger of a stressor leads to the stimulation of the appropriate pathway and the induction of relevant stress response genes. There are several genes involved in different stress responses and activated by different pathways. This is caused by the presence of different transcription-binding sites into the promoter sequences where the equivalent transcription factor (TF) binds. TFs are further defined by the function of regulating transcription nearby sequences that they bind (Hughes & de Boer, 2013) and they are grouped into three classes: (i) Zink $(Zn)^{2+}$stabilized, which is most

abundant in eukaryotic organism; (ii) helix-turned-helix; and (iii) zipper type (Hahn & Young, 2011). (Hahn & Young, 2011). At present, there are 180 known genes designated as TFs in the yeast genome (www.yeastract.com) (Teixeira et al., 2014). Furthermore, the quantity of transcription-binding sites in the promoter region reflects the strength of induction patterns (Estruch, 2000). The induction and regulation of the different stress response pathways for environmental stress, heat and cold shock, osmotic pressure and oxidative stress response will be outlined in the following.

Substantial fractions of responses are not specific to the stimuli than to common environmental changes, these is known as *cross-protection* (Estruch, 2000). Through the **environmental stress response** (**ESR),** approximately 900 genes are altered in the expression level, whereby approximately 600 genes are reduced in expression rate. These genes are mostly associated with a growth-related function and protein synthesis. The remaining genes are induced during ESR, whereby 45% of these genes are uncharacterised. The characterised genes are e.g. related to carbohydrate metabolism, metabolic transport, fatty acid metabolism, protein folding/degradation, DNA damage repair, cell wall modifications, detoxification of ROS, autophagy (see Table 2) (Gasch, 2003; Gasch et al., 2000).

The induction of the particular genes is negatively regulated by the cAMP-protein kinase A (PKA) pathway, where the membrane bound G-coupled receptors respond to external stimuli. Stress conditions reducing interaction of heat shock proteins (e.g. Hsp70p) with the protein kinase Cdc25p are followed by reducing cAMP. This leads to a downregulation of PKA and mediates the transcription genes' transcription factors Yap1p and Msn2p and Msn4p (Estruch, 2000; Martinez-Pastor et al., 1996; Schmitt & McEntee, 1996). Msn2/4p belongs to the zinc-finger family and has the identical DNA binding domain (DBD; CCCCT), which is named stress response element (STRE) (Marchler, Schüller, Adam, & Ruis, 1993; Martinez-Pastor et al., 1996). However, these two TFs vary in their cellular behaviour. A deletion of MSN4 has no influence on the gene expression, whereas a deletion of MSN2 results in a reduced induction pattern of STRE-related genes (Martinez-Pastor et al., 1996). Under un-stressed conditions, the Msn2/4p are phosphorylated and located in the cytosol. Under stress conditions, they become hyper-phosphorylated and translocated to the nucleus (Garreau et al., 2000; Gorner et al., 1998) where they induce the STRE-related genes. Inversely, increasing cAMP reverses the hyper-phosphorylation and triggers the relocation to cytosol (Gasch, 2003).

Table 2: ESR-induced genes and their function

ESR genes	Gene function
Carbohydrate metabolism	
TPS1, TPS2, TSL1	Trehalose 6-phosphate synthase/phosphatase complex
NTH1, ATH1	Trehalases
GSY2, GPH1	Glycogen synthetase, Glycogen phosphorylase
PFK26, FBP26	6-Phosphofructo-2-Kinase, Fructose bisphosphatase
Fatty acid metabolism	
FAA1, PXA2	Fatty acid activation, Peroxisomal ABC-transporter
CAT2	Carnitine Acetyltransferase (exporting)
Respiration	
CIT1, CYC7	Citrat synthase, cytochrome c
COX15, COQ5	Cyctochrom c oxidase, Coenzym Q
Oxidative Stress defence	
TRX2, GRX2	Thioredoxin, Glutaredoxin
ECM38, PRX1	Gamma-glutamyltranspeptidase, Peroxiredoxin
ZWF1, GND2	Glucose-6-phosphate dehydrogenase, 6-Phosphogluconate dehydrogenase (NADH-generating steps)
CTT1, SOD1	Cytosolic catalase, cytosolic superoxide dismutase
HYR1, GPX1	Glutathione peroxidases
CCP1, MCR1	Cytochrome c peroxidase, Cytochrome b5 reductase
Autophagy	
APG1, APG7, AUT1	Autophagy-related proteins and cytoplasm-to-vacuole targeting (CVT) pathway
PMC1	Vacuolar calcium pump
PRC1, YPS6, LAP4, PEP4, PRB1	Vacuolar proteases
PAI3, PB12	Protease inhibitors
Protein folding/degradation	
SSA3, SSA4, SSE2, HSP78	Hsp70 chaperone
HSP12, HSP26, HSP48	Small heat shock proteins
HSP104	Disaggregase, Heat shock protein
UBC5, UBC8, HUL4	Ubiquitin ligation and conjugation
UBI4, UBP15	Polyubiquitin, putative deubiquitinating enzyme

In addition to STRE, there are further transcription-binding sites included in the promoter region of some genes where condition-specific TFs bind; for instance, Hsf1p and Yap1p. Such regulatory systems would confer protection under mild stress, but the activity of these systems would be impaired or become insouciant under severe conditions, thus making the STRE/Msn2/4p system essential for cell survival (Estruch, 2000).

The mentioned Hsf1p is involved in the **heat shock response (HSR)**, which is an evolutionary consistent response for all eukaryotes (Richter et al., 2010). This is a multifaceted regulation system due to the metabolic remodelling, transient cessation of growth and global changes of transcription and it is not only responsible for heat shock but also for cold shock and higher contents of ethanol (Piper, 1995). This is partly triggered by the above-mentioned changes in the membrane fluidity by temperature shifts and ethanol. However, the temperature-sensing mechanism predicted on a membrane-embedded protein compound has not yet been identified (Verghese, Abrams, Wang, & Morano, 2012). Furthermore, the ratio of saturated and unsaturated fatty acids in membrane affects the temperature set point at which the HSR is induced (Carratù et al., 1996). Ethanol tolerance is partly expressed under the same conditions as heat shock. The pre-treatment of cells with heat results in higher ethanol tolerance, but not vise versa. Indeed, a pre-treatment with ethanol results in thermal intolerance (Piper, 1995). Beside the HSP genes listed in Table, 2 the remaining relevant genes of the HSP family (Table 3) are regulated solely by Hsf1p (Boy-Marcotte et al., 1999; A Trott & Morano, 2003), with one exception, HSP30, which is only regulated by Yap1p (Seymour & Piper, 1999). A hypothesis for the induction of the ESR and HSR is not a requirement for survival of the stimulating stress but rather for survival of a subsequent stress and it is named as “acquired stress resistance” (Berry & Gasch, 2008).

The TF Hsf1p is an 833-amino-acids protein and it includes a DBD, a three leucine zipper (LZ) repeats responsible for the trimerisation of the factor, an essential C- terminal transcriptional activation (CTA) domain and – uniquely for yeast – HSF, a transcriptional activation domain at the N-terminus (Hashikawa, Yamamoto, & Sakurai, 2007; Nieto-Sotelo, Wiederrecht, Okuda, & Parker, 1990). The heat shock element (HSE) is a pentameric unit grouped into three distinct classes: i) the “perfect” type HSE comprises three continuously-inverted repeats of the pentameric unit (nTTCnnGAAnnTTCn); ii) the “gap” type [nTTCnnGAAn(5 bp)nGAAn]; and

iii) the “step” type [nTTCn(5 bp)nTTCn(5 bp)nTTCn]. Types ii and iii include a five bp insertion between the consensus sequence to preserve the proper spatial orientation (Hashikawa et al., 2007; Sakurai & Takemori, 2007). In contrast to vertebrates, one yeast HSF pentameric unit binds poorly on HSE and is insufficient to induce transcription, where a trimerisation of HSF increases the DNA binding and stabilises the DNA-protein interaction (Drees, Grotkopp, & Nelson, 1997).

Table 3: Relevant heat shock proteins involved in heat shock response in *Saccharomyces* yeast (Becker & Craig, 1994; Bleoanca & Bahrim, 2013; Boy-Marcotte et al., 1999; Ma & Liu, 2010; Seymour & Piper, 1999)

HSP gene	Physiological function	TF binding site		
		STRE	HSE	YAP
Small HSP´s				
HSP12	Cellular role is not entirely known; possible impact on the initiation of stationary phase and induction of sporulation. Hsp30p may regulate plasma membrane ATPase	7	1	0
HSP26		4	7	0
HSP30		0	0	3
HSP70 family				
SSA3	Cytosolic HSP70 gene, molecular chaperones, binding newly-translated proteins to assist in proper folding and prevent aggregation/misfolding	0	1	0
SSA4	Paralog of SSA3	0	1	0
HSP100 family				
HSP104	Disaggregases; Solubilisation of protein aggregates and degradation of proteins Essential for thermotolerance acquisition. It is expressed constitutively in respiring cells, which do not ferment, entering stationary phase.	3	10	1

One of the five MAP kinases in *S. cerevisiae* regulates the genetic response affected by osmotic pressure, namely the **high osmolarity glycerol (HOG) pathway** (Gustin, Albertyn, Alexander, & Davenport, 1998; Markus J. Tamás & Hohmann, 2003). In order to avoid excessive turgor or cell burst through osmotic stress, *compatible solutes* are synthesised by the yeast cells. These are osmoprotectants, which not affect the physical or biochemical processes and increase internal osmolarity. The sole compatible solute in *S. cerevisiae* is glycerol. The accumulation of trehalose and glycogen is stimulated by general stress and functions as a storage carbohydrate. The genes GPD1 (Glycerol-3-Phosphate Dehydrogenase) and GPP2 (Glycerol-3-Phosphate Phosphatase) mainly affect the synthesis of glycerol during osmotic stress (Rep, Albertyn, Thevelein, Prior, & Hohmann, 1999; M. Rep et al., 1999). Furthermore, for control of cellular glycerol content, the glycerol channel Fps1p is rapidly gated by

osmolarity changes to ensure internal glycerol accumulation (Markus J Tamás et al., 1999). Both stress-relevant genes GPD1 and GPP2 are rapidly and transient induced to 50-fold after stressor occurs. The protein level and enzyme activity of Gpd1p and Gpp2p increase up to 10-fold, depending on the severity of stress (Blomberg, 1995; Blomberg & Adler, 1989; Martijn Rep et al., 1999; M. Rep et al., 1999). and are triggered by the transmembrane histidine phosphotransfer kinase and osmosensor Sln1p (Figure 2). The generated signal is transduced through the MAPK phosphorylation cascade (Ssk2/22p and Pbs2p). The phosphorylated Hog1p translocates from cytoplasm to the nucleus and binds in complex with Hot1p on the promoter region of GPP2 and GPD1 (Aguilera, Randez-Gil, & Prieto, 2007; Alepuz, de Nadal, Zapater, Ammerer, & Posas, 2003). The transcription-binding site of Hot1p has not been determined.

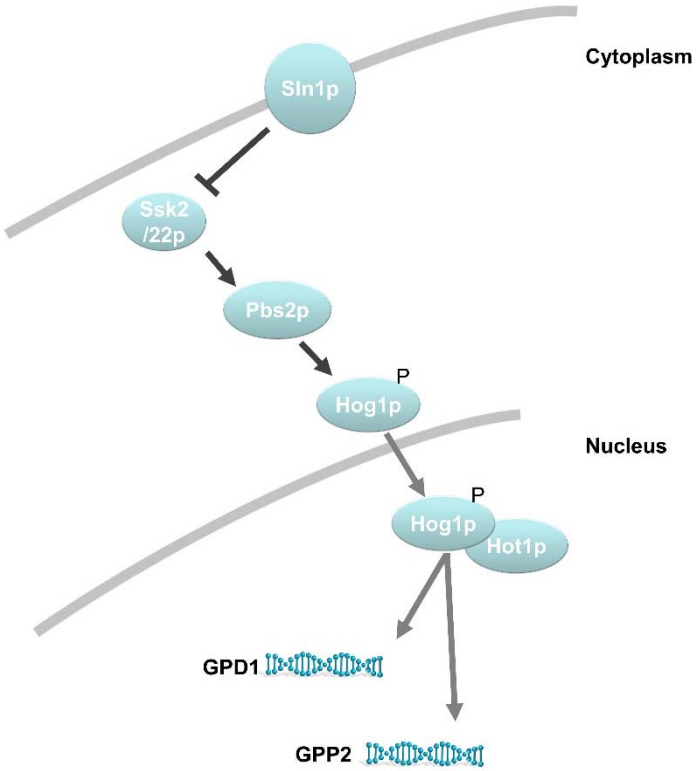

Figure 2: Signal cascade of HOG pathway. Trigger of the membrane bound osmosensor Sln1p. Signal transduced through MAPK phosphorylation cascade, which results in the phosphorylation of Hog1p. Consequently, phosphorylated Hog1p relocate to nucleus and promotes gene expression of inter alia GOD1 and DPP2.

Changes in intracellular concentrations of oxidants activate the **oxidative stress response** by a sophisticated redox sensor. This is provoked by the incomplete reduction of oxygen during propagation. The genomic response to low doses of ROS such as H_2O_2 results in rapid and transient regulation of more than 100 up-regulated

genes and 50 repressed genes (Gasch et al., 2000). The direct activation of gene expression by oxidants is based on the TF Yap1p that co-regulates in association with Skn7p. Yap1p controls the major response regulon to oxidative stress, whereas Skn7p is required for a differential usage of two overlapping sets of genes in distinct stress responses (J. Lee et al., 1999). Yap1p binds to the Yap1p recognition element (YRE) T(T/G)ACTAA in the promoter region of response genes (Fernandes, Rodrigues-Pousada, & Struhl, 1997). (Fernandes et al., 1997). Yap1p contains on the N-Terminus a nuclear location signal and on the C-Terminus a nuclear export signal. In non-stress situations, Yap1p is located in cytosol, whereas the conformation change triggered by intramolecular disulphide bounds in the presence of H_2O_2 results in relocation to the nucleus (Gulshan, Rovinsky, Coleman, & Moye-Rowley, 2005; Kuge et al., 2001). Table 4 shows relevant genes induced by oxidative stress and their regulation by Yap1p and Skn7p. Beside this, genes from the HSP family, drug transporters and genes that are involved in carbohydrate metabolism are also induced by ROS.

Table 4: Relevant genes involved in oxidative stress response and their TF´s in *Saccharomyces* yeast (Gasch et al., 2000; Godon et al., 1998; J. Lee et al., 1999)

Gene	Physiological function	Regulation
Glutathione system		
GSH1	Glutamate-cysteine ligase	Yap1p
GSH2	Glutathione synthetase	Yap1p
GLR1	Glutathione reductase	Yap1p
GPX1/2	Glutathione peroxidase	Yap1p
Thioredoixin system		
TRX2	Thioredoxin 2	Yap1p/Skn7p
TRR1	Thioredoxin reductase 1	Yap1p/Skn7p
TSA1	Thiol peroxidase	Yap1p/Skn7p
Other antioxidants		
CCP1	Cytochrome-c peroxidase	Yap1p/Skn7p
CTA1	Catalase A	Yap1p
SOD1	Superoxide dismutase	Yap1p/Skn7p

At the end of fermentation, ethanol toxicity, the depletion of carbohydrates and nutrient starvation result in the increase of different stress-related genes. Furthermore, the yeast cells enter the stationary phase (Christopher Boulton & Quain, 2008). The metabolism of glycogen and trehalose functions as reserve carbohydrates. Glycogen

accumulates before fermentable sugar is exhausted, whereas trehalose accumulates when cells enter the stationary phase due to nutritional shortage (François & Parrou, 2001; B. R. Gibson et al., 2008). Both reserve carbohydrates are synthesised from Glucose-6-phosphate and UDP-Glucose. Trehalose is synthesised by *trehalose synthase complex* through the genes TPS1, TPS2 (see Table 5), and the largest unit is this complex TPS3 and TSL1 (Bell et al., 1998). Thereby, the regulation of transcription is STRE-dependent and is further controlled by post-translational regulations of the subunits (Parrou, Teste, & François, 1997). The slow degradation of trehalose during the stationary phase is accompanied with the genes NTH1/2 (neutral trehalase) and ATH1 (vacuolar acid trehalose).
The activation of glycogen metabolism is linked to decreasing concentrations of glucose and it is also STRE-dependent (Parrou et al., 1997). The initiation of the metabolism of glycogen is ensured through the self-glycosylating initiator proteins Glg1/2p and glycogen synthetase (GLG1/2) followed by the glycogen-branching enzyme GLC3 (François & Parrou, 2001).

Besides the starvation and ethanol toxicity, at the end of fermentation a decrease in temperature occur due to the transition from fermentation to maturation. The stress response of **cold shock** affects membrane fluidification, cell wall maintenance, osmolyte synthesis, protein-folding support and ROS detoxification (Homma, Iwahashi, & Komatsu, 2003; Kandror, Bretschneider, Kreydin, Cavalieri, & Goldberg, 2004; Murata et al., 2006). In particular, the primary signal is transduced to classical stress pathways and transcription factors (Aguilera et al., 2007) such as the high osmolality glycerol (HOG) pathway (Panadero et al., 2006; A. Trott, Shaner, & Morano, 2005), **cell wall integrity (CWI) pathway** (Corcoles-Saez, Ballester-Tomas, de la Torre-Ruiz, Prieto, & Randez-Gil, 2012), and heat shock response. The cold-sensing signal is triggered by changes in the physical state of membrane, which are monitored by the histidine phosphotransfer kinase and osmosensor Sln1p (Carratù et al., 1996; Panadero et al., 2006). Furthermore, a deletion of the TF Msn2/4p results in rapid death of the yeast cells at 0°C (Kandror et al., 2004) which suggests that **ESR** is involved equally. The involvement of cold shock-relevant genes and the associated TF is summarised in Table 5.

Table 5: Cold-shock relevant genes and the associated pathway in *Saccharomyces* yeast.

Genes	Physiological function
cAMP-PKA	
TPS1	Trehalose-6-Phosphate Synthase
TPS2	Trehalose-6-Phosphate Synthase
HOG Pathway	
GPD1	Glycerol-3-Phosphate Dehydrogenase
GRE1	stress responsive gene; Hydrophilin essential in desiccation-rehydration process
GLO1	Monomeric glyoxalase I
MOX Factors	
TIR1/2/4	Cell wall mannoprotein, TIP1 (Major cell wall mannoprotein with possible lipase activity) related
PAU-Family	Located in subtelomeric regions, serve adaptive purposes
DAN1	Cell wall mannoprotein; similar to Tir1p, Tir2p, Tir3p, and Tir4p

The response to cold shock is regulated in different patterns, depending on the time and temperature (Sahara, Goda, & Ohgiya, 2002; Schade, Jansen, Whiteway, Entian, & Thomas, 2004). Through the industrial application, *Saccharomyces* yeast is exposed to temperature (10-20°C) far below the natural physiological temperature of 25–30°C. Furthermore, storage of industrial yeast proceeds at a very low temperature (0-4°C), whereby the growth is restricted and the vitality and viability is maintained over longer time periods (Hill, 2015).

At the initial state of cold shock, (0-2 h) 323 genes that are involved in phospholipid syntheses (INO1, OLE1, OPI3), transcription (RPA49, NSR1, DBP2), and 94 genes encodes for ribosomal proteins are induced. However, the expression was drastically repressed at longer shock conditions (up to 24 h) at 10°C and 4°C (Sahara et al., 2002; Schade et al., 2004).

With the continuous shock situation (4-24 h) genes of HSPs, reserve carbohydrate synthesis, oxidative stress response and ROS detoxification are induced (Homma et al., 2003; Kandror et al., 2004; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004).

At mid to low temperature, transcriptional machinery is induced for the purpose of growth. This growth stops as the ambient temperature decreases further on. At this stage, freeze protective machinery is activated through the transcription of genes involved in fatty acid desaturation and synthesis of osmoprotectants. These are

followed by the growth arrest at near freezing temperatures with activation of protective response, such as oxidative stress response and HSPs (see Figure 3).

Genes of the HSP family are differentially regulated dependent on temperature and it is suggested that the cellular response at 10°C varies from the response at 4°C (Murata et al., 2006). (Murata et al., 2006). At 10°C, the following HSP genes are highly repressed but are induced at lower temperatures: HSP30, HSP60, HSP78, HSP82, SSA1, SSA2 and HSP 150.

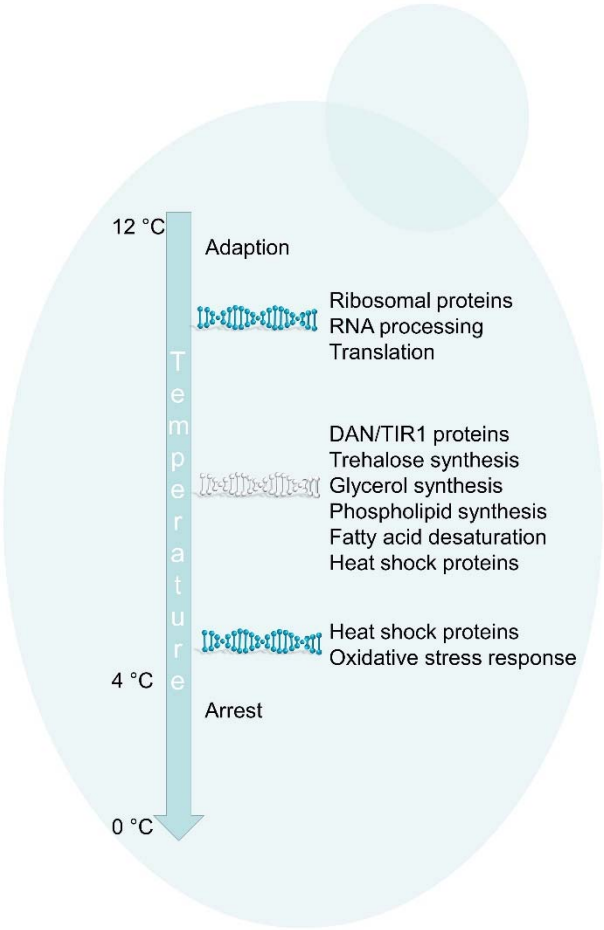

Figure 3: Schematic procedure of sequential induction of gene expression throughout cold adaption until growth arrest of yeast cells (modified according to Aguilera *et al*., 2007).

1.3 Homologues promoters of *Saccharomyces* yeast for metabolic engineering

Yeast are highly adapted on different stressors in a natural environment and due to the domestication on multiple stressors during industrial fermentation processes. Notwithstanding, the ongoing industrialisation and process optimisations in the food and beverage industry request new demands of industrial yeasts. In order to overcome

the absence or unfavourable characteristics of yeast without disturbing the general performance, genetic technology has been used for years. Besides the classical breeding methods, genetic modification by gene technology is highly established primarily due to the enormous knowledge of the genetic constitution of *S. cerevisiae.*
Gene deletion, the integration of heterologous genes, overexpressing of genes or optimised regulation of the gene expression are main focuses in the optimisation of industrial yeasts. For the regulation of the gene expression, constitutive and regulative promoters are well characterised (Table 6). Constitutive promoters transcribe independently of environmental conditions and growth phases and are mostly involved in the central catabolic pathway. In order to compare promoter strength, a comparable experimental setup should be given, e.g. copy number by integrative experiment versus plasmid-based investigations. PARTOW and colleagues published such a promoter screening experiment with an increased number of different constitutive promoters (Partow, Siewers, Bjorn, Nielsen, & Maury, 2010):

$$P_{TEF1} \sim P_{HXT7} > P_{PGK1} > P_{TPI1} \sim P_{TDH3} > P_{PYK1} \sim P_{ADH1}$$

Even with the usage of constitutive promoters, a variation in the expression strength could occur by the metabolic mode (fermentative versus respiratory), which demonstrates the glucose-dependent expression patterns (Partow et al., 2010; Sun-Hee, Hyoun-Kyoung, & Han-Seung, 2012). In particular, the Promoter P_{HXT7} shows strong differences in the regulation, according to the glucose concentration (Partow et al., 2010).
Based on the metabolic burden or the accumulation of toxic substances, high constitutive expression of genes in a pathway could be counterproductive. Therefore, the regulation of gene expression holds main interest with the fine-tuned production of homologous or heterologous proteins. Furthermore, for cell growth without the burden caused by the product formation, decoupling of these two phases is indispensable to ensure a good balance between flux concerning final product and the metabolic requirements of the yeast cell (Hubmann, Thevelein, & Nevoigt, 2014). An ideal inducible promoter for the industrial fermentation of yeast must: (i) be tightly regulated, (ii) be inexpensive to induce, (iii) express at high levels after induction, and (iv) be easy to handle (Nevoigt et al., 2007). Ideally, regulative promoters function in an on-off

Table 6: Commonly-used constitutive promoters for metabolic engineering of *Saccharomyces* yeast (Hirosawa et al., 2004; Iijimalijima & Ogata, 2010; Mumberg, Muller, & Funk, 1995; Partow et al., 2010; Sun et al., 2012)

Promoters	Physiological function	Metabolic mode
ADH1	Alcoholdehydrogenase 1	Glycolysis
CYC1	Cytochrom C	Respiration
ENO2	Endolase	Glycolysis
FBA1	Fructose 1,6-bisphosphate aldolase	Glycolysis
GPM1	Tetrameric phosphoglycerate mutase	Glycolysis/ Gluconeogenesis
HXT7	Glucose transporter	Glycolysis
PDC1	Pyruvat decarboxylase	Glycolysis
PGK1	3-phosphoglycerate kinase	Glycolysis/ Gluconeogenesis
PGI1	Phosphoglucose isomerase	Gluconeogenesis
PYK1	Pyruvate kinase	Glycolysis
TDH2/3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis/ Gluconeogenesis
TEF1/2	Translational elongation factor EF-1 alpha	Translation
TPI1	Triose phosphate isomerase	Glycolysis

switch mode for inducers or repressors by external stimuli or the accumulation of cellular metabolites (Hubmann et al., 2014). The best-known representatives of regulative promoters are members of the GAL family. The promoters $_P$GAL1, $_P$GAL7 and $_P$GAL10 are strongly induced by galactose when glucose is depleted. The interaction of the TFs Gal4p, Gal80p and Gal3p are responsible for the induction (Hahn & Young, 2011; Hawkins & Smolke, 2006; West, Yocum, & Ptashne, 1984). Besides galactose, other sugars such as maltose and sucrose could also induce regulative gene expression; however, glucose repression overacts induction by the respective sugar (Finley, Zhang, Zhong, & Stanyon, 2002; Park, Shiba, Lijima, Kobayashi, & Hishinuma, 1993). This is the limitation for usage of such promoters in mixed substrates such as wort.

Another well-known inducible promoter is $_P$CUP1 (CUP1 encodes for metallothionein), which induces the gene expression in the presence of Cu^{2+} ions in the medium (Farhi et al., 2006). The responsible TF is Cup2p (Labbe & Thiele, 1999). The depletion of nutrient such as inorganic phosphates, methionine or glucose results in the activation of the promoters $_P$PHO5, $_P$MET3 and $_P$ADH2, respectively (K. M. Lee & DaSilva, 2005; Mountain, Bystrom, Larsen, & Korch, 1991; Mumberg, Muller, & Funk, 1994).

The mentioned promoters – regarding whether used for metabolic engineering –are not suitable for usage in food and beverage production, caused by the mixed substrates (e.g. wort and must) and the prohibition of additives. Investigations for usable promoters in a food and beverage fermentation context are occasional.

The usage of bioprocess parameters such as the availability of oxygen or shifts in temperature is also considered as an inductor for gene expression. This results from the benefit of the controllability, whereby small changes in temperature and oxygen are easy to implement into fermentation processes. However, previous research has focused on promoters of the DAN/TIR family for induction under anaerobic conditions In the promoter sequence of all eight DAN/TIR members the anaerobic response elements AR1 (consensus sequence TCGTTYAG) and carrier DAN1, DAN2 and DAN3 were found, as well as AR2 (consensus sequence AAAAATTGTTGA) (Cohen, Sertil, Abramova, Davies, & Lowry, 2001). The gene expression is regulated by the repression factors Mox1/2, Mot1p, Rox1p and activator Mox4 and possibly other TFs (Abramova et al., 2001; Cohen et al., 2001; Sertil, Kapoor, Cohen, Abramova, & Lowry, 2003). However, metabolic or environmental changes associated with anaerobia such as ethanol production, reduction of pH, sterol depletion also lead to gene expression and are not excluded as an inductor (Cohen et al., 2001). Furthermore, the optimisation of regulation properties of the $_P$DAN1 was investigated by simple sequence changes. This demonstrates the possibility of user-specific engineering of existing promoters or the creation of new ones (Nevoigt et al., 2007).

The usage of temperature-regulated gene expression based on several studies with temperature-sensitive SIR3 mutation in haploid *Saccharomyces* yeast (Cheng & Yang, 1996; Kobayashi, Nakazawa, Harashima, & Oshima, 1990; Sledziewski, Bell, Kelsay, & MacKay, 1988). SIR genes (silent information regulator) are the silent copies of the additional mating-type information and encoded by the genes HMR (for **a**-mating) and HML (for **α**-Mating). Concerning to a mutation of SIR3, several mating-type regulatory proteins were synthesised and realised a temperature depending on expression by repression of Mat**a**-specific operator regions (31 bp), which functions as a promoter for the genes of interest (Rine & Herskowitz, 1987). These studies shown the transcription at 25°C and repression at 30-35°C (Kobayashi et al., 1990; Sledziewski et al., 1988) and vice versa (Cheng & Yang, 1996).

1.4 Motivation

The previous chapters provided an overview of the stress responses of yeast during industrial fermentation processes such as brewing, given that the genetic response varies with type and intensity of stress. The industrial procedure is far away from the natural circumstances of yeast - notwithstanding adaption throughout industrialisation - and leads to a gene expression induced by bioprocess parameters. Temperature is a stressor that occurs during fermentation in different phases: transition from propagation to fermentation and at the end of the main fermentation through the transition to maturation (B. R. Gibson et al., 2007). In comparison to ethanol, substrate composition and nutrition availability, temperature is an influenceable stressor, without direct interferences of the product composition (B. R. Gibson et al., 2007; Lagunas, 1993; Piper, 1995). At present, little is known about stress-induced promoters for metabolic engineering, which is characterised by targeted modification of the intermediary metabolism using recombinant techniques (Nielsen, 1998). Concerning the manifold occurrence of stressors during industrial fermentation and the mentioned overlap of stress responses, usable promoters for metabolic engineering are not characterised. Additionally, to find the optimal expression strength for a gene in a metabolic engineering approach, it is best to test different promoters. Furthermore, the usage of the described regulative promoters for gene expression is not allowed in food and beverage production caused by the induction through additives such as copper or galactose (Hahn & Young, 2011; Hawkins & Smolke, 2006; Labbe & Thiele, 1999; West et al., 1984). A tight regulation of promoters for the industrial application is indispensable to avoid interruption of fermentation processes or metabolic burden. This further ensures, that relevant precursors for the synthesis of the metabolic product are available.

Besides the problematic of a tight-regulated induced gene expression by bioprocess parameters, an additional factor is the usage of genetic modified organism in food and beverage production. The declaration of GM food – namely food that is produced by genetically-modified organisms, plants or animals – depends on each individual country´s law. In Europe, for instance, the production of such food is not allowed, whereas in the US there is no differentiation between GMO and non-GMO (Sheldon, 2001). In China, such GM food has to be labelled (MOH, 2002). Furthermore, the consumer acceptance of such products is quite low in Europe ("Eurobarometer 238

"Risk issue"," 2006; Eurobarometer, 2010; "Special Eurobarometer 244b "Europeans in Biotechnology in 2005: Patterns and Trends"," 2006), but relish greater acceptance in USA and developing countries (Ceccoli & Hixon, 2012; Curtis, McCluskey, & Wahl, 2004; Lusk & Rozan, 2005). This low acceptance could be explained by the usage of heterologous genetic material to produce such organisms (Figure 4). In order to overcome this declaration the absolute renouncement of heterologous nucleic acids is indispensable. The term self-cloning implies the enzymatic or mechanic modification, re-arrangement and re-insertion of homologue nucleic acids, and it is – for instance – defined by the European directive 2009/41/EG, and implemented to the German Genetic Engineering Act ("Gesetz zur Regelung der Gentechnik - GenTG"). Furthermore, such self-cloning techniques are equated with classical breeding methods such as cell fusion and mutagenesis. Therefore, the fundamental question of the stress-regulated gene expression of industrial yeast during fermentative food and beverage production has not yet been answered. Such stress-regulated promoters could be a possibility for growth-uncoupled gene induction in industrial application.

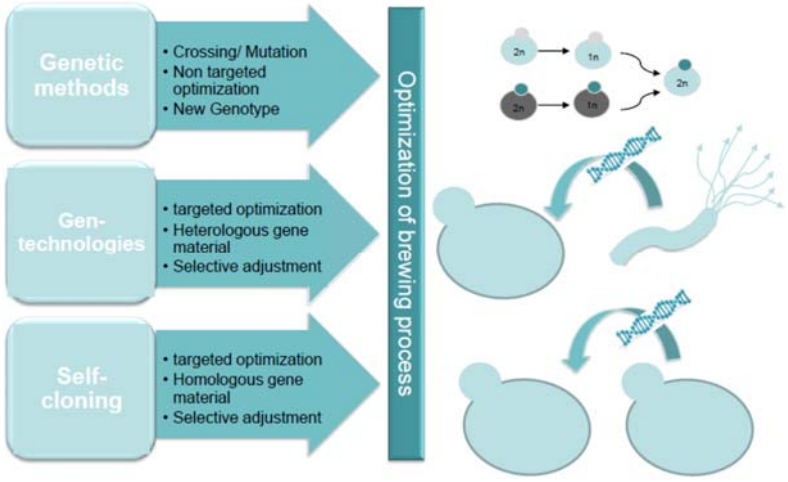

Figure 4: Schematic overview of the differences in the manipulation of industrial yeast.

The main objective of this thesis is the biotechnological generation and evaluation of process optimised *Saccharomyces* yeasts for temperature-induced gene expression. It should be clarified how temperature shifts influence the induction of stress-relevant genes of the industrial yeast *Saccharomyces pastorianus* TUM 34/70 and *Saccharomyces cerevisiae* TUM 68.

Based on this research knowledge, the following hypotheses were formulated:

- *The induction if the stress-related promoters are uncoupled from growth phases and unaffected at higher concentrations of ethanol.*
- *The evaluated promoters of homologous cold and heat shock genes show various induction patterns under equal stress situations and thus a various metabolite profile.*
- *The process-induced induction of the evaluating promoters leads to an overexpression of the target gene.*
- *Throughout "fine tuning" of shock situations (temperature shift and duration), the biosynthesis of the target gene is controllable.*
- *The evaluated shock situations are unaffected by the viability and vitality of the yeast population.*

In order to overcome the unfavourable characteristics of industrial yeast or adaption on the accelerating processes, a subset of regulative homologues promoters of industrial yeasts could offer new possibilities for induced gene expression in food and beverage production. Furthermore, based on a self-cloning procedure, targeting genetic modification of the yeast is possible without the GMO labelling in most countries (aside from the EU).

2. Summary of results (Thesis publications)

The results are summarized in this chapter, followed by copies of the individual publications.

Part I Page 28	Self-cloning brewing yeast: a new dimension in beverage production

Since the mid-1990s, biotechnology has advanced, and there has been an increased focus on using genetically modified yeast in the production of fermented beverages and the manufacturing of bioethanol. Yeast is the primary microorganism for fermented beverages such as beer, wine and sake. However, existing individual strains will not completely fulfill future demands for an efficient and high-quality fermentation. In this case, several research groups have been working on genetic modifications of yeast to create an up-to-date application. Genetically modified organisms (GMO) such as yeast, crops and plants in the food and beverage production are not desired by the consumer. A possible solution to overcome the consumer distaste of products labeled as containing GMO could be the application of self-cloning yeasts. Thus, connotated, the modification of the genome occurs without heterologous DNA. This review is an overview of current research regarding the use of self-cloning yeast in brewing, wine making, baked goods and sake production. The main focus of this paper concerns the possibilities of promoter usage, the construction of self-cloning yeast, and the monitoring of self-cloning yeast.

Part II EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system
Page 41

Targeted induced gene expression for industrial fermentation processes in food and beverage production could fulfill future requirements. Up to now, there is limited data of inducible expression patterns for targeted gene expression under such specific conditions. For the evaluation of temperature induced native promoters, the widely used reporter gene “enhanced green fluorescence protein” (EGFP) by utilizing high throughput systems was applied. Five different promoters of the industrial yeast strain *Saccharomyces cerevisiae* TUM 68 were evaluated ($_P$HSP12, $_P$HSP26, $_P$HSP30, $_P$HSP104, and $_P$SSA3). They are induced during temperature shifts, which may occur in transition of fermentation to maturation. Furthermore, the induction of gene expression affected by different contents of ethanol were investigated, by using synthetic wort that mimics a 12 °P wort. Promoters $_P$HSP30 and $_P$SSA3 showed the highest fluorescence value during temperature shift from 20°C to 10°C. A temperature shift from 20°C to 4°C resulted in highest fluorescence values of $_P$SSA3 and $_P$HSP26. Further, these promoters showed the lowest induction value by ethanol concentrations between 4 and 6%-vol. With this method, it is possible to evaluate native temperature induced promoters for the usage in self-cloning brewing yeast under strict industrial conditions.

Part III Page 48	Induced gene expression in industrial *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70: Evaluation of temperature and ethanol inducible native promoters

Induced gene expression is an important trait in yeast metabolic engineering, but current regulations prevent the use of conventional expression systems, such as galactose and copper, in food and beverage fermentations. This article examines the suitability of temperature-inducible native promoters for use in the industrial yeast strain *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70 under brewing conditions. Ten different promoters were cloned and characterized under varying temperature shifts and ethanol concentrations using a green fluorescent protein reporter. The activities of these promoters varied depending upon the stress conditions applied. A temperature shift to 4°C led to the highest fold changes of $_{P}$SSA3, $_{P}$UBI4 and $_{P}$HSP104 by 5.4, 4.5 and 5.0, respectively. Ethanol shock at 24°C showed marked, concentration-dependent induction of the promoters. Here, $_{P}$HSP104 showed its highest induction at ethanol concentrations between 4% (v/v) and 6% (v/v). The highest fold changes of $_{P}$SSA3 and $_{P}$UBI4 were found at 10% (v/v) ethanol. In comparison, the ethanol shock at a typical fermentation temperature (12°C) leads to lower induction patterns of these promoters. Taken together, the data show that three promoters ($_{P}$HSP104, $_{P}$UBI4 and $_{P}$SSA3) have high potential for targeted gene expression in self-cloning brewing yeast using temperature shifts.

Part IV	Induced expression of the alcohol acetyltransferase gene *ATF1* in
Page 58	industrial yeast *Saccharomyces pastorianus* TUM 34/70

Targeted induced gene expression for industrial fermentation processes in food and beverage production could fulfill future demands. To avoid metabolic burden and disturbances due to the fermentation procedure, induced gene expression is necessary for combating stress, such as that caused by temperature shifts that occur during the transition from fermentation to maturation in the brewing process. The aim of this study was to target gene expression in industrial yeast using stress-responsive promoters and homologs of the selection marker SMR1. Self-cloning strains of the industrial brewing yeast *Saccharomyces pastorianus* TUM 34/70 were constructed to overexpress the alcohol acetyltransferase (ATF1) gene under the control of inducible promoters $_{P}$SSA3, $_{P}$HSP104, and $_{P}$UBI4.

Transcription analysis shows the highest induction after 72 h of shock situation for $_{P}$HSP104 with 1.3-fold and $_{P}$UBI4 with 2.2-fold. Further, at the end of shock situation the concentrations of ethyl acetate were 1.2-fold and 1.3-fold higher than the wild type, for $_{P}$HSP104 and $_{P}$UBI4, respectively. In addition, the influence of the final temperature and temporal sequence of temperature shock to 4°C had a major impact on expression patterns. Therefore, these data show that temperature-induced gene expression of self-cloning industrial yeast could be an option for optimization of the beverage fermentation.

