Ansätze zur Optimierung der Glutathionausbeute in der Hefe *Saccharomyces spp.*

vorgelegt von

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Inhaltsverzeichnis

Zusammenfassung

Glutathion (GSH) ist die am häufigsten vorkommende nicht-proteingebundene Thiolverbindung in Eukaryonten. Die primäre intrazelluläre Funktion von GSH ist es, den Redoxstatus aufrecht zu erhalten und die Zelle vor Oxidationsreaktionen ausgelöst durch z.B. Radikale oder Umwelteinflüsse zu schützen. Nicht nur die physiologische Bedeutung in der Zelle, sondern auch seine industrielle Relevanz machen GSH zum Forschungsgegenstand vieler Studien. Aufgrund seiner antioxidativen Eigenschaften wird GSH in vielen Bereichen industriell genutzt, wie der pharmazeutischen- und kosmetischen Industrie. Aber auch in der Lebensmittelindustrie oder als Nahrungsergänzungsmittel steigt der Bedarf an GSH.

Diese Arbeit befasst sich theoretisch und praktisch mit der bioverfahrenstechnischen Optimierung der GSH-Produktion in *Saccharomyces spp.* und zeigt Wege und Möglichkeiten als auch Lösungsansätze auf. Der theoretische Teil der Arbeit (Kapitel 1 & 2) ist die Einleitung in das Thema Hefe und zeigt, wie vielseitig Hefe eingesetzt werden kann. Darunter fällt auch GSH, wobei im weiteren Verlauf auf die Historie, Biosynthese und physiologische Funktion eingegangen wird. Auch auf Strategien zur GSH-Produktion und auf die derzeitige Patentsituation wird eingegangen.

Der praktische Teil der Arbeit besteht aus 3 unabhängigen Studien mit Fokus auf dem Kultivierungsmedium, dem GSH Induktor sowie der Prozessführung. Für eine effiziente GSH-Produktion ist die Wahl des geeigneten Kultivierungsmediums, unter Berücksichtigung der späteren Anwendung, entscheidend. Bei der industriellen Produktion werden meist Komplexrohstoffe wie Melasse verwendet. Jedoch ist die Melassequalität und Zusammensetzung saisonalen Schwankungen unterworfen. In wissenschaftlichen Studien können diese Qualitätsunterschiede zu erhöhter Prozessvariabilität führen. Deshalb wird, wenn möglich, bevorzugt auf chemisch-definierte (CD) Medien und Feeds zurückgegriffen. In Kapitel 3 wird die Entwicklung eines CD-Mediums/Feeds erläuert. Als Ausgangspunkt für ein CD-Medium diente die elementare Zusammensetzung der Hefe *S. cerevisiae*. Dabei konnte als ideale Stickstoffquelle eine Kombination aus $(NH_4)_2SO_4$ und NH_4Cl gefunden werden, um hohe Biomassen zu erzielen. Für eine verbesserte GSH-Produktivität wurde die Wachstumsrate angepasst. Hierfür wurde eine Ableitung des Chemostats, der sog. A-Stat, verwendet. Schlussendlich konnten alle nicht chemisch definierten Medienkomponenten entfernt bzw. ersetzt werden, ohne Einbußen in der Biomasse- und Produktbildung zu verzeichnen.

Ein ebenso wichtiger Faktor wie das Nährmedium ist die Substanz, welche als Vorstufe oder Präkusor für die Biotransformation zu GSH dient und diese gleichzeitig induziert (hier als Induktor bezeichnet). In Kapitel 4 werden neben Cystein noch 3 weitere Substanzen auf ihre Fähigkeit hin untersucht, die GSH-Biosynthese zu induzieren. Dazu wurden 50mL Kultivierungsröhrchen verwendet, welche mit einer gaspermeablen Membran ausgerüstet waren. Aufgrund der industriellen Relevanz wurden die Untersuchungen auch mit anderen Hefestämmen wie der Brauhefe *S. bayanus* und der probiotischen Hefe *S. boulardii* durchgeführt. Am Ende konnte für alle verwendeten Hefestämme gezeigt werden, dass Cystein-Ethyl-Ester ein hoch effizienter Induktor/Präkursor ist. Es wird vermutet, dass die chemischen Eigenschaften Cystein-Ethyl-Ester befähigen, leichter die Membran zu passieren. Auch konnte gezeigt werden, dass im Vergleich zu Cystein das Verhältnis zwischen Induktor und intrazellulärer GSH-Konzentration um mehr als Faktor 2 erhöht wird.

Die Prozesssteuerung und -optimierung trägt im hohen Maße zur Gesamtwirtschaftlichkeit bei. In Kapitel 5 lag der Fokus auf der Optimierung des Verhältnisses zwischen eingesetztem Cystein und produziertem GSH ($Y_{cys/GSH}$). Zuvor wurde jedoch ein Fed-Batch Verfahren entwickelt, welches hohe Biomassen garantiert. Hierzu wurde mit Hilfe einer Abgasanalyse der respiratorische Quotient (RQ) berechnet. Dieser diente zur Steuerung einer Fütterungspumpe, mit der ein geschlossener, selbst steuernder Regelkreis realisiert wurde. Die sich daraus ergebenen Fütterungsmengen wurden per Waage und Prozessleitsystem aufgezeichnet und konnten mit einem logistischen Regressionsmodel beschrieben werden. Daraus ergab sich ein Fütterungsprofil, welches im weiteren Verlauf der Studie verwendet wurde. Zur Optimierung des $Y_{cys/GSH}$ Ratios wurden unterschiedliche Cystein-Zugabezeitpunkte, der Zugabemodus (dis-/kontinuierlich) und die Cysteinkonzentration untersucht. Dabei wurde das höchste $Y_{cys/GSH}$ Ratio von 0.54 mol/mol erhalten, wenn Cystein in der späten Wachstumsphase und in kleinen Mengen kontinuierlich zugefüttert wird. Dieses Setup resultierte in einer GSH-Konzentration von 1650 mg/L bzw. 1.76% bei einer Biomasse von 99.5 g/L. Diese Prozessstrategie wurde erfolgreich vom Labormaßstab (5-L) in den Pilotmaßstab (150-L) übertragen. Durch Kombination der optimierten Zugabe des Präkusors Cystein und des Repeated-Fed-Batch Modus konnte die Raum-Zeit-Ausbeute signifikant erhöht werden.

Im letzten Teil der Arbeit werden die oben beschriebenen Ergebnisse in einen wirtschaftlichen Kontext gebracht und es wird aufgezeigt, welche Faktoren die Prozessökonomie beeinflussen können. Als Modellbeispiel diente eine traditionelle Backhefeproduktion. Die Hauptkostentreiber sind die Aufwendungen für Rohmaterialien, Energie, Prozesswasser und Entsorgungskosten. Auch wird Bezug genommen auf die Aufarbeitung der angereicherten GSH-Hefen und wie diese durch die Anwendung beeinflusst wird.

Die Herausforderungen an eine nachhaltige Produktion von GSH werden weiter steigen wie auch die weltweite Nachfrage. Um diesen Bedarf decken zu können, müssen die vorhandenen bioprozesstechnischen Verfahren optimiert werden. Generell unterliegen mikrobielle Produktionsprozesse einem hohen Preisdruck, weil die Gewinnmargen klein und die Prozesskosten hoch sind. Gerade die Kosten für Rohstoffe und Energie werden zukünftig stetig ansteigen. Deshalb wird das Interesse an einer effizienten, nachhaltigen und verantwortungsvollen Nutzung der vorhandenen Ressourcen weiter in den Fokus rücken. Diese Arbeit zeigt bioprozesstechnische Lösungsansätze auf, um die zukünftige Nachfrage an GSH und assoziierten Produkten decken zu können.

Summary

Glutathione is the most abundant non-protein thiol compound and tripeptide found in eukaryotes. The primary function of glutathione is to maintain the redox status and to protect the cell against oxidation caused by production of e.g. radicals or environmental influences. It is subject of different studies due to its physiological importance and industrial relevance. Based on its anti-oxidative characteristics, glutathione is used in many industrial areas e.g. pharmaceuticals and cosmetics. Additionally, for dietary supplement- and in food industry an increasing demand can be observed.

To meet this demand, the available bioengineering technologies have to be optimized and raw materials should be used in a sustainable way. Furthermore, it is of high importance to reduce the manufacturing costs at all. This is fortified by low profit margins and high pressure on costs, which is a general challenge in microbial production processes.

This thesis deals theoretically and practically with the bioprocessing optimization of glutathione production in *Saccharomyces spp.* and shows ways and possibilities how this can be achieved and what has to be considered. The theoretical part (chapter 1 & 2) of this thesis introduces the yeast and its applications in general. In the following course of introduction, the history, biosynthesis, physiological function, strategies for glutathione production, strain -mutagenesis, -engineering and -selection of glutathione high producer is discussed. Additionally, the chapter 2 deepens and completes the knowledge in glutathione manufacturing and give an overview about the current patent situation and further glutathione applications.

The practical part consists of three independent studies (chapter 3-5). For an efficient glutathione production, different contributing factors have to be considered. One is the choice of the right cultivation medium that depends also on later cultivation. Generally, for industrial application molasses is traditionally used as main carbon source. Molasses is the raw material of choice for yeast fermentation. It is an affordable byproduct which is produced during sugar production and it exists lot of experience to use molasses for breeding yeast in industrial application. However, molasses suspects seasonal fluctuations in terms of nutrient quality and composition. Therefore, for scientific studies it is preferable to use chemical-defined media and feeds. Chapter 3 addresses the approach how to design a chemical-defined medium systematically. Thereby the medium composition was based on the elemental composition of yeast. By applying a simplex centroid design, a combination of ammonium sulfate and chloride was identified to achieve higher biomasses and productivity. Furthermore, an optimal growth rate for glutathione production was detected by applying the so-called accelerated-stat (A-stat) process control strategy which is a derivation of traditional chemostat. Finally, all complex medium components like yeast extract and peptone could be successfully removed from the applied medium and feed without any disadvantages in growth and productivity. Generally, this CD-medium/feed setup is suitable for R&D purpose to cultivate *Saccharomyces spp*. efficiently.

Another contributing factor especially in glutathione overproduction is the precursor respectively inducer for the glutathione biotransformation. In this context the most investigated component is the amino acid L-cysteine. In chapter 4, three different cysteine derivatives and their effect on glutathione yield were explored. To gather more data in a short time period a

miniaturized and parallelized cultivation system, based on 50-mL shake tubes equipped with a gas-permeable membrane, was successfully applied for initial experiments. Due to the industrial relevance not only *S. cerevisiae*, but also the brewing yeast *S. bayanus* and the probiotic yeast *S. boulardii* was investigated in these studies. Finally, cysteine-ethyl-ester was found as highly efficient inducer for glutathione overproduction for all applied yeasts strains. It is assumed that due to its chemical properties cysteine-ethyl-ether can pass the yeast membrane more efficiently. The ratio between inducer and intracellular glutathione was more than 2 times compared to cysteine.

In chapter 5 it was intented to emphasize the improvement of the cysteine/glutathione ratio ($Y_{cys/GSH}$). Prior, a closed-loop-feedback-control strategy was developed to achieve an optimal feeding and ensure high biomasses. This feeding strategy is based on the evaluation of the respiratory quotient (RQ). Thereby an online RQ-controller is connected to a feeding pump and a balance, which monitores and collects data of the feeding amount. Once the fermentation process was finished a logistic regression model was chosen for fitting the resulted gravimetric values and to define a feeding regime. After the implementation of the feeding scheme, the $Y_{cys/GSH}$ ratio had to be optimized. For this purpose, different time-points for cysteine addition, the mode of addition (bolus/continuous) and the concentration were studied. The highest $Y_{cys/GSH}$ ratio (0.54 mol/mol) was obtained when cysteine is added during the late growth phase and in small proportions continuously. Additionally, this approach resulted in a glutathione concentration of 1650 mg/L and biomass of about 99.5 g/L which corresponds to 1.76% intracellular glutathione content. The results could be successfully transferred into a 150-L pilot scale bioreactor. Through a combination of the optimized cysteine addition strategy and Repeated-Fed-Batch approach the space-time-yield was further improved which could be of high relevance for industrial production.

In the last chapter 6, the gained results and findings are put and discussed in the context to economic perspectives. A traditional baker's yeast production is used as comparative example. Further, the main cost drivers for an industrial glutathione production process in yeast are considered. In particular, this includes the costs of raw materials, energy, personnel process water and the costs of disposals. In addition, methods for processing glutathione enriched yeasts are discussed. Here the final application often decides about the choice of the processing method. It has to be differentiated between glutathione yeast-derived products and glutathione as bulk chemical. Some of these applications and their manufacturer are mentioned.

Finally, it can be stated that the global demand for glutathione will be further increase in all industrial sectors. In parallel, raw material costs becoming constantly higher. Therefore, an efficient, sustainable and responsible management with resources moving into the spotlight. This thesis gives thought-provoking impulses and shows potential solutions to overcome the increasing demand of glutathione and related products.

Danksagung

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Euer Eric

Kapitel 1

Allgemeine Einleitung

1 Einleitung

Mit Beginn der organisierten Landwirtschaft vor mehr als 3000 Jahren im Gebiet des heutigen Ägyptens wurden bereits unbewusst Hefen eingesetzt, um Lebensmittel wie Bier oder Brot herzustellen (Delwen Samuel, 1993; D. Samuel, 1996). Das erste Mal wurden Hefen unter dem Mikroskop im Jahre 1680 von A. van Leeuwenhoek beobachtet und beschrieben. Pasteur publizierte 1857 seine erste Arbeit über die alkoholische Fermentation und Emil Fischer untersuchte zwischen 1884 – 1894 die Stereochemie von Zuckern und deren Aufnahme und Verwertung durch Hefe und formulierte dabei als Erster das Schlüssel-Schloss-Prinzip oder auch Enzym-Substrat-Komplex (Barnett, 2003; Fischer & Thierfelder, 1894). Die erste Starterkultur zum Bierbrauen und zur Weinherstellung wurde von Hansen und Müller-Thurgau ebenfalls Ende des 19. Jahrhunderts vertrieben (Türker, 2014). Heutzutage werden verschiedenste Hefen in unterschiedlichsten Industrien eingesetzt, um Produkte des täglichen Bedarfs zu produzieren (siehe Abbildung 1-1).

