THESIS ON NATURAL AND EXACT SCIENCES B206

Applications of ^{15}N-labeled Yeast Hydrolysates in Metabolic Studies of *Lactococcus lactis* and *Saccharomyces cerevisiae*

KASPAR KEVVAI

TUT
PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Gene Technology

This dissertation was accepted for the defense of the degree of Doctor of Philosophy (Gene Technology) on April 6th, 2016.

Supervisor: Professor Toomas Paalme,
Department of Food Processing,
Tallinn University of Technology (TUT)

Co-supervisor: Associate Professor Ildar Nisamedtinov,
Department of Food Processing,
Tallinn University of Technology (TUT)

Opponents: Adjunct Professor Matti Korhola,
Department of Biosciences,
University of Helsinki, Finland

Senior Scientist Petri-Jaan Lahtvee,
Institute of Technology,
University of Tartu, Estonia

Defense of the thesis: May 27th, 2016

DECLARATION: I hereby declare that this doctoral thesis, submitted for the doctoral degree at TUT, is my original investigation and achievement and has not been submitted for the defense of any academic degree elsewhere.

Kaspar Kevvai

COLOPHON: This thesis was typeset with LaTeX 2ε using André Miede's *classicthesis* style with modifications by Ardo Illaste, David W. Schryer and Kaspar Kevvai to conform with TUT style guidelines. The main font is Libertine. ZapfChancery is used for Latin text. Biolinum is used for *sans* serif text.

ISSN 1406-4723
ISBN 978-9949-23-929-0 (publication)
ISBN 978-9949-23-930-6 (PDF)

European Union
European Social Fund
Investing in your future

LOODUS- JA TÄPPISTEADUSED B206

^{15}N-märgistatud pärmihüdrolüsaatide rakendused *Lactococcus lactis*'e ja *Saccharomyces cerevisiae* ainevahetuse uurimisel

KASPAR KEVVAI

TTÜ
KIRJASTUS

CONTENTS

ABSTRACT

Lactococcus lactis and *Saccharomyces cerevisiae* are among the best studied microbes and an ever increasing number of "omics" datasets are being made publicly available. Although their physiology and metabolism have been extensively investigated, consumption of several important groups of molecules, such as peptides and B-group vitamins have received somewhat less attention. While both of these organisms are known to be capable of utilizing peptides for growth, and the peptide transporters and peptidases are relatively well characterized, a proper, quantitative understanding how these compounds are consumed under industrially relevant growth conditions is currently lacking. Understanding how peptides are utilized is important because they are a major source of nitrogen in certain media for starter culture production as well as in a number of fermentation processes utilized by the food and biotechnology industries involving yeast and lactic acid bacteria.

In this work we present how U-^{15}N-labeled yeast hydrolysates were used in metabolic labeling experiments to characterize the nitrogen metabolism of both *L. lactis* and *S. cerevisiae* and as a source of isotope labeled metabolome standards for analytical purposes. The primary aim was to understand the role of peptides as a source of amino acids in these organisms. Quantitative measurements of the ^{15}N-labeling of the amino acids within the cells and the culture media was carried out using LC-MS and the resulting data was analyzed with the help of metabolic flux models.

The labeling experiments with *L. lactis* demonstrated that when all amino acids are present in both free and peptide-bound forms, both of these forms are consumed in parallel. The labeling data also revealed that an extensive efflux of amino acids from lactococcal cells occurs after their uptake from the medium. In addition, we found that *S. cerevisiae* simultaneously consumes ammonia, free amino acids, and peptides during fermentative growth and a significant proportion of the proteinogenic amino acids within cells originates from peptides. Both organisms were found to clearly display a strong preference for peptides that contain hydrophobic amino acid residues. These results can be applied in the production of yeast hydrolysates whose amino acid and peptide composition has been optimized for a particular bioprocess and/or microorganism.

In addition to the peptide consumption studies, ^{15}N-labeled yeast extracts were also utilized as a source of isotopically labeled internal standards to aid in the LC-MS quantification of various vitamin B_3 derivatives, including nicotinamide riboside, nicotinamide mononucleotide, and NAD^+.

Taken together, our results demonstrate the potential of ^{15}N-labeled yeast hydrolysate as a cost-effective yet powerful tool for metabolic labeling experiments and as a multifunctional internal standard.

KOKKUVÕTE

Lactococcus lactis ja *Saccharomyces cerevisiae* on ühed enim uuritud mikroobid ning nende kohta on lisandumas aina uusi avalikke oomikameetoditel põhinevaid andmestikke. Ehkki kõnealuste organismide füsioloogiat ning ainevahetust on põhjalikult uuritud, on mitmete oluliste molekulide, näiteks peptiidide ning B-rühma vitamiinide roll märksa vähem tähelepanu pälvinud. Kuigi on teada, et mõlemad organismid on võimelised kasutama kasvuks peptiide ning vastavad transportsüsteemid ning peptidaasid on üpriski hästi karakteriseeritud, on aminohapete ja peptiidide kvantitatiivsed tarbimismustrid mitmetes tööstusliku tähtsusega süsteemides siiski ebaselged. Peptiidide tarbimise mõistmine on aga oluline, kuna nad on üheks põhiliseks lämmastikuallikaks mitmetes starterkultuuride söötmetes ning toidu- ja biotehnoloogiatööstuse fermentatsioonides.

Käesolev doktoritöö keskendub U-^{15}N-märgistatud pärmihüdrolüsaatide rakendustele *L. lactis*'e ning *S. cerevisiae* lämmastikumetabolismi kirjeldamisel ning analüütilistes meetodites. Töö põhieesmärk on mõista peptiidide rolli aminohapete allikana nendes organismides. Selleks teostati kvantitatiivsed aminohapete ^{15}N-märgistuse mõõtmised rakkudes ning kasvukeskkonnas LC-MS'iga ning saadud mõõtmistulemusi analüüsiti ainevahetusvoogude mudelite abil.

^{15}N-märgistega eksperimendid *L. lactis*'ega näitasid, et aminohapete olemasolul keskkonnas nii vabal kui peptiidsel kujul tarbitakse neid paralleelselt. Katseandmed näitasid ka seda, et toimub ulatuslik aminohapete väljavool bakterirakkudest pärast nende assimileerimist keskkonnast peptiidsel kujul. Samuti ilmnes, et fermentatiivsel kasvul tarbib *S. cerevisiae* ammoniaaki, vabu aminohappeid ja peptiide paralleelselt ning märkimisväärne hulk valgufraktsiooni lämmastikust pärineb just viimastest. Peptiidide aminohappelise koostise suhtes olid mõlema organismi eelistused võrdlemisi sarnased; selgelt eelisjärjekorras tarbiti hüdrofoobsete aminohapete jääke sisaldavaid peptiide. Saadud uurimistulemusi on võimalik rakendada konkreetsete fermentatsioonide ja/või mikroorganismide tarbeks optimeeritud aminohappelise koostisega pärmihüdrolüsaatide väljatöötamisel ja tootmisel.

Lisaks leidis ^{15}N-märgistatud pärmiekstrakt rakendust ka isotoopmärgistatud sisestandardina mõnede B_3-vitamiini vormide, näiteks nikotiinamiid-ribosiidi, nikotiinamiid-mononukleotiidi ning NAD^+ määramisel LC-MS'i abil.

Kokkuvõtvalt, uurimistulemused illustreerivad ^{15}N-märgistatud pärmihüdrolüsaatide suurt potentsiaali suhteliselt odava, kuid tõhusa vahendina ainevahetuseuuringutes ning multifunktsionaalse sisestandardina.

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

I Kevvai K, Kütt ML, Nisamedtinov I, Paalme T. **Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids.** *Antonie van Leeuwenhoek*, 105(3):511-522, (2014)

II Paalme T, Kevvai K, Vilbaste A, Hälvin K, Nisamedtinov I. **Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture.** *World Journal of Microbiology and Biotechnology*, 30(9):2351-2359, (2014)

III Kevvai K, Kütt ML, Nisamedtinov I, Paalme T. **Simultaneous utilization of ammonia, free amino acids and peptides during fermentative growth of *Saccharomyces cerevisiae*.** *Journal of the Institute of Brewing*, 122(1):110-115, (2016)

ADDITIONAL PUBLICATIONS

IV Orumets K, Kevvai K, Nisamedtinov I, Tamm T, Paalme T. ***YAP1* over-expression in *Saccharomyces cerevisiae* enhances glutathione accumulation at its biosynthesis and substrate availability levels.** *Biotechnology Journal*, 7(4):566-568, (2012)

V Nisamedtinov I, Kevvai K, Orumets K, Arike L, Sarand I, Korhola M, Paalme T. **Metabolic changes underlying the higher accumulation of glutathione in *Saccharomyces cerevisiae* mutants.** *Applied Microbiology and Biotechnology*, 89(4):1029-1037, (2011)

VI Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T. **Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding.** *Applied Microbiology and Biotechnology*, 87(1):175-183, (2011)

VII Nisamedtinov I, Lindsey GG, Karreman R, Orumets K, Koplimaa M, Kevvai K, Paalme T. **The response of the yeast *Saccharomyces cerevisiae* to sudden vs. gradual changes in environmental stress monitored by expression of the stress response protein Hsp12p.** *FEMS Yeast Research*, 8(6):829-838, (2008)

SUMMARY OF AUTHOR'S CONTRIBUTION

I In Publication I, the author conceived the study, designed and carried out the experiments, interpreted the data, and wrote the manuscript.

II In Publication II, the author contributed to designing the study and the experiments, aided in data analysis, and participated in writing the manuscript.

III In Publication III, the author conceived the study, designed and carried out the experiments, interpreted the data, and wrote the manuscript.

LIST OF PRESENTATIONS

I Kevvai K, Nisamedtinov I, Paalme T. **Microbial and cell culture growth-inducing peptides in yeast hydrolysates.** *Poster presentation at Conference of Graduate School "Functional materials and technologies"*,
February, 2012, Tartu, Estonia.

II Kevvai K, Kütt ML, Nisamedtinov I, Paalme T. **Peptide and amino acid consumption patterns of *Lactococcus lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labeled yeast hydrolysate.** *Poster presentation at 5th Congress of European Microbiologists (FEMS2013)*,
July 2013, Leipzig, Germany.

III Kevvai K, Kütt ML, Nisamedtinov I, Paalme T. **Utilization of ^{15}N-labeled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids.** *Poster presentation at Advanced Lecture Course on Systems Biology (SYSBIO2014)*,
March 2014, Innsbruck, Austria.

IV Kevvai K, Kütt ML, Nisamedtinov I, Paalme T. **Utilization of ^{15}N-labeled yeast hydrolysate in the study of amino acid and peptide metabolism of *L. lactis* IL1403 & *S. cerevisiae* S288c.** *Oral presentation at Lallemand Cross-Fertilization Meeting*,
January 2015, Montréal, Canada.

V Kevvai K, Vilbaste A, Hälvin K, Nisamedtinov I, Paalme T. **Uptake and accumulation of B-complex vitamins in yeast.** *Oral presentation at Lallemand Food and Biotech Seminar 6*,
May 2015, Aarhus, Denmark.

ACKNOWLEDGMENTS

The research presented in this dissertation was carried out at the Competence Center of Food and Fermentation Technologies (CCFFT). My sincere gratitude belongs to my supervisor Prof. Toomas Paalme for his guidance, understanding, and encouragement throughout my studies. I am no less indebted to my co-supervisor Dr. Ildar Nisamedtinov. Toomas and Ildar were the ones who sparked my interest in cell biology and biotechnology back when I joined CCFFT as a first year physics student. It has been an amazing journey and I feel very lucky to have had such inspiring, open-minded, and supportive supervisors.

The completion of this thesis wouldn't have been possible, quite literally, without the help of my dear colleague Dr. Mary-Liis Kütt, who carried out some of the most laborious and difficult parts of the work — thank you! I also wish to express my gratitude to all my other wonderful colleagues, past and present, at CCFFT. In particular, I want to thank my co-authors Kerti, Kristel H., and Allan, as well as everyone else in the "yeast group"; Kristel V., Sten, Rain, Mart, and David the for great times in and out of the lab; Gethe, Kadi, Kristi, Minna, Martti, and Aavo for the pure joy of making music with the greatest CCFFT band in the world!

This section would not be complete without thanking the people that are most dear to me, my friends and family. The greatest acknowledgment is saved for my parents Toomas and Sille, and my sister Kadi for the confidence they've had in me and for their support throughout my life.

Financial support for this research was provided by the European Regional Development Fund (projects EU22704 and EU29994) and Estonian Ministry of Education (research grants ETF9189, SF0140090s08, and institutional research funding IUT19-27). These studies were also partially supported by European Social Fund's Doctoral Studies, Internationalisation Programme DoRa as well as Graduate School "Functional materials and technologies" receiving funding from the European Social Fund under project 1.2.0401.09-0079 in University of Tartu, Estonia.

ACRONYMS

ABC	ATP-binding cassette
BCAA	brached-chain amino acid
CEP	cell envelope-associated proteinase
DAL5	gene encoding Dal5p
GlnPQ	glutamine ABC transporter with GlnP and GlnQ subunits, TCDB: 3.A.1.3.25
LAB	lactic acid bacteria
LC-MS	liquid chromatography–mass spectrometry
MDV	mass distribution vector
MS	mass spectrometry
NCR	nitrogen catabolite repression
NMR	nuclear magnetic resonance
NMWL	nominal molecular weight limit
Opp	oligopeptide transport system, TCDB: 3.A.1.5.10
opp-pepO1	operon encoding the Opp system and PepO
Opt	oligopeptide transport system, TCDB: 3.A.1.5.25
OPT1	gene encoding Opt1p
OPT2	gene encoding Opt2p
pepC	gene encoding PepC
pepC	gene encoding PepN
pepX	gene encoding PepX
PDA	photodiode array
prtP	gene encoding PrtP
PTR2	gene encoding Ptr2p
SPS	Ssy1p-Ptr3p-Ssy5p-sensor system
YAN	yeast assimilable nitrogen

CHEMICALS AND ENZYMES

AcaP	amino acid permease, UniProt: F2HJG8
Agp1p	general amino acid permease, UniProt: P25376
Ape1p	vacuolar aminopeptidase 1, UniProt: P14904
Ape2p	aminopeptidase 2, UniProt: P32454
Ape3p	aminopeptidase Y, UniProt: P37302
Aqr1p	probable transporter, UniProt: P53943
AraT	aromatic amino acid specific aminotransferase, UniProt: Q9CJE0

ArcD1	arginine:ornithine antiporter, UniProt: F2HL56
ArcD2	arginine:ornithine antiporter, UniProt: F2HL52
AspB	asparate aminotransferase, UniProt: Q9CEK7
ATP	adenosine-5'-triphosphate, KEGG: C00002
Bap2p	Leu/Val/Ile amino-acid permease, UniProt: P38084
Bap3p	valine amino-acid permease, UniProt: P41815
BcaP	branched-chain amino acid permease, UniProt: S6EX81
BcaT	branched-chain-amino-acid aminotransferase, UniProt: Q9CG22
Can1p	arginine permease, UniProt: P04817
CcpA	catabolite control protein A, UniProt: A2RJC5
CitB	aconitate hydratase, UniProt: Q9CHQ5
CitD	citrate lyase acyl carrier protein, UniProt: Q9CGA9
CitP	citrate-sodium symporter, UniProt: P21608
CodY	transcriptional regulator, UniProt: Q9CJ45
Cpg2	carboxypeptidase G2, UniProt: P06621
Cps1p	carboxypeptidase S, UniProt: P27614
Cup9p	homeobox protein, UniProt: P41817
Dal5p	allantoate permease, UniProt: P15365
Dap2p	dipeptidyl aminopeptidase B, UniProt: P18962
Dip5p	dicarboxylic amino acid permease, UniProt: P53388
DtpT	di-/tripeptide transporter, UniProt: P0C2U2
Fot2p	putative amino acid transporter, UniProt: A0A0C7KNI9
FywP	amino acid permease, UniProt: F2HN33
GABA	gamma-aminobutyic acid, KEGG: C00334
Gap1p	general amino-acid permease, UniProt: P19145
Gdh	glutamate dehydrogenase, UniProt: G1FE36
GlnP	glutamine ABC transporter permease and substrate binding protein, UniProt: Q9CES5
GlnQ	glutamine ABC transporter ATP-binding protein, UniProt: Q9CES4
Gnp1p	high-affinity glutamine permease, UniProt: P48813
HisP	lysine-specific permease, UniProt: K7WL41
Icd	isocitrate dehydrogenase, UniProt: Q9CHQ4
Lyp1p	lysine-specific permease, UniProt: P32487
LysP	lysine specific permease, UniProt: K7VV21
Mep1p	ammonium transporter 1, UniProt: P40260
Mep2p	ammonium transporter 2, UniProt: P41948
Mep3p	ammonium transporter 3, UniProt: P53390
NA	nicotinic acid, KEGG: C00253
NAD^+	nicotinamide adenine dinucleotide, KEGG: C00003
Nam	nicotinamide, KEGG: C00153
NMN	nicotinamide mononucleotide, KEGG: C00455
NR	nicotinamide riboside, KEGG: C03150
OppA	oligopeptide-binding protein, UniProt: Q9CEK0
OppB	oligopeptide transport system permease protein, UniProt: P0A4N7
OppC	oligopeptide transport system permease protein, UniProt: P0A4N9

OppD	oligopeptide transport ATP-binding protein, UniProt: Q07733
OppF	oligopeptide transport ATP-binding protein, UniProt: P0A2V4
Opt1p	oligopeptide transporter 1, UniProt: P40897
Opt2p	oligopeptide transporter 2, UniProt: Q06593
OptA	oligopeptide ABC transporter substrate binding protein, UniProt: Q9CIL2
OptS	OptS, UniProt: Q64K09
Pep4p	saccharopepsin, UniProt: P07267
PepA	glutamyl aminopeptidase, UniProt: Q9CIH3
PepC	aminopeptidase C, UniProt: Q9CEG3
PepDA	(probable) dipeptidase A, UniProt: Q9CIV4
PepDB	(probable) dipeptidase B, UniProt: Q9CFC3
PepE	aminopeptidase E, UniProt: P94870
PepF	oligoendopeptidase F, UniProt: Q9CEV7
PepG	aminopeptidase G, UniProt: P94869
PepI	proline iminopeptidase, UniProt: P46542
PepL	proline iminopeptidase, UniProt: Q48531
PepM	methionine aminopeptidase, UniProt: Q9CHV6
PepN	aminopeptidase N, UniProt: Q9CIQ1
PepO	neutral endopeptidase, UniProt: Q07744
PepP	aminopeptidase P, UniProt: Q9CHN7
PepQ	proline dipeptidase, UniProt: Q9CF32
PepR	proline iminopeptidase, UniProt: F9US06
PepS	aminopeptidase PepS, UniProt: Q9X4A7
PepT	peptidase T, UniProt: Q9CEM7
Pept1	solute carrier family 15 member 1 (rabbit), UniProt: P36836
PepV	dipeptidase, UniProt: Q9CH96
PepX	Xaa-Pro dipeptidyl-peptidase, UniProt: Q9CE01
Pnc1p	nicotinamidase, UniProt: P53184
Prb1p	cerevisin, UniProt: P09232
Prc1p	carboxypeptidase Y, UniProt: P00729
Prd1p	saccharolysin, UniProt: P25375
PrtB	proteinase, UniProt: Q1GA00
PrtH	cell envelope-associated proteinase, UniProt: Q9S4K2
PrtP	PI-type proteinase, UniProt: P16271
PrtR	cell envelope-associated proteinase, UniProt: Q8GC13
PrtS	cell envelope proteinase, UniProt: Q9F8Q4
Ptr2p	peptide transporter, UniProt: P32901
Ptr3p	SPS-sensor component, UniProt: P43606
Put4p	proline-specific permease, UniProt: P15380
SerP1	amino acid permease, UniProt: F2HQ25
SerP2	amino acid permease, UniProt: F2HQ24
SspA	glutamyl endopeptidase, UniProt: P0C1U8
Ssy1p	SPS-sensor component, UniProt: Q03770
Ssy5p	SPS-sensor serine protease component, UniProt: P47002
Tat1p	valine/tyrosine/tryptophan amino-acid permease 1, UniProt: P38085
Tat2p	tryptophan permease, UniProt: P38967
Ubr1p	E3 ubiquitin-protein ligase, UniProt: P19812
Ybt1p	ATP-dependent bile acid permease, UniProt: P32386

THESIS

1

INTRODUCTION

A major challenge for microbial populations is adapting to changing environmental conditions in a way that optimizes a combination of growth and survival. This means responding to changes in the availability of nutrients, accumulation of waste products, toxins, variations in chemical-physical conditions (pH, temperature, osmolarity, gases) and so forth. The cell must maintain an internal environment that is usually markedly different from that outside of it. Due to the second law of thermodynamics, the environment as a whole always tends to move towards greater disorder and the cell's struggle for life boils down to whether it can sustain itself in this natural movement towards chaos. Part of this endeavor is to balance the supply of cellular building blocks and their precursors under diverse environmental conditions.

Assimilation of nitrogen is an essential and central process in all microorganisms. The competing processes of anabolic biosynthetic reactions and catabolic nitrogen source utilization must be coordinated to enable cells to manifest a proper response to the availability of nutrients. It seems as if organisms that are considered well adapted to "rich" environments employ a decidedly different approach to nitrogen supply balancing than more versatile organisms that are well adapted to "poorer" environments. Good examples of organisms that fall into these categories are the lactic acid bacterium *Lactococcus lactis* and the baker's yeast *Saccharomyces cerevisiae*, respectively. The main aim of the work presented in this dissertation is to improve our understanding of these behaviors.

During the last few decades, improvements in genetic manipulation techniques and the proliferation of genome-wide studies has led to a more integrated view of the regulation of nitrogen metabolism. Yet, surprisingly there are significant gaps in our knowledge regarding the mechanisms that regulate the consumption of several important groups of nitrogen compounds, such as peptides and B-group vitamins. This is also true for extensively studied model microorganisms such as *L. lactis* and *S. cerevisiae*. Understanding the utilization of peptides is important because they are a major nitrogen source in certain media for starter culture production as well as in a number of food industry fermentation processes that involve yeast and lactic acid bacteria. It is evident that a considerable amount of research in these fields is currently being conducted, however, due to its proprietary nature these studies are typically not made publicly available [1].

Yeast extract is one of the most widespread sources of essential and growth-promoting compounds in both laboratory and industrial media used to cultivate microorganisms. This mixture contains amino acids, peptides, nucleotides, B-group vitamins, and minerals. Since yeast hydrolysates provide an undefined set of precursor molecules for both biomass and product synthesis, it is difficult to precisely determine the intrinsic product

yields in rich-medium fermentations. To overcome this problem, isotope labeling analysis can provide insights into the contribution of various compounds present in yeast hydrolysates to biomass and product synthesis.

In this work we report on novel applications of U-^{15}N-labeled yeast hydrolysates for the characterization of the nitrogen metabolism of *L. lactis* and *S. cerevisiae*. These hydrolysates can be used to conduct metabolic labeling experiments and for analytical purposes. A review of the current knowledge of peptide and amino acid utilization systems in the two organisms under study is provided followed by a short overview of the principles of isotope tracer analysis and applications of isotope-labeled yeast extracts. Finally, we present the results of the experiments that aimed to study the amino acid, peptide, and B-vitamin metabolism within these organisms.

2

LITERATURE REVIEW

2.1 PEPTIDE AND AMINO ACID METABOLISM OF *Lactococcus lactis*

Lactococcus lactis is a natural inhabitant within both plants and dairies. Various strains of lactococci are used in the production of cheeses and other fermented dairy, as well as vegetable, fruit, cereal, and meat products. Its relatively simple metabolism also makes *L. lactis* an attractive candidate for use as a cell factory [2] and has drawn attention as a potential host for production and delivery of therapeutic proteins [3]. Due to its substantial economic importance, *L. lactis* is the most extensively studied lactic acid bacterium.

A common feature of lactic acid bacteria (LAB) is the production of lactic acid as the major end product of sugar fermentation. In general, they can be characterized as Gram-positive, aerobic or facultatively anaerobic, nonsporulative rods and cocci that are oxidase, catalase, and benzidine negative, lack cytochromes, do not reduce nitrates to nitrite, are gelatinase negative, and are unable to metabolize lactate [1].

The species *Lactococcus lactis* belongs to the class *Bacilli*, the order *Lactobacillales*, and the family *Streptococcaceae*, and is further divided into four subspecies: *lactis*, *cremoris*, *hordniae*, and *tructae* [4]. Bacteria that belong to the *Lactococcus* genus possess relatively small genomes with around 2000 to 2800 protein-encoding genes [5], less than half the number of that encoded by *Saccharomyces cerevisiae*. *Lactococcus lactis* subsp. *lactis* IL1403 was the first lactic acid bacterium whose genome was completely sequenced [6]. The strain IL1403 is a plasmid-free variant of *L. lactis* subsp. *lactis* biovar. *diacetylactis* CNRZ157 (also referred to as IL594; [7, 8]). As of March 2016, the complete genomes of seven *L. lactis* subsp. *lactis* strains from different environmental niches are annotated in the Kyoto Encyclopedia of Genes and Genomes; the sequencing of new strains is ongoing.

The general consensus is that industrial dairy strains have probably evolved from plant strains and have adapted to thrive in milk and related environments [8]. This adaptation has resulted in genome reduction along with gene decay (including nonsense mutations, deletions, truncations and frameshifts), but also acquisition of new genes by horizontal gene transfer. Notably, these alterations have resulted in an inability to synthesize a number of amino acids [9]. Concurrently, industrial dairy strains often carry several plasmids that confer important traits such as the ability to utilize lactose, provide bacteriophage resistance, as well as the expression of extracellular proteinases and peptide uptake systems [10, 11].

In this section an overview of the peptide and amino acid utilization systems within this bacterium is provided.

2.1.1 *Cell-envelope proteinases*

Due to their industrial significance, the most well studied proteolytic systems within LAB are found within dairy strains. The largest sources of nitrogen for dairy LAB are the breakdown products of caseins. Generally, the utilization of caseins starts by their hydrolysis using a cell envelope-associated proteinase (CEP).

There are similarities as well as differences between the CEPs from different LAB species (PrtP of *L. lactis* and *Lactobacillus paracasei*, PrtH of *Lactobacillus helveticus*, PrtR of *Lactobacillus rhamnosus*, PrtS of *Streptococcus thermophilus*, PrtB of *Lactobacillus bulgaricus*); all of them belong to the same family subtilisin family of multi-domain proteases and share some common structural features, including a catalytic serine protease domain, whereas differences in substrate specificity, bacterial anchor and domain organization have been described [12, 13]. Virulent streptococci also possess similar proteinases, although they are generally not utilized for nutritional purposes [12]. In lactococci, the proteinase genes can be either plasmid- or genome-encoded [13, 14]. The presence or absence of a CEP is a species- and strain-dependent trait [13]; *L. lactis* subsp. *lactis* IL1403 used in this study does not encode PrtP [15–17].

Whereas LAB encode several peptide and amino acid transporters and intracellular peptidases (discussed below), with a few notable exceptions, they typically possess only a single CEP [18–21]. The plasmid carrying the CEP gene can occasionally be lost during extended propagation, even though the presence of proteinase results in increased growth rates and biomass yields [22]. It has been proposed that although the expression of CEP places a metabolic burden on the cells, its presence allows them to acquire a relatively larger fraction of peptides before they diffuse away [23]. Such insights are important to understand the dynamics of mixed culture fermentations. Strains that lack PrtP activity are considered to be unable to consume proteins as a source of amino acids and only reach cell densities between 5–25 % relative to proteinase-positive strains in milk [24, 25].

Numerous studies have been carried out to determine the substrate specificities of PrtP from different strains. For example, the action of PrtP from *L. lactis* Wg2 on β-casein results in the generation of various oligopeptides that mostly range between four to 30 amino acid residues in length, along with minimal amounts of smaller peptides and free amino acids [26]. It appears that minor changes in the amino acid sequence of the PrtP variants from various *L. lactis* strains cause slight differences in the casein breakdown specificity [27, 28]. These degradation products are subsequently transported into the cell through the peptide transport systems outlined below.

2.1.2 *Oligopeptide transporters of L. lactis*

Opp is the major oligopeptide transporter in *L. lactis* that belongs to the ATP-binding cassette (ABC) transporter family. It consists of five subunits: two (homologous) integral membrane proteins OppB and OppC, which form the translocation pore, two (homologous) nucleotide-binding domains OppD and OppF, which fuel transport by ATP hydrolysis, and the substrate-binding protein OppA [29], which determines the substrate specificity of the

system [30]. OppA from *L. lactis* binds peptides of widely varied lengths, from four to at least 35 amino residues long.

The substrate specificity and the binding mechanisms of OppA have been extensively studied using combinatorial peptide libraries and competitive inhibition studies [31, 32] as well its crystallization in the presence of substrate peptides [33, 34]. It was found, for example, that proline-rich peptides that contain at least one isoleucine are bound with high affinity, whereas the position of the isoleucine residue is not critical, *i. e.*, the mechanism for peptide selection is based on amino-acid composition rather than the exact sequence [33].

The second, somewhat less well characterized oligopeptide transport system present in most lactococcal strains is Opt. Despite the presence of the respective genes, it was discovered that *L. lactis* IL1403 does not express OppA under any conditions and thus the Opt system is the only known transport system used for the utilization of oligopeptides in this strain [17, 30, 35]. Opt also belongs to the ABC transporter family but differs from Opp by the presence of two peptide-binding proteins OptS and OptA and peptide uptake specificity. Initially, the role of Opt was thought to be limited to the transport of hydrophobic di- and tripeptides [36]. However, later studies implicated the role of Opt in the transport of oligopeptides with three to six amino acid residues [17].

2.1.3 *Di- and tripeptide transporter DtpT of L. lactis*

DtpT belongs to the proton-dependent oligopeptide transporter family [37] and has counterparts in, among other organisms, yeast (Ptr2p, see below). It is a proton-motive-force-driven di- and tripeptide transporter with a wide substrate specificity [38–40], a 12-transmembrane domain protein encoded by a single gene. The physiological roles of DtpT are not fully clear. Because the breakdown of caseins by PrtP results in the generation of oligopeptides with a minimal amount of free amino acids and di-/tripeptides, its nutritional role could be rather minor. Kunji *et al.* [41] reported that inactivation of DtpT does not affect the growth of *L. lactis* on β-casein. In complex microbial consortia of natural environments, the situation might be different.

It has been suggested that DtpT may be utilized for the excretion of di- and tripeptides when they over-accumulate in the cell [42, 43]. One can hypothesize that peptides which cannot be readily and efficiently hydrolyzed in the cell are excreted instead. Due to the reversible nature of DtpT, this may also help to generate additional proton motive force.

Under certain conditions, di- or tripeptides, particularly peptides containing branched-chain amino acids (BCAAs), entering the cell through this transporter serve a signaling function [17, 44, 45]. BCAAs liberated through hydrolysis serve as allosteric regulators of CodY, which is a transcriptional repressor that regulates expression of genes involved in nitrogen metabolism [46] (discussed below). Thus, DtpT seems to serve an important, if non-essential role in the fine-tuned proteolytic machinery of *L. lactis*.

2.1.4 *Peptidases of L. lactis*

The last step in the utilization of peptides for nutritional purposes in the proteolytic pathway is the degradation of the peptides taken up from the medium. Upon entering the cell, peptides are rapidly hydrolyzed into their constituent free amino acids. For this purpose, most species of LAB employ an extensive array of intracellular peptidases.

Lactococcus lactis subsp. *lactis* IL1403 encodes thirteen distinct peptidases. These include endopeptidases (PepO, PepF), aminopeptidases (PepC, PepN, PepM, PepA, PepP), the X-prolyl dipeptidyl aminopeptidase (PepX), tripeptidase (PepT), dipeptidases (PepDA, PepDB, PepV), and prolidase (PepQ) [6, 16]. Members of the PepE/PepG as well as PepI/PepR/PepL peptidase superfamilies seem to be missing in lactococci. Notably, no carboxypeptidase activity has been reported to be present in any LAB strain [13]. The localization of all peptidases identified in lactic acid bacteria is intracellular (none contain signal sequences for excretion or membrane-spanning domains) [43, 47, 48].

More recent genome-wide comparative studies have revealed variations in the expression of a number of peptidases between different LAB species [16], probably resulting from the presence or absence of the respective plasmids and possibly reflects their adaption to a particular environmental niche.

To unravel the physiological roles of peptidases, a number of studies have analyzed single and multiple peptidase knockout mutants of LAB [42, 43, 49–54]. While single peptidase knockouts usually do not display significantly impaired growth rates compared to wild-type strains when grown in milk (the growth defect is even smaller or non-existent in rich media), multiple knockouts may result in severely inhibited growth rates (again, less so in rich media). This indicates that the peptidolytic machinery has some redundancy, or in other words, the peptidases have some overlap in their specificities. In chemically defined media, however, a single peptidase mutant may not be able to grow when an essential amino acid is supplied within a specific peptide. Importantly, intracellular accumulation of peptides that are taken up from the growth medium has been demonstrated in multiple-knockout peptidase mutants [42].

The physiological role of peptidases in LAB is not limited to purely nutritional purposes. For example, PepF may play a role in protein turnover [55]. Likewise, PepS of *S. thermophilus* may be involved in protein turnover and/or maturation [56].

The expression of several peptidases has been shown to be regulated by CodY in response to intracellular branched-chain amino acid levels; see below.

2.1.5 *Free amino acid transport in L. lactis*

LAB also possess an extensive arsenal of free amino acid transporters. In lactococci, these include symport systems (Leu, Ile, Val, Met, Ala, Gly, Ser, Thr, Lys, His, Pro, Tyr, Phe, and Cys), antiport systems (Arg/Orn, Lys), and ABC transporters (Glu and Gln, Asn, Asp, and Pro) [57, 58]. Cloning and functional characterization of several of these transporters (GlnPQ, BcaP, LysP, HisP, SerP1, SerP2, AcaP, and FywP) has been carried out [59–62]. In a number of cases, a single amino acid can enter the cell through more than one transporter, albeit with different affinities [61]. In addition, several amino acids compete with each

other for the same transport system, *e.g.*, Ile, Leu, Val, and Met in the case of BcaP [60, 63]. Some systems, for example, the arginine-ornithine antiporter (ArcD1, ArcD2), have the sole role of supplying the cell with metabolic energy, and are not used for anabolic reactions [58].

The physiological roles of a number of the free amino acid transporters under different growth conditions are not fully clear. On one hand, casein is the main source of amino acids for *L. lactis* during growth in milk [64]. On the other hand, it has been demonstrated that *L. lactis* is able to grow on minimal media containing solely free amino acids as an amino acid source [65]. Furthermore, some strains don't reach their maximum growth rate in milk without amino acid supplements [66].

Importantly, a common phenomenon observed during the consumption of peptides by *L. lactis* is the subsequent excretion of (some) constituent amino acids [64, 67]. The efflux of excess amino acids has been proposed to be coupled to the excretion of a proton, which would result in the generation of metabolic energy in the form of proton motive force [47, 58]. This might serve as an important source of metabolic energy when sugars are depleted or, conversely, during aggressive growth. Notably, recent studies have demonstrated that the proteins responsible for the excretion of amino acids may not necessarily be the same used for high affinity uptake of the same amino acids [61, 62].

While the free amino acid transporters are clearly important for sustaining growth in defined medium, their role in complex media, such as milk is probably multifaceted (as in the case of DtpT). For example, in mixed cultures, amino acids released or secreted by one organism could be utilized by another.

2.1.6 *Regulation of amino acid metabolism in L. lactis*

All strains of *L. lactis* are auxotrophic for a number of amino acids; for strain IL1403 these include leucine, isoleucine, valine, methionine, histidine, and arginine [68]. To respond to changes in the nitrogen composition of the environment, LAB have evolved a regulatory system to maintain a blance of nitrogen species within the cell.