2.1 Self-cloning brewing yeast: a new dimension in beverage production

Eur Food Res Technol (2013) 237:851–863
DOI 10.1007/s00217-013-2092-9

REVIEW PAPER

Self-cloning brewing yeast: a new dimension in beverage production

S. Fischer · S. Procopio · T. Becker

Received: 7 March 2013 / Revised: 30 July 2013 / Accepted: 17 August 2013 / Published online: 19 September 2013

Abstract Since the mid-1990s, biotechnology has advanced, and there has been an increased focus on using genetically modified yeast in the production of fermented beverages and the manufacturing of bioethanol. Yeast is the primary microorganism for fermented beverages such as beer, wine and sake. However, existing individual strains will not completely fulfill future demands for an efficient and high-quality fermentation. In this case, several research groups have been working on genetic modifications of yeast to create an up-to-date application. Genetically modified organisms (GMO) such as yeast, crops and plants in the food and beverage production are not desired by the consumer. A possible solution to overcome the consumer distaste of products labeled as containing GMO could be the application of self-cloning yeasts. Thus, connotated, the modification of the genome occurs without heterologous DNA. This review is an overview of current research regarding the use of self-cloning yeast in brewing, wine making, baked goods and sake production. The main focus of this paper concerns the possibilities of promoter usage and the construction of self-cloning yeast and the monitoring of self-cloning yeast.

Keywords Self-cloning · Beer · Wine · *Saccharomyces cerevisiae* · Industrial application

S. Fischer · S. Procopio (✉) · T. Becker
Lehrstuhl für Brau- und Getränketechnologie, Technische Universität München, Weihenstephaner Steig 20, 85354 Freising, Weihenstephan, Germany
e-mail: Susanne.procopio@wzw.tum.de

S. Fischer
e-mail: susann.fischer@wzw.tum.de

Introduction

The yeast *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* var. *carlsbergensis* are important values which greatly influence the end product of fermented beverages such as beer and wine. Since Louis Pasteur described yeast as a microorganism which converts sugar to ethanol, scientists have overwhelmingly focused on understanding the different genetic processes that occur during fermentation. Currently, *S. cerevisiae* has the status of a eukaryotic model organism in microbiology, biochemistry and genetics. It is classified as GRAS (generally recognized as safe).

In 1996, Goffeau and his co-workers published the whole sequence of *S. cerevisiae* [1]. Since that time, research in the field of genetic engineering of yeast has drastically increased. The applied research is focused on the application of self-cloning yeast in fermented beverage industries like brewing, vinification and sake production.

Saccharomyces spp. causes specific characteristics during the production such as premature or minor flocculation of yeast cells [2, 3], deficient ethanol [4, 5] and osmotic stress tolerance, minor diacetyl degradation [6, 7] minor glycerol production [8, 9] and insufficient carbohydrate transport into the cell such as dextrine and maltotriose [10–13]. These are also summarized by several reviewers [14–17].

To overcome the absence and/or unfavorable characteristics of yeast without affecting its performance, genetic technology is used. For improving industrial yeast strains without using genetically modifying techniques, selected yeast strains with positive characteristics are crossed to generate even better strains [16]. The advantage of genetic technology compared with classical breeding methods is that just one characteristic can be modified, and the

heterologous gene can be expressed, without influencing other properties.

Genetically modified organism (GMO) possesses DNA sequences, which have been altered using genetic engineering techniques such as recombinant DNA technology. DNA molecules can be original or obtained from other organisms followed by a transfer into the organism as well as antibiotic or other selective markers. Organisms with inserted DNA from different species are called transgenic organisms. However, the self-cloning organism does not harbor any foreign DNA molecules in the final strain. The usages of plasmids, which possess foreign DNA sequences for the manipulation procedure, are possible. After which, the foreign DNA sequences must be removed. GM yeast is a processing aid, which has been granted GRAS status by the American Food and Drug Association (FDA) when used as intended. To gain this status, additives must be reviewed by independent government experts [18].

Genetically modified food (GM food) is produced by genetically modified microorganism or genetically modified plants and animals.

In most countries, genetically engineered yeast has no consumer acceptance. The regulation of GMO use in the food and beverage industry is dependent upon each individual country's laws.

A self-cloning organism is modified without heterologous nucleic acids or even nucleic acids from closely related organisms. However, there is no clear definition of close relation. In the case of self-cloning organisms, governments hold diverse opinions in regard to them. The genetic directives (2009/41/EC) of the European Union (EU) describe techniques and methods of genetic modification, such as mutagenesis, cell-fusion (incl. protoplast fusion) and self-cloning as not yielding to a genetically modified microorganism in contained use [19]. Indeed, the usage of self-cloning organism for industrial application is still not in contained use.

In the USA, there is no difference between the labels of GMO and non-GMO [20]. Yeast is a special case, and the FDA classifies it as processing aide. Therefore, beverages produced by GM yeast are not required to be labeled as GM products [21]. The Japanese government does not categorize the self-cloning under the GMO regulation [22]. In 2006, the central government of India, in collaboration with the central committee for food standards added the "Draft Rule 37-E Labeling of Genetically Modified Food" into the prevention of "Food Adulteration Rules" [23]. This rule states that all primary or processed food, food ingredients or food additives derived from a GM food are required to be labeled. Imported food derived from GM food should also indicate the status of approval from its country of origin.

In the case of the *Mercosur* states (an economic and political agreement among several South American states), there is no regulation of labeling from GM food or additives derived from GMO. Indeed, in Brazilian food and ingredient products from animals, which are fed transgenic ingredients, have to be labeled. In the case of Paraguay, there is no specific regulation of GMO in the Law No. 1334/1998 of consumer and user defense. The biosafety national cabinet of Uruguay (GNBIO No. 468/2008) promotes actions leading to the voluntary labeling of GM or non-GM food [24].

In China, the ministry of health (MOH) regulation No. 28 "Administrative Provisions for Genetically Modified Food Hygiene" that food and food additives which are produced by genetically modified plants, animals and even microorganism must be labeled [25]. Self-cloning technology is defined but does not differentiate between GMO and self-cloning [26].

GM food has a higher level of acceptance in the USA than in Europe [27, 28]. In undeveloped countries, there is much greater acceptance of GM foods which coincides with the concern of food availability and nutritional intake [29]. Sixty-two percent of EU citizens do not see any benefit of GM foods [30], and 50 percent of EU citizens are worried about GM food and drinks and deem it morally unacceptable, fundamentally unnatural and a risk for society [31, 32].

The disparity of acceptance between countries results in little knowledge about GM foods [33]. Several research groups pointed out that knowledge is the important factor of the willingness to buy GM foods [27, 34–36]. Different studies show that consumer acceptance of GM food decreases with a mandatory labeling for such products. United States consumers are fairly confident about the current FDA policy, which has approved numerous GM crops. In comparison, the Europe Union has a mandatory policy, which enforces the labeling of GM food [37, 38].

Acceptance of the GM product depends on the type of food product [28, 39]. GM plants and microorganism have a much higher acceptance rate than GM animals and meat [40, 41].

In the case of consumer acceptance of beer, the impact of the appreciation of the beer in relation to the manufacturing process was studied by Caporale and Monteleone [42]. They pointed out that beer produced with GM yeast has a negative impact on consumer appreciation. In contrast to this, the usages of organic or traditional technologies are well respected.

The advantage of self-cloning, such as abdication of drug-resistant markers or the usage of native DNA sequences for optimization of the yeast for different application steps could be a key agreement for consumer application.

Genetically modified and self-cloning yeast in process

The first genetically modified organism authorized for food production in UK was baker's yeast. This strain produced 30 percent more CO_2 than the parental strain [43, 44]. In the case of fermented beverages, the UK commercialized the brewing yeast which contains the *STA2* gene for extracellular production of glycoamylase in 1994 [45]. But neither of these authorized yeasts was used in the industry.

There are only two authorized genetically modified wine yeast strains in Canada and USA, which are still in use. ML 01 is a *prise du mousse* strain which contains the malate transport gene (mae1) from *Schizosaccharomyces pombe* and the malolactic gene from *Oenococcus oeni*. This yeast is able to decarboxylate up to 9.2 g/L malate to equimolar amounts of lactate [46–48] which reduces the production time by omitting the malolactic fermentation. The second authorized strain is ECMo01, a *S. cerevisiae* strain Davis 522 with an additional urea amidolyase from *S. cerevisiae* strain TCY1 (encoded by DUR1,2) which is able to catalyze the hydrolysis of urea. Urea is a precursor of ethyl carbamate (EC), which is suspected to be a mutagen carcinogen to humans [49, 50]. Beer also contains low levels of EC (up to 5 μg/kg) [51, 52]. The reduction in EC in alcoholic beverages, like wine, fortified wines and distilled spirits is important since the EC content is limited in Canada, USA, Czech Republic and France [52]. The usage of a urea hydrolyzing yeast can drastically minimize the problem of high EC content, which could be an important fact for alcoholic beverages.

Table 1 show several yeasts which were modified by self-cloning techniques. In the case of brewing yeast, they focus on the enhancement of glutathione as a main antioxidant, which also correlate with flavor stability [53–55] and the incensement of the sulfite content [56]. Another point is the reduction in proteinase A for a better foam stability, since proteinase A is involved in degradation of foam-active proteins [53]. Further aspects are enhancement of yeast flocculation by overexpression of lectin-like proteins [57], reduction in acetaldehyde as a by-product of ethanol production [54] and reduction in vicinal diketone by metabolization of α-acetolactate and α-aceto-α–hydroxybutyrate [7].

For the application in sake production there are different starting points. One point is the reduction in EC [58]. Another one is the focus on flavor enhancement [59, 60] and the accumulation of stress-relevant amino acids against ethanol stress like proline [61].

For the application of self-cloning baker's yeast research predominantly focus on tolerance to freeze and thaw as well as the viability of the yeast [62–66].

In the case of wine yeast, the existing research focuses on the reduction in acetic acid during wine fermentation [67].

Promoters for self-cloning yeast

For the construction of self-cloning yeast, understanding how to handle the gene expression is necessary. *S. cerevisiae* is well adapted to different stress conditions, such as cryo- and osmo-tolerance as well as tolerance against ethanol and high temperatures [68]. This adaptation is generated from different stress-response genes, which are regulated by transcription-binding sites on the promoter and the transcription factors.

The expression of genes is highly regulated by the promoter's enhancers and silencers. The structure of promoters in eukaryotes is extremely diverse and complex. Promoters are predominantly located upstream of the gene and have different elements, such as core promoter, proximal promoter and distal promoter [69]. The core promoter is the minimal part necessary (approximately 60 bp long DNA sequence) [70] to initiate the transcription with the initiator, BRE element (TFIIB-recognition element, for transcription factor IIB) [71] and occasionally, TATA-boxes [72, 73]. The TATA-box is a stress-sensitive regulatory motif. The TATA-box-binding protein (TBP) is an individual transcription factor and used by all three RNA polymerases [74]. In total, there are 19.2 percent of the TATA-box containing genes in the genome of *S. cerevisiae*. About 50 percent occurs in duplicated genes, resulting in higher expressed variability [75].

In the case of the differential regulation of RNA transcription in yeast, there are different transcription factors involved [76–80]. These promoters are more suitable when expression of genes is needed within a specific part of the fermentation process and for an uninterrupted fermentation process. Another point is the availability of precursors in the specific fermentation stage. Indeed, the intensity of expression is limited by the sensitivity to the inducer such as temperature or ethanol content.

For the construction of yeast with induced and constitutive expressions, there are several promoters from *S. cerevisiae* described in the literature [81, 82]. The application of different promoters in yeast for beer, wine, sake and bioethanol production is described below.

One major research topic is the flocculation of yeast. A lot of work is spent on the induction of native promoters at the end of fermentation. The capability of flocculation is an important characteristic of yeast in the brewing and wine industry. The yeast cells coagulate, settle down and can be separated effectively and economically from the product. The genes FLO1, FLO5, FLO9, FLO 10 and FLO11 are involved [2, 83, 84]. For the induction of the gene expression in the late stationary fermentation phase in wort or synthetic must, the promoter of the gene HSP30 (Heat shock protein) is used [85–89]. During the exponential phase of fermentation, the promoter is strongly repressed.

Table 1 Self-cloning yeast for industrial application

Genes	Gene function	Gene manipulation	Product benefit/Phenotype	Ref.
Brewing yeast				
FLO1	Lectin-like protein	Integration (URA3 locus)	Increased flocculation	[57]
URA3	Orotidine-5′-phosphate decarboxylase	Reintegration	Higher ethanol production	
GSH1	-Glutamylcysteine synthetase	Integration (Overexpression)	Increase in glutathione and foam stability in beer	[53]
PEP4	Proteinase A	Disruption		
GSH1	-Glutamylcysteine synthetase	Integration (Overexpression)	Decrease in acetaldehyde, increase in glutathione	[54]
CUP1	Metallothionein	Integration (Overexpression)	Copper resistance as selection marker	
ADH2	Alcohol dehydrogenase	Disruption		
$_{P}$TDH3	Promoter of glyceraldehyde-3-phosphate dehydrogenase	Replacement in $_{P}$SSU1	Increased sulfite concentration	[56]
$_{P}$SSU1	Promoter of plasma membrane sulfite pump		Decreased hydrogen sulfite, MBT and 2M3 MB	
ILV2	α-Acetohydroxyacid synthetase	Disruption	Decrease in diacetyl content, increase in glutathione	[55]
GSH1	-Glutamylcysteine synthetase	Integration (Overexpression)	Copper resistance as selection marker	
CUP1	Metallothionein	Integration (Overexpression)		
ILV2	α-Acetohydroxyacid synthetase	Replaced by SMR1B	Decrease in vicinal diketone (VDK) precursors	[7]
ILV5	Bifunctional acetohydroxyacid reductoisomerase	Integration	Sulfometuron methyl (SM) resistance	
SMR1B	Sulfometuron methyl-resistant marker (point mutation of ILV2)			
Sake yeast				
$_{P}$TDH3	Promoter of glyceraldehyde-3-phosphate dehydrogenase	Replacement in $_{P}$ATF1	Increase in isoamyl acetate and decrease in acetic acid	[59]
$_{P}$ATF1	Promoter of alcohol acetyltransferase1			
FAS2	α Subunit of fatty acid synthetase	Point Mutation	Resistance to cerulenin	[60]
		Gly → Ser at 1250th Codon	Increase in ethyl caproate	
PRO1	- Glutamyl-kinase	Integration	Increased proline accumulation	[61]
PUT1	Proline oxidase	Disruption		
DUR1,2	Urea amidolyase	Integration (Overexpression)	Increased reduction in ethyl carbamate	[58]
URA3	Orotidine-5′-phosphate decarboxylase	Disruption	Expression increased 9.3 - 12.8 fold	
Baker's yeast				
PRO1	- Glutamyl-kinase	Replacement (with L150T Allele – less sensitive)	Accumulation of proline and trehalose	[62]
NTH1	Neutral trehalase	Disruption	Enhanced freeze tolerance Enhanced fermentation ability	
PRO1	- Glutamyl-kinase	Replacement (with L150T Allele – less sensitive)	Increase intercellular ROS and NO level	[63]
MPR1	*N*-Acetyltransferase	Replacement (with F65L Allele – thermo tolerant)	Increase intracellular proline content	
PUT1	Proline oxidase	Disruption		
MSN2	Transcriptional activator	Integration	Higher freeze-draw tolerance Enhanced intracellular trehalose level Enhanced fermentation ability	[64]
PRO1	- Glutamyl-kinase	Replacement with L150T	Higher cell viability	[65]
PUT1	Proline oxidase	Disruption	Lower intracellular oxidation level Enhance fermentation ability	
PRO1	- Glutamyl-kinase	Replacement with L150T and D145 N Allele	Enhance intracellular proline	[66]
PUT1	Proline oxidase	Disruption	Higher fermentation ability	
Wine yeast				
YAP1	Basic leucine zipper transcription factor	Chemical mutagenesis, result in stop codon in C-terminus	Less production of acetic acid during fermentation	[67]

This promoter is controlled by nutritional limitation and ethanol content, whereas the ethanol content is the stronger inducer [90].

Another promoter which has been studied for stationary phase induction is the promoter of the alcohol dehydrogenase 2 [87, 91]. However, $_{p}$ADH2 shows only the stationary phase induction when it is modified. CUNHA and coworkers (2006) fused the native core promoter of the gene FLO5 into the regulatory region of the $_{p}$ADH2. In the presence of glucose, the transcription is fully eliminated. The promoter of the gene TPS1 is studied by Li et al. [92]. This gene encodes for the trehalose-6-phosphate synthetase 1. The gene cassette with $_{p}$TPS1 upstream of the gene FLO1 had been transformed in non-flocculation yeast. The flocculation is triggered by 3 percent (v/v) ethanol and is completed by 8 percent (v/v) ethanol [92].

The promoter of the maltose transporter gene AGT1 (alias MAL11) from the ale strain has a strong expression of maltose and has only a low but significant expression of glucose [93]. In lager strains, the expression of the AGT1 promoter is weak in comparison with the ale strain, but has a strong repression through glucose. This differentiates the result of that from some of the Mig1 and MAL-activator binding sites, which are located in the promoter region of the ale strain.

To improve the flavor content in sake, the promoter of the cell wall protein Sed1p was fused upstream of the ATF2 gene [94]. The expression increased to 2.7-fold in the stationary phase in comparison with log phase [95].

The use of constitutive promoters for gene expression in application for brewing, wine and sake yeast has been comprehensively studied. There are three promoters that are widely used. The promoter of the triose-phosphate dehydrogenase (PTDH3) has a high-level activity during the glucose consumption and is used to enhance the sulfate efflux by replacing the native promoter of the plasma membrane sulfite pump (SSU1) [56] and the reduction in haze particles [96]. PPGK1 primarily induce the 3-phosphoglycerate kinase and is applied for the enhancement of urea degradation [50, 58, 97] enhancement of flocculation [92] and for the reduction in caloric content in beer [98]. The promoter of the alcohol dehydrogenase 1 is used for the recombinant expression of lipid transfer protein (LTP1) from barley for enhancement of foam stability in beer [99]. The strongest inducers are PPGK1and PTDH3 (alias PGPD); in contrast, PADH1 has only a fifth of the activity [100, 101].

For pointed expression of different genes in industrial yeast for wine, beer and sake production, more knowledge of different inducible promoters is needed. Different expression analyses show that some genes are highly regulated, e.g., genes of stress response [82], genes which are involved in carbohydrate utilization, or genes that are involved in sugar transmembrane transport [102]. PIPER and colleagues described that a minimum of 4 percent ethanol is needed for the notable induction of HSP expression. However, a subset of HSP genes shows ideal expression at different ethanol concentrations [82]. HSP26, HSP70 and HSP104 are highly induced by ethanol concentration between 4 and 10 percent. Indeed, by the ethanol concentration of 8 percent, the expression of HSP70 and HSP104 are stronger than by heat shock. HSP12 shows an optimal expression of the ethanol concentration between 4 and 6 percent with a maximal in 12-fold expression. The maximal expression induced by heat shock results in a 61-fold increase.

There is a sequential utilization of carbohydrates [103] and a regulation of genes which encodes for the correlated carbon transporters. Due to this, it is possible to use these promoters for an inducible expression of a specific medium, such as must, wort or sake must, cause of the different carbon sources. In the case of wort fermentation, some genes that are involved in sugar transmembrane transporter activity show specific transcription profiles. The hexose transporter, encoded by HXT5, has the highest transcription toward the end of fermentation. HXT2 has a broad peak in expression between 60 and 80 h. Genes which are involved in transmembrane transport of maltose and maltotriose peak in transcription after 60 and 80 h for MPH2/MPH3 and MAL11, respectively [102, 104].

Some genes that are involved in glycolysis show differential expression. ADH1 and ADH2, for example, are the highest level of expressed and occurs between 60 and 80 h of fermentation [102]. Present transcription analysis of *S. cerevisiae* can give some possibilities for medium-specific transcription regulation or even for general identification of noteworthy native promoters [89, 90, 105].

Most of the studies of promoters and gene expression are based on laboratory strains. A direct transfer of the experiments' results with lab strains to the phenotype of industrial strains fermenting in wort or must is not possible. Laboratory strains are often auxotrophic mutants (*leu2, ura3* etc.) and haploid. In contrast, industrial yeasts are diploid or even polyploidy, genetically diverse and often with low or no sporulation competence [17]. The manipulations of such industrial yeast are quite different to that of laboratory strains, justifying the necessity for the integration of genes/promoter in the genome or even the usage of a drug as a selection marker for plasmid transformation. However, the use of industrial genetically modified yeast for the production of food and beverages is indispensable [17].

The fermentation rate and resistance to stress induced by fermentation are lower in laboratory strains in comparison with industrial strains [21, 106, 107]. In the case of diploid laboratory strains, there are better fermentation conditions according to the haploid laboratory strain [108]. However,

an extrapolation of laboratory small-scale fermentation conditions to commercial large-scale fermentation conditions is possible [109].

For independent growth expression of genes, there are only a few promoters available. In microarray analysis of different expression patterns of yeast, *S. cerevisiae* demonstrates that the most regulated expression of genes is dependent on the growth phase and even medium consumption [102, 105]. Several stress-relevant genes, e.g., from the heat shock protein gene family and cold shock-inducible protein genes [82, 89, 110–113] are strongly regulated by shifts in temperature. Some genes of the heat shock protein family are also induced by ethanol, which was mentioned above. The highest expression of these genes occurs due to the increasing ethanol content (7–10 vol %) [82].

Possible selection markers for self-cloning

During the transformation process, only a small proportion of cells are transformed. For this reason, it is necessary to select the cells that transformed in a rapid and efficient way. For application of selection marker in industrial yeast, auxotrophic markers are seldom used. This occurs primarily because of the diploidity or even polyploidity of the industrial strains. HASHIMOTO and colleagues were able to isolate auxotrophic mutants of diploid Sake yeast strains after UV mutagenesis for usage of auxotrophic selection markers, such as Arg(−), Leu(−), His(−), Met(−), Lys(−), Trp(−) and Ura(−) [114]. A problem with using auxotrophic strains is that the growth rate in the nutrient-supplemented media is not categorical and physiologically equivalent to complemented transformants [115]. Another point is that description of the amino acids' concentration for the selection medium in literature is too variable [116].

The usage of drug resistance, such as G418 [117], chloramphenicol [118] and glyphosate [119], is only applied by heterologous gene expression. In the final strain, the foreign DNA must be completely eliminated for the self-cloning yeast. The selection of gene or marker eliminating is called counter selection [120]. BOECKE and colleagues [121] used the counter-selection system with URA3 as the selection marker, *ura3*$^{-}$ mutant and 5-fluoroorotic acid (5-FOA). When establishing industrial yeast without drug-resistant markers and foreign DNA, a two-step gene replacement method is used [59, 122]. The plasmid contains resistant markers for transformation selection, a galactose-inducible growth inhibitor sequence (GAL10p::GINII) for counter selection [10, 123] and modified sequence (for example by point mutation of the wild-type gene, which result in the target gene). The first step is the integration of the linearized plasmid into the target locus of the genome. After the integration into the genome, a repeated sequence of wild-type gene and a modified target gene which surrounds the plasmid sequence is to be found. The second step is the loss of the plasmid sequence by homolog recombination between a wild-type gene and target gene resulting in either a wild-type strain or in a self-cloning strain (Fig. 1).

Further, there are some native genes or even mutants which are applicable for the selection. The semi-dominant selection marker CUP1 encodes a copper-binding metallothionein resulting in copper resistance due to the overexpression of this gene [53, 54, 124, 125]. Another selection marker is the SMR1-410, which determines the resistance against sulfometuron methyl [126, 127]. These are phenotypically distinct allele of ILV2 mutant. ILV2 encodes for the acetolactate synthetase. There is a single C to T transition mutation on nucleotide 574, which results in a proline to serine change at position 192. The mutant SMR1B results in a single C–T transition mutation in the proline to leucine change at position 192 [128]. Similar to SMR1-410, the SMR1B gene leads to a resistance of sulfometuron methyl and can be utilized as a dominant selection in industrial yeast.

Actual cloning technology for construction of self-cloning yeast

As previously mentioned, the construction procedure of self-cloning yeast differs from conventional cloning techniques. The following section explores different methods of constructing yeast without leaving a trace of foreign DNA.

The seamless gene-deletion protocol is used for systematic repeated gene modification without leaving any traces of foreign DNA sequences and scars on the chromosome and removal of the selection marker [129]. This is possible, due to the repeating of short sequences (30–40 bp) of an adjacent region to the target locus which is surrounded by the selection marker after integration into the chromosome (Fig. 2a). After transformation and integration in the chromosomal locus, the transformation construct deletes the unwanted gene. Due to the repeated sequences, the homolog recombination results in the scarless loss of the marker. Therefore, the usage of selection markers originating from other genera, such as the dominant selection marker amdS derived from *Aspergillus nidulan,* is possible. This marker codes for the acetamidase, which confers the yeast *S. cerevisiae* to utilize acetamide as a sole nitrogen source. The direct repeat of the flaking short sequences of the amdSYM marker allows the efficient and scarless recombinative excision. The loss of the marker cassette is detectable by the growth in the presence of fluoroacetamide [130].

The gene cassette including promoter, gene of interest and terminator must be flanked by an identical sequence

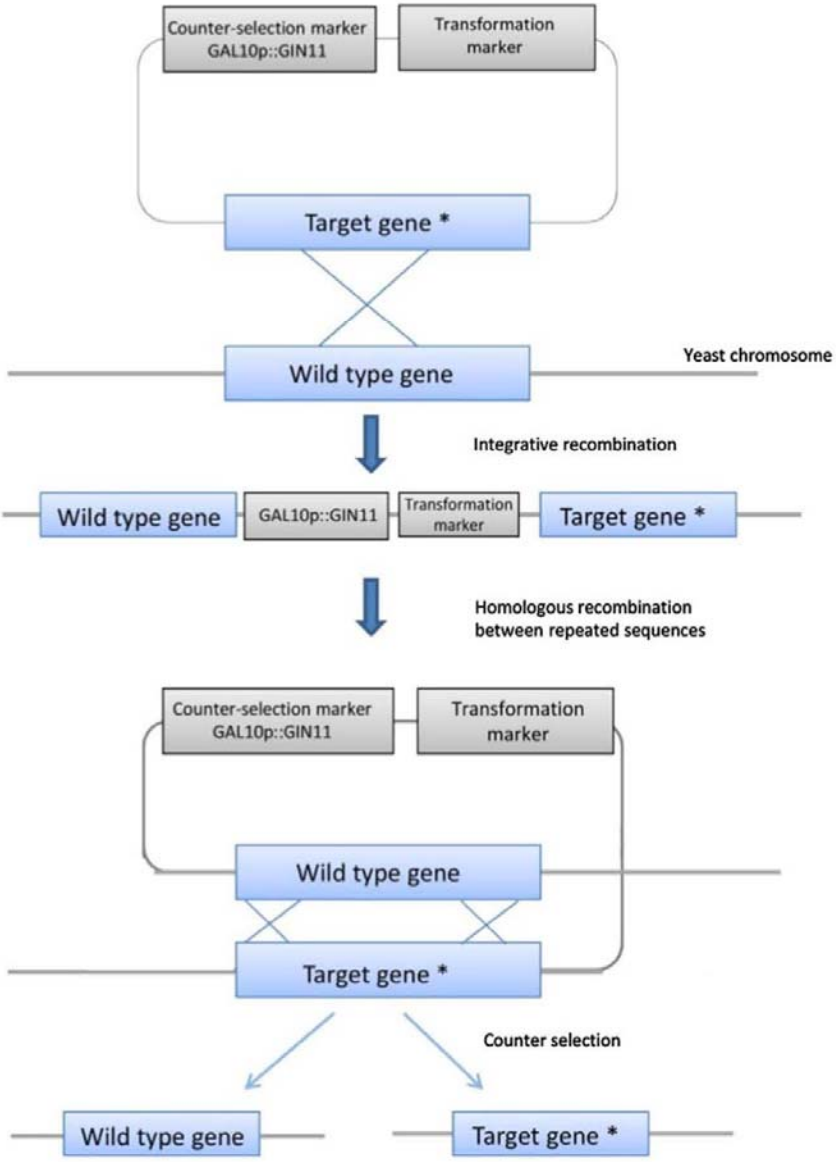

Fig. 1 Two-step gene replacement. *First step* plasmid integration, which contain transformation marker, counter-selection marker and the target gene which is mutated, into chromosome sequence. *Second step* homologs recombination between wild-type gene and target gene which results in counter-selection strain without any heterologous DNA sequence (modified according to [122])

of a gene in the genome (as example URA3, LEU2 or even HO-locus) for the overexpression of genes to occur. The integration in the genome requires the gene cassette to be separated from the vector backbone via restriction and gel purification or amplification through PCR. In this instance, it is possible that the vector contains foreign DNA or even selective markers, which will separate before transformation. The gene cassette will be integrated into the genome through homolog recombination (Fig. 2b) [53, 57, 61]. In the case of the URA3 disruption, the mutant is not sensitive to 5′Fluoorotic acid (5′FOA) which is converted to a toxic compound (5′fluoro-UMP) by Ura3p [121].

Another possible procedure is to integrate the gene cassette into a gene with an unwanted function (such as PEP4

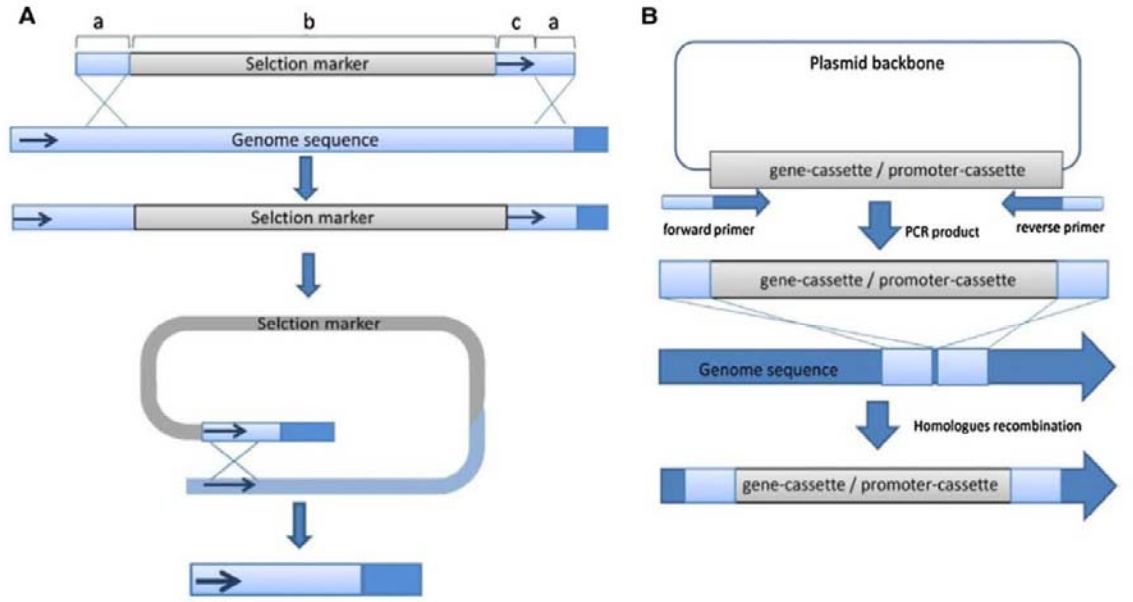

Fig. 2 Schematic diagram **A** of the seamless gene-deletion procedure (modified according to [129, 130]). Transformation construct: **a** 50–55 bp for homolog recombination; **b** selection marker module; **c** 30–40 bp for seamless marker recovery. First step is the integration of transformation construct into the locus chromosome by homolog recombination. Second step is the loss of the selection marker module by homolog recombination of the repeated short sequence (*arrows*). **b** Schematic diagram for integration of gene/promoter cassette into genome by homolog recombination. It would result in a self-cloning strain

[53], PUT1 [61] and ILV2 [55]) which results in the deletion of the unwanted gene. The selection is possible by using the copper resistant marker CUP1.

The two-step gene replacement protocol is used for promoter replacement [59], which was described in the section above. Another example of promoter replacement according to Iijima and Ogata is the construction of a plasmid containing a URA3 gene (as selection marker) and the promoter of TDH3 from *S. cerevisiae* [56]. This fraction was amplified via PCR. The forward primer contains a 59 bp sequence of the 5′SSU1 promoter region (target for replacement in the genome) and the reverse primer a 26 bp region of the 5′SSU1 coding sequence (Fig. 2b). The promoter cassette was integrated through homolog recombination into the genome.

Monitoring and survival of GMO's in the environment and fermented beverages

The release of yeast into environment during production of fermented beverages is unavoidable. In the case of beer production, the sewage is contaminated, and yeast is used as animal food. Furthermore, there could be remaining yeast cells in bottled beer and naturally in top fermenting beer. During wine production, there is also a contamination of sewage and pomace is used as fertilizer for vineyards. Permanent establishment of commercial wine yeast in vineyards (especially grape, soil, leaves and grapes) shows only limited dissemination during a short period of time (36 month) [131]. Since genetically modified yeast became available, some studies have monitored the amount of persistence in the environment.

There are several methods available for the detection of genetically modified DNA in food (Table 2). All the methods listed require the prior knowledge of distinguishing which specific GMO will be monitored [132]. In the special case of complex medium wine, elements from wine-related microorganisms, which are still present during the production procedure, occur naturally in the final product. PCR-based methods can give false-positive results [133]. Particularly, the available genetically modified wine yeast ML 01 carries two different heterologous genes from *Schizosaccharomyces pombe* and *Oenococcus oeni*.

Leon and colloquies published a method in 2011 for detecting genetically modified yeast in wine via multiplex PCR linked with capillary gel electrophoresis using laser-induced fluorescence (PCR-CGE-LIF) [134].

Schoeman and colloquies [135] analyzed the behavior of the yeast LKA1, a *S. cerevisiae* VIN 13 which carries the

Table 2 Summary of methods that specifically detect recombinant DNA or its products by food-stuff (modified according to [132])

Parameters	Protein-based			DNA-based			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very High	High	High
Duration	2 days	30–90 min	10 min	6 h	1, 5 days	2 days	1 days
Gives quantitative results	No	Yes	No	No	No	Yes	Yes
Suitable for field tests	No	Yes	Yes	No	No	Yes	Yes
Employed mainly in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

alpha amylase from *Lipomyces kononenkoae* [136]. This strain and the parental equivalent are able to survive in an artificially constructed sand soil and form stable biofilms. The GM yeast does not integrate successfully in the mixed-biofilm community or disrupt the community [135]. Grossmann and co-workers analyzed the persistence of three different GM yeasts compared with the parental strain. They pointed out that GM yeast behave as well as parental strain. In a greenhouse simulation, GM yeast becomes a part of the yeast flora on grapes. In addition, they demonstrated that GM yeast is able to survive in bottled wine [137].

Freeze-tolerant self-cloning and genetically modified baker's yeast were verified in simulated environment (sources water and soil). The decrease in GM and self-cloning viable cells were nearly equal or higher than the cells of the wild type. The disruption of the ATH1 gene that encodes for acid trehalase results in a freeze-tolerance phenotype and does not promote survival in natural environments [138].

The assessment of genetically modified and self-cloning yeast needs more long-term studies. There is limited knowledge about the persistence in the environment and interaction with existing microflora of fermentation cellar.

Conclusion

Saccharomyces cerevisiae is highly adaptable on the production of fermented beverages. Indeed for cost, time and efficient up-to-date application, the yeast has some unfavorable properties. Self-cloning yeast could be the answer, because only homolog DNA or DNA from closely related organism are inserted or modified.

This benefit of self-cloning yeast and the usage of only homolog DNA are disadvantageous. The usage of promoters and selection markers is strictly limited. A concern is the rare knowledge of properties of promoters under industrial conditions. For the gene expression of specific fermentation stages, it is necessary to know which parameters and which conditions induce the expression. Another point is that the fermentation medium wort and must are highly diverse, and the yeast is highly stressed during the fermentation [102].

Several self-cloning yeast strains have been constructed and studied (Table 1). In the case of brewing, the research does not focus on the bottom fermenting yeast *Saccharomyces pastorianus* var. *carlsbergensis* for genetical modification. Notwithstanding, 90 percent of this strain contributes to the world wide beer market [139, 140]. The bottom fermenting yeast is an allotetraploid hybrid of the top fermenting strain *S. cerevisiae* and *Saccharomyces eubayanus* [141]. Dunn and Sherlock [142] pointed out that there may have been two independent origins of *S. pastorianus* var. *carlsbergensis* strains. These circumstances alone complicate the research, and therefore, further investigations are necessary.