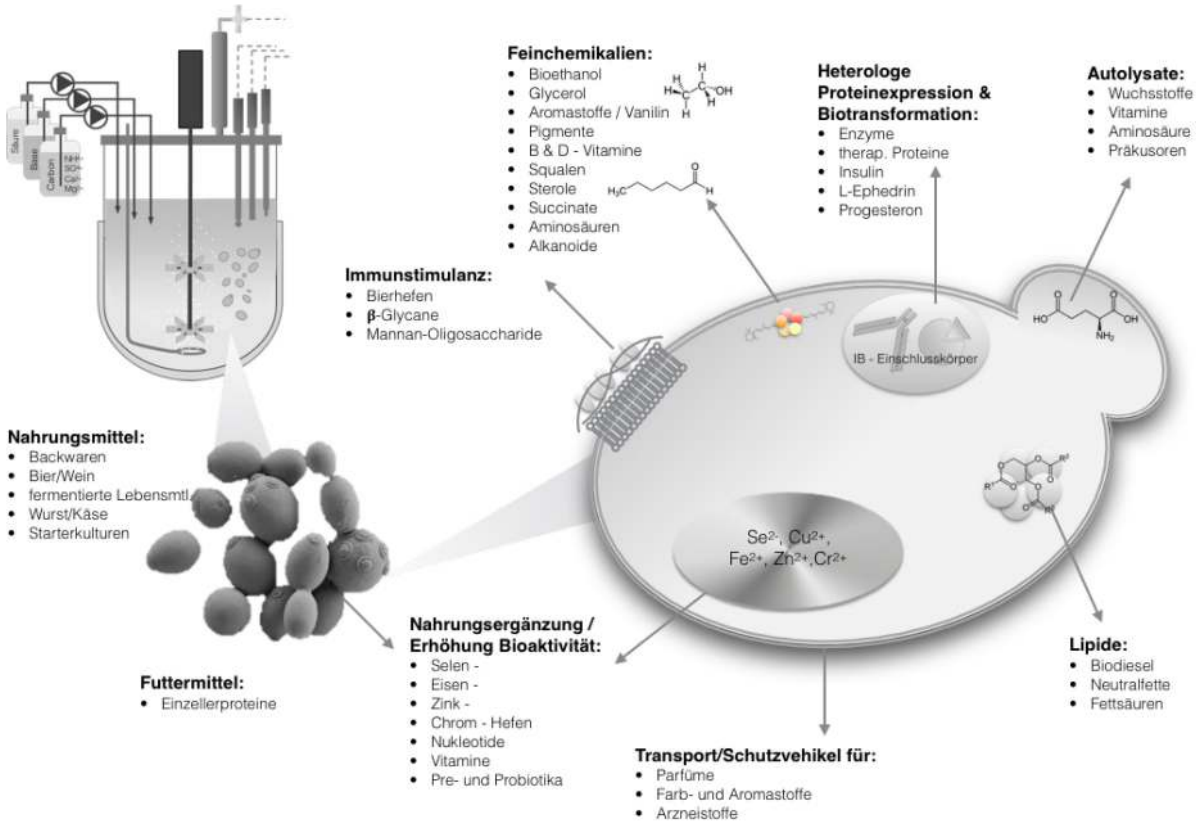

Abbildung 1-1 Überblick von ausgesuchten biotechnologisch erzeugten Produkten aus Hefe.

1.1 Allgemeine Applikationen von Hefe

Die Chemie- und Pharmaindustrie stellt mit Hefen, wie zum Beispiel *Saccharomyces spp., Candida spp., Yarrowia lipolytica, Rhodotorula spp. Cryptococcus spp., Lipomyces spp.* und

Kluyveromyces spp. verschiedenste Produkte und Feinchemikalien wie Vitamine, Aminosäuren, Zucker- und Fettalkohole, organische Säuren, Aromastoffe, Ergosterol, Ephedrin, Biodiesel, Ethanol, Pigmente oder Farbstoffe biotechnologisch her. Aber auch viel größere Moleküle, darunter eine Vielzahl von funktionellen Enzymen, Insulin und auch therapeutische Proteine, werden mit gentechnisch-veränderten Hefen wie zum Beispiel *Pichia spp. in vivo* synthetisiert.

In der Tierzucht werden Hefen (*Saccharomyces spp.*) und Hefeinhaltsstoffe dem Futter beigemischt, um den Mineralstoff-, Protein-, Aminosäuren- und Vitamingehalt zu erhöhen (Bekatorou, Psarianos, & Koutinas, 2006; Ferreira, Pinho, Vieira, & Tavarela, 2010; Shurson, 2018). Separierte Hefeschale, hergestellt aus Bierabfallhefen, mit hohem Gehalt an β-Glukan und Mannan-Oligosacchariden wird verwendet, um das Immunsystem (Stimulanz) der Tiere zu entlasten und von Toxinen zu befreien oder die Verdauung zu unterstützen.

Hefelysate und Extrakte werden oft als Wuchsstofflieferant zu Fermentationsmedien supplementiert und finden ebenfalls breite Anwendung im Bereich der Lebensmittelindustrie. Dabei werden Hefeextrakte am häufigsten als Geschmacksverstärker oder Modulatoren verwendet (Ferreira et al., 2010). Aber auch in Nahrungsergänzungsmitteln wird Hefeextrakt als Protein, Nukleotid- und B-Vitaminquelle beigemischt (http://www.leibergmbh.de, abgerufen 07/2019).

Des Weiteren werden Hefen eingesetzt, welchen (*S. cerevisiae var. boulardii, Kluyveromyces spp.*) nachweislich eine gesundheitlich fördernde Wirkung (probiotisch) zugesprochen wird (Kelesidis & Pothoulakis, 2012; Palma et al., 2015). Aufgrund der robusten Struktur und durch die hohe Zellkonzentration bei Einnahme (oral) passieren sie den Magen und können im Darm Stoffe sekretieren oder Viren/Viruspartikel absorbieren und bei Darmerkrankungen zu einer schnelleren Genesung des Patienten führen (Hatoum, Labrie, & Fliss, 2012). Vermehrt werden diese Hefen als Kombinationspräparate mit probiotischen Milchsäurebakterien Patienten verschrieben, welche eine Antibiotikatherapie bekommen, um die Darmflora zu unterstützen. Mit Ausnahme von chemischen und biopharmazeutischen Produkten machen Hefen, die zur Herstellung von Brot und Bäckereiprodukten benutzt werden, an Volumen und Marktwert die größte Marge aus, gefolgt sowohl von Hefelysaten und Extrakten als auch von Hefen, welche zur Herstellung von Gärgetränken verwendet werden. Größter Produzent und gleichzeitig Konsument von Backhefe ist Europa, gefolgt von Asien. Ein Markt mit ebenfalls hohen Marktvolumen ist der von Bioethanol. Dabei stammen 80% des weltweit produzierten Bioethanols aus Brasilien und Nordamerika.

Insgesamt betrug das jährliche Marktvolumen (2017) für Hefen der Gattung *Saccharomyces* und den zugehörigen Produkten geschätzte 7.6 Milliarden USD und wird 2022 auf geschätzte 10.7 Milliarden USD anwachsen. Darin enthalten sind Bäcker-, Bier-, Wein-, und Futterhefe, Bioethanol, Mineralhefen, probiotische Hefen sowie Autolysate, die als Geschmacksverstärker oder Fermentationssupplement verwendet werden. Allerdings liegen die Preise für Hefe mit

Ausnahme von Autolysaten und Extrakten in dem Segment durchschnittlich bei ca. 1 USD pro kg Hefe (McWilliams, 2017). Unter dieses Preissegment fallen auch die sogenannten „angereicherte Hefen“ (*engl. enriched-yeasts*). Dazu zählen Hefen, die mittels biotechnologischer Verfahren, also durch Propagation oder durch Reaktivierung des Stoffwechsels von bereits vorhandener Hefebiomasse und ggf. durch Zugabe von speziellen Substraten, Elementen oder Präkusoren, Stoffe intrazellulär akkumulieren bzw. mit diesen angereichert werden.

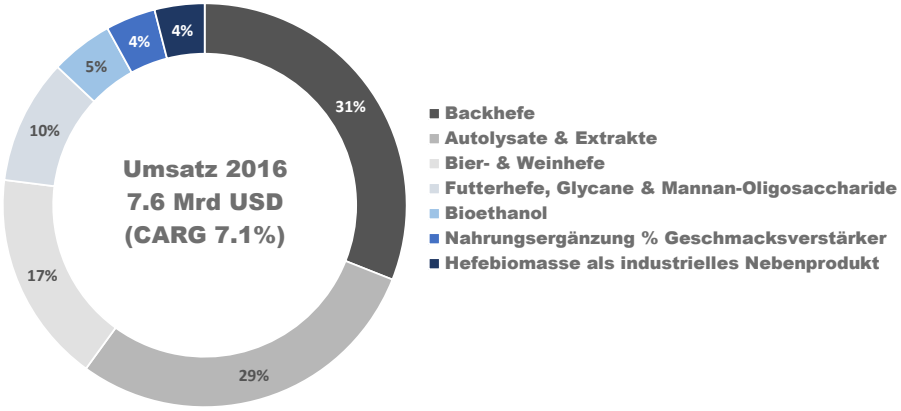

Abbildung 1-2 Globale Marktübersicht zu Hefe & Hefeprodukte modifiziert nach McWilliams (2017)

Spurenelemente wie Zink, Kupfer, Eisen oder Selen, welche für sich genommen in höheren Konzentrationen eine toxische Wirkung auf den menschlichen Organismus hätten, werden in Hefen z.B. in der Vakuole gespeichert bzw. detoxifiziert oder an Aminosäuren, Proteine oder Zellwandbestandteile gebunden (Mrvcic, Stanzer, Stehlik-Tomas, Skevin, & Grba, 2007; Shariatmadari, Kamranazad, & Karimi torshizi, 2014; Stehlik-Tomas & Zetic, 2004; Szulc-Musioł, Szulc-Musioł, Ryszka, & Dolińska, 2016; Tompkins, Renard, & Kiuchi, 2007). Diese „gebundenen“ Spurenelemente habe im Vergleich zu anderen Präparaten (organische Salze) eine erhöhte Bioverfügbarkeit. (Fairweather-Tait, Collings, & Hurst, 2010; Kyyaly, Powell, & Ramadan, 2015; Oraby, Allababidy, & Ramadan, 2015; Sabatier et al., 2017; Sapota et al., 2014; Stroobants et al., 2009).

Ein weiteres Verfahren ist die Mikroverkapselung mit Hefen. Hierbei werden diese mit Substanzen wie Aromen, Antioxidantien oder Lebensmittelfarbstoffen angereichert und die Hefe selbst dient als Schutzmatrix. Entsprechende Applikationen wurden in den Übersichtsartikeln von Pham-Hoang et al. (2013) und Đorđević et al. (2014) zusammengefasst.

Bei der Biotransformation werden Präkusoren zielgerichtet enzymatisch umgewandelt und das Produkt intrazellulär akkumuliert oder ins Medium sekretiert. Ein Beispiel hierfür ist die Biokonversion von Cystein, Glycin und Glutamat zu Glutathion, worauf in den nächsten Abschnitten umfassend eingegangen wird.

1.2 Glutathion - Historie

Glutathion ist das am meisten in eukaryotischen Organismen vorkommende, nicht-RNA-codierte, thiolhaltige Tripeptid mit vielen biochemischen Funktionen. Auch in prokaryotischen Systemen konnte, bis auf wenige Ausnahmen, Glutathion detektiert werden (Bachhawat, et al., 2009). Im Jahr 1888 wurde Glutathion vom Wissenschaftler J. De Rey-Pailhade erstmalig in Hefe nachgewiesen (J. De Rey-Pailhade, 1888). 1929 konnte Hopkins nachweisen, dass es sich um ein Tripeptid handelt (Hopkins, 1929). Sechs Jahre später wurde die Struktur von Glutathion durch Harington und Mead aufgeklärt und Bloch beschrieb den biosynthetischen Stoffwechselweg von Glutathion in Leberzellen (1949). Weitere 21 Jahre später, 1970, formulierte Meister den γ-Glutamylzyklus. Heute werden GSH und GSH angereicherte Hefen in Arzneimitteln, Pharma- und Nahrungsmittel- sowie Getränkeprodukten eingesetzt. Hierbei stehen verschiedene Charakteristika und Eigenschaften des Moleküls im Vordergrund: der Schutz vor Oxidation als Radikalfänger, die Funktion als starkes Reduktionsmittel und die Eigenschaft als Geschmacksmodulator. Spezifische GSH-Applikationen sind im Kapitel 2 aufgeführt.

1.3 Glutathion - Synthese

Glutathion ist nicht in den normalen Proteinstoffwechsel eingebunden. Die reduzierte Form des Glutathions (GSH) besteht aus drei Aminosäuren: L-Glutamat, L-Cystein und Glycin. Die Besonderheit dieses niedrig-molekularen Moleküls (307.22 g/mol) besteht in der speziellen γ-Peptidbindung zwischen L-Glutamat und L-Cystein, welche die Hydrolyse durch Amino-Peptidasen verhindert (Anderson, 1998). Diese Aminosäure und insbesondere die enthaltene Thiolgruppe (SH-Gruppe) ist ausschlaggebend für die herausragende Funktion des GSHs in der Zelle. Dabei erfolgt die Biosynthese in 2 enzymatischen Schritten, wobei pro Syntheseschritt jeweils 1 ATP verbraucht wird. In Abbildung 1-2 sind die einzelnen Kondensationsschritte der Synthese des GSHs visualisiert. Die Energie in Form von ATP und die Substrate bzw. Präkusoren L-Cystein, L-Glutamat und Glycin stammen dabei aus der Glykolyse, dem Citratzyclus und dem Sulfat-Assimilierungsstoffwechsel. In den Übersichtartikeln von Bachhawat, et al., (2009) und Stipanuk, (2004) sind die entsprechenden Stoffwechselwege übersichtlich skizziert. Ein weiteres Merkmal bei der Synthese von GSH ist die post-transkriptionale Regulation der Glutamatcysteinligase durch GSH selbst. Hierbei kommt es zu einer nicht allosterischen Feedback-Inhibierung, wobei GSH die Bindung des Enzyms für L-Glutamat und L-Cystein blockiert. Diese Regulation stellt ein Problem bei der Biotransformation von Cystein zu GSH dar und ist somit von entscheidender Bedeutung für die Entwicklung von Produktionsstrategien.