In *L. lactis*, expression of a number of genes of the proteolytic system was shown to be dependent on the peptide content of the growth medium [17, 44, 69]. The transcription of *prtP*, *pepX*, *pepC*, *pepC*, and *opp-pepO1* were shown to be strongly repressed in a peptide-rich medium [45]. On the other hand, many of the respective proteins, including OppA, PepO, PepN, PepC, PepF, as well as OptS were shown to be up-regulated in nitrogen-poor environments [70].

It has been demonstrated that CodY, a global transcriptional regulator of the proteolytic system in *L. lactis*, controls the expression of a number of genes involved in the uptake and biosynthesis of amino acids and in the regulation of their intracellular pools [71, 72]. It was established that BCAAs Ile, Leu, and Val released from small peptides are effectors of CodY [46, 73–75]. In particular, branched-chain amino acids stimulate the binding of CodY to the so-called CodY-box sequence [75].

The role of other (potential) nitrogen regulatory mechanisms is probably narrower. The expression of several subunits of the proteolytic systems, such as PepF [70] and PepP

[45], has been suggested to be independent of CodY. Transcription of the latter appears to be regulated by the carbon source through a CcpA-like regulator.

An important consideration from a practical standpoint (such as flavor development) is the ability of a particular LAB strain to degrade amino acids. The first step of the catabolism of most amino acids (as well as the last step of their biosynthesis) is catalyzed by aminotransferases. The genome sequence of *L. lactis* IL1403 encodes (at least) 12 aminotransferases [6], two of which, AraT and BcaT, have been purified and characterized [71]. These enzymes catalyze the transfer of the amino group of amino acids to an alpha-ketoacid acceptor, α-ketoglutarate being the preferred cosubstrate [76–78]. The process is often hampered by the limited availability of α-ketoglutarate [79]. A few strains of *L. lactis* are able to synthesize α-ketoglutarate from glutamate *via* the glutamate dehydrogenase (Gdh) reaction. Importantly, *L. lactis* IL1403 lacks the *gdh* gene [6] and has also been experimentally shown to lack Gdh activity [80]. Alternatively, α-ketoglutarate could be synthesized from citrate *via* the pathway that requires citrate permease (CitP), citrate lyase (CitD), and aspartate aminotransferase (AspB), or *via* the pathway requiring aconitase (CitB) and isocitrate dehydrogenase (Icd). The latter pathway is not functional or is functional at a very low level [81], whereas the former is only used by *L. lactis* subsp. *lactis* biovar. *diacetylactis* [79].

2.2 PEPTIDE AND AMINO ACID METABOLISM OF *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, commonly known as baker's yeast, is a unicellular fungus and a classic model organism used extensively in genetics and molecular biology studies. It has been found to occupy a wide variety of environmental niches, including wine, beer, plants, soil, as well as the tissues of birds and mammals. In addition to its traditional applications in food and beverage fermentation, it is also used for the industrial production of various chemicals, including pharmaceuticals.

The genome of *S. cerevisiae* was the first to be completely sequenced from a eukaryotic organism, and contains around 6000 protein-encoding genes [82]. The original genomic sequence and its annotation have been publicly available since 1996 and since then, a large number of changes, corrections, and additions have been incorporated [83]. The reference sequence is that of strain S288c, a common haploid laboratory strain with a long history of use in genetic and molecular biology studies. S288c is a contrived strain with a complex genealogy that has been obtained through numerous crosses. Approximately 88 % of its gene pool originates from strain EM93, which was isolated by Emil Mrak in 1938 from a rotting fig from California's Central Valley.

Like most wild-type yeasts, and in stark contrast with *L. lactis*, *S. cerevisiae* S288c has minimal nutrient demands, only requiring biotin, a source of carbon and nitrogen, as well as some salts and trace elements [84]. Sugars are used as the main carbon and energy source by most yeasts. After glycolysis, respiration and fermentation are the two major pathways that make use of pyruvate for further energy production. A large variety of compounds, for example, ammonia, urea, free amino acids, and small peptides can be used as sources of nitrogen. With some strain variability, all L-amino acids, except lysine, histidine, and cysteine, can be utilized as the sole nitrogen source for growth [85, 86]. These compounds can be used either directly for biosynthetic purposes, for the

generation of ammonium through deamination, or serve as substrates for transaminases that transfer amino groups to α-ketoglutarate to form glutamate, which can then act as a donor of amino groups during the synthesis of most amino acids. The assimilation preferences of peptides in the presence of free amino acids and ammonia in *S. cerevisiae* have remained rather unclear. In this section, an overview of the current knowledge of peptide, amino acid, and ammonia utilization systems of baker's yeast is given.

2.2.1 *Extracellular proteolytic activity*

Unlike many pathogenic yeasts, such as *Candida albicans* and *Candida tropicalis* [87], as well as several non-*Saccharomyces* wine yeasts [88] that have been shown to be producers of extracellular proteinases, reports on the proteinases secreted by *S. cerevisiae* are rather sporadic and somewhat ambiguous. With some noteworthy exceptions, most screens for extracellular proteolytic activity have produced negative results [87, 89]. Bilinski *et al.* [90] found two protease-positive *S. cerevisiae* strains (out of 120) when screening for useful proteases for brewing applications. Sturley and Young [91] isolated an extracellular proteolytic activity-producing laboratory strain of *S. cerevisiae*. In that particular case, the trait was related to aberrant secretion of intracellular proteases. Rosi *et al.* [92] reported that 17 of 94 tested *Saccharomyces* wine strains displayed extracellular proteolytic activity. In that case, it is possible that proteases were released as the yeast autolyzed over the 15-day incubation period. Release of proteases, in particular proteinase A (Pep4p), into beer has also been reported [93–96]. In a more recent study [97], a novel extracellular pepsin-like aspartic acid protease secreted by living, healthy cells of a *S. cerevisiae* strain isolated from Pinot noir grapes was characterized. pH and nutrient-dependent degradation of heterologous proteins has also been reported [98, 99]. The proteases involved were proposed to be cell-bound, rather than secreted into the medium.

2.2.2 *Di- and tripeptide transporters of S. cerevisiae*

Ptr2p is the main transporter responsible for the uptake of di- and tripeptides [100–102]. It belongs to the proton-dependent oligopeptide transporter family [37, 103] and is related to lactococcal DtpT. Ptr2p is an integral membrane protein with twelve trans-membrane domains that functions as a proton symporter. Ito *et al.* [104] characterized the substrate specificity of Ptr2p using a dipeptide library and showed that it displays comparatively higher affinity towards dipeptides containing residues of aromatic (Phe, Trp and Tyr) and branched-chain amino acids (Ile, Leu and Val), as well as Met.

The second transporter responsible for di-/tripeptide uptake is Dal5p. Initially described as an allantoate and ureidosuccinate permease, it was later found to also transport peptides, although with lower affinity [105, 106]. Dal5p belongs to the major facilitator superfamily [107] and seems to be particularly important for growth on peptides containing N-terminal Ser, Gly, and Ala [105, 106]. Homann *et al.* [105] studied the ability of *S. cerevisiae* strains of various origins (lab, vineyard, and clinical isolates) to utilize peptides as well as their sensitivity to a toxic alanyl-ethionine dipeptide. The observed variations

could be attributed to the different activities of Ptr2p and Dal5p within those strains, and the different specificities of these transporters are thus, in a way, complementary.

Notably, Gap1p (general amino acid permease) and not the "regular" di-/tripeptide transporters Ptr2p or Dal5p (or the oligopeptide transporter Opt1p, see below) was shown to transport certain gamma-glutamyl dipeptides in yeast [108]. These dipeptides could be used as a nitrogen source for growth, but their uptake was highest at very low pH values, accompanied by a rapid drop of cytosolic pH.

Release of small peptides (and amino acids) has been demonstrated during fermentative growth (before autolysis) with ammonia as the nitrogen source has been demonstrated [109]. Intracellular proteolysis has been associated with nutritional stress, including nitrogen or glucose starvation. The exact mechanism of peptide exodus is unclear, but the involvement of Ptr2p cannot be ruled out. Studies of the mammalian homologue (Pept1) expressed in *Xenopus* oocytes revealed that the transporter is effectively reversible, producing strong outward currents when a large intracellular concentration of dipeptides is encountered [110, 111]. This property could help cells maintain membrane potential under such conditions. A similar role can be attributed to the lactococcal DtpT.

2.2.3 *Oligopeptide transporters of S. cerevisiae*

In addition to di- and tripeptides, *S. cerevisiae* is also known to be able to utilize oligopeptides with four to five (perhaps more), amino acid residues. The uptake of these peptides is mediated by Opt1p (also known as Gsh11p, Hgt1p) and, possibly, by the closely related Opt2p [102, 112, 113]. These transporters belong to the oligopeptide transporter family and are not to be confused with the Opt system of *L. lactis* which belongs to the ABC transporter superfamily. No ATP-dependent oligopeptide carriers (such as the lactococcal Opp or Opt) have been identified in yeast or other eukaryotes [103]. Recent studies have identified novel oligopeptide transporters Fot1p-Fot2p in certain *S. cerevisiae* wine strains [114] that could confer fitness in an enological niche. Notably, those transporters are believed to be acquired through horizontal gene transfer [115], in a somewhat similar manner to the process of adaptation of *L. lactis* dairy strains. The *FOT* genes are not present in *S. cerevisiae* S288c.

Compared to the di-/tripeptide transporters, the oligopeptide transporters have been studied somewhat less extensively. Whereas homologues of Ptr2p have been identified in almost all known organisms (including DtpT of *L. lactis*), the distribution of oligopeptide transporter family members seems to be more restricted, with eukaryotic members found only in fungi and plants [116].

Opt1p functions as a plasma membrane-embedded proton symporter of tetra- and pentapeptides and has a broad substrate specificity [112, 117]. Its expression was shown to be dependent on the sulfur content of the medium [113, 118]. An additional, and perhaps a more important role of Opt1p seems to be the uptake of glutathione and its conjugates [119]. Opt2p, on the other hand, seems to be predominantly localized in the membranes of peroxisomes and its major function seems to be the maintenance of glutathione redox homeostasis and drug detoxification [120, 121]. The physiological role of the these proteins is, thus, multifaceted and dependent on particular growth conditions. It seems,

however, that oligopeptide transport might be a coincidental role rather than the primary one. Recent investigations have shed light on the mechanistic workings of Opt1p [122, 123].

2.2.4 *Intracellular peptide degradation*

Due to the compartmentalized nature of eukaryotic cells, pinpointing the exact location of cellular events, including peptidolysis, poses a challenge and, compared to *L. lactis*, the fate of exogenously supplied peptides in yeast is somewhat less clear.

A major proteolytic complex in the cytosol of eukaryotic cells is the proteasome that is mainly involved in the regulation of the concentration of particular proteins and the degradation of misfolded proteins through ubiquitination. Small exogenously supplied peptides are not suitable substrates because the minimal length of the C-terminal tail of ubiquitin directed to proteasome seems to be around 20 amino acid residues [124, 125]. Degradation of polypeptides in the proteasome results in the generation of peptides with approximately 3 to 23 amino acid residues, with an average length of either 7 or 8 amino acids [126]. Obviously, other peptidases in the cytosol or vacuoles are responsible for further lysis of these degradation products as well as exogenously supplied peptides.

The vacuole with its array of peptidases is usually considered to be the primary site of non-specific proteolysis in yeast cells [127, 128]. The yeast vacuole contains seven well-characterized proteases: Pep4p aspartyl endoprotease, Prb1p serine endoprotease, Prc1p serine carboxypeptidase, Cps1p Zn metallo-carboxypeptidase, Ape1p Zn metallo-aminopeptidase, Ape3p metalloprotease, and Dap2p serine protease. While autophagy has been suggested to be the main mechanism of bulk translocation of protein material from the cytosol into the vacuole [129], particularly under conditions of nutrient stress, the uptake routes of small peptides are not fully understood. Considering the fact that more than a fourth of all proteins with vacuolar localization function as transporters [130], it is likely that some of those have peptide transport capabilities. Cai *et al.* [131], for example, provided evidence that the ABC transporter Ybt1p may facilitate the translocation of peptides from the cytosol into the vacuole. Failure of Ybt1p mutant to do so resulted in increased concentrations of exogenously supplied toxic peptides in the cytosol.

The role of cytosolic peptidases is rather unclear and has received considerably less attention over the years. Cai *et al.* [131] showed that mutations in a cytosolic zinc metalloendopeptidase Prd1p [132] results in a decreased ability to utilize a dipeptide (His-Leu) for growth. Similarly, the Ape2p aminopeptidase seems to be involved in the cellular supply of leucine from leucine-containing dipeptides [133]. It is possible that most exogenously supplied di- and tripeptides are broken down in the cytosol and never reach the vacuoles.

2.2.5 *Amino acid and ammonia transport in S. cerevisiae*

S. cerevisiae encodes an extensive array of amino acid transporters with varying specificities. The compartmentalized architecture of yeast cells means that, in addition to the

plasma membrane transporters, numerous transport proteins are localized at the membranes or intracellular organelles, particularly those of vacuoles and mitochondria.

All known amino acid import systems that function at the plasma membrane are proton symporters [134] and most of these belong to the yeast amino acid transporter family of the amino acid-polyamine-organocation superfamily. The substrate specificities of these proteins vary widely, from all L-amino acids (and some D-amino acids, as well as β-alanine, polyamines, GABA, and some peptides) in the case of Gap1p (K_m~4–1000 μM for various substrates) to essentially a single amino acid in case of, *e.g.*, arginine for Can1p (K_m~10 μM) or lysine for Lyp1p (K_m~25 μM), and a number of permeases that transport several amino acids. Individual amino acids are, thus, transported by several different systems that function redundantly [135], albeit over a range of kinetic specificities (K_m~10–1000 μM). This, together with differential regulation of those systems (discussed below) allows the cells to adapt to a variety of environments. Notably, in contrast to *L. lactis*, no plasma membrane amino acid import systems belong to the ABC transporter superfamily.

In addition to amino acids, there are import systems for various other nitrogenous compounds. Of particular interest are the ammonium permeases Mep1p, its paralog Mep3p, and Mep2p which belong to the ammonia channel transporter family. Mep2p displays the highest affinity for NH_4^+ (K_m~1.4–2.1 μM), followed by Mep1p (K_m~5–10 μM) and Mep3p, whose affinity is much lower (K_m~1.4–2.1 mM) [136]. It was later shown that the transporters also differ in their transport mechanisms and optimal operating pH [137].

The excretion of amino acids from living cells has been documented for yeast grown in defined media under stress conditions, for example, upon entry into early stationary phase [109] and under potassium limitation [138]. Importantly, the most readily excreted amino acids (Ala, Val, Pro, Glu, and Gln) were the same in both studies, which implies the involvement of similar cellular response mechanisms. The physiological causes, however, are probably different from those of the similar phenomenon observed in the case *L. lactis*. The exact mechanisms of amino acid efflux in those studies are unclear, although the phenomenon has been studied extensively in some bacteria [139]. The only known transporter with amino acid export capabilities in yeast is the multidrug resistance protein Aqr1p that functions as a proton antiporter [140]. A similar role has also been implicated for Gap1p under certain conditions [141]. In general, excretion of amino acids seems to be a much less common phenomenon in yeast compared to either *E. coli* or *L. lactis* and underlines an important difference in how these organisms deal with excess amino acids. Exodus of carbon skeletons following deamination as well as an ability to store large amounts of amino acids in the vacuole have been proposed as explanations of these differences [142, 143]. Several permeases have been characterized that catalyze the transport of amino acids across the vacuolar membrane by either proton antiport (into the vacuole) or proton symport (out of the vacuole); the basic amino acids (His, Arg, and Lys) are sequestered in the vacuole, whereas the acidic amino acids (Asp and Glu) are excluded [144].

2.2.6 *Regulation of amino acid metabolism in S. cerevisiae*

Yeast has evolved an intricate regulatory system that responds to the availability and quality of nitrogen sources in the environment. It is significantly more complex than its lactococcal counterpart and cellular response to specific environmental conditions results from the integration of signals from multiple pathways (which can be either inductive or repressive). These pathways, as well as the relevant nutrient sensors, have been covered in detail in excellent reviews [145–148].

S. cerevisiae preferentially uses nitrogen sources that allow for more rapid growth through a general regulatory mechanism termed nitrogen catabolite repression (NCR). This suprapathway mechanism functions to ensure the preferential utilization of "preferred" nitrogen sources when they are available, and, conversely, the indiscriminate use of "non-preferred" sources in the absence of a "preferred" nitrogen source [149]. Amino acid permeases with broad substrate specificities, Gap1p and Agp1p, as well as proline permease Put4p, arginine permease Can1p, dicarboxylic amino acid permease Dip5p, and the ammonium permeases Mep1p, Mep2p, and Mep3p, are all under NCR control. Additionally, the peptide transporters Ptr2p, Dal5p, as well as Opt1p and Opt2p appear to be targets of NCR [147, 150, 151]. It has also been shown that several peptidases in yeast are under NCR control [152–154], although carbon source and phase of growth are also major influencers of cellular peptidase activity [109, 155]. In particular, the activity of peptidases increases dramatically as cells enter stationary phase.

Another mechanism involving the Ssy1p-Ptr3p-Ssy5p (SPS) sensor [156–158] works by sensing the ratio of intra- and extracellular amino acids and regulates the expression of several amino acid permeases, thereby enabling amino acids to induce their own uptake. In particular, Agp1p, Bap2p, Bap3p, Gnp1p, Tat1p, and Tat2p are controlled by the SPS sensor [147]. Importantly, *PTR2* [159] as well as *OPT1* [113] were found to be upregulated by the presence of amino acids in the growth medium, with Leu and Trp being particularly potent inducers. This regulatory mechanism is probably tailored to sense the (likely) presence of peptides in the environment through their breakdown products, that is, free amino acids. It was later shown that tryptophan induces *PTR2 via* the SPS system [160].

In addition to SPS regulation, the expression of *PTR2* is positively regulated by dipeptides with basic (Arg, His, or Lys) and bulky hydrophobic (Ile, Leu, Phe, Trp, or Tyr) N-terminal residues. These peptides bind to Ubr1p to allosterically activate Ubr1p-mediated degradation of the *PTR2* repressor Cup9p [161]. This generates a positive feedback loop whereby peptides accelerate their own uptake, in stark contrast with the regulatory mechanisms in *L. lactis*, where peptides that contain BCAA residues repress the expression of peptide uptake systems. Apparently, the lifetime of such peptides in the cytosol is long enough to bind to Ubr1p. Similarly to *PTR2*, the expression of *OPT2* is also down-regulated by the Cup9p, while the expression of *OPT1* seems to be independent of Cup9p [113]. *DAL5*, on the other hand, seems to be up-regulated by Cup9p [106]. A genome-wide screen performed by Cai *et al.* [131] revealed several other potential regulatory mechanisms of di- and tripeptide utilization in yeast.

In short, peptide and amino acid uptake capacity results from the integration of a multitude of signals (repressive or inductive), whereby the cells (optimally) adapt to the environmental conditions.

2.3 ^{15}N TRACER ANALYSIS

Optimization of cellular processes and growth conditions requires an in-depth understanding of the reactions that support both growth and product formation. Metabolic flux analysis is an integrated approach of experimental and computational techniques for comprehensive and quantitative understanding of biochemical reaction networks with a particular relevance in metabolic engineering and systems biology [162, 163]. In its simplest form, metabolic flux analysis is based solely on a stoichiometric reaction model and substrate consumption and product secretion rates. However, when it comes to networks containing two (or more) pathways that lead to the same metabolite or contain futile cycles, this simple approach is often not adequate. More elaborate methods for the analysis of the biochemical networks that operate within cells are based on the use of stable isotope-labeled substrates. This is carried out by feeding ^{13}C- or, less often, ^{15}N-labeled substrates to the cells followed by the analysis of culture samples using either nuclear magnetic resonance (NMR) or mass spectrometry (MS) which provides information about the labeling patterns of both the metabolic intermediates and the end products of metabolism. Interpretation of these labeling patterns can be used to quantify intracellular metabolic fluxes, reveal the compartmentation of metabolic reactions and determine the relative nutrient contributions to the production of a particular metabolite.

To simplify metabolic flux analysis, experiments are typically conducted at metabolic steady state and the culture samples are collected and analyzed after the end products have reached an isotopic steady state, that is, the labeling of the substrate and measured metabolite pools have reached equilibrium. However, nonstationary analysis provides more information and is useful for studying industrially relevant cultivation techniques such as batch and fed-batch that never reach an isotopic steady state [164], or biological systems that do not reach it in a reasonable time such as many plant systems. For example, in labeling experiments involving mammalian (and often bacterial) cells, exchange of metabolites (including amino acids) with the growth media delays the intracellular isotopic steady state usually beyond the possibility of maintaining metabolic steady state [165]. A rigorous framework for non-stationary metabolic flux analysis has been established [166]. Implementing this approach in practice is, however, both time and data intensive and more streamlined approaches are sometimes utilized [167, 168]. In many cases, direct interpretation of labeling patterns without formal flux analysis can be sufficient to provide information on relative pathway activities or nutrient contributions to the production of metabolites [169].

Regardless of the chosen data analysis approach, one of the prerequisites is the accurate measurement of labeling patterns within the targeted metabolites. The labeling patterns of metabolites can be analyzed by NMR or MS methods. A major advantage of MS-based approaches is that a comparatively small amount of sample is required, which results in easy handling, cost-efficiency, and provides the possibility to study metabolic fluxes in very small reactors [170]. An important step in the workflow of the analysis of ^{13}C and ^{15}N labeling patterns of metabolites is considering the presence of naturally occurring isotopes in the analytes. This is equally important in both tracer studies and when labeled compounds are used as internal standards. Various approaches have been proposed to tackle this issue [171–173], however, there is no agreement about which

correction method should be used and different correction algorithms provide different corrected values [174].

Although ^{13}C is by far the most widely used isotope in metabolic labeling studies (since all bioorganic molecules contain carbon atoms and carbon transfers in biochemical reactions follow well-documented rules), ^{15}N (as well as ^{2}H and ^{18}O) can often be an attractive alternative, in particular with regards to cost [175]. The obvious downside of labeling with ^{15}N is that its use is restricted to nitrogen-containing compounds. One clear advantage, however, is that it can be used in organisms that are able to utilize inorganic nitrogen sources (*e.g.*, ammonia), such as yeasts and plants [176, 177]. ^{15}N-labeling was elegantly utilized already in the 1960s in the classic studies of yeast nitrogen metabolism that highlighted the central role of glutamate and glutamine and shed light on the extensive transamination network that operates within cells [178, 179]. With the increasing resolution of mass-spectrometers, analysis of the cometabolism of carbon and nitrogen is possible by feeding the cells ^{13}C- and ^{15}N-labeled substrates simultaneously [180]. Although the application of stable isotope labeling in (semi-) rich media is rather rare, such experiments can provide valuable insight into the role of various nutrients under industrially relevant cultivation conditions [181–183]. Such approaches will be addressed in the following section.

2.4 APPLICATIONS OF LABELED YEAST HYDROLYSATES

Yeast hydrolysates are extensively used in biotechnology as media supplements. Isotopically labeled yeast hydrolysates have found use as cost-effective components in culture media, for example, within labeled protein expression systems based on insect and mammalian cell lines [184–186] and even for multicellular organisms, such as *Drosophila melanogaster* [187, 188]. *E. coli* and algal (including *Spirulina*) extracts have also been used in similar applications.

Because yeast hydrolysates can provide undefined building blocks for both biomass and product synthesis, it is difficult to precisely calculate the intrinsic product yields in rich-medium fermentations. To overcome this problem, ^{13}C- (or ^{15}N-) analysis can provide insights into the carbon (nitrogen) contribution from yeast hydrolysates to product biosynthesis [189]. This approach has been used to elucidate the role of yeast extract in isobutanol fermentations with heterologous *E. coli* [182, 183].

Labeled yeast extracts can also be used as internal standards in mass-spectrometry, which is the central analytical tool within a high-throughput metabolomics workflow that includes sampling, quenching, extraction, and analysis [190].

2.5 THE ROLE OF NUCLEOTIDES IN YEAST HYDROLYSATES

In this work we focus on elucidating the roles of peptides and free amino acids in the growth of *L. lactis* and *S. cerevisiae*. However, nucleotides also play a central part in nitrogen metabolism by either participating directly in the metabolic reactions or passively as regulatory species. Because yeast hydrolysates generally contain significant amounts

of nucleotides and related compounds [191], further research regarding their roles in the growth of microbes is warranted.

Yeast can synthesize nucleotides *de novo*, whereas certain lactic acid bacteria require an external source of the respective precursors [192]. As an alternative to biosynthesis, most organisms are capable of exploiting exogenous nucleotide sources. To accomplish this, the nucleotide precursors must pass the cell membrane, which requires cellular transporters. Normally, phosphorylated compounds are not subject to transport, and thus the utilization of nucleotides is dependent on the activity of extracellular phosphatases. Extracellular phosphatase activities have been reported for both lactic acid bacteria [193] and yeasts [194].

While organisms such as yeast or *E. coli* can utilize exogenous nucleotides as a carbon and energy source, lactic acid bacteria cannot [192]. The amino group in both cytidine and adenosine can be utilized as a nitrogen source and many lactic acid bacteria have the respective deaminases [195]. Nevertheless, the main function of nucleotide salvage pathways in lactic acid bacteria seems to be to the utilization of nucleobases and nucleosides for nucleotide synthesis. In any case, addition of certain nucleotides to the growth medium can result in increased bacterial growth rates [196]. In a potential follow-up study, the protocol presented in this work could be applied to elucidate aspects of nucleotide metabolism. Ultimately, a more rigorous approach using mass and energy balances and possibly dynamic simulation of the metabolic reactions should be conducted in future studies where yeast extracts are used.

3

AIMS OF THIS DISSERTATION

The general aim of the work presented in this dissertation is to gain insight into the amino acid and peptide metabolism of *Lactococcus lactis* and *Saccharomyces cerevisiae* using an integrated approach involving the utilization of ^{15}N-labeled yeast hydrolysate. Specifically, the aims are as follows:

I Establish a protocol for the production of uniformly ^{15}N-labeled yeast hydrolysate of consistent and high quality for metabolic labeling experiments and analytical purposes.

II Measure the consumption patterns of free and peptide-bound amino acids by *L. lactis* grown on a medium containing ^{15}N-labeled yeast hydrolysate and to gain insight into the role of peptides as a source of amino acids.

III Determine the role of peptides as a nitrogen source under conditions of fermentative growth of *S. cerevisiae*.

4

MATERIALS AND METHODS

Detailed descriptions of the materials and methods applied are available in the publications. The following sections are intended to make the material more accessible.

4.1 STRAINS, INOCULA, AND CULTURE MEDIA

Lactococcus lactis subsp. *lactis* strain IL1403 and *Saccharomyces cerevisiae* strain S288c were used in the experiments. Inocula were prepared using freeze-dried (*L. lactis*) and glycerol (*S. cerevisiae*) stock cultures stored at -80 ℃ which were pre-grown twice on chemically defined base media.

4.1.1 *Culture medium for L. lactis*

A chemically defined medium based on the GIBCO™ F-12 Nutrient Mixture (Invitrogen Corporation, Carlsbad, CA) with some modifications [197] was used as a base medium which was supplemented with 2 $g \cdot L^{-1}$ of ^{15}N-labeled yeast hydrolysate. The concentration of yeast hydrolysate was chosen in order to achieve close-to-equal molar concentrations of peptide-bound and free amino acids in the culture medium. Composition of the base medium was as follows ($mg \cdot L^{-1}$): D-glucose – 3500; biotin – 0.305; choline chloride – 9.8; D-pantothenate – 0.65; folic acid – 1.21; niacinamide – 0.325; pyridoxine hydrochloride – 0.642; riboflavin – 0.326; thiamine hydrochloride – 0.51; vitamin B_{12} – 0.98; myo-inositol – 12.6; $CaCl_2$ – 28; $CuSO_4 \cdot 5H_2O$ – 0.272; $FeSO_4 \cdot 7H_2O$ – 0.71; $MgCl_2$ – 58; KCl – 157; NaCl – 5580; Na_3PO_4 – 99; $ZnSO_4 \cdot 7H_2O$ - 1; hypoxanthine-Na – 3; linoleic acid – 0.1; lipoic acid – 0.1; phenol red – 0.8; putrescine·2HCl – 0.1; Na-pyruvate – 77; thymidine – 0.5. The amino acid concentrations in the medium are provided in Table 1.

Table 1. Amino acid composition of the medium used in the experiments with *L. lactis*.

Amino acid	Base medium [197] (μM)	Yeast hydrolysate free[1] (μM)	Yeast hydrolysate bound[1] (μM)
Ala	875	373	1383
Arg	1062	113	479
Asn	560	62	1026[2]
Asp	541	58	
Cys	264	28	N/D[3]
Gln	903	54	1386[2]
Glu	476	201	
Gly	773	103	992
His	387	23	342
Ile	778	68	545
Leu	1578	148	937
Lys	1081	101	819
Met	275	27	138
Phe	521	57	354
Pro	799	59	611
Ser	1551	96	887
Thr	638	65	643
Trp	78	5	N/D[3]
Tyr	160	32	159
Val	913	124	787

[1] Calculated based on the concentration of added yeast hydrolysate (2 $g \cdot L^{-1}$)
[2] Asp + Asn, Glu + Gln
[3] Not determined due to degradation during analysis

4.1.2 *Culture medium for S. cerevisiae*

A chemically defined medium based on synthetic grape juice medium described by Jiranek *et al.* [198], was used as a base medium after diluting by fourfold. The main fermentation experiments were carried out on the same medium supplemented with ammonium chloride and yeast hydrolysate (2 $g \cdot L^{-1}$). The composition of the base medium was as follows ($g \cdot L^{-1}$): D-glucose – 50; NH_4Cl – 0.750 or $^{15}NH_4Cl$ – 0.764; K-tartarate – 1.25; L-malic acid – 0.75; citric acid – 0.05; KH_2PO_4 – 0.285; $MgSO_4 \cdot 7H_2O$ – 0.308; $CaCl_2 \cdot H_2O$ – 0.11. The medium also contained the following trace elements and vitamins ($mg \cdot L^{-1}$): $MnCl_2 \cdot 4H_2O$ – 0.1982; $ZnCl_2$ – 0.1355; $FeCl_2$ – 0.032; $CuCl_2$ – 0.0136; H_3BO_3 – 0.0057; $Co(NO_3)_2 \cdot 6H_2O$ – 0.0291; $NaMoO_4 \cdot 2H_2O$ – 0.0242; KIO_3 – 0.0108; myo-inositol – 100; pyridoxine·HCl – 2; nicotinic acid – 2; Ca-pantothenate – 1; thiamin·HCl – 0.5; p-amino benzoic acid – 0.2; riboflavin – 0.2; biotin – 0.125; folic acid – 0.2. The amino acid concentrations in the medium are provided in Table 2.

Table 2. Amino acid composition of the medium used in the experiments with *S. cerevisiae*.

Amino acid	Base medium (μM)	Yeast hydrolysate free[1] (μM)	Yeast hydrolysate bound[1] (μM)
Ala	281	835	733
Arg	1078	66	298
Asn	250	40	786[2]
Asp	658	11	
Cys	103	21	N/D[3]
Gln	340	20	1129[2]
Glu	850	314	
Gly	167	82	818
His	155	24	195
Ile	382	26	355
Leu	573	64	577
Lys	343	46	501
Met	252	20	136
Phe	227	32	274
Pro	1087	26	389
Ser	952	54	554
Thr	735	45	478
Trp	123	8	N/D[3]
Tyr	28	25	219
Val	427	75	538

[1] Calculated based on the concentration of added yeast hydrolysate (2 $g \cdot L^{-1}$)
[2] Asp + Asn, Glu + Gln
[3] Not determined due to degradation during analysis

4.2 REACTOR SET-UP, CULTIVATION CONDITIONS, AND SAMPLING ROUTINES

All cultivation experiments were carried out in 1 L Biobundle bioreactors (Applikon, Schiedam, the Netherlands) controlled by Applikon EZ-control bio-controllers together with BioXpert XP cultivation software (Applikon). The reactors were equipped with pH, temperature, and gas control.

Batch cultivations with *L. lactis* were carried out under anaerobic conditions (100 $mL \cdot min^{-1}$ N_2 blanketing) at 34 ℃ and pH 6.4 (maintained by titration with 1 M NaOH) at an agitation speed of 300 rpm. Prior to inoculation with 1 vol% of washed overnight culture, the medium was sparged with N_2.

Batch cultivations with *S. cerevisiae* were carried out under micro-aerobic conditions (surface aeration with 100 $mL \cdot min^{-1}$ air) at 30 ℃ and pH 4.0 (maintained by titration with 2 M NaOH) at an agitation speed of 500 rpm. The cultures were inoculated with 1 vol% of washed overnight culture.

Culture samples were withdrawn at 1 h intervals and collected on ice. The samples were centrifuged (20,000·g, 4 min, 4 ℃) and their supernatants were collected and stored at -20 ℃ until analysis. The biomass pellet was washed twice using ice cold 0.9 % NaCl solution and stored at -20 ℃ until analysis.

4.3 PRODUCTION OF ^{15}N-LABELED YEAST HYDROLYSATE

The ^{15}N-labeled yeast hydrolysate used in the experiments was produced by means of controlled proteolysis from *S. cerevisiae* S288c biomass grown in glucose-limited fed-batch on a minimal medium containing biotin based on the formulation described by Nisamedtinov *et al.* [199] with ^{15}N-labeled (98 %) ammonium chloride (Sigma, USA) as the nitrogen source. The yeast biomass was freeze-dried and hydrolyzed using a Promod 144GL protease preparation (Biocatalysts, Cardiff, UK). The extract was separated from the insoluble fraction by centrifugation. To remove any residual protease activity, the soluble fraction was further purified using Amicon Ultra 15 centrifugal filter devices (Millipore Corp., MA) with a 10,000 NMWL cut-off (by centrifugation for 45 min at 4000·g). The resulting hydrolysate was freeze-dried. The lack of proteinase activity was confirmed using a Calbiochem Protease Assay Kit (Millipore Corp., USA).

4.4 ANALYTICAL METHODS

4.4.1 *Biomass concentration and metabolites*

The concentration of biomass in the reactor was determined on the basis of the optical density of the culture medium (λ = 600 nm) and related to the dry weight of the biomass (determined gravimetrically; biomass conversion factors K_{Lla} = 0.372±0.005 and K_{Sce} = 0.290±0.005 g dwt·L^{-1}·AU$_{600}^{-1}$ for *L. lactis* and *S. cerevisiae*, respectively).

The concentration of glucose, lactate, formate, acetate, glycerol, and ethanol in the culture media was determined in the culture supernatants using a liquid chromatograph (Alliance 2795 system, Waters Corp., Milford, MA) equipped with a BioRad HPX-87H column (Hercules, CA); isocratic elution was applied using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL·min^{-1} at 35 ℃. A refractive index detector (model 2414; Waters Corp.) was used for detection and quantification.

Amino acid concentrations in the biomass and culture medium and the respective mass spectra were determined using an LCT Premier LC-MS system (Waters Corp.). Samples were derivatised with AccQ-Fluor reagent and loaded on AccQ-Tag™ Ultra columns (2.1×100 mm) at 55 ℃ (flow rate 0.3 mL·min^{-1}) connected to a PDA detector (λ = 260 nm) and a mass-spectrometer. Amino acids were separated using a 20 min gradient from 0.1 to 59.6 % B (A: AccQTag Ultra eluent A, B: AccQTag Ultra eluent B; Waters Corp.), The mass-spectrometer was operated in positive ionization mode at 300 ℃ solvation temperature, 120 ℃ source temperature and 2.5 kV capillary voltage. Full mass spectra were acquired over the range of 100–1000 m/z. MassLynx V 4.1 software (Waters Corp.) was used for data processing.