In the case of self-cloning brewing yeast, strain there is a large area for scientific development and research for application under industrial conditions. There is a versatile usage of self-cloning yeast in the beer industry. A focal point could be the enhancing yeast's adaptability during the brewing process to help create a method, which is both, economical and time efficient. Another point could be the reduction in ethanol content of beer without the loss of volatile flavor compounds. For this type of research, it is necessary to evaluate different native promoters for growth-independent expression.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

References

1. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. Science 274(5287):546
2. Verstrepen KJ, Derdelinckx G, Verachtert H, Delvaux FR (2003) Yeast flocculation: what brewers should know. Appl Microbiol Biotechnol 61(3):197–205

3. Panteloglou AG, Bell AE, Ma F (2010) Effect of high-hydrostatic pressure and pH on the rheological properties of gum arabic. Food Chem 122(4):972–979
4. Birch RM, Walker GM (2000) Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. Enzyme Microb Technol 26(9–10):678–687
5. Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA (2010) The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol 109(1):13–24
6. Duong CT, Strack L, Futschik M, Katou Y, Nakao Y, Fujimura T, Shirahige K, Kodama Y, Nevoigt E (2011) Identification of Sc-type ILV6 as a target to reduce diacetyl formation in lager brewers' yeast. Metab Eng 13(6):638–647
7. Kusunoki K, Ogata T (2012) Construction of self-cloning bottom-fermenting yeast with low vicinal diketone production by the homo-integration of ILV5. Yeast 29(10):435–442
8. Cambon B, Monteil V, Remize F, Camarasa C, Dequin S (2006) Effects of GPD1 overexpression in *Saccharomyces cerevisiae* commercial wine yeast strains lacking ALD6 genes. Appl Environ Microbiol 72(7):4688–4694
9. Remize F, Sablayrolles JM, Dequin S (2000) Re-assessment of the influence of yeast strain and environmental factors on glycerol production in wine. J Appl Microbiol 88(3):371–378
10. Akada R, Hirosawa I, Kawahata M, Hoshida H, Nishizawa Y (2002) Sets of integrating plasmids and gene disruption cassettes containing improved counter-selection markers designed for repeated use in budding yeast. Yeast 19(5):393–402
11. Dietvorst J, Londesborough J, Steensma HY (2005) Maltotriose utilization in lager yeast strains: MTT1 encodes a maltotriose transporter. Yeast 22(10):775–788
12. Zheng X, Damore T, Russell I, Stewart GG (1994) Factors influencing maltotriose utilization during brewery wort fermentations. J Am Soc Brew Chem 52(2):41–47
13. Cousseau FEM, Alves SL, Trichez D, Stambuk BU (2013) Characterization of maltotriose transporters from the *Saccharomyces eubayanus* subgenome of the hybrid *Saccharomyces pastorianus* lager brewing yeast strain Weihenstephan 34/70. Lett Appl Microbiol 56(1):21–29
14. Schuller D, Casal M (2005) The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. Appl Microbiol Biotechnol 68(3):292–304
15. Fleet GH (2008) Wine yeasts for the future. FEMS Yeast Res 8(7):979–995
16. Steensels J, Snoek T, Meersman E, Picca Nicolino M, Aslankoohi E, Christiaens JF, Gemayel R, Meert W, New AM, Pougach K, Saels V, van der Zande E, Voordeckers K, Verstrepen KJ (2012) Selecting and generating superior yeasts for the brewing industry. Cerevisia 37(2):63–67
17. Akada R (2002) Genetically modified industrial yeast ready for application. J Biosci Bioeng 94(6):536–544
18. USFDA Generally Recognized as Safe (GRAS). http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/ucm2006850.htm. Accessed April 2013
19. Directives 2009/41/EC (2009) Off J Eur Union
20. Sheldon IM (2001) Regulation of biotechnology: will we ever "freely" trade GMOs? Paper presented at the 77th EAAE seminar/NJF seminar no. 325, Helsinki
21. Swiegers JH, Capone DL, Pardon KH, Elsey GM, Sefton MA, Francis IL, Pretorius IS (2007) Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. Yeast 24(7):561–574
22. Hino A (2002) Safety assessment and public concerns for genetically modified food products: the Japanese experience. Toxicol Pathol 30(1):126–128
23. Gruère GP, Rao SR (2007) A review of international labeling policies of genetically modified food to evaluate India's proposed rule. AGBioForum J Agrobiotechnol Manage Econ 10(1):51–64
24. BIOTECSUR (2008) Inventory of biotechnological rules and regulations in the MERCOSUR. http://docs.biotecsur.org/informes/en/inventario/4_normativa_ms.pdf. Accessed April 2013
25. MOH (2002) Ministry of health regulation; order no. 28 administrative provisions for genetically modified food hygiene
26. Wang ZY, Zhang F, He XP, Zhang BR (2006) A survey and outlook of research in breeding of industrial brewing yeast by self-cloning technique. Microbiol China 33(6):138–141
27. Ceccoli S, Hixon W (2012) Explaining attitudes toward genetically modified foods in the European Union. Int Polit Sci Rev 33(3):301–319
28. Lusk JL, Rozan A (2005) Consumer acceptance of biotechnology and the role of second generation technologies in the USA and Europe. Trends Biotechnol 23(8):386–387
29. Curtis KR, McCluskey JJ, Wahl TI (2004) Consumer acceptance of genetically modified food products in the developing world. AGBioForum J Agrobiotechnol Manage Econ 7(1 and 2):70–75
30. Special Eurobarometer 244b Europeans in biotechnology in 2005: patterns and trends (2006) European commission. http://ec.europa.eu/public_opinion/index_en.htm
31. Eurobarometer 238 "Risk issue" (2006) European commission. http://ec.europa.eu/public_opinion/index_en.htm
32. Eurobarometer 73.1 Biotechnology (2010) European commission. http://ec.europa.eu/public_opinion/index_en.htm
33. PEW (2003) Initiative on food and biotechnology-survey conducted August 5–10
34. Chen M-F, Li H-L (2007) The consumer's attitude toward genetically modified foods in Taiwan. Food Qual Prefer 18(4):662–674
35. Martinez-Poveda A, Molla-Bauza MB, del Campo Gomis FJ, Martinez LM-C (2009) Consumer-perceived risk model for the introduction of genetically modified food in Spain. Food Policy 34(6):519–528
36. Lusk JL, House LO, Valli C, Jaeger SR, Moore M, Morrow JL, Traill WB (2004) Effect of information about benefits of biotechnology on consumer acceptance of genetically modified food: evidence from experimental auctions in the United States, England, and France. Eur Rev Agric Econ 31(2):179–204
37. Loureiro ML, Hine S (2004) Preferences and willingness to pay for GM labeling policies. Food Policy 29(5):467–483
38. Lusk JL, Rozan A (2008) Public policy and endogenous beliefs: the case of genetically modified food. J Agric Resour Econ 33(2):270–289
39. Tenbült P, De Vries NK, van Breukelen G, Dreezens E, Martijn C (2008) Acceptance of genetically modified foods: the relation between technology and evaluation. Appetite 51(1):129–136
40. Šorgo A, Jaušovec N, Jaušovec K, Puhek M (2011) The influence of intelligence and emotions on the acceptability of genetically modified organisms
41. Han JH, Harrison RW (2007) Factors influencing urban consumers' acceptance of genetically modified foods. Rev Agric Econ 29(4):700–719
42. Caporale G, Monteleone E (2004) Influence of information about manufacturing process on beer acceptability. Food Qual Prefer 15(3):271–278
43. Tomlinson N (1998) Worldwide regulatory issues: legislation and labeling. In: Roller S, Harlander S (eds) Genetic modification in the food industry—a strategy for food quality improvement. Blackie Academic and Professional, London
44. van Roojian R, Klaassen P (1998) Backer's yeast. In: Roller S, Harlander S (eds) Genetic modification in rh food industry—a strategy for food quality improvement. Blackie Academic and Professional, London

45. Hammond JRM (1995) Genetically-modified brewing yeasts for the twenty first century. Progress to date. Yeast 11(16):1613–1627
46. Husnik JI, Delaquis PJ, Cliff MA, van Vuuren HJJ (2007) Functional analyses of the malolactic wine yeast ML01. Am J Enol Vitic 58(1):42–52
47. Main GL, Threlfall RT, Morris JR (2007) Reduction of malic acid in wine using natural and genetically enhanced microorganisms. Am J Enol Vitic 58(3):341–345
48. Husnik JI, Volschenk H, Bauer J, Colavizza D, Luo ZL, van Vuuren HJJ (2006) Metabolic engineering of malolactic wine yeast. Metab Eng 8(4):315–323
49. USFDA (2006) Agency response letter GRAS notice no. GRN 000175. http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm154604.htm. Accessed June 2012
50. Coulon J, Husnik JI, Inglis DL, van der Merwe GK, Lonvaud A, Erasmus DJ, van Vuuren HJJ (2006) Metabolic engineering of *Saccharomyces cerevisiae* to minimize the production of ethyl carbamate in wine. Am J Enol Vitic 57(2):113–124
51. Zimmerli B, Schlatter J (1991) Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. Mutation Res Gen Toxicol 259(3–4):325–350
52. EFSA (2007) Ethyl carbamate and hydrocyanic acid in food and beverages scientific opinion of the panel on contaminants (question no EFSA-Q-2006-076). EFSA J 551:1–44
53. Wang ZY, He XP, Zhang BR (2007) Over-expression of GSH1 gene and disruption of PEP4 gene in self-cloning industrial brewer's yeast. Int J Food Microbiol 119(3):192–199
54. Wang ZY, Wang JJ, Liu XF, He XP, Zhang BR (2009) Recombinant industrial brewing yeast strains with ADH2 interruption using self-cloning GSH1 + CUP1 cassette. FEMS Yeast Res 9(4):574–581. doi:10.1111/j.1567-1364.2009.00502.x
55. Wang ZY, He XP, Liu N, Zhang BR (2008) Construction of self-cloning industrial brewing yeast with high-glutathione and low-diacetyl production. Int J Food Sci Technol 43(6):989–994
56. Iijima K, Ogata T (2010) Construction and evaluation of self-cloning bottom-fermenting yeast with high SSU1 expression. J Appl Microbiol 109(6):1906–1913
57. Ishida-Fujii K, Goto S, Sugiyama H, Takagi Y, Saiki T, Takagi M (1998) Breeding of flocculent industrial alcohol yeast strains by self-cloning of the flocculation gene FLO1 and repeated-batch fermentation by transformants. J Gen Appl Microbiol 44(5):347–353
58. Dahabieh MS, Husnik JI, van Vuuren HJJ (2010) Functional enhancement of Sake yeast strains to minimize the production of ethyl carbamate in Sake wine. J Appl Microbiol 109(3):963–973
59. Hirosawa I, Aritomi K, Hoshida H, Kashiwagi S, Nishizawa Y, Akada R (2004) Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. Appl Microbiol Biotechnol 65(1):68–73
60. Aritomi K, Hirosawa I, Hoshida H, Shiigi M, Nishizawa Y, Kashiwagi S, Akada R (2004) Self-cloning yeast strains containing novel FAS2 mutations produce a higher amount of ethyl caproate in Japanese sake. Biosci Biotechnol Biochem 68(1):206–214
61. Takagi H, Matsui F, Kawaguchi A, Wu H, Shimoi H, Kubo Y (2007) Construction and analysis of self-cloning sake yeasts that accumulate proline. J Biosci Bioeng 103(4):377–380
62. Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012) Simultaneous accumulation of proline and trehalose in industrial baker's yeast enhances fermentation ability in frozen dough. J Biosci Bioeng 113(5):592–595
63. Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012) Enhancement of the proline and nitric oxide synthetic pathway improves fermentation ability under multiple baking-associated stress conditions in industrial baker's yeast. Microb Cell Fact 11
64. Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012) Overexpression of the transcription activator Msn2 enhances the fermentation ability of industrial baker's yeast in Frozen Dough. Biosci Biotechnol Biochem 76(3):624–627
65. Sasano Y, Haitani Y, Ohtsu I, Shima J, Takagi H (2012) Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough. Int J Food Microbiol 152(1–2):40–43
66. Kaino T, Tateiwa T, Mizukami-Murata S, Shima J, Takagi H (2008) Self-cloning baker's yeasts that accumulate proline enhance freeze tolerance in doughs. Appl Environ Microbiol 74(18):5845–5849
67. Cordente AG, Cordero-Bueso G, Pretorius IS, Curtin CD (2013) Novel wine yeast with mutations in YAP1 that produce less acetic acid during fermentation. FEMS Yeast Res 13(1):62–73
68. Hohmann S, Mager WH (2003) Yeast stress responses. Springer, Berlin
69. Allison LA (2007) Fundamental molecular biology. Blackwell Publishing, New York
70. Miller G, Hahn S (2006) A DNA-tethered cleavage probe reveals the path for promoter DNA in the yeast preinitiation complex. Nat Struct Mol Biol 13(7):603–610
71. Lagrange T, Kapanidis AN, Tang H, Reinberg D, Ebright RH (1998) New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB. Genes Dev 12(1):34–44
72. Benoist C, Chambon P (1981) In vivo sequence requirements of the SV40 early promoter region. Nature 290(5804):304–310
73. Basehoar AD, Zanton SJ, Pugh BF (2004) Identification and distinct regulation of yeast TATA box-containing genes. Cell 116(5):699–709
74. Cormack BP, Struhl K (1992) The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. Cell 69(4):685–696
75. Zou YY, Huang W, Gu ZL, Gu X (2011) Predominant gain of promoter TATA box after gene duplication associated with stress responses. Mol Biol Evol 28(10):2893–2904
76. MartinezPastor MT, Marchler G, Schuller C, MarchlerBauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). EMBO J 15(9):2227–2235
77. Conlin LK, Nelson HCM (2007) The natural osmolyte trehalose is a positive regulator of the heat-induced activity of yeast heat shock transcription factor. Mol Cell Biol 27(4):1505–1515
78. de Boer CG, Hughes TR (2012) YeTFaSCo: a database of evaluated yeast transcription factor sequence specificities. Nucleic Acids Res 40(D1):D169–D179
79. Boy-Marcotte E, Lagniel G, Perrot M, Bussereau F, Boudsocq A, Jacquet M, Labarre J (1999) The heat shock response in yeast: differential regulations and contributions of the Msn2p/Msn4p and Hsf1p regulons. Mol Microbiol 33(2):274–283
80. Singh DP, Fatma N, Kimura A, Chylack LT Jr, Shinohara T (2001) LEDGF binds to heat shock and stress-related element to activate the expression of stress-related genes. Biochem Biophys Res Commun 283(4):943–955
81. Da Silva NA, Srikrishnan S (2012) Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. FEMS Yeast Res 12(2):197–214
82. Piper PW, Talreja K, Panaretou B, Moradasferreira P, Byrne K, Praekelt UM, Meacock P, Recnacq M, Boucherie H (1994) Induction of major heat-shock proteins of

Saccharomyces cerevisiae, including plasma-membrane HSP30, by ethanol levels above a critical threshold. Microbiology-(UK) 140:3031–3038
83. Penacho V, Blondin B, Valero E, Gonzalez R (2012) Flocculation and transcriptional adaptation to fermentation conditions in a recombinant wine yeast strain defective for KNR4/SMI1. Biotechnol Prog 28(2):327–336
84. Goossens K, Willaert R (2010) Flocculation protein structure and cell-cell adhesion mechanism in *Saccharomyces cerevisiae*. Biotechnol Lett 32(11):1571–1585
85. Verstrepen KJ, Derdelinckx G, Delvaux FR, Winderickx J, Thevelein JM, Bauer FF, Pretorius IS (2001) Late fermentation expression of FLO1 in *Saccharomyces cerevisiae*. J Am Soc Brew Chem 59(2):69–76
86. Govender P, Domingo JL, Bester MC, Pretorius IS, Bauer FF (2008) Controlled expression of the dominant flocculation genes FLO1, FLO5, and FLO11 in *Saccharomyces cerevisiae*. Appl Environ Microbiol 74(19):6041–6052
87. Govender P, Bester M, Bauer FF (2010) FLO gene-dependent phenotypes in industrial wine yeast strains. Appl Microbiol Biotechnol 86(3):931–945
88. Govender P, Kroppenstedt S, Bauer FF (2011) Novel wine-mediated FLO11 flocculation phenotype of commercial *Saccharomyces cerevisiae* wine yeast strains with modified FLO gene expression. FEMS Microbiol Lett 317(2):117–126
89. Donalies UEB, Stahl U (2001) Phase-specific gene expression in *Saccharomyces cerevisiae*, using maltose as carbon source under oxygen-limiting conditions. Curr Genet 39(3):150–155
90. Riou C, Nicaud JM, Barre P, Gaillardin C (1997) Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13(10):903–915
91. Cunha AF, Missawa SK, Gomes LH, Reis SF, Pereira GAG (2006) Control by sugar of *Saccharomyces cerevisiae* flocculation for industrial ethanol production. FEMS Yeast Res 6(2):280–287
92. Li Q, Zhao XQ, Chang AK, Zhang QM, Bai FW (2012) Ethanol-induced yeast flocculation directed by the promoter of TPS1 encoding trehalose-6-phosphate synthase 1 for efficient ethanol production. Metab Eng 14(1):1–8
93. Vidgren V, Kankainen M, Londesborough J, Ruohonen L (2011) Identification of regulatory elements in the AGT1 promoter of ale and lager strains of brewer's yeast. Yeast 28(8):579–594
94. Sahara H, Kotaka A, Kondo A, Ueda M, Hata Y (2009) Using promoter replacement and selection for loss of heterozygosity to generate an industrially applicable sake yeast strain that homozygously overproduces isoamyl acetate. J Biosci Bioeng 108(5):359–364
95. Shimoi H, Kitagaki H, Ohmori H, Iimura Y, Ito K (1998) Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. J Bacteriol 180(13):3381–3387
96. Omura F, Nakao Y, Teranishi T, Fujita A (2009) High expression levels of cell wall protein Cwp2p decrease the turbidity of fresh lager beer by reducing the size of Haze particles. J Am Soc Brew Chem 67(3):135–140
97. Dahabieh MS, Husnik JI, van Vuuren HJJ (2009) Functional expression of the DUR3 gene in a wine yeast strain to minimize ethyl carbamate in chardonnay wine. Am J Enol Vitic 60(4):537–541
98. Wang JJ, Wang ZY, Liu XF, Guo XN, He XP, Wensel PC, Zhang BR (2010) Construction of an industrial brewing yeast strain to manufacture beer with low caloric content and improved flavor. J Microbiol Biotechnol 20(4):767–774
99. Zhang HB, Ruan H, Li WF, Zhang W, Su ZR, He GQ, Chen QH (2011) Construction of recombinant industrial S. cerevisiae strain with barley lipid-transfer protein 1 secretion capability and lower PrA activity. Eur Food Res Technol 233(4):707–716
100. Partow S, Siewers V, Bjorn S, Nielsen J, Maury J (2010) Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. Yeast 27(11):955–964
101. Mumberg D, Muller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156(1):119–122
102. Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD, Smart KA (2007) Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol Rev 31(5):535–569
103. Lagunas R (1993) Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 104(3–4):229–242
104. Gibson BR, Boulton CA, Box WG, Graham NS, Lawrence SJ, Linforth RST, Smart KA (2008) Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. Yeast 25(8):549–562
105. Backhus LE, DeRisi J, Brown PO, Bisson LF (2001) Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions. FEMS Yeast Res 1(2):111–125
106. Harsch MJ, Lee SA, Goddard MR, Gardner RC (2010) Optimized fermentation of grape juice by laboratory strains of *Saccharomyces cerevisiae*. FEMS Yeast Res 10(1):72–82
107. Pizarro F, Vargas FA, Agosin E (2007) A systems biology perspective of wine fermentations. Yeast 24(11):977–991
108. Schehl B, Muller C, Senn T, Heinisch JJ (2004) A laboratory yeast strain suitable for spirit production. Yeast 21(16):1375–1389
109. Rossouw D, Jolly N, Jacobson D, Bauer FF (2012) The effect of scale on gene expression: commercial versus laboratory wine fermentations. Appl Microbiol Biotechnol 93(3):1207–1219
110. Murata Y, Homma T, Kitagawa E, Momose Y, Sato MS, Odani M, Shimizu H, Hasegawa-Mizusawa M, Matsumoto R, Mizukami S, Fujita K, Parveen M, Komatsu Y, Iwahashi H (2006) Genome-wide expression analysis of yeast response during exposure to 4 degrees C. Extremophiles 10(2):117–128
111. Werner-Washburne M, Stone DE, Craig EA (1987) Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol Cell Biol 7(7):2568–2577
112. Homma T, Iwahashi H, Komatsu Y (2003) Yeast gene expression during growth at low temperature. Cryobiology 46(3):230–237
113. Kondo K, Inouye M (1991) TIP1, a cold shock-inducible gene of *Saccharomyces cerevisiae*. J Biol Chem 266(26):17537–17544
114. Hashimoto S, Ogura M, Aritomi K, Hoshida H, Nishizawa Y, Akada R (2005) Isolation of auxotrophic mutants of diploid industrial yeast strains after UV mutagenesis. Appl Environ Microbiol 71(1):312–319
115. Pronk JT, Steensma HY, vanDijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12(16):1607–1633
116. Cakar ZP, Sauer U, Bailey JE (1999) Metabolic engineering of yeast: the perils of auxotrophic hosts. Biotechnol Lett 21(7):611–616
117. Kodama Y, Fukui N, Ashikari T, Shibano Y, Moriokafujimoto K, Hiraki Y, Nakatani K (1995) Improvement of maltose fermentation efficiency—constitutive expression of mal genes in brewing yeasts. J Am Soc Brew Chem 53(1):24–29
118. Hadfield C, Cashmore AM, Meacock PA (1986) An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *escherichia coli*. Gene 45(2):149–158
119. Kunze G, Bode R, Rintala H, Hofemeister J (1989) Heterologous gene-expression of the glyphosate resistance marker and its application in yeast transformation. Curr Genet 15(2):91–98

120. Sikorski RS, Boeke JD (1991) In vitro mutagenesis and plasmid shuffling—from cloned gene to mutant yeast. Method Enzymol 194:302–318
121. Boeke JD, Lacroute F, Fink GR (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast—5-fluoro-orotic acid resistance. Mol Gen Genet 197(2):345–346
122. Akada R, Matsuo K, Aritomi K, Nishizawa Y (1999) Construction of recombinant sake yeast containing a dominant FAS2 mutation without extraneous sequences by a two-step gene replacement protocol. J Biosci Bioeng 87(1):43–48
123. Kawahata M, Amari S, Nishizawa Y, Akada R (1999) A positive selection for plasmid loss in *Saccharomyces cerevisiae* using galactose-inducible growth inhibitory sequences. Yeast 15(1):1–10
124. Wang DL, Wang ZY, Liu N, He XP, Zhang BR (2008) Genetic modification of industrial yeast strains to obtain controllable NewFlo flocculation property and lower diacetyl production. Biotechnol Lett 30(11):2013–2018
125. Zhang JG, Liu XY, He XP, Guo XN, Lu Y, Zhang BR (2011) Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the FPS1 aquaglyceroporin gene. Biotechnol Lett 33(2):277–284
126. Casey GP, Xiao W, Rank GH (1988) A convenient dominant selection marker for gene-transfer in industrial strains of *Saccharomyces* yeast—SMRI encoded resistance to the herbicide sulfometuron methyl. J Inst Brew 94(2):93–97
127. Saerens SMG, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM, Delvaux FR (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Appl Environ Microbiol 74(2):454–461
128. Xie Q, Jimenez A (1996) Molecular cloning of a novel allele of SMR1 which determines sulfometuron methyl resistance in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 137(2–3):165–168
129. Akada R, Kitagawa T, Kaneko S, Toyonaga D, Ito S, Kakihara Y, Hoshida H, Morimura S, Kondo A, Kida K (2006) PCR-mediated seamless gene deletion and marker recycling in *Saccharomyces cerevisiae*. Yeast 23(5):399–405
130. Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P (2013) amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. FEMS Yeast Res 13(1):126–139
131. Cordero-Bueso G, Arroyo T, Serrano A, Valero E (2011) Remanence and survival of commercial yeast in different ecological niches of the vineyard. FEMS Microbiol Ecol 77(2):429–437
132. Ahmed FE (2002) Detection of genetically modified organisms in foods. Trends Biotechnol 20(5):215–223
133. Bauer FF, Dequin S, Pretorius IS, Schoeman H, Wolfaardt GM, Schroder MB, Grossmann M (2004) The assessment of the environmental impact of genetically modified wine yeast strains. Bulletin De L'oiv no. 881–882:515–528
134. Leon C, Garcia-Canas V, Gonzalez R, Morales P, Cifuentes A (2011) Fast and sensitive detection of genetically modified yeasts in wine. J Chromatogr A 1218(42):7550–7556
135. Schoeman H, Wolfaardt GM, Botha A, van Rensburg P, Pretorius IS (2009) Establishing a risk-assessment process for release of genetically modified wine yeast into the environment. Can J Microbiol 55(8):990–1002
136. Moses SBG, Otero RRC, La Grange DC, van Rensburg P, Pretorius IS (2002) Different genetic backgrounds influence the secretory expression of the LKA1-encoded Lipomyces kononenkoae alpha-amylase in industrial strains of *Saccharomyces cerevisiae*. Biotechnol Lett 24(8):651–656
137. Grossmann M, Kiessling F, Singer J, Schoeman H, Schroder MB, von Wallbrunn C (2011) Genetically modified wine yeasts and risk assessment studies covering different steps within the wine making process. Ann Microbiol 61(1):103–115
138. Ando A, Suzuki C, Shima J (2005) Survival of genetically modified and self-cloned strains of commercial baker's yeast in simulated natural environments: environmental risk assessment. Appl Environ Microbiol 71(11):7075–7082
139. Saerens SMG, Duong CT, Nevoigt E (2010) Genetic improvement of brewer's yeast: current state, perspectives and limits. Appl Microbiol Biotechnol 86(5):1195–1212
140. Kodama Y, Kielland-Brandt M, Hansen J (2006) Lager brewing yeast. In: Sunnerhagen P, Piskur J (eds) Comparative genomics, vol 15., Topics in current geneticsSpringer, Berlin, pp 145–164
141. Libkind D, Hittinger CT, Valerio E, Goncalves C, Dover J, Johnston M, Goncalves P, Sampaio JP (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc Natl Acad Sci U S A 108(35):14539–14544
142. Dunn B, Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. Genome Res 18(10):1610–1623

2.2 EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system

LWT - Food Science and Technology 68 (2016) 556–562

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system

S. Fischer*, C. Engstler, S. Procopio, T. Becker

Chair of Brewing and Beverage Technology, Technische Universität München, Weihenstephaner Steig 20, 85354 Freising, Germany

ARTICLE INFO

Article history:
Received 14 July 2015
Received in revised form
7 December 2015
Accepted 11 December 2015
Available online 15 December 2015

Keywords:
Industrial yeast
EGFP
Temperature shift
Heat shock protein

ABSTRACT

Targeted induced gene expression for industrial fermentation processes in food and beverage production could fulfill future requirements. Up to now, there is limited data of inducible expression patterns for targeted gene expression under such specific conditions. For the evaluation of temperature induced native promoters, the widely used reporter gene "enhanced green fluorescence protein" (EGFP) by utilizing high throughput systems was applied. Five different promoters of the industrial yeast strain *Saccharomyces cerevisiae* TUM 68 were evaluated ($_P$HSP12, $_P$HSP26, $_P$HSP30, $_P$HSP104, and $_P$SSA3). They are induced during temperature shifts, which may occur in transition of fermentation to maturation. Furthermore, the induction of gene expression affected by different contents of ethanol were investigated, by using synthetic wort which mimics a 12 °P wort. Promoters $_P$HSP30 and $_P$SSA3 showed the highest fluorescence value during temperature shift from 20 °C to 10 °C. A temperature shift from 20 °C to 4 °C, resulted in highest fluorescence values of $_P$SSA3 and $_P$HSP26. Further, these promoters showed the lowest induction value by ethanol concentrations between 4 and 6%-vol. With this method, it is possible to evaluate native temperature induced promoters for the usage in self-cloning brewing yeast under strict industrial conditions.

1. Introduction

The application of self-cloning *Saccharomyces cerevisiae* yeast in the field of beverage production has been intensively studied. Modified yeast is desired because a self-cloning yeast must not be declared as genetically modified yeast (GM yeast), which depends on the state legislation (Fischer, Procopio, & Becker, 2013). Present applications of industrial self-cloning yeast are based on constitutive regulated gene expression or gene replacement (Hirosawa et al., 2004; Iijimalijima & Ogata, 2010; Ishida-Fujii et al., 1998; Kusunoki & Ogata, 2012; Wang, He, Liu, & Zhang, 2008; Wang, He, & Zhang, 2007). Regulated expression systems such as galactose (West, Yocum, & Ptashne, 1984) or cupper inducible systems (Farhi et al., 2006; Labbe & Thiele, 1999) are not conceivable for the use in food and beverage production. Due to harmfulness and the proscription of additives which are regulated in the union list of food additives (commission regulation No. 1129/2011).

During the industrial fermentation process of beverages there are temperature shifts. The initiation of fermentation and even the transition from fermentation to maturation is accompanied by changes in temperature. The industrial yeast *S. cerevisiae* is adapted to different stressors such as high concentration of ethanol, osmotic pressure, limitation in nutrition and temperature cycling. This temperature shift leads to a modification of specific genetic regulation. The genetic response of cold shock results in membrane fluidification, cell wall maintenance, osmolyte synthesis, protein folding support and, ROS detoxification. In particular, the primary signal is transduced to classical stress pathways and transcription factors (Aguilera, Randez-Gil, & Prieto, 2007). The high osmolality glycerol (HOG) pathway responds to numerous extracellular stimuli and is even involved in the expression of a subset of cold induced genes after cold shock (Aguilera et al., 2007; Panadero, Pallotti, Rodriguez-Vargas, Randez-Gil, & Prieto, 2006; Trott, Shaner, & Morano, 2005). The cell wall integrity (CWI) pathway has an important part in the cold shock response as well (Corcoles-Saez, Ballester-Tomas, de la Torre-Ruiz, Prieto, & Randez-Gil, 2012). Moreover, changes in membrane fluidity influenced the primary signal, which triggers the cold shock response (Aguilera et al., 2007). Genes involved in glycogen and trehalose synthesis are

Abbreviations: EGFP, enhanced green fluorescence protein; YEPD, Yeast extract peptone dextrose; ROS, Reactive Oxygen Species.

* Corresponding author.
E-mail address: Susann.Fischer@tum.de (S. Fischer).

http://dx.doi.org/10.1016/j.lwt.2015.12.020
0023-6438/

induced during cold-shock, suggesting that biosynthesis and accumulation of these reserve carbohydrates are necessary for adaptation and survival under cold stress (Kandror, Bretschneider, Kreydin, Cavalieri, & Goldberg, 2004; Murata, Homma, Kitagawa, Momose, Sato, Odani et al., 2006). Indeed, members of heat shock protein (HSP) family are strictly induced after heat shock (Piper, Talreja, Panaretou, Moradasferreira, Byrne, Praekelt et al., 1994; Yamamoto, Maeda, Ikeda, & Sakurai, 2008) and some of them are also highly induced by higher concentrations of ethanol and even cold shock (Homma, Iwahashi, & Komatsu, 2003; Izawa, Kita, Ikeda, & Inoue, 2008; Murata et al., 2006). These proteins function as molecular chaperones which refold damaged proteins and protect thermally damaged proteins from aggregation and contribute to cell wall restructuration and synthesis of compatible solutes (Verghese, Abrams, Wang, & Morano, 2012).

For a reliable regulation of gene expression a subset of promoters is needed that operates independently of fermentation process by a strictly regulated and powerful induction of the target gene. Up to now, little attention has been paid to the induction patterns and the intensity of temperature induced promoters for industrial application. Especially, the composition of complex industrial fermentation media such as wort or grape must, could influence such gene regulations. The aim of the present work was the application of a high-throughput method for the evaluation of temperature induced promoters of *S. cerevisiae* TUM 68 under brewing conditions. Unlike previous studies on cold-shock inducible gene regulation, the fermentation temperature and maturation temperature are based on specific industrial parameters and industrial fermentation media. Furthermore, industrial strains are much more complex in the genetic architecture and available research data from laboratory yeast cannot by simply extrapolated (Steensels et al., 2014). For analysis of gene regulation during temperature shifts under brewing conditions, the reporter system EGFP was used. This reporter system was chosen due to its advantages, such as auto-fluorescence, non-invasive *in situ* detection and the modesty regarding cultivation media. A synthetic medium which mimics a 12 °P wort was used, which has an equivalent amino acid content and carbohydrate composition. The frequency of initiation is correlated to promoter strength and is visualized by the detection of fluorescence. Quantitative reporting property has been demonstrated (Attfield, Choi, Veal, & Bell, 2001; Brown & Lostroh, 2008; Lu, Bentley, & Rao, 2004). The yeast promoters for the evaluation of induction conditions were grouped as HSP family, which are known for induction by heat- and cold shock. The choice is further justified by the fact of different transcription factor binding sites which are harboring into the sequence of the promoter (Table 1). For further investigation of suitable evaluated promoters, the integration of a complete gene cassette is preferred in contrast to a replacement of HSP-genes by the reporter gene.

Table 1
STRE, HSE and Yap consensus sites of the promoter regions of Hsf1p-, Msn2p/4p- and YAP-dependent genes (Boy-Marcotte et al., 1999; Ma & Liu, 2010).

Promoter	STRE (CCCT)	HSE (NGAANNTTC)	YAP (TTACTAA)
$_P$HSP12	7	1	0
$_P$HSP26	4	7	0
$_P$HSP30	0	0	3
$_P$HSP104	3	10	1
$_P$SSA3	0	1	0

2. Materials and methods

2.1. Strains, plasmids and cultivation conditions

The ale yeast *S. cerevisiae* TUM 68 was used as the host for yeast transformation. The promoter expression cassette was inserted into the URA3 locus. The constructed recombinant strains for the promoter screening are listed in Table 2. The recombinant strains are selected as geneticin-sulfate (G418) resistant strains. *Escherichia coli* DH5α were used for plasmid construction. *E. coli* strains was cultivated at 37 °C on Lauria-Bertani (LB) medium (Green & Sambrook, 2012) supplemented with ampicillin (100 mg/l) or kanamycin (50 mg/l) if necessary. Yeast was grown at 25 °C in YEPD (10 g/l yeast extract; 20 g/l peptone; 20 g/l glucose) supplemented with G418 (200 mg/l) if necessary. Standard synthetic wort medium was used for yeast fermentation ability and shock conditions and composed of (g/l): yeast nitrogen base LoFlo w/o amino acids (Formedium, UK), 6.9; K_2HPO_4,1.3; glucose,12; maltose, 74; maltotriose, 17; fructose, 2.5; sucrose, 4; glycine, 0.04; alanine, 0.12; valine, 0.13; leucine, 0.17; isoleucine, 0.08; serine, 0.07; threonine, 0.08; asparagine, 0.16; glutamine, 0.11; aspartic acid, 0.9; glutamic acid, 0.1; cysteine, 0.01; methionine, 0.02; lysine, 0.14; arginine, 0.15; histidine, 0.05; phenylalanine, 0.15; tyrosine, 0.12; tryptophan, 0.02; proline, 0,36 and if necessary adjust the pH value with 20% lactic acid to pH 5.4 (Sacher, 2006). To minimize the background fluorescence yeast nitrogen base (LoFlo) without riboflavin and folic acid was used.

2.2. DNA Manipulation and plasmid construction

Plasmid DNA was prepared from *E. coli* by the alkali lyse method. Genomic yeast DNA was prepared by glass bead preparation (Amberg, Burke, & Strathern, 2005). Table 3 shows all the primer for amplifying the promoters. The cloning vector pEGFP was extended with kanamycin resistance marker (KanMX) from pUG6 which was amplified by PCR and digested with restriction enzymes *EagI/AatII* and inserted into the *EagI* and *AatII* site of pEGFP to generated plasmid pEGFP-KanMX. The terminator region of CYC1 was amplified from *S. cerevisiae* TUM 68 using primers CYC1-L and CYC1-R with restriction site *EagI/BglII* and these fragments were ligated into the *EagI* and *BglII* site of pEGFP-KanMX by T4 Ligase (Thermo Scientific, Germany) to construct the plasmid pEGFP-CYC1ter-KanMX. Promoters were amplified by PCR and sequenced for observation of differences in the sequence in compression to database of S288c. After the PCR product was obtained, it was digested with restriction enzymes *SphI/SmaI* (or *NheI/SmaI* was used for pSSA3 and pHSP26) and cloned by ligation into the plasmid pEGFP-KanMX. Flow diagrams for construction of transformation cassettes shown in Fig. 1.

2.3. Transformation and screening

Rubidium chloride competent *E. coli* cells were transformed by the heat shock method. The promoter expression cassette was amplified by PCR with URA3-FL-L and URA3-FL-R used proofreading Pwo-Polymerase (Preqlab, Germany) for transformation into host strain. By those primers, the transformation cassette was elongated on both sites with 25 bp homolog sequence to target locus URA3. The transformation was performed with a purified DNA fragment (PCR cycle pure Kit; Peqlab, Germany) using lithium acetate (LiAc) method as described by (Gietz & Schiestl, 2007). The recombinant strains were selected on YEPD contained 200 mg/l G418. PCR was carried out to amplify the transformation cassette from the whole genome of equivalent recombinant strain. The PCR product was sequenced (GATC, Germany) and squared. For genetic

Table 2
Strains and plasmids used in this study.

Strain or plasmids	Relevant genotype	Reference or source
Strains		
E. coli DH5α	supE44 ΔlacU169(φ 80lacZΔM15) hsdR17 recAl endAl gyrA96 thi-1 relAl	Invitrogen
TUM 68	Wild type *Saccharomyces cerevisiae* industrial strain	Own stock
TUM 68-pHSP12	Promoter of the Heat Shock Protein, plasma membrane protein	This study
TUM 68-pHSP26	Promoter of the small Heat Shock Protein	This study
TUM 68-pHSP30	Promoter of the Heat shock Protein, negative regulator of the H(+)-ATPase Pma1p	This study
TUM 68-pHSP104	Promoter of the heat Shock Protein, Disaggregase	This study
TUM 68-pSSA3	Promoter of the Heat Shock Protein, ATPase	This study
Plasmids		
pUG6	Cloning vector *AmpR, KanMX*	Euroscarf
pEGFP	Cloning vector *EGFP, Amp*	Clontech
peGFP-CYC1ter-KanMX	Recombined plasmid, *KanMX, EGFP, CYC1-terminator*	This study
pSSA3-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing EGFP gene by $_p$SSA3	This study
pHSP12-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing EGFP gene by $_p$HSP12	This study
pHSP26-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing EGFP gene by $_p$HSP26	This study
pHSP30-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing EGFP gene by $_p$HSP30	This study
pHSP104-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing EGFP gene by $_p$HSP104	This study

Table 3
Oligonucleotide primers used in PCR purification.

Primers	Sequence 5′ (3′
P_{HSP12}-L	GCATGCTTTTTTTGTCCAGGTGGAGTG (SphI)
P_{HSP12}-R	CCCGGGGACATTGTTGTATTTAGTTTTTT (XmaI)
P_{HSP26}-L	GCTAGCGTTGGACTTTTTTTAATATAACC (NheI)
P_{HSP26}-R	CCCGGGGTTAATTTGTTTAGTTTGTTTGT (XmaI)
P_{HSP30}-L	CGGCATGCGTTTATTCGAATACCCAATTAG (SphI)
P_{HSP30}-R	GTCCCGGGTTTGAAATTTGTTGTTTTTAGT (XmaI)
P_{HSP104}-L	TGGCATGCGCTAGCTCAGCCGGA (SphI/NheI)
P_{HSP104}-R	ACCCCGGGCATATATTCTGTATATTTTATGGTACGTG (XmaI)
P_{SSA3}-L	ACGCTAGCAATTCAAGTAATTATTTTGGGG (NheI)
P_{SSA3}-R	TTCCCGGGTTTTCTTTGTAGCGTTTAGT (XmaI)
CYC1-L	GGCGGCCGATGTAATTAGTTATGTCACG (EagI)
CYC1-R	CGAGATCTGCAGCTTGCAAATTAAAGC (BglII)
KanMX-L	CGGCCGTAGGTCTAGAGATCT (EagI)
KanMX-R	GACGTCATTAAGGGTTCTCGAG (AatII)
URA3-FL-L	CGAAAGCTACATATAAGGAACGTGCTGACCATGATTACGCCAAG
URA3-FL-R	AAATATGCTTCCCAGCCTGCTTTTCTTCCCCGAAAAGTGCCACCT

stability all recombinant strains were inoculated on YEPD plates without G418, grown for 24 h by 25 °C and repeated five times. After the fifth transfer and incubation the yeast colonies were inoculated on YEPD plates with G418 (200 and 400 mg/l) and incubated for 2 day at 25 °C. Additionally, after 72 h of shock situation, the recombinant strains were plated on YEPD Plates with 300 mg/l G418 to test the resistance again. The consolidation of the gene cassette into the genome was analyzed by amplification of the gene cassette and sequencing.

2.4. Fermentation and fluorescence detection

Fermentations have been carried out in science laboratory-scale (10 ml) and high-throughput-scale (96 well multitier plates, black with clear bottom; Biozyme, Germany) with 12 °P synthetic wort at 20 °C. The cells were cropped, washed with sterile distilled water and pitched into the synthetic wort at a ratio 15 × 10^6 cells/ml. Fermentation were conducted in high-throughput-scale in four replicates at 20 °C and the temperature shifts were performed at the beginning and end of the stationary phase of fermentation. The temperature shifts to 10 °C and near freezing 4 °C were conducted for 72 h. Ethanol shock carried out at 20 °C with ethanol concentrations between 4 and 6%-vol. in synthetic wort. Fluorescence observation during fermentation and after shock situation was performed with the Synergy H4 Hybrid Microplate reader (Biotek, Germany). Read by extinction of 485/20 nm and emission at 525/9 nm and 585/9 nm by a gain of 120. The optical density was measured at 600 nm and correlated to the cell number. The fluorescence value was normalized to the cell number to obtain the relative fluorescence unit per cell (RFU). The RFU of the equivalent GFP expressing yeast were normalized to initial shock situation (time point 0) and the autofluorescence of the host strain (Lichten, White, Clark, & Swain, 2014).

2.5. Data analysis and statistical data processing

The fluorescence of the recombinant strain was corrected by the measured autofluorescence of the host strain, according to (Lichten et al., 2014). The autofluorescence r_a is the ratio of fluorescence at 585 nm and fluorescence at 525 nm of the host strain at the optical density of the recombinant strain. The optical density at 600 nm of the host strain are fitted over the whole time by cubic polynomial when necessary. At each time point the r_a, the fluorescence data (fitted over the time by cubic polynomial when necessary) of the recombinant strain, and the optical density of each time point is needed for the computation of the fluorescence per cell (Eq. (1)). The data analysis is performed with OriginLab.

$$g = \frac{r_a f_{525} - f_{585}}{r_a - r_g} \quad (1)$$

Significant differences between the expression values of equivalent promoters were determined by ANOVA. The statistical level of significances was set at $P \leq 0.05$.