1.4 Glutathion - Lokalisierung

Die durchschnittliche intrazelluläre GSH-Konzentration beträgt 0.5-10 mmol/L (Meister & Tate, 1976). Thommel (1976) ermittelte im Rahmen seiner Dissertation unterschiedliche

GSH-Konzentrationen in Abhängigkeit der Hefespezies im Bereich von 0.11 – 0.57%. GSH wird in Säugertierzellen in 3 Hauptreservoirs gespeichert.

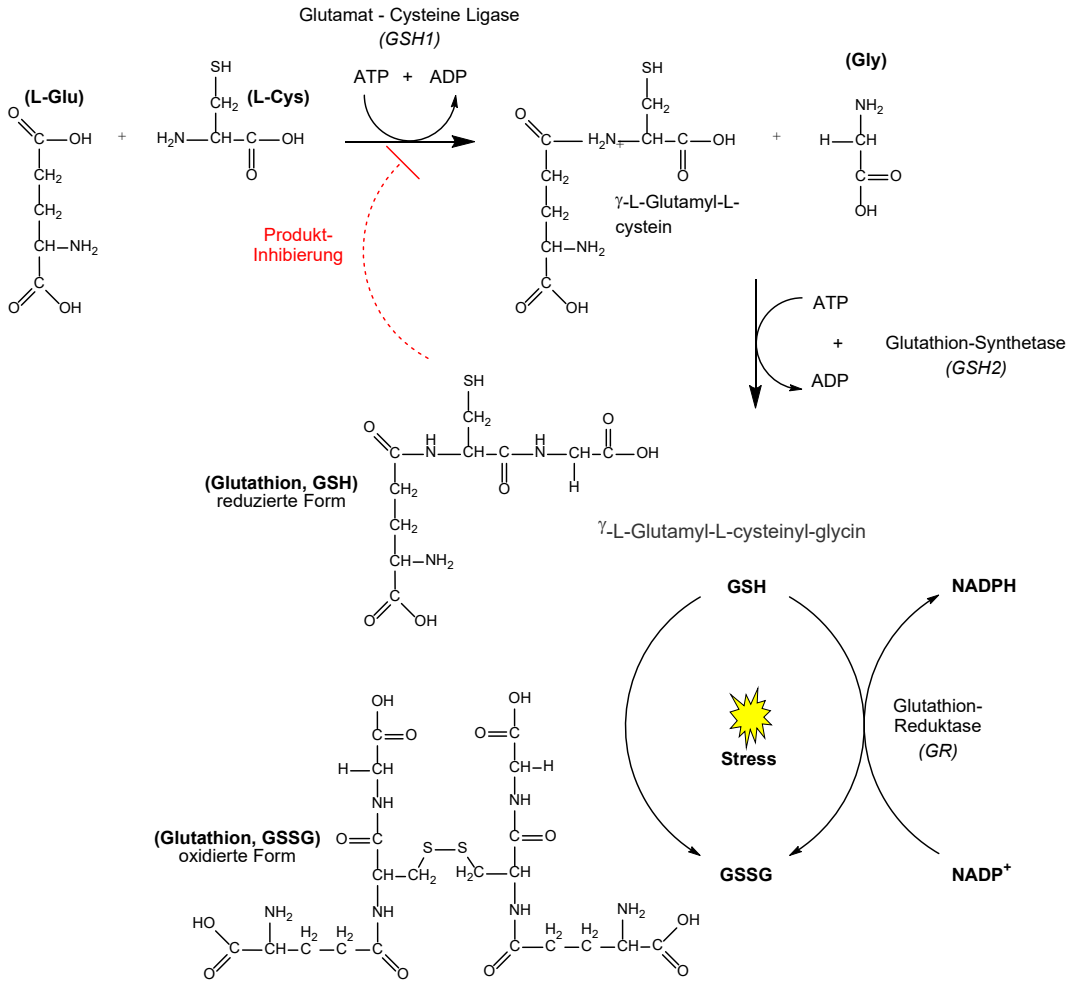

Abbildung 1-3 Schematische Darstellung der Biosynthese und des Redoxsystems von Glutathion in der Hefe *S. cerevisiae*.

Dabei ist der größte Teil ca. 80-85% im Zytosol, 10-15% in den Mitochondrien und der kleinste Teil im endoplasmatischen Retikulum lokalisiert (Lu, 2013). In *S. cerevisiae* konnte Zechmann et al. (2011) per quantitativer Immun-Elektronenmikroskopie die höchsten Konzentrationen an GSH in den Mitochondrien detektieren, gefolgt vom Zytosol, Nucleus und Vakuole.

1.5 Glutathion - Zelluläre Funktion

GSH ist Teil des sogenannten γ-Glutamyl-Zyklus. Dieser wird detailliert beschrieben von Meister & Tate (1976), Penninckx (2002) und Wu, Fang, Yang, Lupton, & Turner (2004). In diesem γ-Glutamyl-Zyklus herrscht ein Gleichgewicht zwischen Biosynthese und Degradation. Ein Grund für den enzymatischen Abbau ist, dass GSH als Stickstoff-, Schwefel- oder

Aminosäurequelle dient. Speziell L-Cystein als essentielle Schwefelquelle ist in geringen Konzentrationen toxisch, jedoch in gebundener Form nicht, und kann bei Bedarf durch Degradation von GSH bereit gestellt werden (Elkens, Jaspers, & Penninckx, 1991; Mehdi & Penninckx, 1997; Miyake et al., 1999). Eine weitere Funktion von GSH ist u.a. der Transport von extrazellulären Aminosäuren in die Zelle (Meister & Tate, 1976). Dabei wird GSH durch die Glutamyltransferase gespalten und der γ-L-Glutamyl-Rest an eine extrazelluläre Aminosäure gebunden (During-Olsen, Regenberg, Gjermansen, Kielland-Brandt, & Hansen, 1999; Kaur & Bachhawat, 2007; Payne & Payne, 1984; Penninckx, Jaspers, & Wiame, 1980). Neue wissenschaftliche Erkenntnisse hinterfragen den Aufbau des von Orlowski & Meister (1970) postulierten γ-Glutamyl-Zykluses und dessen funktionelle Rolle und zeigen Alternativen auf bzw. erweitern Zyklus zu einem Glutathion-Zyklus (Bachhawat & Yadav, 2018).

Zur Detoxifizierung von Xenobiotika, Metall- und Schwermetallen wie Cadmium, Arsen, Quecksilber, Silber und Blei wird ebenfalls das GSH-System von der Zelle verwendet (Hammond, Lee, & Ballatori, 2001). Diese Metallionen interagieren mit den Carboxyl- oder Thiolgruppen von Proteinen aufgrund ihrer ionophoren Eigenschaften (Mendoza-Cozatl, Loza-Tavera, Hernandez-Navarro, & Moreno-Sanchez, 2005). Auch können Metallionen wie Eisen oder Kupfer mit Peroxiden reagieren. Diese sogenannte Fenton-Haber-Reaktion und das Produkt sind hoch reaktive Hydroxylradikale (OH•) (Halliwell, 2006). Bei der Detoxifizierung bzw. Neutralisierung von Metallionen oder Xenobiotika wird, katalysiert durch das Enzym Glutathiontransferase (*GTT1 & 2*), die Thiolgruppe des GSHs dehydriert und das Metallion an den Schwefelrest gebunden. Das dabei entstandene S-Konjugat wird in die Vakuole per ABC-Transporter (*YCF1*) geschleust und entweder gespeichert oder abgebaut und ist somit neutralisiert (Z. S. Li, Szczypka, Lu, Thiele, & Rea, 1996). Auch die extrazelluläre Ausschleusung der S-Konjugate per ABC-Transporter *GEX1* oder *YOR1* ist möglich. GSH bildet ebenfalls S-Konjugate mit freien Protein-Thiolgruppen. Dieser Vorgang wird als S-Glutathionylation bezeichnet. Die dabei gebildeten reversiblen Disulfide schützen das Protein vor Oxidation und Inaktivierung durch Radikale (Perrone, Tan, & Dawes, 2008). Diese S-Konjugate werden auch in der Humanmedizin als Biomarker (S-glutathionyliertes Hämoglobin) und Hinweis auf oxidativen Stress verwendet (Bursell & King, 2000; Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013).

Eine ebenfalls wichtige zelluläre Funktion des GSHs ist die Neutralisierung von oxidativem Stress. Harman (1956) formulierte als Erster die Theorie, dass toxische Nebenprodukte des respiratorischen Stoffwechsels sowohl für die Alterung von Zellen als auch für viele menschliche Krankheiten verantwortlich sein könnten. Diese Nebenprodukte werden auch als reaktive Sauerstoffspezies (*engl. reactive oxygen species, ROS*) bezeichnet. Kommt es zur vermehrten Bildung von ROS in der Zelle, ausgelöst durch unterschiedlichste Umweltbedingungen oder einfach durch die Zellatmung, und können die dabei entstandenen Radikale nicht ausreichend durch Antioxidantien bzw. Radikalfänger neutralisiert werden, resultiert daraus ein Ungleichgewicht im Redoxpotential der Zelle und man spricht von

oxidativem Stress (Ayer, Gourlay, & Dawes, 2014; Farrugia & Balzan, 2012; Turrens, 2003). Auch über Umwelteinflüsse kann oxidativer Stress induziert werden, wie z.B. durch Temperaturänderung, Schwermetalle, Gifte/Xenobiotika, osmotischen Druck und Nährstoffmangel (Muthukumar & Nachiappan, 2010; Ribeiro, Corte-Real, & Johansson, 2006; Sugiyama, Kawamura, Izawa, & Inoue, 2000; Turrens, 2003).

Zu hohe Konzentrationen an ROS können zu einer beschleunigten Alterung der Zelle führen oder Proteine, Lipide und Erbgut (DNA) irreparabel schädigen (Bilinski, 1991; Falcone & Mazzoni, 2016; Lam, Aung-Htut, Lim, Yang, & Dawes, 2011). Deshalb hat die Zelle unterschiedliche Schutzmechanismen entwickelt (Halliwell & Gutteridge, 2015). Ein Großteil der natürlich entstehenden ROS wird, wie bereits erwähnt, in der inneren Mitochondrienmembran während der Re-oxidierung der Reduktionsäquivalente (NAD+/NADH; FAD+/FADH2) und der finalen Übertragung der Elektronen auf den Akzeptor Sauerstoff in der Atmungskette gebildet (Halliwell, 2006; Herrero, Ros, Belli, & Cabiscol, 2008). Ungefähr 1-2% der Elektronen werden dabei nicht korrekt auf den Sauerstoff übertragen, sondern unvollständig zu ROS reduziert (Chance, Sies, & Boveris, 1979; Ostermann & Rödel, 2006). Turrens (2003) gibt dazu in seinem Übersichtsartikel unterschiedliche potentielle Quellen für die Entstehung von Superoxidanionen innerhalb der Atmungskette an.

Zur Neutralisation der ROS verfügt die Zelle über mehr oder weniger ineinandergreifende und parallel operierende enzymatische und nicht-enzymatische Systeme. Enzymatische Systeme sind die Superoxiddismutasen (*SOD1, 2*), die Katalasen (*CTT1 & CTA1*) und Peroxidasen (*GPX1-3, GRX 1-5 & TRX 1-3*). Nicht-enzymatische Substanzen sind Erythroascorbat, Tocopherol, Carotinoide und GSH (Frei, 1994; Grant, 2001; Grant, Perrone, & Dawes, 1998; Herrero et al., 2008; Jamieson, 1998). Bei der Inaktivierung von oxidativem Stress ist GSH mit einem Redoxpotential von -240 mV fundamentaler Bestandteil der Stressantwort.

Dabei fungiert GSH t als Katalysator bzw. Elektronendonator und oxidiert selbst (GSSG, siehe Abbildung 1-3). Die Reduktion und Reaktivierung von GSH erfolgt durch die Glutathionreduktase (*GLR1*) unter Verbrauch bzw. Oxidation von NADPH. Beide Formen, sowohl red. GSH als auch ox. GSSG (Verhältnis: 25 - 100:1) bilden also mit NADP+/NADPH ein Redoxsystem (siehe Abbildung 1-3) (Ask, Mapelli, Hock, Olsson, & Bettiga, 2013; Bachhawat et al., 2009; Drakulic et al., 2005). Madeo et al. (1999) konnte die Bedeutung von GSH demonstrieren, in dem er in *S. cerevisiae* die γ-Glutamyl-L-Cystein-Ligase (*GSH1*) deletierte, wodurch in der Hefe ohne exogene Zugabe von GSH Apoptose ausgelöst wurde.

Kommt es zu Störungen oder Dysregulationen des zellulären Redox-Status und speziell im GSH-Stoffwechsel, können schwere Krankheitsbilder beim Menschen auftreten. Im Gegensatz zu malignen Zellen sind die meisten anderen Glutathion-assoziierten Krankheiten mit einem zellulären GSH-Mangel verbunden. Leberzirrhose, HIV-Infektion, verschiedene Atemwegserkrankungen, Diabetes, zystische Fibrose, kardiovaskuläre-, entzündliche-, immunologische-, metabolische- und neurodegenerative Krankheiten wie Alzheimer und

Parkinson sind hier zu nennen (Aoyama & Nakaki, 2013; Cantin, North, Hubbard, & Crystal, 1987; Ho et al., 2013; Traverso et al., 2013; Wallace, 2012; Z. Wang, Zhang, & Tan, 2010; Yang, Magilnick, Xia, & Lu, 2008; Yuan & Kaplowitz, 2009). Wissenschaftler und Mediziner arbeiten daran, diesen GSH-Mangel durch entsprechende Substanzen und Diäten exogen zu manipulieren.

1.6 Glutathion - Stammentwicklung

Das Kultivierungsmedium ist eine der tragenden Säulen für eine erfolgreiche Prozessentwicklung und Herstellung von bioverfahrens- und biotechnologischen Produkten. Mit Bezug zur GSH-Produktion wird auf die Medienentwicklung in Kapitel 2 und 3 eingegangen. Die Übersichtartikel von Connors (2003); Hahn-Hagerdal et al. (2005); Kennedy & Krouse (1999); Zhang & Greasham (1999) sind zum Thema Medienentwicklung zu empfehlen. Ebenso wichtig wie das Kultivierungsmedium ist die Wahl des Produktionsstammes.