4.4.2 *^{15}N-labeling patterns of amino acids*

To determine the contribution of different nitrogen sources to the accumulation of amino acids in the cells, analysis of the fractional labeling of the amino acids in the cells and the culture media was carried out. The labeling patterns of amino acids (or any metabolite) are represented by mass distribution vectors (MDVs) that describe the fractional abundance of each isotopologue normalized to the sum of all possible isotopologues [170]. MDVs are obtained from the measured fractional abundances of the measured metabolite ions by correcting for naturally occurring isotopes using a correction matrix as described below. No positional labeling information (resolving the isotopomers) was obtained in the present work.

A linear system for an amino acid with n nitrogen atoms that are subject to isotope labeling (*i. e.*, excluding the nitrogen atoms of the derivatization reagent) can be formulated as follows:

$$\begin{bmatrix} i_0 \\ i_1 \\ \vdots \\ i_n \\ \vdots \\ i_{n+k} \end{bmatrix} = \begin{bmatrix} c_{00} & c_{01} & \cdots & c_{0n} \\ c_{10} & c_{11} & \cdots & c_{1n} \\ \vdots & \vdots & & \vdots \\ c_{n0} & c_{n1} & \cdots & c_{nn} \\ \vdots & \vdots & & \vdots \\ c_{(n+k)0} & c_{(n+k)1} & \cdots & c_{(n+k)n} \end{bmatrix} \begin{bmatrix} m_0 \\ m_1 \\ \vdots \\ m_n \end{bmatrix}. \tag{4.1}$$

$\mathbf{i} = [i_0 \dots i_{n+k}]^T$ is the vector containing normalized ion counts (fractional abundances of the measured ions). $\mathbf{C} = [c_{ij}] \in \mathbb{R}^{(n+k)\times n}$ is the correction matrix. The columns of the correction matrix are populated with the theoretical isotopic distributions of the ions of all possible nitrogen labeling states (*i. e.*, when 0 to n nitrogen atoms are ^{15}N) of the derivatised amino acids. These were calculated using the algorithm described by Rockwood and Van Orden [200] using the following natural isotope abundances: ^{2}H (0.0156 atom %), ^{13}C (1.082 atom %), ^{15}N (0.366 atom %), ^{17}O (0.038 atom %), ^{18}O (0.204 atom %), ^{33}S (0.749 atom %), ^{34}S (4.197 atom %), and ^{36}S (0.015 atom %) [164]. $\mathbf{m} = [m_0 \dots m_n]^T$ is the mass distribution vector after correcting for naturally occurring isotopes. The MDVs $\mathbf{m}$ of all amino acids were obtained by solving the system (Eq. 4.1) using a least squares fit with the additional constraint $\sum_j m_j = 1$ (the sum of their elements equals one). k denotes additional measured ion abundances beyond n (here, k=2).

The developed mass spectrometry method was validated for its accuracy to measure isotopologue distributions by measuring the mass spectra of the amino acids in both the unlabeled and ^{15}N-labeled yeast extracts used in the experiments.

To determine the fraction of nitrogen within each amino acid nitrogen that originated from labeled nutrients, the MDV of an ion cluster is converted into a summed fractional labeling:

$$\varphi = \sum_{j=0}^{n} m_j \cdot \frac{j}{n}, \tag{4.2}$$

where n is the number of nitrogen atoms in the amino acid (without the derivatization group), j denotes the isotopologues, m_j is the relative abundance of the ion species with an m/z value of $M+1+j$.

Interpretation of these results and their application in metabolic models is discussed in detail within the publications.

5

RESULTS AND DISCUSSION

A summary of the major results from Publication I, II, and III is provided below and some additional findings are discussed in the following sections.

One of the underpinnings of the approach used in this research is the use of uniformly ^{15}N-labeled yeast hydrolysate of consistently high quality. The *in vivo* synthesis of U-^{13}C metabolites using microorganisms cultivated on U-^{13}C substrates for use as multifunctional internal standards is well established [190, 201]. An alternative, albeit much less commonly used strategy is U-^{15}N-labeled biomass for the same applications. The latter has the obvious drawback of being useful only for nitrogen-containing compounds. However, the cost of substrate for lab-scale production of U-^{15}N-labeled *Saccharomyces cerevisiae* biomass is an order of magnitude lower than that of U-^{13}C-labeled biomass ($^{15}NH_4Cl$ and of U-^{13}C-labeled glucose, respectively).

In this work a protocol for the production of U-^{15}N-labeled yeast hydrolysate from the biomass of *S. cerevisiae* S288c laboratory strain was established (see Materials and Methods for details).

The hydrolysate was used as an internal standard for the quantification of B-group vitamers using mass-spectrometry (Publication II), as well as a complex substrate in fermentation experiments with *Lactococcus lactis* and *Saccharomyces cerevisiae* with the aim of gaining insight into the roles of peptides as a source of amino acids in these organisms (Publication I, Publication III).

5.1 UPTAKE AND ACCUMULATION OF B-GROUP VITAMERS IN *Saccharomyces cerevisiae* IN ETHANOL-STAT FED-BATCH CULTURE (PUBLICATION II)

Baker's and brewer's yeasts are well known for their high content of B-group vitamins and are often used as nutritional supplements. B vitamins exist as sets of structurally related chemical compounds termed vitamers. Differentiation between these forms is crucial because vitamers exhibit different bioavailability and stability [202, 203]. Moreover, the absolute and relative concentrations of vitamers and their biosynthetic intermediates reflect the metabolic and physiological state of the cells and tissues, potentially having implications in human longevity and health [204, 205]. Thus, quantification of the so-called vitamin metabolome is becoming increasingly important. For this purpose, rapid and reliable analytical methods are required.

5.1.1 *Utilization of ^{15}N-labeled yeast extract for the characterization of B-group vitamin accumulation in the fed-batch culture of S. cerevisiae reveals dynamic changes in individual vitamer levels*

In the present work, the accumulation of thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine vitamers in *S. cerevisiae* grown in fed-batch was studied. A switch to a vitamin-enriched feeding medium during cultivation was applied and the concentrations of vitamers in the cells and the culture medium were determined by LC-MS combined with a stable isotope dilution assay [206, 207]. The method relies on isotope labeled internal standards, but commercial availability of certain pure isotope labeled vitamers is limited (unavailable or prohibitively expensive). When available, commercial labeled vitamers were used. For the quantification of several vitamers, including nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), and nicotinamide adenine dinucleotide (NAD^+), we made use of ^{15}N-labeled yeast extract. The concentration of labeled vitamers in the extract was determined by LC-MS using unlabeled internal standards. We then followed the dynamic changes in the intra- and extracellular B_3 (and other B) vitamer levels during vitamin feeding in the fed-batch fermentation.

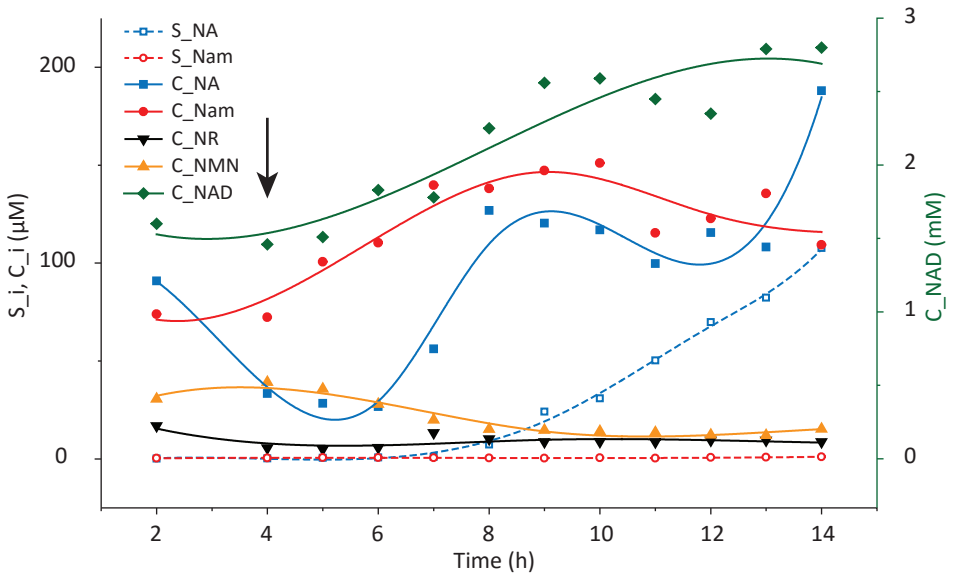

Figure 1. Accumulation of vitamin B_3 in *S. cerevisiae* 210NG during fed-batch cultivation with a switch to a nicotinamide-enriched feeding medium. S_{NA}, S_{Nam} – concentration of nicotinic acid and nicotinamide in the culture medium, respectively (µM); C_{NA}, C_{Nam}, C_{NR}, C_{NMN} – concentration of nicotinic acid, nicotinamide, nicotinamide riboside, and nicotinamide mononucleotide in the cells, respectively (µM); C_{NAD} – concentration of NAD^+ in the cells (mM); the *arrow* denotes the switch to Nam-enriched medium.

NAD^+ is an essential cofactor in both cellular redox reactions and energy metabolism, the *de novo* synthesis of which in yeast can occur from tryptophan *via* kynurenine or *via*

the NAD^+ salvage pathway from nicotinamide (Nam), nicotinic acid (NA), or nicotinamide riboside (NR) [208]. It must be pointed out that the cellular concentration of B_3 vitamers varies from ~1 µM to ~1 mM and every vitamer can be converted to one or more other metabolites. Thus, assessment of the levels of a single B_3 vitamer without a quantitative measurement of the entire NAD^+ metabolome can be misleading.

The cultivation was started on a medium containing 80 µM Nam and later (from 4 h onwards) switched to one that contained 30 times more Nam. During the cultivation, all exogenously supplied Nam was taken up by the cells. Vitamin feeding was accompanied by a prompt increase in intracellular NAD^+ and Nam concentrations and, later, excretion of NA from the cells (Figure 1). Elevated Nam concentrations in the cells under glucose-limited conditions have been associated with accelerated cell aging [204, 209]. This suggest that following uptake, to mitigate its potentially harmful effects, excess Nam is converted into NA *via* the nicotinamidase (Pnc1p) reaction. These results also agree with the findings of Belenky *et al.* [210] who found that Nam taken up by cells was converted to NA and then excreted from the cells. Alternatively, and possibly simultaneously, the elevated levels of NAD^+ could counter the potentially inhibitory effects of Nam.

The ability to quantitatively and reliably monitor the intra- and extracellular vitamer levels is, thus, crucial for understanding physiological responses in the cells to environmental changes. The ^{15}N-labeled yeast extract was found to be a suitable source of internal standards for the MS-based determination of NAD^+, NMN, and NR. The development of methods for the determination of flavin adenine dinucleotide, flavin adenine mononucleotide, pantotheine, coenzyme A, and thiamine pyrophosphate using internal standards form labeled yeast extracts is ongoing. The main problems to be overcome are the relatively low concentrations of certain vitamers in the (labeled) extracts and associated sensitivity issues, as well as low sensitivity of MS detection for coenzyme A.

5.2 UTILIZATION OF ^{15}N-LABELLED YEAST HYDROLYSATE IN *Lactococcus lactis* IL1403 CULTURE INDICATES CO-CONSUMPTION OF PEPTIDE-BOUND AND FREE AMINO ACIDS WITH SIMULTANEOUS EFFLUX OF FREE AMINO ACIDS (PUBLICATION I)

Most LAB are fastidious organisms that require an external source of amino acids and other nutrients. The supply of amino acids is facilitated by both a range of enzymes that degrade proteins and peptides, as well as transport systems for free amino acids and peptides. The uptake and subsequent hydrolysis of peptides is believed to be energetically more favorable than the uptake of individual amino acids *via* their dedicated transport systems [54]. However, the cells' capacity to transport peptides is limited and different peptides compete for the same peptide transport systems.

In this work, *Lactococcus lactis* subsp. *lactis* IL1403 was grown in medium containing unlabeled free amino acids and ^{15}N-labeled yeast hydrolysate to gain insight into the role of peptides as an amino acid source under conditions with an abundance of free amino acids (Figure 2; see also Figure 1 in Publication I). A mathematical model was set up to estimate the fluxes of free and peptide-derived amino acids into and out of the intracellular amino acid pool. The model relies on the following assumptions: (i) all amino acids used for growth are obtained from the culture medium, *i. e.*, no (significant) *de novo* synthesis occurs; (ii) no extracellular hydrolysis of peptides occurs during the experiment; (iii) amino acid turnover and interconversion can be considered negligible.

5.2.1 *Peptides and amino acids are used in parallel by L. lactis*

First, we analyzed the fractional labeling of the amino acids both in the culture medium and in the biomass during growth (Figure 3 in Publication I). Next, we used this labeling data to investigate the relative incorporation of free and peptide-bound amino acids into the biomass during growth. Co-consumption of free and peptide-bound forms occurs for all amino acids, probably in order to optimally utilize the available cellular transport (and biosynthetic) systems, and no obvious differences were observed between amino acids that are considered essential (Leu, Ile, Val, Met, His, and Arg) and non-essential for growth (Figure 4 in Publication I). These results suggested that the incorporation rate of a particular amino acid into biomass is dependent on its availability in a readily assimilated form. Nevertheless, significant differences were observed in the consumption profiles of several amino acids, however, this was not found to correlate with amino acid auxotrophies of the IL1403 strain.

Glutamine and glutamate (Glx), as well as asparagine and aspartate (Asx)[1], for example, exhibited a consistently low incorporation of peptide-bound forms. Analysis of the labeling patterns showed that a large part of the intracellular glutamate and aspartate pools are synthesized from free glutamine and asparagine, respectively. Conversely, the rate of incorporation of methionine from peptide-bound forms was very high during the first few hours of the cultivation, possibly compensating for its inhibited uptake in free form.

1 Glutamine and glutamate, asparagine and aspartate are pooled during acid hydrolysis of biomass.

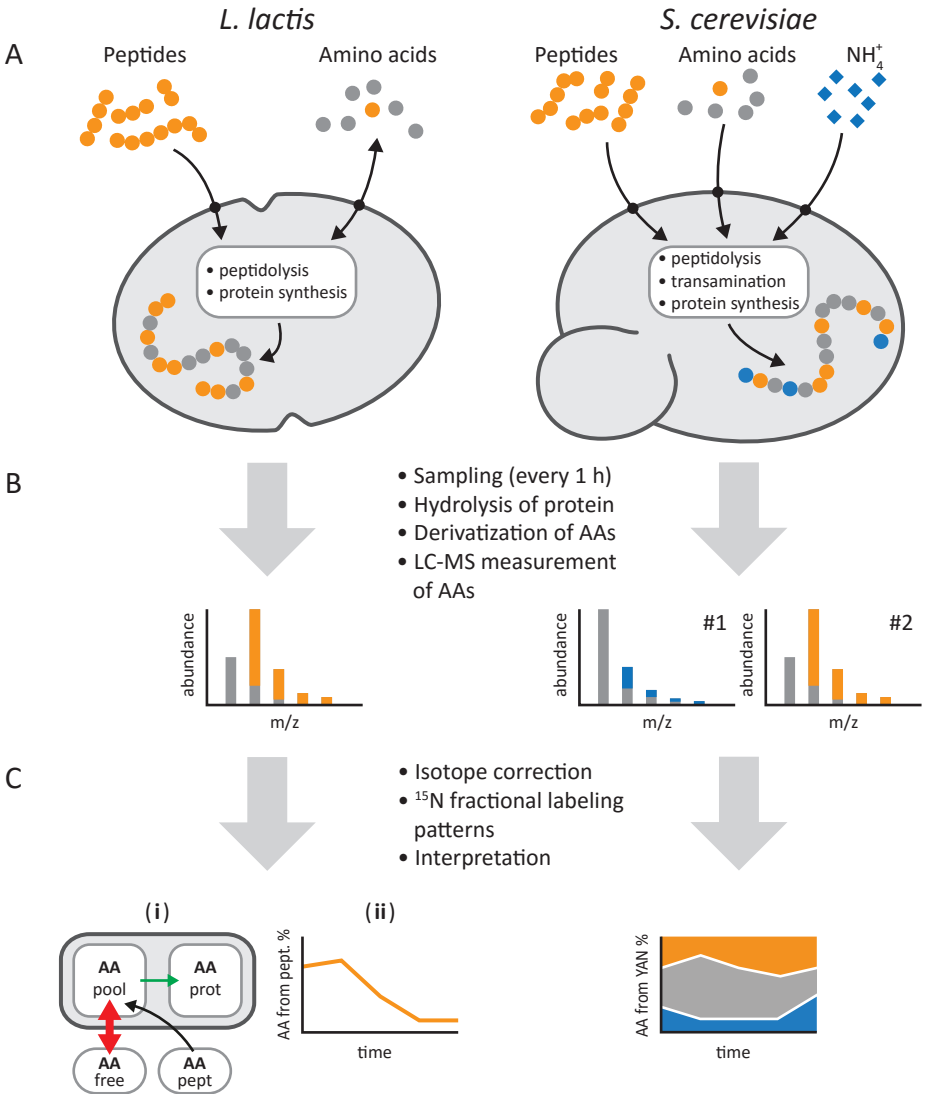

Figure 2. Interpretation of ^{15}N-labeling patterns in the experiments with *L. lactis* IL1403 and *S. cerevisiae* S288c. **A** *L. lactis* and *S. cerevisiae* were grown in batch cultures on defined media containing unlabeled free amino acids (and ammonia in the case of *S. cerevisiae*) supplemented with yeast hydrolysate (a source of peptides). In the experiment with *L. lactis*, yeast hydrolysate was ^{15}N-labeled, whereas in the two experiments with *S. cerevisiae*, either ammonia *or* yeast hydrolysate was ^{15}N-labeled. **B** Hourly samples from the labeling experiments were analyzed using LC-MS. Complementary datasets were obtained from the two experiments with *S. cerevisiae* using different labeled substrates. **C** For *L. lactis* the fluxes into and out of the intracellular amino acid pool were estimated (i); fraction of each amino acid obtained from peptides during growth is also calculated (ii). For *S. cerevisiae* fractional contribution of the three YAN sources to each amino acid in the cellular protein fraction is calculated.

With the exception of free arginine, no peptide-bound or free amino acids were exhausted during the cultivation (Figure 2 in Publication I). Despite this, we observed a decrease in the incorporation of peptide-bound forms for most amino acids during the later stages of cultivation. This could be explained by the exhaustion of the most readily transported peptides. In contrast, the consumption of peptide-bound forms of some amino acids, in particular histidine, lysine, and proline, increased during cultivation. The physiological reason for this behavior is unclear.

5.2.2 *Extensive exchange of amino acids with the extracellular medium occurs in L. lactis*

We noticed that the ^{15}N-enrichment of all free amino acids (except glutamate) in the culture medium increased during cultivation (Figure 3 in Publication I) and concluded that this was mainly caused by extensive and reversible exchange of the intra- and extracellular amino acid pools. This behavior could result from the over-accumulation of peptide-derived amino acids in the cells which are subsequently excreted into the culture medium. This is similar to what has been observed by authors who studied the growth of *L. lactis* in milk [64, 67]. Our calculations indicate that many amino acids are excreted in quantities that far exceed the amount required for the formation of biomass (Figure 3). Such high exchange fluxes may appear surprising at first, but similar behavior was also observed by Nicolae *et al.* [165] in a CHO-K1 cell line, where, as an example, the serine secretion flux was up to 35 times higher than its net uptake flux. The efflux of excess amino acids has been proposed to be coupled to the excretion of a proton, which would provide a means for the generation of metabolic energy in the form of proton motive force [61]. Therefore, such bidirectional fluxes of amino acids may help balance the intracellular amino acid pools under growth in rich media where the influx rate is higher than that of the *de novo* synthesis pathways and the feed-back control mechanisms of the *de novo* synthesis cannot prevent over-accumulation of amino acids. The exact transporters responsible for amino acid efflux in *L. lactis* are, however, unknown.

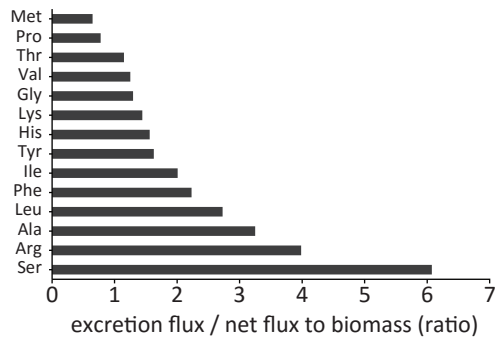

Figure 3. Ratio of the amount of amino acids excreted during growth and the amount of amino acids used for biomass synthesis during the batch cultivation of *L. lactis* IL1403 on a synthetic medium supplemented with ^{15}N-labeled yeast hydrolysate. Data from 6 h sampling point; see Figure 3 in Publication I for additional details.

5.3 SIMULTANEOUS UTILIZATION OF AMMONIA, FREE AMINO ACIDS AND PEPTIDES DURING FERMENTATIVE GROWTH OF *Saccharomyces cerevisiae* (PUBLICATION III)

The assimilation preferences of peptides in the presence of free amino acids and ammonia in yeast are rather unclear and different industrial strains have very different nitrogen requirements. Understanding the utilization of peptides by *S. cerevisiae* is important because peptides are one of the major nitrogen sources in several food fermentation processes involving yeast, including brewing.

In this work, *S. cerevisiae* was grown in batch cultures using media with the same chemical composition but differing in terms of isotopically labeled substrates ($^{15}NH_4Cl$ or ^{15}N-labeled yeast hydrolysate). The fractional labeling of intra- and extracellular amino acids was measured to determine which yeast assimilable nitrogen (YAN) sources (ammonia, free amino acids, and peptides) cells prefer under conditions of fermentative growth (Figure 2; see also Figure 1 in Publication III).

5.3.1 *A significant proportion of cellular nitrogen originates from yeast hydrolysate*

Based on the isotope labeling data for individual amino acids in the two batch experiments, we determined the relative contributions of each of the three YAN sources to the synthesis of the yeast protein fraction (Figure 3 in Publication III). The amino acid enrichment patterns obtained in the experiment using labeled ammonia ($^{15}NH_4Cl$) indicated the extent of their *de novo* synthesis. On the other hand, the amino acid enrichment patterns obtained using labeled yeast hydrolysate indicated the relative preference of peptide-bound amino acids compared to free amino acids. Our results suggest that co-consumption of ammonia, free amino acids and peptides by yeast occurs during fermentative growth and a significant proportion of the nitrogen in the cellular protein fraction can originate from peptides (approximately 40 % under our experimental conditions), even in the presence of ammonia and free amino acids. While we observed major differences in the distribution of label that originated from ammonia (*e.g.*, high for Glu/Gln, Pro; low for Lys, His), the contribution of nitrogen derived from yeast hydrolysate was relatively uniform across all amino acids.

While *L. lactis* seems to use excretion to avoid over-accumulation or imbalance of amino acids in the cells, this strategy seems to play a much lesser role in yeast. It has been suggested that over-accumulated amino acids could partly leave the cells as deaminated derivatives [211]. In any case, expulsion of excess amino acids or their breakdown products seems to be a better strategy than inhibition of peptide uptake to avoid amino acid over-accumulation; scaling back the latter process would compromise the ability to rapidly take up potentially useful amino acids.

5.3.2 *Peptides containing hydrophobic amino acid residues are preferentially taken up by both S. cerevisiae and L. lactis*

Having established the relative contributions of peptide-bound and free amino acids for the synthesis of cellular proteins, we then analyzed the preferences of the cells for various peptides. Figure 4 presents the residual concentrations (relative to initial concentrations) of peptide-bound amino acids in the culture medium at the penultimate sampling points of each experiment (6 h and 10 h in the case of *L. lactis* and *S. cerevisiae*, respectively). With some exceptions, the consumption patterns were rather similar in the two organisms. For example, in both organisms glutamine and glutamate as well as asparagine and aspartate were consumed relatively less extensively than the branched chain amino acids Ile, Leu, and Val, as well as Arg and Met. These results are in a good agreement with the findings of Ito *et al.* [104] who studied the substrate specificity of the major di-/tripeptide transporter Ptr2p of *S. cerevisiae* and concluded that it displays comparatively higher affinity towards dipeptides containing residues of aromatic or branched-chain amino acids. Likewise, Detmers *et al.* [31] demonstrated, that while the differences in affinity of the lactococcal OppA for particular side chains is relatively small, hydrophobic residues are generally favored, whereas glycine, proline, and negatively charged residues lower the binding affinity. Taken together, these results can be leveraged to produce yeast hydrolysates with optimized composition for a particular bioprocess and microorganism (see below).

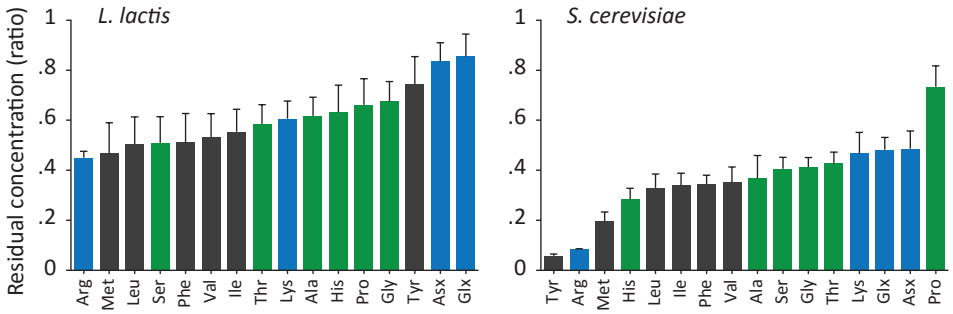

Figure 4. Relative residual concentrations of peptide-bound amino acids in the culture medium of the batch cultivations of *L. lactis* IL1403 (left) and *S. cerevisiae* S288c (right) at the penultimate sampling points of each cultivation (10 h and 6 h, respectively). The *bars* are color-coded according to the hydrophobicity of the corresponding amino acids (based on the classification of the WebLogo program, http://weblogo.berkeley.edu/): hydrophilic (Arg, Asn, Asp, Gln, Glu, Lys) – *blue*; neutral (Ala, Gly, His, Pro, Ser, Thr) – *green*; hydrophobic (Ile, Leu, Met, Phe, Tyr, Val) – *dark grey*. The *error bars* represent relative deviations from the mean of two independent experiments.

5.4 HOW CAN WE APPLY THESE RESULTS TO OPTIMIZE THE COMPOSITION OF YEAST HYDROLYSATES?

The global market for yeast products reached $5.8 billion in 2013 and is expected to grow to $9.2 billion by the year 2019 [212]. Optimization of the specific bioactivity and production processes of yeast hydrolysates is, thus, of major economic interest.

The composition of the final extract can be influenced at various stages of the production process. Firstly, it has been shown that different yeast species and strains differ in their biomass composition, including the relative abundance of amino acids [213, 214]. The cellular amino acid composition (proteome) can also be influenced to a certain degree through the adjustment of the cultivation conditions, including the choice of carbon and nitrogen sources [215, 216]. Secondly, choice of hydrolytic enzymes is a crucial step. As shown by us and other authors, amino acid composition of the peptides present in the hydrolysates determines how readily they are taken up through the respective transport systems. Careful choice of peptidases could thus make it possible to maximize the occurrence of preferred amino acids (for example, BCAAs and aromatic amino acids) in the composition of peptides while minimizing the occurrence of non-preferred ones (for example, Glu and Asp), which are more readily transported in free form.

For instance, a combination of endo- and exopeptidase treatments could be utilized to target the liberation of Glu residues from proteins subjected to hydrolysis. Glutamyl endopeptidase SspA specifically cleaves the peptide bond after Glu and, to a lesser extent, Asp. Glu-specific peptidases, such as glutamate carboxypeptidase Cpg2, or non-specific carboxypeptidases can then be used to remove the exposed C-terminal Glu from the resulting peptides. Strong glutamyl enopeptidase activity has been measured in commercial proteinase preparations Alcalase (Novozymes) [217] and SP 446 (Novo Nordisk) [218]. Purified bacterial glutamate carboxypeptidase preparations are available [219] and several commercial preparations, including Flavorpro 192 (Biocatalysts) and Flavourzyme (Novozymes), have appreciable albeit non-specific carboxypeptidase activities [217, 220]. The production and testing of optimized hydrolysates is, however, beyond the scope of the present work.

In addition to the strategies above, fractionation and/or purification of the resultant hydrolysate to concentrate the most bio-active compounds might be feasible in specific cases.

SUMMARY

6

CONCLUSIONS

An integrated approach involving the utilization of ^{15}N-labeled yeast hydrolysate was developed and implemented to study the amino acid and peptide metabolisms of both *Lactococcus lactis* and *Saccharomyces cerevisiae*. The main conclusions of the work presented in this dissertation are listed below.

I The labeling data reveal that *L. lactis* simultaneously consumes both free and peptide bound amino acids when both of these forms are readily available.

II Extensive efflux of amino acids occurs in *L. lactis* after their initial uptake from the culture medium.

III Co-consumption of ammonia, free amino acids, and peptides by *S. cerevisiae* during fermentative growth takes place and a significant proportion of the proteinogenic amino acids within cells can originate from peptides.

IV Both *L. lactis* and *S. cerevisiae* display a preference for peptides that contain hydrophobic amino acid residues.

V The ^{15}N-labeled yeast hydrolysate was found to be suitable for the determination of NAD^+, nicotinamide mononucleotide, and nicotinamide riboside and was shown to be an efficient tool for the determination of the derivatives of nicotinamide using LC-MS.

BIBLIOGRAPHY

BIBLIOGRAPHY

[1] Carr FJ, Chill D, Maida N. The lactic acid bacteria: a literature survey. *Crit Rev Microbiol* 28(4):281–370, 2002.

[2] Gaspar P, Carvalho AL, Vinga S, Santos H, Neves AR. From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria. *Biotechnol Adv* 31(6):764–788, 2013.

[3] Wells J. Mucosal vaccination and therapy with genetically modified lactic acid bacteria. *Annu Rev Food Sci Technol* 2:423–445, 2011.

[4] Pérez T, Balcázar JL, Peix A, Valverde A, Velázquez E, de Blas I, Ruiz-Zarzuela I. *Lactococcus lactis* subsp. *tructae* subsp. nov. isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). *Int J Syst Evol Microbiol* 61(8):1894–8, 2011.

[5] Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sci U S A* 103(42):15611–6, 2006.

[6] Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* 11(5):731–53, 2001.

[7] Chopin A, Chopin MC, Moillo-Batt A, Langella P. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* 11(3):260–3, 1984.

[8] Kelly WJ, Ward LJH, Leahy SC. Chromosomal diversity in *Lactococcus lactis* and the origin of dairy starter cultures. *Genome Biol Evol* 2(1):729–44, 2010.

[9] Cavanagh D, Fitzgerald GF, McAuliffe O. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol* 47:45–61, 2015.

[10] Siezen RJ, Renckens B, van Swam I, Peters S, van Kranenburg R, Kleerebezem M, de Vos WM. Complete sequences of four plasmids of *Lactococcus lactis* subsp. *cremoris* SK11 reveal extensive adaptation to the dairy environment. *Appl Environ Microbiol* 71(12):8371–82, 2005.

[11] Górecki RK, Koryszewska-Bagińska A, Gołębiewski M, Żylińska J, Grynberg M, Bardowski JK. Adaptative potential of the *Lactococcus lactis* IL594 strain encoded in its 7 plasmids. *PLoS One* 6(7):e22238, 2011.

[12] Siezen RJ. Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie Van Leeuwenhoek* 76(1-4):139–55, 1999.

[13] Savijoki K, Ingmer H, Varmanen P. Proteolytic systems of lactic acid bacteria. *Appl Microbiol Biotechnol* 71(4):394–406, 2006.

[14] Nissen-Meyer J, Lillehaug D, Nes IF. The plasmid-encoded lactococcal envelope-associated proteinase is encoded by a chromosomal gene in *Lactococcus lactis* subsp. *cremoris* BC101. *Appl Environ Microbiol* 58(2):750–3, 1992.

[15] Xie Y, Chou Ls, Cutler A, Weimer B. DNA Macroarray profiling of *Lactococcus lactis* subsp. *lactis* IL1403 gene expression during environmental stresses. *Appl Environ Microbiol* 70(11):6738–47, 2004.

[16] Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genomics* 11:36, 2010.

[17] Lamarque M, Aubel D, Piard JCC, Gilbert C, Juillard V, Atlan D. The peptide transport system Opt is involved in both nutrition and environmental sensing during growth of *Lactococcus lactis* in milk. *Microbiology* 157(6):1612–1619, 2011.

[18] Kok J, van Dijl JM, van der Vossen JM, Venema G. Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. *Appl Environ Microbiol* 50(1):94–101, 1985.

[19] Xu FF, Pearce LE, Yu PL. Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 and construction of a new lactococcal vector pFX1. *Arch Microbiol* 154(1):99–104, 1990.

[20] Law J, Vos P, Hayes F, Daly C, de Vos WM, Fitzgerald G. Cloning and partial sequencing of the proteinase gene complex from *Lactococcus lactis* subsp. *lactis* UC317. *J Gen Microbiol* 138(4):709–18, 1992.

[21] Pederson JA, Mileski GJ, Weimer BC, Steele JL. Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. *J Bacteriol* 181(15):4592–7, 1999.

[22] Teusink B, Bachmann H, Molenaar D. Systems biology of lactic acid bacteria: a critical review. *Microb Cell Fact* 10(Suppl 1):S11, 2011.

[23] Bachmann H, Molenaar D, Kleerebezem M, van Hylckama Vlieg JET. High local substrate availability stabilizes a cooperative trait. *ISME J* 5(5):929–32, 2011.

[24] Thomas TD, Pritchard GG. Proteolytic enzymes of dairy starter cultures. *FEMS Microbiol Lett* 46(3):245–268, 1987.

[25] Kok J, Buist G. Genetics of Proteolysis in *Lactococcus lactis*. In: B Wood, P Warner (eds.) *Genetics of Lactic Acid Bacteria*, pp. 189–223. Springer US, Boston, MA, 2003.

[26] Juillard V, Laan H, Kunji ER, Jeronimus-Stratingh CM, Bruins AP, Konings WN. The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes beta-casein into more than one hundred different oligopeptides. *J Bacteriol* 177(12):3472–8, 1995.

[27] Kunji ER, Fang G, Jeronimus-Stratingh CM, Bruins aP, Poolman B, Konings WN. Reconstruction of the proteolytic pathway for use of beta-casein by *Lactococcus lactis*. *Mol Microbiol* 27(6):1107–18, 1998.

[28] Børsting MW, Qvist KB, Brockmann E, Vindeløv J, Pedersen TL, Vogensen FK, Ardö Y. Classification of *Lactococcus lactis* cell envelope proteinase based on gene sequencing, peptides formed after hydrolysis of milk, and computer modeling. *J Dairy Sci* 98(1):68–77, 2015.

[29] Tynkkynen S, Buist G, Kunji E, Kok J, Poolman B, Venema G, Haandrikman A. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. *J Bacteriol* 175(23):7523–32, 1993.

[30] Doeven MK, Kok J, Poolman B. Specificity and selectivity determinants of peptide transport in Lactococcus lactis and other microorganisms. *Mol Microbiol* 57(3):640–9, 2005.

[31] Detmers FJ, Lanfermeijer FC, Abele R, Jack RW, Tampe R, Konings WN, Poolman B. Combinatorial peptide libraries reveal the ligand-binding mechanism of the oligopeptide receptor OppA of Lactococcus lactis. *Proc Natl Acad Sci U S A* 97(23):12487–92, 2000.

[32] Helinck S, Charbonnel P, Foucaud-Scheunemann C, Piard JC, Juillard V. Charged casein-derived oligopeptides competitively inhibit the transport of a reporter oligopeptide by *Lactococcus lactis*. *J Appl Microbiol* 94(5):900–7, 2003.

[33] Berntsson RPA, Doeven MK, Fusetti F, Duurkens RH, Sengupta D, Marrink SJ, Thunnissen AMWH, Poolman B, Slotboom DJ. The structural basis for peptide selection by the transport receptor OppA. *EMBO J* 28(9):1332–40, 2009.