3. Results

3.1. Expression vector sets for promoter screening

For evaluation of the expression pattern of stress related promoters, five different promoters were selected for the induction of EGFP expression. As mentioned above, gene expression of laboratory yeast under stress situation, such as heat and cold shock of HSP30, HSP12, HSP26, HSP104 are well characterized (Homma et al., 2003; Murata et al., 2006; Piper et al., 1994; Schade, Jansen, Whiteway, Entian, & Thomas, 2004). For each individual promoter, an expression cassette was constructed, including the promoter of interest, EGFP, CYC1-terminator, and G418 resistance marker. The obtained plasmids are shown in Table 2. Further, the gene cassette was amplified by proofreading PWO-polymerase at which the 5′and 3′end of the gene-cassette was elongated with

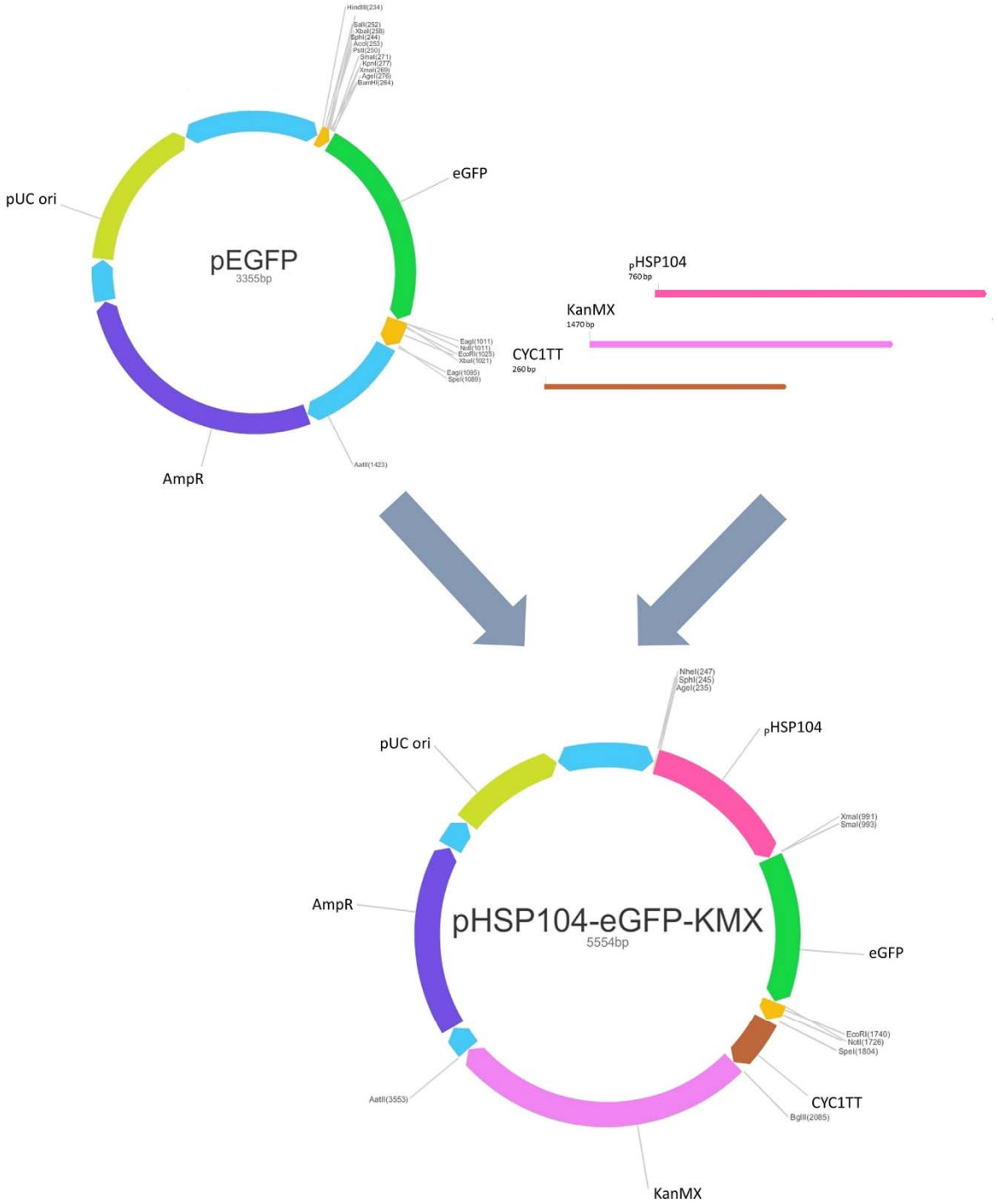

Fig. 1. Flow diagram: Construct of the transformation cassette for integration into the target locus *URA3* by elongation on 5′ and 3′ end of the cassette with 25 bp homologous sequence of target locus.

25 bp homologous sequence to target locus *URA3*. The host strain, was transformed with the elongated gene cassette. Because of the

homologous sequence of the DNA fragment, homologues recombination between the gene cassette and chromosomal DNA in TUM 68 occurred. The recombinant strains was selected on YEPD complemented with 300 mg/l G418. The obtained strains are shown in Table 2.

3.2. Genetic stability and sequence analysis

Recombinant yeast strains was successfully grown on YEPD plates with 300 mg/l and 400 mg/l G418 after incubation without selection pressure for 5 times. These indicated that the gene cassette with the "geneticin resistance protein" gene was inserted into the genome of all recombinant strains. These resulted in resistance against G418 and indicated that these strains were genetically stable. Further, the promoter in combination with EGFP were amplified by PCR and sequenced.

3.3. Fermentation test

The fermentation in 96-Well plates (black with clear bottom; Biozyme, Germany) were carried out at 20 °C in 12°P synthetic wort. All recombinant strains showed identical cell growth compared to the host strain (Fig. 2a). The disruption of one copy of the URA3 gene into the industrial strain TUM 68 (Donhauser, Springer, & Vogeser, 1990), which encode for Orotidine-5'-phosphate (OMP) decarboxylase had no influence on cell growth. The correlation of cell count (Mio/ml) and optical density is shown in Fig. 2b. The ratio of fluorescence emitted at 525 nm and 585 nm (r_a) are plotted versus cell count. The corrected cell count is the fundament for the calculation of fluorescence per cell.

3.4. Induction affected by cold shock

Principal, the evaluation of induction patterns of these promoters focused on cold shock scenarios. In the brewing processes, during the transition from fermentation to maturation a temperature shift is occurs, as the average maturation temperature at 4 °C. The shock situation was performed from 20 °C to 10 °C and 4 °C over a time frame from 72 h in synthetic wort. Table 4 shows the fluorescence change of EGFP under control of the different yeast promoters at each time point after exposure to 10 °C and 4 °C.

For all five evaluated promoters, it was clearly observed that both temperature shifts led to induction of the EGFP expression. A shift from 20 °C to 10 °C resulted in a steady increment of fold change for all recombinant strains. The highest ratio of fluorescence was detected by pSSA3 and pHSP30 with 6.8 and 5.8 respectively. The exposure to 4 °C led to higher fold changes at the beginning of the shock in comparison to 10 °C. However, the exposure to 10 °C led to a higher increase of the induction over the time frame. The maximum of fluorescence at 4 °C exposure were detected after 48 h for pHSP12, pHSP30 and pHSP104 with fold changes of 4.4, 5.1 and 3.1 respectively. pHSP26 and pSSA3 reached the maximum of fold change at the end of shock situation with 5.0 and 5.9.

Table 4
Fluorescence changes of EGFP under control of the different yeast promoters at each time point after exposure to 10 °C and 4 °C.

Strain	6 h	12 h	24 h	36 h	48 h	72 h
Temperature shift to 10 °C						
pHSP12	2.3	2.5	2.9	2.9	3.3	3.9
pHSP26	2.6	3.0	3.6	3.9	4.3	5.1
pHSP30	3.0	3.4	4.0	4.4	5.0	5.8
pHSP104	2.3	2.6	3.2	3.4	3.8	4.9
pSSA3	3.2	3.5	4.4	4.6	5.6	6.8
Temperature shift to 4 °C						
pHSP12	3.5	3.4	3.6	4.1	4.4	4.1
pHSP26	3.6	4.0	4.5	4.7	4.9	5.0
pHSP30	3.8	4.1	4.4	4.7	5.1	4.7
pHSP104	2.4	2.4	2.6	2.8	3.1	3.0
pSSA3	4.6	4.8	5.4	5.7	5.7	5.9

Results are shown as fold change to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 15%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

3.5. Induction through ethanol shock

As mentioned above, promoters of the HSP-family are also induced by different ethanol concentration. Especially during industrial wort fermentation ethanol concentrations above 5%-vol. do occur. To verify the influence of ethanol on the induction patterns of these promoters under brewing conditions, ethanol concentration of 4 and 6%-vol. were selected. Thus, recombinant strains and host strain were inoculated with 15×10^6 cells/ml in synthetic wort and complemented with equivalent amount of ethanol and induction patterns were analyzed over 24 h by 20 °C. Table 5 shows the fold-changes of fluorescence over the time frame.

Ethanol concentrations of 4%-vol. have also an influence to the

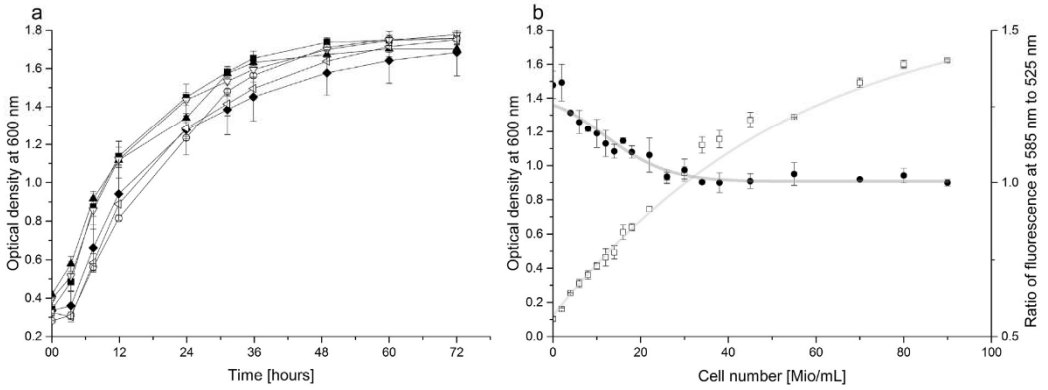

Fig. 2. a Cell growth during fermentation by 20 °C in synthetic wort (■WT; ○ pHSP12; ▲ pHSP26; ▽ pHSP30; ◆ pHSP104; ◁ pSSA3). b Standard curve of optical density at 600 nm and normalized ratio (r_a) of fluorescence detected by extinction of 488 nm and emission at 585 nm and 525 nm. Host strain inoculated in 12 °P synthetic word (❒ optical density; ● r_a).

Table 5
Fluorescence changes of EGFP under control of the different native yeast promoters after exposure to different ethanol contents in synthetic wort by 20 °C over 24 h.

Strain	4%-vol.			6%-vol.		
	6 h	12 h	24 h	6 h	12 h	24 h
pHSP12	2.0	2.5	3.1	1.1	1.2	1.4
pHSP26	−1.5	−1.2	−1.1	−1.6	−1.7	−1.5
pHSP30	2.0	2.8	4.0	1.1	1.1	1.5
pHSP104	1.9	2.7	3.2	−1.2	1.2	1.4
pSSA3	1.2	1.8	2.2	1.0	1.7	2.1

Results are shown as fold change to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

induction of the promoters, more than 6%-vol. However, the promoters pHSP26 and pSSA3 showed the lowest value at 4%-vol. ethanol with −1.1 and 2.2, respectively. In contrast, the highest values were observed for pHSP104 and pHSP30 at 4%-vol. with 3.2 and 4.0, respectively.

4. Discussion

The method applied in this study enables to characterize native promoters of industrial yeast by mimicking industrial conditions in high throughput experiments. Inducible native promoters of industrial *Saccharomyces* yeast are necessary for the construction of self-cloning yeast for food and beverage production by intended induction. There are limited opportunities to induce gene expression independent of the process parameters or composition of the fermentation-medium. The evaluated promoters are involved in stress response and are screened during industrial temperature conditions. The promoters for application of this method are chosen due to the high content of regulative motifs into the sequence (Table 1). Due to that fact, expression of HSP genes depends on the activation of different transcription factors and the equivalent binding sites (Verghese et al., 2012).

The results showed that under selected conditions, promoters of this ale yeast are induced in a relevant amount. Especially, industrial fermentation conditions lead to numerous stressful situations for yeast (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). The yeast transcribed the genes differently according to the environmental changes. During adaption to 10 °C and 4 °C the total RNA content did not change (Kandror et al., 2004) but the content of mRNA of HSP12, HSP26, HSP30 and HSP104 increased (Homma et al., 2003; Murata et al., 2006; Schade et al., 2004). However, the knowledge of EGFP synthesis in comparison to mRNA level could be relevant to characterize the promoter activity under such stressful conditions. The results show a higher induction activity by a temperature shift from 20 °C to 10 °C in comparison to a temperature shift from 20 °C to 4 °C. HSP12 and HSP26 are induced drastically after the cessation of the exponential phase, where HSP26 occurred at an earlier stage (Praekelt & Meacock, 1990; Welker, Rudolph, Frenzel, Hagn, Liebisch, Schmitz et al., 2010). SSA3 and HSP104 are slightly expressed by temperatures at 23 °C (Werner-Washburne, Stone, & Craig, 1987) and in fermenting cells (Sanchez, Taulien, Borkovich, & Lindquist, 1992) respectively. The main focus for application of this method is the observation of the induction level during cold shocks. Generally, cold shocks leads to higher induction patterns in comparison to ethanol stress of the evaluated promoters. The fold change in fluorescence detection by exposure to 4 °C of pHSP12, pHSP30 and pHSP104 shows similar tendencies to the observation of Murata and colleagues (Murata et al., 2006). The highest expression of pHSP12 is shown at time point 48 h with a fold change of 4.4. pHSP104 shows nearly constant values over the measurement, where pHSP30 has the highest fold change at 48 h with 5.1 as maximum. The temperature shift from 20 °C to 10 °C shows higher fluorescence values by pHSP30, pHSP104 and pSSA3. Sahara and colleagues pointed out that in the gene expression during the temperature shift from 30 °C to 10 °C, only HSP26 and HSP12 are up-regulated in late phase (4 h after exposure) and HSP30 was down-regulated (Sahara, Goda, & Ohgiya, 2002). HSP12, HSP26 and HSP104 are classified as late cold response, since there are up-regulated (≥2 fold) after 12 h of exposure (Schade et al., 2004). The increases of the fold change by fluorescence detection are lower after the first 12 h and rise with time (Table 4). The gene expression of SSA3 is up-regulated by exposure to 4 °C (Homma et al., 2003) and our results indicated that pSSA3 has the highest activity in direct comparison by both cold shock scenarios.

A further approach for the qualification of this analytical method are the investigations of the induction levels by different ethanol contents (Table 5). The maximal expression of HSP-genes are observed by higher contents of ethanol in laboratory strains (Piper et al., 1994). Our results shows that under 20 °C in synthetic wort with 4%-vol. ethanol have a higher induction of pHSP12, pHSP30 and pHSP104 than by 6%-vol. ethanol. Indeed, the decline of induction by 6%-vol. of pHSP12 and pHSP30 are also described in previous studies (Piper et al., 1994). In contrast, the promoters pSSA3 and pHSP26 have the lowest induction by these both ethanol concentrations and therefore they are suitable for the induction by the transmission to maturation. We observed that the activity of five different promoters varied with the temperature shifts and ethanol shocks. The overall ranking of the promoter activities is as follows:

Cold shock to 10 °C: pSSA3 ~ pHSP30 > pHSP26 ~ pHSP104 > pHSP12
Cold shock to 4 °C: pSSA3 > pHSP26 ~ pHSP30 > pHSP12 > pHSP104

To evaluate the activity of the studied promoters at different temperature shifts, results show that pSSA3 has the strongest induction by shifts to cold temperature and a low induction at ethanol concentrations between 4 and 6%-vol.

In conclusion, with this analytical method it is possible to describe promoter activity in high-throughput by mimicking stressful industrial fermentation conditions. The stress response of the yeast to varying effects during the fermentation are now predictable. Furthermore, this method has the potential to study gene expression during industrial fermentation by different strains aimed for the selection of improved adaption on stressful conditions such as a different composition of carbon sources or the adaption to high contents of ethanol for the bioethanol production.

Acknowledgment

This work was supported by the DFG (Deutsche ForschungsGemeinschaft, BE 2245/12-1).

References

Aguilera, J., Randez-Gil, F., & Prieto, J. A. (2007). Cold response in Saccharomyces cerevisiae: new functions for old mechanisms. *Fems Microbiology Reviews, 31*(3), 327–341.
Amberg, D., Burke, D., & Strathern, J. (2005). *Methods in yeast genetics: a cold spring harbor laboratory course manual* (2005 Edition). Cold Spring: Cold Spring Harbor Laboratory Press.
Attfield, P. V., Choi, H. Y., Veal, D. A., & Bell, P. J. L. (2001). Heterogeneity of stress gene expression and stress resistance among individual cells of Saccharomyces cerevisiae. *Molecular Microbiology, 40*(4), 1000–1008.

Boy-Marcotte, E., Lagniel, G., Perrot, M., Bussereau, F., Boudsocq, A., Jacquet, M., et al. (1999). The heat shock response in yeast: differential regulations and contributions of the Msn2p/Msn4p and Hsf1p regulons. *Molecular Microbiology, 33*(2), 274–283.
Brown, D., & Lostroh, C. P. (2008). Inferring gene expression dynamics from reporter protein levels. *Biotechnology Journal, 3*(11), 1437–1448.
Corcoles-Saez, I., Ballester-Tomas, L., de la Torre-Ruiz, M. A., Prieto, J. A., & Randez-Gil, F. (2012). Low temperature highlights the functional role of the cell wall integrity pathway in the regulation of growth in Saccharomyces cerevisiae. *Biochemical Journal, 446*, 477–488.
Donhauser, S., Springer, R., & Vogeser, G. (1990). Identifizierung und Klassifizierung von Brauereihefen durch Chromosomenanalyse mit der Pulsfeldgelelektrophorese. *Monatsschrift Fur Brauwissenschaft, 12*, 392–400.
Farhi, M., Dudareva, N., Masci, T., Weiss, D., Vainstein, A., & Abeliovich, H. (2006). Synthesis of the food flavoring methyl benzoate by genetically engineered Saccharomyces cerevisiae. *Journal of Biotechnology, 122*(3), 307–315.
Fischer, S., Procopio, S., & Becker, T. (2013). Self-cloning brewing yeast: a new dimension in beverage production. *European Food Research and Technology, 237*(6), 851–863.
Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *Fems Microbiology Reviews, 31*(5), 535–569.
Gietz, R. D., & Schiestl, R. H. (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocol, 2*(1), 35–37.
Green, M. R., & Sambrook, J. (2012). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
Hirosawa, I., Aritomi, K., Hoshida, H., Kashiwagi, S., Nishizawa, Y., & Akada, R. (2004). Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. *Applied Microbiology and Biotechnology, 65*(1), 68–73.
Homma, T., Iwahashi, H., & Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. *Cryobiology, 46*(3), 230–237.
Iijimalijima, K., & Ogata, T. (2010). Construction and evaluation of self-cloning bottom-fermenting yeast with high SSU1 expression. *Journal of Applied Microbiology, 109*(6), 1906–1913.
Ishida-Fujii, K., Goto, S., Sugiyama, H., Takagi, Y., Saiki, T., & Takagi, M. (1998). Breeding of flocculent industrial alcohol yeast strains by self-cloning of the flocculation gene FLO1 and repeated-batch fermentation by transformants. *Journal of General and Applied Microbiology, 44*(5), 347–353.
Izawa, S., Kita, T., Ikeda, K., & Inoue, Y. (2008). Heat shock and ethanol stress provoke distinctly different responses in 3 '-processing and nuclear export of HSP mRNA in Saccharomyces cerevisiae. *Biochemical Journal, 414*, 111–119.
Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D., & Goldberg, A. L. (2004). Yeast Adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and Certain molecular chaperones. *Molecular Cell, 13*(6), 771–781.
Kusunoki, K., & Ogata, T. (2012). Construction of self-cloning bottom-fermenting yeast with low vicinal diketone production by the homo-integration of ILV5. *Yeast, 29*(10), 435–442.
Labbe, S., & Thiele, D. J. (1999). Copper ion inducible and repressible promoter systems in yeast. *Expression of Recombinant Genes in Eukaryotic Systems, 306*, 145–153.
Lichten, C. A., White, R., Clark, I. B. N., & Swain, P. S. (2014). Unmixing of fluorescence spectra to resolve quantitative time-series measurements of gene expression in plate readers. *Bmc Biotechnology, 14*.
Lu, C., Bentley, W. E., & Rao, G. (2004). A High-Throughput approach to promoter study using green fluorescent protein. *Biotechnology Progress, 20*(6), 1634–1640.
Ma, M., & Liu, L. Z. (2010). Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in Saccharomyces cerevisiae. *BMC microbiology, 10*(1), 169.
Murata, Y., Homma, T., Kitagawa, E., Momose, Y., Sato, M. S., Odani, M., et al. (2006). Genome-wide expression analysis of yeast response during exposure to 4 degrees C. *Extremophiles, 10*(2), 117–128.
Panadero, J., Pallotti, C., Rodriguez-Vargas, S., Randez-Gil, F., & Prieto, J. A. (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in Saccharomyces cerevisiae. *Journal of Biological Chemistry, 281*(8), 4638–4645.
Piper, P. W., Talreja, K., Panaretou, B., Moradasferreira, P., Byrne, K., Praekelt, U. M., et al. (1994). Induction of major heat-shock proteins of Saccharomyces cerevisiae, including plasma-membrane HSP30, by ethanol levels above a critical threshold. *Microbiology-Sgm, 140*, 3031–3038.
Praekelt, U. M., & Meacock, P. A. (1990). HSP12, a new Small heat-shock gene of Saccharomyces-Cerevisiae - analysis of Structure, regulation and function. *Molecular & General Genetics, 223*(1), 97–106.
Sacher, B. (2006). Hefecharakterisierung anhand brautechnologischer Verhaltensmuster. In *3. Weihenstephaner Hefesymposium*. Germany: Freising-Weihenstephan.
Sahara, T., Goda, T., & Ohgiya, S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *Journal of Biological Chemistry, 277*(51), 50015–50021.
Sanchez, Y., Taulien, J., Borkovich, K. A., & Lindquist, S. (1992). HSP104 is required for tolerance to many forms of stress. *Embo Journal, 11*(6), 2357–2364.
Schade, B., Jansen, G., Whiteway, M., Entian, K. D., & Thomas, D. Y. (2004). Cold adaptation in budding yeast. *Molecular Biology of the Cell, 15*(12), 5492–5502.
Steensels, J., Snoek, T., Meersman, E., Nicolino, M. P., Voordeckers, K., & Verstrepen, K. J. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. *Fems Microbiology Reviews, 38*(5), 947–995.
Trott, A., Shaner, L., & Morano, K. A. (2005). The molecular chaperone Sse1 and the growth control protein kinase Sch9 collaborate to remalate protein kinase a activity in Saccharomyces cerevisiae. *Genetics, 170*(3), 1009–1021.
Verghese, J., Abrams, J., Wang, Y. Y., & Morano, K. A. (2012). Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiology and Molecular Biology Reviews, 76*(2), 115–158.
Wang, Z. Y., He, X. P., Liu, N., & Zhang, B. R. (2008). Construction of self-cloning industrial brewing yeast with high-glutathione and low-diacetyl production. *International Journal of Food Science and Technology, 43*(6), 989–994.
Wang, Z. Y., He, X. P., & Zhang, B. R. (2007). Over-expression of GSH1 gene and disruption of PEP4 gene in self-cloning industrial brewer's yeast. *International Journal of Food Microbiology, 119*(3), 192–199.
Welker, S., Rudolph, B., Frenzel, E., Hagn, F., Liebisch, G., Schmitz, G., et al. (2010). Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function. *Molecular Cell, 39*(4), 507–520.
Werner-Washburne, M., Stone, D. E., & Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. *Mol Cell Biol, 7*(7), 2568–2577.
West, R. W., Yocum, R. R., & Ptashne, M. (1984). Saccharomyces cerevisiae GAL1-GAL10 divergent promoter region - Location and function of the upstream activating sequence UASG. *Molecular and Cellular Biology, 4*(11), 2467–2478.
Yamamoto, N., Maeda, Y., Ikeda, A., & Sakurai, H. (2008). Regulation of thermotolerance by stress-induced transcription factors in Saccharomyces cerevisiae. *Eukaryotic Cell, 7*(5), 783–790.

2.3 Induced gene expression in industrial *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70: Evaluation of temperature and ethanol inducible native promoters

FEMS Yeast Research, 16, 2016, fow014

doi: 10.1093/femsyr/fow014
Advance Access Publication Date: 15 February 2016
Research Article

RESEARCH ARTICLE

Induced gene expression in industrial *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70: evaluation of temperature and ethanol inducible native promoters

S. Fischer*, C. Engstler, S. Procopio and T. Becker

Chair of Brewing and Beverage Technology, Technische Universität München, Weihenstephaner Steig 20, D-85354 Freising, Germany

*Corresponding author: Lehrstuhl fur Brau- und Getranketechnologie, Technische Universität München, Weihenstephaner Steig 20, D-85354 Freising, Germany. Tel: +49-8161-71-3687; Fax: +49-8161-71-3883; E-mail: susann.fischer@tum.de

One sentence summary: Evaluation of 10 temperature induced promoters of industrial lager yeast for induced gene-expression showed three potential promoters (pHSP104, pSSA3 and pUBI4) for application in self-cloning brewing yeast.

Editor: Isak Pretorius

ABSTRACT

Induced gene expression is an important trait in yeast metabolic engineering, but current regulations prevent the use of conventional expression systems, such as galactose and copper, in food and beverage fermentations. This article examines the suitability of temperature-inducible native promoters for use in the industrial yeast strain *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70 under brewing conditions. Ten different promoters were cloned and characterized under varying temperature shifts and ethanol concentrations using a green fluorescent protein reporter. The activities of these promoters varied depending upon the stress conditions applied. A temperature shift to 4°C led to the highest fold changes of $_{p}$SSA3, $_{p}$UBI4 and $_{p}$HSP104 by 5.4, 4.5 and 5.0, respectively. Ethanol shock at 24°C showed marked, concentration-dependent induction of the promoters. Here, $_{p}$HSP104 showed its highest induction at ethanol concentrations between 4% (v/v) and 6% (v/v). The highest fold changes of $_{p}$SSA3 and $_{p}$UBI4 were found at 10% (v/v) ethanol. In comparison, the ethanol shock at a typical fermentation temperature (12°C) leads to lower induction patterns of these promoters. Taken together, the data show that three promoters ($_{p}$HSP104, $_{p}$UBI4 and $_{p}$SSA3) have high potential for targeted gene expression in self-cloning brewing yeast using temperature shifts.

Keywords: lager yeast; promoter strength; temperature shift; enhanced green fluorescence protein; induced gene expression

INTRODUCTION

Studies on inducible homologous promoters for the regulation of gene expression in self-cloning yeasts are limited. Existing research focuses mainly on induction triggered by additives such as copper and galactose (West, Yocum and Ptashne 1984, Labbe and Thiele 1999, Farhi *et al.* 2006) or induction that occurs during sequential utilization of carbohydrates (Lagunas 1993). The addition of substances such as copper or galactose is not permitted for the food and beverage industry; their use is regulated in the union list of food additives (Commission Regulation No. 1129/2011). In addition, the carbohydrate and free amino nitrogen (FAN) compositions of the wort differ from stock to stock

Received: 14 December 2015; **Accepted:** 10 February 2016

(Lea, Piggott and Piggott 2003). Therefore, the use of promoters induced by these conditions is not favourable.

The stress responses of yeast and their associated gene regulation could be an option for successfully targeting the induction of gene expression during the industrial fermentation processes. Industrial yeasts adapt to different stresses during the fermentation process, including stresses like osmotic pressure, insufficient supply of FAN, temperature shifts and elevated concentrations of ethanol, under industrial conditions in particular (Gibson *et al.* 2007). This adaption is generated by different stress-response genes, which are regulated by transcription-factor binding sites on their promoters and transcription factors, including the stress response element, which binds Msn2/4 transcription factors; the heat shock element, which is controlled by Hsf1; and the AP-1-responsive element, which link to the transcription factors Gcn4 and Yap (Estruch 2000; Kandror *et al.* 2004; Aguilera, Randez-Gil and Prieto 2007; Ma and Liu 2010; Schade *et al.* 2004). Few studies have focused on gene regulation during shifts to cold or near freezing temperatures (Kondo and Inouye 1991; Kowalski, Kondo and Inouye 1995; Sahara, Goda and Ohgiya 2002; Becerra *et al.* 2003; Homma, Iwahashi and Komatsu 2003; Schade *et al.* 2004; Murata *et al.* 2006) or high temperatures (Estruch 2000; Izawa *et al.* 2008). Genes involved in trehalose and glycogen production are upregulated during shifts to low temperature (TPS1 and TPS2), as are genes associated with cell wall mannoproteins (TIP-related genes). A ≥2-fold increase in the induction of TPS1, TPS2, UBI4 and SSA3 has been reported after a temperature shift to 10°C (Sahara, Goda and Ohgiya 2002). Exposure to 4°C results in a >2-fold upregulation of TIR1, TIR2, TPS1, TIP1 and SSA3 (Homma, Iwahashi and Komatsu 2003). Further analysis of gene expression after temperature shifts to near freezing (4°C) shows the highest expression of TIR1 and TIR2 at the beginning of the shock situation (with a fold change of 9.0 and 6.2, respectively, after 6 h), with much smaller fold changes of 3.2 and 1.9, respectively, after 48 h (Murata *et al.* 2006). Moreover, TIR1 and TIR2 are strongly induced by a decrease in temperature, which is consistent with their low basal expression during fermentation (Kowalski, Kondo and Inouye 1995). Apart from these, genes from the heat shock protein (HSP) family are also induced during cold shock (Homma, Iwahashi and Komatsu 2003; Murata *et al.* 2006; Izawa *et al.* 2008). These proteins function as molecular chaperones to refold damaged proteins, protect thermally damaged proteins from aggregation and contribute to cell wall restructuring (Verghese *et al.* 2012). A higher concentration of ethanol also leads to stress that is associated with induced gene expression. A minimum of 4% (v/v) ethanol has been reported to be needed for a notable induction of HSP expression (Piper *et al.* 1994; Piper 1995). However, subsets of HSP genes show ideal expression patterns at different ethanol concentrations (Piper *et al.* 1994). Further, the promoter of TPS1 has been used for ethanol-induced yeast flocculation (Li *et al.* 2012).

Self-cloning yeasts offer different advantages for industrial application during food and beverage production. For example, research on wort fermentation has been focused on enhanced flocculation after fermentation (Ishida-Fujii *et al.* 1998), reduced maturation time (Kusunoki and Ogata 2012) and enhanced glutathione content and foam stability (Wang, He and Zhang 2007; Wang *et al.* 2008, 2009). In contrast to genetic modification, self-cloning does not result in genetically modified organisms (Fischer, Procopio and Becker 2013). Due to self-cloning, only homologous nucleic acids are utilized. In case of brewing yeast, research does not focus heavily on the lager yeast strain *Saccharomyces pastorianus* var. *carlsbergensis*; however, this strain contributes to 90% of the worldwide beer market (Kodama, Kielland-Brandt and Hansen 2006; Saerens, Duong and Nevoigt 2010). The lager yeast is an allotetraploid hybrid of the ale yeast *S. cerevisiae* and an *S. eubayanus* strain (Bing *et al.* 2014) and due to the genetically differences to the ale yeast more investigation is necessary.

In the present study, a total of 10 different native promoters of temperature-induced genes were evaluated during the brewing process. In addition to promoters of the HSP-gene family (Fischer *et al.* 2016), promoters of the TIP-related gene family were also considered due to the variety of results concerning temperature shifts to near freezing by laboratory yeasts, as mentioned above. In addition to the induction of these promoters by different temperature shifts, the influence of different ethanol contents was also investigated. To understand gene regulation during these stress situations, an enhanced green fluorescence protein (EGFP)-based method was used under industrial fermentation conditions (Fischer *et al.* 2016).

MATERIALS AND METHODS

Strains, plasmids and cultivation conditions

The lager yeast *S. pastorianus* var. *carlsbergensis* TUM 34/70 was used as the cloning host for yeast transformation. A promoter expression cassette was inserted into the URA3 locus. The recombinant strains constructed for promoter screening are shown in Table 1. The recombinant strains were selected using geneticin sulphate (G418) resistance. *Escherichia coli* DH5α was used for plasmid construction and was cultivated at 37°C in Luria–Bertani (LB) medium (Green and Sambrook 2012) supplemented with ampicillin (100 mg L^{-1}) or kanamycin (50 mg L^{-1}) when necessary. Yeasts were grown at 24°C in yeast extract peptone dextrose (YEPD; 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) supplemented with G418 (200 mg L^{-1}) when necessary. Standard synthetic wort (12°P) medium (Procopio *et al.* 2013), which was used to assess yeast fermentation ability and shock conditions, was composed of (g L^{-1}): yeast nitrogen base LoFlo w/o amino acids (Formedium, UK), 6.9; K_2HPO_4, 1.3; glucose, 12; maltose, 74; maltotriose, 17; fructose, 2.5; sucrose, 4; glycine, 0.04; alanine, 0.12; valine, 0.13; leucine, 0.17; isoleucine, 0.08; serine, 0.07; threonine, 0.08; asparagine, 0.16; glutamine, 0.11; aspartic acid, 0.9; glutamic acid, 0.1; cysteine, 0.01; methionine, 0.02; lysine, 0.14; arginine, 0.15; histidine, 0.05; phenylalanine, 0.15; tyrosine, 0.12; tryptophan, 0.02 and proline, 0.36, and if necessary, the pH value was adjusted to pH 5.4 with 20% lactic acid.

DNA manipulation and plasmid construction

Plasmid DNA was prepared from *E. coli* using the alkaline lysis method. Genomic yeast DNA was prepared using the glass bead method (Amberg, Burke and Strathern 2005). The primers used in this study are shown in Table 2. Promoters were amplified from the genomic DNA of the brewer's yeast *S. cerevisiae* TUM 68 and *S. pastorianus* var. *carlsbergensis* TUM 34/70 with equivalent primers using the polymerase chain reaction (PCR), followed by restriction fragment length polymorphism analysis to detect differences in the sequence. After the PCR product was obtained, it was digested with the restriction enzymes *Sph*I and *Sma*I (*Nhe*I and *Sma*I was used for $_P$SSA3 and $_P$HSP26) and cloned into the plasmid pEGFP-KanMX (Fischer *et al.* 2016).

Table 1. Strains and plasmids used in this study.

Strain or plasmids	Relevant genotype	Reference or source
Strains		
Escherichia coli DH5α	supE44 ΔlacU169(φ 80lacZΔM15) hsdR17 recAl endAl gyrA96 thi-1 relAl	Invitrogen
TUM 34/70	Wild type *S. pastorianus* var. *carlsbergensis*	Own stock
TUM 34/70-pTIP1	Promoter of the temperature shock-inducible protein	This study
TUM 34/70-pTPS1	Promoter of Trehalose-6-phosphate Synthase	This study
TUM 34/70-pTIP2	Promoter of Trehalose-6-phosphate Phosphatase	This study
TUM 34/70-pTIR1	Promoter related to TIP1	This study
TUM 34/70-pTIR2	Promoter related to TIP1	This study
TUM 34/70-pHSP12	Promoter of the heat shock protein, plasma membrane protein	This study
TUM 34/70-pHSP26	Promoter of the small heat shock protein	This study
TUM 34/70-pHSP30	Promoter of the heat shock protein, negative regulator of the H(+)-ATPase Pma1p	This study
TUM 34/70-pHSP104	Promoter of the heat shock protein, Disaggregase	This study
Plasmids		
pUG6	Cloning vector *Amp, KanMX*	Euroscarf
pEGFP	Cloning vector *eGFP, Amp*	Clontech
peGFP-CYC1ter-KanMX	Recombined plasmid, *KanMX, eGFP, CYC1-terminator*	Fischer *et al.* (2016)
pTIP1-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{TIP1}	This study
pTPS1-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{TPS1}	This study
pTIR1-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{TIR1}	This study
pTIR2-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{TIR2}	This study
pUBI4-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{UBI4}	This study
pSSA3-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{SSA3}	Fischer *et al.* (2016)
pHSP12-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{HSP12}	Fischer *et al.* (2016)
pHSP26-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{HSP26}	Fischer *et al.* (2016)
pHSP30-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{HSP30}	Fischer *et al.* (2016)
pHSP104-eGFP-CYC1ter-KanMX	Recombined plasmid peGFP-CYC1ter-KanMX, expressing eGFP gene by P_{HSP104}	Fischer *et al.* (2016)

Transformation and screening

Rubidium chloride-competent *E. coli* cells were transformed using the heat-shock method. For yeast transformation, the promoter expression cassette was amplified using PCR (with URA3-FL-L and URA3-FL-R primers) with the proofreading Pwo polymerase (Preqlab, Germany). Through the use of those primers, the transformation cassettes were elongated on both sites with 25 bp of sequence homologous to the target locus URA3. The transformation was performed with purified DNA fragments (PCR cycle pure Kit; Peqlab, Germany) using the lithium acetate (LiAc) method, as previously described (Gietz and Schiestl 2007). The recombinant strains were selected on YEPD plates containing 200 mg L^{-1} G418. PCR was carried out to amplify the transformation cassettes from the whole genome of equivalent recombinant strains. The PCR products were sequenced (GATC, Germany) and squared. For genetic stability testing, all recombinant yeast strains were grown on YEPD plates without G418 for 24 h at 24°C. This process was repeated five times. After the fifth transfer and incubation, the yeast colonies were grown on YEPD plates containing G418 (200 and 400 mg L^{-1}) for two days at 24°C. To ensure an equal integration event in the URA3 locus of the allotetraploid yeast strain, transformed yeasts were plated on YEPD plates with increasing G418 content (1000–3500 mg L^{-1}) and incubated for two days at 24°C.

Sampling during fermentation and shock situation

Fermentation and shock situations (four replicates) were carried out in high-throughput scale (black, clear-bottom 96-well microtiter plates (Biozyme, Germany) with standard synthetic wort medium (12°P) at 12°C. The population of recombinant strains and their host were added to every well (15 × 10^6 cells mL^{-1}). Temperature shifts to near freezing (4°C) were performed for up to 72 h at different growth phases. At the beginning of the fermentation and at the end of stationary phase [≈5°P residual extract and 3% (v/v) ethanol,] the temperature was shifted from 12°C to 4°C. Furthermore, the influences of genetic responses to different concentration of ethanol [4% (v/v), 6% (v/v), 8% (v/v) and 10% (v/v)] were also investigated.

Fluorescence observations were performed during fermentation and after shock situations using a Synergy H4 Hybrid Microplate reader (Biotek, Germany) with excitation at 485/20 nm and emission at 525/9 nm and 585/9 nm (Gain 120). The optical density (OD) measured at 600 nm was correlated to the cell number (Fischer *et al.* 2016). The fluorescence value was normalized to the cell number value to obtain the relative fluorescence units per cell (RFU). The RFU of the equivalent GFP-expressing yeast was normalized to the beginning of the shock situation and to the autofluorescence of the host strain (Lichten *et al.* 2014).

Data analysis and statistical data processing

The fluorescence of the recombinant strain was corrected by the measured autofluorescence of the host strain as previously described (Lichten *et al.* 2014). The autofluorescence r_a is the ratio of the fluorescence at 585 nm to the fluorescence at 525 nm of the host strain measured at the OD of the recombinant strains. The symbol g denotes fluorescence from the protein tag and the symbol f denotes the fluorescence measurement at the indicated wavelength. The OD at 600 nm of the host strain was fitted over the whole time, using a cubic polynomial when necessary. At each time point, the r_a, the fluorescence data of the

Table 2. Oligonucleotide primers used in PCR amplification.

Primers	Sequence 5′→ 3′
P_{TIP1}-L	GCATGCAAGCTTATCATTTCTGGTGTT (SphI)
P_{TIP1}-R	CGGATCCGATGGTCTGATGCTCTTTTTG (BamHI)
P_{TPS1}-L	CGGCATGCGATTCTTGATGAATTTTACGA (SphI)
P_{TPS1}-R	GCCCCGGGTTAATAAGTCTGTATGTG (XmaI)
P_{TIR1}-L	CGGCATGCTCTTAATTCAAATAAGCACTG (SphI)
P_{TIR1}-R	CACCCGGGGCCATTTTTAATTATTGTAGT (XmaI)
P_{TIR2}-L	CGGCATGCGAAAGTTAAAACAATCGATTA (SphI)
P_{TIR2}-R	GACCCGGGCATTTTTTTTGTTATAGTTGAA (XmaI)
P_{UBI4}-L	TAGCATGCAGGATTTTCAGGTTCAGGAT (SphI)
P_{UBI4}-R	TGCCCGGGATCTATTAGTTAAAGTAAAGTG (XmaI)
P_{HSP12}-L	GCATGCTTTTTTTGTCCAGGTGGAGTG (SphI)
P_{HSP12}-R	CCCGGGGACATTGTTGTATTTAGTTTTTT (XmaI)
P_{HSP26}-L	GCTAGCGTTGGAGTTTTTTTAATATAACC(NheI)
P_{HSP26}-R	CGCGGGGTTAATTTGTTTAGTTTGTTTGT (XmaI)
P_{HSP30}-L	CGGCATGCGTTTATTGGAATAGCCAATTAG (SphI)
P_{HSP30}-R	GTCCCGGGTTTGAAATTTGTTGTTTTTAGT (XmaI)
P_{HSP104}-L	TGGCATGCGCTAGCTCAGCCGGA (SphI/NheI)
P_{HSP104}-R	ACCCCGGGGCATATATTCTGTATATTTTATGGTACGTG (XmaI)
P_{SSA3}-L	ACGCTAGCAATTCAAGTAATTATTTTGGGG (NheI)
P_{SSA3}-R	TTCCCGGGTTTTCTTTGTAGCGTTTAGT (XmaI)
CYC1-L	GGCGGCCGATGTAATTAGTTATGTCACG (EagI)
CYC1-R	CGAGATCTGCAGCTTGCAAATTAAAGC (BglII)
KanMX-L	GGGCCGTAGGTCTAGAGATCT (EagI)
KanMX-R	GACGTCATTAAGGGTTCTCGAG (AatII)
URA3-FL-L	CGAAAGCTACATATAAGGAACGTGCTGACGATGATT ACGCCAAG
URA3-FL-R	AAATATGCTTCCCAGCCTGCTTTTCTTCCCCGAAA AGTGCCACCT

recombinant strain, and the OD was needed for the computation of the fluorescence per cell (Eq. 1). The data analysis was performed with the OriginLab software (ORIGIN® 2015).

$$g = \frac{r_a f_{525} - f_{585}}{r_a - r_g} \quad (1)$$

All experiments were repeated at least four times, and all data are reported at the mean ± SD. The statistical significance of the differences between the expression values of equivalent promoters was determined by analysis of variance (ANOVA). The statistical level of significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Expression vector sets for promoter screening

Ten promoters were chosen to evaluate the expression patterns of stress-related promoters from the yeast *S. pastorianus* var. *carlsbergensis* TUM 34/70. The gene cassette, which contained the promoter of interest, EGFP, the CYC1-terminator and the geneticin sulphate resistance marker, was amplified from the 5′ and 3′ end of the gene cassette with primers elongated with sequences homologous to the URA3 locus using the proof-reading Pwo polymerase. After transformation, all recombinant strains showed 100% stability over five re-inoculations and incubation for 24 h at 24°C without additional antibiotic (data not shown). Further, the copy number of the gene cassettes inserted in the URA3 locus was assessed by increasing the G418 content (1000–3500 mg L^{-1}). All strains exhibited growth at 3000 mg L^{-1} G418, indicating an equal copy number (Parekh, Shaw and Wittrup 1996).