Bei der Stammentwicklung bzw. Auswahl ist es wichtig, sich die Frage zu stellen, was das finale Produkt sein soll. Hinsichtlich GSH-Hefen bleibt zu überlegen, ob die GSH angereichte Hefe selbst das Produkt ist und im Lebensmittelsektor oder in der Futtermittelindustrie eingesetzt wird, oder ob GSH das Produkt ist, welches später aus den Hefen extrahiert und gereinigt wird und als Feinchemikalie verkauft werden soll.

Im ersten Fall würde man sich aufgrund der gesetzlichen Bestimmungen und der geringen gesellschaftlichen Akzeptanz gegenüber GVOs in Nahrungsmittelketten auf natürliche vorkommende Hefestämme konzentrieren oder diese Stämme als Ausgangspunkt für ungerichtete Mutagenese (*engl. random mutagenesis*) verwenden. Die erzeugten Stämme wären nicht deklarationspflichtig. Hamada et al. (1982), Lai et al. (2008) und Lang-Hinrichs und Jansen-Weismann (1993) z.B. haben durch ungerichtete Mutagenese per Methylnitronitrosoguanidin (MNNG), Ethylmethansulfonat (EMS) und/oder starker UV-Strahlung und anschließender Selektion der Mutanten die GSH-Konzentration auf 3.9% bzw. 3.4% bzw. 6.6% (w/w) steigern können. Aber auch andere Wissenschaftler konnten durch ungerichtete Mutagenese und anschließende Selektion Stämme mit hoher GSH-Konzentration selektieren (Nishiuchi et al., 2012; Shang et al., 2008; Wang et al., 2011). Im zweiten Fall hingegen, bei der das GSH von der Hefe getrennt und gereinigt wird, können zielgerichtet Mutationsmethoden angewendet werden (*engl. genetic or metabolic engineering*). Hierbei werden spezifische Gene oder Genabschnitte auf chromosomaler Ebene verändert und modifiziert oder neue eingebracht. Die auf diesem Wege hergestellten Stämme wären laut EU-Richtlinie 2001/18/EG deklarationspflichtig. Wissenschaftler überexprimierten, deletierten und kombinierten zentrale Enzyme der GSH-Synthese, des γ-Glutamyl-Zyklus oder des Schwefelmetabolismus. Auch die Überexpression von Transkriptionsfaktoren, welche für die Regulation des Schwefelstoffwechsels verantwortlich sind, wurden untersucht, um eine Steigerung der GSH-Produktion zu erreichen (Hara et al., 2012; Ubiyvovk, Ananin, Malyshev, Kang, & Sibirny, 2011). Des Weiteren wurde ein Stamm designt, der intrazelluläres GSH in

das Kultivierungsmedium ausschleust, um die Feedback-Inhibierung der Glutamatcysteinligase (*GSH1*) zu umgehen (Kiriyama, Hara, & Kondo, 2012). Jedoch sind mit all den in diesem Abschnitt genannten genetisch manipulierten Hefen nur geringe Biomassen und GSH-Konzentrationen generierbar. Eine Ausnahme bildet der Prozess von Ubiyvovk et al. (2011), in dem Biomassen von 72 g/L erreicht und die intrazelluläre GSH-Konzentration von 1.3% auf 3.1% gesteigert wurde.

Die klassische ungerichtete Mutagenese, wie oben beschrieben, hat einige Nachteile: eine kleine Populationsdiversität, Akkumulation von ungewünschten Mutanten und die Kultivierungsbedingungen sind mit dem finalen Prozess nicht vergleichbar (Patzschke et al., 2015). Aber auch die zielgerichtet genetische Manipulation hat Nachteile. Oft sind Stoffwechselwege und deren Regulation kompliziert und selten sind alle Informationen z.B. von metabolischen Flussanalysen vorhanden (Bailey et al., 2002; Cakar, Turanli-Yildiz, Alkim, & Yilmaz, 2012). Diese Wissensdefizite führen, trotz adäquater Vorbereitung, oft zu Rückschlägen und Misserfolgen.

Eine alternative Strategie zu den o.g. Verfahren der Stammentwicklung ist die sogenannte adaptive Evolution (*engl. evolutionary engineering oder adaptive evolution*). Die Theorie dieser Strategie ist einfach, es ist das Imitieren der Natur durch zufällige Mutation (Cakar et al., 2012). Durch fortwährende Kultivierung über viele Generationen eines Stammes oder einer Population kommt es zu natürlich vorkommenden Mutationen. Die Mutationsrate, oder auch Evolutionsrate genannt, beträgt bei *S. cerevisiae* ca. 10^{-11}-10^{-10} pro Generation und Basenpaaren (Lang & Murray, 2008). Um diese Rate zu erhöhen werden oft mutagene Subtanzen wie EMS, MNNG, Nitrosoguanidin (NTG) oder Diethylsulfonat (DES) verwendet, wodurch sich sowohl die Mutationsrate um Faktor 100 als auch die genetische Diversität steigern lässt. Ein weiterer Faktor ist der Selektionsdruck, wobei dieser so gewählt werden sollte, dass sich der gewünschte Phänotyp über mehrere Generationen durchsetzt und chromosomal fixiert wird. Oft werden diese Selektionsstrategien im Bioreaktor bzw. Chemostat durchgeführt.

Der Vorteil im Gegensatz zu der früher verwendeten Plattenselektion ist, dass sich über viele Generationen hinweg ein gewünschter, an industrielle Bedürfnisse angepasster Phänotyp (Toleranzen gegenüber Kultivierungsbedingungen, Resistenzen gegen toxischen Komponenten, Verwertung von Substraten, Erhöhung der Produktivität etc.) entwickelt hat. Ausführlichere Erläuterungen zum Thema adaptive Evolution können den Übersichtartikeln von Cakar et al. (2012) und Winkler & Kao (2014) entnommen werden.

Das beschriebene Verfahren fand Anwendung bei Mezzetti, De Vero & Giudici (2014) zur Selektion von *S. cerevisiae* Stämmen zur Weinherstellung. Dieser führt in seinem Artikel aus, dass die Geschmacksstabilität von Wein durch Oxidation von Aromastoffen negativ beeinflusst wird. Durch sexuelle Rekombination und spätere Selektion durch hohe toxische Konzentrationen an Molybdat konnte der GSH-Gehalt sowohl in der Hefe als auch im Wein signifikant gesteigert werden. Patzschke et al. (2015) konnte durch Mutagenese mit MNNG und

späterer Selektion durch Acrolein nach mehreren Zyklen zwei Stämme mit einem intrazellulären GSH-Gehalt von 3.9% respektive 5.9% isolieren. Fraglich ist, ob Stämme mit derart hohen GSH-Konzentrationen auch genetisch stabil sind. Bis dato sind die selektierten Stämme in keiner relevanten industriellen Bioreaktorkultivierung, also im Kubikmeter-Maßstab verwendet worden. Die bisher größte publizierte Glutathionherstellung erfolgte in einem 120 m^3-Reaktor der Firma Kyowa Hakko Kogyo Co. Ltd. (Sakato & Tanaka, 1992). Dabei wurden Konzentrationen von 2360 mg/L GSH erreicht.

1.7 Glutathion - Produktion

Die GSH-Produktion erfolgt auf unterschiedliche Weise: durch chemische Synthese, enzymatisch oder fermentativ. Die chemische Synthese von Glutathion wurde in den 50iger Jahren kommerzialisiert. Allerdings war die Herstellung zeitaufwendig, kompliziert und ineffektiv. Zudem lag das Endprodukt als Razemat vor und war somit teilweise optisch inaktiv (Bachhawat et al., 2009; Y. Li, Wei, & Chen, 2004).

Das Problem der Razematbildung kann durch Verwendung der für die Glutathionsynthese spezifischen Enzyme Glutamatcysteinligase (Gsh1p) und Glutathionsynthetase (Gsh2p) unter Zugabe von ATP, Magnesium (Cofaktor), Phosphatpuffer (pH-Stabilisation) und Calcium umgangen werden. Zusätzlich werden die 3 Präkusoren L-Glutamat, L-Cystein und Glycin ebenfalls benötigt. Auch können zur enzymatischen Synthese ganzen Zellen, also Hefen oder Bakterien verwendetet werden. Dazu muss jedoch die Zellmembran zuvor mit Detergenzien, Chemikalien oder durch physikalische Methoden z.B. mit „Einfrieren – Auftauen“ Prozedere permeabilisiert werden. Der Vorteil der enzymatischen Synthese liegt in höheren Produktkonzentrationen und kürzeren Produktionszyklen (W. Li, Li, & Ye, 2010). Ein weiterer Vorteil dieser Methode ist die kontinuierliche Abführung des Produkts oder die Herstellung in zwei separaten Phasen. Dabei wird in der ersten Phase nur L-Glutamat und L-Cystein dazugegeben. Das Produkt des ersten Syntheseschritts ist γ-L-Glutamyl-L-Cystein. Im finalen Schritt wird Glycin supplementiert, was zur Synthese von GSH durch die Glutathionsynthetase führt. Bei beiden Methoden wird jeweils die Feedback-Hemmung der Glutamatcysteinligase (*GSH1*) umgangen.

Die dritte Methode zur GSH-Produktion ist aus industrieller Sicht die Methode der Wahl, die kontrollierte Kultivierung von Hefen oder Bakterien im Bioreaktor. Die Firma Kyowa Hakko Kogyo Co. Ltd. fing in den 70ziger Jahren des letzten Jahrhunderts an GSH aus Backhefe (0.1 – 1%) zu extrahieren. *S. cerevisiae* als auch *C. utilis* sind die am häufigsten verwendeten Organismen zur Produktion von GSH im industriellen Umfeld (Alfafara, C. G. et al., 1992) . Dennoch wurden vor kurzem zwei Prozesse mit *E. coli* entwickelt, die sehr effizient hinsichtlich ihrer Ausbeute und Produktkonzentration sind (C. Wang, Zhang, Wu, Li, & Ye, 2015; Zhang et al., 2016). Es bleibt jedoch zu bedenken, dass bei der Verwendung von gramnegativen Bakterien die in der Zellmembran enthaltenen Lipopolysaccharide bzw. Endotoxine stark

abgereichert werden müssen, da diese im menschlichen Körper zu starken immunologischen Reaktionen führen können.

Die Palette von bioverfahrenstechnischen Entwicklungen von Kultivierungsprozessen zur Produktion von Glutathion ist mannigfaltig. Die meisten Optimierungen befassen sich mit der Reduktion von Stoffwechselnebenprodukten und deren Monitoring, um effizient hohe Biomassen zu erreichen. Hierbei sollte die Hefe aerob kultiviert und Sauerstofflimitierungen vermieden werden (aerob-fermentativ oder fermentativ). Aufgrund der Prozessökonomie und steril-technischen Gegebenheiten wird die GSH-Produktion ausschließlich im Fed-Batch Verfahren durchgeführt. Dabei ist es egal, für welchen Parameter man sich zur Regelung des Prozesses entscheidet. Prinzipiell gilt für eine erfolgreiche Produktion von GSH in Hefen, dass der Prozess aus zwei Phasen besteht:

Die erste Phase ist die Propagation der Hefe, um eine möglichst hohe Biomasse zu erzeugen. Hierfür werden im industriellen Umfeld meist Melasse als C-Quelle und Maisquellwasser, Hefeextrakt, Ammoniumsulfat und Ammoniak als N-Quelle verwendet. Dabei besteht häufig die Möglichkeit, die Stickstoffquelle gleichzeitig als pH-Korrekturmittel zu nutzen.

Im zweiten Abschnitt des Prozesses, also im Übergang zwischen Wachstumsphase und stationärer Phase, erfolgt die Zugabe der Präkusoren für GSH. Um die Glykolyse bzw. die ATP-Generierung aufrecht zu erhalten wird weiterhin Nährlösung in den Bioreaktor gepumpt. Für einen ökonomisch-effizienten Prozess ist es wichtig, eine optimale Ausbeute der Präkusoren, insbesondere die Biotransformation von Cystein zu Glutathion zu erhalten. Im Kapitel 5 wird dies systematisch untersucht. Hierbei wurden als Faktoren der Zugabezeitpunkt, die Konzentration und Zugabeprofil von Cystein variiert. Natürlich gibt es auch andere Verfahren, um die Ausbeute und Produktion von GSH zu optimieren. Produktionsverfahren, Kontrollstrategien, unterschiedliche Nährstoffquelle und Präkusor sind im Kapitel 2 tabellarisch und umfassend aufgeführt.

Die Ökonomie des Herstellungsprozesses ist abhängig von der Prozesszeit sowie den Energie-, Personal, Medien- und Abwasserkosten. Tendenziell sollten sich die Produktionszeiten zwischen 24h und max. 48h bewegen. Es gibt jedoch die Möglichkeit durch „Repeated-Fed-Batch“ Verfahren, also durch eine direkte Wiederholung des Prozesses, die Bioreaktorrüstzeit sowie die Anzucht der Hefen zu sparen. Der Artikel von Koutinas, Chatzifragkou, Kopsahelis, Papanikolaou & Kookos (2014) gibt hierzu eine gute Übersicht.

1.8 Prozessbetriebsarten

Im folgenden Abschnitt wird auf die in dieser Arbeit eingesetzten Betriebsarten zur Kultivierung von Hefe kurz eingegangen. Dabei werden die Unterschiede zwischen den Prozessführungen verdeutlicht sowie deren Vor- und Nachteile kurz erläutert.

Auf Massenbilanzierung, formalkinetische Ansätze und die daraus resultierenden mathematischen Modelle der in diesem Abschnitt aufgeführten Prozessbetriebsarten wird

bewusst verzichtet und auf die Literatur von Chmiel (2011); Doran (1995); Enfors & Häggström (2000) verwiesen.

1.8.1 Satzbetrieb – „Batch“

Beim sogenannten Satzbetrieb sind alle Komponenten in ausreichender Konzentration im Kultivierungsmedium vorhanden und abgesehen von vernachlässigbar geringen Mengen Titrations- und Antischaummittel werden dem System keine Flüssigkeiten zugeführt. Die Biomassekonzentration wächst exponentiell uneingeschränkt mit maximaler Wachstumsrate. Dies geht solange, bis es zu Limitierungen von spezifischen Nährstoffkomponenten (auch Sauerstoff) oder zur Anhäufung von inhibieren Stoffwechselmetaboliten kommt. Dies ist ein signifikanter Nachteil dieser einfachen Prozessführung.