[34] Berntsson RPA, Thunnissen AMWH, Poolman B, Slotboom DJ. Importance of a hydrophobic pocket for peptide binding in lactococcal OppA. *J Bacteriol* 193(16):4254–6, 2011.

[35] Lamarque M, Charbonnel P, Aubel D, Piard JC, Atlan D, Juillard V. A multifunction ABC transporter (Opt) contributes to diversity of peptide uptake specificity within the genus *Lactococcus*. *J Bacteriol* 186(19):6492–500, 2004.

[36] Foucaud C, Kunji ER, Hagting A, Richard J, Konings WN, Desmazeaud M, Poolman B. Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- and tripeptides. *J Bacteriol* 177(16):4652–7, 1995.

[37] Steiner HY, Naider F, Becker JM. The PTR family: A new group of peptide transporters. *Mol Microbiol* 16(5):825–834, 1995.

[38] Smid EJ, Driessen AJ, Konings WN. Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis*. *J Bacteriol* 171(1):292–8, 1989.

[39] Hagting A, Kunji ER, Leenhouts KJ, Poolman B, Konings WN. The di- and tripeptide transport protein of *Lactococcus lactis*. A new type of bacterial peptide transporter. *J Biol Chem* 269(15):11391–9, 1994.

[40] Fang G, Konings WN, Poolman B. Kinetics and substrate specificity of membrane-reconstituted peptide transporter DtpT of *Lactococcus lactis*. *J Bacteriol* 182(9):2530–5, 2000.

[41] Kunji ER, Hagting A, De Vries CJ, Juillard V, Haandrikman AJ, Poolman B, Konings WN. Transport of beta-casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. *J Biol Chem* 270(4):1569–74, 1995.

[42] Kunji ER, Mierau I, Poolman B, Konings WN, Venema G, Kok J. Fate of peptides in peptidase mutants of *Lactococcus lactis*. *Mol Microbiol* 21(1):123–31, 1996.

[43] Mierau I, Kunji ER, Venema G, Kok J. Casein and peptide degradation in lactic acid bacteria. *Biotechnol Genet Eng Rev* 14(1):279–301, 1997.

[44] Marugg JD, Meijer W, van Kranenburg R, Laverman P, Bruinenberg PG, de Vos WM. Medium-dependent regulation of proteinase gene expression in *Lactococcus lactis*: control of transcription initiation by specific dipeptides. *J Bacteriol* 177(11):2982–9, 1995.

[45] Guédon E, Renault P, Ehrlich SD, Delorme C. Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptide supply. *J Bacteriol* 183(12):3614–22, 2001.

[46] Guédon E, Serror P, Ehrlich SD, Renault P, Delorme C. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Mol Microbiol* 40(5):1227–39, 2001.

[47] Kunji ER, Mierau I, Hagting A, Poolman B, Konings WN. The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek* 70(2-4):187–221, 1996.

[48] Christensen JE, Dudley EG, Pederson JA, Steele JL. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* 76(1-4):217–46, 1999.

[49] Mayo B, Kok J, Bockelmann W, Haandrikman A, Leenhouts KJ, Venema G. Effect of X-prolyl dipeptidyl aminopeptidase deficiency on *Lactococcus lactis*. *Appl Environ Microbiol* 59(7):2049–55, 1993.

[50] Mierau I, Haandrikman aJ, Velterop O, Tan PST, Lennhouts KL, Konings WN, Venema G, Kok J. Tripeptidase gene (*pepT*) of *Lactococcus lactis*: Molecular cloning and nucleotide sequencing of *pepT* and construction of a chromosomal deletion mutant. *J Bacteriol* 176(10):2854–2861, 1994.

[51] Monnet V, Nardi M, Chopin A, Chopin MC, Gripon JC. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. *J Biol Chem* 269(51):32070–6, 1994.

[52] Mierau I, Kunji ER, Leenhouts KJ, Hellendoorn MA, Haandrikman AJ, Poolman B, Konings WN, Venema G, Kok J. Multiple-peptidase mutants of *Lactococcus lactis* are severely impaired in their ability to grow in milk. *J Bacteriol* 178(10):2794–803, 1996.

[53] Guinec N, Nardi M, Matos J, Gripon JC, Monnet V. Modulation of casein proteolysis by lactococcal peptidase gene inactivation. *Int Dairy J* 10(9):607–615, 2000.

[54] Christensen JE, Steele JL. Impaired growth rates in milk of *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acid supplements. *J Bacteriol* 185(11):3297–306, 2003.

[55] Nardi M, Renault P, Monnet V. Duplication of the *pepF* gene and shuffling of DNA fragments on the lactose plasmid of *Lactococcus lactis*. *J Bacteriol* 179(13):4164–71, 1997.

[56] Thomas S, Besset C, Courtin P, Rul F. The role of aminopeptidase PepS in the growth of *Streptococcus thermophilus* is not restricted to nitrogen nutrition. *J Appl Microbiol* 108(1):148–57, 2010.

[57] Konings WN, Poolman B, Driessen AJ. Bioenergetics and solute transport in lactococci. *Crit Rev Microbiol* 16(6):419–76, 1989.

[58] Konings WN. The cell membrane and the struggle for life of lactic acid bacteria. *Antonie Van Leeuwenhoek* 82(1-4):3–27, 2002.

[59] Schuurman-Wolters GK, Poolman B. Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*. An ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. *J Biol Chem* 280(25):23785–90, 2005.

[60] den Hengst CD, Groeneveld M, Kuipers OP, Kok J. Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (CtrA). *J Bacteriol* 188(9):3280–9, 2006.

[61] Trip H, Mulder NL, Lolkema JS. Cloning, expression, and functional characterization of secondary amino acid transporters of *Lactococcus lactis*. *J Bacteriol* 195(2):340–50, 2013.

[62] Noens EEE, Lolkema JS. Physiology and substrate specificity of two closely related amino acid transporters, SerP1 and SerP2, of *Lactococcus lactis*. *J Bacteriol* 197(5):951–8, 2015.

[63] Driessen AJ, de Jong S, Konings WN. Transport of branched-chain amino acids in membrane vesicles of *Streptococcus cremoris*. *J Bacteriol* 169(11):5193–200, 1987.

[64] Juillard V, Le Bars D, Kunji ER, Konings WN, Gripon JC, Richard J. Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. *Appl Environ Microbiol* 61(8):3024–30, 1995.

[65] Jensen PR, Hammer K. Minimal requirements for exponential growth of *Lactococcus lactis*. *Appl Environ Microbiol* 59(12):4363–6, 1993.

[66] Hugenholtz J, Dijkstra M, Veldkamp H. Amino acid limited growth of starter cultures in milk. *FEMS Microbiol Lett* 45(4):191–198, 1987.

[67] Cretenet M, Laroute V, Ulvé V, Jeanson S, Nouaille S, Even S, Piot M, Girbal L, Le Loir Y, Loubière P, Lortal S, Cocaign-Bousquet M. Dynamic analysis of the *Lactococcus lactis* transcriptome in cheeses made from milk concentrated by ultrafiltration reveals multiple strategies of adaptation to stresses. *Appl Environ Microbiol* 77(1):247–57, 2011.

[68] Zhang G, Mills Da, Block DE. Development of chemically defined media supporting high-cell-density growth of lactococci, enterococci, and streptococci. *Appl Environ Microbiol* 75(4):1080–7, 2009.

[69] Meijer W, Marugg JD, Hugenholtz J. Regulation of proteolytic enzyme activity in *Lactococcus lactis*. *Appl Environ Microbiol* 62(1):156–61, 1996.

[70] Gitton C, Meyrand M, Wang J, Caron C, Trubuil A, Guillot A, Mistou MY. Proteomic signature of *Lactococcus lactis* NCDO763 cultivated in milk. *Appl Environ Microbiol* 71(11):7152–63, 2005.

[71] Chambellon E, Yvon M. CodY-regulated aminotransferases AraT and BcaT play a major role in the growth of *Lactococcus lactis* in milk by regulating the intracellular pool of amino acids. *Appl Environ Microbiol* 69(6):3061–8, 2003.

[72] Guédon E, Sperandio B, Pons N, Ehrlich SD, Renault P. Overall control of nitrogen metabolism in *Lactococcus lactis* by CodY, and possible models for CodY regulation in Firmicutes. *Microbiology* 151(Pt 12):3895–909, 2005.

[73] Petranovic D, Guédon E, Sperandio B, Delorme C, Ehrlich D, Renault P. Intracellular effectors regulating the activity of the *Lactococcus lactis* CodY pleiotropic transcription regulator. *Mol Microbiol* 53(2):613–21, 2004.

[74] den Hengst CD, Curley P, Larsen R, Buist G, Nauta A, van Sinderen D, Kuipers OP, Kok J. Probing direct interactions between CodY and the *oppD* promoter of *Lactococcus lactis*. *J Bacteriol* 187(2):512–21, 2005.

[75] den Hengst CD, van Hijum SaFT, Geurts JMW, Nauta A, Kok J, Kuipers OP. The *Lactococcus lactis* CodY regulon: identification of a conserved cis-regulatory element. *J Biol Chem* 280(40):34332–42, 2005.

[76] Yvon M, Thirouin S, Rijnen L, Fromentier D, Gripon JC. An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. *Appl Environ Microbiol* 63(2):414–9, 1997.

[77] Yvon M, Chambellon E, Bolotin A, Roudot-Algaron F. Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. *Appl Environ Microbiol* 66(2):571–7, 2000.

[78] Van Kranenburg R, Kleerebezem M, Van Hylckama Vlieg J, Ursing BM, Boekhorst J, Smit Ba, Ayad EHE, Smit G, Siezen RJ. Flavour formation from amino acids by lactic acid bacteria: Predictions from genome sequence analysis. In: *Int. Dairy J.*, vol. 12, pp. 111–121, 2002.

[79] Tanous C, Gori A, Rijnen L, Chambellon E, Yvon M. Pathways for alpha-ketoglutarate formation by *Lactococcus lactis* and their role in amino acid catabolism. *Int Dairy J* 15(6-9):759–770, 2005.

[80] Tanous C, Kieronczyk A, Helinck S, Chambellon E, Yvon M. Glutamate dehydrogenase activity: A major criterion for the selection of flavour-producing lactic acid bacteria strains. *Antonie van Leeuwenhoek, Int J Gen Mol Microbiol* 82(1-4):271–278, 2002.

[81] Lapujade P, Cocaign-Bousquet M, Loubiere P. Glutamate Biosynthesis in *Lactococcus lactis* subsp. *lactis* NCDO 2118. *Appl Environ Microbiol* 64(7):2485–9, 1998.

[82] 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. Life with 6000 genes. *Science* 274(5287):546, 563–7, 1996.

[83] Engel SR, Dietrich FS, Fisk DG, Binkley G, Balakrishnan R, Costanzo MC, Dwight SS, Hitz BC, Karra K, Nash RS, Weng S, Wong ED, Lloyd P, Skrzypek MS, Miyasato SR, Simison M, Cherry JM. The reference genome sequence of *Saccharomyces cerevisiae*: then and now. *G3 (Bethesda)* 4(3):389–98, 2014.

[84] Mortimer RK, Johnston JR. Genealogy of principal strains of the yeast genetic stock center. *Genetics* 113(1):35–43, 1986.

[85] Watson TG. Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino-acid nitrogen source. *J Gen Microbiol* 96(2):263–8, 1976.

[86] Large PJ. Degradation of organic nitrogen compounds by yeasts. *Yeast* 2(1):1–34, 1986.

[87] Ogrydziak DM. Yeast extracellular proteases. *Crit Rev Biotechnol* 13(1):1–55, 1993.

[88] Strauss MLA, Jolly NP, Lambrechts MG, Rensburg PV. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *J Appl Microbiol* 91:182–190, 2001.

[89] Nelson G, Young TW. Yeast extracellular proteolytic enzymes for chill-proofing beer. *J Inst Brew* 92(6):599–603, 1986.

[90] Bilinski CA, Russell I, Stewart GG. Applicability of yeast extracellular proteinases in brewing: physiological and biochemical aspects. *Appl Environ Microbiol* 53(3):495–9, 1987.

[91] Sturley SL, Young TW. Extracellular protease activity in a strain of *Saccharomyces cerevisiae*. *J Inst Brew* 94(1):23–27, 1988.

[92] Rosi I, Costamagna L, Birtuccion M. Screening for extracellular acid protease production by wine yeasts. *J Inst Brew* 93(4):322–324, 1987.

[93] Maddox IS, Hough JS. Proteolytic enzymes of *Saccharomyces carlsbergensis*. *Biochem J* 117(5):843–52, 1970.

[94] Ormrod IHL, Lalor EF, Sharpe FR. The release of yeast proteolytic enzymes into beer. *J Inst Brew* 97(6):441–443, 1991.

[95] Wang ZY, He GQ, Liu ZS, Ruan H, Chen QH, Xiong HP. Purification of yeast proteinase A from fresh beer and its specificity on foam proteins. *Int J Food Sci Technol* 40(8):835–840, 2005.

[96] Lekkas C, Hill A, Taidi B, Hodgson J, Stewart G. The role of small wort peptides in brewing fermentations. *J Inst Brew* 115(2):134–139, 2009.

[97] Younes B, Cilindre C, Villaume S, Parmentier M, Jeandet P, Vasserot Y. Evidence for an extracellular acid proteolytic activity secreted by living cells of *Saccharomyces cerevisiae* PlR1: impact on grape proteins. *J Agric Food Chem* 59(11):6239–46, 2011.

[98] Kang Ha, Choi ES, Hong WK, Kim JY, Ko SM, Sohn JH, Rhee SK. Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 53(5):575–82, 2000.

[99] Görgens JF, van Zyl WH, Knoetze JH, Hahn-Hägerdal B. Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. *Appl Microbiol Biotechnol* 67(5):684–91, 2005.

[100] Island MD, Perry JR, Naider F, Becker JM. Isolation and characterization of *S. cerevisiae* mutants deficient in amino acid-inducible peptide transport. *Curr Genet* 20(6):457–63, 1991.

[101] Perry JR, Basrai Ma, Steiner HY, Naider F, Becker JM. Isolation and characterization of a *Saccharomyces cerevisiae* peptide transport gene. *Mol Cell Biol* 14(1):104–15, 1994.

[102] Hauser M, Narita V, Donhardt AM, Naider F, Becker JM. Multiplicity and regulation of genes encoding peptide transporters in *Saccharomyces cerevisiae*. *Mol Membr Biol* 18(1):105–12, 2001.

[103] Daniel H, Spanier B, Kottra G, Weitz D. From bacteria to man: archaic proton-dependent peptide transporters at work. *Physiology (Bethesda)* 21:93–102, 2006.

[104] Ito K, Hikida A, Kawai S, Lan VTT, Motoyama T, Kitagawa S, Yoshikawa Y, Kato R, Kawarasaki Y. Analysing the substrate multispecificity of a proton-coupled oligopeptide transporter using a dipeptide library. *Nat Commun* 4:2502, 2013.

[105] Homann OR, Cai H, Becker JM, Lindquist SL. Harnessing natural diversity to probe metabolic pathways. *PLoS Genet* 1(6):e80, 2005.

[106] Cai H, Hauser M, Naider F, Becker JM. Differential regulation and substrate preferences in two peptide transporters of *Saccharomyces cerevisiae*. *Eukaryot Cell* 6(10):1805–13, 2007.

[107] Hellborg L, Woolfit M, Arthursson-Hellborg M, Piskur J. Complex evolution of the DAL5 transporter family. *BMC Genomics* 9:164, 2008.

[108] Rubio-Texeira M, Van Zeebroeck G, Thevelein JM. Peptides induce persistent signaling from endosomes by a nutrient transceptor. *Nat Chem Biol* 8(4):400–8, 2012.

[109] Alexandre H, Heintz D, Chassagne D, Guilloux-Benatier M, Charpentier C, Feuillat M. Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. *J Ind Microbiol Biotechnol* 26(4):235–40, 2001.

[110] Kottra G, Frey I, Daniel H. Inhibition of intracellular dipeptide hydrolysis uncovers large outward transport currents of the peptide transporter PEPT1 in *Xenopus* oocytes. *Pflügers Arch Eur J Physiol* 457(4):809–20, 2009.

[111] Renna MD, Oyadeyi AS, Bossi E, Kottra G, Peres A. Functional and structural determinants of reverse operation in the pH-dependent oligopeptide transporter PepT1. *Cell Mol Life Sci* 68(17):2961–75, 2011.

[112] Hauser M, Donhardt AM, Barnes D, Naider F, Becker JM. Enkephalins are transported by a novel eukaryotic peptide uptake system. *J Biol Chem* 275(5):3037–41, 2000.

[113] Wiles AM, Cai H, Naider F, Becker JM. Nutrient regulation of oligopeptide transport in *Saccharomyces cerevisiae*. *Microbiology* 152(Pt 10):3133–45, 2006.

[114] Damon C, Vallon L, Zimmermann S, Haider MZ, Galeote V, Dequin S, Luis P, Fraissinet-Tachet L, Marmeisse R. A novel fungal family of oligopeptide transporters identified by functional metatranscriptomics of soil eukaryotes. *ISME J* 5(12):1871–80, 2011.

[115] Marsit S, Sanchez I, Galeote V, Dequin S. Horizontally acquired oligopeptide transporters favor adaptation of *Saccharomyces cerevisiae* wine yeast to enological environment. *Environ Microbiol* 63(1):9–14, 2015.

[116] Gomolplitinant KM, Saier MH. Evolution of the oligopeptide transporter family. *J Membr Biol* 240(2):89–110, 2011.

[117] Osawa H, Stacey G, Gassmann W. *ScOPT1* and *AtOPT4* function as proton-coupled oligopeptide transporters with broad but distinct substrate specificities. *Biochem J* 393(Pt 1):267–75, 2006.

[118] Miyake T, Kanayama M, Sammoto H, Ono B. A novel cis-acting cysteine-responsive regulatory element of the gene for the high-affinity glutathione transporter of *Saccharomyces cerevisiae*. *Mol Genet Genomics* 266(6):1004–11, 2002.

[119] Bourbouloux A, Shahi P, Chakladar A, Delrot S, Bachhawat AK. Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 275(18):13259–65, 2000.

[120] Aouida M, Khodami-Pour A, Ramotar D. Novel role for the *Saccharomyces cerevisiae* oligopeptide transporter Opt2 in drug detoxification. *Biochem Cell Biol* 87(4):653–61, 2009.

[121] Elbaz-Alon Y, Morgan B, Clancy A, Amoako TNE, Zalckvar E, Dick TP, Schwappach B, Schuldiner M. The yeast oligopeptide transporter Opt2 is localized to peroxisomes and affects glutathione redox homeostasis. *FEMS Yeast Res* 14(7):1055–67, 2014.

[122] Kaur J, Srikanth CV, Bachhawat AK. Differential roles played by the native cysteine residues of the yeast glutathione transporter, Hgt1p. *FEMS Yeast Res* 9(6):849–66, 2009.

[123] Thakur A, Bachhawat AK. Charged/Polar-residue scanning of the hydrophobic face of transmembrane domain 9 of the yeast glutathione transporter, hgt1p, reveals a conformationally critical region for substrate transport. *G3 (Bethesda)* 5(5):921–9, 2015.

[124] Verhoef LGGC, Heinen C, Selivanova A, Halff EF, Salomons FA, Dantuma NP. Minimal length requirement for proteasomal degradation of ubiquitin-dependent substrates. *FASEB J* 23(1):123–33, 2009.

[125] Shabek N, Herman-Bachinsky Y, Buchsbaum S, Lewinson O, Haj-Yahya M, Hejjaoui M, Lashuel HA, Sommer T, Brik A, Ciechanover A. The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. *Mol Cell* 48(1):87–97, 2012.

[126] Nussbaum aK, Dick TP, Keilholz W, Schirle M, Stevanović S, Dietz K, Heinemeyer W, Groll M, Wolf DH, Huber R, Rammensee HG, Schild H. Cleavage motifs of the yeast 20S proteasome beta subunits deduced from digests of enolase 1. *Proc Natl Acad Sci U S A* 95(21):12504–9, 1998.

[127] Van Den Hazel HB, Kielland-Brandt MC, Winther JR. Review: biosynthesis and function of yeast vacuolar proteases. *Yeast* 12(1):1–16, 1996.

[128] Hecht KA, O'Donnell AF, Brodsky JL. The proteolytic landscape of the yeast vacuole. *Cell Logist* 4(1):e28023, 2014.

[129] Horst M, Knecht EC, Schu PV. Import into and degradation of cytosolic proteins by isolated yeast vacuoles. *Mol Biol Cell* 10(9):2879–89, 1999.

[130] Li SC, Kane PM. The yeast lysosome-like vacuole: endpoint and crossroads. *Biochim Biophys Acta* 1793(4):650–63, 2009.

[131] Cai H, Kauffman S, Naider F, Becker JM. Genomewide screen reveals a wide regulatory network for di/tripeptide utilization in *Saccharomyces cerevisiae*. *Genetics* 172(3):1459–76, 2006.

[132] Büchler M, Tisljar U, Wolf DH. Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase (metalloendopeptidase, EP 24.15). *Eur J Biochem* 219(1-2):627–39, 1994.

[133] Hirsch HH, Suárez Rendueles P, Achstetter T, Wolf DH. Aminopeptidase yscII of yeast. Isolation of mutants and their biochemical and genetic analysis. *Eur J Biochem* 173(3):589–98, 1988.

[134] Horák J. Yeast nutrient transporters. *Biochim Biophys Acta* 1331(1):41–79, 1997.

[135] Regenberg B, Düring-Olsen L, Kielland-Brandt MC, Holmberg S. Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. *Curr Genet* 36(6):317–28, 1999.

[136] Marini AM, Soussi-Boudekou S, Vissers S, Andre B. A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol* 17(8):4282–93, 1997.

[137] Boeckstaens M, André B, Marini AM. Distinct transport mechanisms in yeast ammonium transport/sensor proteins of the Mep/Amt/Rh family and impact on filamentation. *J Biol Chem* 283(31):21362–70, 2008.

[138] Hess DC, Lu W, Rabinowitz JD, Botstein D. Ammonium toxicity and potassium limitation in yeast. *PLoS Biol* 4(11):e351, 2006.

[139] Burkovski A, Krämer R. Bacterial amino acid transport proteins: occurrence, functions, and significance for biotechnological applications. *Appl Microbiol Biotechnol* 58(3):265–74, 2002.

[140] Velasco I, Tenreiro S, Calderon IL, André B. *Saccharomyces cerevisiae* Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryot Cell* 3(6):1492–503, 2004.

[141] Gresham D, Usaite R, Germann SM, Lisby M, Botstein D, Regenberg B. Adaptation to diverse nitrogen-limited environments by deletion or extrachromosomal element formation of the *GAP1* locus. *Proc Natl Acad Sci U S A* 107(43):18551–6, 2010.

[142] Nisbet TM, Payne JW. Peptide uptake in *Saccharomyces cerevisiae*: characteristics of transport system shared by di- and tripeptides. *J Gen Microbiol* 115(1):127–133, 1979.

[143] Nisbet T. *Aspects of peptide transport in saccharomyces cerevisiae and streptococcus spp.* PhD thesis, Durham University, 1980.

[144] Sekito T, Fujiki Y, Ohsumi Y, Kakinuma Y. Novel families of vacuolar amino acid transporters. *IUBMB Life* 60(8):519–25, 2008.

[145] Schneper L, Düvel K, Broach JR. Sense and sensibility: nutritional response and signal integration in yeast. *Curr Opin Microbiol* 7(6):624–30, 2004.

[146] Zaman S, Lippman SI, Zhao X, Broach JR. How *Saccharomyces* responds to nutrients. *Annu Rev Genet* 42:27–81, 2008.

[147] Ljungdahl PO, Daignan-Fornier B. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. *Genetics* 190(3):885–929, 2012.

[148] Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M, Thevelein JM. Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 38(2):254–99, 2014.

[149] Magasanik B, Kaiser Ca. Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* 290(1-2):1–18, 2002.

[150] Chisholm VT, Lea HZ, Rai R, Cooper TG. Regulation of allantoate transport in wild-type and mutant strains of *Saccharomyces cerevisiae*. *J Bacteriol* 169(4):1684–90, 1987.

[151] Marzluf GA. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* 61(1):17–32, 1997.

[152] Naik RR, Nebes V, Jones EW. Regulation of the proteinase B structural gene *PRB1* in *Saccharomyces cerevisiae*. *J Bacteriol* 179(5):1469–74, 1997.

[153] Coffman JA, Cooper TG. Nitrogen GATA factors participate in transcriptional regulation of vacuolar protease genes in *Saccharomyces cerevisiae*. *J Bacteriol* 179(17):5609–13, 1997.

[154] Hofman-Bang J. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. *Mol Biotechnol* 12(1):35–73, 1999.

[155] Jones EW. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 266(13):7963–6, 1991.

[156] Forsberg H, Ljungdahl PO. Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Curr Genet* 40(2):91–109, 2001.

[157] Wu B, Ottow K, Poulsen P, Gaber RF, Albers E, Kielland-Brandt MC. Competitive intra- and extracellular nutrient sensing by the transporter homologue Ssy1p. *J Cell Biol* 173(3):327–31, 2006.

[158] Ljungdahl PO. Amino-acid-induced signalling via the SPS-sensing pathway in yeast. *Biochem Soc Trans* 37(Pt 1):242–7, 2009.

[159] Didion T, Regenberg B, Jørgensen MU, Kielland-Brandt MC, Andersen Ha. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. *Mol Microbiol* 27(3):643–50, 1998.

[160] Xia Z, Turner GC, Hwang CS, Byrd C, Varshavsky A. Amino acids induce peptide uptake via accelerated degradation of CUP9, the transcriptional repressor of the PTR2 peptide transporter. *J Biol Chem* 283(43):28958–68, 2008.

[161] Turner GC, Du F, Varshavsky A. Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. *Nature* 405(6786):579–83, 2000.

[162] Christensen B, Nielsen J. Metabolic network analysis. A powerful tool in metabolic engineering. In: B Sonnleitner (ed.) *Advances in Biochemical Engineering/Biotechnology*, vol. 66, pp. 209–231, 1999.

[163] Nanchen A, Fuhrer T, Sauer U. Determination of Metabolic Flux Ratios From ^{13}C-Experiments and Gas Chromatography-Mass Spectrometry Data. In: W Weckwerth (ed.) *Methods in Molecular Biology*, vol. 358, pp. 177–197. Humana Press, 2007.

[164] Antoniewicz MR, Kraynie DF, Laffend LA, González-Lergier J, Kelleher JK, Stephanopoulos G. Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of *E. coli* producing 1,3-propanediol. *Metab Eng* 9(3):277–92, 2007.

[165] Nicolae A, Wahrheit J, Bahnemann J, Zeng AP, Heinzle E. Non-stationary ^{13}C metabolic flux analysis of Chinese hamster ovary cells in batch culture using extracellular labeling highlights metabolic reversibility and compartmentation. *BMC Syst Biol* 8(1):50, 2014.

[166] Nöh K, Wiechert W. Experimental design principles for isotopically instationary ^{13}C labeling experiments. *Biotechnol Bioeng* 94(2):234–51, 2006.

[167] Jensen NBS, Christensen B, Nielsen J, Villadsen J. The simultaneous biosynthesis and uptake of amino acids by *Lactococcus lactis* studied by ^{13}C-labeling experiments. *Biotechnol Bioeng* 78(1):11–6, 2002.

[168] Shlomi T, Fan J, Tang B, Kruger WD, Rabinowitz JD. Quantitation of cellular metabolic fluxes of methionine. *Anal Chem* 86(3):1583–91, 2014.

[169] Buescher JM, Antoniewicz MR, Boros LG, Burgess SC, Brunengraber H, Clish CB, DeBerardinis RJ, Feron O, Frezza C, Ghesquiere B, Gottlieb E, Hiller K, Jones RG, Kamphorst JJ, Kibbey RG, Kimmelman AC, Locasale JW, Lunt SY, Maddocks ODK, Malloy C, Metallo CM, Meuillet EJ, Munger J, Nöh K, Rabinowitz JD, Ralser M, Sauer U, Stephanopoulos G, St-Pierre J, Tennant DA, Wittmann C, Vander Heiden MG, Vazquez A, Vousden K, Young JD, Zamboni N, Fendt SM. A roadmap for interpreting ^{13}C metabolite labeling patterns from cells. *Curr Opin Biotechnol* 34:189–201, 2015.

[170] Wittmann, Heinzle. Mass spectrometry for metabolic flux analysis. *Biotechnol Bioeng* 62(6):739–750, 1999.

[171] Lee WN, Byerley LO, Bergner EA, Edmond J. Mass isotopomer analysis: theoretical and practical considerations. *Biol Mass Spectrom* 20(March):451–458, 1991.

[172] Fernandez Ca, Des Rosiers C, Previs SF, David F, Brunengraber H. Correction of ^{13}C mass isotopomer distributions for natural stable isotope abundance. *J Mass Spectrom* 31(3):255–262, 1996.

[173] van Winden WA, Wittmann C, Heinzle E, Heijnen JJ. Correcting mass isotopomer distributions for naturally occurring isotopes. *Biotechnol Bioeng* 80(4):477–9, 2002.

[174] Crown SB, Antoniewicz MR. Publishing ^{13}C metabolic flux analysis studies: a review and future perspectives. *Metab Eng* 20:42–8, 2013.

[175] Lafaye A, Labarre J, Tabet JC, Ezan E, Junot C. Liquid chromatography-mass spectrometry and ^{15}N metabolic labeling for quantitative metabolic profiling. *Anal Chem* 77(7):2026–33, 2005.

[176] Harada K, Fukusaki E, Bamba T, Sato F, Kobayashi A. *In vivo* ^{15}N-enrichment of metabolites in suspension cultured cells and its application to metabolomics. *Biotechnol Prog* 22(4):1003–11, 2006.

[177] Yoneyama T, Fujimori T, Yanagisawa S, Hase T, Suzuki A. ^{15}N tracing studies on in vitro reactions of ferredoxin-dependent nitrite reductase and glutamate synthase using reconstituted electron donation systems. *Plant Cell Physiol* 56(6):1154–61, 2015.

[178] Sims AP, Folkes BF. A kinetic study of the assimilation of ^{15}N-ammonia and the synthesis of amino acids in an exponentially growing culture of candida utilis. *Proc R Soc London Ser B, Biol Sci* 159:479–502, 1964.

[179] Jones M, Pragnell MJ, Pierce JS. Absorption of amino acids by yeasts from a semi-defined medium simulating wort. *J Inst Brew* 75(6):520–536, 1969.

[180] Blank LM, Desphande RR, Schmid A, Hayen H. Analysis of carbon and nitrogen co-metabolism in yeast by ultrahigh-resolution mass spectrometry applying ^{13}C- and ^{15}N-labeled substrates simultaneously. *Anal Bioanal Chem* 403(8):2291–305, 2012.

[181] Christiansen T, Christensen B, Nielsen J. Metabolic network analysis of *Bacillus clausii* on minimal and semirich medium using ^{13}C-labeled glucose. *Metab Eng* 4(2):159–69, 2002.

[182] Xiao Y, Feng X, Varman AM, He L, Yu H, Tang YJ. Kinetic modeling and isotopic investigation of isobutanol fermentation by two engineered *Escherichia coli* strains. *Ind Eng Chem Res* 51(49):15855–15863, 2012.

[183] Xiao Y, Ruan Z, Liu Z, Wu SG, Varman AM, Liu Y, Tang YJ. Engineering *Escherichia coli* to convert acetic acid to free fatty acids. *Biochem Eng J* 76:60–69, 2013.

[184] Egorova-Zachernyuk TA, Bosman GJ, Pistorius AM, DeGrip WJ. Production of yeastolates for uniform stable isotope labelling in eukaryotic cell culture. *Appl Microbiol Biotechnol* 84(3):575–81, 2009.

[185] Meola A, Deville C, Jeffers SA, Guardado-Calvo P, Vasiliauskaite I, Sizun C, Girard-Blanc C, Malosse C, van Heijenoort C, Chamot-Rooke J, Krey T, Guittet E, Pêtres S, Rey FA, Bontems F. Robust and low cost uniform ^{15}N-labeling of proteins expressed in *Drosophila* S2 cells and *Spodoptera frugiperda* Sf9 cells for NMR applications. *J Struct Biol* 188(1):71–8, 2014.

[186] Opitz C, Isogai S, Grzesiek S. An economic approach to efficient isotope labeling in insect cells using homemade ^{15}N-, ^{13}C- and ^{2}H-labeled yeast extracts. *J Biomol NMR* 62(3):373–85, 2015.

[187] Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RHA, Heck AJR. Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nat Biotechnol* 21(8):927–31, 2003.

[188] Gouw JW, Pinkse MWH, Vos HR, Moshkin Y, Verrijzer CP, Heck AJR, Krijgsveld J. *In vivo* stable isotope labeling of fruit flies reveals post-transcriptional regulation in the maternal-to-zygotic transition. *Mol Cell Proteomics* 8(7):1566–78, 2009.

[189] Varman AM, He L, You L, Hollinshead W, Tang YJ. Elucidation of intrinsic biosynthesis yields using ^{13}C-based metabolism analysis. *Microb Cell Fact* 13(1):42, 2014.

[190] Neubauer S, Haberhauer-Troyer C, Klavins K, Russmayer H, Steiger MG, Gasser B, Sauer M, Mattanovich D, Hann S, Koellensperger G. U^{13}C cell extract of *Pichia pastoris*–a powerful tool for evaluation of sample preparation in metabolomics. *J Sep Sci* 35(22):3091–105, 2012.

[191] Smith JS, Hillier AJ, Lees GJ, Jago GR. The nature of the stimulation of the growth of *Streptococcus lactis* by yeast extract. *J Dairy Res* 42(01):123, 1975.

[192] Kilstrup M, Hammer K, Ruhdal Jensen P, Martinussen J. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* 29(3):555–90, 2005.

[193] Akuzawa R, Fox P. Purification and characterization of an acid phosphatase from cell membrane fraction of *Lactococcus lactis* ssp. *lactis* 303. *Food Res Int* 31(2):157–165, 1998.

[194] Oshima Y. The phosphatase system in *Saccharomyces cerevisiae*. *Genes Genet Syst* 72(6):323–34, 1997.

[195] Martinussen J, Hammer K. Powerful methods to establish chromosomal markers in *Lactococcus lactis*: an analysis of pyrimidine salvage pathway mutants obtained by positive selections. *Microbiology* 141 (Pt 8)(8):1883–90, 1995.

[196] Martinussen J, Wadskov-Hansen SLL, Hammer K. Two nucleoside uptake systems in *Lactococcus lactis*: competition between purine nucleosides and cytidine allows for modulation of intracellular nucleotide pools. *J Bacteriol* 185(5):1503–8, 2003.

[197] Lahtvee PJ, Adamberg K, Arike L, Nahku R, Aller K, Vilu R. Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. *Microb Cell Fact* 10(1):12, 2011.

[198] Jiranek V, Langridge P, Henschke PA. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *Am J Enol Vitic* 46(1):75–83, 1995.

[199] Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T. Glutathione accumulation in ethanol-stat fed-batch culture of Saccharomyces cerevisiae with a switch to cysteine feeding. *Appl Microbiol Biotechnol* 87(1):175–83, 2010.

[200] Rockwood AL, Van Orden SL. Ultrahigh-speed calculation of isotope distributions. *Anal Chem* 68(13):2027–30, 1996.

[201] Mashego MR, Wu L, Van Dam JC, Ras C, Vinke JL, Van Winden WA, Van Gulik WM, Heijnen JJ. MIRACLE: mass isotopomer ratio analysis of U-^{13}C-labeled extracts. A new method for accurate quantification of changes in concentrations of intracellular metabolites. *Biotechnol Bioeng* 85(6):620–8, 2004.