Fermentation patterns of recombinant strains

Fermentations were carried out in black, clear-bottom 96-well plates at 24°C in 12°P synthetic wort inoculated with 15×10^6 cells mL^{-1} of *S. pastorianus* var. *carlsbergensis* TUM 34/70. All recombinant strains showed identical cell growth (Fig. 1a). This indicated that the characteristics of the different recombinant strains were comparable to those of the host strain. The disruption of one copy of the URA3 gene, which encodes orotidine-5′-phosphate (OMP) decarboxylase, and transformation into the allotetraploid strain TUM 34/70 (Walther, Hesselbart and Wendland 2014) had no influence on cell growth. The cell growth in the presence of ethanol decreased in proportion to the concentration of ethanol added (Fig. 1b).

Induction affected by cold shock

To determine if induction of EGFP expression is affected by cold shock, the yeasts (15×10^6 cells mL^{-1}) were added to synthetic wort and fermented at 12°C. First, the shock was performed in an exponential growth phase [approximately 0.4% (v/v)]. All recombinant strains showed the highest induction 24 h after initiation of the shock condition; $_P$TPS1 had the highest fold change among the promoters of the TIP-family (Fig. 2a). As previously reported (Kowalski, Kondo and Inouye 1995), exposure to 10°C resulted in drastically increased numbers of transcripts of TIP-related-proteins. After exposure to 4°C, the fold change of the $_P$TIR1 expression was similar to that of $_P$TIR2. The promoter $_P$TIP1 showed a lower fold change in expression than those of $_P$TIR1 and $_P$TIR2. Higher expression of TIR1 than of TIR2 was also observed after a temperature shift from 25°C to 4°C (Murata *et al.* 2006). The fluorescence patterns of the promoters from the HSP-family were clearly higher than these from cold shock related genes; $_P$HSP104 and $_P$HSP26 showed the highest induction, with fold changes of 5.2 and 5.0, respectively (Fig. 2a).

To determine whether the induction affected by cold shock during growth at 12°C differed from the exponential phase to the late stationary phase, we analysed induction of the recombinant strains at the end of fermentation [≈ 5°P residual extract and 3% (v/v) ethanol]. It was interesting to investigate the introduction of the shock situation at the end of fermentation rather than induction during the exponential phase because of relevance of this for the transition from fermentation to maturation in the industrial brewing process (Fig. 2b). The induction of the promoter of TPS1 decreased in fold change after 12 h compared with introducing the shock situation in the exponential growth phase where the induction continued to rise until 24 h. A similar effect has been reported for TIP1 expression (Kondo and Inouye 1991), but does not conform to the trend found in our results for $_P$TIP1. Further, slightly increased induction of all TIP-family promoters was observed; $_P$TIR1 had the highest induction (2.8-fold) under these conditions, relative to that in the earlier shock situation. The shock at the end of fermentation led to higher induction patterns for the HSP-family, and $_P$SSA3 (5.4-fold) and $_P$HSP104 (5.0-fold) showed the highest fold change in induction. A drastic increase in SSA3 gene regulation by a shift from 25°C to 4°C has also been reported previously (Homma, Iwahashi and Komatsu 2003). The activity of $_P$HSP104 resulted in a higher fold change for both shock situations, which is similar to the results seen for $_P$SSA3. Previous studies have also shown increased SSA3 expression in response to a shift to 4°C, however, lower expression

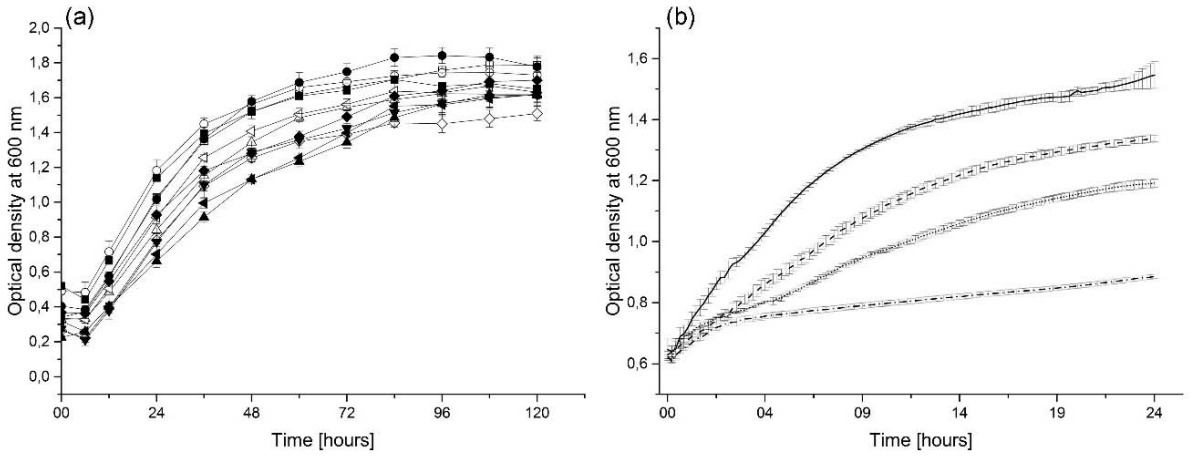

Figure 1. (a) Cell growth during fermentation in synthetic wort at 12°C (□ WT; ○ pTIP1; △ pTPS1; ◇ pTIR1; ◁ pTIR2; ■ pHSP12 ● pHSP26; ▲ pHSP30; ▼ pHSP104; ♦ pSSA3; ▲ pUBI4), (b) Cell growth of host strain under different ethanol concentrations in synthetic wort [— 4% (v/v); 6% (v/v); 8% (v/v); 10% (v/v)].

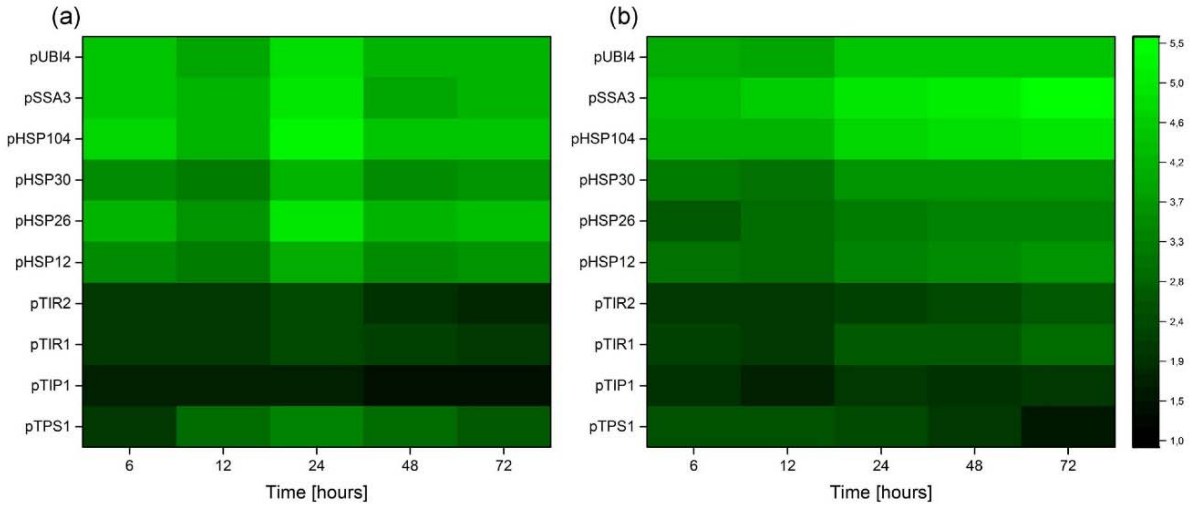

Figure 2. Heat map of fluorescence changes of EGFP under control of the different yeast promoters at each time point after temperature shift from 12°C to 4 C. (a) Fluorescence detection at initiation of fermentation. (b) Fluorescence detection at end of fermentation. Results are shown as ratio to time point 0 h of four replicates, values are indicated by colour bar. Standard derivation were typically about 10% and never exceed 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

was observed for HSP104 under this condition (Homma, Iwahashi and Komatsu 2003; Murata *et al.* 2006). Indeed, exposure to mild preconditioning stress could result in a certain degree of tolerance toward the same stress (Estruch 2000; Yamamoto *et al.* 2008; Morano, Grant and Moye-Rowley 2012). That is also consistent with the report that a temperature shift from 20°C to 4°C leads to minor induction of pHSP104 (Fischer *et al.* 2016) in comparison to a fermentation temperature at 12°C what results in a mild preconditioning stress.

The influence of ethanol on induction

Another interesting aspect in a practical point of view, is whether or not the induction is affected by various ethanol concentrations, which should be investigated to exclude the possibility that the induction of the gene expression by cold shock at the end of fermentation is actually triggered by ethanol. As seen with temperature shifts, the presence of ethanol leads to an accumulation of transcription factor Msn2/4p in the nucleus, which induces the expression of stress-related genes that harbour the STRE element (Martinez-Pastor *et al.* 1996; Gorner *et al.* 1998). Exposure to ethanol also leads to an increase in trehalose accumulation in lager and ale yeast cells (Odumeru *et al.* 1993) and enhanced membrane rigidity (Alexandre, Rousseaux and Charpentier 1994). To visualize the effect of ethanol alone on the selected promoters, the shock situation was performed at the optimal growth temperature (24°C) of the lager yeast strain (Fig. 3) and further under fermentation temperature (12°C; Table 4). Ethanol concentrations above 5% (v/v) do occur in this process, especially during industrial fermentation. Therefore, recombinant strains (15×10^6 cells mL^{-1}) were added to synthetic wort that was supplemented with equivalent amounts of ethanol [4% (v/v), 6% (v/v), 8% (v/v) and 10% (v/v)] and induction patterns were analysed over 24 h. The effects of

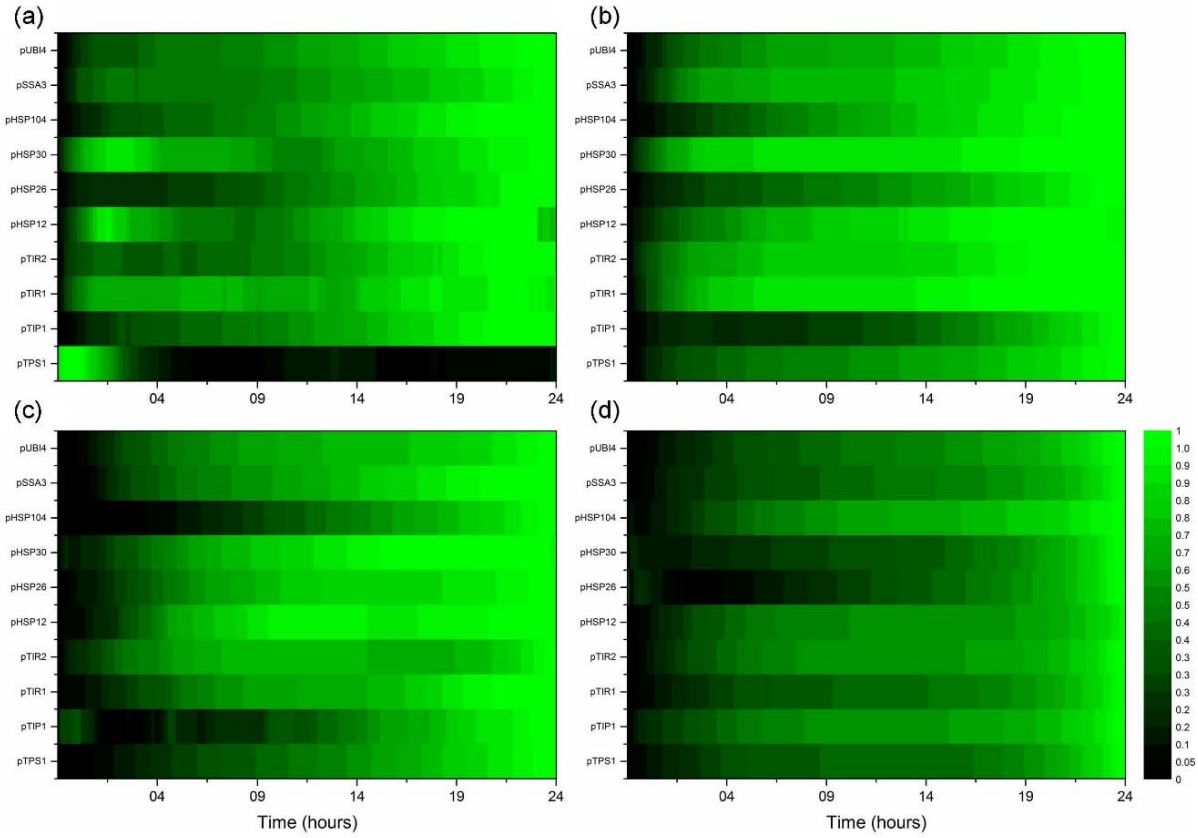

Figure 3. Heat map of fluorescence per cell over the time-series at 24°C. (a) Fluorescence detection by ethanol shock 4% (v/v). (b) Fluorescence detection by ethanol shock 6% (v/v). (c) Fluorescence detection ethanol shock 8% (v/v). (d) Fluorescence detection ethanol shock 10% (v/v). Data are normalized to the maximum value of each fluorescence expression under the different promoters. The normalized level of fluorescence detection is indicated by the colour bar. Standard derivation ware typically about 10% and never exceeded 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

Table 3. Fluorescence changes of EGFP under control of the different yeast promoters at selected time points after exposure to different ethanol contents in synthetic wort over 24 h at 24°C.

	4% (v/v)			6% (v/v)			8% (v/v)			10% (v/v)		
Strain	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
$_{P}$TPS1	−1.7	−1.7	−2	2.1	2.4	3.4	1.4	1.7	2.5	1.3	1.4	1.9
$_{P}$TIP1	5.7	7.7	12.4	1.1	1.2	1.8	1.0	1.2	1.6	1.4	1.4	1.7
$_{P}$TIR1	2.2	2.2	2.6	2.0	2.0	2.1	1.4	1.6	1.9	1.5	1.7	2.7
$_{P}$TIR2	1.5	1.7	2.3	1.5	1.6	1.7	1.4	1.4	1.6	1.7	1.8	2.4
$_{P}$HSP12	1.1	1.2	1.2	1.5	1.6	1.7	1.3	1.4	1.4	1.6	1.7	2.3
$_{P}$HSP26	1.3	1.6	2.3	1.4	1.7	2.2	1.3	1.4	1.6	1.0	1.0	1.2
$_{P}$HSP30	1.6	1.4	1.9	1.7	1.7	1.8	1.3	1.4	1.5	1.2	1.2	1.7
$_{P}$HSP104	2.0	2.5	3.4	2.8	4.0	5.4	1.2	1.6	2.4	1.3	1.4	1.6
$_{P}$SSA3	1.4	1.5	1.8	1.7	1.8	2.0	1.2	1.3	1.4	1.6	1.9	2.9
$_{P}$UBI4	1.5	1.6	1.9	1.9	2.1	2.6	1.4	1.6	1.9	1.5	1.8	2.6

Results are shown as ratio to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

various ethanol concentrations on cell growth are displayed in Fig. 1b. Cell growth was drastically affected by increasing amounts of ethanol. Fig. 3 shows the fluorescence per cell, normalized to the maximum of each strain, as a function of time at 24°C. The induction patterns of each strain differed at various ethanol concentrations. The promoter $_{P}$TPS1 showed maximum fluorescence at the beginning of the shock situation when 4% (v/v) ethanol was applied. In contrast, the maximum for this promoter was reached at the end of the shock situation with 6%–10% (v/v) ethanol. The overall maximum fluorescence value

Table 4. Fluorescence changes of EGFP under control of the different yeast promoters at selected time points after exposure to different ethanol contents in synthetic wort over 12 h at 12°C.

	4% (v/v)			6% (v/v)			8% (v/v)			10% (v/v)		
Strain	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
pTPS1	1.1	−1.5	−2.0	1.3	1.0	−1.5	−1.1	−1.5	−2.3	1.2	1.1	−1.1
pTIP1	1.2	−1.5	−2.4	1.4	1.1	−1.7	−1.1	−1.5	−2.4	1.3	1.2	−1.1
pTIR1	1.2	−1.6	−2.5	1.3	−1.1	−1.8	−1.2	−1.9	−2.9	1.3	1.1	−1.2
pTIR2	1.3	−1.5	−2.3	1.4	1.0	−1.6	−1.4	−2.3	−3.2	1.2	1.1	−1.2
pHSP12	−1.1	−1.5	−2.6	−1.1	−1.5	−2.4	−1.4	−1.8	−2.4	1.2	1.0	−1.1
pHSP26	−1.1	−1.4	−2.4	−1.0	−1.3	−1.9	−1.4	−1.7	−2.3	1.2	1.0	−1.2
pHSP30	−1.0	−1.2	−2.3	1.2	−1.1	−1.8	−2.0	−2.6	−3.3	1.2	1.1	−1.2
pHSP104	−1.2	−1.4	−2.6	1.1	−1.3	−2.1	−1.6	−2.2	−3.1	1.1	−1.1	−1.3
pSSA3	−1.1	−1.4	−2.4	1.1	−1.2	−1.7	−1.6	−1.8	−2.7	−1.1	−1.2	−1.4
pUBI4	−1.1	−1.4	−2.5	1.2	−1.2	−1.8	−1.7	−2.1	−3.1	−1.1	−1.2	−1.4

Results are shown as ratio to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 15%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

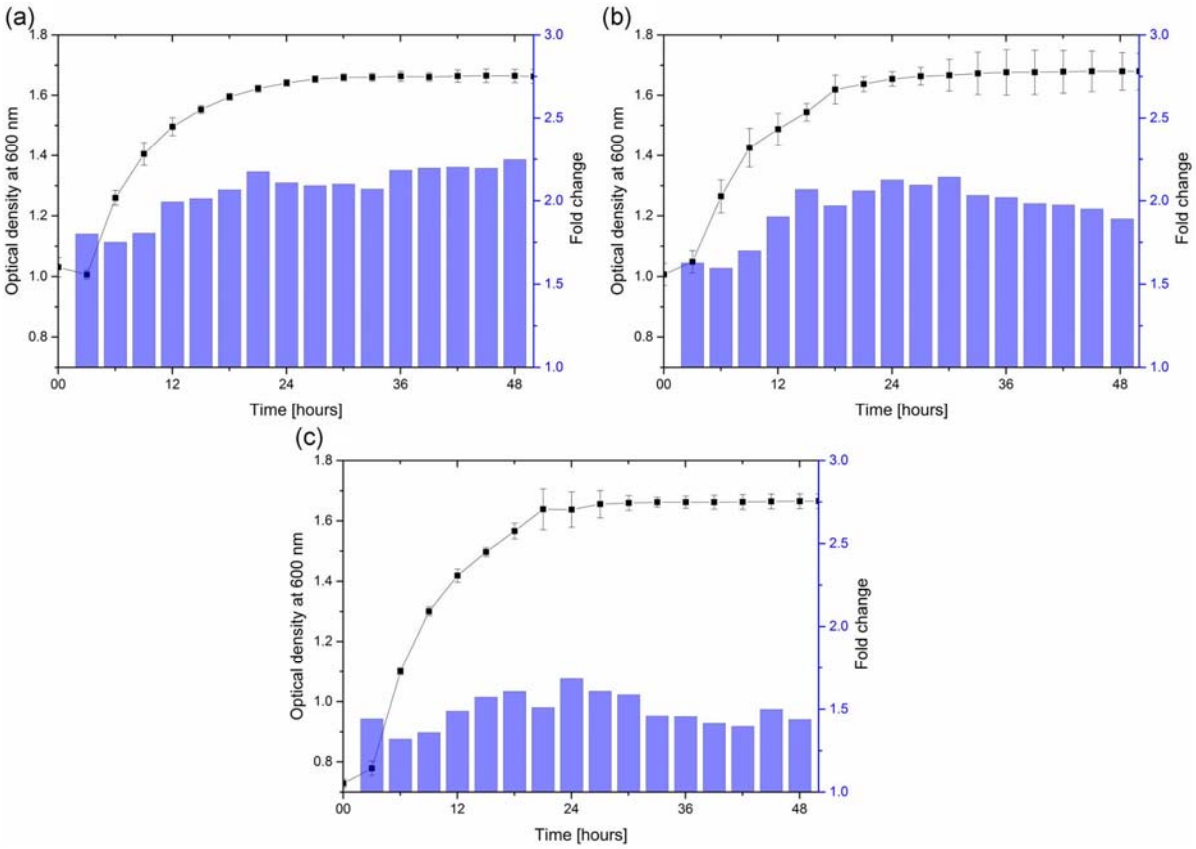

Figure 4. Cell growth of recombinant yeast over 50 h at 24°C (curve) and fold-change of fluorescence during fermentation in columns, relatively to time point at 0 h. (a) pHSP104; (b) pSSA3; (c) pUBI4. Results of fold-changes are shown as ratio to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

for pTIR1 was detected at 8% (v/v) and 10% (v/v) ethanol, and this fluorescence value increased with time, whereas at 4% (v/v) and 6% (v/v) ethanol, the fluorescence values were constant. The fluorescence value for pHSP12 reached its maximum directly after exposure to 4% (v/v) ethanol, while higher concentrations of ethanol [6% (v/v) and 8% (v/v)] resulted in maximal fluorescence values at 10 h. Similar induction trends were seen at 4%–8% (v/v) ethanol with pHSP26, and maximum induction was observed directly after the shock exposure at 10% (v/v) ethanol. By the induction of pHSP30 started at the beginning of shock situation upon adding 4% (v/v) or 6% (v/v) ethanol, in contrast to the results seen with 8% (v/v) and 10% (v/v) ethanol. The maximum fluorescence values for pTIP1, pHSP104, pSSA3 and pUBI4 occurred at 24 h for all ethanol shock treatments.

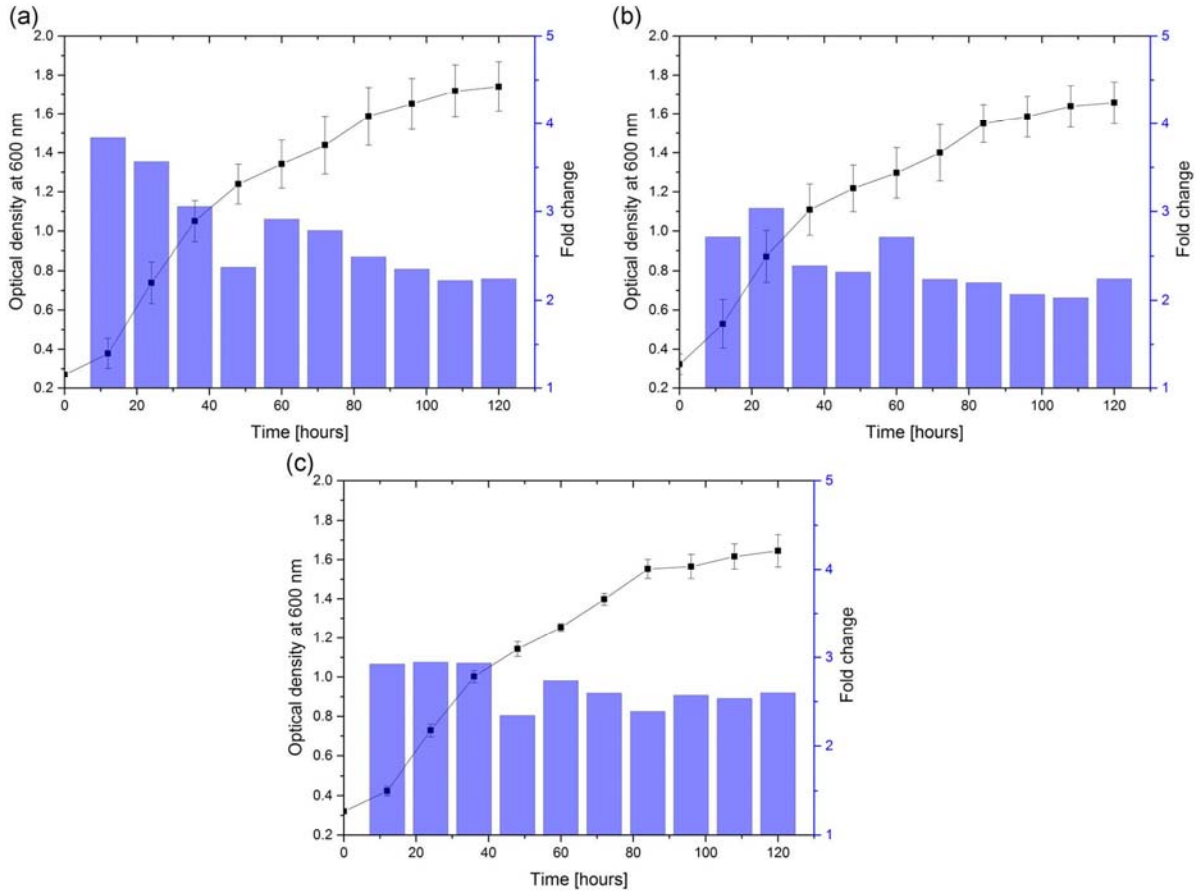

Figure 5. Cell growth of recombinant yeast over 72 h at 12°C (curve) and fold-change of fluorescence during fermentation in columns, relatively to time point at 0 h. (a) $_{P}$HSP104; (b) $_{P}$SSA3; (c) $_{P}$UBI4. Results of fold-changes are shown as ratio to time point 0 h of four replicates. Standard derivation were typically about 10% and never exceed 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

To compare the activity of the different promoters, the fold changes at selected time points are displayed in Table 3. The highest activity among the promoters of the TIP-family was observed at 4% (v/v) ethanol, where the maximum value was reached after 24 h (12.9-fold for $_{P}$TIP1 and 2.6-fold for $_{P}$TIR1). In contrast, the promoters of HSP-family showed highest activities at higher concentrations of ethanol. Notably, $_{P}$HSP12, $_{P}$SSA3 and $_{P}$UBI4 showed their highest actives at 10% (v/v), while $_{P}$HSP26 and $_{P}$HSP30 displayed their highest at 6% (v/v). Our results are consistent with the observations of Piper and colleagues (Piper *et al.* 1994), with the acceptation of $_{P}$HSP104, for which we observed the highest activity at ethanol concentrations of 6% (v/v), instead of 8% (v/v).

During cold shock, a decrease in membrane fluidity triggers the cold shock response pathway (Aguilera, Randez-Gil and Prieto 2007), which enhances the fluidity of the membrane. In contrast, the presence of ethanol leads to an increase of fluidity of the membrane and triggers the pathways that enhance membrane rigidity by increasing the ergosterol and trehalose content, which antagonize the inhibitory effect of ethanol stress (Wang *et al.* 2015). Due to these events, the effect of the ethanol content on the induction patterns might by rather different at 12°C (Table 4). Because of the rapid induction that occurred directly after the shock situation and the decrease in induction after 12 h, the 3 h time point is shown instead of the 24 h time point (Table 4), which contrasts with the ethanol shock at 24°C (Table 3). In general, the induction patterns at 12°C were lower than those at 24°C. The promoters of TPS1 and TIP-related genes showed slight induction after 3 h of exposure to 4% (v/v) ethanol. In comparison, these promoters showed slightly higher induction that persisted till 6 h at 6% (v/v) ethanol, and then, the induction decreased after 12h. The promoters $_{P}$HSP30, $_{P}$HSP104, $_{P}$SSA3 and $_{P}$UBI4 also showed induction at 6% (v/v) ethanol. At 8% (v/v) ethanol, no increase in fluorescence was detected over the time frame tested; the rapid induction of the promoters, which is also observed at other ethanol contents, followed by a faster decrease in induction could be the reason for this. On the other hand, the induction affected by 10% (v/v) ethanol was detectable for nearly all promoters, except for $_{P}$UBI4 and $_{P}$SSA3.

The inductions of $_{P}$SSA3 and $_{P}$UBI4 caused by ethanol treatment (Tables 3 and 4) were clearly lower than the highest inductions affected by cold shock (Table S1, Supporting Information). Therefore, these promoters are suitable for targeted induction by cold shock. Further, the greatest induction of the promoter of HSP104 was also affected by cold shock but also shows higher activity at 24°C with 4% (v/v) and 6% (v/v) ethanol, giving fold changes of 3.4 and 5.4, respectively. In contrast, the

ethanol shock at 12°C shows lower induction of pHSP104 as well (Table 4), which concludes that this promoter are also for main interest in targeting gene expression under such industrial conditions.

Induction during fermentation

As mentioned above, pHSP104, pSSA3 and pUBI4 showed high induction during cold shock, with fold changes of 5.0, 5.4 and 4.4 respectively (Fig. 2; Table S1, Supporting Information). Additionally, pHSP104 showed a fold change of 5.4 at 4%–6% (v/v) ethanol and 24°C (Table 3). In comparison, the induction affected at varied ethanol concentrations at 12°C show for these three promoters lower induction (Table 3). Indeed, stress-related genes such as HSP12, HSP30 and UBI4 show transition-phase induction during fermentation (Riou *et al.* 1997). Therefore, induction was observed during fermentation at 24°C (Fig. 4) and 12°C (Fig. 5). When fermentation was conducted at 24°C, pHSP104 showed a maximum change in its induction of 2.1-fold during the transition to stationary phase. These levels of induction were gradually reduced, except for those of pSSA3. With pSSA3, the induction increased slightly after transition to the stationary phase, with a maximum change of 2.25-fold. Minor steady-state expression was observed for pUBI4. However, the induction patterns observed after fermentation for more than 120 h at 12°C show slightly higher fold changes (Fig. 5). The promoter of HSP104 showed the highest fold change at the transition from lag phase to log phase (3.8-fold) and decreased at a nearly constant rate to 2.2 at the end of the fermentation (120 h). At log phase, an increase in induction was also observed with pSSA3, reaching a maximum of 3.0 and then decreasing slightly to 2.2 after 120 h. The induction of pUBI4 during fermentation at 12°C showed its highest value (2.9-fold) from the start of fermentation until 36 h. Thereafter, the induction fluorescence decreased slightly, to 2.2-fold, at the end of fermentation.

CONCLUSION

The availability of promoters suitable for induced gene expression in industrial applications related to food and beverage production is limited. The present study evaluated, for the first time temperature-inducible native promoters from lager yeast under conditions similar to those encountered during brewing. The induction patterns of 10 different promoters were evaluated under optimal growth temperature and stress situations that could occur during industrial brewing fermentations, such as temperature shifts that occur during the transition from fermentation to maturation and higher ethanol content. When being cold shocked, pHSP104, pSSA3 and pUBI4 showed the highest activity. Further, the induction initiated by ethanol was weak in pSSA3 and pUBI4. In comparison, the induction of pHSP104 was affected more by the ethanol content when it reached 6% (v/v) at 24°C. Steady-state expression during fermentation should be as limited as possible. In this case, pUBI4 showed the lowest tendency for steady-state expression, compared with that of pHSP104 and pSSA3 at optimal growth temperature. Beside these, the steady-state expression under fermentation temperature (12°C) are for all three promoters slightly higher, but compared to cold-shock lower expression are observed. These three native promoters show high potential for induced gene expression in self-cloning brewing yeast.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

ACKNOWLEDGEMENTS

We thank Roland Kerpes for useful discussions.

FUNDING

This work was supported by the DFG (Deutsche ForschungsGemeinschaft, BE 2245/12-1).

Conflict of interest. None declared.

REFERENCES

Aguilera J, Randez-Gil F, Prieto JA. Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *Fems Microbiol Rev* 2007;**31**:327–41.

Alexandre H, Rousseaux I, Charpentier C. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and Kloeckera apiculata. *FEMS Microbiol Lett* 1994;**124**:17–22.

Amberg D, Burke D, Strathern J. *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2005 Edition (Cold Spring)*, DOI citeulike-article-id:571213. New York: Cold Spring Harbor Laboratory Press, 2005.

Becerra M, Lombardia LJ, Gonzalez-Siso MI *et al.* Genome-wide analysis of the yeast transcriptome upon heat and cold shock. *Comp Funct Genomics* 2003;**4**:366–75.

Bing J, Han P-J, Liu W-Q *et al.* Evidence for a Far East Asian origin of lager beer yeast. *Curr Biol* 2014;**24**:R380–R1.

Estruch F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *Fems Microbiol Rev* 2000;**24**:469–86.

Farhi M, Dudareva N, Masci T *et al.* Synthesis of the food flavoring methyl benzoate by genetically engineered *Saccharomyces cerevisiae*. *J Biotechnol* 2006;**122**:307–15.

Fischer S, Engstler C, Procopio S *et al.* EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system. *Food Science Technol* 2016;**68**:556–62.

Fischer S, Procopio S, Becker T. Self-cloning brewing yeast: a new dimension in beverage production. *Eur Food Res Technol* 2013;**237**:851–63.

Gibson BR, Lawrence SJ, Leclaire JPR *et al.* Yeast responses to stresses associated with industrial brewery handling. *Fems Microbiol Rev* 2007;**31**:535–69.

Gietz RD, Schiestl RH. Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2007;**2**:35–7.

Gorner W, Durchschlag E, Martinez-Pastor MT *et al.* Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev* 1998;**12**:586–97.

Green MR, Sambrook J. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 2012.

Homma T, Iwahashi H, Komatsu Y. Yeast gene expression during growth at low temperature. *Cryobiology* 2003;**46**:230–7.

Ishida-Fujii K, Goto S, Sugiyama H *et al.* Breeding of flocculent industrial alcohol yeast strains by self-cloning of the flocculation gene FLO1 and repeated-batch fermentation by transformants. *J Gen Appl Microbiol* 1998;**44**:347–53.

Izawa S, Kita T, Ikeda K *et al.* Heat shock and ethanol stress provoke distinctly different responses in 3′-processing and nuclear export of HSP mRNA in *Saccharomyces cerevisiae*. *Biochem J* 2008;**414**:111–9.

Kandror O, Bretschneider N, Kreydin E *et al.* Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. *Mol Cell* 2004;**13**:771–81.

Kodama Y, Kielland-Brandt M, Hansen J. Lager brewing yeast. In: Sunnerhagen P, Piskur J (eds.) Comparative Genomics Vol. 15, Berlin: Springer, 2006, 145–64.

Kondo K, Inouye M. TIP1, a cold-shock-inducible gene of *Saccharomyces cerevisiae*. *J Biol Chem* 1991;**266**:17537–44.

Kowalski LRZ, Kondo K, Inouye M. Cold-shock induction of a family of TIP1-related proteins associated with the membrane in Saccharomyces cerevisiae. *Mol Microbiol* 1995;**15**:341–53.

Kusunoki K, Ogata T. Construction of self-cloning bottom-fermenting yeast with low vicinal diketone production by the homo-integration of ILV5. *Yeast* 2012;**29**:435–42.

Labbe S, Thiele DJ. Copper ion inducible and repressible promoter systems in yeast. In: Schmidt MC, Glorioso JC (eds). *Expression of Recombinant Genes in Eukaryotic Systems*. University of Virginia1999,145–53.

Lagunas R. Sugar transport in *Saccharomyces cerevisiae*. FEMS *Microbiol Lett* 1993;**104**:229–42.

Lea AGH, Piggott JR, Piggott JR. *Fermented Beverage Production*: Dordrecht, Netherlands: Springer, 2003.

Li Q, Zhao XQ, Chang AK *et al.* Ethanol-induced yeast flocculation directed by the promoter of TPS1 encoding trehalose-6-phosphate synthase 1 for efficient ethanol production. *Metab Eng* 2012;**14**:1–8.

Lichten CA, White R, Clark IBN *et al.* Unmixing of fluorescence spectra to resolve quantitative time-series measurements of gene expression in plate readers. *BMC Biotech* 2014;**14**, DOI: 10.1186/1472-6750-14-11.

Ma M, Liu LZ. Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. *BMC Microbiol* 2010;**10**:169.

Martinez-Pastor MT, Marchler G, Schuller C *et al.* The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *Embo J* 1996;**15**:2227–35.

Morano KA, Grant CM, Moye-Rowley WS. The Response to Heat Shock and Oxidative Stress in *Saccharomyces cerevisiae*. *Genetics* 2012;**190**:1157–95.

Murata Y, Homma T, Kitagawa E *et al.* Genome-wide expression analysis of yeast response during exposure to 4 degrees C. *Extremophiles* 2006;**10**:117–28.

Odumeru JA, D'Amore T, Russell I *et al.* Alterations in fatty acid composition and trehalose concentration of Saccharomyces brewing strains in response to heat and ethanol shock. *J Ind Microbiol* 1993;**11**:113–9.

Parekh RN, Shaw MR, Wittrup KD. An integrating vector for tunable, high copy, stable integration into the dispersed Ty delta sites of *Saccharomyces cerevisiae*. *Biotechnol Prog* 1996;**12**:16–21.

Piper PW. The heat-shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol Lett* 1995;**134**:121–7.

Piper PW, Talreja K, Panaretou B *et al.* Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma-membrane HSP30, by ethanol levels above a critical threshold. *Microbiology-(UK)* 1994;**140**:3031–8.

Procopio S, Krause D, Hofmann T *et al.* Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression. *Food Science Technol* 2013;**51**:423–32.

Riou C, Nicaud JM, Barre P *et al.* Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast* 1997;**13**:903–15.

Saerens SMG, Duong CT, Nevoigt E. Genetic improvement of brewer's yeast: current state, perspectives and limits. *Appl Microbiol Biot* 2010;**86**:1195–212.

Sahara T, Goda T, Ohgiya S. Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J Biol Chem* 2002;**277**:50015–21.

Schade B, Jansen G, Whiteway M *et al.* Cold adaptation in budding yeast. *Mol Biol Cell* 2004;**15**:5492–502.

Verghese J, Abrams J, Wang YY *et al.* Biology of the heat shock response and protein chaperones: budding yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiol Mol Biol Rev* 2012;**76**:115–58.

Walther A, Hesselbart A, Wendland J. Genome sequence of *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast. *G3: Genes—Genomes—Genetics* 2014;**4**:783–93.

Wang YF, Zhang SX, Liu HQ *et al.* Changes and roles of membrane compositions in the adaptation of *Saccharomyces cerevisiae* to ethanol. *J Basic Microbiol* 2015;**55**:1417–26.

Wang ZY, He XP, Liu N *et al.* Construction of self-cloning industrial brewing yeast with high-glutathione and low-diacetyl production. *Int J Food Sci Technol* 2008;**43**:989–94.

Wang ZY, He XP, Zhang BR. Over-expression of GSH1 gene and disruption of PEP4 gene in self-cloning industrial brewer's yeast. *Int J Food Microbiol* 2007;**119**:192–9.

Wang ZY, Wang JJ, Liu XF *et al.* Recombinant industrial brewing yeast strains with ADH2 interruption using self-cloning GSH1+CUP1 cassette. *FEMS Yeast Res* 2009;**9**:574–81.

West RW, Yocum RR, Ptashne M. Saccharomyces cerevisiae GAL1 - GAL10 divergent promoter region - Location and function of the upstream activating sequance UASG. *Mol Cell Biol* 1984;**4**:2467–78.

Yamamoto N, Maeda Y, Ikeda A *et al.* Regulation of thermotolerance by stress-induced transcription factors in *Saccharomyces cerevisiae*. *Eukaryot Cell* 2008;**7**:783–90.