Trotzdem wird diese Prozessführung aufgrund ihrer simplen Durchführbarkeit oft für Hochdurchsatz-Screenings im Bereich Medienentwicklung, Substanz- und Stamm-Screening eingesetzt. Weitere Gründe, warum oft im Forschungsbereich der Satzbetrieb verwendet wird, sind die mangelnde Verfügbarkeit oder auch das limitierte Budget für adäquate Fed-Batch Systeme. Schnell/Oft werden für solche Systeme Preise im sechsstelligen Bereich je nach Ausstattung und Durchsatz aufgerufen, wobei die Kosten für Verbrauchsmittel und Maintenance nicht inkludiert sind. Der Platzbedarf für solche Systeme ist ebenfalls nicht außer Acht zu lassen. Deshalb wird oft auf erschwingliche Systeme wie Schüttelkolben zurückgegriffen. Durch Kombination mit einem enzymbasierten Freisetzungssystem für Glucose kann somit auch ein Überflussmetabolismus umgangen werden (Glazyrina et al., 2012). Aber nicht nur klassische Schüttelkolben, sondern auch 50 mL-Zentrifugationsröhrchen haben sich als kostengünstige Lösung bewährt, um Effekte aufzuzeigen (Anderlei et al., 2009; De Jesus et al., 2004; Xiaowei Zhang et al., 2009; X. Zhang et al., 2009). Ein weiterer Vorteil dieser Prozessführung ist das reduzierte Kontaminationsrisiko, da keine Nährstoffe zugeführt werden. Im industriellen Bereich werden solche Verfahren eingesetzt, wo eine hohe Ausbeute pro Substrateinheit erzielt wird und bei denen die Mikroorganismen hohe anfängliche Substratkonzentration tolerieren.

1.8.2 Zulaufverfahren - „Fed-Batch“

Das Zulaufverfahren ist eine der Schüsseltechnologien zur effizienten Produktion biotechnologischer Erzeugnisse von Feinchemikalien und Sekundärmetaboliten über Antibiotika bis hin zu komplexen Molekülen wie monoklonalen Antikörpern. Sie alle können mit diesen Verfahren hergestellt werden (Lee, Lee, Park, & Middelberg, 1999). Wie bereits in einem US-Patent (US 1449105) von Hayduck (1923) erwähnt, geht es im Zulaufverfahren um die Zugabe von konzentrierter Nährstofflösung zu einer wachsenden Kultur, um die metabolische Aktivität des Organismus zu steuern und Substratinhibierungen zu vermeiden.

Im Gegensatz zum Satzverfahren sollte die initiale Substratkonzentration im Reaktor relativ gering sein. Durch Zulauf des Substrats nimmt das Volumen im Bioreaktor stetig zu. Der

kontrollierte Substratzulauf erlaubt es, spezifische Reaktionsraten unter einem kritischen Niveau bzw. die Wachstumsrate auf einen optimalen Wert einzustellen. Katabolitrepression oder ein Überflussmetabolismus (*engl. overflow metabolism*) wird durch eine solche „substratlimitierende Prozessführung“ umgangen (Sonnleitner & Kappeli, 1986). Einbußen in der Biomassekonzentration oder Produktqualität und Ausbeute können die Folgen solcher Effekte sein. Des Weiteren können durch die Steuerung der Zulaufrate verfahrenstechnische Limitierungen hinsichtlich der Kühlung und der Sauerstoffversorgung vermieden werden. Dies führt dazu, dass in kürzester Zeit hohe Biomassekonzentrationen erzielt werden können, was bei wachstumsgekoppelten Produkten zu einer erhöhten Produktmenge führt. Ebenfalls lassen sich durch das Zulaufverfahren sekundäre Metabolite durch eine zweistufige Strategie (Wachstumsphase und anschließender Produktionsphase) herstellen.

Vorteilhaft ist ebenfalls eine effiziente zeitliche Auslastung und die volumetrische Produktivität der Bioreaktoren, was in Kombination mit den erhöhten Produktmengen, wie sie im Zulaufverfahrens erzielt werden, die Fixkosten gegenüber dem Satzbetrieb reduziert. Dem gegenüber steht das erhöhte Kontaminationsrisiko durch die Dosage von unterschiedlichsten Nährlösungen und die längere Prozesszeit sowie die zusätzlichen Instandhaltungskosten für Pumpen und Regelungsequipment sowie Lager- und Medientanks.

1.8.3 Kontinuierliche Prozessführung – Chemostat und A-Stat

Bei der Betriebsart A-stat handelt es sich um eine Ableitung des Chemostats. Letztere ist dadurch charakterisiert, dass die Wachstumsrate über die Verdünnungsrate gesteuert wird bzw. beide äquivalent sind (*engl. steady state*). Nährstoffe werden mit einer bestimmten Rate in den Reaktor gepumpt und gleichzeitig verbrauchtes Medium inklusive Zellen/Zellprodukten abgezogen. Dabei bleiben das Kulturvolumen und die Biomassekonzentration im Reaktor über den Prozess konstant. Dies ist auch gleichzeitig der Vorteil dieser Betriebsart, da im Gegensatz zum Satzbetrieb kein Sekundärwachstum sowie Stressantworten aufgrund mehrerer gleichzeitiger Limitationen entstehen (Hoskisson & Hobbs, 2005). Die dadurch vorherrschenden idealisierten Bedingungen wie sie im Chemostat herrschen, lassen sich nicht im Zulaufbetrieb realisieren. Deshalb wird diese Verfahrensweise oft im wissenschaftlichen Bereich für Flussanalysen (*engl. flux analysis*) oder Genexpressionsstudien eingesetzt. Der Chemostat kann auch für die Stammcharakterisierung und Medienentwicklung genutzt werden, oder um den Einfluss physikalischer Parameter wie dem pH-Wert, die Sauerstoffsättigung oder die Temperatur zu untersuchen. Ein großer Vorteil der kontinuierlichen Prozessführung im industriellen Umfeld ist die hohe Raum-Zeit-Ausbeute (engl. space-time-yield, STY). Hier kurz aufgezeigt die theoretische Reihenfolge der Raum-Zeit-Ausbeute für unterschiedliche Betriebsarten: Batch << Fed-Batch << Chemostat << Perfusion.

Wie bereits oben erwähnt ist der A-Stat eine Modifikation des Chemostats. Das Verfahren wurde erstmals von Paalme & Vilu (1992) beschrieben, um Stämme hinsichtlich ihres Wachstums und der respiratorischen Kapazität zu charakterisieren. Beim A-Stat wird die

Verdünnungsrate mit einer kontinuierlichen Beschleunigung erhöht bzw. reduziert. Dadurch lässt sich praktisch eine Vielzahl an Einstellungen untersuchen. Die optimale spezifische Wachstumsrate ist ein wichtiger Zielparameter bei der Entwicklung von Produktionsverfahren im Zulaufbetrieb. Im Kapitel 3 untersuchten Schmacht, Lorenz, Stahl, & Senz (2017) mit dem A-Stat den Einfluss der spezifischen Wachstumsrate auf die GSH-Bildung zur Entwicklung und Optimierung eines Zulaufverfahrens mit chemisch definiertem Medium.

Jedoch ist der verfahrenstechnische Aufwand solcher Systeme im Allgemeinen hoch und es existieren signifikant weniger Produktionsprozesse im kontinuierlichen als im Fed-Batch-Betrieb. Der Grund hierfür ist zum einen der hohe steril-technische Aufwand und das dazu benötigte Equipment und zum anderen die aus Qualitätsmanagementsicht unklare Chargenrückverfolgbarkeit. Ebenfalls schwierig ist der hohe Durchsatz an Medium, was bei der Planung solcher Anlagen unbedingt bedacht werden sollte. Deshalb lohnen sich diese Verfahren meist nur bei hohen Gewinnmargen und/oder wenn eine Feedbackinhibierung des sekretierten Produktes auf die Zellen existiert.

1.9 Ziel und Struktur der Arbeit

Der Fokus dieser Dissertationsarbeit liegt in der Verfahrensentwicklung zur intrazellulären Anreicherung und Produktion von Glutathion mit speziell zuvor selektierten *Saccharomyces spp*. Hefen. In insgesamt 6 Kapiteln werden unterschiedliche bioverfahrenstechnische Ansätze und Strategien zur Optimierung der Glutathionausbeute aufgezeigt und die erzielten Ergebnisse vorgestellt, erläutert und umfassend diskutiert.

1.9.1 Kapitel 1 - Allgemeine Einleitung und Überblick zur GSH Lokalisation, Funktion, Biosynthese und bioverfahrenstechnische Produktion

Im ersten Kapitel wird eine umfassende Einleitung zum Thema Glutathion gegeben. Zuvor erfolgt eine kurze historische Übersicht. Beispiele zur Verwendung von Hefen im Allgemeinen sowie eine rudimentäre wirtschaftliche Einordnung von Produkten aus Hefe werden aufgezeigt. Im Weiteren werden die geschichtlichen Aspekte des GSHs und dessen Entdeckung kurz abgehandelt. Umfassend wird auf die Glutathion-Biosynthese, die Lokalisation und die zelluläre Funktion des Tripeptids eingegangen. Auch die Stammentwicklung zur Überproduktion von GSH in Hefe wird umfassend behandelt. Das Ende dieses Kapitels bildet der Abschnitt der Glutathionherstellung explizit in Hefe und die in dieser Arbeit verwendeten Kultivierungsmodi im Bioreaktor werden kurz erklärt.

1.9.2 Kapitel 2 - Überblick zur GSH Produktion, Prozesssteuerung und Patentsituation

Microbial production of glutathione / World Journal of Microbiology and Biotechnology Vol. 33 No. 6, 106 (2017) / https://doi.org/10.1007/s11274-017-2277-7

Das Kapitel 2 komplettiert das vorhergehende Kapitel 1. Der Übersichtsartikel betrachtet nicht nur die verschiedenen Produktionsmodi von GSH mit Hefe und deren Besonderheiten hinsichtlich Kultivierungsmedien, Prozesssteuerung, Stammentwicklung und Ausbeute, sondern gibt auch einen Überblick zur mikrobiellen Produktion von GSH. Des Weiteren wird die Verwendung in der Kosmetik-, Lebensmittel- und Getränkeindustrie aufgelistet und zeigt auf, wie verbreitet GSH eingesetzt wird. Zuletzt wird auf die momentane Patentsituation umfassend eingegangen.

1.9.3 Kapitel 3 - Medienentwicklung

Medium optimization based on yeast's elemental composition for glutathione production in Saccharomyces cerevisiae / Journal of Bioscience and Bioengineering Vol. 123 No. 5, 555-561, 2017 / https://doi.org/10.1016/j.jbiosc.2016.12.011

In Kapitel 3 wird die Bedeutung der Medienentwicklung thematisiert und aufgezeigt. Dabei wird auf die Vor- und Nachteile von chemisch definierten und komplexen Medien kurz eingegangen. Basierend auf der elementaren Zusammensetzung von Backhefe also *S. cerevisiae* wurde ein chemisch definiertes Medium erfolgreich entwickelt. Ebenfalls Teil der Medienentwicklung war die Untersuchung von verschiedenen Stickstoffquellen und deren Effekt auf die Biomassebildung und GSH-Produktion. Um eine optimale Wachstumsrate für einen späteren Fed-Batch Prozess zu ermitteln wurde das A-stat Verfahren (*engl. accelerated-stat*), eine Modifikation des Chemostats, verwendet. Am Ende stand ein chemisch definiertes Medium zur GSH-Produktion, das zuverlässig für Forschungszwecke eingesetzt werden kann, aber auch industriellen Ansprüchen genügt.

1.9.4 Kapitel 4 - GSH Induktion mit Cysteinderivaten in drei Hefestämmen

Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in Saccharomyces spp. / Enzyme and Microbial Technology 93 (2016) 122–131 / https://doi.org/10.1016/j.enzmictec.2016.08.004

In Kapitel 4 werden drei Cysteinderivate und deren potentielle Effekte auf die GSH-Produktion untersucht. Ziel war es, eine effiziente Alternative zum Cystein zu finden, welches bereits industriell zur Induktion der GSH-Produktion in Hefen eingesetzt wird. Aufgrund der industriellen Relevanz wurde nicht nur die Backhefe *S. cerevisiae,* sondern auch die Bierhefe *S. bayanus* und die probiotische Hefe *S. boulardii* verwendet. Um die Faktoren zu Beginn der Studie und die daraus resultierende Anzahl an Faktorkombinationen parallel untersuchen zu können, musste ein neues Kultivierungssystem in parallelisierten 50 mL-Ansätzen etabliert werden. Mit diesem System konnten erfolgreich Faktoren identifiziert werden, die einen positiven Effekt auf die GSH-Produktion zeigten. Die finale Verifikation der Effekte wurde im klassisch gerührten Bioreaktor im Batch Verfahren durchgeführt. Dabei konnte erfolgreich Cystein-Ethyl-Ester (CEE) für alle verwendeten Hefestämmen als effizienter Induktor zur

GSH-Produktion bestätigt werden. Es wird postuliert, dass aufgrund des Ethyl-Ester-Rests das CEE im Vergleich zu Cystein schneller von der Hefe aufgenommen werden kann. Dies könnte die sehr erfolgreiche Ausbeute erklären, bei der das Verhältnis zwischen Induktor und intrazellulären GSH mit CEE mehr als doppelt so hoch war als mit Cystein.

1.9.5 Kapitel 5 - Fed-Batch Prozessentwicklung und Effizienzsteigerung des Cystein/Glutathion Ratio

Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of Saccharomyces cerevisiae / Journal of Biotechnology Vol. 216, 131–139, (2015) / http://dx.doi.org/10.1016/j.jbiotec.2015.10.016

Kapitel 5 befasst sich umfassend mit der Fed-Batch Prozessentwicklung zur Produktion von Glutathion in der Hefe *S. cerevisiae*. Als Grundlage zur Prozessentwicklung diente das Medium WMIX, welches mit Hefeextrakt supplementiert wurde, um höhere Biomassen zu erreichen und ggf. Nährstofflimitationen zu vermeiden. Um eine optimale Fütterungsstrategie zu entwickeln wurde mit Hilfe einer Abgasanalyse die CO_2 (CER, *engl. carbon evolution rate*) Produktion und die O_2 Aufnahmerate (OUR, engl. *oxygen uptake rate*) bestimmt und per online Datenanalyse der respiratorische Quotient (RQ) berechnet. Der Quotient wurde dazu benutzt eine Fütterungspumpe zu steuern, welche gravimetrisch die Fütterungsmengen aufzeichnete. Durch diese „closed loop feedback control“ Steuerung war es möglich, in kurzer Zeit ein optimales Fütterungsprofil zu generieren, welches Biomassen von bis zu 100 g/L innerhalb von 48h ermöglicht.