[202] Freisleben A, Schieberle P, Rychlik M. Specific and sensitive quantification of folate vitamers in foods by stable isotope dilution assays using high-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 376(2):149–56, 2003.

[203] Gregory JF. Accounting for differences in the bioactivity and bioavailability of vitamers. *Food Nutr Res* 56:1–12, 2012.

[204] Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Sinclair DA. Nicotinamide and *PNC1* govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* 423(6936):181–5, 2003.

[205] Trammell SA, Brenner C. Targeted, LCMS-based metabolomics for quantitative measurement of NAD^+ metabolites. *Comput Struct Biotechnol J* 4:e201301012, 2013.

[206] Hälvin K, Paalme T, Nisamedtinov I. Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay. *Anal Bioanal Chem* 405(4):1213–22, 2013.

[207] Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T. Stability of B-complex vitamins and dietary fiber during rye sourdough bread production. *J Cereal Sci* 57(1):30–38, 2013.

[208] Bieganowski P, Brenner C. Discoveries of nicotinamide riboside as a nutrient and conserved *NRK* genes establish a Preiss-Handler independent route to NAD^+ in fungi and humans. *Cell* 117(4):495–502, 2004.

[209] Gallo CM, Smith DL, Smith JS. Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol Cell Biol* 24(3):1301–12, 2004.

[210] Belenky P, Stebbins R, Bogan KL, Evans CR, Brenner C. Nrt1 and Tna1-independent export of NAD^+ precursor vitamins promotes NAD^+ homeostasis and allows engineering of vitamin production. *PLoS One* 6(5):e19710, 2011.

[211] Woodward JR, Cirillo VP. Amino acid transport and metabolism in nitrogen-starved cells of *Saccharomyces cerevisiae*. *J Bacteriol* 130(2):714–23, 1977.

[212] *Yeasts, Yeast Extracts, Autolysates and Related Products: The Global Market*. Tech. rep., BCC Research, 2014.

[213] Martini AE, Miller MW, Martini A. Amino acid composition of whole cells of different yeasts. *J Agric Food Chem* 27(5):982–4, 1979.

[214] Sarwar G, Shah BG, Mongeau R, Hoppner K. Nucleic acid, fiber and nutrient composition of inactive dried food yeast products. *J Food Sci* 50(2):353–357, 1985.

[215] Moat AG, Ahmad F, Alexander JK, Barnes IJ. Alteration in the amino acid content of yeast during growth under various nutritional conditions. *J Bacteriol* 98(2):573–8, 1969.

[216] Albers E, Larsson C, Lidén G, Niklasson C, Gustafsson L. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Appl Environ Microbiol* 62(9):3187–95, 1996.

[217] Merz M, Claaßen W, Appel D, Berends P, Rabe S, Blank I, Stressler T, Fischer L. Characterization of commercially available peptidases in respect of the production of protein hydrolysates with defined compositions using a three-step methodology. *J Mol Catal B Enzym* 127:1–10, 2016.

[218] Dambmann C, Mortensen SB, Budtz P, Eriksen S. Method for hydrolyzing proteins with gluyasp specific protease, 1999.

[219] Sherwood RF, Melton RG. Glutamate Carboxypeptidase. In: AJ Barrett, ND Rawlings, JF Woessner (eds.) *Handbook of Proteolytic Enzymes*, pp. 1576–1581. Elsevier, 3rd ed., 2013.

[220] Kilcawley K, Wilkinson M, Fox P. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. *Enzyme Microb Technol* 31(3):310–320, 2002.

CURRICULUM VITAE

Curriculum vitae

PERSONAL DATA

Name	Kaspar Kevvai
Date & place of Birth	2 June 1986, Tartu, Estonia
Citizenship	Estonian
E-mail	kaspar.kevvai@gmail.com
Phone	+372 51 38 470

WORK EXPERIENCE

March 2006 – present
Competence Center of Food and Fermentation Technologies, Tallinn, Estonia
Researcher

June 2012 – present
Bioexpert AS, Tallinn, Estonia
Service engineer

EDUCATION

2011 – present
Tallinn University of Technology, Tallinn, Estonia

PhD Chemistry and gene technology
Thesis: Applications of ^{15}N-labeled yeast hydrolysates in metabolic studies of *Lactococcus lactis* and *Saccharomyces cerevisiae*
Advisors: Prof. Toomas Paalme & Assoc. Prof. Ildar Nisamedtinov

2008 – 2010
Tallinn University of Technology

MSc Engineering physics / Applied mathematics
Thesis: Numerical simulation of densification front propagation in cellular materials using finite volume method

2005 – 2008
Tallinn University of Technology

BSc Engineering physics
Thesis: Yeast cultivation in ethanol-stat

SPECIAL COURSES

March 2014
SYSBIO2014 Advanced Lecture Course on Systems Biology
Innsbruck, Austria

August 2011
Basics in Interpretation of Mass Spectra
Nurmijärvi, Finland

TEACHING AND SUPERVISING

April – May 2012
March – April 2011
March – April 2010
University of Helsinki, Finland

Teaching assistant
Carried out practical hands-on courses on fermentation technology (ETT330 Fermentation Technology for graduate students).

2015
Polina Anisimova

MSc thesis co-supervisor
Thesis: Development of high protein yeast and yeast extract

OTHER INFORMATION

Honors

2008 · Estonian Revelia Academic Fund – Harald Raudsepp Scholarship
2007 · Development Fund of TUT – AS Saku Brewery Scholarship
2007 · Rotalia Foundation – Bruno and Helge Laan Scholarship
2006 · Development Fund of TUT – AS Hansapank Scholarship

Languages

Estonian · Mother tongue
English · Fluent
Russian · Intermediate
French · Basic

Elulookirjeldus

ISIKUANDMED

Nimi	Kaspar Kevvai
Sünniaeg & -koht	2. juuni 1986, Tartu, Eesti
Kodakondsus	Eesti
E-mail	kaspar.kevvai@gmail.com
Telefon	+372 51 38 470

TEENISTUSKÄIK

märts 2006 – . . .
AS Toidu- ja fermentatsioonitehnoloogia arenduskeskus, Tallinn
Teadur

juuni 2012 – . . .
Bioexpert AS, Tallinn
Hooldusinsener

HARIDUSKÄIK

2011 – . . .
Tallinna Tehnikaülikool

PhD Keemia ja geenitehnoloogia
Väitekiri: ^{15}N-märgistatud pärmihüdrolüsaatide rakendused *Lactococcus lactis*'e ja *Saccharomyces cerevisiae* ainevahetuse uurimisel
Juhendajad: Prof. Toomas Paalme & Dots. Ildar Nisamedtinov

2008 – 2010
Tallinna Tehnikaülikool

MSc Tehniline füüsika / Rakendusmatemaatika
Väitekiri: Tihenemisfrontide leviku numbriline simuleerimine tsellulaarsetes tahkistes lõplike mahtude meetodil

2005 – 2008
Tallinna Tehnikaülikool

BSc Tehniline füüsika
Väitekiri: Pärmi kultiveerimine etanoolstaadis

TÄIENDUSÕPE

märts 2014
SYSBIO2014 Advanced Lecture Course on Systems Biology
Innsbruck, Austria

august 2011
Basics in Interpretation of Mass Spectra
Nurmijärvi, Soome

ÕPETAMINE JA JUHENDAMINE

aprill – mai 2012
märts – aprill 2011
märts – aprill 2010
Helsingi Ülikool

Assistent Fermentatsioonitehnoloogia alase kursuse ETT330 (Fermentation technology) läbiviimine.

2015
Polina Anisimova

MSc kaasjuhendaja Väitekiri: Kõrge proteiinisisaldusega pärmi ja pärmiekstrakti arendus

MUU INFORMATSIOON

Tunnustused

2008 · Estonian Revelia Academic Fund – Harald Raudsepa nimeline stipendium
2007 · TTÜ arengufond – AS Saku Õlletehase stipendium
2007 · Rotalia Fond – Bruno ja Helge Laane nimeline stipendium
2006 · TTÜ arengufond – AS Hansapanga stipendium

Keelteoskus

EESTI · emakeel
INGLISE · kõrgtase
VENE · kesktase
PRANTSUSE · algtase

APPENDICES

PUBLICATION I

Kevvai K, Kütt ML, Nisamedtinov I, Paalme T

Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids

Antonie Van Leeuwenhoek, 105(3):511-522, (2014)

Antonie van Leeuwenhoek (2014) 105:511–522
DOI 10.1007/s10482-013-0103-2

ORIGINAL PAPER

Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids

Kaspar Kevvai · Mary-Liis Kütt · Ildar Nisamedtinov · Toomas Paalme

Received: 5 November 2013 / Accepted: 19 December 2013 / Published online: 4 January 2014

Abstract *Lactococcus lactis* subsp. *lactis* IL1403 was grown in medium containing unlabelled free amino acids and ^{15}N-labelled yeast hydrolysate to gain insight into the role of peptides as a source of amino acids under conditions where free amino acids are abundant. A mathematical model was composed to estimate the fluxes of free and peptide-derived amino acids into and out of the intracellular amino acid pool. We observed co-consumption of peptides and free amino acids and a considerable efflux of most free amino acids during growth. We did not observe significant differences between the peptide consumption patterns of essential and non-essential amino acids, which suggests that the incorporation of a particular amino acid is more dependent on its availability in a readily assimilated form than the organism's auxotrophy for it. For most amino acids the contribution of peptide-bound forms to the formation of biomass was initially between 30 and 60 % with the remainder originating from free amino acids. During the later stages of fermentation we observed a decrease in the utilization of peptide-bound amino acids, thus indicating that the more readily assimilated peptides are gradually exhausted from the medium during growth.

Keywords ^{15}N-labelling · *Lactococcus lactis* · Yeast hydrolysate · Peptides · Amino acids

List of symbols

C^i_{pool}; C^i_{prot}	Concentration of amino acid *i* in the intracellular pool of free amino acids and in the protein fraction, respectively (mol g^{-1}_{DW})
C^i_{AA}	Concentration of free amino acid *i* in the culture medium (mol L^{-1})
φ^i_{pool}; φ^i_{prot}	Fractional labelling of amino acid *i* in the intracellular pool of free amino acids and in the protein fraction, respectively
φ^i_{pep}; φ^i_{AA}	Fractional labelling of peptide-bound and free amino acid *i* in the culture medium, respectively

Electronic supplementary material The online version of this article (doi:10.1007/s10482-013-0103-2) contains supplementary material, which is available to authorized users.

K. Kevvai (✉) · M.-L. Kütt · I. Nisamedtinov
Competence Centre of Food and Fermentation Technologies, Akadeemia tee 15a, 12618 Tallinn, Estonia
e-mail: kaspar.kevvai@tftak.eu

K. Kevvai
Department of Chemistry, Tallinn University of Technology, Tallinn, Estonia

M.-L. Kütt · I. Nisamedtinov · T. Paalme
Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia

I. Nisamedtinov
Lallemand Inc., Montreal, QC, Canada

$r^i_{pep}; r^i_{AA}; r^i_{-AA}; r^i_{prot}$	Flux of peptide-bound amino acid *i* from the culture medium into the intracellular pool; of free amino acid *i* from the culture medium into the intracellular pool; of *i* from the intracellular pool into the culture medium; of *i* from the intracellular pool into the protein fraction (mol g^{-1}_{DW} h^{-1})
X	Concentration of biomass in the culture medium (g_{DW} L^{-1})
$\mu = \frac{dX}{Xdt}$	Specific growth rate (h^{-1})
P^i	Fraction of amino acid *i* obtained from peptides during growth
$\Delta_t C^i_X$	Concentration of amino acid *i* required for biomass synthesis (mol L^{-1})
$\Delta_t C^i_{-AA}$	Concentration change of free amino acid *i* in the culture medium as a result of its efflux from the cell (mol L^{-1})
Y_{XGlc}	Biomass yield relative to glucose consumption (g_{DW} g^{-1})
t	Experiment time (h)

Introduction

Lactic acid bacteria (LAB) are an important group of microorganisms widely used in both the food industry and in the production of various metabolic products and recombinant proteins. They are fastidious organisms that require an external source of essential amino acids and other nutrients. The supply of amino acids is facilitated by both a well-developed proteolytic system comprised of enzymes that degrade proteins and peptides, and transport systems for free amino acids and peptides.

In *Lactococcus lactis*, the cell wall-bound PrtP is considered to be the only proteinase responsible for the extracellular breakdown of proteins into peptides. These range from 4 to 30 residues with a minimal amount of smaller peptides and free amino acids (Juillard et al. 1995a; Konings 2002). The *prtP* genes can either be plasmid- or genome-encoded (Nissen-Meyer et al. 1992; Savijoki et al. 2006). However, some strains, including the plasmid free strain IL1403 used in this study, do not encode PrtP (Xie et al. 2004; Lamarque et al. 2011).

All strains of *L. lactis* are auxotrophic for a number of amino acids; for IL1403 these include leucine, isoleucine, valine, methionine, histidine, and arginine (Zhang et al. 2009). Both these as well as nonessential amino acids are transported into the cells in free and peptide-bound forms. In *lactococci*, the uptake of free amino acids is accomplished by means of symport systems (Leu, Ile, Val, Met, Ala, Gly, Ser, Thr, Lys, His, Pro, Tyr, Phe, and Cys), antiport systems (Arg/Orn, Lys), and ABC transporters (Glu and Gln, Asn, Asp, and Pro) (Konings et al. 1989; Konings 2002). In recent years, several of those (GlnPQ, BcaP, LysP, HisP, AcaP, and FywP) have been cloned and characterized (Schuurman-Wolters and Poolman 2005; den Hengst et al. 2006; Trip et al. 2012). In spite of the large body of knowledge about the free amino acid transport systems, the uptake mechanisms for a number of amino acids remain poorly understood. Also, several amino acids may compete with each other for the same transport system, e.g., Ile, Leu, Val, and Met in the BcaP system (Driessen et al. 1987; den Hengst et al. 2006).

Three peptide transport systems have been described in *L. lactis*: DtpT, Opp, and Opt. DtpT is a proton motive force (PMF)-driven transporter that catalyses the uptake of di- and tripeptides (Doeven et al. 2005). The Opp system belongs to the superfamily of ABC transporters and consists of five proteins (OppA, OppB, OppC, OppD, and OppF). It mediates the transport of peptides from 4 to at least 18 or, according to some data, up to 35 amino acid residues. The substrate specificity of the Opp system is determined by the receptor protein OppA (Tynkkynen et al. 1993; Detmers et al. 2000). Despite the presence of *opp* genes it was discovered that *L. lactis* IL1403 does not express OppA under any conditions and thus the Opt system is the only known transport system used for the utilization of oligopeptides in this strain (Doeven et al. 2005; Lamarque et al. 2004, 2011). Opt also belongs to the ABC transporter family but differs from Opp by the presence of two peptide-binding proteins OptS and OptA and peptide uptake specificity. Initially, the role of Opt was thought to be limited to the transport of hydrophobic di- and tripeptides (Foucaud et al. 1995). However, later studies implicated the role of Opt in the transport of oligopeptides with three to six amino acid

residues (Lamarque et al. 2011). After uptake into the cell, peptides are broken down into free amino acids by various intracellular peptidases that include endo-, amino-, tri- and dipeptidases (Christensen et al. 1999; Christensen and Steele 2003).

Peptides are usually considered nutritionally superior to free amino acids since the uptake of oligopeptides is believed to be energetically more favourable than the uptake of individual amino acids via their dedicated transport systems (Christensen and Steele 2003). In addition, a number of amino acids, released in excess during the hydrolysis of oligopeptides, are excreted from cells via specific amino acid transporters (Konings 2002). Because this efflux may occur by proton symport transporters it could also contribute to the generation of PMF. The capacity of cells to transport peptides is limited and, similar to some free amino acids, different peptides compete for the same transport systems (Smid and Konings 1990; Helinck et al. 2003). Thus, balancing the supply of amino acids and peptides during bioprocessing may improve the overall efficiency.

Yeast hydrolysates are an excellent source of amino acids, peptides, vitamins, and other nutrients and are often used as complex additives in the industrial cultivation of LAB. However, the performance of each type of hydrolysate is highly dependent on the nutritional requirements of a particular species or strain (Ummadi and Curic-Bawden 2010). Additionally, lot-to-lot variations of the same hydrolysate may also significantly influence the performance of a bioprocess, despite their apparently stable composition (Lobo-Alfonso et al. 2010). The reasons for such variations are poorly understood. Moreover, we currently have limited knowledge regarding the interplay of the various components within the proteolytic systems of LAB during growth in industrially relevant media (Benthin and Villadsen 1996), which often makes it challenging to optimize the media composition for a given bioprocess with a specific LAB species.

The aim of the present work was to measure the consumption patterns of free and peptide-bound amino acids by *L. lactis* subsp. *lactis* IL1403 grown on a medium containing a mixture of free amino acids supplemented with ^{15}N-labelled yeast hydrolysate and then use these consumption measurements in a flux model (Fig. 1) to gain further insight into the role of peptides as a source of amino acids under conditions where free amino acids are abundant.

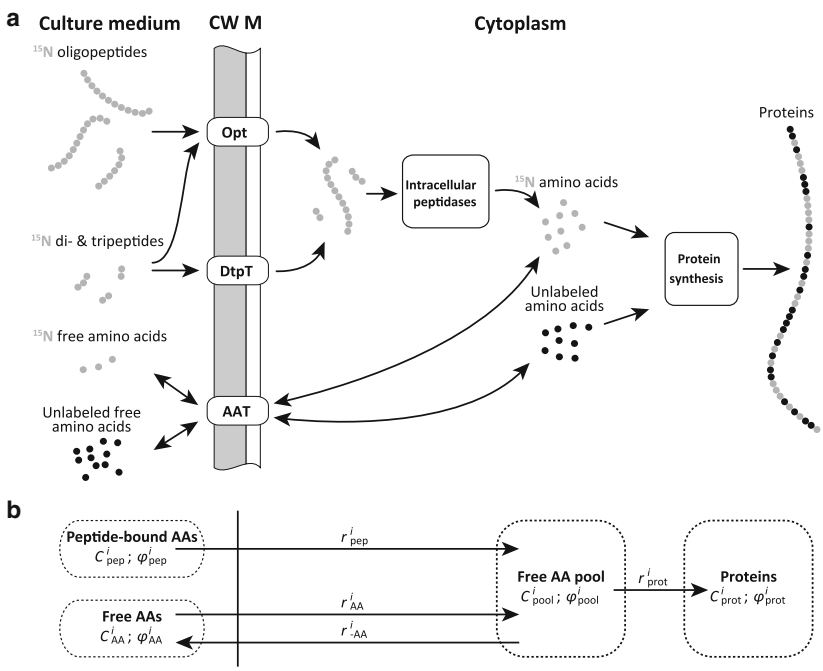

Fig. 1 (**a**) Simplified scheme of the amino acid and peptide utilization systems of *Lactococcus lactis* IL1403. *CW* cell wall, *M* cellular membrane, *Opt* ABC transporter for di-, tri-, and oligopeptides; *DtpT* ion-linked transporter for di- and tripeptides; *AAT* refers to the various free amino acid transporters. Note that efflux of amino acids may occur after their uptake in peptide-bound and free forms. The strain IL1403 lacks cell wall-bound proteinases. (**b**) Schematic representation of the flux model. See the "List of symbols" section for the relevant nomenclature

Materials and methods

Strain and culture media

The *Lactococcus lactis* subsp. *lactis* strain IL1403 used in this study was kindly provided by Dr. Ogier from INRA (Jouy-en-Josas, France). Inoculum was prepared using a freeze-dried stock culture stored at −80 °C which was pre-grown twice on the base medium without yeast hydrolysate.

A chemically defined medium based on the GIBCO™ F-12 Nutrient Mixture (Invitrogen Corporation, Carlsbad, CA) with some modifications (Lahtvee et al. 2011) was used as a base medium which was supplemented with 2 g L^{-1} of ^{15}N-labelled yeast hydrolysate. The concentration was chosen in order to achieve close-to-equal molar concentrations of peptide bound and free amino acids in the culture medium. Refer to Online Resource 1 for detailed medium composition.

Production of ^{15}N-labelled yeast hydrolysate

The ^{15}N-labelled yeast hydrolysate used in the experiments was produced by means of controlled proteolysis from *Saccharomyces cerevisiae* (strain S288c) biomass grown in fed-batch on a synthetic medium (Nisamedtinov et al. 2010) with ^{15}N-labelled (98 %) ammonium chloride (Sigma, USA) as the sole nitrogen source. The yeast biomass was freeze-dried and hydrolysed using a Promod 144GL protease preparation (Biocatalysts, Cardiff, UK). The extract was separated from the insoluble fraction by centrifugation. To remove any residual protease activity, the soluble fraction was further purified using Amicon Ultra 15 centrifugal filter devices (Millipore Corp., MA) with a 10,000 NMWL cut-off (by centrifugation for 45 min at 4,000×*g*). The resulting hydrolysate was freeze-dried. The lack of proteinase activity was confirmed using a Calbiochem Protease Assay Kit (Millipore Corp., USA).

Cultivation system and sampling routines

The cultivation system is comprised of a 1 L Biobundle bioreactor (Applikon, Schiedam, the Netherlands) controlled by an ADI 1030 biocontroller (Applikon) together with BioXpert NT software (Applikon). Cultivations were carried out under anaerobic conditions (surface aeration with N_2) at 34 °C and pH 6.4 (maintained by titration with 1 M NaOH) at an agitation speed of 300 rpm.

The concentration of biomass in the reactor was determined on the basis of the optical density of the culture medium (measured at 600 nm; biomass conversion factor $K = 0.372 \pm 0.005$ g L^{-1} AU_{600}^{-1}). Culture samples were withdrawn at 1 h intervals and collected on ice. The samples were centrifuged (20,000×*g*, 4 min, 4 °C); supernatants were collected and stored at −20 °C until analysis. The biomass pellet was washed twice using ice cold 0.9 % NaCl solution and stored at −20 °C until analysis.

Determination of the size distribution of peptides

The size distribution of peptides in the culture medium was characterized using size-exclusion chromatography (SEC). A Waters 1515 HPLC system (Waters Corp., Milford, MA) equipped with a Superdex Peptide 10/300GL column (GE Healthcare, NJ) and a Waters 2414 Refractive Index detector was used for analysis. Isocratic elution was applied using 0.1 N ammonium acetate (pH 5) as a mobile phase. Data acquisition was carried out using Breeze 3.30 software (Waters Corp.). The size distribution of culture medium components was determined based on the elution time of following molecular markers: alpha-lactalbumin (14,146 Da), aprotinin (6,512 Da), nisin (3,354 Da) and glutathione (307 Da).

Amino acid analyses with UPLC/ESI–MS

Amino acid concentrations in the biomass and culture medium and the respective mass spectra were determined using LCT Premier LC–MS system (Waters Corp.). Samples were derivatised with AccQ-Fluor reagent and loaded on AccQ-Tag™ Ultra columns (2.1 × 100 mm) at 55 °C (flow rate 0.3 mL min^{-1}) connected to a PDA detector ($\lambda = 260$ nm). Amino acids were separated using a 20 min gradient from 0.1 to 59.6 % B (A: AccQTag Ultra eluent A, B: AccQTag Ultra eluent B; Waters Corp.), sprayed directly into the mass-spectrometer operated in positive ionisation mode at 300 °C solvation temperature, 120 °C source temperature and 2.5 kV capillary voltage. Full mass spectra were acquired over the range of 100–1,000 *m*/*z*. MassLynx V 4.1 software (Waters Corp.) was used for data processing.

For measuring protein- and peptide-bound amino acid concentrations in the biomass and culture medium, the samples were hydrolysed with 6 M HCl containing 1 % phenol for 24 h at 105 °C. After hydrolysis the dried samples were dissolved in a methanol-MilliQ water mixture, filtered through Millex 0.2 µm filters (Millipore Corp.) and the free amino acids in the hydrolysate were analysed as described above. The concentrations of peptide-bound amino acids in the culture medium were calculated by subtracting the concentrations of free amino acids from the concentrations of total amino acids.

Calculation of fractional labelling of amino acids

Theoretical isotopic distributions (i.e., the relative abundances of ion species in the mass spectra) of labelled and unlabelled AccQ-Fluor derivatised amino acids were calculated using the algorithm described by Rockwood and Van Orden (1996).

The fractional labelling of each amino acid in the biomass and in the culture medium (i.e., the content of the ^{15}N-labelled amino acid relative to the total content of the respective amino acid) at each sampling point was estimated using least squares regression to fit a measured spectrum by theoretical isotope distribution patterns.

Analysis of peptide consumption patterns

Using the measured enrichment data, a mathematical model was developed to estimate the fluxes of free and peptide-bound amino acids (Fig. 1b; Eq. 1a–1f). The model assumes that no extracellular hydrolysis of peptides occurs during growth and the magnitude of de novo synthesis and turnover of amino acids is negligible.

The system of model equations is presented below:

$$\frac{d(C^i_{\text{pool}} \times \varphi^i_{\text{pool}} \times X)}{dt} = \left(r^i_{\text{pep}} \times \varphi^i_{\text{pep}} + r^i_{\text{AA}} \times \varphi^i_{\text{AA}} - r^i_{-\text{AA}} \times \varphi^i_{\text{pool}} - r^i_{\text{prot}} \times \varphi^i_{\text{pool}}\right) \times X; \quad (1a)$$

$$\frac{d(C^i_{\text{pool}} \times X)}{dt} = \left(r^i_{\text{pep}} + r^i_{\text{AA}} - r^i_{-\text{AA}} - r^i_{\text{prot}}\right) \times X; \quad (1b)$$

$$\frac{d(C^i_{\text{prot}} \times \varphi^i_{\text{prot}} \times X)}{dt} = r^i_{\text{prot}} \times \varphi^i_{\text{pool}} \times X; \quad (1c)$$

$$\frac{d(C^i_{\text{prot}} \times X)}{dt} = r^i_{\text{prot}} \times X; \quad (1d)$$

$$\frac{d(C^i_{\text{AA}} \times \varphi^i_{\text{AA}} \times X)}{dt} = \left(r^i_{-\text{AA}} \times \varphi^i_{\text{pool}} - r^i_{\text{AA}} \times \varphi^i_{\text{AA}}\right) \times X; \quad (1e)$$

$$\frac{d(C^i_{\text{AA}} \times X)}{dt} = \left(r^i_{-\text{AA}} - r^i_{\text{AA}}\right) \times X; \quad (1f)$$

Every model parameter is time dependent and all six sub-equations represent distinct material balances for amino acid i. Two balances were composed for each of three distinct pools of amino acids. These were, the intracellular pool of free amino acids (Eq. 1a, 1b), the amino acids in the protein fraction (Eq. 1c, 1d), and the extracellular pool of free amino acids (Eq. 1e, 1f). In each pool, separate balances were composed for labelled amino acids (Eq. 1a, 1c, 1e) and unlabelled amino acids (Eq. 1b, 1d, 1f).

The discrete measured data points for biomass concentration (X), the concentration (C) of each amino acid, and fractional labelling (φ) of each amino acid in each of the three pools were smoothed and fitted with cubic splines; the resulting continuous functions were used to solve for all model fluxes (r) as a function of time. The resulting solution forms the basis for further calculations.

The fraction of amino acids obtained from the peptides during growth (P^i) can be expressed as a ratio of the flux of peptide-bound amino acid i and the total flux of the respective amino acid into the intracellular amino acid pool:

$$P^i = \frac{r^i_{\text{pep}}}{r^i_{\text{AA}} + r^i_{\text{pep}}}. \quad (2)$$

The concentration of amino acid i required for biomass synthesis during the experiment can be expressed as follows:

$$\Delta_t C^i_X = \int_0^t C^i_{\text{prot}} \times \mu dt. \quad (3)$$

Concurrently, the concentration change of free amino acid i in the culture medium as a result of its efflux from the cell can be expressed as follows:

$$\Delta_t C^i_{-\mathrm{AA}} = \int_0^t r^i_{-\mathrm{AA}} \times X dt. \quad (4)$$

Results

The effect of yeast hydrolysate on the growth characteristics of *L. lactis* IL1403

To see the effect of the addition of yeast hydrolysate on the growth of *L. lactis*, batch experiments at $T = 34$ °C, pH 6.4 on a synthetic medium with and without the added hydrolysate were carried out. The addition of yeast hydrolysate resulted in a significantly higher maximum specific growth rate of the bacteria ($\mu_{max} = 0.86 \pm 0.02$ h^{-1} compared with $\mu_{max} = 0.62 \pm 0.01$ h^{-1}; average values of two independent experiments ± standard deviation). Chromatography analyses of the culture media suggested that in both experiments, growth ceased upon the exhaustion of glucose (see Fig. A1 in Online Resource 1 for growth curves). Growth in the medium supplemented with yeast hydrolysate also led to a higher final yield of biomass relative to glucose consumption than in the pure base medium, $Y_{X\mathrm{Glc}} = 0.22 \pm 0.01$ g g^{-1} and $Y_{X\mathrm{Glc}} = 0.14 \pm 0.01$ g g^{-1}, respectively.

Amino acid consumption profiles

In the cultivation with added ^{15}N-labelled yeast hydrolysate, the concentration of free and peptide-bound amino acids in the culture medium were determined at hourly intervals and presented in Fig. 2a. Asparagine, glutamine and arginine were the most intensively consumed free amino acids. Free arginine in particular was completely exhausted at the end of the experiment. The concentration of free serine, threonine, methionine, leucine, and isoleucine also decreased, whereas the concentration of ornithine, glycine, aspartate, and glutamate increased. The increasing concentration of ornithine along with the decreasing concentration of arginine indicates the activity of the arginine–ornithine antiport. Changes in the concentration of other free amino acids were within the range of measurement error. The concentration of all measured peptide-bound amino acids decreased during the experiment (Fig. 2b).

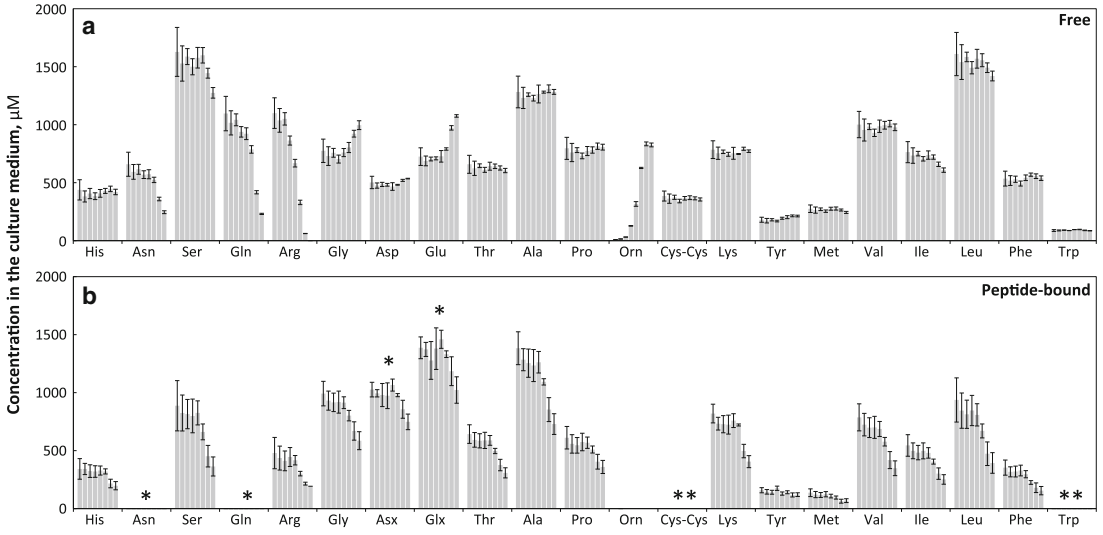

Fig. 2 Concentration of free (**a**) and peptide-bound (**b**) amino acids in the culture medium during the batch cultivation of *L. lactis* IL1403 on a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. Data from one of the two independent experiments is shown; each *column* indicates the concentration at hourly sampling points; measurements were carried out in triplicate and the *error bars* represent standard deviation. *asterisk* Glutamate and glutamine, asparagine and aspartate were pooled after hydrolysis; *double asterisk* cysteine and tryptophan were degraded during hydrolysis. The concentration of peptide-bound amino acids is expressed as the differences between the concentration of free and total (free plus bound) amino acids

^{15}N-labelling patterns of amino acids

In order to better understand the role of free and peptide-bound amino acids during growth, analyses of their labelling patterns in both the biomass and the culture medium were carried out. It was found that, with the exception of glutamate, the fractional labelling of all free amino acids in the culture medium increased during the experiment (Fig. 3a, dashed lines). This could result from either (a) extracellular hydrolysis of ^{15}N-labelled peptides (unlikely, because *L. lactis* IL1403 lacks cell wall-bound proteases); (b) efflux of labelled amino acids from the cell (due to excessive accumulation). Considering the latter scenario, the estimated amount of the excreted amino acid (Eq. 4) was in the same order of magnitude with what was theoretically required for biomass production (Eq. 3; see Fig. 3b for tyrosine and histidine as examples; refer to Fig. A2 in Online Resource 1 for other amino acids). From the second sampling point onwards, the fractional labelling of amino acids in the biomass were higher than of those in the culture medium (Fig. 3a), which suggests that the amino acids used for growth were at least partially obtained from (labelled) peptides.

Based on the labelling data presented above, the fractions of amino acids used for biomass production obtained from peptides were then calculated (Eq. 2; Fig. 4). For most amino acids the contribution of peptide-bound forms to the formation of biomass was initially between 30 and 60 % with the remainder originating from free amino acids. During the later stages of fermentation we observed a decrease in the utilization of amino acids originating from peptide-bound forms thus indicating that the more readily assimilated peptides are gradually exhausted from the medium during the cultivation.

Methionine was largely derived from peptide-bound forms during the first hours of both experiments. This suggests that the uptake of this amino acid in free form was inhibited. Both glutamine/glutamate and asparagine/aspartate (Glx and Asx, respectively) exhibited a consistently low incorporation of peptide-bound forms. This agrees with our observation that large amounts of free glutamine and asparagine were consumed (Fig. 2a). During growth, proline, histidine, and lysine were found to increasingly originate from peptide-bound forms.

Estimation of the size of consumed peptides

To estimate the upper size limit of the peptides consumed during the experiment, size-exclusion chromatography (SEC) analysis was carried out on samples of the culture media. The changes in the molecular weight distribution of the culture medium are illustrated in Fig. 5. The results indicated that the yeast hydrolysate used in the experiments contained compounds with molecular weights up to approximately 5,000 Da. However, significant consumption of media components with molecular weight of up to approximately 1,700 Da (corresponding to 15 amino acid residues in case of peptides) occurred.

Discussion

In this study we investigated the role of free and peptide-bound amino acids during the growth of *L. lactis* in a synthetic medium containing a mixture of free amino acids supplemented with ^{15}N-labelled yeast hydrolysate.