2.4 Induced expression of the alcohol acetyltransferase gene ATF1 in industrial yeast *Saccharomyces pastorianus* TUM 34/70

Received: 21 November 2017 | Revised: 17 April 2018 | Accepted: 20 April 2018
DOI: 10.1002/yea.3319

RESEARCH ARTICLE

WILEY Yeast

Induced expression of the alcohol acetyltransferase gene *ATF1* in industrial yeast *Saccharomyces pastorianus* TUM 34/70

S. Fischer | K.R. Büchner | T. Becker

Technical University of Munich, Institute of Brewing and Beverage Technology, Research Group Beverage and Ceral Biotechnology, Weihenstephaner Steig 20, 85354 Freising, Germany

Correspondence
S. Fischer, Technical University of Munich, Institute of Brewing and Beverage Technology, Research Group Beverage and Ceral Biotechnology, Weihenstephaner Steig 20, 85354 Freising, Germany.
Email: susann.fischer@tum.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: BE 2245/12-1

Abstract

Targeted induced gene expression for industrial fermentation processes in food and beverage production could fulfill future demands. To avoid metabolic burden and disturbances owing to the fermentation procedure, induced gene expression is necessary for combating stress, such as that caused by temperature shifts that occur during the transition from fermentation to maturation in the brewing process. The aim of this study was to target gene expression in industrial yeast using stress-responsive promoters and homologues of the selection marker *SMR1*. Self-cloning strains of the industrial brewing yeast *Saccharomyces pastorianus* TUM 34/70 were constructed to overexpress the alcohol acetyltransferase (*ATF1*) gene under the control of inducible promoters $_{p}$*SSA3*, $_{p}$*HSP104* and $_{p}$*UBI4*. Transcription analysis shows the highest induction after 72 h of shock situation for $_{p}$*HSP104* with 1.3-fold and $_{p}$*UBI4* with 2.2-fold. Further, at the end of shock situation the concentrations of ethyl acetate were 1.2- and 1.3-fold higher than the wild type for $_{p}$*HSP104* and $_{p}$*UBI4*, respectively. In addition, the influence of the final temperature and temporal sequence of temperature shock to 4°C had a major impact on expression patterns. Therefore, these data show that temperature-induced gene expression of self-cloning industrial yeast could be an option for optimization of the beverage fermentation.

KEYWORDS

ATF1, fermentation, induced gene expression, industrial brewing yeast, self-cloning, stress response

1 | INTRODUCTION

To overcome the unfavourable characteristics of industrial yeast or adaption on the accelerating fermentation processes, metabolic engineering in yeast is of major interest for industrial applications. It is important to avoid accumulation of toxic products and/or metabolic burden because it drains energy reserves and dilutes molecular factors required for gene transcription and translation. Thus, fine-tuning of gene expression is indispensable (Nevoigt, 2008). To accomplish this goal, precise and regulated gene expression using effective regulatory gene promoters has recently been investigated (Govender, Domingo, Bester, Pretorius, & Bauer, 2008; Hubmann, Thevelein, & Nevoigt, 2014; Verstrepen et al., 2001) and intensively reviewed (Da Silva & Srikrishnan, 2012). However, regulated expression systems such as galactose (West, Yocum, & Ptashne, 1984) or copper-inducible systems (Farhi et al., 2006; Labbe & Thiele, 1999) are not conceivable for use in food and beverage production, because of the EU's food additive regulation (Commission regulation no. 1129/2011).

The industrial fermentation procedure in food and beverages is far from the natural circumstances of the yeast and leads to gene expression induced by bioprocess parameters. Such stress-induced gene expression could combine the advantages of metabolic engineering and applicability in industrial beverage fermentations. External stimuli, such as ethanol toxicity, malnutrition, osmotic pressure and changes in temperature, induce the expression of transcription factors that trigger the expression of stress-response genes by binding to transcription factor-binding sites in their promoters (Estruch, 2000; Kajiwara, Aritomi, Suga, Ohtaguchi, & Kobayashi, 2000; Morano, Grant, &

Moye-Rowley, 2012; Murata et al., 2006; Panadero, Pallotti, Rodriguez-Vargas, Randez-Gil, & Prieto, 2006; Werner-Washburne, Stone, & Craig, 1987). For example, transcription factors Msn2/4, Hsf1, Gcn4 and Yap bind to the stress-response element, the heat-shock element and the Activator Protein-1 (AP-1) responsive element, respectively (Aguilera, Randez-Gil, & Prieto, 2007; Kandror, Bretschneider, Kreydin, Cavalieri, & Goldberg, 2004; Ma & Liu, 2010; Schade, Jansen, Whiteway, Entian, & Thomas, 2004). During industrial fermentation, different stress conditions may arise sequentially and/or simultaneously (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). First, the pitching of the yeast into the fermentation medium exposes the yeast to high concentrations of osmotically active substances, in particular, glucose, fructose, sucrose, maltose and maltotriose. Such hypertonic conditions lead to an efflux of water from the cell, reduction of the water availability and diminished turgor pressure (Gibson et al., 2007; Hohmann, 2002; Tamás & Hohmann, 2003). Nutrient limitations, which could result in reduction of fermentation efficiency or starvation, occur at different stages of the fermentation, with the highest stress phenomena at the end of fermentation provoked by the simultaneous appearance of ethanol toxicity (Boulton, Singleton, Bisson, & Kunkee, 2013). During the fermentation, higher amounts of ethanol are produced. Ethanol is toxic to organisms at concentrations as low as 2% (v/v), and influences membrane fluidity by changing the levels of hexadecanoic, octadecanoic and palmitoleic acids to enhance the membrane fluidity and reduction of the intracellular water activity (Kajiwara, Suga, Sone, & Nakamura, 2000). Temperature shifts occur at the beginning (pitching) and at the end of fermentation by the switch from fermentation to maturation. All changes in temperature are recognized as a stress by the yeast cell (Piper, OrtizCalderon, Holyoak, Coote, & Cole, 1997).

Response to heat shock is evolutionarily conserved across all eukaryotes (Richter, Haslbeck, & Buchner, 2010). This is a multifaceted regulation system that involves metabolic remodelling, transient cessation of growth and global changes in transcription. It enables response to not only heat shock but also cold shock and increase in ethanol content (Piper, 1995), and is triggered by changes in membrane fluidity (Panadero et al., 2006). A minimum of 4% (v/v) ethanol is needed for a notable induction of heat-shock protein (*HSP*) genes (Piper, 1995; Piper et al., 1994). However, the temperature sensing mechanism was predicted on a membrane-embedded protein compound that has not yet been identified (Verghese, Abrams, Wang, & Morano, 2012).

The response to cold shock is regulated by different mechanisms depending on the time and temperature (Sahara, Goda, & Ohgiya, 2002; Schade et al., 2004). A decrease in temperature results in upregulation of genes involved in trehalose and glycogen production (*TPS1* and *TPS2*) and genes associated with cell wall mannoproteins (*TIP*-related genes). A 2-fold or greater increase in the induction of *TPS1*, *TPS2*, *UBI4* and *SSA3* genes has been reported after a decrease in temperature to 10°C (Sahara et al., 2002). Similarly, exposure to 4°C results in >2-fold upregulation of *TIR1*, *TIR2*, *TPS1*, *TIP1* and *SSA3* genes (Homma, Iwahashi, & Komatsu, 2003). Moreover, *TIR1* and *TIR2* are strongly induced by a decrease in temperature, which is consistent with their low basal expression during fermentation (Kowalski, Kondo, & Inouye, 1995). Additionally, *HSP* genes are also induced during cold shock (Homma et al., 2003; Izawa, Kita, Ikeda, & Inoue, 2008; Murata et al., 2006). Cellular response in yeast at 10°C varied from that at 4°C, suggesting that gene regulation is temperature dependent (Murata et al., 2006). Furthermore, the promoter of *HSP30* gene has also been used successfully for induced flocculation in the stationary phase of fermentation (Govender et al., 2008; Verstrepen et al., 2001).

Promoters of temperature-sensitive genes are an excellent choice for targeted gene expression in industrial processes. We previously evaluated gene promoters from ale yeast (*Saccharomyces cerevisiae* TUM 68) and lager yeast (*Saccharomyces pastorianus* TUM 34/70) for their induction patterns under brewing conditions (Fischer, Engstler, Procopio, & Becker, 2016a, 2016b). Ten different promoters from the *HSP* gene family, *TIP*-related gene family and *Ubiquitin* gene were investigated. The highest induction patterns were observed for three promoters, including $_{p}$*UBI4*, $_{p}$*SSA3* and $_{p}$*HSP104*, in response to temperature shifts, analogous to those that occur during the transition from fermentation to maturation in the brewing process.

In this study, we further evaluated these three promoters for targeted gene expression of *alcohol acetyltransferase* (*ATF1*) gene in the industrial lager yeast. The gene *ATF1*, which encodes for alcohol acetyltransferase (Atf1p) (Fujii et al., 1994; Mason & Dufour, 2000; Verstrepen et al., 2003), was chosen on account of the fact that volatile metabolites such as higher alcohols and esters contribute fundamentally to the quality of fermented beverages such as beer and wine (Belda et al., 2017; Pires, Teixeira, Branyik, & Vicente, 2014). In particular, esters are more relevant, owing to the low odour threshold value, whereas isoamyl acetate is the more desirable ester for beer flavour. They are mainly formed via the intracellular enzymatic condensation reaction of Atf1p, Atf2p and Eat1p between ethanol or a fusel alcohol (e.g. isoamyl alcohol, 1-propanol, isobutanol, hexanol) and acetylCoA (Kruis et al., 2017; Park, Shaffer, & Bennett, 2009). Further, principal olfactory alcohols responsible for the typical beer aroma as well as precursors for acetate esters include 1-propanol, isobutanol and isoamyl alcohol (Meilgaard, 1975). These are directly related to amino acid metabolism (valine, leucine and threonine, respectively) and generated via the Ehrlich pathway, where amino acids are transaminated by mitochondrial and cytosolic branched-chain amino acid aminotransferases (Eden, Van Nedervelde, Drukker, Benvenisty, & Debourg, 2001; Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Romagnoli, Luttik, Kotter, Pronk, & Daran, 2012). The expression levels of *ATF1* significantly affect the production of acetate esters, and further, synthase activities is linked to high sugar concentration in wort and nitrogen compounds (Engan, 1970; Verstrepen et al., 2003). The nitrogen metabolism is directly linked to the production of higher alcohols which leads, in turn, to higher concentrations of the equivalent acetate ester (Calderbank & Hammond, 1994). Furthermore, temperature conditions influence the acetate ester production significantly, where ethyl acetate and phenyl ethyl acetate are produced in their maximal concentrations at 20°C, for instance (Hammond, 1993). Higher fermentation temperatures result in higher expression levels of *ATF1* (Saerens, Verbelen, Vanbeneden, Thevelein, & Delvaux, 2008) and the highest activity of the synthase is at 30°C and reduced to 20% of activity at 4°C (Yoshioka & Hashimoto, 1981).

Owing to the usage of only homologous nucleic acids, the constructed yeast strains did not result in genetically modified organisms. It is to be declared as self-cloning yeast, depending on the state legislation (Fischer, Procopio, & Becker, 2013).

2 | MATERIALS AND METHODS

2.1 | Strains, plasmids and cultivation conditions

Lager yeast *S. pastorianus* TUM 34/70 comprises two nuclear subgenomes originating from *S. cerevisiae* and *Saccharomyces eubayanus* and was used as the cloning host for yeast transformation. A self-cloning expression cassette was inserted into the *Sc-URA3* locus. In order to ensure integration into the *Sc-URA3* locus, the primer and homologous overhangs of the gene cassette were compared with the specific sequences of the *URA3* gene of *S. cerevisiae* and *S. pastorianus* (Casaregola, Nguyen, Lapathitis, Kotyk, & Gaillardin, 2001). Self-cloning strains for screening are summarized in Table 1. Recombinant strains were selected on sulfometuron methyl (SM) media. The *Escherichia coli* DH5α strain was used for plasmid construction and was grown at 37°C on Luria Bertani media (Green & Sambrook, 2012). Recombinant *E. coli* cells were selected on Luria Bertani media supplemented with ampicillin (100 mg L^{-1}) or kanamycin (50 mg L^{-1}). Yeast was grown at 24°C in YEPD media (10 g L^{-1} yeast extract, 20g L^{-1} peptone and 20g L^{-1} glucose) or for selection of self-cloning strains on minimal media (7 g L^{-1} yeast nitrogen base without amino acids and 20g L^{-1} glucose) supplemented with SM (40 mg L^{-1}) when necessary. Standard synthetic wort (12°P) medium (Procopio, Krause, Hofmann, & Becker, 2013) was used for testing yeast fermentation ability and shock conditions in 2 L EBC standard tall tubes (0.5 × 150 cm). The composition of synthetic wort (12°P) medium was as follows (g L^{-1}): yeast nitrogen base without amino acids (Sigma Aldrich, Germany), 6.9; K_2HPO_4, 1.3; glucose, 12; maltose, 74; maltotriose, 17; fructose, 2.5; sucrose, 4; glycine, 0.04; alanine, 0.12; valine, 0.13; leucine, 0.17; isoleucine, 0.08; serine, 0.07; threonine, 0.08; asparagine, 0.16; glutamine, 0.11; aspartic acid, 0.9; glutamic acid, 0.1; cysteine, 0.01; methionine, 0.02; lysine, 0.14; arginine, 0.15; histidine, 0.05; phenylalanine, 0.15; tyrosine, 0.12; tryptophan, 0.02; and proline, 0.36, with pH adjusted to 5.4 using 20% lactic acid. Wort for fermentation and shock situation was prepared from Bavaria pilsner malt extract (Weyermann, Bamberg, Germany) and dissolved to 12°P.

2.2 | DNA manipulation and plasmid construction

Plasmid DNA was isolated from *E. coli* by alkaline lysis of cells. Genomic yeast DNA was isolated using glass beads (Amberg, Burke, & Strathern, 2005). For the construction of the expression cassettes EC-GA1, EC-GA4 and EC-GA5 (Table 1), promoter sequences and the *ATF1* gene were PCR-amplified from genomic DNA of lager yeast TUM 34/70 using gene-specific primers (Table 2). The selection marker *SMR1* was PCR-amplified from the plasmid pCP-2-4-10 (Casey, Xiao, & Rank, 1988; Govender et al., 2008). Amplified PCR products were purified using the Monarch PCR & DNA Cleanup Kit and digested with *DpnI* restriction endonuclease (New England Biolabs, Frankfurt/Main, Germany) when necessary to avoid DNA methylation. A schematic for the assembling of expression cassettes is shown in Figure 1. Subsequently, the gene cassette was integrated into the vector p44K (Ponchon et al., 2013) and transformed in *E. coli*.

2.3 | Transformation and screening

Rubidium chloride-competent *E. coli* cells were transformed by the heat-shock method. For yeast transformation, expression cassettes (EC-GA1, 5.3 kb; EC-GA4, 5.4 kb; EC-GA5, 5.6kb) were PCR-amplified using primer pairs $_P$SSA3/URA3-R, $_P$HSP104/URA3-R, $_P$UBI4/URA3-F and SMR1/URA3-R, respectively (Table 2) with the proofreading Q5-Polymerase (New England Biolabs, Frankfurt/Main, Germany). These primer pairs add 25 BP of *URA3* homologous sequence at either end of the expression cassette. PCR products were purified (Monarch PCR & DNA Cleanup Kit; New England Biolabs, Frankfurt/Main, Germany) and transformed into *Sc-URA3* locus in the lager yeast using the lithium acetate method as described in Gietz and Schiestl (2007).

TABLE 1 Strains and plasmids used in this study

Strain or plasmids	Relevant genotype	Reference or source
Strains		
E. coli DH5α	*supE44 ΔlacU169(φ 80lacZΔM15) hsdR17 recAl endAl gyrA96 thi-1 relAl*	Invitrogen
E. coli DH5α–GA1	p44K -$_P$SSA3-ATF1-CYC1-SMR1	This study
E. coli DH5α–GA4	p44K–$_P$HSP104-ATF1-CYC1-SMR1	This study
E. coli DH5α–GA5	p44K–$_P$UBI4-ATF1-CYC1-SMR1	This study
TUM 34/70	Wild-type *Saccharomyces pastorianus* var. *carlsbergensis*	Own Stock
TUM 34/70 GA1	TUM 34/70- $_P$SSA3-ATF1-CYC1-SMR1	This study
TUM 34/70 GA4	TUM 34/70- $_P$HSP104-ATF1-CYC1-SMR1	This study
TUM 34/70 GA5	TUM 34/70- $_P$UBI4-ATF1-CYC1-SMR1	This study
Plasmids		
p44K	Cloning vector AmpR	Ponchon et al. (2013),
pCP-2-410	Cloning vector URA3, SMR1	Casey et al. (1988)
EC-GA1	p44K–$_P$SSA3-ATF1-CYC1-SMR1	This study
EC-GA4	p44K–$_P$HSP104-ATF1-CYC1-SMR1	This study
EC-GA5	p44K–$_P$UBI4-ATF1-CYC1-SMR1	This study

TABLE 2 Primers used in this study

Primers	Sequence (5′ → 3′)	Underlined sequence
$_{p}$SSA3-F	acacatatgggccatggcactagtggatccAATTCAAG-TAATTATTTTGGGGAG	Overlap to p44K
$_{p}$SSA3-R	atcgatttcattcatTTTTCTTTGTAGCGTTTAGTAC	Overlap to ATF1
$_{p}$HSP104-F	acacatatgggccatggcactagtggatccTCAGCCGGAACCTAAATTG	Overlap to p44K
$_{p}$HSP104-R	atcgatttcattcatATATTCTGTATATTTTATGGTACGTG	Overlap to ATF1
$_{p}$UBI4-F	acacatatgggccatggcactagtggatccAGGATTTTCAGGTTCAGG	Overlap to p44K
$_{p}$UBI4-R	atcgatttcattcatATCTATTAGTTAAAGTAAAGTGGGAG	Overlap to ATF1
ATF1-F-G1	acgctacaaagaaaaATGAATGAAATCGATGAGAAAAATC	Overlap to $_{p}$SSA3
ATF1-F-G4	aaatatacagaatatATGAATGAAATCGATGAGAAAAATC	Overlap to $_{p}$HSP104
ATF1-F-G5	cttttaactaatagatATGAATGAAATCGATGAGAAAAATC	Overlap to $_{p}$UBI4
ATF1-R	cataactaattacatCTAAGGGCCTAAAAGGAG	Overlap to CYC1TT
CYC1TT-F	cttttaggcccttagATGTAATTAGTTATGTCACGC	Overlap to ATF1
CYC1TT-R	agccaagccggtaccGCAGCTTGCAAATTAAAGC	Overlap to SMR1
SMR1-F	taatttgcaagctgcGGTACCGGCTTGGCTTCA	Overlap to CYC1TT
SMR1-R	gtgatggtgatgatgggtaccggcggccgcAGCTTGCAATTTTTGACGGC	Overlap to p44K
p44K-F	GGATCCACTAGTGCCATGGCCC	
p44K-F	GCGGCCGCCGGTACCCAT	
$_{p}$SSA3/URA3-F	CGAAAGCTACATATAAGGAACGTGCaattcaagtaattattttggggag	$_{p}$SSA3-specific
$_{p}$HSP104/URA3-F	CGAAAGCTACATATAAGGAACGTGCtcagccggaacctaaattg	$_{p}$HSP104-specific
$_{p}$UBI4/URA3-F	CGAAAGCTACATATAAGGAACGTGCaggattttcaggttcagg	$_{p}$UBI4-specific
SMR1/URA3-R	AAATATGCTTCCCAGCCTGCTTTTCagcttgcaatttttgacggc	SMR1-specific
Sc-URA3-F	CGAAAGCTACATATAAGGAACGTGC	
Sc-URA3-R	AAATATGCTTCCCAGCCTGCTTTTC	
ATF-Q-L	GTACGAGGAGGATTACCA	qPCR
ATF-Q-R	ATGATCTCGGTGACAAC	qPCR
TAF10-Q-L	GAGGAGATTCTAGAGATGATGGACAG	qPCR
TAF10-Q-R	GTAGTCTATTACTGCATCGGGAATG	qPCR
UBC6-Q-L	GTGATTACCACCCTGATACTTGG	qPCR
UBC6-Q-R	ACCCGTTCAAAATGGTTGAG	qPCR
TCF1-Q-L	CAGACACTCCAGGCGGTATT	qPCR
TCF1-Q-R	ACCACGGTATTCTTTTTCCATC	qPCR

The entries in bold are the main primers used for the construction and the main Strains used in this study

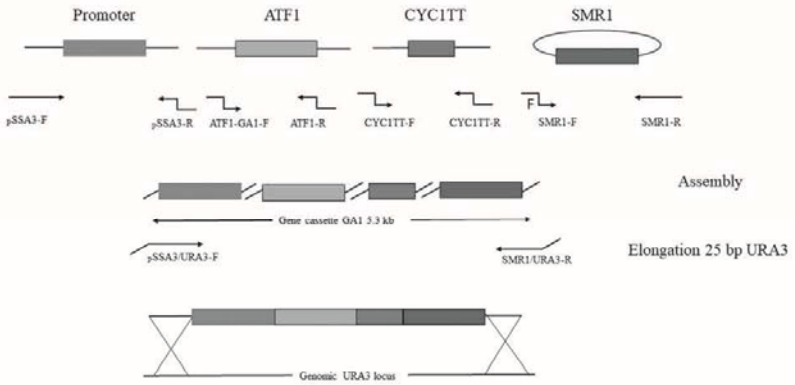

FIGURE 1 Schematic showing the construction of the expression cassette for self-cloning brewing yeast TUM 34/70 GA1 strain

The recombinant strains GA1, GA4 and GA5 were selected on minimal media containing SM (40 mg L^{-1}). Transformed cassettes were PCR-amplified (Q5-Polymerase, New England Biolabs, Frankfurt/Main, Germany) from the genome of the recombinant yeast strains with primer **ATF1-Q-L** and **ATF1-Q-R**, confirmed by sequencing (GATC, Konstanz, Germany), and squared. For genetic stability, all self-cloning yeast strains were inoculated on minimal media without SM and grown for 24 h at 24°C. This was repeated five times. After the fifth transfer and incubation, the yeast colonies were inoculated on minimal media with SM (70 mg L^{-1}) and incubated for 3 days at

24°C. To ensure an equal integration event in the *Sc-URA3* locus of the self-cloning yeast strain, transformed yeasts were plated on minimal media plates with increasing SM content (20–70 mg L^{-1}) and incubated for 2 days at 24°C.

2.4 | Construct copy number assay

Copy number was quantified using qPCR from total DNA extracts of wild-type TUM 34/10 and self-cloning strains. Quantitative PCR was performed on LightCycler 480 II (Roche Diagnostics, Mannheim, Germany), using Blue S'Green qPCR Mix from Biozym (Hess. Oldendorf, Germany), following the manufacturer's instructions with an annealing of 58°C and 30 ng of total DNA per 10 µL reaction volume. Primer ATF-Q-L/R (Table 2) was used for copy number estimation and compared with *UBC6* gene (UBC6-Q-L/R). The standard curve was established using *ATF1* gene from TUM34/70 with serial dilution with concentration of 5×10^1 to 1×10^8.

2.5 | Fermentation and shock conditions

Yeast precultures were shaken overnight at 24°C in test tubes with 10 mL of unhopped malt extract medium (Weyermann, Bamberg, Germany; 12°P). After 16 h of growth, this 10 mL overnight culture was used to inoculate 200 mL of unhopped malt extract medium in 300 mL Erlenmeyer flasks fixed with parafilm. The second preculture was shaken at 24°C for 48 h followed by inoculation in 2 L medium in a Duran bottle and shaken at 24°C for further 48 h under semi-anaerobic conditions.

A total of 15×10^6 viable cells mL^{-1} were fermented at 12°C for 168 h and shocked for 120h at 4°C in 2 L scale (diameter 5 cm × height 150 cm). Additional fermentation and shock situations were tested on self-cloning and wild-type yeast strains under different scenarios (Table 4) at a 300 mL (diameter 3.8 cm × height 30 cm) scale, where a faster temperature shift is feasible. Samples (40 mL) were taken at intervals for up to 288 h after pitching and immediately cooled on ice. Medium and cells were separated by centrifugation at 0°C. Cell pellets for RNA isolation were flash-frozen in liquid nitrogen and stored at −30°C. All fermentations were repeated at least three times, and all data were reported at the mean value ± SD. Significant differences between the induction values of equivalent strain were determined by ANOVA. The statistical level of significances was set at $p \leq 0.05$.

2.6 | Analysis of yeast growth during fermentation and shock situation

The course of fermentation and yeast growth was monitored. Samples were periodically withdrawn and suspensions were diluted to an appropriate volume. Cell density was measured at OD_{600}. Subsequently, yeast and media were separated by centrifugation for 1000 rpm for 10 min at 4°C (Eppendorf, Hamburg, Germany). The alcohol content, cell density and pH of the media were measured using a DMA 4500 Alcolyser Plus density analyser (Anton Paar, Graz, Austria) and pH meter, respectively.

2.7 | Analysis of volatile compounds by gas chromatography coupled with flame ionization detection

Headspace gas chromatography coupled with flame ionization detection was used for measuring higher alcohols and esters in the fermentation products. Samples (5 mL) were collected in 15 mL precooled glass tubes, which were immediately closed and placed on ice. The samples were analysed using a calibrated Hewlette Packard 6890 gas chromatograph equipped with a headspace sampler (HP 7694; Hewlette Packard, Waldbronn, Germany) and with an HP-5 column (crosslinked with 5% Phe and 95% Me-Si; length, 50 m; inside diameter, 0.32 mm; and layer thickness, 0.52 mm; Waldbronn, Germany). Samples were heated for 20 min at 65°C in the headspace autosampler. The injection block and flame ionization detector temperatures were kept constant at 150 and 250°C, respectively; helium was used as the carrier gas. The oven temperature was held at 50°C for 11 min, then increased to 120°C at a rate of 10°C per min, held for 5 min, then increased to 220°C at a rate of 20°C per min, and finally held at 220°C. The results were analysed using the Agilent Technologies Chemstation Rev. A.10.01 software.

Diacetyl were quantified by gas chromatography–flame ionization detection (Hewlett-Packard 5890 Series II Plus) with a Hewlett Packard 7673 A automatic sampler (HP Inc., Böblingen, Germany) based on the method previously reported (Krahl, Zarnkow, Stürmer, & Becker, 2009). Compounds were separated using two capillary columns with different polarities. Column I was a 60 m HP Innovax Polyethylene glycol and column II was a 60 m HP5 column both with 0.25 mm film thickness and 0.25 mm internal diameter. Carrier gas was hydrogen at a constant flow of 3.8 mL/min (split 1:10) and the injection volume was 3 mL at 250°C. The following temperature programme was applied: 60°C for 4 min, increasing at 5°C/min to 220°C, with 30 min hold. Concentrations were calculated from internal calibrations with commercial reference substances.

2.8 | Transcription analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and further purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was conducted following the RevertAid Reverse Transcriptase protocol with oligo(dT) and random hexamer primers (Thermo Fischer Scientific, Darmstadt, Germany). Quantitative analysis was performed by RT-qPCR. The *UBC6* (ubiquitin–protein ligase activity) and *TCF1* (RNA Pol transcription factor activity) genes were used as references in gene expression analysis (Teste, Duquenne, Francois, & Parrou, 2009). *ATF1* primers were designed according to Saerens et al. (2008). mRNA quantification was conducted using Blue S'Green qPCR Mix (Biozym, Hess. Oldendorf, Germany) in the LightCycler 480 II (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The following conditions were used for the amplification: initial denaturation for 60 s at 95°C, amplification by 40 cycles of 10 s at 95°C, 25 s at the optimal annealing temperature for each primer pair, and 10 s at 72°C elongation temperature. For the relative quantification of gene expression, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was used.

The values represent the average of three independent biological and three technical replicates. The results were statistically evaluated using one-way ANOVA ($p < 0.05$) followed by the Fischer test.

3 | RESULTS AND DISCUSSION

3.1 | Confirmation of self-cloning brewing yeast

Expression cassettes for temperature-induced self-cloning strains of bottom-fermenting yeast TUM 34/70 were constructed as shown in Figure 1. In addition to the three promoters *pSSA3*, *pHSP104* and *pUBI4*, which were evaluated in previous work for high induction efficiency affected by cold shock (Fischer et al., 2016b), the *ATF1* and the dominant selection marker *SMR1* were assembled and integrated in the previously described vector p44K (Ponchon et al., 2013). To ensure that only homologous gene material is present in the expression cassette, the vectors were isolated and sequenced after transformation. To obtain stable, single-copy integration of the inducible expression cassette into the *Sc-URA3* locus, 25 bp overhangs homologous to *Sc-URA3* were created at either end of the gene cassette via PCR. Homologous recombination between the endogenous *Sc-URA3* and 25 bp overhangs ensured stable integration of the expression cassette into the yeast genome. Recombinant, self-cloning strains were selected on minimal media supplemented with SM. Post-transformation, all recombinant strains showed 100% stability over five 24 h inoculation and incubation cycles at 24°C without additional SM (data not shown). To assess copy numbers of the gene cassette inserted in the *Sc-URA3* locus, the concentration of SM was increased from 20 to 70 mg L^{-1}. All strains exhibited growth at 70 mg L^{-1}, indicating an equal copy number of cassettes (Parekh, Shaw, & Wittrup, 1996). The further investigation to determine the copy number by qPCR of the genomic DNA confirmed the result (Figure 2).

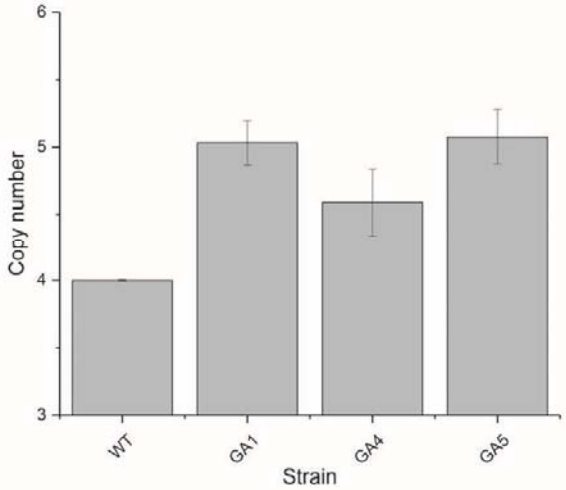

FIGURE 2 Copy number per cell. Copy number of Sc-*ATF1* were determined by qPCR of genomic DNA in wild-type and self-cloning strains. Results were normalized to wild type. Error bars represent standard deviations among tested technical replicates. Three technical replicates were tested for each strain

3.2 | Fermentation patterns of self-cloning yeast strains

Wild-type and self-cloning strains of lager yeast (GA1, GA4 and GA5) were fermented at 12°C in 12°P synthetic wort medium inoculated with 15×10^6 cells mL^{-1}. All self-cloning strains (except of GA4) produced identical fermentation patterns (Figure 3). GA4 shows deviations in the reduction of the extract as well as in the alcohol content after the shock, which, however, were not significant. This indicated that characteristics of different self-cloning strains were comparable with those of the host strain. The disruption of one copy of the *URA3* gene, which encodes for orotidine-5′-phosphate decarboxylase, and transformation into the allotetraploid strain TUM 34/70 (Nakao et al., 2009; Walther, Hesselbart, & Wendland, 2014) had no influence on cell growth.

3.3 | Induction affected by cold shock

Acetate esters, such as ethyl acetate and isoamyl acetate, are the main class of flavour-active metabolites in alcoholic fermented beverages and a product of Atf1p and Atf2p catalysed condensation reaction of a higher alcohol and acetyl-CoA (Fujii et al., 1994; Mason & Dufour, 2000; Verstrepen, Derdelinckx, et al., 2003; Verstrepen, Van Laere, et al., 2003). The acetate esters were generated throughout the fermentation and shock condition (Figure 4). Compared with the wild-type strain, self-cloning strains GA5 and GA4 generated significantly more ethyl acetate ($p < 0.05$). By the end of the experiment, ethyl acetate levels for GA5 and GA4 were 17 and 15.4 mg L^{-1}, which were 1.3- and 1.2-fold higher than the wild type, respectively. The expression level of *ATF1* (Table 3) was evaluated right before the temperature shift to 4°C (time point 0 h) until the end of shock situation. The expression rose after 24 h of shock situation for GA5 and had the highest level after 72 h with 1.3-fold for GA4 and 2.2-fold for GA5. Although ethanol is a potent inducer of stress-related promoters (Fischer et al., 2016a, 2016b; Piper et al., 1994), its level did not reach the critical threshold of 4% (v/v) when the temperature was shifted to 4°C. Furthermore, the influence of induction during fermentation at 12°C was also observed for these three promoters (Fischer et al., 2016b). Upon temperature of 12°C, no significant difference was observed in ethyl acetate levels between self-cloning strains and wild type ($p < 0.05$), indicating that higher content of ethyl acetate results from the induction of *pHSP104* and *pUBI4* through cold shock to 4°C. Indeed, no significant differences were detected for isoamyl acetate at the end of the shock situation (data not shown). However, the concentrations with an average of 0.65 mg L^{-1} were at the limit of the detection and taste threshold.

The concentrations of the precursor of isoamyl acetate, isoamyl alcohol and the other relevant higher alcohols, isobutanol and 1-propanol, were determined (Figure 5). Although no significant differences were detected in the alcohol levels at 72 h after the transition to 4°C, comparable amounts of propanol (Figure 5a) and isoamyl alcohol (Figure 5c) were determined for self-cloning strains at the end-point of sampling (120 h of shock situation) compared with those for the wild type. The concentration of isobutanol (Figure 5b) at the sampling end-point was significantly higher in self-cloning than in

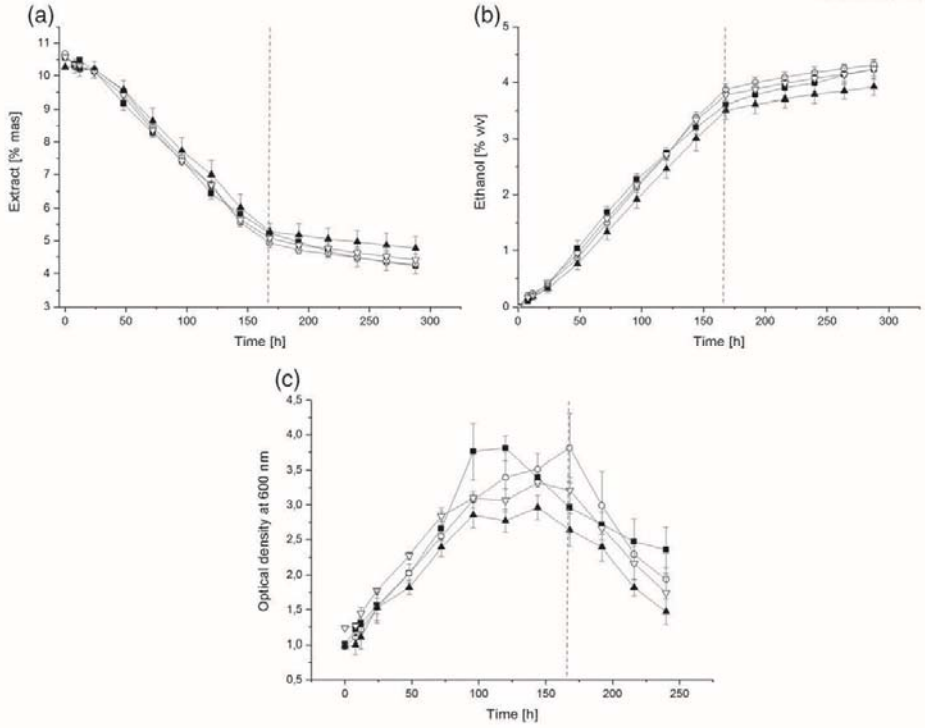

FIGURE 3 Analysis of fermentation parameters of wild-type (WT) and self-cloning yeast strains. Fermentation parameters including extract (a), ethanol (b) and optical density (OD_{600}) (c) were monitored for wild-type (■) and self-cloning yeast strains, GA1 (○), GA4 (▲) and GA5 (▽) grown in synthetic wort (12°P) at 12°C in EBC tall tubes. Error bars indicate standard deviation of three independent replicates, vertical dotted line indicates the beginning of the shock situation

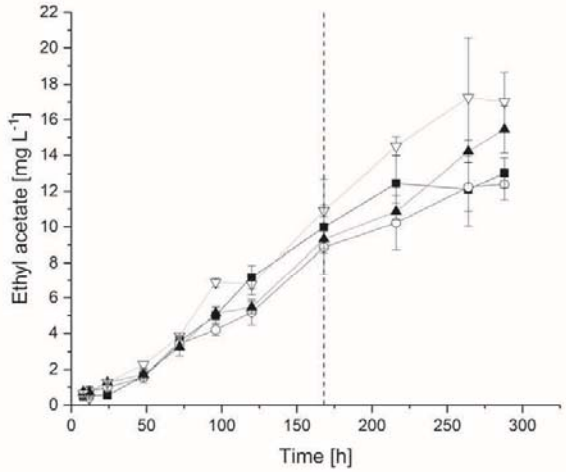

FIGURE 4 Determination of esters produced during fermentation. Production of (a) ethyl acetate and (b) isoamyl acetate synthesized in EBC tall tubes by WT and self-cloning yeast strains was measured fermented synthetic wort. WT (■), GA1 (○), GA4 (▲) and GA5 (▽). Error bars indicate standard deviation of three independent replicates, vertical dotted line indicates the beginning of the shock situation

TABLE 3 Relative transcription profile of ATF1 by self-cloning yeast strains after shock condition

	Time (h)			
Strain	0	24	72	96
WT	1.00 (± 0.17)	0.90 (± 0.13)	0.77 (± 0.29)	1.18 (± 0.20)
GA1	1.01 (± 0.18)	1.00 (± 0.26)	0.95 (± 0.15)	0.89 (± 0.08)
GA4	1.01 (± 0.15)	0.88 (± 0.24)	1.02 (± 0.02)	0.74 (± 0.12)
GA5	1.00 (± 0.11)	**1.23** (± 0.22)	**1.67** (± 0.32)	0.97 (± 0.06)

Results are the averages of three independent fermentations and three technical replicates. Significant differences ($p < 0.05$) are indicated in bold.

wild-type yeast strains. The content increased significantly after the shock situation. This is consistent with the result of a previous report by Lilly, Lambrechts, and Pretorius (2000) showing significantly higher amounts of isobutanol during wine fermentation with yeast overexpressing *ATF1* than with the wild-type strain, but the exact mechanism is not known yet.

Effects of the final maturation temperature and faster cooling rate were further investigated on the self-cloning yeast strain GA5 compared with the wild-type strain (Table 4). The yeast strain GA5 showed a significantly higher amount of ethyl acetate than the wild-type strain under shock scenarios 1 and 2 (Table 5). Under the shock scenario 1,

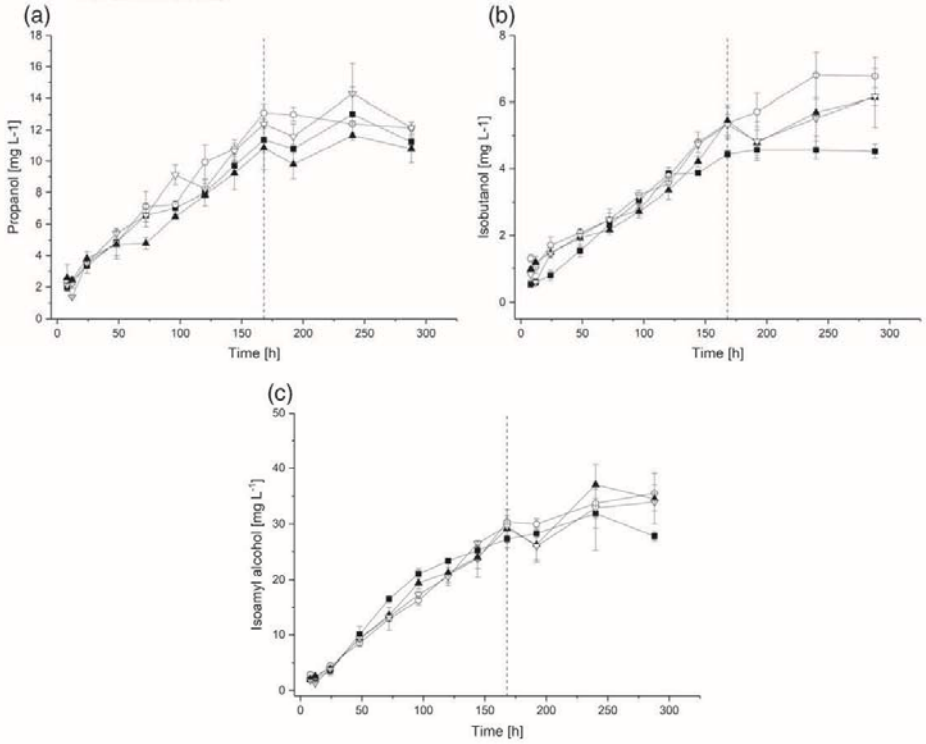

FIGURE 5 Evaluation of higher alcohols produced during fermentation. Production of (a) propanol, (b) isobutanol and (c) isoamyl alcohol synthesized in EBC tall tubes by WT and self-cloning yeast strains was measured fermented synthetic wort. WT (■), GA1 (○), GA4 (▲) and GA5 (▽). Error bars indicate standard deviation of three independent replicates, vertical dotted line indicate the beginning of the shock situation

TABLE 4 Scenarios for fermentation and shock situations

Fermentation	Duration	Shock	Duration
Scenario 1			
12 °C	72 h	4 °C	48 h
12 °C	72 h	4 °C	96 h
Scenario 2			
12 °C	72 h	6 °C	48 h
12 °C	72 h	6 °C	96 h
Scenario 3			
20 °C	24 h	6 °C	48 h
20 °C	24 h	6 °C	96 h

ethyl acetate concentration increased to 4.29 mg L^{-1} after 96 h, which corresponded to a 2.9-fold increase and was 1.6-fold higher than the WT. A temperature shift from 12 to 6°C resulted in slightly higher fold changes of ethyl acetate production after 96 h with 1.4-fold for GA5. No significant differences in the production of isoamyl acetate were detected. No significant differences were detected by a temperature shift from 20 to 6°C. Indeed, exposure to mild preconditioning stress could result in a certain degree of tolerance towards the same stress (Estruch, 2000; Morano et al., 2012; Yamamoto, Maeda, Ikeda, & Sakurai, 2008). That is also consistent with the report that a temperature shift from 20 to 4°C leads to minor induction of temperature-induced promoters (Fischer et al., 2016a) in comparison with a fermentation temperature at 12°C which results in a mild preconditioning stress.