Ein weiterer integraler Bestandteil und weiteres Ziel war die Erhöhung und Optimierung des auf den Gesamtprozess bezogenen Cystein/GSH Ratios. Hierfür wurden unterschiedliche Zeitpunkte, Cysteinkonzentrationen und Zugabemodi des Induktors (bolus / kontinuierlich) untersucht. Es stellte sich heraus, dass eine späte Zugabe von Cystein die Biomasseentwicklung weniger beeinflusst als eine Zugabe während der Wachstumsphase. Gleiches trifft auf die finale GSH-Konzentration zu. Auch konnte beobachtet werden, dass die Zugabe von kleineren Mengen an Cystein zu höheren GSH/Cystein Ratio führt und umgekehrt. Die Kombination dieser Ergebnisse führte zu einem finalen Setup, in dem Cystein kontinuierlich in geringer Rate in den Bioreaktor zugegeben wurde. Diese Verfahrensweise resultierte in einer Biomasse von 99.5 g/L. Darüber hinaus konnte die höchste GSH-Konzentration von 1650 mg/L (1.76%) gemessen werden und das GSH/Cystein Ratio wurde signifikant auf 0.54 mol/mol erhöht.

Das entwickelte Prozessformat wurde erfolgreich im 150-L Bioreaktor bestätigt. Um die Raum-Zeit Ausbeute zu optimieren wurde zudem ein Repeated Fed-Batch Verfahren etabliert, welches von industrieller Relevanz ist.

1.9.6 Kapitel 6 - Diskussion

Im letzten Kapitel dieser Arbeit werden die erzielten Ergebnisse zusammenfassend diskutiert. Der Fokus der Diskussion liegt auf der Glutathionproduktion und beleuchtet die

Einflussfaktoren zur effizienten Produktion von GSH, insbesondere in wirtschaftlicher Hinsicht. Des Weiteren wird auf die Prozessierung der GSH angereicherten Hefen eingegangen und die sich daraus ergebenden Produkte und Beispiele werden genannt.

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Kapitel 2

Microbial production of glutathione - Review

Microbial production of glutathione

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2 Microbial production of glutathione

2.1 Abstract

Glutathione (GSH) is a non-coding tripeptide thiol with several important regulative and protective functions in eukaryotes and in most prokaryotes. The primary function of GSH is to maintain the redox potential of the cell, which is directly connected to GSH concentration, and to prevent cellular damages caused by reactive oxygen species (ROS) or toxic heavy metals. Due to its antioxidant character, it is widely used in pharmaceutical, cosmetic and food industry. There have been different strategies to optimize GSH yield and productivity in bacteria and yeasts by means of metabolic and evolutionary engineering, media optimization and bioprocess engineering. The fed-batch procedure with yeasts of the genera Saccharomyces and Candida is still common method for industrial production. However, for an economic bioprocess production of GSH key factors like media costs, strain performance and process scalability are essential. Beside the extraction and purification of GSH as bulk product, GSH-enriched yeast cells are used for food and beverage applications, as well. This review outlines current applications of microbially produced GSH and illustrates current developments and strategies for its production.

2.2 Introduction

It was already stated e.g. by Anschau et al. (2013); Meister & Anderson (1983) there are too many scientific contributions on glutathione to read or cite them all. A current search on glutathione in publication titles via PubMed (https://www.ncbi.nlm.nih.gov/pubmed/; search term: glutathione [Title]; 20.01.2017) yielded 26493 results underpinning its prominence. This review seeks to show current applications of microbial GSH and up-to-date developments and strategies for its production, whereby the different aspects of its fermentative production are focused.

The non-coding tripeptide thiol glutathione (γ-glutamyl-cysteinyl-glycine; GSH), consisting of the three amino acids L-glutamate, L-cysteine and glycine (see Figure 2-1), occurs in most pro- and eukaryotic organisms in concentrations of 0.2-10 mM (Anderson, 1998). In eukaryotes, it is synthesized via two adenosine triphosphate (ATP) dependent reactions (Figure 2-1), whereby the first one is rate-limiting due to the feedback inhibition of γ-glutamyl-L-cysteine synthetase (GCS) by GSH (Lu, 2009). In combination with its oxidized form glutathione disulfide (GSSG), exhibiting a ratio of 30-100:1, both act as a pivotal redox couple (E0 = -240 mV), which maintains the redox status of the cell, by e.g. preventing the cell from damage by reactive oxygen species (ROS) or heavy metals, attracting attention for diverse medical or industrial applications (Ask et al., 2013; Ayer et al., 2014; Bachhawat et al., 2009; Drakulic et al., 2005; Stephen & Jamieson, 1997).

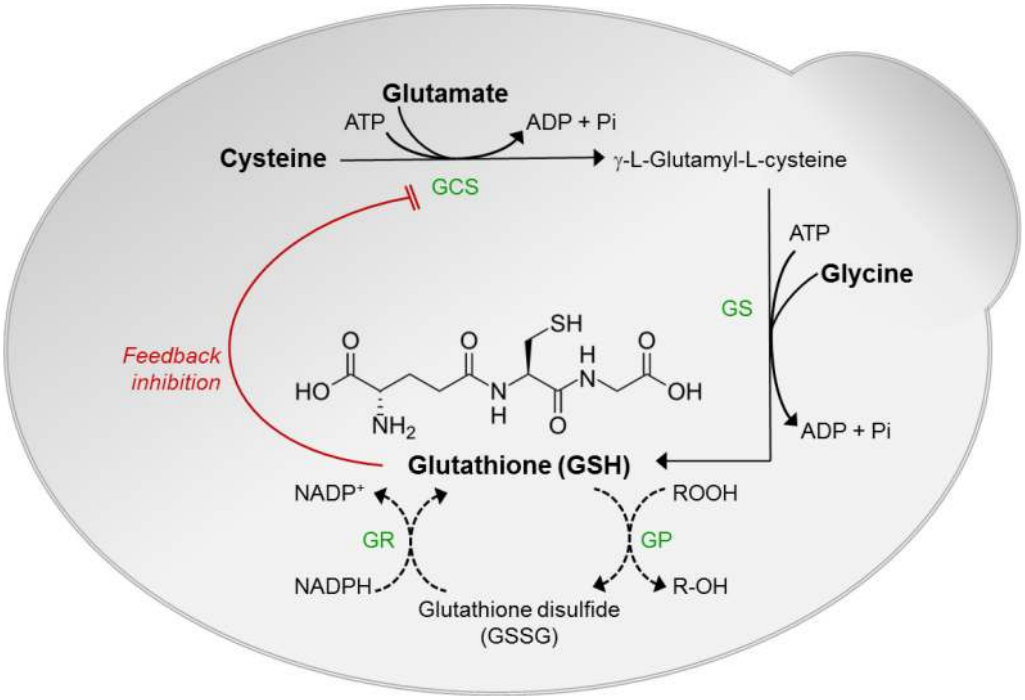

Figure 2-1 **Most relevant steps of glutathione metabolism in *S. cerevisiae*. Enzymes stated in green: GCS: γ-glutamyl-cysteine synthase, GS: glutathione synthetase, GR: glutathione reductase, GP: glutathione peroxidase**

2.3 Application of glutathione

It is supposed that the worldwide annual turnover of yeast-derived products, including GSH containing yeasts and GSH purified out of it, will exceed 9 billion dollars by the year 2019 (Marz, 2014). Per year, more than 200 tons of pure crystalline GSH are produced worldwide (Orumets et al., 2012). The interest in glutathione and derived products is based on the reductive character and the resulting diverse possibilities of medical and industrial application. Main characteristic features of the tripeptide GSH arise from its particular γ-glutamyl linkage, preventing it from cleavage of most peptidases, and the sulfhydryl (SH) group providing its participation as reductant at chemical reactions. Additionally, this tripeptide represents a stable sulfur storage and can serve as cysteine source via the γ-glutamyl cycle (Elskens et al., 1991; Lu, 2013). On cellular physiological level, main functions of GSH can be categorized in the antioxidative activities, that protects e.g. cells from ROS mediated damage, the detoxification of heavy metals and xenobiotics, and the immune boosting effect coupled with white blood cell production in mammalian cells (Perricone et al., 2009; Wu et al., 2004).

It is not surprising that an imbalance of GSH is linked to a wide range of pathologies, for instance diabetes mellitus, neurodegenerative disorders, HIV, cancer or aging, which are mainly based on disturbed protection against oxidative stress on the cellular level. Thus, GSH can serve

as an interesting biomarker for detection of disorders as well as a potential drug or drug precursor for specific treatments, respectively (Perricone et al., 2009; Townsend et al., 2003).

Glutathione is a natural antioxidant with several properties that can be easily linked to health promoting effects, thus, it is intensively marketed in diverse functional supplements for oral consumption or cosmetic application. However, the bioavailability of pure GSH is controversially discussed in literature. Witschi et al. (1992) e.g. studied the oral application of 0.15 mmol/kg GSH as a single dose but did not see any short term effects within 270 min after administration. In general, the activity of the hepatic γ-glutamyltransferase, which cleaves GSH is believed to be the cause of the absence of an increase in circulating GSH (Witschi et al., 1992). Musatti et al. (2014) conducted in vitro gastrointestinal digestion and absorption assays employing Caco-2 and HT29-MTX cell lines, but GSH transport from apical to the basolateral compartment was below 10 %. However, a recent study by Richie et al. (2015) gave evidence that the daily intake of oral GSH was effective in increasing body compartment stores within 6 months. To overcome the issue of bioavailability via intestinal uptake, further forms of GSH administration were studied as well. For instance, Schmitt et al. (2015) showed the superiority of sublingual GSH compared to oral GSH, Buonocore et al. (2016) studied an orobuccal formulation of GSH with positive results and Zarka & Bridge (2017) successfully used oral γ-glutamylcysteine as an alternative to GSH. Next to a broad offer of health promoting pills, the usage of GSH as skin whitener is very prominent Table 2-1. GSH and GSH-enriched yeast cells are also used to improve technical properties of food or beverage products, e.g. as aroma stabilizer, flavor enhancer or baking agent (Mezzetti et al., 2014; Rodriguez et al., 2004; Suas, 2008; Ueda et al., 1997). In the latter case, GSH-enriched yeasts are used to alter disulfide bonds in the protein network of wheat dough with the effect of dough weakening and modified baking properties (Wieser, 2007). Within the European Union, food additives have to be declared according to Annex II of Regulation (EC) No. 1333/2008 with so-called E-numbers. The advantage of inactivated GSH enriched yeasts, e.g. compared to cysteine (E920), is no need of declaration with an E-number and thus the possibility to provide a "clean label" (Suas, 2008). The different areas of GSH application are also illustrated in Table 2-1.

Table 2-1 Different applications and modes of action of glutathione and related products.

Industry	Application	Mode of action	Reference(s)
Beverage	Beer	Stabilization of taste, antioxidant	Chen, Y. et al. (2012)
Beverage	GSH enriched yeast in wine	Stabilization of taste, antioxidant	Ortiz-Julien (2005), Mezzetti et al. (2014), Mao et al. (2016)
Beverage	Extract of reduced GSH in yellow rice wine	Antioxidant	Xia et al. (2013)

Industry	Application	Mode of action	Reference(s)
Food	Dough modifier	Substitution of L-cysteine[a], dough-relaxation	en.angleyeast.com[b], organobalance.de[b], Perrone et al. (2005)
Food	Tasting agent	Taste modulator	Ueda et al. (1997)
Food	GSH enriched nutrient porridge	Skin protection	Zhang, Q. (2016)
Food	Rice noodles	Nourishing faces, skin protection	Lyu (2016)
Food	Yogurt	Antioxidant, aging-delaying effects	Ye et al. (2016)
Food	Chewing gum	Dispelling freckles, beautifying skin	Wang, Q. (2016)
Food, Packaging	Food packaging film with enhanced GSH stability	Protection of oxidation	Lee, H. G. et al. (2015)
Feed	Feed supplement	Animal health-enhancing	Perrone et al. (2005)
Feed	GSH enriched yeast as feed supplement	Growth promotion	Xu et al. (2016)
Health	Functional capsules, dietary supplement	Antioxidant, detoxification	Crum (2011), europharmausa.com[b]
Health	GSH containing capsules	Alleviating hangover, liver protection	Chu (2013), Fu (2015)
Cosmetics	GSH enriched yeast extract supplemnent	Inhibition of melanin production	Nakagawa et al. (2016)
Cosmetics	Whitening cosmetic, skin cream	Skin whitener, anti-aging drug	Wang, Z. (2014) Watanabe et al. (2014)
Cosmetics	Tooth gel, mouth rinse	Reduction of ROS	Hersh (1999)
Medicine	Eyedropper, eye health supplement	Prevention and treatment of eye-related conditions or diseases	Thiermann (2010), Saucedo & Ambati (2016)
Biotechnology	Cell culture media	Antioxidant	sigmaaldrich.com[b]

[a] Compared to L-cysteine (E920) there is no need of declaration of GSH with an E-number according to Annex II of Regulation (EC) No. 1333/2008.

[b] Date of inquiry: 20.01.2017

2.4 Traditional production of GSH

After its discovery by de Ray-Pailhade in 1888 (Meister, 1988), the chemical structure of GSH was elucidated by Hopkins in 1929, who also prepared it from biological extracts (Hopkins, 1929). Harington and Mead described the chemical synthesis of GSH in 1935, which was commercialized in the 1950s (Harington & Mead, 1935; Li, Y. et al., 2004). However, as only the L-form is physiologically relevant, alternative methods for GSH production were

mandatory. Followed by the disclosure of the biosynthesis of GSH by Bloch (1949), the way was paved for enzymatic and microbial GSH production.