First, we analysed changes in the fractional labelling of the amino acids in the culture medium and biomass during growth. Two independent experiments indicated that, with the exception of glutamate, the ^{15}N-enrichment of all free amino acids in the culture medium increased during cultivation (Fig. 3a). This could be explained by extra- and/or intracellular hydrolysis of the labelled peptides. Extracellular hydrolysis is unlikely because strain IL1403 does not encode the cell wall-bound proteinase PrtP (Xie et al. 2004; Lamarque et al. 2011) and potential residual proteolytic activity in the yeast hydrolysate was removed by ultrafiltration (10 kDa cut-off). It could also be speculated that peptides were partially hydrolysed by intracellular peptidases leaking into the culture medium from lysed cells or, e.g., by the surface housekeeping proteinase HtrA (Poquet et al. 2000). However, a significant degree of autolysis is very unlikely under our experimental conditions and the role of HtrA is also probably negligible (Kok and Buist 2003). Thus, it can be assumed that the increasing fractional labelling was mainly caused by the over-accumulation of peptide-derived amino acids in the cells and subsequent excretion of the excess of those amino acids into the culture medium—a

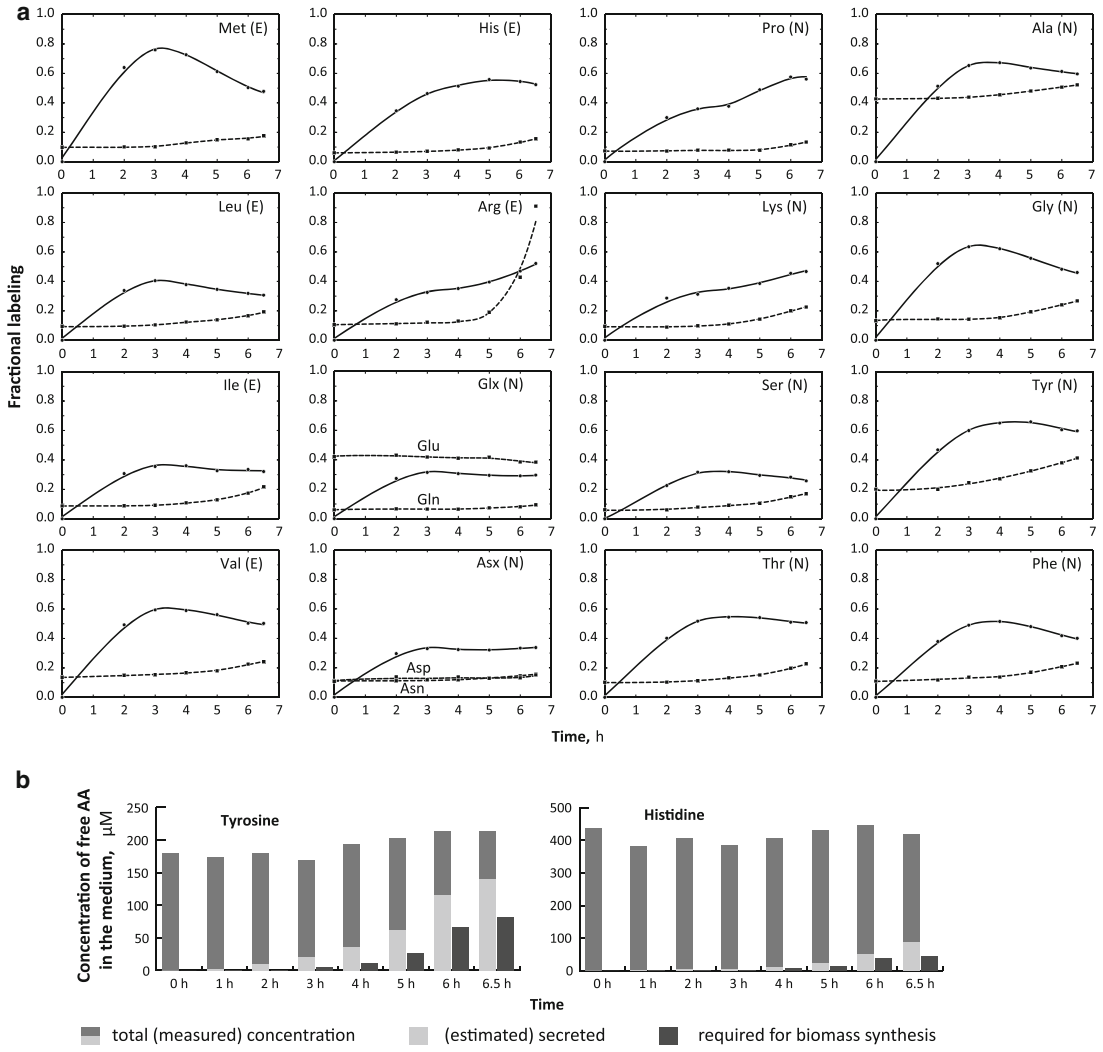

Fig. 3 **a** Fractional labelling of free amino acids in the culture medium (*dashed lines*) and biomass (*solid lines*) determined during the batch cultivation of *L. lactis* IL1403 on a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate; *E* and *N* denote whether the amino acid is considered essential or non-essential for *L. lactis* IL1403, respectively. **b** Concentration of free tyrosine and histidine in the culture medium. The *total column height* corresponds to the (measured) concentration of the respective amino acid (Fig. 2a) whereas the *light grey area* indicates the (estimated) concentration of secreted amino acid (Eq. 4), and the *black columns* indicate the theoretical concentration of amino acids required for the synthesis of biomass during the experiment (Eq. 3). Data from one of the two independent experiments is shown

phenomenon that has also been observed by authors who studied the growth of *L. lactis* in milk (Juillard et al. 1995b; Cretenet et al. 2011). Assuming that extracellular proteolytic activities were negligible, our calculations indicate that many amino acids are excreted in quantities that exceed the amount required for the formation of biomass (Fig. 3b). The efflux of excess amino acids has been proposed to be coupled to the excretion of a proton, which would result in the generation of metabolic energy in the form of proton motive force (Trip et al. 2012), and provide an additional means to maintain intracellular pH. The

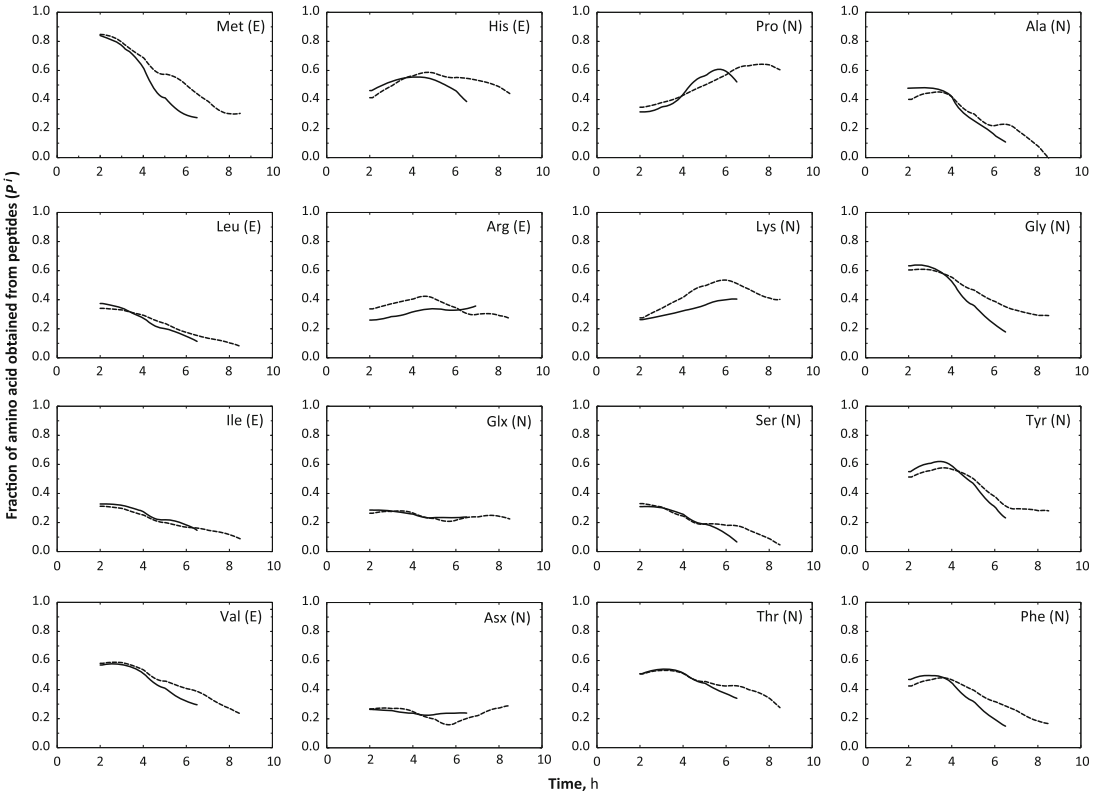

Fig. 4 Fraction of amino acids obtained from peptides during the growth of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. *Glx* Glutamine + Glutamic acid; *Asx* Asparagine + Aspartic acid. *E* and *N* denote whether the amino acid is considered essential or non-essential for *L. lactis* IL1403, respectively. Data from two independent experiments (indicated by the *solid* and *dashed lines*) with different inoculum sizes is shown

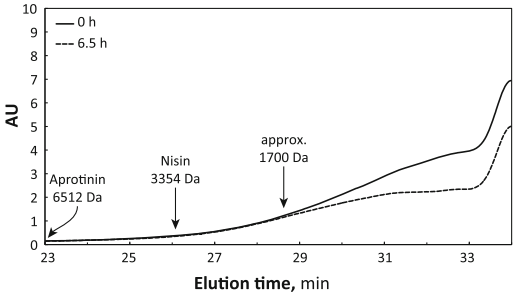

Fig. 5 Size-exclusion chromatograms of the culture medium collected during the cultivation of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. *Solid* and *dashed lines* show chromatograms at the beginning and end of the experiment, respectively. The *arrows* denote the elution times of two marker peptides, and the upper size limit of media components consumed during growth. Data from one of the two independent experiments is shown

PMF generated in this process could be used to bring in other amino acids and/or peptides. Thus, bidirectional fluxes of amino acids may help balance the intracellular amino acid pools under conditions where the de novo synthesis pathways and their feed-back control mechanisms are not operative.

Excluding extracellular hydrolysis, we estimated the size of peptides utilized during growth. Because the Opt system is the only known transporter in *L. lactis* IL1403 responsible for the uptake of oligopeptides, our results suggest that this system is capable of transporting peptides with a molecular weight up to approximately 1,700 Da, i.e., containing up to 15 amino acid residues (Fig. 5). This extends the findings of Lamarque et al. (2011). They concluded, based on studies with synthetic peptides, that the Opt system in IL1403 can transport peptides containing between

three and six amino acid residues. Because of the combinatorial nature of using synthetic peptides, it is possible that the apparent size limit observed by Lamarque and colleagues may be due to a limited number of tests. Our results suggest that much longer peptides can enter the cell.

Next, we studied the relative incorporation of free and peptide-bound amino acids into biomass. Our calculations assume that all amino acids used for growth are obtained from the culture medium. Jensen et al. (2002) confirmed that under conditions of amino acid surplus in the cultivation media, only aspartate is synthesized de novo by *L. lactis* in considerable amounts (likely from glucose via oxaloacetate). We observed that the consumption patterns of both essential and non-essential amino acids were similar, and co-consumption of free and peptide-bound forms occurs for all amino acids (Fig. 4). This suggests that the incorporation of a particular amino acid is more dependent on its availability in a readily assimilated form than the organism's auxotrophy for it. Nevertheless, the consumption profiles of amino acids between peptide-bound and free forms are noticeably different.

Glutamine and glutamate (Glx; Fig. 4), for example, exhibit a consistently low incorporation of peptide-bound forms. These amino acids are both translocated by the ATP-driven transporter GlnPQ, with glutamate taken up in its protonated form (i.e., as glutamic acid) (Poolman et al. 1987a; Schuurman-Wolters and Poolman 2005). Accordingly, under our culture conditions (pH 6.4), the apparent affinity of GlnPQ for glutamate is an order of magnitude lower than for glutamine, which results in the preferential translocation of the latter into the cells. Interestingly, glutamate was the only amino acid that exhibited no increase in the ^{15}N-enrichment in the cultivation medium during growth (Fig. 3a). This, together with its increasing concentration (Fig. 2a) and the observed low rate of incorporation from peptide-derived forms, suggest that a large part of the intracellular glutamate pool is synthesized from free glutamine (which displayed low albeit slightly increasing fractional labelling in the culture medium; Fig. 3a) and oxoglutarate via the glutamate synthase (gltB) reaction. Excess glutamate, in turn, was then excreted into the culture medium, resulting in an overall increase in the concentration of this amino acid during growth. The efflux of glutamate via the recently characterized AcaP transporter (Trip et al. 2012) may also result in the generation of PMF. The rate of incorporation of peptide-bound forms of asparagine and aspartate was also relatively low (Asx; Fig. 4). Like glutamic acid, aspartic acid is believed to be taken up only in its protonated form and the respective transport kinetics is thus dependent on pH (Poolman et al. 1987b). Thus, one could also speculate that proteinogenic aspartate was (partially) synthesized from asparagine via an asparaginase (ansB) catalysed reaction. These findings suggest that, despite the abundance of glutamate and aspartate (in both free and peptide-bound forms), efficient growth might not be achieved with this strain in case of insufficient supply of free glutamine and asparagine.

In contrast, the rate of incorporation of methionine in peptide-bound forms was over 0.8 during the first hours of the cultivation (Fig. 4). The uptake of free methionine is believed to occur via the secondary amino acid transporter BcaP that is specific for branched-chain amino acids (BCAAs) and (to a lesser extent) methionine (den Hengst et al. 2006). Because the concentrations of free BCAAs in the culture medium were several times higher than that of methionine (and the affinity of BcaP for methionine is lower) its uptake in free form was most likely inhibited by BCAAs. The observed decrease of the incorporation of peptide-bound methionine in the later stages of the cultivation could be explained by the exhaustion of the most readily assimilated methionine-containing peptides. Interestingly, the incorporation of peptide-bound BCAAs into the protein fraction (Fig. 4) was roughly proportional to the affinity constants of the BcaP system (K_t values 6.5, 8.0 and 12 μM for leucine, isoleucine and valine, respectively; Driessen et al. 1987), which is below 0.4 for Leu and Ile, and around 0.6 for Val during the first hours of the experiment. This further supports the notion that in cases where a transport system is shared, the less actively transported amino acids are more readily obtained from peptides. Glycine and alanine are also believed to enter the cell through a shared transport system (K_t values 52 and 330 μM for alanine and glycine, respectively; Konings et al. 1989), and their consumption profiles can be explained using the same principle, although the effect is much less pronounced.

The consumption of peptide-bound forms of a number of amino acids, in particular histidine, lysine, and proline, increased during cultivation (Fig. 4). The exact cause of this behaviour is unclear. The growth environment in batch culture is constantly changing

and transitions in the availability of various peptides could result in a situation where competitive inhibition results in the favoured translocation of peptides with certain characteristics. Helinck et al. (2003) found, for example, that charged casein-derived oligopeptides competitively inhibit the transport of a reporter oligopeptide in *L. lactis*. The increased consumption of peptide-bound amino acids can also be explained by down-regulation of the respective free amino acid transporters. Determining the mechanisms that lead to these peculiar peptide and free amino acid consumption patterns are beyond the scope of this work.

The labelling data reveal that when all amino acids are readily available in both free and peptide-bound forms, co-consumption of these forms takes place, possibly in order to optimally utilize the available cellular transport systems. Our data also indicate that an extensive efflux of amino acids occurs after their uptake from the culture medium. The exact physiological rationale of this phenomenon remains to be elucidated and could be revealed by conducting an accurate mass and energy balance in future studies. The labelling data also suggest that optimization of cultivation conditions and media composition may be possible. One could, for instance, vary both the concentration and composition of free amino acids and/or the labelled yeast hydrolysate in fed-batch or continuous culture experiments while acquiring accurate consumption profiles of free and peptide-bound amino acids. The relative consumption as a function of growth efficiency could be used to adjust the process used to produce yeast hydrolysates to create targeted and optimal yeast extracts for the industrial cultivation of a given microorganism.

Acknowledgments Financial support for this research was provided by the European Regional Development Fund (Project EU29994) and Estonian Ministry of Education (Research Grant SF0140090s08). The authors would like to thank Dr. Kaarel Adamberg (Tallinn University of Technology), and Dr. David Schryer (University of Tartu) for critical revision of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Benthin S, Villadsen J (1996) Amino acid utilization by *Lactococcus lactis* subsp. *cremoris* FD1 during growth on yeast extract or casein peptone. J Appl Microbiol 80:65–72

Christensen JE, Steele JL (2003) Impaired growth rates in milk of *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acid supplements. J Bacteriol 185:3297–3306

Christensen JE, Dudley EG, Pederson JA, Steele JL (1999) Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 76:217–246

Cretenet M, Laroute V, Ulvé V, Jeanson S, Nouaille S, Even S, Piot M, Girbal L, Le Loir Y, Loubière P, Lortal S, Cocaign-Bousquet M (2011) Dynamic analysis of the *Lactococcus lactis* transcriptome in cheeses made from milk concentrated by ultrafiltration reveals multiple strategies of adaptation to stresses. Appl Environ Microbiol 77:247–257

den Hengst CD, Groeneveld M, Kuipers OP, Kok J (2006) Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (CtrA). J Bacteriol 188:3280–3289

Detmers FJ, Lanfermeijer FC, Abele R, Jack RW, Tampe R, Konings WN, Poolman B (2000) Combinatorial peptide libraries reveal the ligand-binding mechanism of the oligopeptide receptor OppA of *Lactococcus lactis*. Proc Natl Acad Sci USA 97:12487–12492

Doeven MK, Kok J, Poolman B (2005) Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms. Mol Microbiol 57:640–649

Driessen AJ, de Jong S, Konings WN (1987) Transport of branched-chain amino acids in membrane vesicles of *Streptococcus cremoris*. J Bacteriol 169:5193–5200

Foucaud C, Kunji ER, Hagting A, Richard J, Konings WN, Desmazeaud M, Poolman B (1995) Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- and tripeptides. J Bacteriol 177:4652–4657

Helinck S, Charbonnel P, Foucaud-Scheunemann C, Piard JC, Juillard V (2003) Charged casein-derived oligopeptides competitively inhibit the transport of a reporter oligopeptide by *Lactococcus lactis*. J Appl Microbiol 94:900–907

Jensen NBS, Christensen B, Nielsen J, Villadsen J (2002) The simultaneous biosynthesis and uptake of amino acids by *Lactococcus lactis* studied by (13)C-labeling experiments. Biotechnol Bioeng 78:11–16

Juillard V, Laan H, Kunji ER, Jeronimus-Stratingh CM, Bruins AP, Konings WN (1995a) The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes beta-casein into more than one hundred different oligopeptides. J Bacteriol 177(12):3472–3478

Juillard V, Le Bars D, Kunji ER, Konings WN, Gripon JC, Richard J (1995b) Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. Appl Environ Microbiol 61:3024–3030

Kok J, Buist G (2003) Genetics of Proteolysis in *Lactococcus lactis*. In: Wood B, Warner P (eds) Genetics of lactic acid Bacteria. Springer, New York, pp 189–223

Konings WN (2002) The cell membrane and the struggle for life of lactic acid bacteria. Antonie Van Leeuwenhoek 82:3–27

Konings WN, Poolman B, Driessen AJ (1989) Bioenergetics and solute transport in *lactococci*. Crit Rev Microbiol 16:419–476

Lahtvee PJ, Adamberg K, Arike L, Nahku R, Aller K, Vilu R (2011) Multi-omics approach to study the growth efficiency

and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. Microb Cell Fact 10:1–12

Lamarque M, Charbonnel P, Aubel D, Piard JC, Atlan D, Juillard V (2004) A multifunction ABC transporter (Opt) contributes to diversity of peptide uptake specificity within the genus *Lactococcus*. J Bacteriol 186:6492–6500

Lamarque M, Aubel D, Piard JC, Gilbert C, Juillard V, Atlan D (2011) The peptide transport system Opt is involved in both nutrition and environmental sensing during growth of *Lactococcus lactis* in milk. Microbiology 157:1612–1619

Lobo-Alfonso J, Price P, Jayme D (2010) Benefits and limitations of protein extracts. In: Pasupuleti VK, Demain AL (eds) Protein hydrolysates in biotechnology. Springer, New York, pp 55–78

Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T (2010) Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding. Appl Microbiol Biotechnol 87:175–183

Nissen-Meyer J, Lillehaug D, Nes IF (1992) The plasmid-encoded lactococcal envelope-associated proteinase is encoded by a chromosomal gene in *Lactococcus lactis* subsp. *cremoris* BC101. Appl Environ Microbiol 58:750–753

Poolman B, Smid EJ, Konings WN (1987a) Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*. J Bacteriol 169:2755–2761

Poolman B, Driessen AJ, Konings WN (1987b) Regulation of solute transport in streptococci by external and internal pH values. Microbiol Rev 51:498–508

Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A (2000) HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol Microbiol 35:1042–1051

Rockwood AL, Van Orden SL (1996) Ultrahigh-speed calculation of isotope distributions. Anal Chem 68:2027–2030

Savijoki K, Ingmer H, Varmanen P (2006) Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 71:394–406

Schuurman-Wolters GK, Poolman B (2005) Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*. An ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. J Biol Chem 280:23785–23790

Smid EJ, Konings WN (1990) Relationship between utilization of proline and proline-containing peptides and growth of *Lactococcus lactis*. J Bacteriol 172:5286–5292

Trip H, Mulder NL, Lolkema JS (2012) Cloning, expression, and functional characterization of secondary amino acid transporters of *Lactococcus lactis*. J Bacteriol 195:340–350

Tynkkynen S, Buist G, Kunji E (1993) Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J Bacteriol 175:7523–7532

Ummadi M, Curic-Bawden M (2010) Use of protein hydrolysates in industrial starter culture fermentations. In: Pasupuleti VK, Demain AL (eds) Protein hydrolysates in biotechnology. Springer, New York, pp 91–114

Xie Y, Chou L, Cutler A, Weimer B (2004) DNA macroarray profiling of *Lactococcus lactis* subsp. *lactis* IL1403 gene expression during environmental stresses. Appl Environ Microbiol 70:6738–6747

Zhang G, Mills DA, Block DE (2009) Development of chemically defined media supporting high-cell-density growth of *lactococci*, enterococci, and streptococci. Appl Environ Microbiol 75:1080–1087

PUBLICATION II

Paalme T, Kevvai K, Vilbaste A, Hälvin K, Nisamedtinov I

Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture

World Journal of Microbiology and Biotechnology, 30(9):2351-2359, (2014)

World J Microbiol Biotechnol (2014) 30:2351–2359
DOI 10.1007/s11274-014-1660-x

ORIGINAL PAPER

Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture

T. Paalme · K. Kevvai · A. Vilbaste · K. Hälvin · I. Nisamedtinov

Received: 3 February 2014 / Accepted: 22 April 2014 / Published online: 30 April 2014

Abstract The uptake and accumulation of the B-group vitamins thiamine, riboflavin, nicotinamide, pantothenic acid and pyridoxine in *Saccharomyces cerevisiae* was studied by gradually increasing the specific dosage of vitamins in an ethanol-stat fed-batch culture. Thiamine, nicotinamide, pantothenic acid, and pyridoxine were almost completely taken up at low vitamin dosages. Thiamine was determined to be the major accumulating form of vitamin B_1 while most of the assimilated nicotinamide and pantothenic acid accumulated in cofactor forms. Despite the obvious uptake of pyridoxine, accumulation of B_6 vitamers was not observed. In contrast with the other vitamins studied, riboflavin began accumulating in the culture medium immediately after vitamin addition was initiated. By the end of the experiment, the apparent uptake of all vitamins exceeded their accumulation in the cells. Variations in the growth rate of yeast at different vitamin dosages demonstrate the importance of balancing the vitamins in the media during cultivation.

T. Paalme · A. Vilbaste · K. Hälvin · I. Nisamedtinov
Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia

K. Kevvai · A. Vilbaste · K. Hälvin · I. Nisamedtinov (✉)
Competence Centre of Food and Fermentation Technologies, Akadeemia tee 15a, 12618 Tallinn, Estonia
e-mail: inisamedtinov@lallemand.com

K. Kevvai
Department of Chemistry, Tallinn University of Technology, Tallinn, Estonia

I. Nisamedtinov
Lallemand, Inc., Montréal, QC, Canada

Keywords B-group vitamins · *Saccharomyces cerevisiae* · Fed-batch · LC–MS · Stable isotope dilution assay

Introduction

Baker's and brewer's yeasts are well known for their high content of B-group vitamins, which also justifies their use as nutritional supplements. Most wild-type strains of *Saccharomyces cerevisiae* are prototrophic for all B-group vitamins. The exception is biotin, the synthesis of which is a variable trait (Hall and Dietrich 2007). In addition to de novo synthesis, a number of vitamers can also be taken up from the growth environment. Considering the complexity of the vitamin synthesis pathways, the uptake of vitamins from the extracellular medium would allow cells to save intracellular resources for other biosynthetic processes.

Vitamin B_1 occurs in yeast as free thiamine and its phosphoesters thiamine phosphate, thiamine pyrophosphate, and thiamine triphosphate, with thiamine pyrophosphate being the main bioactive form. Thiamine is synthesized from pyridoxine and histidine, the precursors of its pyrimidine unit (Tazuya et al. 1989, 1993), and glycine and ribulose 5-phosphate, the precursors of its thiazole unit (Linnett and Walker 1968; White and Spenser 1979, 1982). In addition to de novo synthesis, yeasts can readily take up thiamine from the extracellular environment by the plasma membrane transporter Thi7p ($K_m = 0.18$ μM) (Enjo et al. 1997). In addition, low-affinity transport of thiamine via the nicotinamide riboside tranporter Nrt1p (Thi71p) (Enjo et al. 1997; Belenky et al. 2008; Li et al. 2010) as well as via Thi72p (Mojzita and Hohmann 2006) has been reported. The expression of genes encoding the enzymes involved in the metabolism of

thiamine (*THI* genes), including *THI7*, are coordinately repressed by exogenous thiamine and induced in its absence, whereas thiamine pyrophosphate seems to serve as a corepressor (Nishimura et al. 1991, 1992; Nosaka et al. 2005).

The de novo synthesis of riboflavin (vitamin B_2) and the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) occurs from guanosine 5′-triphosphate and ribulose 5-phosphate over a number of consecutive reactions catalysed by Rib1-7p, ATP-dependent riboflavin kinase (Fmn1p), and FAD synthase (Fad1p) (Oltmanns and Bacher 1972; Santos et al. 1995; Reihl and Stolz 2005). The uptake of riboflavin from the growth medium can occur by facilitated diffusion via the plasma membrane transporter Mch5p ($K_m = 17$ μM). In contrast with riboflavin auxotrophic strains, the uptake of riboflavin by commercial baker's yeast strains has been shown to be relatively poor, even under conditions where its extracellular concentration exceeded the K_m value by several times (Perl et al. 1976). Years later, it was suggested that the expression of *MCH5* is regulated by the intracellular flavin content (Reihl and Stolz 2005).

Nicotinamide adenine dinucleotides NAD^+ and $NADP^+$ are essential cofactors for cellular redox reactions and energy metabolism, the de novo synthesis of which can occur from tryptophan via kynurenine (Bedalov et al. 2003) or via the NAD^+ salvage pathway from the B_3 vitamers nicotinic acid and nicotinamide. In *S. cerevisiae,* the uptake of nicotinic acid from the culture medium is mediated by the high affinity permease Tna1p ($K_m = 1.7$ μM), the expression of which is inversely related to the extracellular concentration of nicotinic acid (Llorente and Dujon 2000). No transporters with nicotinamide affinity have been identified (Belenky et al. 2011).

Pantothenic acid (B_5) serves as a precursor for the synthesis of coenzyme A (CoA) in a five-step pathway with some organism-specific variations. The respective enzymes in yeast (Cab1-5p) have been identified (Olzhausen et al. 2009; Ruiz et al. 2009). *S. cerevisiae* is able to perform de novo synthesis of pantothenic acid from β-alanine and pantoate, which are derived from methionine and valine, respectively (White et al. 2001). *S. cerevisiae* can also acquire pantothenic acid from the surrounding medium by the proton symporter Fen2p ($K_m = 3.5$ μM). The transport rate has been shown to be modulated by the content of pantothenate in the growth medium, with maximal rate occurring at low concentrations (Stolz and Sauer 1999).

Pyridoxal 5-phosphate (PLP), the bioactive form of vitamin B_6, has multiple roles as a versatile cofactor of enzymes that almost exclusively function in the metabolism of amino compounds (Stolz and Vielreicher 2003). The B_6 vitamers pyridoxine, pyridoxine 5-phosphate, pyridoxamine, pyridoxamine 5-phosphate, and pyridoxal can all be converted to PLP via the PLP salvage pathway. The only known carrier of pyridoxine, pyridoxal, and pyridoxamine in *S. cerevisiae* is the proton symporter Tpn1p, which has high affinity for pyridoxine ($K_m = 0.55$ μM) (Stolz and Vielreicher 2003). Expression of *TPN1* is transcriptionally regulated, and is highly induced when either the pyridoxine concentration in the growth medium drops below the threshold concentration needed to support maximal growth, or in response to nitrogen depletion (Gasch et al. 2000).

Among other environmental factors, the assimilation of vitamins may also depend on the growth rate, the sources of carbon and nitrogen, and the state of metabolism (i.e., fermentative or respiratory growth), as occurs with the assimilation of riboflavin (Perl et al. 1976; Reihl and Stolz 2005). The majority of studies that measure B-group vitamin accumulation in yeast have focused on the uptake of one or two particular vitamins in shake flask cultures; studies which utilize industrially relevant fed-batch conditions are scarce. In this work we study the simultaneous assimilation of five B-group vitamins (thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine) by *S. cerevisiae* in an ethanol-stat cultivation using gradually increasing specific dosage of vitamins.

Materials and methods

Fed-batch cultivation with continuous addition of B-complex vitamins

This study utilizes commercial baker's yeast strain *S. cerevisiae* 210NG, kindly provided by AS Salutaguse Pärmitehas (Estonia). A 7 L "BioBench" fermenter (Applikon, The Netherlands), equipped with pH, pO_2, O_2, CO_2, and temperature sensors was used for cultivation. The ethanol concentration was measured online using a "Chemgard" infrared gas monitor (Mine Safety Appliances, USA) and cultivation conditions were controlled using the cultivation control software "BioXpert" (Applikon). Two variable speed pumps (Cole Parmer, USA) were used to pump the feeding media with and without the mixture of B-group vitamins. The feeding media vessels were placed on digital scales (Sartorius, Germany) to precisely determine the pumping rates. Cultivation was carried out at 30 °C and pH 4.75, maintained by titration with 1 M NH_4OH. The dissolved oxygen concentration (pO_2) was controlled at min 20 % of air saturation by adjusting the ratio of air and oxygen in the aeration gas mixture while the reactor was continuously stirred at 800 rpm.

The compositions of the cultivation media were based on those described previously by Nisamedtinov et al. (2010),

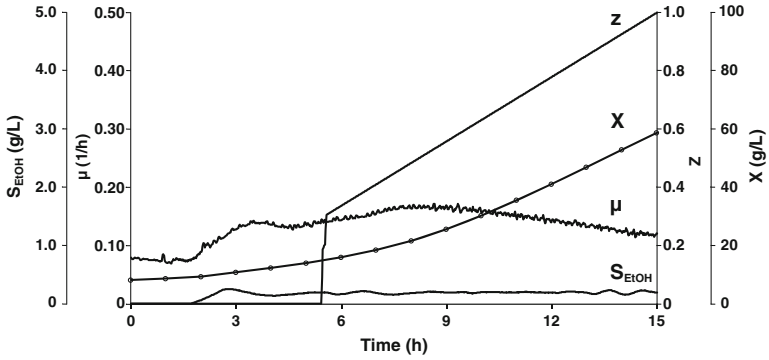

Fig. 1 Ethanol-stat cultivation of *S. cerevisiae* 210NG with gradual increase of B-group vitamins concentration in the feeding medium. *z*—the fraction of vitamin supplemented medium (*Medium 2*) in the feeding medium (in relative units); *X*—biomass concentration (g_{DW}/L), μ—specific growth rate (1/h); S_{EtOH}—ethanol concentration in the cultivation medium (g/L)

and modified in terms of vitamin content. The *Batch medium* contained 50 g/L glucose, 0.5 g/L NaCl, 0.5 g/L $CaCl_2$, 3 g/L KH_2PO_4, 2.5 g/L NH_4Cl, 0.8 g/L $MgSO_4{\cdot}7H_2O$, 1.9 mg/L $ZnSO_4{\cdot}7H2O$, 1.9 mg/L $FeSO_4{\cdot}7H_2O$, 0.6 mg/L $CuSO_4{\cdot}5H_2O$, 0.5 mg/L $MnSO_4{\cdot}H_2O$, 0.4 mg/L $CaCl_2{\cdot}H_2O$, 0.1 mg/L $CoSO_4{\cdot}7H_2O$, 25 μg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and vitamins (μM): biotin 2, myo-inositol 165. Upon completion of the batch phase and before the start of the fed-batch the following mineral salts were additionally added into the cultivation medium: 6 g NH_4Cl, 34 g KH_2PO_4 and 12 g $MgSO_4 \times 7H_2O$. Two feeding media (*Medium 1* and *Medium 2*) were used in the fed-batch phase of the experiment. Both media contained 400 g/L glucose, 30 mg/L $CaCl_2{\cdot}2H_2O$, 15 mg/L $ZnSO_4{\cdot}7H2O$, 15 mg/L $FeSO_4{\cdot}7H_2O$, 5 mg/L $CuSO_4{\cdot}5H_2O$, 4 mg/L $MnSO_4{\cdot}H_2O$, 3 mg/L $CaCl_2{\cdot}H_2O$, 1 mg/L $CoSO_4{\cdot}7H_2O$, 0.2 mg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. *Medium 1* contained (μM): biotin 2, myo-inositol 1400; *Medium 2* contained, in addition to biotin and myo-inositol, also (μM): thiamine 180, riboflavin 50, nicotinamide 2750, pantothenic acid, and pyridoxine 120. The vessel of *Medium 2* was covered with aluminium foil in order to prevent light-induced degradation of riboflavin.

The experiment was started by inoculating the *Batch medium* (2 L) with 20 mL of culture grown without vitamins (except biotin and myo-inositol). The yeast was then grown in pH- and pO_2-controlled batch culture until nearly all ethanol was consumed. Subsequently, the fed-batch phase was initiated using *Medium 1* in ethanol-stat mode (ethanol set-point, $S^{set}_{EtOH} = 0.2$ g/L) as described by Nisamedtinov et al. (2010). These conditions allow for cultivation near the maximum respiratory growth rate ($\mu \approx \mu_{crit}$). After stabilization of the culture (as indicated by constant μ), a gradual increase in the specific dosage of vitamins was applied by increasing the fraction of *Medium 2* in the feed from 30 to 100 % over 10 h (Fig. 1).

Sampling routines and determination of vitamer concentrations

The concentrations of vitamers in the biomass and culture medium were determined by LC–MS combined with a stable isotope dilution assay method (Hälvin et al. 2013; Mihhalevski et al. 2013). When available, commercial isotope labelled vitamers where used. For quantification of a number of vitamers (nicotinamide riboside, nicotinamide mononucleotide, and NAD^+) we used ^{15}N-labelled yeast hydrolysate, prepared as described by Kevvai et al. (2014). The vitamers were extracted from the freeze-dried hydrolysate with hot ammonium formate (0.05 M, 70 °C, pH 4.5) followed by separation in a Superdex™ Peptide 10/300 GL size-exclusion column (GE Healthcare, USA). The concentration of labelled vitamers was determined by LC–MS using unlabelled internal standards.

Culture samples were withdrawn from the fermenter at 1 h intervals. To determine the extracellular vitamin concentration, 1 mL of sample was immediately centrifuged at 18,000×*g* for 3 min, the supernatant was filtered through 0.2 μm filter and stored at −40 °C until analysis. For analysis of intracellular vitamer concentrations, the biomass samples (10 mL) were promptly quenched in 40 mL of 60 % MeOH-MilliQ solution (−40 °C) as described by Villas-Boas et al. (2005). The quenched samples were centrifuged at 18,000×*g* for 3 min. The yeast biomass pellets were washed twice with 60 % MeOH-MilliQ solution (−40 °C). The washed pellets were freeze-dried and stored at −40 °C until analysis. Vitamin extraction from lyophilized yeast samples (50 mg in five replicates) was carried out in 1 mL of 70 °C 0.05 M ammonium formate buffer (pH 4.5) for 30 min. The sample extracts were centrifuged at 18,000×*g* for 5 min at room temperature and the supernatants were filtered through 0.2 μm filters (Millipore, USA).