The dominant selection marker *SMR1* encodes for the α-acetolactate synthase, which synthesizes α-acetolactate from pyruvate (Smolke, 2009). This α-acetolactate is converted non-enzymatically to diacetyl. For this reason, the content of diacetyl was also measured (Table 5). Only the fermentation at 12°C and shock at 6°C showed a significantly higher diacetyl content compared with the wild-type strain. In the other fermentations no significant effects could be detected.

As already mentioned, the activity of Atf1p is also temperature dependent. This could be the reason for the weak effect of ethyl acetate and isoamyl acetate production under the shock condition. Additionally, enzyme activity is reported to be inversely proportional to the temperature, e.g. enzyme activity at 6°C cannot be expected to be higher than that at 4°C (Yoshioka & Hashimoto, 1981).

Therefore, in addition to the temporal sequence of temperature shifts, the final temperature is important for the induction of the signal cascade (Murata et al., 2006; Panadero et al., 2006; Schade et al., 2004). Further, the duration of shock situation had an influence on the induction patterns of *pUBI4* (Sahara et al., 2002;

TABLE 5 Final concentrations of aromatic compounds in self-cloning yeast strain GA5 and wild-type (WT) yeast after 48 and 96 h of various shock scenarios

Compound (mg L^{-1})	WT	GA5	WT	GA5	WT	GA5
	0 h		48 h		96 h	
Scenario 1						
Ethanol [% (v/v)]	1.6	1.3	2.2	1.8	2.5	2.2
Propanol	3.9	4.5	4.4	5.0	3.6	5.9
Isobutanol	1.7	1.5	2.0	2.2	1.7	2.7
Isoamyl alcohol	13.1	14.5	15.6	15.5	12.9	**18.7**
Ethyl acetate	1.2	1.5	2.0	2.2	2.7	**4.3**
Isoamyl acetate	0.0	0.1	0.0	**0.1**	0.0	**0.2**
Diacetyl					0.45	0.40
Scenario 2						
Ethanol [% (v/v)]	0.6	0.7	0.7	0.9	1.0	1.2
Propanol	5.4	6.4	6.4	6.2	6.0	6.6
Isobutanol	1.5	**2.4**	2.0	**2.7**	2.0	3.1
Isoamyl alcohol	10.0	11.8	12.9	13.0	12.3	13.6
Ethyl acetate	1.7	2.2	3.9	4.6	4.1	**5.8**
Isoamyl acetate	0.1	0.1	0.2	0.2	0.2	0.2
Diacetyl					0.21	**0.48**
Scenario 3						
Ethanol [% (v/v)]	0.6	0.7	1.2	1.1	1.4	1.7
Propanol	3.9	4.4	5.2	5.2	4.6	5.5
Isobutanol	1.4	**2.0**	1.6	2.5	1.8	2.7
Isoamyl alcohol	9.3	9.4	10.7	11.0	11.1	12.1
Ethyl acetate	1.9	2.3	4.4	5.1	5.6	8.5
Isoamyl acetate	0.1	0.1	0.3	0.3	0.4	0.4
Diacetyl					0.27	0.34

Results are averages of three independent fermentations. Significant differences ($p < 0.05$) are indicated in bold. Standard deviations were typically ~15% of the values and never exceed 30%.

Schade et al., 2004). This is consistent with our results that higher fold changes were reached after 48 h of shock situation. Thus, the final temperature and temporal sequence of the cold shock have a major impact on gene regulation by the stress-related promoter *UBI4*.

4 | CONCLUSION

Induced gene expression of industrial yeast is highly relevant for food and beverage production. In addition to establishing methods and selection markers for constructing yeast strains, we examined the induction efficiency of three temperature-inducible homologous promoters by the overexpression of *ATF1* in the industrial lager yeast *S. pastorianus*. Fermentations were performed by mimicking industrial trials with different shock scenarios. Data clearly showed that temperature shock induced gene expression in self-cloning yeast strains, resulting in an increased production of ethyl acetate. The promoters $_p$*UBI4* and $_p$*HSP104* (strain GA5 and GA4, respectively) showed significantly higher production of ethyl acetate, whereas $_p$*UBI4* showed the highest fold change. This is due to the higher transcription rate of *ATF1* by the promoter $_p$*UBI4*. However, different shock scenarios, where temperature decreased to 6 and 4°C, did not result in an increase in ethyl acetate production, indicating that, in addition to the shock situation itself, the final temperature has a significant effect on induction of the evaluated promoters. A steady-state induction was not detected during the fermentation, which was reflected by the absence of significant differences in ethyl acetate level before the shock situation. In summary, homologous promoters $_p$*HSP104* and $_p$*UBI4* may be suitable for induced gene expression in industrial fermentation and combine the advantages of the genetic modification of yeast and its application in food and beverage production.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche ForschungsGemeinschaft (BE 2245/12-1).

COMPLIANCE WITH ETHICAL STATEMENTS

No conflict of interest is declared. This article does not contain any studies with human or animal subjects.

ORCID

S. Fischer http://orcid.org/0000-0002-8526-2362

REFERENCES

Aguilera, J., Randez-Gil, F., & Prieto, J. A. (2007). Cold response in *Saccharomyces cerevisiae*: New functions for old mechanisms. *FEMS Microbiology Reviews*, *31*(3), 327–341.

Amberg, D., Burke, D., & Strathern, J. (2005). *Methods in yeast genetics: A Cold Spring Harbor Laboratory course manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., & Moreno-Arribas, M. V. (2017). Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules*, *22*(2), 189.

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (2013). *Principles and practices of winemaking*. Berlin: Springer Science.

Calderbank, J., & Hammond, J. R. M. (1994). Influence of higher alcohol availability on ester formation by yeast. *Journal of the American Society of Brewing Chemists*, *52*(2), 84–90.

Casaregola, S., Nguyen, H. V., Lapathitis, G., Kotyk, A., & Gaillardin, C. (2001). Analysis of the constitution of the beer yeast genome by PCR, sequencing and subtelomeric sequence hybridization. *International Journal of Systematic and Evolutionary Microbiology*, *51*(Pt 4), 1607–1618.

Casey, G. P., Xiao, W., & Rank, G. H. (1988). A convenient dominant selection marker for gene transfer in industrial strains of Saccharomyces yeast: SMRI encoded resistance to the herbicide sulfometuron methyl. *Journal of the Institute of Brewing*, *94*(2), 93–97.

Da Silva, N. A., & Srikrishnan, S. (2012). Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *12*(2), 197–214.

Eden, A., Van Nederveldе, L., Drukker, M., Benvenisty, N., & Debourg, A. (2001). Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast. *Applied Microbiology and Biotechnology*, *55*(3), 296–300.

Engan, S. (1970). Wort composition and beer flavour. I. The influence of some amino acids on the formation of higher aliphatic alcohols and esters. *Journal of the Institute of Brewers*, *76*(3), 254–261.

Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews*, *24*(4), 469–486.

Farhi, M., Dudareva, N., Masci, T., Weiss, D., Vainstein, A., & Abeliovich, H. (2006). Synthesis of the food flavoring methyl benzoate by genetically engineered *Saccharomyces cerevisiae*. *Journal of Biotechnology*, *122*(3), 307–315.

Fischer, S., Engstler, C., Procopio, S., & Becker, T. (2016a). EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system. *LWT – Food Science and Technology*, *68*, 556–562.

Fischer, S., Engstler, C., Procopio, S., & Becker, T. (2016b). Induced gene expression in industrial *Saccharomyces pastorianus* var. carlsbergensis TUM 34/70: Evaluation of temperature and ethanol inducible native promoters. *FEMS Yeast Research*, *16*(3), 1–10.

Fischer, S., Procopio, S., & Becker, T. (2013). Self-cloning brewing yeast: A new dimension in beverage production. *European Food Research and Technology*, *237*(6), 851–863.

Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, W., & Hamachi, M. (1994). Molecular-cloning, sequence-analysis, and expression of the yeast alcohol acetyltransferase gene. *Applied and Environmental Microbiology*, *60*(8), 2786–2792.

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, *31*(5), 535–569.

Gietz, R. D., & Schiestl, R. H. (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols*, *2*(1), 35–37.

Govender, P., Domingo, J. L., Bester, M. C., Pretorius, I. S., & Bauer, F. F. (2008). Controlled expression of the dominant flocculation genes FLO1, FLO5, and FLO11 in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *74*(19), 6041–6052.

Green, M. R., & Sambrook, J. (2012). *Molecular cloning: A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hammond, J. R. M. (1993). 2 – Brewer's yeasts A2 – Rose, Anthony H. In J. S. Harrison (Ed.), *The yeasts* (2nd ed.) (pp. 7–67). San Diego, CA: Academic Press.

Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology*, *74*(8), 2259–2266.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Reviews*, *66*(2), 300–372.

Homma, T., Iwahashi, H., & Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. *Cryobiology*, *46*(3), 230–237.

Hubmann, G., Thevelein, J. M., & Nevoigt, E. (2014). Natural and modified promoters for tailored metabolic engineering of the yeast *Saccharomyces cerevisiae*. In V. Mapelli (Ed.), *Yeast metabolic engineering: Methods and protocols* (pp. 17–42). New York: Springer.

Izawa, S., Kita, T., Ikeda, K., & Inoue, Y. (2008). Heat shock and ethanol stress provoke distinctly different responses in 3′-processing and nuclear export of HSP mRNA in *Saccharomyces cerevisiae*. *Biochemical Journal*, *414*, 111–119.

Kajiwara, S., Aritomi, T., Suga, K., Ohtaguchi, K., & Kobayashi, O. (2000). Overexpression of the OLE1 gene enhances ethanol fermentation by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *53*(5), 568–574.

Kajiwara, S., Suga, K., Sone, H., & Nakamura, K. (2000). Improved ethanol tolerance of *Saccharomyces cerevisiae* strains by increases in fatty acid unsaturation via metabolic engineering. *Biotechnology Letters*, *22*(23), 1839–1843.

Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D., & Goldberg, A. L. (2004). Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. *Molecular Cell*, *13*(6), 771–781.

Kowalski, L. R. Z., Kondo, K., & Inouye, M. (1995). Cold-shock induction of a family of TIP1-related proteins associated with the membrane in *Saccharomyces cerevisiae*. *Molecular Microbiology*, *15*(2), 341–353.

Krahl, M., Zarnkow, M., Stürmer, F., & Becker, T. (2009). Flavor stability of alternative malt-based beverages. *MBAA Technical Quarterly*, *46*, 4.

Kruis, A. J., Levisson, M., Mars, A. E., van der Ploeg, M., Garcés Daza, F., Ellena, V., … Weusthuis, R. A. (2017). Ethyl acetate production by the elusive alcohol acetyltransferase from yeast. *Metabolic Engineering*, *41*(suppl C), 92–101.

Labbe, S., & Thiele, D. J. (1999). [8] Copper ion inducible and repressible promoter systems in yeast. In *Methods in Enzymology* (Vol. 306) (pp. 145–153). USA: Elsevier.

Lilly, M., Lambrechts, M. G., & Pretorius, I. S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Applied and Environmental Microbiology*, *66*(2), 744–753.

Ma, M., & Liu, Z. L. (2010). Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *87*(3), 829–845.

Mason, A. B., & Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast*, *16*(14), 1287–1298.

Meilgaard, M. C. (1975). Flavor chemistry of beer: part ii: flavour and threshold of 239 aroma volatiles. *MBAA TQ*, *12*(3), 151–168.

Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics*, *190*(4), 1157–1195.

Murata, Y., Homma, T., Kitagawa, E., Momose, Y., Sato, M. S., Odani, M., … Iwahashi, H. (2006). Genome-wide expression analysis of yeast response during exposure to 4 degrees C. *Extremophiles*, *10*(2), 117–128.

Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., … Ashikari, T. (2009). Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research*, *16*(2), 115–129.

Nevoigt, E. (2008). Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, *72*(3), 379–412.

Panadero, J., Pallotti, C., Rodriguez-Vargas, S., Randez-Gil, F., & Prieto, J. A. (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *281*(8), 4638–4645.

Parekh, R. N., Shaw, M. R., & Wittrup, K. D. (1996). An integrating vector for tunable, high copy, stable integration into the dispersed Ty delta sites of *Saccharomyces cerevisiae*. *Biotechnology Progress*, *12*(1), 16–21.

Park, Y. C., Shaffer, C. E., & Bennett, G. N. (2009). Microbial formation of esters. *Applied Microbiology and Biotechnology*, *85*(1), 13–25.

Piper, P. W. (1995). The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters*, *134*(2–3), 121–127.

Piper, P. W., OrtizCalderon, C., Holyoak, C., Coote, P., & Cole, M. (1997). Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H^+-ATPase. *Cell Stress and Chaperones*, *2*(1), 12–24.

Piper, P. W., Talreja, K., Panaretou, B., Moradasferreira, P., Byrne, K., Praekelt, U. M., … Boucherie, H. (1994). Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma-membrane HSP30, by ethanol levels above a critical threshold. *Microbiology-Sgm*, *140*, 3031–3038.

Pires, E. J., Teixeira, J. A., Branyik, T., & Vicente, A. A. (2014). Yeast: The soul of beer's aroma – A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology*, *98*(5), 1937–1949.

Ponchon, L., Catala, M., Seijo, B., El Khouri, M., Dardel, F., Nonin-Lecomte, S., & Tisne, C. (2013). Co-expression of RNA–protein complexes in *Escherichia coli* and applications to RNA biology. *Nucleic Acids Research*, *41*(15), e150.

Procopio, S., Krause, D., Hofmann, T., & Becker, T. (2013). Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression. *Lwt – Food Science and Technology*, *51*(2), 423–432.

Richter, K., Haslbeck, M., & Buchner, J. (2010). The heat shock response: Life on the verge of death. *Molecular Cell*, *40*(2), 253–266.

Romagnoli, G., Luttik, M. A., Kotter, P., Pronk, J. T., & Daran, J. M. (2012). Substrate specificity of thiamine pyrophosphate-dependent 2-oxo-acid decarboxylases in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *78*(21), 7538–7548.

Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., & Delvaux, F. R. (2008). Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Applied Microbiology and Biotechnology*, *80*(6), 1039–1051.

Sahara, T., Goda, T., & Ohgiya, S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *Journal of Biological Chemistry*, *277*(51), 50015–50021.

Schade, B., Jansen, G., Whiteway, M., Entian, K. D., & Thomas, D. Y. (2004). Cold adaptation in budding yeast. *Molecular Biology of the Cell*, *15*(12), 5492–5502.

Smolke, C. (2009). *The metabolic pathway engineering handbook: Fundamentals* (Vol. 1). Boca Raton, FL: CRC Press.

Tamás, M. J., & Hohmann, S. (2003). The osmotic stress response of *Saccharomyces cerevisiae*. In S. Hohmann, & W. H. Mager (Eds.), *Yeast stress responses* (pp. 121–200). Berlin: Springer.

Teste, M.-A., Duquenne, M., Francois, J. M., & Parrou, J.-L. (2009). Validation of reference genes for quantitative expression analysis by real-time RT-PCR in Saccharomyces cerevisiae. *BioMed Central Molecular Biology*, 10. http://doi.org/10.1186/1471-2199-1110-1199

Verghese, J., Abrams, J., Wang, Y. Y., & Morano, K. A. (2012). Biology of the heat shock response and protein chaperones: Budding yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiology and Molecular Biology Reviews*, *76*(2), 115–158.

Verstrepen, K. J., Derdelinckx, G., Delvaux, F. R., Winderickx, J., Thevelein, J. M., Bauer, F. F., & Pretorius, I. S. (2001). Late fermentation expression of FLO1 in *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*, *59*(2), 69–76.

Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Pretorius, I. S., Thevelein, J. M., & Delvaux, F. R. (2003). The *Saccharomyces cerevisiae* alcohol acetyl transferase gene ATF1 is a target of the cAMP/PKA and FGM nutrient-signalling pathways. *FEMS Yeast Research*, *4*(3), 285–296.

Verstrepen, K. J., Van Laere, S. D. M., Vanderhaegen, B. M. P., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., … Delvaux, F. R. (2003). Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Applied and Environmental Microbiology*, *69*(9), 5228–5237.

Walther, A., Hesselbart, A., & Wendland, J. (2014). Genome sequence of *Saccharomyces carlsbergensis*, the World's First Pure Culture Lager Yeast. *G3: Genes|Genomes|Genetics*, *4*(5), 783–793.

Werner-Washburne, M., Stone, D. E., & Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, *7*(7), 2568–2577.

West, R. W., Yocum, R. R., & Ptashne, M. (1984). *Saccharomyces cerevisiae* GAL1 – GAL10 divergent promoter region – Location and function of the upstream activating sequance UASG. *Molecular and Cellular Biology*, *4*(11), 2467–2478.

Yamamoto, N., Maeda, Y., Ikeda, A., & Sakurai, H. (2008). Regulation of thermotolerance by stress-induced transcription factors in *Saccharomyces cerevisiae*. *Eukaryotic Cell*, *7*(5), 783–790.

Yoshioka, K., & Hashimoto, N. (1981). Ester Formation by Alcohol Acetyltransferase from Brewers' Yeast. *Agricultural and Biological Chemistry*, *45*(10), 2183–2190.

How to cite this article: Fischer S, Büchner KR, Becker T. Induced expression of the alcohol acetyltransferase gene *ATF1* in industrial yeast *Saccharomyces pastorianus* TUM 34/70. *Yeast*. 2018;1–11. https://doi.org/10.1002/yea.3319

3. Discussion

Through the knowledge of yeast genome since 1996, gene regulation and modification have also influenced the industrial yeast application. Industrial fermentations correlate with several stressors as mentioned in detail in the introduction for the beer fermentation. Existing research has mostly focused on the understanding of gene regulation during fermentation e.g. for the synthesis of volatile compounds or off-flavour, stress responses and the influence of the fermentation process itself (Aguilera et al., 2007; Gibson et al., 2008; Hohmann, 2002; Homma et al., 2003; S. M. G. Saerens, Delvaux, Verstrepen, & Thevelein, 2010; Verstrepen, Derdelinckx, et al., 2003; Verstrepen, Van Laere, et al., 2003). Furthermore, genome-based breeding for classical genetic modification on industrial yeast strains is problematic based on the global change of genotype and phenotype and the reduced property of sporulation (Bilinski, Russell, & Stewart, 1986), caused by the higher ploidy levels in industrial strains. Nevertheless, establishing targeted gene expression is not possible with these methods. This also applies for genetic modification of industrial organisms (for food and beverage production), caused by the harmfulness and prohibition of additives to the process.

The ability to adapt to different environmental changes is the reason for the success story of *Saccharomyces* yeast for decades. Besides the fermentation stressors (malnutrition, osmotic pressure and ethanol), *Saccharomyces* yeast are highly adapted to temperature shifts. The corresponding genes are well known and their regulation was studied under laboratory conditions (Aguilera et al., 2007; Hohmann & Mager, 2003; Homma et al., 2003; Kandror et al., 2004; Murata et al., 2006; Piper, 1995; Piper et al., 1997; Sahara et al., 2002; Schade et al., 2004). The present work provides a fundamental contribution to understand the stress-related gene regulations and the interaction of different kinds of stressors, that occurs during industrial fermentation teamed with the allotetraploid hybrid *S. pastorianus* var. *carlsbergensis*. The induction characteristics of stress related promoters were evaluated with the aim of induced gene expression of ATF1 provoked by the bioprocess-parameter temperature.

In an initial experimental set up (Chapter 2.2), a method for the evaluation of promoter strength of the non-hybrid industrial *Saccharomyces cerevisiae* TUM 68 yeast by mimic industrial brewing conditions in high throughput was established. Therefore, five different promoters of the heat shock family were cloned up-stream of the reporter

gene EGFP. This procedure allowed a non-invasive detection of the promoter induction. Furthermore, the usage of synthetic wort complements the industrial mimicry.

For high throughput measurements of fluorescence, it is important to correct the fluorescence of other sources, such as the auto-fluorescence of each yeast cell and media. Especially for longer time measurements, auto-fluorescence depends not only on the size or stage of population growth, but also media composition at every time point through the depletion of nutrients and excreted products by the yeast cells. This correction was conducted via spectral unmixing of auto-fluorescence and GFP-based florescence by using two wavelengths (525nm and 585nm) (Lichten, White, Clark, & Swain, 2014).

The influence of a temperature shift from 20 to 4 and 10°C to the induction conditions is shown in Table 4 - Chapter 2.2. as a fold change to time point 0h. Both temperature shifts led to an induction of the EGFP expression. The shift to 10°C results in the highest expression at 72h by pSSA3 and pHSP30, with 6.8 and 5.8, respectively. Further, pHSP12, pHSP26, and pHSP104 grouped to late cold shock response (Schade et al., 2004) show slight fluorescence patterns and rise with the time after 12 hours. The expression patterns of pHSP12, pHSP30 and pHSP104 by temperature shift to 4°C are consistent with existing literature (Murata et al., 2006).

Furthermore, the induction patterns influenced by different contents of ethanol also leads to already-described induction patterns. A minimum content of ethanol is needed for the induction of HSP genes (Piper et al., 1994) and leads to increased fluorescence patterns of the evaluated promoters, except pHSP26. There are higher fold changes measured by 4%(v/v) in comparison to 6%(v/v) ethanol. Further, a decline of induction is detected by pHSP12 and pHSP30, which was also described by PIPER and colleagues.

Summarising, the method is sufficiently sensible, reproducible and useful for time series detection in high throughput measurements of EGFP expression under industrial conditions. Furthermore, investigations into promoter strength and induction conditions in a stress full environment based on RNA hides the translational modifications.

In order to evaluate most suitable homologous promoters of the industrial yeast *S. pastorianus* var. *carlsbergensis* TUM 34/70, the method was investigated during fermentation, ethanol stress and cold shock (Chapter 2.3).

Besides the high induction efficiency by temperature shocks, other stressors should not or only minimally initiate the induction. Especially when different stressors occur simultaneously – for instance, at the end of fermentation with higher concentrations of ethanol and shifts in temperature by transition to maturation – an overlap of stress responses emerges and a prediction of the stress response is complicated (Y. Wang et al., 2015). The low temperature leads to an enhanced membrane fluidity, while by contrast higher concentrations of ethanol lead to enhanced rigidity of the membrane (Aguilera et al., 2007; Alexandre, Ansanay-Galeote, Dequin, & Blondin, 2001; Y. F. Wang et al., 2015) and trehalose accumulation (Odumeru, D'Amore, Russell, & Stewart, 1993). Therefore, the influence of varied concentrations of ethanol were investigated under optimal and fermentation temperature (Table 3 – Chapter 2.3 and Table 4 – Chapter 2.3, respectively). The promoters of the TIP-related family show the highest induction at an ethanol concertation of 4% (v/v), whereby promoters of the HSP family show higher induction by higher ethanol concentrations. $_{P}$HSP12, $_{P}$SSA3 and $_{P}$UBI4 show the highest induction patterns at 10% (v/v), whereas $_{P}$HSP26 and $_{P}$HSP30 show the highest induction at 6% (v/v) which is consistent with PIPER et al (Piper et al., 1994). In direct comparison, the induction patterns by ethanol shock at 12°C are generally lower.

In principle, the influence of growth phases on the induction pattern effected by cold shock was investigated. All promoters show the highest induction after 24h after exposure to 4°C at the beginning of the stationary phase (Figure 5a and Figure 2a – Chapter 2.3). $_{P}$TIP1 shows the highest induction in the TIP-related family. Nevertheless, members of the HSP family show the highest induction patterns of all evaluated promoters, with $_{P}$HSP104 (fold change of 5.2) and $_{P}$HSP26 (fold change 5.0). In order to explore the dependency of growth phases where a residual extract of approximately 5 °P exists, temperature shifts were applied in the same conditions (Figure 5b and Figure 2b – Chapter 2.3.). Up to that moment, the yeast underlie further stressors, such as higher concentrations of ethanol (≈ 4%(v/v)), proceeded depletion of nutrients, and through sequential uptake of carbohydrates, only polysaccharides are available. In contrast to the fluorescence patterns at the beginning of the stationary phase, the fluorescence increases over time. Further, a categorization into three induction groups could be applied. The Promoters of the TIP-related family shows again the lowest induction patterns. The promoters $_{P}$HSP12, $_{P}$HSP26 and $_{P}$HSP30, all members of the small HSP´s, show equivalent induction patters to the temperature

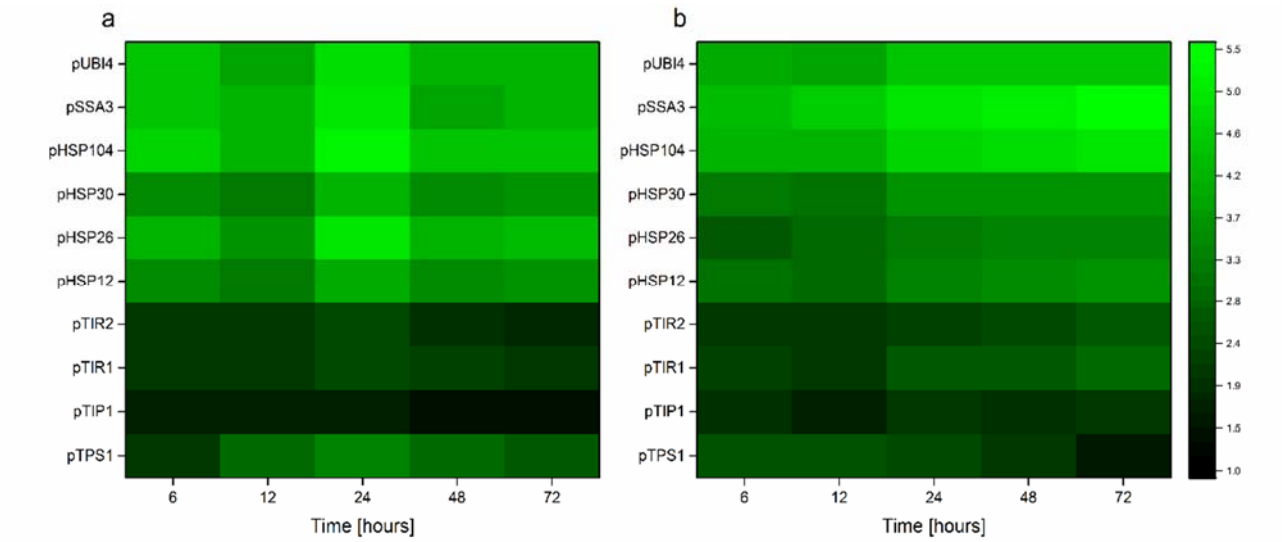

Figure 5: Heat map of fluorescence changes of EGFP under control of the different yeast promoters at each time point after temperature shift from 12°C to 4°C. **a:** Fluorescence detection at initiation of fermentation. **b:** Fluorescence detection at end of fermentation. Results are shown as ratio to time point 0 h of four replicates; values are indicated by color bar. Standard derivations were typically about 10% and never exceeded 20%. All promoters presented show a statically significant change in activity to each other, as determined by one-way ANOVA ($P < 0.05$).

shift at beginning of stationary phase. The highest induction patterns were observed from pHSP104, pSSA3 and pUBI4 with 5.0 und 5.4 and 4.4, respectively. However, the results are controversial in relation to existing literature, where HSP104 shows the lowest expression and SSA3 an enhanced expression under similar conditions (Homma et al., 2003; Murata et al., 2006). Due to these results, the hypothesis that the

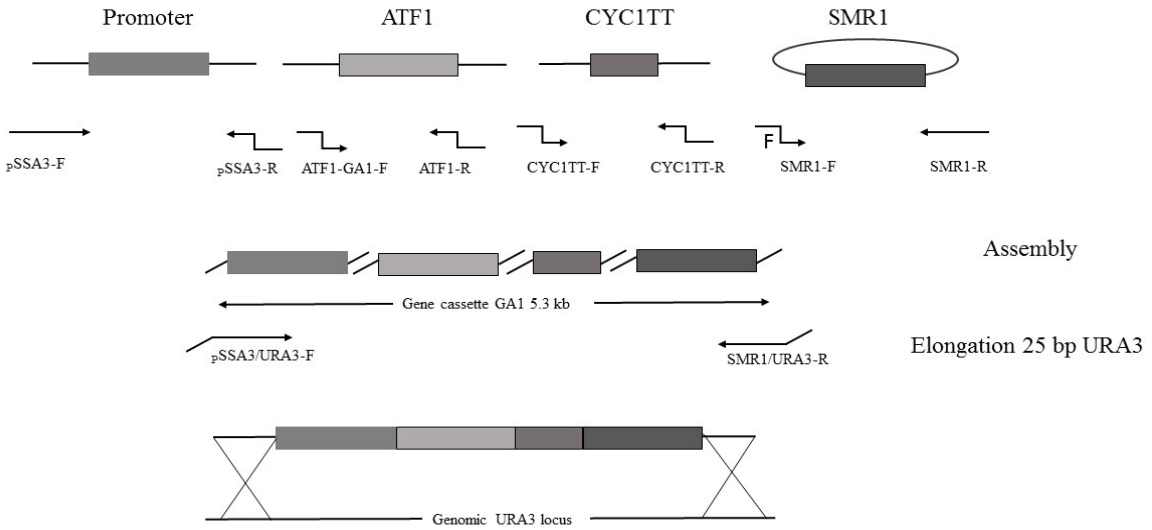

Figure 6: Schematic diagram of the construction of self-cloning brewing yeast TUM 34/70 GA1 by assembling the gene cassette, with the promoter pSSA3, the target gene ATF1, terminator CYC1TT and the resistant marker SMR1 and integration into the URA3 locus via homologues recombination.

induction is uncoupled on growth phases could be refuted. Furthermore, the hypothesis that under equal stress conditions the induction patterns of different stress-related promoters varied could verified.

In summary, $_P$HSP104, $_P$SSA3 and $_P$UBI4 show the most suitability for induction throughout cold shock teamed with low induction at typical ethanol concentrations (4-6%v/v). The transition phase during fermentation also affect the gene regulation of these stress related genes (Riou, Nicaud, Barre, & Gaillardin, 1997). Therefore, the induction patterns affected by fermentation were investigated, where $_P$HSP104 and $_P$SSA3 show the highest fold change by transition from the lag to log phase at optimal growth temperature (24°C). By contrast, at fermentation temperature, a decrease of the fluorescence value could be detected over the time.

These three promoters were evaluated with the most induction efficiency and were used for the construction of the self-cloning gene cassette. In order to ensure that only homologous nucleic acid is present, the different gene cassettes were assembled followed by integration into the genome of the lager yeast strain TUM 34/70 (Figure 5 and Chapter 2.4). The assembling based on an isothermal *in-vitro* recombination of multiple overlapping DNA molecules (Figure 6 and Figure 1 – Chapter 2.4) processed by 5´ exonuclease, a DNA polymerase and a DNA ligase in a single reaction (D. G. Gibson et al., 2009).

For efficient selection of the self-cloning yeast strain, a homologous selection marker is necessary or a heterologous selection marker has to be removed by e.g. counter selection (Figure 1 – Chapter 2.1) (Akada, Hirosawa, Kawahata, Hoshida, & Nishizawa, 2002; Kawahata, Amari, Nishizawa, & Akada, 1999). The main benefit is the reusability of the selection marker. Relating to the lower efficiency of the method and the planned unique integration of the gene cassette, a homologous selection marker was chosen. There are two homologous genes of *Saccharomyces* yeast that are suitable as a selection marker: CUP1 and SMR1-410. The semi-dominant CUP1 encodes for a copper-binding metallothionein resulting in copper resistance by the overexpression of this gene (D. L. Wang, Wang, Liu, He, & Zhang, 2008; Z. Y. Wang, He, & Zhang, 2007; Z. Y. Wang, Wang, Liu, He, & Zhang, 2009; Zhang et al., 2011). The expression of SMR1-410 results in increased resistance to the sulfonylurea herbicide sulfometuron methly (SM). This selection marker is distinct to the ILV2 gene of the yeast, which encodes for the acetolactate synthetase with a single point mutation on nucleotide 574 (cytosine to thymine) resulting in a proline to serine change at

position 192 (Casey, Xiao, & Rank, 1988; Xie & Jimenez, 1996). The dominant marker SMR1-410 was chosen for the construction of the self-cloning gene cassette as a result of the semi-dominance of CUP1 and the existing resistance against minor concentrations of cupper of the industrial yeast TUM 34/70 and TUM 68 (data not shown), which results in a small slot of ideal concentration for selection.

The gene ATF1 – which encodes for alcohol acetyltransferase (AATase) (Fujii et al., 1994; Mason & Dufour, 2000; Verstrepen, Van Laere, et al., 2003) – was chosen give that volatile metabolites such as higher alcohols and esters contribute fundamentally to the quality of fermented beverages such as beer and wine (Belda et al., 2017; Pires, Teixeira, Branyik, & Vicente, 2014). Especially esters are more relevant, due to the low odour threshold value. They are mainly formed via the intracellular enzymatic condensation reaction of AATaseI and AATaseII between ethanol (or e.g. isoamyl alcohol, 1-propanol, isobutanol, hexanol) and acetylCoA (Figure 7). Besides the knowledge of the gene function and ethyl acetate formation, ATF1 has been used several times in overexpressing experiments, including for industrial strains (Fujii et al., 1994; Mason & Dufour, 2000; Verstrepen, Van Laere, et al., 2003).

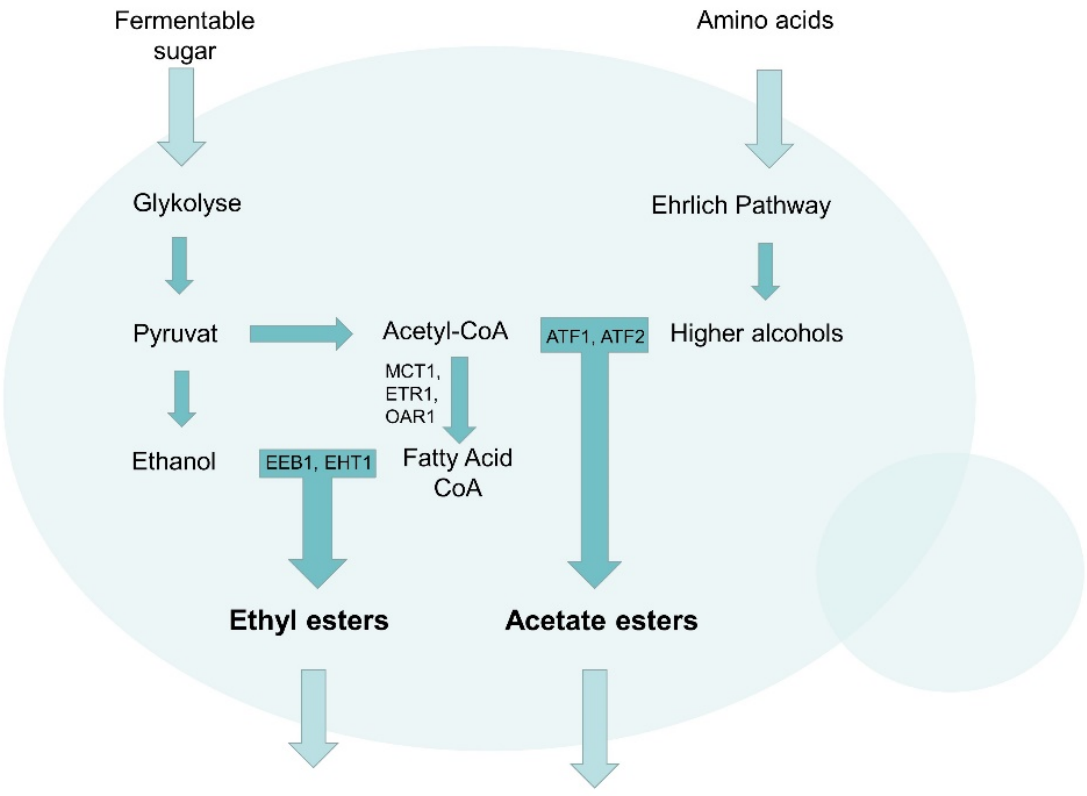

Figure 7: Simplified metabolic pathway of the volatile acetate and ethyl esters in *Saccharomyces* yeast and the genes involved. ATF1 and ATF2 are responsible for the acetyl transfer to ethanol or isoamyl alcohol, where ATF1 is more important. EEB1 and the paralog EHT1 are responsible for the acyl transfer to ethanol for the medium-chain fatty acid ethyl ester biosynthesis during fermentation.

Fermentations with the three different self-cloning yeasts (GA1 – $_{P}$SSA3; GA4 – $_{P}$HSP104; GA5 – $_{P}$UBI4) were carried out in a 2 L EBC standard tall tube (Ø 5 cm × h 150 cm) with synthetic wort to mimic industrial conditions (Chapter 2.4). Transcription analysis shows the highest induction after 72 h of shock situation for $_{P}$HSP104 1.3-fold and $_{P}$UBI4 with 2.2-fold. Furthermore, for GA4 and GA5 a significant different ethyl acetate production was measured at the end of fermentation with 1.2 and 1.3-fold, respectively. At the temperature shock (0 h), no significant difference was observed ($P < 0.05$), which indicates that the higher content on ethyl acetate results from the induction of the stress related promoters $_{P}$HSP104 and $_{P}$UBI4 through cold shock. For GA5, the content of ethyl acetate (2.9-fold) increased with a twice-as-fast temperature shift from 12°C to 4°C. Significant amounts of isoamyl acetate were detected, but even in low concentrations. Further, a shock to 6°C resulted in a lower induction efficiency with 1.4-fold in comparison to wild type. Similar results were observed by a stronger shift of temperature (20°C to 6°C).

These results indicate the higher relevance of a fast temperature change to the final temperature. As well as the impact of temperature near the freezing point (4°C). Besides the expression of ATF1, which is significantly teamed with the production of ethyl and isoamyl acetate (Verstrepen, Derdelinckx, et al., 2003), the enzyme activity also holds prime importance and is significantly influenced by temperature (S. M. Saerens, Verbelen, Vanbeneden, Thevelein, & Delvaux, 2008). The highest enzyme activity of ATF1 was determined at 30°C and reduced to 20% at 4°C (Yoshioka & Hashimoto, 1981). Additionally, enzyme activity is reported to be inversely proportional to the temperature, e.g. enzyme activity at 6°C cannot be expected to be higher than at 4°C (Yoshioka & Hashimoto, 1981). This could be the reason for the weak effect of ethyl acetate and isoamyl acetate production under the shock condition.

The hypothesis that the evaluated promoters leads to an overexpression of the target gene could be verified. A fine-tuning of gene expression is principally possible by fastness of cooling or the final temperature and thus this hypothesis could also be verified through this study. However, these results are not adaptable to all enzymes and the coding genes. Especially for enzymes that are not inhibited by low temperatures, they could have a significantly higher activity and substrate reaction due to overexpression. The extent to which fine-tuning has an impact on very strong activity must first be overhauled.