The enzymatic synthesis of GSH is naturally high specific and promises high yields as only the supply of the minimal needed substances, i.e. amino acids, ATP and enzymes, is necessary, which simplifies purification as well. These approaches were followed especially in the late 70s to the 80s as reviewed by Li, Y. et al. (2004). However, up to now, the transfer to industrial scale was not achieved as ATP is costly and efficient ATP regeneration systems were lacking. Nevertheless, the enzymatic synthesis of GSH returned as a research topic (e.g. Chen, J. L. et al. (2013); Li, W. et al. (2010)). A very recent publication by Zhang, X. et al. (2017) showed promising results of 8.76 g/L GSH in 5 h applying a bifunctional glutathione synthetase of *Streptococcus sanguinis* and a polyphosphate ATP regeneration system in 1 mL-scale.

Nonetheless, the microbial production of GSH via fermentation of affordable carbon sources is still the state of the art and will be more intensely reviewed in the following.

2.5 Microbial fermentative GSH production

The organisms of choice for GSH production have been yeast cells, especially *Candida utilis* and *Saccharomyces cerevisiae* (Bachhawat et al., 2009; Li, Y. et al., 2004); Table 2-2. In wild type strains of these species, the GSH concentration is already rather high and varies between 0.1 % - 1 % of dry weight (Bachhawat et al., 2009; Li, Y. et al., 2004). Furthermore, advantages such as the ability to grow fast to high cell densities using low-price ordinary media add economic value to these fermentative approaches applying the mentioned strains (Bachhawat et al., 2009). In addition, in contrast to some bacteria, there is no risk of endotoxins within the finished product.

Due to the high industrial interest in efficient GSH production caused by the broad range of its applicability, various fields, such as medium optimization, biotransformation or genetic engineering, were in the focus of research (Table 2-2).

Table 2-2 Different points of investigation for the enhancement of biomass and glutathione concentration in microbial GSH production.

Studied substances/parameters	Strain	Process mode	Biomass (max.)	Glutathione (max.)		Reference
			(g/L)	(mg/L)	(%)	
Precursors: Cys, Glu, Gly (independently)	*Saccharomyces cerevisiae* KY 5711	Batch	~ 2.5	~ 45	~ 1.8	Alfafara, C. G. et al. (1992)
Precursor: Cys	*S. cerevisiae* KY 5711	Batch	~ 10.0	~ 210	~ 2.1	Alfafara, C. G. et al. (1992)
Feedforward/feedback control system	*S. cerevisiae* KY 6186	Fed-Batch	63.8	2360	3.7	Sakato & Tanaka (1992)
C-source, N-source, mineral salts	*S. cerevisiae* CCRC 21727	Batch	9.5	125	n.a.	Liu et al. (1999)
Temperature shift from 30 to 26 °C	*Candida utilis* WSH 02-08	Batch	15.4	385	2.5	Wei, G. et al. (2003)
C-source, N-source, mineral salts, Cys	*S. cerevisiae* FF-8	Batch	9.0	204	2.3	Cha et al. (2004)
Precursors: Cys, Glu, Gly, Ser	*S. cerevisiae* T65	Fed-Batch	133.0	1875	1.4	Wen, S. H. et al. (2004)
Precursors: Cys, Glu, Gly	*S. cerevisiae* T65	Batch	19.8	329	1.7	Wen, S. H. et al. (2005)
Concentration of C-, N- source, mineral salts, temperature	*S. cerevisiae* CBS 1171	Batch	10.5	n.a.	1.9	Rollini & Manzoni (2006)
Precursors: Cys, Glu, Gly	*S. cerevisiae* T65	Fed-Batch	133.3	2190	1.6	Wen, S. et al. (2006)
Temperature, agitation rate, initial pH, inoculum concentration, glucose concentration	*S. cerevisiae* ATCC 7754	Batch	9.1	155	1.7	Santos et al. (2007)
RQ, precursors: Cys, Glu, Gly	*S. cerevisiae* G-14	Fed-Batch	132.0	2020	1.5	Wang, Z. et al. (2007)
C-source, N-source, mineral salts, vitamin	*S. cerevisiae* T65	Batch	8.0	75	0.9	Zhang, T. et al. (2007)

Studied substances/parameters	Strain	Process mode	Biomass (max.)	Glutathione (max.)		Reference
			(g/L)	(mg/L)	(%)	
DO control, precursor: Cys	*C. utilis* WSH 02-08	Fed-Batch	106.0	1767	1.7	Liang, G.-B. et al. (2008)
Low-pH shift, precursor: Cys	*C. utilis* WSH 02-08	Fed-Batch	80.8	1730	2.1	Liang, G., Du, G., et al. (2008)
Precursors: Cys, Glu, Gly, ATP addition	*C. utilis* WSH 02-08	Fed-Batch	100.6	2043	2.0	Liang, G., Liao, X., et al. (2008)
Feedback control of glucose feeding rate, precursor: Cys	*S. cerevisiae* GE-2	Fed-Batch	110.0	2280	2.1	Shang et al. (2008)
Temperature shift from 30 to 26 °C, precursor: Cys	*C. utilis* WSH 02-08	Fed-Batch	36.4	1313	3.8	Wei, Gongyuan et al. (2008)
Precursors: Cys, Glu, Gly, temperature shift	*S. cerevisiae* T65	Fed-Batch	~ 118.0	~ 1700	~ 1.4	Xiong et al. (2008)
GMO strain, precursors: Cys, Glu, Gly	*Pichia pastoris* D18	Fed-Batch	98.2	4150	4.2	Fei et al. (2009)
H_2O_2-induced oxidative stress	*C. utilis* WSH 02-08	Batch	14.2	328	2.3	Liang et al. (2009)
Precursors: Cys, Glu, Gly, ATP addition, SDS addition	*C. utilis* WSH 02-08	Fed-Batch	101.0	2485	2.5[a]	Liang et al. (2010)
Polynomial glucose feeding strategy	*C. utilis* SZU 07-01	Fed-Batch	80.0	825	1.0	Nie, W. et al. (2005)
Ethanol-stat, precursors: Cys-, Gly-feeding	*S. cerevisiae* LYCC7048	Fed-Batch	~ 80.0	~ 810	~ 1.0	Nisamedtinov et al. (2010)
Cell permeabilization via detergents, precursors: Cys, Gly	*S. cerevisiae* GB Italy (commercial), i.a.	Batch	100.0	3200	3.2[a]	Rollini et al. (2010)
Precursors: Cys, Glu, Gly, coupled with H_2O_2 stresses	*C. utilis* WSH 02-08	Fed-Batch	112.3	2448	2.2	Wang, B. et al. (2010)
RQ-feeding, precursors: Cys, Glu, Gly,	*S. cerevisiae* T65	Fed-Batch	126.0	2100	1.7	Xiong et al. (2010)
GMO strain	*Hansenula polymorpha* MOXp-GSH2	Fed-Batch	72.0	2270	3.2	Ubiyvovk et al. (2011)
Precursors: Cys, Glu, Gly	*S. cerevisiae* 26-2	Fed-Batch	105.0	2250	2.1	Wang, M. et al. (2012)

Studied substances/parameters	Strain	Process mode	Biomass (max.)	Glutathione (max.)		Reference
			(g/L)	(mg/L)	(%)	
Precursors: Cys, Met	*C. utilis* CCTCC M 209298	Batch	15.5	428	2.8	Wang, Y. et al. (2012)
C-source, precursors: Cys, Glu, Gly	*S. cerevisiae* ATCC 7754	Batch	25.0	236	0.9	Anschau et al. (2013)
GMO strain, GSH oxidation	*S. cerevisiae* GCIΔGLR1	Batch	n.a.	n.a.	3.9[a]	Kiriyama et al. (2012)
Post-fermentative production via compressed yeast, precursors: Cys, Glu, Gly, Ser, medium composition	*S. cerevisiae* Zeus (commercial), i.a.	Batch	50.0	1020	2.0[a]	Musatti et al. (2013)
Chicken feather protein hydrolysate as medium supplement	*S. cerevisiae*	Batch	17.1	271	1.6	Taskin (2013)
Precursors: Cys, Met, citrate addition	*C. utilis* CCTCC M 209298	Batch	14.0	345	2.5	Wang, Y. et al. (2013)
GMO strain, GSH oxidation	*S. cerevisiae* GCIΔGLR1/ERO1	Batch	3.2	119	3.7[a]	Hara et al. (2015)
RQ-feeding, precursor: Cys-feeding	*S. cerevisiae* Sa-07346	Fed-Batch	99.5	1651	1.8	Lorenz et al. (2015)
Evolutionary engineering	*S. cerevisiae* A4-19-13	Batch	4.6	270	5.9	Patzschke et al. (2015)
GMO strain with bifunctional glutathione synthetase gshF from Streptococcus thermophilus, precursors: Cys, Glu, Gly	*Escherichia coli* BL21(pUC18-gshF)	Fed-Batch	31.9	15210	47.7	Wang, C. et al. (2015)
On-line μ feedback control, precursors: Cys, Glu, Gly	*S. cerevisiae* T65	Fed-Batch	122.0	1900	1.6	Xiong et al. (2015)
Precursor: cysteine ethyl ester	*Saccharomyces bayanus* Sa-00645	Batch	21.9	853	3.9	Lorenz et al. (2016)
Growth rate control, medium composition, precursor: Cys-feeding	*S. cerevisiae* Sa-07346	Fed-Batch	90.1	1459	1.6	Schmacht et al. (2017)
GMO strain with bifunctional glutathione synthetase gshF from *Streptococcus thermophilus*, DO control, precursors: Cys, Glu, Gly, glucose feeding	*E. coli* JM109(pTrc99A-gshF)	Fed-Batch	28.0	11300	40.4	Wang, D. et al. (2016)

Studied substances/parameters	Strain	Process mode	Biomass (max.)	Glutathione (max.)		Reference
			(g/L)	(mg/L)	(%)	
Genome shuffling, UV irradiation and nitrosoguanidine mutagenesis	*S. cerevisiae* YSF2-19	Batch	11.3	319	2.8	Yin et al. (2016)
GMO strain with bifunctional glutathione synthetase gshF from *Actinobacillus succinogenes*, precursors: Cys, Glu, Gly	*E. coli* ZJ12345	Fed-Batch	6.8	5870	86.3	Zhang, J. et al. (2016)

[a]Total glutathione (sum of GSH and GSSG)

2.5.1 Aspects for the fermentative production process – medium composition

General approaches to achieve high GSH concentrations are high cell densities as well as high intracellular GSH accumulation (Li, Y. et al., 2004). A key factor to achieve these objectives is the medium composition. Liu et al. (1999); Rollini and Manzoni (2006) and Zhang, T. et al. (2007) used design of experiments (DoE) approaches to investigate different medium components and their optimal concentration, respectively. Cha et al. (2004) studied different C-, N-sources and mineral salts as well to find optimal production conditions. Moreover, the application of alternative substrates could lead to economically efficient GSH production. For instance, Yoshida et al. (2011) used an engineered S. cerevisiae strain, which expressed amylases for direct utilization of starch and thus avoided the occurrence of the Crabtree effect. The Crabtree effect arises, if glucose is metabolized under oxido–reductive conditions, i.e. it is partly metabolized respiratory and partly fermentative with ethanol as an end product as the respiratory capacity is limited (Sonnleitner & Kappeli, 1986). This overflow metabolism limits the yield of product out of glucose since ethanol is formed. Further, Taskin (2013) studied chicken feather hydrolysate as a cheap source of nitrogen and mineral salts as well as cysteine. Recently, Schmacht et al. (2017) developed a chemically defined medium based on yeast's elemental composition, which was suited for high cell density cultivation exhibiting high intracellular GSH concentrations at the same time.

In most cases, complex media are used for GSH production regarding economic aspects, as their preparation is simple and all nutritional demands are covered with few ingredients. However, chemically defined media exhibit some advantages, such as high process reproducibility, robustness, a less challenging and faster scale-up from laboratory to industrial scale as well as a facilitated downstream processing (Zhang, J. & Greasham, 1999). They could also be used for metabolism studies in order to explore promising production pathways, especially if industrial relevant high biomasses are achieved (Schmacht et al., 2017). Moreover, especially the sulfur content in the medium is believed to be a key factor for GSH production, which can be seen from different GSH/cysteine yields (Lorenz et al., 2016). Those might result from low sulfur availability in the medium, due to consumption of GSH by the cells as a sulfur source via the γ-glutamyl-cycle (Elskens et al., 1991; Lorenz et al., 2016). There is still potential to optimize production media that combine high reproducibility as well as low-cost complex compounds regarding bioeconomy aspects.

Table 2-3 Different feeding strategies for the production of glutathione enriched yeasts under high cell density conditions.