To determine the concentrations of free B-group vitamins, 1 mL aliquots of diluted sample extracts (dilution factor 5) were added into LC–MS vials containing isotope labelled internal standards and injected into LCT Premier LC–MS (Waters Corp., Milford, MA, USA). To determine nicotinamide riboside, nicotinamide mononucleotide, and NAD^+, 1 mL aliquots of diluted sample extracts (dilution factor 25) were added into LC–MS vials containing purified ^{15}N-labelled yeast hydrolysate and injected into the LC–MS. To determine the total concentration of vitamins, 2 mL aliquots of diluted sample extracts (dilution factor 5) were treated with 0.17 mg of potato acid phosphatase (Sigma, P1146) at 37 °C for 18 h. After incubation, the samples were centrifuged at 18,000×*g* for 5 min at room temperature, filtered through 0.2 μm filters, and analysed using LC–MS–TOF as described in Hälvin et al. (2013). The following ions were detected (*m*/*z*): thiamine—$[M]^+ = 265.11$, labelled thiamine—$[M]^+ = 268.12$, riboflavin—$[M + H]^+ = 377.15$, labelled riboflavin—$[M + H]^+ = 383.16$, nicotinamide—$[M + H]^+ = 123.06$, labelled nicotinamide—$[M + H]^+ = 127.08$, nicotinic acid—$[M + H]^+ = 124.04$, labelled nicotinic acid—$[M + H]^+ = 128.06$, pantothenic acid—$[M + H]^+ = 220.12$, labelled pantothenic acid—$[M + H]^+ = 224.13$, pyridoxal—$[M + H]^+ = 168.07$, labelled pyridoxal—$[M + H]^+ = 171.09$, pyridoxine—$[M + H]^+ = 170.08$, labelled pyridoxine—$[M + H]^+ = 174.10$, NAD^+—$[M]^+ = 664.08$, labelled NAD^+—$[M]^+ = 671.08$, NR^+—$[M]^+ = 255.09$, labelled NR^+—$[M]^+ = 257.09$, nicotinamide mononucleotide—$[M + H]^+ = 335.06$, labelled nicotinamide mononucleotide—$[M + H]^+ = 337.06$.

Calculation of culture parameters

The specific dosage of vitamer *i* [F_i (*t*)], mol/g_{DW} which shows the amount of a given vitamer added per unit of dry biomass in the fermenter was calculated as follows:

$$F_i(t) = \frac{\int_{t_0}^{t} S_i^F \cdot z(t) \cdot \text{pump}(t)dt}{X(t) \cdot V(t)}, \quad (1)$$

where S_i^F (M) is the concentration of vitamer *i* in the vitamin-supplemented feeding medium (*Medium 2*), $z(t)$ is the fraction of *Medium 2* in the feeding medium, pump(*t*) (L/h) is the total flow rate of the feeding medium into the reactor, $V(t)$ (L) is the current volume of the culture medium, and $X(t)$ (g/L) is the dry biomass concentration in the culture medium, t_0(h) is the fermentation time corresponding to the start of vitamin addition, *t*(h) is the current fermentation time.

The apparent specific uptake rate of vitamer *i* [$U_i(t)$, mol/(g_{DW}h)] shows the amount of vitamer *i* assimilated by the cells per unit dry biomass per unit time and was calculated as follows:

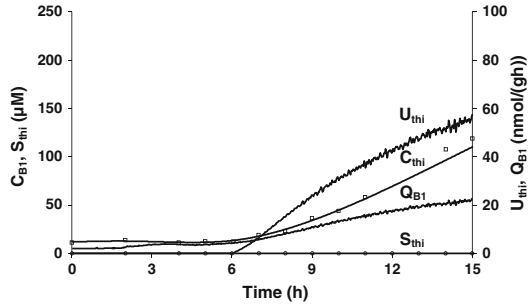

Fig. 2 Accumulation of vitamin B_1 in *S. cerevisiae* 210NG as the concentration of thiamine in the feeding medium was gradually increased. C_{thi}—concentration of thiamine in the biomass (μM); U_{thi}—apparent specific uptake rate of thiamine [nmol/(g_{DW}h)]; S_{thi}—concentration of thiamine in the culture medium (μM); Q_{B1}—specific accumulation rate of vitamin B_1 in the biomass [nmol/(g_{DW}h)]

$$U_i(t) = \mu(t) \cdot \left(F_i(t) - \frac{S_i(t)}{X(t)}\right), \quad (2)$$

where $\mu(t)$ (1/h) is the specific growth rate,

$$\mu = \frac{d(X \cdot V)}{X \cdot Vdt}. \quad (3)$$

The intracellular molar concentration of vitamer *i* (C_i, M) was calculated with the assumption that the intracellular water space in the yeast cells is constant at $v = 2.1$ mL/g_{DW} (Okada and Halvarson 1964).

The specific accumulation rate of vitamer *i* in the dry biomass [Q_i, mol/(g_{DW}h)] was calculated as follows:

$$Q_i = \mu \cdot C_i \cdot v. \quad (4)$$

Results

The experiment presented herein used ethanol-stat fed-batch culture which simulates the industrial production conditions of yeast for the purpose of studying the effect of increasing the specific dosage of B-group vitamins (Eq. 1) on the intracellular accumulation of each respective vitamer (Eq. 4). After all ethanol was consumed in the batch phase, the culture was switched to ethanol-stat fed-batch mode. The specific growth rate ($\mu \approx \mu_{crit}$) in the fed-batch after stabilization on a vitamin-free feeding medium (*Medium 1*) was 0.14 1/h and increased to 0.17 1/h with the gradual increase in the concentration of thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine in the feeding medium (Fig. 1). At higher specific dosages of vitamins and at higher biomass concentration, a slow decrease in the specific growth rate was observed.

The intracellular concentration of thiamine (C_{thi}) during growth on the vitamin-free medium (henceforth referred to as the 'de novo concentration') was 12.1 μM (Fig. 2;

Table 1 Intracellular concentrations of vitamers in an ethanol-stat culture on vitamin free medium and their maximum intracellular concentrations observed at the highest vitamer dosage at 15 h ($C_i^{\text{de novo}}$ and C_i^{15h}, respectively), the concentration of vitamers in the culture medium at the end of the experiment (S_i^{15h}), the maximum apparent uptake rate of vitamers (U_i^{15h}), the specific vitamin accumulation rate during growth without vitamins and at the highest vitamer dosage ($Q_i^{\text{de novo}}$, Q_i^{15h}, respectively)

Vitamer/vitamin	$C_i^{\text{de novo}}$ (μM)	C^{15h} (μM)	S^{15h} (μM)	U^{15h} nmol/(g_{DW}h)	$Q_i^{\text{de novo}}$ nmol/(g_{DW}h)	Q_i^{15h} nmol/(g_{DW}h)
Thiamine	12.1 ± 0.5	105 ± 18	<0.1	57	4	23
Total B_1 (Thi + ThiP + ThiPP)	13.0 ± 1.3	113 ± 21	<0.1	57	4	26
Riboflavin	5.9 ± 0.8	7.6 ± 1.8	4.0 ± 0.1	17	3	4
Total B_2 (Riboflavin + FMN + FAD)	40.8 ± 3.3	45.4 ± 2.6	4.0 ± 0.1	17	10	13
Nicotinamide (NAM)	59.6 ± 3.4	161 ± 17	0.5 ± 0.1	905	17	37
Nicotinic acid (NA)	9.7 ± 0.9	70.3 ± 8.6	8.4 ± 0.6	0	3	16
Nicotinamide riboside (NR)	8.3 ± 1.2	12.5 ± 3.6	N/A	0	2	3
Nicotinamide mononucleotide (NMN)	70.7 ± 4.8	106 ± 14	N/A	0	20	24
NAD	922 ± 53	2,152 ± 52	N/A	0	265	493
Total B_3 (NAM + NA + NR + NMN + NAD)	1,080 ± 63	2,497 ± 96	8.9 ± 0.6	905	307	573
Pantothenic acid	5.8 ± 0.6	62.7 ± 16.6	5.5 ± 0.1	101	2	13
Total B_5	N/A	N/A	N/A	101	N/A	N/A
Pyridoxine (PN)	0[a]	0[a]	10.1 ± 0.1	35	0	0
Pyridoxal (PL)	9.3 ± 1.2	16.5 ± 3.5	0[a]	0	3	4
PL + PLP	18.2 ± 1.6	25.1 ± 3.3	0[a]	0	5	7
Total B_6	N/A	N/A	N/A	35	N/A	N/A

N/A not analysed

[a] Below detection limit

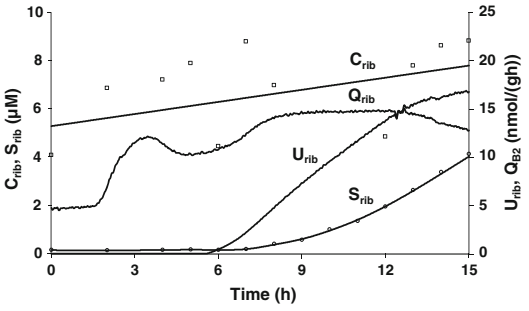

Fig. 3 Accumulation of vitamin B_2 in *S. cerevisiae* 210NG as the concentration of riboflavin in the feeding medium was gradually increased. C_{rib}—concentration of riboflavin in the biomass (μM); U_{rib}—apparent specific uptake rate of riboflavin [nmol/(g_{DW}h)]; S_{rib}—concentration of riboflavin in the culture medium (μM); Q_{B2}—specific accumulation rate of vitamin B_2 in the biomass [nmol/(g_{DW}h)]

Table 1), and accounted for over 90 % of the total de novo synthesized B_1 vitamers. This ratio remained largely unchanged over the course of the entire experiment. With an increase in the specific thiamine dosage, the intracellular vitamin B_1 concentration increased proportionally. The extracellular concentration of thiamine remained negligible ($S_{thi} < 0.1$ μM) throughout the experiment, which indicates its active uptake by the Thi7p transporter ($K_m = 0.18$ μM; Iwashima et al. 1973; Enjo et al. 1997). The apparent specific uptake rate of thiamine (U_{thi}) was over two times higher than the calculated accumulation rate of vitamin B_1 (Q_{B1}) in the cells.

The de novo concentration of riboflavin in the cells (C_{rib}) was around 5.9 μM (Fig. 3; Table 1) and approximately 20 % of the total B_2 vitamers determined. In contrast to thiamine, we did not observe an accumulation of riboflavin or the other B_2 vitamers in the cells while the riboflavin concentration in the feed was increased, even though the apparent uptake rate (U_{rib}) increased.

The de novo concentration of B_3 vitamers and cofactors was found to be two orders of magnitude higher than the other vitamins studied (1,080 μM; Table 1). The majority of the total B_3 pool existed as cofactor NAD^+ (922 μM). The concentrations of other B_3 vitamers were significantly lower: 9.7 μM for nicotinic acid (C_{NA}), 59.6 μM for nicotinamide (C_{NAM}), 8.3 μM for nicotinamide riboside (C_{NR}) and 70.7 μM for nicotinamide mononucleotide (C_{NMN}). With a gradual increase in the concentration of nicotinamide in the feed, the total intracellular concentration of B_3 vitamers and cofactors increased to 2,497 μM, mainly due to an increase in the concentration of NAD^+ (not illustrated). The extracellular concentration of nicotinamide

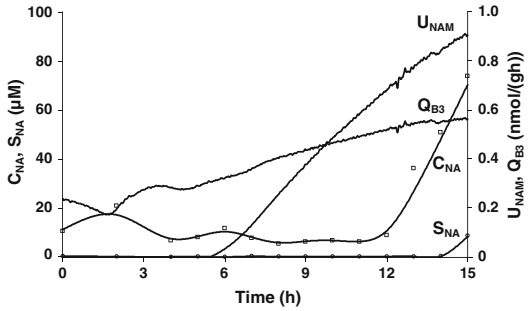

Fig. 4 Accumulation of vitamin B_3 in *S. cerevisiae* 210NG as the concentration of nicotinamide in the feeding medium was gradually increased. C_{NA}—concentration of nicotinic acid in the biomass (μM); U_{NAM}—apparent specific uptake rate of nicotinamide [nmol/(g_{DW}h)]; S_{NA}—concentration of nicotinic acid in the culture medium (μM); Q_{B3}—specific accumulation rate of vitamin B_3 in the biomass [nmol/(g_{DW}h)]

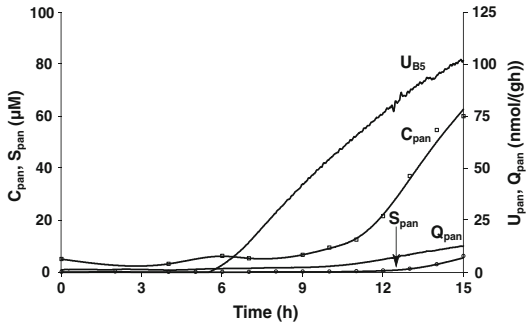

Fig. 5 Accumulation of pantothenic acid in *S. cerevisiae* 210NG as its concentration in the feeding medium was gradually increased. C_{pan}—concentration of pantothenic acid in the biomass (μM); U_{pan}—apparent specific uptake rate of pantothenic acid [nmol/(g_{DW}h)]; S_{pan}—concentration of pantothenic acid in the culture medium (μM); Q_{pan}—specific accumulation rate of pantothenic acid in the biomass [nmol/(g_{DW}h)]

remained close to zero over the course of the entire experiment (S_{NAM}, Fig. 4), suggesting that it was actively transported into the cells. Notably, we observed that nicotinic acid accumulated in the culture medium (S_{NA}) at high specific nicotinamide dosages. The specific uptake rate of nicotinamide (U_{NAM}) was higher than the accumulation rate of total vitamin B_3 (Q_{B3}).

The de novo concentration of pantothenic acid (C_{pan}) was 5.8 μM (Fig. 5; Table 1). The concentrations of other B_5 vitamers and cofactors (pantetheine and coenzyme A) were not determined in the present study, which at least

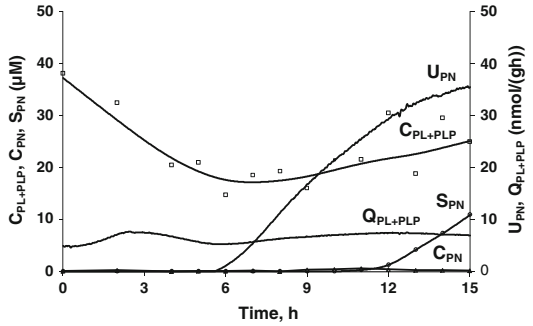

Fig. 6 Accumulation of vitamin B_6 (pyridoxal and pyridoxal phosphate) in *S. cerevisiae* 210NG as the pyridoxine concentration in the feeding medium was gradually increased. C_{PL+PLP}—concentration of pyridoxal and pyridoxal phosphate in the biomass (μM); C_{PN}—concentration of pyridoxine in the biomass (μM); U_{PN}—apparent specific uptake rate of pyridoxine [nmol/(g_{DW}h)]; S_{PN}—concentration of pyridoxine in the culture medium (μM); Q_{PL+PLP}—specific accumulation rate of pyridoxal and pyridoxal phosphate in the biomass [nmol/(g_{DW}h)]

partly explains why its apparent uptake rate (U_{pan}) was an order of magnitude higher than the accumulation rate of vitamin B_5 (Q_{B5}). As additional pantothenic acid was added in the feed, its extracellular concentration remained low (S_{pan} = 5.5 μM), suggesting near complete uptake. Despite this, its accumulation over the de novo concentration in the cells was only observed 3 h after the start of vitamin addition. At high specific dosages of pantothenic acid, we observed its accumulation in the culture medium, which suggests either saturation or feed-back repression of its transport system.

From the B_6 vitamers only pyridoxal and its phosphorylated form were determined to be above the detection limit in the biomass. Of these, pyridoxal phosphate accounted for nearly 50 % of the B_6 vitamer pool; the intracellular concentration of pyridoxine remained below the detection limit over the course of the entire experiment. The concentration of pyridoxal and pyridoxal phosphate (C_{PL+PLP}) at the end of the batch phase was over 35 μM (Fig. 6; Table 1). Notably, with the switch to the ethanol-stat culture without vitamins, the concentration of this pool decreased by approximately twofold to 18.2 μM. During the first stages (6–10 h) of vitamin feeding, the pyridoxine concentration in the culture medium was low, which suggests near-complete uptake. At higher specific dosages, it was found to accumulate in the medium. The specific uptake rate of pyridoxine (U_{PN}) was approximately five times higher than the specific accumulation rate of pyridoxal and pyridoxal phosphate (Q_{PL+PLP}).

Discussion

In the present work the effect of the gradual addition of B-group vitamins (thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine) on their rate of assimilation in *S. cerevisiae* was studied. An ethanol-stat fed-batch culture was used, simulating the conditions often applied in industrial baker's yeast production. The results suggest that while supplementation of the culture with these vitamins is not strictly required, adding them in optimal concentrations can increase the productivity of the process by increasing the specific growth rate. This positive effect can be related to the reduced amount of cellular resources required for de novo synthesis of vitamins and cofactors, as well as the biosynthetic enzymes. Although we only observed a modest increase in the maximum respiratory growth rate (0.03 1/h), it is possible that a more profound effect could be observed while increasing the dosage of vitamins at a reduced rate due to better adaptation. In addition, the relative concentration of some vitamins in the feeding medium may not have been optimal. For example, the de novo concentration of vitamin B_3 in the cells was approximately 100 times higher than that of vitamin B_1, however, the concentration of nicotinamide in the feed was only ten times higher than that of thiamine. It is possible that such imbalances in vitamin concentrations may lead to specific limitations or inhibitory effects.

At high specific vitamin dosages, the specific growth rate started to decrease slowly. This decrease could have been caused by over-accumulation of vitamins and cofactors. However, no growth inhibition could be demonstrated using shake flask experiments with the same vitamin concentrations as used in *Medium 2* (results not illustrated). Thus, the observed decreasing growth rate at high vitamin concentrations was most likely due to the inhibitory effects related to high biomass concentration. We have previously shown that at biomass concentrations higher than 50 g_{DW}/L, a slow and consistent decrease in the specific growth rate occurred (Nisamedtinov et al. 2010).

The gradual increase in the specific dosage of B-group vitamins clearly increased the total intracellular concentration of B_1, B_3, and B_5 vitamers and cofactors, whereas no remarkable accumulation of B_2 and B_6 vitamers over de novo values were observed (Table 1). During vitamin addition, a marked increase in the specific accumulation rates (Eq. 4) relative to the respective de novo values was observed for both B_1 and B_3 vitamers as well as for pantothenic acid (approximately six, two and sixfold, respectively). These observed differences in vitamin and cofactor accumulation rates could be even greater because the maximum values of intracellular vitamin and cofactor concentrations were not reached in the present study.

Although no nicotinamide transporters in *S. cerevisiae* have been identified, its low extracellular concentration ($S_{NAM}^{15h} = 0.5$ μM) compared to its concentration in the cells ($C_{NAM}^{15h} = 160$ μM) suggests that an active transporter should be involved. Interestingly, the intracellular concentration of nicotinamide exceeded that of nicotinic acid by nearly seven times during growth without vitamins, whereas the concentrations of these two vitamers became very similar during vitamin addition (Table 1; Fig. 4). This indicates that following its uptake, nicotinamide was converted into nicotinic acid in the nicotinamidase reaction and then used for NAD^+ synthesis. The observed increase in both the intra- and extracellular nicotinic acid concentrations indicates possible feed-back inhibition or saturation of the NAD^+ synthesis pathway. These results also fit with the findings of Belenky et al. (2011) who found that nicotinamide taken up by cells was readily converted to nicotinic acid and the excess of the latter was excreted from the cells in a Tna1p independent manner. Nicotinic acid may be excreted to mitigate toxic effects caused by its overaccumulation.

Saccharomyces cerevisiae was long considered a pantothenate auxotroph (Stolz and Sauer 1999; Leonian and Lilly 1942). However, White et al. (2001) showed its ability to synthesize it via a pathway involving methionine-derived spermine. The growth of yeast observed in this experiment with a vitamin-free feeding medium supports this fact. Uptake of pantothenic acid occurred immediately after its concentration in the feeding medium was increased; its intracellular concentration, however, started to increase only several hours later. This could be explained by the prompt conversion of imported pantothenic acid into coenzyme A, the concentrations of which in yeast, according to data of Seifar et al. (2013), are in the range of 200 μM, which is several times higher than the intracellular concentrations of pantothenic acid measured in our study (~6–60 μM). It is likely that the intracellular pantothenic acid concentration started to increase only after the coenzyme A pools were saturated.

Intracellular pyridoxine levels remained below detection limit throughout the experiment, suggesting its prompt conversion into other B_6 vitamers. However, we did not observe a remarkable increase in the intracellular concentrations of either pyridoxal or pyridoxal phosphate with an increase in pyridoxine dosage. This could result from partial condensation of pyridoxal phosphate with the amino acid substrate to form a Schiff base, a uniform component of all pyridoxal phosphate catalysed enzyme reactions (Eliot and Kirsch 2004). It is possible that pyridoxal from such aldimines may not have been released with the acid phosphatase treatment used in the present study. At higher specific dosages, we observed an accumulation of pyridoxine in the culture medium. It can be speculated that this

was due to saturation or down-regulation of Tpn1p. Stolz and Vielreicher (2003) showed a 15-fold difference in the expression of *TPN1* in pyridoxine free medium compared to a medium containing 2 μM of pyridoxine. Interestingly, we observed a gradual decrease in the intracellular concentration of total pyridoxal (C_{PL+PLP}) after the switch from batch to ethanol-stat fed-batch. The reasons behind this phenomenon remain unclear. It is possible that the decrease was induced by the switch from relatively slow growth on ethanol in the final stages of batch to higher growth rates in the ethanol-stat culture.

The intracellular concentration of riboflavin and related cofactors did not increase significantly with an increase in the specific dosage of riboflavin even though its apparent uptake rate reached a value close to that of its de novo accumulation rate (Fig. 6). By the end of the experiment the extracellular concentration of riboflavin (S_{rib}^{15h}) reached 4.0 μM which was close to the intracellular concentration (7.6 μM). Considering that the riboflavin translocation into the cells is believed to occur only by facilitated diffusion via Mch5p ($K_m = 17$ μM) (Reihl and Stolz 2005) its poor uptake into the cells under these experimental conditions could be expected. It is also possible that the concentration of free riboflavin was overestimated due to endogenous pyrohosphatase activitiy which could have released it from flavin nucleotides during sample preparation. Because *S. cerevisiae* wild type strains are invariably riboflavin prototrophs, our findings, together with previous studies, demonstrate the poor ability of *S. cerevisiae* to transport and accumulate riboflavin (Perl et al. 1976). This raises a general question regarding the physiological roles of plasma membrane transporter Mch5p.

A common feature of all vitamins studied in the present work, with the exception of riboflavin, was that their specific accumulation rates were lower than their respective apparent uptake rates. This was most probably due to an underestimation of vitamin and cofactor accumulation. Underestimation of vitamin and cofactor accumulation can occur due to losses during sample processing or due to vitamers that remain undetermined due to technical reasons. The latter was clearly the case for vitamin B_5, and most likely also B_6, where coenzyme A and protein-bound pyridoxal phosphate were not determined. However, underestimation of the accumulation of the other vitamins was probably not significant. Although the catabolic pathways of vitamins and cofactors are not described in yeast, vitamin turnover may also be a partial reason for the low recovery in some cases. Overestimation of vitamin uptake could result from the instability of vitamins in the culture medium, e.g. in case of light-induced degradation of riboflavin. Nevertheless, according to the information available about the stability of vitamins used in the present study (Troy and Beringer 2006), their degradation in the cultivation medium was most likely negligible.

The present study revealed significant differences in the uptake and accumulation between the B-group vitamins studied. The reasons underlying these differences remain to be elucidated. While in some cases this can be explained by the fact that some related compounds were simply not measured in the present work, in other cases it raises important questions regarding the fate of vitamers in *S. cerevisiae*. New (enzymatic) techniques probably need to be developed to determine all the vitamers in each group. Alternatively, vitamin catabolism may play a much more significant role than has previously been assumed. While our approach does not necessarily reveal the role of individual vitamins with regards to the observed changes in specific growth rate, the smooth increase in the dosage of specific vitamins is, nevertheless, a useful tool to study the accumulation of vitamers under industrially relevant conditions.

Acknowledgments This work was supported by the European Regional Development Fund (project EU29994), institutional research funding IUT1927 of the Estonian Ministry of Education and Research and Estonian Science Foundation research grant ETF9189. The authors would like to thank Dr. David Schryer (University of Tartu) for critical revision of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Bedalov A, Hirao M, Posakony J, Nelson M, Simon JA (2003) NAD^+-dependent deacetylase Hst1p controls biosynthesis and cellular NAD^+ levels in *Saccharomyces cerevisiae*. Mol Cell Biol 23:7044–7054

Belenky PA, Moga TG, Brenner C (2008) *Saccharomyces cerevisiae* YOR071C encodes the high affinity nicotinamide riboside transporter Nrt1. J Biol Chem 283:8075–8079

Belenky P, Stebins R, Bogan KL, Evans CR, Brenner C (2011) Nrt1 and Tna1-independent export of NAD^+ precursor vitamins promotes NAD^+ homeostasis and allows engineering of vitamin production. PLoS ONE 6:e19710

Eliot AC, Kirsch JF (2004) Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. Annu Rev Biochem 73:383–415

Enjo F, Nosaka K, Ogata M, Iwashima A, Nishimura H (1997) Isolation and characterization of a thiamin transport gene, *THI10*, from *Saccharomyces cerevisiae*. J Biol Chem 272:19165–19170

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257

Hall C, Dietrich FS (2007) The reacquisition of biotin prototrophy in *Saccharomyces cerevisiae* involved horizontal gene transfer, gene duplication and gene clustering. Genetics 177(4): 2293–2307

Hälvin K, Paalme T, Nisamedtinov I (2013) Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay. Anal Bioanal Chem 405:1213–1222

Iwashima A, Nishino H, Nose Y (1973) Carrier-mediated transport of thiamine in baker's yeast. Biochim Biophys Acta 330:222–234

Kevvai K, Kütt ML, Nisamedtinov I, Paalme T (2014) Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids. Antonie Van Leeuwenhoek. doi:10.1007/s10482-013-0103-2

Leonian LH, Lilly VG (1942) The effect of vitamins on ten strains of *Saccharomyces cerevisiae*. Am J Bot 29:459–464

Li M, Petteys BJ, McClure JM, Valsakumar V, Bekiranov S, Frank EL, Smith JS (2010) Thiamine biosynthesis in *Saccharomyces cerevisiae* is regulated by the NAD^+-dependent histone deacetylase Hst1. Mol Cell Biol 30:3329–3341

Linnett PE, Walker J (1968) Biosynthesis of thiamine. Incorporation experiments with ^{14}C-labelled substrates and with [^{15}N]glycine in *Saccharomyces cerevisiae*. Biochem J 109:161–168

Llorente B, Dujon B (2000) Transcriptional regulation of the *Saccharomyces cerevisiae* DAL5 gene family and identification of the high affinity nicotinic acid permease TNA1 (YGR260w). FEBS Lett 475:237–241

Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T (2013) Stability of B-complex vitamins and dietary fiber during rye sourdough bread production. J Cereal Sci 57:30–38

Mojzita D, Hohmann S (2006) Pdc2 coordinates expression of the THI regulon in the yeast *Saccharomyces cerevisiae*. Mol Genet Genomics 276(2):147–161

Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T (2010) Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding. Appl Microbiol Biotechnol 87:175–183

Nishimura H, Kawasaki Y, Nosaka K, Kaneko Y, Iwashima A (1991) A constitutive thiamine metabolism mutation, thi80, causing reduced thiamine pyrophosphokinase activity in Saccharomyces *cerevisiae*. J Bacteriol 173:2716–2719

Nishimura H, Kawasaki Y, Kaneko Y, Nosaka K, Iwashima A (1992) A positive regulatory gene, *THI3*, is required for thiamine metabolism in *Saccharomyces cerevisiae*. J Bacteriol 174:4701–4706

Nosaka K, Onozuka M, Konno H, Kawasaki Y, Nishimura H, Sano M, Akaji K (2005) Genetic regulation mediated by thiamin pyrophosphate-binding motif in *Saccharomyces cerevisiae*. Mol Microbiol 58:467–479

Okada H, Halvarson HO (1964) Uptake of α-thioethyl D-glucopyranoside by *Saccharomyces cerevisiae* I. The genetic control of facilitated diffusion and active transport. Biochim Biophys Acta 82:538–546

Oltmanns O, Bacher A (1972) Biosynthesis of Riboflavine in *Saccharomyces cerevisiae*: the role of genes *RIB1* and *RIB*7. J Bacteriol 110:818–822

Olzhausen J, Schubbe S, Schuller HJ (2009) Genetic analysis of coenzyme A biosynthesis in the yeast *Saccharomyces cerevisiae*: identification of a conditional mutation in the pantothenate kinase gene CAB1. Curr Genet 55(2):163–173

Perl M, Kearney EB, Singer TP (1976) Transport of riboflavin into yeast cells. J Biol Chem 251:3221–3228

Reihl P, Stolz J (2005) The monocarboxylate transporter homolog Mch5p catalyzes riboflavin (vitamin B_2) uptake in *Saccharomyces cerevisiae*. J Biol Chem 280:39809–39817

Ruiz A, Gonzalez A, Munoz I, Serrano R, Abrie JA, Strauss E, Arino J (2009) Moonlighting proteins Hal3 and Vhs3 form a heteromeric PPCDC with Ykl088w in yeast CoA biosynthesis. Nat Chem Biol 5(12):920–928

Santos MA, García-Ramírez JJ, Revuelta JL (1995) Riboflavin Biosynthesis in *Saccharomyces cerevisiae* cloning, characterization, and expression of the *RIB5* gene encoding riboflavin synthase. J Biol Chem 270:437–444

Seifar RM, Ras C, Deshmmukh AT, Bekers KM, Saurez-Mendez CA, da Cruz ALB, van Gulik WM, Hejinen JJ (2013) Quantitative analysis of intracellular coenzymes in *Saccharomyces cerevisiae* using ion pair reversed phase ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr A 1311:115–120

Stolz J, Sauer N (1999) The fenpropimorph resistance gene *FEN2* from *Saccharomyces cerevisiae* encodes a plasma membrane H1-pantothenate symporter. J Biol Chem 274:18747–18752

Stolz J, Vielreicher M (2003) Tpn1p, the plasma membrane vitamin B_6 transporter of *Saccharomyces cerevisiae*. J Biol Chem 278:18990–18996

Tazuya K, Yamada K, Kumaoka H (1989) Incorporation of histidine into the pyrimidine moiety of thiamin in *Saccharomyces cerevisiae*. Biochim Biophys Acta 990:73–79

Tazuya K, Yamada K, Kumaoka H (1993) Pyridoxine is a precursor of the pyrimidine moiety of thiamin in *Saccharomyces cerevisiae*. Biochem Mol Biol Int 30:893–899

Troy DB, Beringer P (2006) Remington: the science and practice of pharmacy. Lippincott Williams & Wilkins, Philadelphia

Villas-Boas SG, Mas S, Akesson M, Smedsgaad J, Nielsen J (2005) Mass spectrometry in metabolome analysis. Mass Spectrom Rev 24:613–646

White RL, Spenser ID (1979) Thiamin biosynthesis in *Saccharomyces cerevisiae*. Origin of carbon-2 of the thiazole moiety. Biochem J 179:315–325

White RL, Spenser ID (1982) Thiamin biosynthesis in yeast. Origin of the five-carbon unit of the thiazole moiety. J Am Chem Soc 104:4934–4943

White WH, Gunyuzlu PL, Toyn JH (2001) *Saccharomyces cerevisiae* is capable of de Novo pantothenic acid biosynthesis involving a novel pathway of β-alanine production from spermine. J Biol Chem 276:10794–10800

PUBLICATION III

Kevvai K, Kütt ML, Nisamedtinov I, Paalme T

Simultaneous utilization of ammonia, free amino acids and peptides during fermentative growth of *Saccharomyces cerevisiae*

Journal of the Institute of Brewing, 122(1):110-115, (2016)

Research article

Institute of Brewing & Distilling

Received: 26 February 2015 Revised: 3 August 2015 Accepted: 8 September 2015 Published online in Wiley Online Library: 6 January 2016

(wileyonlinelibrary.com) DOI 10.1002/jib.298

Simultaneous utilization of ammonia, free amino acids and peptides during fermentative growth of *Saccharomyces cerevisiae*

Kaspar Kevvai,[1,2]* Mary-Liis Kütt,[1,2] Ildar Nisamedtinov[1,2,3] and Toomas Paalme[2]

The efficiency of nitrogen use by yeast is one of the key determinants of the successful completion of alcoholic fermentations. In this work the growth of *Saccharomyces cerevisiae* S288c in a synthetic medium containing ammonia and free amino acids, supplemented with yeast hydrolysate, was studied. Experiments with $^{15}NH_4Cl$ and ^{15}N-labelled yeast hydrolysate were carried out to gain insight into which of these three classes of assimilable nitrogen sources yeast cells prefer. Co-consumption of all three sources was observed; approximately 40% of the total nitrogen in the yeast protein fraction originated from yeast hydrolysate, while free amino acids and ammonia contributed 40 and 20%, respectively. The results indicate that several amino acids are more readily obtained from peptides, most likely when the uptake of their free forms is competitively inhibited and/or repressed. During the second half of each fermentation, a decrease in the incorporation of yeast hydrolysate-derived nitrogen was observed. These results highlight the nutritional role of peptides in various yeast fermentations.

Addtional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: ^{15}N-labelling; yeast hydrolysate; yeast assimilable nitrogen

Introduction

The yeast *Saccharomyces cerevisiae* is capable of synthesizing all amino acids *de novo* and can utilize a variety of nitrogen sources, including free amino acids, small peptides, and ammonia, which together are referred to as yeast assimilable nitrogen (YAN). Once taken up, these compounds can be used either directly for biosynthetic purposes, for generation of ammonium through deamination, or serve as substrates for transaminases that transfer amino groups to α-ketoglutarate to form glutamate *(1)*. The latter can then act as a donor of amino groups during the synthesis of most amino acids. Ammonia and amino acids can be classified based on their ability to sustain high or low specific growth rates, or based on their preference by yeast cells in the presence of other nitrogen sources *(2,3)*. The assimilation preferences of peptides in the presence of free amino acids and ammonia in this organism have remained rather unclear despite a wealth of knowledge regarding the function of the peptide transport systems *(4–8)*.

Understanding the utilization of peptides by *S. cerevisiae* is important because peptides are one of the major nitrogen sources in several food fermentation processes involving yeast, including brewing and winemaking. Wort naturally contains a wide variety of peptides which, together with amino acids and ammonia, are formed during the proteolysis of malt proteins *(9)*. During wine production, various 'inactive dry yeast' preparations are accepted as yeast nutrients which provide additional YAN compounds, including peptides, to the fermenting must *(10)*. Despite growing interest in yeast fermentation aids in the wine industry, many of their mechanisms of action remain unclear. Moreover, different industrial strains of *S. cerevisiae* have strongly varying nitrogen requirements *(11)*, complicating the assessment of optimum doses of additional YAN sources.

In this study we aimed to determine which YAN sources (ammonia, free amino acids and peptides) *S. cerevisiae* cells prefer under conditions of fermentative growth in a synthetic medium supplemented with yeast hydrolysate. We used a novel method that makes use of ^{15}N-labelled media components ($^{15}NH_4Cl$ and ^{15}N-labelled yeast hydrolysate) to determine the YAN consumption preferences (Fig. 1).

Materials and methods

Strain and culture media

S. cerevisiae strain S288c was used in all experiments. Inoculum was prepared using a freeze-dried stock culture stored at −80°C which was pre-grown on a chemically defined medium (based on the synthetic grape juice medium described by Jiranek et al. *(12)*, but diluted fourfold; see Supporting Information for detailed medium composition), containing 194 mg_{eqv} N L^{-1} in the form of free amino acids. The main fermentation experiments were carried out on the same medium supplemented with ammonium chloride (196 mg_{eqv} N L^{-1}) and yeast hydrolysate (2 g L^{-1}; 170 mg_{eqv} N L^{-1} in the form of free and peptide-bound amino acids).