However, what should be emphasised is the suitability of stress-induced recovery for industrial use, as the yeasts have no negative influence on vitality due to their strong adaption to the different stress situations. Furthermore, the integration of such expression cassettes in genes with unwanted function e.g. Proteinase A, which results in a reduced foam stability, combines two or more features.

Brewer's yeast in particular has been increasingly researched for several years. This is due, the low diversity of the bottom fermented yeast and the conversion of consumers to individual products. The research on the suitability of wild yeasts for wort fermentation is also used in research and pilot breweries. The extent to which consortia fermentations meet the expectations of brewers and consumers will probably be reported in the near future.

In summary, temperature is an effective inducer for homologous gene expression in industrial yeast. Furthermore, temperature is the only stressor that is treatable without directly influencing the process. $_{P}$HSP104 and $_{P}$UBI4 have been identified as the most competent homologous temperature inducible promoters for the industrial brewing yeast *S. pastorianus* TUM 34/70. Further, the study shows the importance of the rapid temperature change and the final temperature for the efficient induction and fine-tuning of gene expression. Although self-cloning yeasts can be used to *tailor* industrial fermentations to fulfil new product requirements, and no danger to consumers and the environment is expected, current use under European law is not allowed. It is also important to evaluate the consumer acceptance of such products in food and beverage production.

4. References

Abramova, N. E., Cohen, B. D., Sertil, O., Kapoor, R., Davies, K. J. A., & Lowry, C. V. (2001). Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in Saccharomyces cerevisiae. *Genetics, 157*(3), 1169-1177.

Aguilera, J., Randez-Gil, F., & Prieto, J. A. (2007). Cold response in Saccharomyces cerevisiae: new functions for old mechanisms. *FEMS Microbiology Reviews, 31*(3), 327-341.

Akada, R., Hirosawa, I., Kawahata, M., Hoshida, H., & Nishizawa, Y. (2002). Sets of integrating plasmids and gene disruption cassettes containing improved counter-selection markers designed for repeated use in budding yeast. *Yeast, 19*(5), 393-402.

Alepuz, P. M., de Nadal, E., Zapater, M., Ammerer, G., & Posas, F. (2003). Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. *EMBO Journal, 22*(10), 2433-2442.

Alexandre, H., Ansanay-Galeote, V., Dequin, S., & Blondin, B. (2001). Global gene expression during short-term ethanol stress in Saccharomyces cerevisiae. *FEBS Letters, 498*(1), 98-103.

Arneborg, N., Hoy, C. E., & Jorgensen, O. B. (1995). The effect of ethanol and specific growth rate on the lipid content and composition of Saccharomyces cerevisiae grown anaerobically in a chemostat. *Yeast, 11*(10), 953-959.

Attfield, P. V., & Bell, P. J. L. (2003). Genetics and classical genetic manipulations of industrial yeasts. In J. H. de Winde (Ed.), *Functional Genetics of Industrial Yeasts* (pp. 17-55). Berlin, Heidelberg: Springer Berlin Heidelberg.

Barlow, S., Chesson, A., Collins, J., Dybing, E., Albert Flynn, Claudia Fruijtier-Pölloth, . . . Vannier., P. (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *The EFSA Journal, 587*, 1-16.

Barnett, J. A. (2000). A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850-1880. *Yeast, 16*(8), 755-771.

Bauer, F. F., & Pretorius, I. S. (2000). Yeast stress response and fermentation efficiency: How to survive the making of wine - A review. *South African Journal of Enology and Viticulture, 21*(Sp. Iss. SI), 27-51.

Becker, J., & Craig, E. A. (1994). Heat-shock proteins as molecular chaperones. *European Journal of Biochemistry, 219*(1-2), 11-23.

Belanger, K. D., Larson, N., Kahn, J., Tkachev, D., & Ay, A. (2016). Microarray Analysis of Gene Expression in Saccharomyces cerevisiae kap108Δ Mutants upon Addition of Oxidative Stress. *G3: Genes|Genomes|Genetics, 6*(4), 1131-1139.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., & Moreno-Arribas, M. V. (2017). Microbial Contribution to Wine Aroma and Its Intended Use for Wine Quality Improvement. *Molecules, 22*(2), 189.

Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., . . . Thevelein, J. M. (1998). Composition and functional analysis of the Saccharomyces cerevisiae trehalose synthase complex. *Journal of Biological Chemistry, 273*(50), 33311-33319.

Berry, D. B., & Gasch, A. P. (2008). Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Molecular Biology of the Cell, 19*(11), 4580-4587.

Bilinski, C. A., Russell, I., & Stewart, G. G. (1986). Analysis of sporulation in brewer's yeast: induction of tetrad formation. *Journal of the Institute of Brewing, 92*(6), 594-598.

Bing, J., Han, P.-J., Liu, W.-Q., Wang, Q.-M., & Bai, F.-Y. (2014). Evidence for a Far East Asian origin of lager beer yeast. *Current Biology, 24*(10), R380-R381.

Bleoanca, I., & Bahrim, G. (2013). Overview on Brewing Yeast Stress Factors. *Romanian Biotechnological Letters, 18*(5), 8560.

Blomberg, A. (1995). Global changes in protein synthesis during adaptation of the yeast Saccharomyces cerevisiae to 0.7 M NaCl. *Journal of Bacteriology, 177*(12), 3563-3572.

Blomberg, A., & Adler, L. (1989). Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of Saccharomyces cerevisiae. *Journal of Bacteriology, 171*(2), 1087-1092.

Boulton, C., & Quain, D. (2008). *Brewing yeast and fermentation*: John Wiley & Sons.

Boulton, C., & Quain, D. (2013). *Brewing Yeast and Fermentation*: Wiley.

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (2013). *Principles and practices of winemaking*: Springer Science.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., . . . Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology, 154*(3), 87-97.

Boy-Marcotte, E., Lagniel, G., Perrot, M., Bussereau, F., Boudsocq, A., Jacquet, M., & Labarre, J. (1999). The heat shock response in yeast: differential regulations and contributions of the Msn2p/Msn4p and Hsf1p regulons. *Molecular Microbiology, 33*(2), 274-283.

Carratù, L., Franceschelli, S., Pardini, C. L., Kobayashi, G. S., Horvath, I., Vigh, L., & Maresca, B. (1996). Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. *Proceedings of the National Academy of Sciences of the United States of America, 93*(9), 3870-3875.

Casey, G. P., Xiao, W., & Rank, G. H. (1988). A convenient dominant selection marker for gene transfer in industrial strains of Saccharomyces yeast: SMRI encoded resistance to the herbicide sulfometuron methyl. *Journal of the Institute of Brewing, 94*(2), 93-97.

Ceccoli, S., & Hixon, W. (2012). Explaining attitudes toward genetically modified foods in the European Union. *International Political Science Review, 33*(3), 301-319.

Cheng, C., & Yang, S. T. (1996). Dynamics and modeling of temperature-regulated gene product expression in recombinant yeast fermentation. *Biotechnology and Bioengineering, 50*(6), 663-674.

Cohen, B. D., Sertil, O., Abramova, N. E., Davies, K. J. A., & Lowry, C. V. (2001). Induction and repression of DAN1 and the family of anaerobic mannoprotein genes in Saccharomyces cerevisiae occurs through a complex array of regulatory sites. *Nucleic Acids Research, 29*(3), 799-808.

Corcoles-Saez, I., Ballester-Tomas, L., de la Torre-Ruiz, M. A., Prieto, J. A., & Randez-Gil, F. (2012). Low temperature highlights the functional role of the cell wall integrity pathway in the regulation of growth in Saccharomyces cerevisiae. *Biochemical Journal, 446*, 477-488.

Corran, H. S. (1975). *A history of brewing*: David & Charles.

Curtis, K. R., McCluskey, J. J., & Wahl, T. I. (2004). Consumer Acceptance of Genetically Modified Food Products in the Developing World. *AGBioForum - The Journal of Agrobiotechnology Management and Economics, 7*(1&2), 70-75.

Drees, B. L., Grotkopp, E. K., & Nelson, H. C. (1997). The GCN4 leucine zipper can functionally substitute for the heat shock transcription factor's trimerization domain. *Journal of Molecular Biology, 273*(1), 61-74.

Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews, 24*(4), 469-486.

Eurobarometer 238 "Risk issue". (2006). In. http://ec.europa.eu/public_opinion/index_en.htm: European Commision.

Eurobarometer. (2010). 73.1 Biotechnology. In. http://ec.europa.eu/public_opinion/index_en.htm: European Commision.

Farhi, M., Dudareva, N., Masci, T., Weiss, D., Vainstein, A., & Abeliovich, H. (2006). Synthesis of the food flavoring methyl benzoate by genetically engineered Saccharomyces cerevisiae. *Journal of Biotechnology, 122*(3), 307-315.

Fernandes, L., Rodrigues-Pousada, C., & Struhl, K. (1997). Yap, a novel family of eight bZIP proteins in Saccharomyces cerevisiae with distinct biological functions. *Molecular and Cellular Biology, 17*(12), 6982-6993.

Finley, R. L., Jr., Zhang, H., Zhong, J., & Stanyon, C. A. (2002). Regulated expression of proteins in yeast using the MAL61-62 promoter and a mating scheme to increase dynamic range. *Gene, 285*(1-2), 49-57.

François, J., & Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. *FEMS Microbiology Reviews, 25*(1), 125-145.

Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, W., & Hamachi, M. (1994). Molecular-cloning, sequence-analysis, and expression of the yeast alcohol acetyltransferase gene. *Applied and Environmental Microbiology, 60*(8), 2786-2792.

Garreau, H., Hasan, R. N., Renault, G., Estruch, F., Boy-Marcotte, E., & Jacquet, M. (2000). Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in Saccharomyces cerevisiae. *Microbiology-Uk, 146*, 2113-2120.

Gasch, A. P. (2003). The environmental stress response: a common yeast response to diverse environmental stresses. In S. Hohmann & P. Mager (Eds.), *Yeast Stress Responses* (Vol. 1): Springer-Verlag Heidelberg.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., . . . Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell, 11*(12), 4241-4257.

Gibson, Lawrence, S. J., Boulton, C. A., Box, W. G., Graham, N. S., Linforth, R. S., & Smart, K. A. (2008). The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. *FEMS Yeast Research, 8*(4), 574-585.

Gibson, B. R., Boulton, C. A., Box, W. G., Graham, N. S., Lawrence, S. J., Linforth, R. S. T., & Smart, K. A. (2008). Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. *Yeast, 25*(8), 549-562.

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews, 31*(5), 535-569.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Meth, 6*(5), 343-345.

Gietz, R. D., & Schiestl, R. H. (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols, 2*(1), 35-37.

Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., . . . Labarre, J. (1998). The H2O2 stimulon in Saccharomyces cerevisiae. *Journal of Biological Chemistry, 273*(35), 22480-22489.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., . . . Oliver, S. G. (1996). Life with 6000 genes. *Science, 274*(5287), 546-&.

Gorner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., . . . Schuller, C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes and Development, 12*(4), 586-597.

Guilliermond, A. (2003). *Yeasts: Culture, Identification, and Microbiology*: Wexford College Press.

Gulshan, K., Rovinsky, S. A., Coleman, S. T., & Moye-Rowley, W. S. (2005). Oxidant-specific folding of Yap1p regulates both transcriptional activation and nuclear localization. *Journal of Biological Chemistry, 280*(49), 40524-40533.

Gustin, M. C., Albertyn, J., Alexander, M., & Davenport, K. (1998). MAP Kinase Pathways in the YeastSaccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews, 62*(4), 1264-1300.

Hahn, S., & Young, E. T. (2011). Transcriptional Regulation in Saccharomyces cerevisiae: Transcription Factor Regulation and Function, Mechanisms of Initiation, and Roles of Activators and Coactivators. *Genetics, 189*(3), 705-736.

Hashikawa, N., Yamamoto, N., & Sakurai, H. (2007). Different mechanisms are involved in the transcriptional activation by yeast heat shock transcription factor through two different types of heat shock elements. *Journal of Biological Chemistry, 282*(14), 10333-10340.

Hawkins, K. M., & Smolke, C. D. (2006). The regulatory roles of the galactose permease and kinase in the induction response of the GAL network in Saccharomyces cerevisiae. *Journal of Biological Chemistry, 281*(19), 13485-13492.

Hill, A. (2015). *Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*: Elsevier Science & Technology.

Hirosawa, I., Aritomi, K., Hoshida, H., Kashiwagi, S., Nishizawa, Y., & Akada, R. (2004). Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. *Applied Microbiology and Biotechnology, 65*(1), 68-73.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Reviews, 66*(2), 300-372.

Hohmann, S., & Mager, W. H. (2003). *Yeast Stress Responses*: Springer.

Homma, T., Iwahashi, H., & Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. *Cryobiology, 46*(3), 230-237.

Hou, J., Tyo, K. E., Liu, Z., Petranovic, D., & Nielsen, J. (2012). Metabolic engineering of recombinant protein secretion by Saccharomyces cerevisiae. *FEMS Yeast Research, 12*(5), 491-510.

Hubmann, G., Thevelein, J. M., & Nevoigt, E. (2014). Natural and Modified Promoters for Tailored Metabolic Engineering of the Yeast Saccharomyces cerevisiae. In V. Mapelli (Ed.), *Yeast Metabolic Engineering: Methods and Protocols* (pp. 17-42): Springer New York.

Hughes, T. R., & de Boer, C. G. (2013). Mapping Yeast Transcriptional Networks. *Genetics, 195*(1), 9-36.

Iijimalijima, K., & Ogata, T. (2010). Construction and evaluation of self-cloning bottom-fermenting yeast with high SSU1 expression. *Journal of Applied Microbiology, 109*(6), 1906-1913.

Kajiwara, S., Suga, K., Sone, H., & Nakamura, K. (2000). Improved ethanol tolerance of Saccharomyces cerevisiae strains by increases in fatty acid unsaturation via metabolic engineering. *Biotechnology Letters, 22*(23), 1839-1843.

Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D., & Goldberg, A. L. (2004). Yeast Adapt to Near-Freezing Temperatures by STRE/Msn2,4-Dependent Induction of Trehalose Synthesis and Certain Molecular Chaperones. *Molecular Cell, 13*(6), 771-781.

Kawahata, M., Amari, S., Nishizawa, Y., & Akada, R. (1999). A positive selection for plasmid loss in Saccharomyces cerevisiae using galactose-inducible growth inhibitory sequences. *Yeast, 15*(1), 1-10.

Kobayashi, H., Nakazawa, N., Harashima, S., & Oshima, Y. (1990). A system for temperature-controlled expression of a foreign gene with dual mode in Saccharomyces cerevisiae. *Journal of Fermentation and Bioengineering, 69*(6), 322-327.

Kuge, S., Arita, M., Murayama, A., Maeta, K., Izawa, S., Inoue, Y., & Nomoto, A. (2001). Regulation of the Yeast Yap1p Nuclear Export Signal Is Mediated by Redox Signal-Induced Reversible Disulfide Bond Formation. *Molecular and Cellular Biology, 21*(18), 6139-6150.

Kumar, A., & Snyder, M. (2001). Emerging technologies in yeast genomics. *Nat Rev Genet, 2*(4), 302-312.

Kurtzman, C., Fell, J. W., & Boekhout, T. (2011). *The yeasts: a taxonomic study*: Elsevier.

Labbe, S., & Thiele, D. J. (1999). Copper ion inducible and repressible promoter systems in yeast. In *Expression of Recombinant Genes in Eukaryotic Systems* (Vol. 306, pp. 145-153).

Lagunas, R. (1993). Sugar transport in Saccharomyces cerevisiae. *FEMS Microbiology Letters, 104*(3–4), 229-242.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., & Toledano, M. B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *Journal of Biological Chemistry, 274*(23), 16040-16046.

Lee, K. M., & DaSilva, N. A. (2005). Evaluation of the Saccharomyces cerevisiae ADH2 promoter for protein synthesis. *Yeast, 22*(6), 431-440.

Lichten, C. A., White, R., Clark, I. B. N., & Swain, P. S. (2014). Unmixing of fluorescence spectra to resolve quantitative time-series measurements of gene expression in plate readers. *BMC Biotechnology, 14*.

Lusk, J. L., & Rozan, A. (2005). Consumer acceptance of biotechnology and the role of second generation technologies in the USA and Europe. *Trends in Biotechnology, 23*(8), 386-387.

Ma, M., & Liu, L. Z. (2010). Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in Saccharomyces cerevisiae. *BMC Microbiology, 10*(1), 169.

Marchler, G., Schüller, C., Adam, G., & Ruis, H. (1993). A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *The EMBO Journal, 12*(5), 1997-2003.

Martinez-Pastor, M. T., Marchler, G., Schuller, C., MarchlerBauer, A., Ruis, H., & Estruch, F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO Journal, 15*(9), 2227-2235.

Mason, A. B., & Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast, 16*(14), 1287-1298.

McGovern, P. E. (2013). *Ancient wine: the search for the origins of viniculture*: Princeton University Press.

McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., . . . Wang, C. (2004). Fermented beverages of pre- and proto-historic China. *Proceedings of the National Academy of Sciences of the United States of America, 101*(51), 17593-17598.

MOH. (2002). Ministry of Health regulation; Order No. 28 Administrative Provisions for Genetically Modified Food Hygiene. Retrieved from http://law.pharmnet.com.cn/laws/detail_469.html

Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The Response to Heat Shock and Oxidative Stress in Saccharomyces cerevisiae. *Genetics, 190*(4), 1157-1195.

Mountain, H. A., Bystrom, A. S., Larsen, J. T., & Korch, C. (1991). Four major transcriptional responses in the methionine/threonine biosynthetic pathway of Saccharomyces cerevisiae. *Yeast, 7*(8), 781-803.

Mumberg, D., Muller, R., & Funk, M. (1994). Regulatable promoters of Saccharomyces cerevisiae – comparison of transcriptional activity and their use heterologous expression. *Nucleic Acids Research, 22*(25), 5767-5768.

Mumberg, D., Muller, R., & Funk, M. (1995). Yeast vectors for the controlled Expression of heterologous proteins in differnt genetic backgrounds. *Gene, 156*(1), 119-122.

Murata, Y., Homma, T., Kitagawa, E., Momose, Y., Sato, M. S., Odani, M., . . . Iwahashi, H. (2006). Genome-wide expression analysis of yeast response during exposure to 4 degrees C. *Extremophiles, 10*(2), 117-128.

Nevoigt, E. (2008). Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews, 72*(3), 379-412.

Nevoigt, E., Fischer, C., Mucha, O., Matthaus, F., Stahl, U., & Stephanopoulos, G. (2007). Engineering promoter regulation. *Biotechnology and Bioengineering, 96*(3), 550-558.

Nielsen, J. (1998). Metabolic engineering: techniques for analysis of targets for genetic manipulations. *Biotechnology and Bioengineering, 58*(2-3), 125-132.

Nieto-Sotelo, J., Wiederrecht, G., Okuda, A., & Parker, C. S. (1990). The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions. *Cell, 62*(4), 807-817.

Odumeru, J. A., D'Amore, T., Russell, I., & Stewart, G. G. (1993). Alterations in fatty acid composition and trehalose concentration ofSaccharomyces brewing strains in response to heat and ethanol shock. *Journal of Industrial Microbiology, 11*(2), 113-119.

Ostergaard, S., Olsson, L., & Nielsen, J. (2000). Metabolic engineering of Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews, 64*(1), 34-50.

Panadero, J., Pallotti, C., Rodriguez-Vargas, S., Randez-Gil, F., & Prieto, J. A. (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in Saccharomyces cerevisiae. *Journal of Biological Chemistry, 281*(8), 4638-4645.

Park, Y. S., Shiba, S., Lijima, S., Kobayashi, T., & Hishinuma, F. (1993). Comparison of three different promoter systems for secretory alpha-amylase production in fed-batch cultures of recombinant Saccharomyces cerevisiae. *Biotechnology and Bioengineering, 41*(9), 854-861.

Parrou, J. L., Teste, M.-A., & François, J. (1997). Effects of various types of stress on the metabolism of reserve carbohydrates in Saccharomyces cerevisiae: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology, 143*(6), 1891-1900.

Partow, S., Siewers, V., Bjorn, S., Nielsen, J., & Maury, J. (2010). Characterization of different promoters for designing a new expression vector in Saccharomyces cerevisiae. *Yeast, 27*(11), 955-964.

Pena-Castillo, L., & Hughes, T. R. (2007). Why are there still over 1000 uncharacterized yeast genes? *Genetics, 176*(1), 7-14.

Piper, P. W. (1995). The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters, 134*(2-3), 121-127.

Piper, P. W., OrtizCalderon, C., Holyoak, C., Coote, P., & Cole, M. (1997). Hsp30, the integral plasma membrane heat shock protein of Saccharomyces

cerevisiae, is a stress-inducible regulator of plasma membrane H+-ATPase. *Cell Stress and Chaperones, 2*(1), 12-24.

Piper, P. W., Talreja, K., Panaretou, B., Moradasferreira, P., Byrne, K., Praekelt, U. M., . . . Boucherie, H. (1994). Induction of major heat-shock proteins of Saccharomyces cerevisiae, including plasma-membrane HSP30, by ethanol levels above a critical threshold. *Microbiology-Sgm, 140*, 3031-3038.

Pires, E. J., Teixeira, J. A., Branyik, T., & Vicente, A. A. (2014). Yeast: the soul of beer's aroma--a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology, 98*(5), 1937-1949.

Querol, A., & Bond, U. (2009). *The complex and dynamic genomes of industrial yeasts* (Vol. 293).

Reinheitsgebot, D. (2016). Bierbrauen: vom Zufallsprodukt zur Handwerkskunst.

Rep, M., Albertyn, J., Thevelein, J. M., Prior, B. A., & Hohmann, S. (1999). Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in Saccharomyces cerevisiae. *Microbiology, 145*(3), 715-727.

Rep, M., Reiser, V., Gartner, U., Thevelein, J. M., Hohmann, S., Ammerer, G., & Ruis, H. (1999). Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. *Molecular and Cellular Biology, 19*(8), 5474-5485.

Richter, K., Haslbeck, M., & Buchner, J. (2010). The Heat Shock Response: Life on the Verge of Death. *Molecular Cell, 40*(2), 253-266.

Rine, J., & Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. *Genetics, 116*(1), 9-22.

Riou, C., Nicaud, J. M., Barre, P., & Gaillardin, C. (1997). Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast, 13*(10), 903-915.

Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., & Delvaux, F. R. (2008). Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Applied Microbiology and Biotechnology, 80*(6), 1039-1051.

Saerens, S. M. G., Delvaux, F. R., Verstrepen, K. J., & Thevelein, J. M. (2010). Production and biological function of volatile esters in Saccharomyces cerevisiae. *Microbial Biotechnology, 3*(2), 165-177.

Sahara, T., Goda, T., & Ohgiya, S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *Journal of Biological Chemistry, 277*(51), 50015-50021.

Sakurai, H., & Takemori, Y. (2007). Interaction between heat shock transcription factors (HSFs) and divergent binding sequences: binding specificities of yeast HSFs and human HSF1. *Journal of Biological Chemistry, 282*(18), 13334-13341.

Schade, B., Jansen, G., Whiteway, M., Entian, K. D., & Thomas, D. Y. (2004). Cold adaptation in budding yeast. *Molecular Biology of the Cell, 15*(12), 5492-5502.

Schmitt, A. P., & McEntee, K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America, 93*(12), 5777-5782.

Schopfer, P., & Brennicke, A. (2011). *Pflanzenphysiologie*: Spektrum Akademischer Verlag.

Sertil, O., Kapoor, R., Cohen, B. D., Abramova, N., & Lowry, C. V. (2003). Synergistic repression of anaerobic genes by Mot3 and Rox1 in Saccharomyces cerevisiae. *Nucleic Acids Research, 31*(20), 5831-5837.

Seymour, I. J., & Piper, P. W. (1999). Stress induction of HSP30, the plasma membrane heat shock protein gene of Saccharomyces cerevisiae, appears not to use known stress-regulated transcription factors. *Microbiology-Uk, 145*, 231-239.

Sheldon, I. M. (2001). *Regulation of biotechnology: Will we ever "freely" trade GMOs?* Paper presented at the 77th EAAE Seminar/ NJF Seminar No. 325, Helsinki.

Sledziewski, A. Z., Bell, A., Kelsay, K., & MacKay, V. L. (1988). Construction of Temperature-Regulated Yeast Promoters Using the Mat[alpha]2 Repression System. *Nat Biotech, 6*(4), 411-416.

Special Eurobarometer 244b "Europeans in Biotechnology in 2005: Patterns and Trends". (2006). In. http://ec.europa.eu/public_opinion/index_en.htm: European Commision.

Strickland, B. R. (2001). The Gale encyclopedia of psychology.

Sun-Hee, K., Hyoun-Kyoung, C., & Han-Seung, S. (2012). Physicochemical properties and antioxidant activities of commercial vinegar drinks in Korea. *Food Science and Biotechnology, 21*(6), 1729-1734.

Sun, J., Shao, Z. Y., Zhao, H., Nair, N., Wen, F., Xu, J. H., & Zhao, H. M. (2012). Cloning and characterization of a panel of constitutive promoters for applications in pathway engineering in Saccharomyces cerevisiae. *Biotechnology and Bioengineering, 109*(8), 2082-2092.

Tamás, M. J., & Hohmann, S. (2003). The osmotic stress response of Saccharomyces cerevisiae. In S. Hohmann & W. H. Mager (Eds.), *Yeast Stress Responses* (pp. 121-200): Springer Berlin Heidelberg.

Tamás, M. J., Luyten, K., Sutherland, F. C. W., Hernandez, A., Albertyn, J., Valadi, H., . . . Ramos, J. (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Molecular Microbiology, 31*(4), 1087-1104.

Teixeira, M. C., Monteiro, P. T., Guerreiro, J. F., Gonçalves, J. P., Mira, N. P., dos Santos, S. C., . . . Sá-Correia, I. (2014). The YEASTRACT database: an upgraded information system for the analysis of gene and genomic transcription regulation in Saccharomyces cerevisiae. *Nucleic Acids Research, 42*(D1), D161-D166.

Toledano, M. B., Delaunay, A., Biteau, B., Spector, D., & Azevedo, D. (2003). Oxidative stress responses in yeast. In S. Hohmann & W. H. Mager (Eds.), *Yeast Stress Responses* (pp. 241-303). Berlin, Heidelberg: Springer Berlin Heidelberg.

Trott, A., & Morano, K. (2003). The yeast response to heat shock. In S. Hohmann & P. Mager (Eds.), *Yeast Stress Response* (Vol. 1): Springer-Verlag Heidelberg.

Trott, A., Shaner, L., & Morano, K. A. (2005). The molecular chaperone Sse1 and the growth control protein kinase Sch9 collaborate to remalate protein kinase a activity in Saccharomyces cerevisiae. *Genetics, 170*(3), 1009-1021.

Underhill, A. P. (2002). Craft production and social change in northern China. In *Craft Production and Social Change in Northern China* (pp. 241-258): Springer.

Vallee, B. L. (1998). Alcohol in the western world. *Scientific American, 278*(6), 80-85.

Verghese, J., Abrams, J., Wang, Y. Y., & Morano, K. A. (2012). Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (Saccharomyces cerevisiae) as a Model System. *Microbiology and Molecular Biology Reviews, 76*(2), 115-158.

Verstrepen, K. J. (2017). *Studying the Yeasts of Yesterday to Generate the Beer Yeasts of Tomorrow*. Paper presented at the 2017 ASBC Annual Meeting, Fort Myers, Florida.

Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Pretorius, I. S., Thevelein, J. M., & Delvaux, F. R. (2003). The Saccharomyces cerevisiae

alcohol acetyl transferase gene ATF1 is a target of the cAMP/PKA and FGM nutrient-signalling pathways. *FEMS Yeast Research, 4*(3), 285-296.

Verstrepen, K. J., Van Laere, S. D. M., Vanderhaegen, B. M. P., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., . . . Delvaux, F. R. (2003). Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Applied and Environmental Microbiology, 69*(9), 5228-5237.

Wang, D. L., Wang, Z. Y., Liu, N., He, X. P., & Zhang, B. R. (2008). Genetic modification of industrial yeast strains to obtain controllable NewFlo flocculation property and lower diacetyl production. *Biotechnology Letters, 30*(11), 2013-2018.

Wang, Q. M., Liu, W. Q., Liti, G., Wang, S. A., & Bai, F. Y. (2012). Surprisingly diverged populations of Saccharomyces cerevisiae in natural environments remote from human activity. *Molecular Ecology, 21*(22), 5404-5417.

Wang, Y. F., Zhang, S. X., Liu, H. Q., Zhang, L., Yi, C. F., & Li, H. (2015). Changes and roles of membrane compositions in the adaptation of Saccharomyces cerevisiae to ethanol. *Journal of Basic Microbiology, 55*(12), 1417-1426.

Wang, Z. Y., He, X. P., & Zhang, B. R. (2007). Over-expression of GSH1 gene and disruption of PEP4 gene in self-cloning industrial brewer's yeast. *International Journal of Food Microbiology, 119*(3), 192-199.

Wang, Z. Y., Wang, J. J., Liu, X. F., He, X. P., & Zhang, B. R. (2009). Recombinant industrial brewing yeast strains with ADH2 interruption using self-cloning GSH1+CUP1 cassette. *FEMS Yeast Research, 9*(4), 574-581.

Werner-Washburne, M., Stone, D. E., & Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. *Molecular and Cellular Biology, 7*(7), 2568-2577.

West, R. W., Yocum, R. R., & Ptashne, M. (1984). Saccharomyces cerevisiae GAL1 - GAL10 divergent promoter region - Location and function of the upstream activating sequance UASG. *Molecular and Cellular Biology, 4*(11), 2467-2478.

Wyman, J. (1862). Spontaneous generation. *British Medical Journal, 2*, 311–312.

Xie, Q., & Jimenez, A. (1996). Molecular cloning of a novel allele of SMR1 which determines sulfometuron methyl resistance in Saccharomyces cerevisiae. *FEMS Microbiology Letters, 137*(2-3), 165-168.

Yoshioka, K., & Hashimoto, N. (1981). Ester Formation by Alcohol Acetyltransferase from Brewers' Yeast. *Agricultural and Biological Chemistry, 45*(10), 2183-2190.

Zhang, J. G., Liu, X. Y., He, X. P., Guo, X. N., Lu, Y., & Zhang, B. R. (2011). Improvement of acetic acid tolerance and fermentation performance of Saccharomyces cerevisiae by disruption of the FPS1 aquaglyceroporin gene. *Biotechnology Letters, 33*(2), 277-284.

5. Appendix

5.1 Reviewed paper

1. Fischer, S. Büchner, K. R., Becker, T.: Induced expression of the alcohol acetyltransferase gene ATF1 in industrial yeast *Saccharomyces pastorianus* TUM 34/70. Yeast (2018). doi: 10.1002/yea.3319

2. Nsogning Dongmo, S., Kollmannsberger, H., Fischer, S., Becker, T.: Exploration of high gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages. International Journal of Food Science and Technology (2018). doi:10.1111/ijfs.13760

3. Nsogning Dongmo, S., Fischer, S., Becker, T.: Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity. Food Microbiology 73 (2018): 245-253

4. Tokpohozin, S. E., Fischer, S., Becker, T.: Assessment of malting and mash bio-acidification on the turnover of sorghum cyanogenic glucoside and protein hydrolysis improvement. LWT-Food Science and Technology 90 (2018): 303-309

5. Kerpes, R., Fischer, S., Becker, T.: The production of gluten-free beer: Degradation of hordeins during malting and brewing and the application of modern process technology focusing on endogenous malt peptidases. Trends in Food Science & Technology 67 (2017): 129-138.

6. Kupetz, M., Zeh, A., Fischer, S., Becker, T.: "Investigation of Filtration-inhibitory Substances in German Wheat Beer. Brewing Science 70 (2017): 1-8.

7. Steiner, J., Franke, K., Kießling, M., Fischer, S., Töpfl, S., Heinz, V., & Becker, T.:Influence of hydrothermal treatment on the structural modification of spent grain specific carbohydrates and the formation of degradation products using model compounds. Carbohydrate Polymers 184 (2017): 315-322.

8. Tokpohozin, S. E., Waldenmaier, J.F.T., Fischer, S., Becker, T.: Polyphasic characterization of lactic acid bacteria isolated from Beninese sorghum beer starter. LWT-Food Science and Technology 80 (2017): 51-58

9. Tokpohozin, S.E., Fischer, S., Sacher, B., Becker, T.: ß-D-glucosidase as “Key enzyme” for sorghum cyanogenic glucoside (dhurrin) removal and beer bioflavouring. Food and Chemical Toxicology 97 (2016): 217-223

10. Tokpohozin, S. E. Lauterbach, A. Fischer, S. Jürgen, B. Sacher, B. Becker, T.: Phenotypical and Molecular Characterization of Yeast Content in the starter of “Tchoukoutou”, a Beninese African Sorghum Beer. European Food and research Technology 242 (2016): 2147–2160

11. Fischer, S., C. Engstler, S. Procopio and T. Becker: EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system. LWT-Food Science and Technology 68 (2016): 556-562.

12. Fischer, S., C. Engstler, S. Procopio and T. Becker: Induced gene expression in industrial *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70: evaluation of temperature and ethanol inducible native promoters. FEMS Yeast Res 16 (2016): 556-562

13. Fischer, S., S. Procopio and T. Becker: Self-cloning brewing yeast: a new dimension in beverage production. European Food Research and Technology 237 (2013): 851-863.

5.2 Non-reviewed publication

14. Eigenfeld, M., Fischer, S., Becker, T.: Stress macht Falten! Auch bei Hefen? Brauwelt 10 (2018): 262-265

15. Fischer, S., Weiß, S., Becker, T.: Hefelagerung – Einfluss auf die Gäraktivität und Vitalität, Brauwelt 37/38 (2017): 1118-1122.

16. Nsogning Dongmo, S. Danner, S., Fischer, S., Becker, T.: Verlängerung der Fermentation von Milchsäurebakterien in Malzwürze, in Neues von Wissenschaftszentrum Weihenstephan“, Brauwelt 18-19 (2017): 552-553

17. Nsogning Dongmo, S. Hunter, I., Fischer, S., Becker, T.: Leistungssteigerung von Milchsäurebakterien, in „Neues von Wissenschaftszentrum Weihenstephan“, Brauwelt 14 (2017) 408-409.

18. Kerpes, R., Fischer, S., Becker, T.: Glutenfreies Gerstenmalzbier - das Potential der Gerstenmalzenzyme. Brauwelt, 36 (2016): 1028-1031.

19. Kupetz, M., Pohler, D., Fischer, S., Becker, T.: Das volle Spektrum der ß-Glucane. Brauwelt 31-32 (2016): 893-897

20. Steiner, J., Fischer, S., Becker, T.: Alkoholfreie Getränke auf Basis von Biertrebern. Brauwelt 49 (2015): 1491-1494.

21. Fischer, S., Byalkov, Y., Procopio, S., Becker, T.: *Saccharomyces eubayanus* und die untergärige Hefe, Brauwelt 41 (2015): 1195-1197.

22. Fischer, S. Procopio, S., Becker, T.: Gentechnisch modifizierte Hefen: Fluch oder Segen? Brauwelt 24-25 (2014): 735-737.

5.3 Oral presentations

1. Fischer, S., Becker, T.: Hefelagerung – Einfluss auf Gäraktivität und Vitalität, 50. Technologisches Seminar, Freising, Germany, 2017-02-15.

2. Fischer, S., Becker T.: Self-cloning brewing yeast – a new dimension of beverage production?, World Brewing Congress, Denver, USA, 2016-08-14

3. Steiner, J., Fischer, S., Becker, T.: Brewer's spent grain - waste material as potential raw material for healthy diet. World Brewing Congress, Denver, USA, 2016-08-14.

4. Kupetz, M., Fischer, S., Becker, T.: Challenges in beer membrane filtration – impact of volatiles on filtration performance of polymer membranes. World Brewing Congress, Denver, USA, 2016-08-17.

5. Tokpohozin, E.S., Fischer, S., Becker, T.: Biodiversity of Yeast and lactic acid bacteria Population isolated from Beninese African Sorghum Beer starter, Young Scientists Symposium on Malting, Brewing and Distilling, Chico, California, USA 2016-04-21.

6. Kerpes, R., Fischer, S., Becker, T.: Der Einsatz endogener Enzyme aus Gerste zur Herstellung von glutenfreiem Bier, 3. DLG-Forum FoodTec, Frankfurt a.M., Germany, 2016-04-20.

7. Kerpes, R., Fischer, S., Becker, T.: Glutenfreies Bier: Einsatz malzeigener Proteasen zur Detoxifikation glutenhaltiger Würze, 49. Technologisches Seminar, Freising, Germany, 2016-02-17.

8. Osen, R., Nsogning D., S., Sacher, B., Procopio, S., Toelstede S., Fritsch, S., Fischer, S.: Brauen mal anders - Entwicklung eines pflanzlichen Proteinerfrischungsgetränks für die vegane Ernährung, Jahrestagung Trend Vegan – Stand der Forschung 2015, Freising, Germany, 2015-10-27./28.

9. Fischer, S., Procopio, S., Becker, T.: Hefephysiologie und Genetik bezogen auf das Aromaprofiling in Bier, 3. Seminar Hefe und Mikrobiologie, Forschungszentrum Weihenstephan, Freising, Germany, 2015-03-25./26.

10. Fischer, S., Procopio, S., Becker, T.: *Saccharomyces eubayanus*: Fermentationseigenschaften im Vergleich zum *Saccharomyces cerevisiae* Stamm TUM 68 und *Saccharomyces pastorianus* Stamm TUM 34/70, 48. Technologisches Seminar, Freising, Germany, 2015-02-11.

11. Fischer, S. Procopio, S., Becker, T.: Self-cloning brewing yeast: new possibilities for optimized wort fermentation, Young Scientist Symposium, Ghent, Belgium, 2014-10-29.

12. Fischer, S. Procopio, S., Becker, T.: Einsatz selbstklonierter Hefen: Prozessoptimierung für fermentierte Getränke, GDL-Fachtagung, Frankfurt-Rodgau, Germany, 2014-10-17.

13. Sieber, E., Fischer, S., Zhang, Z., Heinisch, J., von Wallbrunn, C.: Genetics of ester synthesis in *Hanseniaspora uvarum* during fermentation of wine, ASEV National Conference in Portland, USA, 2012-06-18/22.

14. Fischer, S., Sieber, E., Zhang, Z., Heinisch, J., von Wallbrunn, C.: The genetics of ester synthesis in *Hanseniaspora uvarum* during winemaking, VAAM-Jahrestagung, Tübingen, Germany, 2012-03-18/22.

5.4 Poster presentations with first authorship

15. Fischer, S., Becker, T.: Induced gene expression: Evaluation of temperature and ethanol inducible native promoters for self-cloning brewing yeast. Belgian Brewing Conference 2015, Leuven, Belgium, 2015-09-06.

5.5 Curriculum Vitae

Susann Fischer

Dipl.- Ing (FH); M. Sc.

Born on 15th September 1983 in Leipzig, Germany

WORK EXPERIENCE

09/15 - dato	Technische Universität München, Institute of Brewing and Beverage Technology, Freising, Germany ***Group leader workgroup beverage and cereal biotechnology***
05/12 – 01/17	Technische Universität München, Institute of Brewing and Beverage Technology, Workgroup beverage biotechnology, Freising, Germany ***Phd Student*** Topic: Temperature induced gene expression in self-cloning brewing yeast
08/10 - 12/11	Department of Microbiology and Biochemistry University of Applied Science Wiesbaden – Research Center Geisenheim, Germany ***Master Thesis***: Molecular biological analysis of aroma relevant genes of *Hanseniaspora uvarum* Supervision: Dr. v. Wallbrunn, Prof. Dr. Schnell
08/06 – 01/08	Department of Microbiology and Biochemistry University of Applied Science Wiesbaden – Research Center Geisenheim, Germany ***Diploma Thesis***: Analysis of air contamination with fungal spores in different wineries during the harvest season of the year 2006 and 2007 Supervision: Dr. v. Wallbrunn, Prof. Dr. Großmann

EDUCATION

10/09 – 03/12	Master of Science in Enology and Wine technology, Justus Liebig Universität Gießen, Germany
10/04 – 07/08	Dipl. Ing (FH) in Viticulture and Enology University of Applied Science Wiesbaden – Research Center Geisenheim, Germany
08/03 – 07/04	High School Graduation, Berufliche Schulen Rheingau, Geisenheim, Germany
08/00 – 07/03	Alternance education for Winemaker, Pfalz, Germany