* Correspondence to: K. Kevvai, Competence Centre of Food and Fermentation Technologies, Tallinn, Estonia. E-mail: kaspar.kevvai@tftak.eu

[1] Competence Centre of Food and Fermentation Technologies, Tallinn, Estonia

[2] Tallinn University of Technology, Tallinn, Estonia

[3] Lallemand Inc., Montréal, QC, Canada

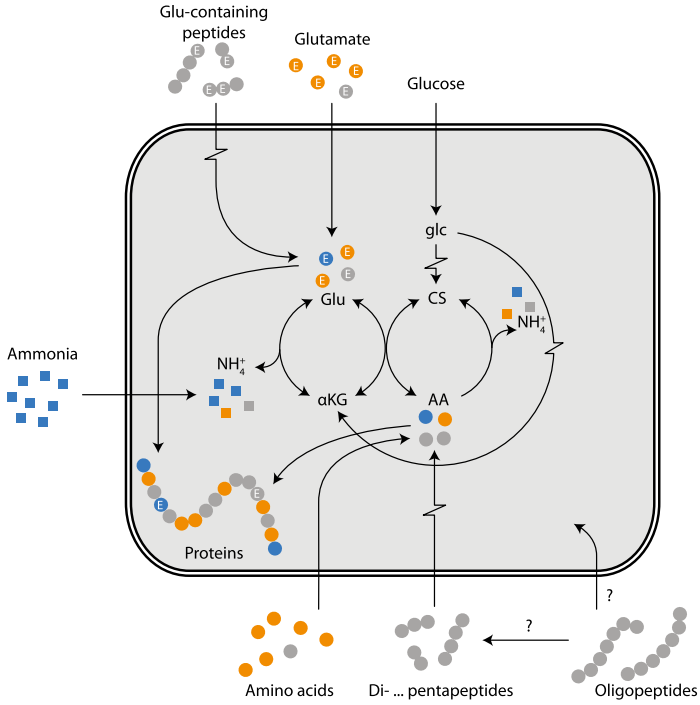

Figure 1. Simplified scheme of the ammonia, amino acid, and peptide utilization systems of *Saccharomyces cerevisiae*. CS, Carbon skeleton of amino acid; αKG, α-ketoglutarate; AA, any amino acid; E, glutamate. Arrows with breaks indicate processes with more than one step; unknown processes are denoted with question marks; colours are used to differentiate between nitrogen originating from ammonia (blue), free amino acids (orange) and yeast hydrolysate (grey). In two experiments, either ammonia or yeast hydrolysate was ^{15}N-labelled. Monitoring the incorporation of labelled amino acids into yeast protein fraction allows one to estimate the relative contribution of different nitrogen sources.

Two experiments were performed, each with the same medium composition and different isotopically labelled compounds: (a) $^{15}NH_4Cl$ + unlabelled free amino acids + unlabelled yeast hydrolysate; and (b) ^{15}N-labelled yeast hydrolysate + unlabelled NH_4Cl + unlabelled free amino acids. Both the labelled and unlabelled yeast hydrolysates used in the experiments were prepared according to the same protocol *(13)*.

Cultivation system and sampling routines

All cultivations were carried out in 1 L Biobundle bioreactors (Applikon, Schiedam, the Netherlands) controlled by Applikon EZ-control biocontrollers together with BioXpert XP software (Applikon). Fermentation experiments were carried out at 30°C and pH 4.0 (maintained by titration with 2 M NaOH) at an agitation speed of 500 rpm. Surface aeration was used (100 mL min^{-1} air) to provide micro-aerobic conditions.

The concentration of biomass in each reactor was determined on the basis of the optical density of the culture medium (measured at 600 nm; biomass conversion factor $K = 0.290 \pm 0.005$ g L^{-1} AU_{600}^{-1}). Culture samples were withdrawn at 1 h intervals and collected on ice. The samples were centrifuged (20,000*g*, 4 min, 4°C); their supernatants were collected and stored at −20°C until analysis. The biomass pellet was washed twice using ice-cold 0.9% NaCl solution and stored at −20°C until analysis.

Determination of the size distribution of peptides

The size distribution of peptides in the culture medium was characterized using size-exclusion chromatography. Analyses were performed using a Waters 515 HPLC pump (Waters Corp., Milford, MA, USA), a Superdex Peptide 10/300GL column (GE Healthcare, NJ, USA) and a Waters 2414 Refractive Index detector. Isocratic elution was applied using 0.1 M ammonium acetate (pH 5) as the mobile phase. Data acquisition was carried out using Empower software (build 1154; Waters Corp.). The size distribution of culture medium components was determined based on the elution time of the following molecular markers: alpha-lactalbumin (14,146 Da), aprotinin (6512 Da), nisin (3354 Da) and glutathione (307 Da).

Amino acid analyses with UPLC/ESI-MS

Amino acid concentrations in the biomass and culture medium and their respective mass spectra were determined using LCT Premier LC-MS system (Waters Corp.). Samples were derivatized with AccQ-Fluor reagent and loaded on AccQ-Tag™ Ultra columns (2.1 × 100 mm) at 55°C (flow rate 0.3 mL min^{-1}) connected to a PDA detector ($\lambda = 260$ nm). Amino acids were separated using a 20 min gradient from 0.1 to 59.6% B (A, AccQTag Ultra eluent A; B, AccQTag Ultra eluent B; Waters Corp.), sprayed directly into the mass spectrometer operated

in positive ionization mode at 300°C solvation temperature, 120°C source temperature and 2.5 kV capillary voltage. Full mass spectra were acquired over the range of 100–1000 *m/z*. Data processing was performed using MassLynx V 4.1 software (Waters Corp.).

To measure the combined protein- and peptide-bound amino acid concentrations in both the biomass and culture medium, samples were hydrolysed with 6 M HCl that contained 1% phenol for 24 h at 105°C. After hydrolysis, the samples were dried and dissolved in a methanol-MilliQ water mixture and filtered through Millex 0.2 μm filters (Millipore Corp.); the amino acids in this permeate were analysed as described above. The concentration of each peptide-bound amino acid in the culture medium was calculated by subtracting its free concentration from the total concentration determined after acid hydrolysis.

Determination of proteinase activity in samples

The proteinase activity in the cell-free culture supernatants was determined using a Calbiochem Protease Assay Kit (Millipore Corp., USA) according to manufacturer's instructions.

Analysis of YAN consumption patterns

Theoretical relative abundances of ion species in the mass spectra of ^{15}N-labelled and unlabelled (naturally labelled) AccQ-Fluor-derivatized amino acids were calculated using the algorithm described by Rockwood and Van Orden *(14)*. The fractional labelling of each amino acid in the biomass and in the culture medium (i.e. the content of the ^{15}N-labelled amino acid relative to the total content of the respective amino acid) at each sampling point was estimated by performing a least squares regression to fit the measured spectrum to the theoretical isotope distribution patterns.

The fractional contributions of yeast hydrolysate $\left(f^i_{YH}\right)$, ammonia $\left(f^i_{NH3}\right)$, and free amino acids $\left(f^i_{AA}\right)$ to the accumulation of amino acid *i* in the cells at each sampling point *t* were calculated as follows:

$$f^i_{YH}(t) = \frac{\varphi^i_{YH}(t)}{\varphi^i_{max}(t)}; \quad (1)$$

$$f^i_{NH3}(t) = \frac{\varphi^i_{NH3}(t)}{\varphi^i_{max}(t)}; \quad (2)$$

$$f^i_{AA}(t) = 1 - \left(f^i_{YH}(t) + f^i_{NH3}(t)\right), \quad (3)$$

where $\varphi^i_{YH}(t)$ and $\varphi^i_{NH3}(t)$ are the fractional labelling of amino acid *i* in the cells at sampling point *t* in the experiment with ^{15}N-labelled yeast hydrolysate and in the experiment with $^{15}NH_4Cl$, respectively, and $\varphi^i_{max}(t)$ is the maximum theoretical fractional labelling of amino acid *i* at sampling point *t*.

Results and discussion

Two batch fermentations with *S. cerevisiae* were carried out using media with the same chemical composition that contained different isotopically labelled substrates ($^{15}NH_4Cl$ or ^{15}N-labelled yeast hydrolysate). No significant differences were observed between the maximum specific growth rates and growth yields of the two cultures ($\mu_{max} = 0.63 \pm 0.01$ h^{-1} and $Y_{XGlc} = 0.140 \pm 0.002$ g g^{-1}, respectively; average values of the experiments ± average absolute deviation). Chromatography analyses of the culture media revealed that glucose was still present in the medium at the end of both fermentation experiments (Fig. A1 in the Supporting Information). Thus, the observed decrease in the average specific growth rate during the final hours of the cultivation could have resulted from either depletion of readily assimilable nitrogen sources, micro-nutrients such as vitamins, and/or accumulation of fermentation products, in particular, ethanol (concentration at 11 h = 17.2 ± 0.3 g L^{-1}).

The concentrations of both free and peptide-bound amino acids in the culture medium were determined at hourly intervals. Almost

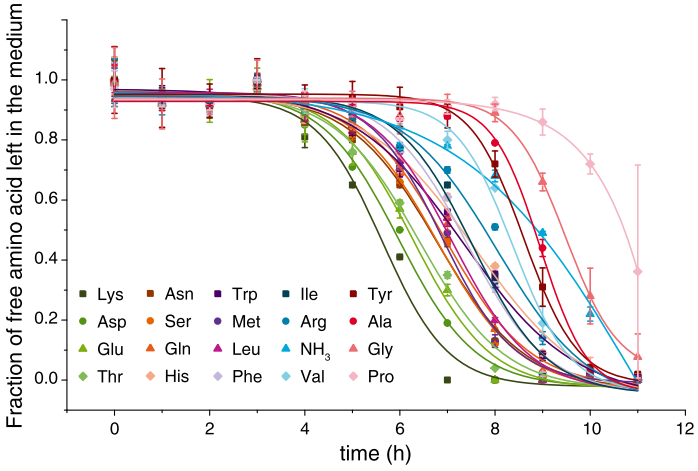

Figure 2. Consumption of free amino acids and ammonia by *S. cerevisiae* S288c during batch cultivation on a synthetic medium supplemented with NH_4Cl and yeast hydrolysate. The residual concentration of each amino acid and ammonia expressed as fractions of their maximum concentrations are shown for each hourly sampling point. Each point is the mean value from the two experiments with different labelled media components and the error bars represent relative deviations from the mean.

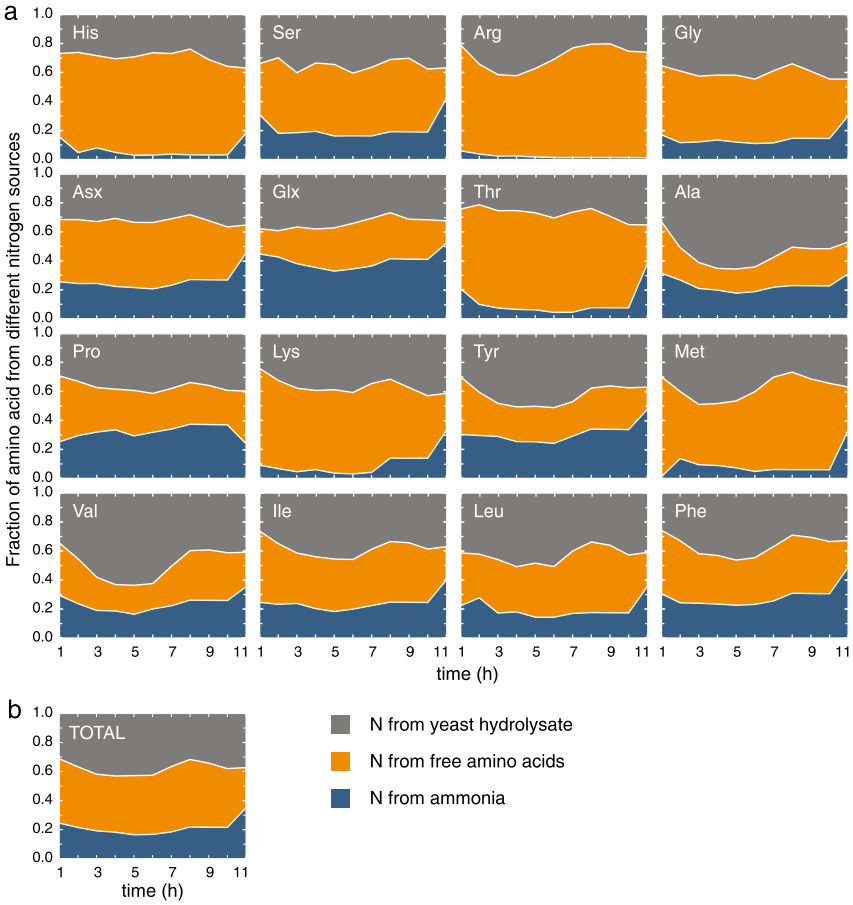

Figure 3. Fractional contribution of three different YAN sources at each sampling point for (a) each amino acid in the protein fraction (b) the sum of all amino acids in the biomass. The fractions of yeast hydrolysate (grey area), free amino acids (orange area) and ammonia (blue area) were calculated from the results of two batch cultivations of *S. cerevisiae* S288c using synthetic media with the same chemical composition that contained different isotopically labelled substrates ($^{15}NH_4Cl$ or ^{15}N-labelled yeast hydrolysate).

all free amino acids were completely exhausted from the medium between 7 h and 11 h after the start of each cultivation, regardless of the labelled substrate used (Fig. 2). The order of depletion generally agreed with results presented in earlier studies, for example, lysine and aspartate are typically amongst the first to be depleted while the bulk consumption of proline and alanine begins several hours later *(15,16)*. The concentration of all measured peptide-bound amino acids in the culture medium decreased during each experiment; however, unlike their free counterparts, none of them were depleted (data not illustrated).

Based on the isotope labelling data for individual amino acids in the two batch experiments, we determined the relative contributions of each of the three YAN sources (i.e. ammonia, free amino acids and peptide-bound amino acids) to the synthesis of the yeast protein fraction (Fig. 3). The amino acid enrichment patterns obtained in the experiment using labelled ammonia ($^{15}NH_4Cl$) allowed us to monitor the net incorporation of free ammonia into the protein fraction in the cells (Fig. 3; blue areas), which indicates the extent of their *de novo* synthesis. As expected, the highest incorporation of extracellular ammonia was observed in glutamate + glutamine (Glx) because these two amino acids act as a nexus of the *de novo* amino acid biosynthesis reactions in the cells. In contrast, some amino acids, specifically His and Lys, as well as Thr, Arg, and Met, exhibited a comparatively low incorporation of free ammonia. This can be explained by the fact that the transporters of the first two are known to be constitutively expressed, regardless of the presence of various nitrogen sources in the medium *(17)*. Likewise, Jones *et al. (18)* showed that methionine in the protein is derived to a large extent from exogenous methionine when present in the medium. A sharp increase in the contribution of labelled ammonia to the synthesis of most amino acids was observed during the final hour of the cultivation. This agrees with the observation that most free amino acids were almost or completely exhausted from the culture medium in the final hour when the increased utilization of (labelled) ammonia took place (Fig. 2). A notable exception is Pro, which displayed a decrease of ammonia incorporation during 11 h of fermentation. In the end of the experiment the cells began absorbing exogenous proline

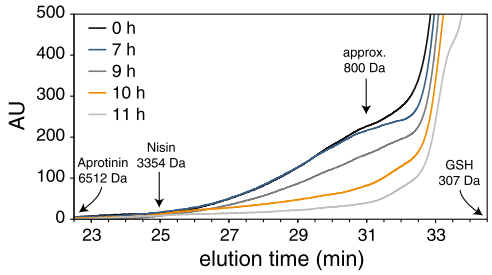

Figure 4. Size-exclusion chromatograms of the culture medium collected during the cultivation of *S. cerevisiae* S288c in a synthetic medium supplemented with unlabelled NH_4Cl and ^{15}N-labelled yeast hydrolysate. The lines show chromatograms at the beginning (0 h), at 7, 9 and 10 h, and the end (11 h) of the experiment. The arrows denote the elution times of three marker peptides and the elution point of media components with molecular weights of ~800 Da.

when most other amino acids were depleted. Indeed, derepression of proline uptake has been associated with the depletion of preferred nitrogen sources *(19)*. It must be emphasized that nitrogen derived from amino acids (Fig. 3; orange areas) represents the contribution of all free amino acids that participate in trans- and deamination reactions in the cells.

In contrast to free ammonia, the contribution of nitrogen derived from yeast hydrolysate to the yeast protein fraction was relatively uniform across all amino acids (Fig. 3; grey areas). The concentration of most free amino acids in labelled yeast hydrolysate was between 5 and 10% of the bound forms (Table A1, Supporting Information), which suggests that the observed nitrogen incorporation patterns result mainly from the consumption of peptides. Notably, there was a significant decline in the contribution of yeast hydrolysate-derived nitrogen to intracellular amino acids between hours 6 and 8 during the experiment. This is most evident in the profiles of Met, Arg, and branched chain amino acids, but can also be observed in the entire protein fraction (Fig. 3b). This suggests that the most readily absorbed peptides were gradually exhausted from the culture medium and the relative contribution of (unlabelled) free amino acids to protein synthesis increased (Fig. 3; orange areas). Nevertheless, consumption of peptides continued until the end of each experiment and even increased after the depletion of several free amino acids.

We assume that peptide-derived amino acids in the cells participate in the same trans- and deamination reactions as their free counterparts, that is, following its uptake, only a fraction of each amino acid is incorporated into protein intact *(18)*. Because this results in a random distribution of the label, it is difficult to determine which amino acids are preferentially transported into the cells in peptide-bound forms using our experimental technique and cultivation conditions. Nevertheless, larger proportion of yeast hydrolysate-derived nitrogen in comparison to that derived from free amino acids could, at least partly, indicate that the respective amino acid moieties are more readily transported into the cell in peptide-bound forms rather than as free amino acids. Prominent examples of such behaviour include Val (and to a lesser extent the other branched chain amino acids), Tyr and Phe. These findings agree with those reported by Ito *et al. (7)*, who studied the substrate (dipeptide) specificity of the major yeast peptide transporter (Ptr2p) and found that the transporter displayed relatively higher affinities towards peptides containing aromatic and branched chain amino acids. It has also been suggested that the branched chain amino acid transporters Bap2p and Bap3p display higher affinity towards Ile and Leu compared with Val *(15)*, which could result in preferential incorporation of peptide-bound Val moieties into the biomass. We have previously proposed that in the cases of shared transport systems in *Lactococcus lactis*, the less actively transported amino acids are more readily obtained from peptides *(13)*.

To estimate the sizes of the peptides consumed during the experiments, size-exclusion chromatography analysis was carried out on samples of the culture media. The change in the molecular weight distribution of the culture medium over time is provided in Fig. 4. During the first 7 h, media components with molecular weights of up to approximately 800 Da were consumed. This corroborates earlier findings that yeast is able to take up peptides comprising up to five, or perhaps six, amino acid residues *(6,20)*. In this phase of the cultivation, the concentration of larger peptides remained virtually unchanged. However, with the exhaustion of many free amino acids after that point and probably the exhaustion of the most readily assimilated peptides as indicated in Figs. 2 and 3, larger peptides began to be consumed. Uptake of peptides comprising more than five amino acid residues by yeast has not been reported to date. Thus, extracellular hydrolytic activity could have provided additional peptides with suitable length and composition for transport. Indeed, proteolytic activity corresponding to ~0.1 U trypsin equivalent was detected in the supernatants of cell free culture supernatants at 11 h; no activity was detected at earlier sampling points. We also observed the appearance of void volume (high molecular weight) peaks in the size-exclusion chromatograms from 9 h onwards (not illustrated), which could result from either extracellular proteases or high molecular weight cell lysis products.

The knowledge regarding extracellular proteases secreted by *S. cerevisiae* is somewhat ambiguous. Most screens for extracellular proteolytic activity have produced negative results *(21,22)*. However, several authors have found evidence of secreted proteases by wine yeasts *(23,24)*. The exact enzymes responsible for this extracellular proteolytic activity have yet to be identified and this observed trait seems to be strain-dependent. These studies do not explicitly conclude that the proteases are specifically secreted or simply the result of intracellular and/or vacuolar peptidases leaking into the culture medium. The exact cause of the extracellular proteolysis we observe requires further study. It is clear, however, that this phenomenon may play an important role in various settings where yeast is cultivated in the presence of oligopeptides.

In this study we focused our efforts on elucidating the contribution of ammonia, free amino acids, and peptides for the synthesis of protein during fermentative growth of *S. cerevisiae*. Even though it is well established that peptides play a role in yeast fermentations, their contribution to biomass formation in the presence of other YAN compounds has remained rather unclear. Our results suggest that co-consumption of ammonia, free amino acids and peptides by yeast during fermentative growth takes place and a significant proportion of the proteinogenic amino acids within cells can originate from peptides, even in the presence of what has been supposed as 'preferred' nitrogen sources, such as ammonia and free amino acids.

Acknowledgements

This work was supported by the European Regional Development Fund (project EU29994) and Estonian Ministry of Education (project IUT19-27). The authors would like to thank Dr David Schryer (University of Tartu) for critical revision of the manuscript. We have no conflict of interest to declare.

References

1. Ljungdahl, P. O., and Daignan-Fornier, B. (2012) Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*, *Genetics 190*, 885–929.
2. Boer, V. M., Tai, S. L., Vuralhan, Z., Arifin, Y., Walsh, M. C., Piper, M. D. W., de Winde, J. H., Pronk, J. T., and Daran, J.-M. (2007) Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures, *FEMS Yeast Res. 7*, 604–620.
3. Magasanik, B., and Kaiser, C. A. (2002) Nitrogen regulation in *Saccharomyces cerevisiae*, *Gene 290*, 1–18.
4. Cai, H., Kauffman, S., Naider, F., and Becker, J. M. (2006) Genomewide screen reveals a wide regulatory network for di/tripeptide utilization in *Saccharomyces cerevisiae*, *Genetics 172*, 1459–1476.
5. Cai, H., Hauser, M., Naider, F., and Becker, J. M. (2007) Differential regulation and substrate preferences in two peptide transporters of *Saccharomyces cerevisiae*, *Eukaryot. Cell 6*, 1805–1813.
6. Hauser, M., Narita, V., Donhardt, A. M., Naider, F., and Becker, J. M. (2001) Multiplicity and regulation of genes encoding peptide transporters in *Saccharomyces cerevisiae*, *Mol. Membr. Biol. 18*, 105–112.
7. Ito, K., Hikida, A., Kawai, S., Lan, V. T. T., Motoyama, T., Kitagawa, S., Yoshikawa, Y., Kato, R., and Kawarasaki, Y. (2013) Analysing the substrate multispecificity of a proton-coupled oligopeptide transporter using a dipeptide library, *Nat. Commun. 4*, 2502.
8. Wiles, A. M., Cai, H., Naider, F., and Becker, J. M. (2006) Nutrient regulation of oligopeptide transport in *Saccharomyces cerevisiae*, *Microbiology 152*, 3133–3145.
9. Lekkas, C., Hill, A., Taidi, B., Hodgson, J., and Stewart, G. G. (2009) The role of small wort peptides in brewing fermentations, *J. Inst. Brew. 115*, 134–139.
10. Pozo-Bayón, Á. M., Andújar-Ortiz, I., and Moreno-Arribas, M. V. (2009) Scientific evidences beyond the application of inactive dry yeast preparations in winemaking, *Food Res. Int. 42*, 754–761.
11. Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F. N., and Guillamon, J. M. (2012) Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must, *Food Microbiol. 31*, 25–32.
12. Jiranek, V., Langridge, P., and Henschke, P. A. (1995) Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium, *Am. J. Enol. Vitic. 46*, 75–83.
13. Kevvai, K., Kütt, M.-L., Nisamedtinov, I., and Paalme, T. (2014) Utilization of (15)N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids, *Antonie Van Leeuwenhoek 105*, 511–522.
14. Rockwood, A. L., and Van Orden, S. L. (1996) Ultrahigh-speed calculation of isotope distributions, *Anal. Chem. 68*, 2027–2030.
15. Crépin, L., Nidelet, T., Sanchez, I., Dequin, S., and Camarasa, C. (2012) Sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: A model based on kinetic and regulation characteristics of nitrogen permeases, *Appl. Environ. Microbiol. 78*, 8102–8111.
16. Jones, M., and Pierce, J. S. (1964) Absorption of amino acids from wort by yeasts, *J. Inst. Brew. 70*, 307–315.
17. ter Schure, E. G., van Riel, N. A., and Verrips, C. T. (2000) The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*, *FEMS Microbiol. Rev. 24*, 67–83.
18. Jones, M., Pragnell, M. J., and Pierce, J. S. (1969) Absorption of amino acids by yeasts from a semi-defined medium simulating wort, *J. Inst. Brew. 75*, 520–536.
19. Poole, K., Walker, M. E., Warren, T., Gardner, J., McBryde, C., de Barros Lopes, M., and Jiranek, V. (2009) Proline transport and stress tolerance of ammonia-insensitive mutants of the *PUT4*-encoded proline-specific permease in yeast, *J. Gen. Appl. Microbiol. 55*, 427–439.
20. Osawa, H., Stacey, G., and Gassmann, W. (2006) ScOPT1 and AtOPT4 function as proton-coupled oligopeptide transporters with broad but distinct substrate specificities, *Biochem. J. 393*, 267–275.
21. Nelson, G., and Young, T. (1986) Yeast extracellular proteolytic enzymes for chill-proofing beer, *J. Inst. Brew. 92*, 599–603.
22. Ogrydziak, D. M. (1993) Yeast extracellular proteases, *Crit. Rev. Biotechnol. 13*, 1–55.
23. Rosi, I., Costamagna, L., and Birtuccion, M. (1987) Screening for extracellular acid protease(s) production by wine yeasts, *J. Inst. Brew. 93*, 322–324.
24. Younes, B., Cilindre, C., Villaume, S., Parmentier, M., Jeandet, P., and Vasserot, Y. (2011) Evidence for an extracellular acid proteolytic activity secreted by living cells of *Saccharomyces cerevisiae* PlR1: Impact on grape proteins, *J. Agric. Food Chem. 59*, 6239–6246.

Supporting information

Addtional supporting information may be found in the online version of this article at the publisher's web-site.

RECENT DISSERTATIONS DEFENDED AT TUT IN NATURAL AND EXACT SCIENCES

B137 **Katri Muska.** Study of Composition and Thermal Treatments of Quaternary Compounds for Monograin Layer Solar Cells. 2012.
B138 **Ranno Nahku.** Validation of Critical Factors for the Quantitative Characterization of Bacterial Physiology in Accelerostat Cultures. 2012.
B139 **Petri-Jaan Lahtvee.** Quantitative Omics-level Analysis of Growth Rate Dependent Energy Metabolism in *Lactococcus lactis*. 2012.
B140 **Kerti Orumets.** Molecular Mechanisms Controlling Intracellular Glutathione Levels in Baker's Yeast *Saccharomyces cerevisiae* and its Random Mutagenized Glutathione Over-Accumulating Isolate. 2012.
B141 **Loreida Timberg.** Spice-Cured Sprats Ripening, Sensory Parameters Development, and Quality Indicators. 2012.
B142 **Anna Mihhalevski.** Rye Sourdough Fermentation and Bread Stability. 2012.
B143 **Liisa Arike.** Quantitative Proteomics of *Escherichia coli*: From Relative to Absolute Scale. 2012.
B144 **Kairi Otto.** Deposition of In_2S_3 Thin Films by Chemical Spray Pyrolysis. 2012.
B145 **Mari Sepp.** Functions of the Basic Helix-Loop-Helix Transcription Factor TCF4 in Health and Disease. 2012.
B146 **Anna Suhhova.** Detection of the Effect of Weak Stressors on Human Resting Electroencephalographic Signal. 2012.
B147 **Aram Kazarjan.** Development and Production of Extruded Food and Feed Products Containing Probiotic Microorganisms. 2012.
B148 **Rivo Uiboupin.** Application of Remote Sensing Methods for the Investigation of Spatio-Temporal Variability of Sea Surface Temperature and Chlorophyll Fields in the Gulf of Finland. 2013.
B149 **Tiina Kriščiunaite.** A Study of Milk Coagulability. 2013.
B150 **Tuuli Levandi.** Comparative Study of Cereal Varieties by Analytical Separation Methods and Chemometrics. 2013.
B151 **Natalja Kabanova.** Development of a Microcalorimetric Method for the Study of Fermentation Processes. 2013.
B152 **Himani Khanduri.** Magnetic Properties of Functional Oxides. 2013.
B153 **Julia Smirnova.** Investigation of Properties and Reaction Mechanisms of Redox-Active Proteins by ESI MS. 2013.
B154 **Mervi Sepp.** Estimation of Diffusion Restrictions in Cardiomyocytes Using Kinetic Measurements. 2013.
B155 **Kersti Jääger.** Differentiation and Heterogeneity of Mesenchymal Stem Cells. 2013.
B156 **Victor Alari.** Multi-Scale Wind Wave Modeling in the Baltic Sea. 2013.
B157 **Taavi Päll.** Studies of CD44 Hyaluronan Binding Domain as Novel Angiogenesis Inhibitor. 2013.
B158 **Allan Niidu.** Synthesis of Cyclopentane and Tetrahydrofuran Derivatives. 2013.
B159 **Julia Geller.** Detection and Genetic Characterization of *Borrelia* Species Circulating in Tick Population in Estonia. 2013.
B160 **Irina Stulova.** The Effects of Milk Composition and Treatment on the Growth of Lactic Acid Bacteria. 2013.
B161 **Jana Holmar.** Optical Method for Uric Acid Removal Assessment During Dialysis. 2013.
B162 **Kerti Ausmees.** Synthesis of Heterobicyclo[3.2.0]heptane Derivatives *via* Multicomponent Cascade Reaction. 2013.
B163 **Minna Varikmaa.** Structural and Functional Studies of Mitochondrial Respiration Regulation in Muscle Cells. 2013.
B164 **Indrek Koppel.** Transcriptional Mechanisms of BDNF Gene Regulation. 2014.
B165 **Kristjan Pilt.** Optical Pulse Wave Signal Analysis for Determination of Early Arterial Ageing in Diabetic Patients. 2014.
B166 **Andres Anier.** Estimation of the Complexity of the Electroencephalogram for Brain Monitoring in Intensive Care. 2014.
B167 **Toivo Kallaste.** Pyroclastic Sanidine in the Lower Palaeozoic Bentonites – A Tool for Regional Geological Correlations. 2014.

B168 **Erki Kärber.** Properties of ZnO-nanorod/In_2S_3/$CuInS_2$ Solar Cell and the Constituent Layers Deposited by Chemical Spray Method. 2014.

B169 **Julia Lehner.** Formation of Cu_2ZnSnS_4 and $Cu_2ZnSnSe_4$ by Chalcogenisation of Electrochemically Deposited Precursor Layers. 2014.

B170 **Peep Pitk.** Protein- and Lipid-rich Solid Slaughterhouse Waste Anaerobic Codigestion: Resource Analysis and Process Optimization. 2014.

B171 **Kaspar Valgepea.** Absolute Quantitative Multi-omics Characterization of Specific Growth Rate-dependent Metabolism of *Escherichia coli*. 2014.

B172 **Artur Noole.** Asymmetric Organocatalytic Synthesis of 3,3'-Disubstituted Oxindoles. 2014.

B173 **Robert Tsanev.** Identification and Structure-Functional Characterisation of the Gene Transcriptional Repressor Domain of Human Gli Proteins. 2014.

B174 **Dmitri Kartofelev.** Nonlinear Sound Generation Mechanisms in Musical Acoustic. 2014.

B175 **Sigrid Hade.** GIS Applications in the Studies of the Palaeozoic Graptolite Argillite and Landscape Change. 2014.

B176 **Agne Velthut-Meikas.** Ovarian Follicle as the Environment of Oocyte Maturation: The Role of Granulosa Cells and Follicular Fluid at Pre-Ovulatory Development. 2014.

B177 **Kristel Hälvin.** Determination of B-group Vitamins in Food Using an LC-MS Stable Isotope Dilution Assay. 2014.

B178 **Mailis Päri.** Characterization of the Oligoadenylate Synthetase Subgroup from Phylum Porifera. 2014.

B179 **Jekaterina Kazantseva.** Alternative Splicing of *TAF4*: A Dynamic Switch between Distinct Cell Functions. 2014.

B180 **Jaanus Suurväli.** Regulator of G Protein Signalling 16 (RGS16): Functions in Immunity and Genomic Location in an Ancient MHC-Related Evolutionarily Conserved Synteny Group. 2014.

B181 **Ene Viiard.** Diversity and Stability of Lactic Acid Bacteria During Rye Sourdough Propagation. 2014.

B182 **Kristella Hansen.** Prostaglandin Synthesis in Marine Arthropods and Red Algae. 2014.

B183 **Helike Lõhelaid.** Allene Oxide Synthase-lipoxygenase Pathway in Coral Stress Response. 2015.

B184 **Normunds Stivrinš.** Postglacial Environmental Conditions, Vegetation Succession and Human Impact in Latvia. 2015.

B185 **Mary-Liis Kütt.** Identification and Characterization of Bioactive Peptides with Antimicrobial and Immunoregulating Properties Derived from Bovine Colostrum and Milk. 2015.

B186 **Kazbulat Šogenov.** Petrophysical Models of the CO_2 Plume at Prospective Storage Sites in the Baltic Basin. 2015.

B187 **Taavi Raadik.** Application of Modulation Spectroscopy Methods in Photovoltaic Materials Research. 2015.

B188 **Reio Põder.** Study of Oxygen Vacancy Dynamics in Sc-doped Ceria with NMR Techniques. 2015.

B189 **Sven Siir.** Internal Geochemical Stratification of Bentonites (Altered Volcanic Ash Beds) and its Interpretation. 2015.

B190 **Kaur Jaanson.** Novel Transgenic Models Based on Bacterial Artificial Chromosomes for Studying BDNF Gene Regulation. 2015.

B191 **Niina Karro.** Analysis of ADP Compartmentation in Cardiomyocytes and Its Role in Protection Against Mitochondrial Permeability Transition Pore Opening. 2015.

B192 **Piret Laht.** B-plexins Regulate the Maturation of Neurons Through Microtubule Dynamics. 2015.

B193 **Sergei Žari.** Organocatalytic Asymmetric Addition to Unsaturated 1,4-Dicarbonyl Compounds. 2015.

B194 **Natalja Buhhalko.** Processes Influencing the Spatio-temporal Dynamics of Nutrients and Phytoplankton in Summer in the Gulf of Finland, Baltic Sea. 2015.

B195 **Natalia Maticiuc.** Mechanism of Changes in the Properties of Chemically Deposited CdS Thin Films Induced by Thermal Annealing. 2015.

B196 **Mario Öeren.** Computational Study of Cyclohexylhemicucurbiturils. 2015.

B197 **Mari Kalda.** Mechanoenergetics of a Single Cardiomyocyte. 2015.

B198 **Ieva Grudzinska.** Diatom Stratigraphy and Relative Sea Level Changes of the Eastern Baltic Sea over the Holocene. 2015.

B199 **Anna Kazantseva.** Alternative Splicing in Health and Disease. 2015.

B200 **Jana Kazarjan.** Investigation of Endogenous Antioxidants and Their Synthetic Analogues by Capillary Electrophoresis. 2016.

B201 **Maria Safonova.** SnS Thin Films Deposition by Chemical Solution Method and Characterization. 2016.

B202 **Jekaterina Mazina.** Detection of Psycho- and Bioactive Drugs in Different Sample Matrices by Fluorescence Spectroscopy and Capillary Electrophoresis. 2016.