Strain	Biomass	Glutathione		Control-type	Controlled conditions	C-Source	Reference
	(g/L)	(mg/L)	(%)	(FF/FB)			
Saccharomyces cerevisiae KY 6186	63.8	2360	3.7	FF/FB	Model-based feeding	Molasses	Sakato & Tanaka (1992)
S. cerevisiae T65	133.0	1875	1.4	FB	Glucose-feeding	Glucose	Wen, S. H. et al. (2004)
S. cerevisiae T65	133.3	2190	1.6	FB	Glucose-feed based on ethanol concentration	Glucose	Wen, S. et al. (2006)
S. cerevisiae G-14	132.0	2020	1.5	FB	RQ<1; glucose-feed based on ethanol concentration	Glucose/malt liquor/sugar cane molasses	Wang, Z. et al. (2007)
Candida utilis WSH 02-08	106.0	1767	1.7	FF	µ-exponential feeding → constant linear feeding & DO control	Glucose	Liang, G.-B. et al. (2008)
C. utilis WSH 02-08	80.8	1730	2.1	FF	µ-exponential feeding	Glucose	Liang, G., Du, G., et al. (2008)
C. utilis WSH 02-08	100.6	2043	2.0	FF	µ-exponential feeding → constant linear feeding & DO control	Glucose	Liang, G., Liao, X., et al. (2008)
S. cerevisiae GE-2	110.0	2280	2.1	FB	Periodic pulse feedback control	Glucose	Shang et al. (2008)
S. cerevisiae T65	~ 118.0	~ 1700	~ 1.4	FB	Feedback glucose control	Glucose	Xiong et al. (2008)
Pichia pastoris D18	98.2	4150	4.2	FF	Constant linear feeding	Glucose	Fei et al. (2009)
C. utilis WSH 02-08	101.0	2485	2.5[a]	FF	µ-exponential feeding → constant linear feeding & DO control	Glucose	Liang et al. (2010)
C. utilis SZU 07-01	80.0	825	1.0	FF	Linear, exponential, polynomial feeding	Glucose	Nie, M. et al. (2010)

Strain	Biomass	Glutathione		Control-type	Controlled conditions	C-Source	Reference
	(g/L)	(mg/L)	(%)	(FF/FB)			
S. cerevisiae LYCC7048	~ 80.0	~ 810	~ 1.0	FB	Adaptive proportional-integral algorithm for ethanol-stat	Sucrose	Nisamedtinov et al. (2010)
C. utilis WSH 02-08	112.3	2448	2.2	FF	µ-exponential feeding → constant linear feeding & DO-c.	Glucose	Wang, B. et al. (2010)
S. cerevisiae T65	126.0	2100	1.7	FB	RQ-based feeding	Glucose	Xiong et al. (2010)
Hansenula polymorpha MOXp-GSH2	72.0	2270	3.2	FB	DO-stat-based feeding	Methanol/glucose	Ubiyvovk et al. (2011)
S. cerevisiae 26-2	105.0	2250	2.1	FB	Glucose-feed based on ethanol concentration	Glucose	Wang, M. et al. (2012)
S. cerevisiae ATCC 7754	25.0	236	0.9	FF	Constant linear feeding	Molasses	Anschau et al. (2013)
S. cerevisiae Sa-07346	99.5	1650.7	1.8	FB → FF	RQ-based → glucose feeding based on logistic function	Glucose	Lorenz et al. (2015)
Escherichia coli BL21(pUC18-gshF)	31.9	15210	47.7	FF	µ-feeding	Glucose	Wang, C. et al. (2015)
S. cerevisiae T65	122.0	1900	1.6	FB	Feeding based on µ-online control	Glucose	Xiong et al. (2015)
S. cerevisiae T65	113.0	1200	1.1	FB	Glucose-feed based on ethanol concentration	Glucose	Xiong et al. (2015)
S. cerevisiae Sa-07346	90.1	1459	1.6	FF	A-Stat → µ-exponential feeding	Glucose	Schmacht et al. (2017)
E. coli JM109(pTrc99A-gshF)	28.0	11300	40.4	FF	µ-exponential feeding	Glucose	Wang, D. et al. (2016)

FF: feed forward control; FB: feedback control; RQ: respiratory quotient; DO: dissolved oxygen; [a] total glutathione (sum of GSH and GSSG)

2.5.2 Aspects for the fermentative production process – process control

The most common process strategy for GSH production in an industrial scale is the fed-batch mode for high cell densities, although, alternatives such as chemostat or repeated fed-batch approaches are in the focus of research as well (Lorenz et al., 2015; Schmacht et al., 2015). There are two dominant strategies for process control in GSH production, i.e. feed forward control based on the C-source feeding profile and feedback control based on the ethanol concentration e.g. (Liang, G.-B. et al., 2008; Wen, S. H. et al., 2004). The most significant driver for these two strategies is to prevent the formation of byproducts due to overflow metabolism caused by surplus of carbon source under aerobic conditions, which manifests as the Crabtree effect. Therefore, often respiratory quotient (RQ)-based feeding strategies are followed as well in order to keep maximum productivity without any overflow metabolism e.g. (Wang, Z. et al., 2007; Xiong et al., 2010). A compilation of published microbial GSH production processes and the therein applied feeding strategies under high cell density conditions is given in Table 2-3.

2.5.3 Aspects for the fermentative production process – Biotransformation

An efficient GSH production is dependent on an effective biotransformation of the precursor amino acids. Therefore, many researchers extensively studied their addition (see Table 2-2). Thereby, the most popular strategy was the single shot addition of cysteine, glutamate and glycine. Additionally, combinations of other inducing amino acids, such as methionine, serine, or cysteine ethyl ester were also applied (Lorenz et al., 2016; Wang, Y. et al., 2012; Wang, Y. et al., 2013; Wen, S. H. et al., 2004). However, the limiting GSH precursor is cysteine (Anderson, 1998), which was also confirmed e.g. by Alfafara, C. G. et al., (1992) and Wang, Y. et al. (2012). Hence, supplementation of only cysteine in combination with other techniques, such as DO control to avoid oxidation, temperature shift, low pH shift for extracellular excretion, or constant cysteine feeding for elevated incorporation, was applied as well (Liang, G.-B. et al., 2008; Liang, G., Du, G., et al., 2008; Lorenz et al., 2015; Shang et al., 2008; Wei, Gongyuan et al., 2008). Thereby, concentrations always were significantly increased in comparison to controls without precursor addition. Nevertheless, the efficiency of the usage of the precursor substances was rather seldom taken into account (Lorenz et al., 2015) and thus leaves capability for further research. Aspects for the fermentative production process – engineering of strains

The production strain is a crucial parameter for an efficient GSH manufacture. Usually, strains already exhibiting high intracellular GSH concentrations are taken as a platform for further optimization. Thereby, the optimization of the strain itself, e.g. by means of evolutionary or metabolic engineering, as well as the optimization of the influence of the medium and process control parameters on the strain behavior has to be taken into consideration. The number of

publications presenting genetically modified strains for GSH production has risen recently (Table 2-2). Patzschke et al. (2015) used evolutionary engineering to improve GSH production in *S. cerevisiae* and achieved intracellular concentrations of 5.9 % under lab-scale bioreactor conditions. Fei et al. (2009) and Ubiyvovk et al. (2011) used the unconventional strains *Pichia pastoris* and *Hansenula polymorpha* for genetic engineering and achieved high cell densities as well as high intracellular GSH contents. Furthermore, research was done on the effective transformation of reduced GSH in the oxidized form (GSSG) due to its enhanced stability (Hara et al., 2012; Kiriyama et al., 2012). Thereby, different thiol oxidase genes were over-expressed which improved the glutathione production in *S. cerevisiae* strains to 3.9 % and 3.7 %, respectively. Moreover, in the early past, genetically engineered strains of *Escherichia coli* were used that exhibited different bifunctional glutathione synthetases, which avoid product inhibition of the GCS (Figure 2-1) (Wang, C. et al., 2015; Wang, D. et al., 2016; Zhang, J. et al., 2016). Thereby, incomparably high concentrations of 5870 mg/L to 15210 mg/L GSH were achieved. However, the usage of such genetically modified organisms (GMOs) is more likely to be applied for bulk GSH production itself than for food applications such as e.g. GSH enriched yeasts as consumers usually do not accept GMOs in food products. Moreover, problems can occur as bacteria like *E. coli* may include endotoxins and the usage of GMOs e.g. in the European Union is subject to obligatory declaration (2001/18/EG). In general, with increased adaption of the strain properties to high GSH productivity in a certain fermentation process, the stability of these features under large scale conditions becomes more challenging. Thus, the scalability of a production strain is mandatory. In conclusion, the selection and development of the production strain has to be carefully considered with regard to the later application of the product and the final production process.

2.5.4 Current patent situation of microbial GSH production

Industrial activities and innovations of technologies and processes are partially reflected by existing patents of the topic. To illustrate the current situation of microbial GSH production, an additional patent search was conducted, whereby strategies and trends for microbial GSH production were identified (see Table 2-4). Considering the submitted patents from 2010 to 2017, it is conspicuous that the majority of contributions are from Asiatic countries, especially from China. It also stands out that the engineering of high GSH producing strains is one of the main strategies for current process optimization approaches.

Table 2-4 Current strategies and trends for microbial GSH production shown in patents from 2010 to 2017*

Patent number	Strategy	Microorganism(s)	Reference
CN101824451 (A)	Three level fed-batch with glycerol and sorbitol as carbon-source; no ethanol accumulation	*P. pastoris*	Hongtao (2010)
KR20100095829 (A)	Provision of a mutant by irradiating UV of *S. cerevisiae* for overexpression of GSH	*S. cerevisiae* 54-8m(KACC 93070P)	Lee, W. S. et al. (2010)
KR20100040589 (A)	Enhanced GSH yield by improved fed-batch culture of yeast *S. cerevisiae* FF-08 at 0.78 bar of inner pressure	*S. cerevisiae* FF-08	Cho, Y. S. (2010)
CN102134556 (A)	Culture medium composition includes inositol and nicotinamide which increase the growing speed of GSH productive yeast and simultaneously increase the accumulated amount of GSH	*Candida, Debaryomyces, Rhodotorula, Torulopsis, Saccharomyces, Hansenula, Schizosaccharomyces, Pichia, Kluyveromyces, Phaffia*	Qijiu (2011)
JP2011234645 (A)	Addition of beneficial compounds to the fermentation medium produced from an aromatic amino acid by the yeast metabolism (e.g. phenylpyruvic acid, phenylacetaldehyde and phenylethanol obtained from phenylalanine)	*Saccharomyces, Candida, Debaryomyces, Rhodotorula, Torulopsis, Hansenula, Schizosaccharomyces, Pichia, Kluyveromyces, Phaffia*	Tomita et al. (2011)
CN102653722 (A)	Culture conditions, feeding strategy and related techniques are optimized to obtain a GSH-enriched efficient dough yeast product	Dough yeast	Cai & Changgao (2012)
CN101921712 (B)	Transformation by a comprehensive biological breeding technology to obtain a *Phaffia rhodozyma* strain for high-yield astaxanthin and byproduct-glutathione by means of genetic and engineering control to finally reduce environmental pollution	*Phaffia rhodozyma*	Pei & Huang (2012)
EP2501823 (A1)	Production of extracellular GSH by addition of an oxidizing agent for promoting the production of GSH by the microorganism and addition of a modifying agent for the opening of an ion channel of the microorganism for discharge of GSH	Wild-type or mutated microorganisms, such as *S. cerevisiae* L5267, *C. utilis*, aquatic algae strains, *Escherichia coli*	Versari et al. (2012)

Patent number	Strategy	Microorganism(s)	Reference
KR101150148 (B1)	Yeast mutant strain which contains a large amount of GSH	*C. utilis* SY8-M2-1	(Cho, D. W. et al., 2012)
JP2012213361 (A)	Enhanced GSH production by screening a cadmium-resistant strain and further a cycloheximide-resistant strain of *S. cerevisiae* and/or mutation treatment	Baker's yeast	(Fukabori et al., 2012)
ZA201202077 (B)	Conversion of cystine to cysteine and/or GSH comprising contacting cystine with a microorganism	*Trichoderma, Aspergillus, Saccharomyces, Kluyveromyces, Candida, Clostridia, Escherichia, Methanobacterium, Methanosarcina*	(Noordam, 2013)
CN102559529 (B)	Inclusion of the bacterial *Vitreoscilla* hemoglobin gene vgb in *S. cerevisiae* for improved oxygen utilizing capability resulting in higher GSH yields	*S. cerevisiae* CGMCC 5758 101-V	(Wang, F. et al., 2013)
CN103695506 (A)	Optimization of inoculum size, pH adding time and adding volume of cysteine	*S. cerevisiae, Candida and Pichia*	(Zhu & Huang, 2014)
CN102586369 (B)	Genetically engineered *E. coli* were used that exhibited different bifunctional glutathione synthetases, resulting in no product inhibition of the γ-glutamyl-cysteine synthase	*E. coli* BL21 / pET28a-gshF or JM109 / pTrc99a_gshF	(Li, Z. et al., 2014)
US8647839 (B2)	Utilization of a microorganism with a higher activity of transport proteins passing intracellular GSH to the outside of cells, and a higher activity of a protein involved in GSH or γ-glutamylcysteine biosynthesis	*E. coli*	(Kazuhiko & Yoshiyuki, 2014)
JP5634051 (B2)	GMO with suppression of GSH feedback inhibition and improved GSH production	Yeast	(Suzuki et al., 2014)
KR101612421 (B1)	Provision of the GSH overexpressing strain *S. cerevisiae* BB-01 (KCTC 18262P)	*S. cerevisiae* BB-01	(Kim, 2016)
UA111511 (C2)	Transformation of *Hansenula polymorpha* with pUC19/prGAP_MET4/NTC vector comprising additional replicas of gene encoding first GSH biosynthesis enzyme and gene encoding transcriptional activator of cysteine biosynthesis genes	*H. polymorpha*	(Kurylenko et al., 2016)

Patent number	Strategy	Microorganism(s)	Reference
CN105838706 (A)	Fusion of protoplasts of *H. polymorpha* and *Schizosaccharomyces pombe* with high temperature resistance, ethanol tolerance and sodium molybdate tolerance to obtain the GSH high-yield strains	*H. polymorpha, Schizosaccharomyces pombe*	(Qian et al., 2016)
CN105238797 (A)	Inclusion of gshF genes of *Streptococcus agalactiae* into *P. pastoris* and *S. cerevisiae* to obtain dual-function GSH synthetase which is not affected by feedback inhibition of GSH and has a high catalytic efficiency	*P. pastoris, S. cerevisiae*	(Xu et al., 2016)

* Shown are relevant patents from a patent search via the European Patent Office with the following search terms: glutathione production (title); glutathione yeast(title); glutathione bacteria(title and abstract); glutathione coli(title and abstract) within the years 2010-2017 (date of inquiry: April 2017).

2.6 Conclusion

After around 40 years of research in the field of microbial GSH production, this topic is still of high importance in research and industry due to the broad applicability of glutathione itself and glutathione enriched yeasts for food and health applications. Historically, mainly yeasts were used as microbial producers, however, within the last five years, many metabolically engineered bacteria were presented that show high GSH production concentrations. However, as it is affordable to use low-priced raw materials and run smart process strategies to achieve efficient biotransformation yields, there is still potential for improvements for an economic microbial GSH production.

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Kapitel 3

Medium optimization based on yeast's elemental composition for glutathione production in *Saccharomyces cerevisiae*

Medium optimization based on yeast's elemental composition for glutathione production in *Saccharomyces cerevisiae*

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3 Medium optimization based on yeast's elemental composition for glutathione production in *Saccharomyces cerevisiae*

3.1 Abstract

The production of glutathione (GSH) or GSH enriched yeast is still in the focus of research driven by a high industrial interest. In this study, an optimal growth rate for GSH production via *S. cerevisiae* Sa-07346 was investigated. To further improve the fermentation process in a way that it is independent of lots, the influence of different WMIX medium compositions on biomass and GSH production was studied. Thereby, the fermentation medium was adjusted based on yeast's elemental composition. The resulting chemically defined fermentation medium led to high cell densities in fed-batches. Therefore, it has the potential to be applied successfully for other high cell density yeast fermentation processes. As cysteine is the key component for GSH production, different cysteine addition strategies were studied and finally, a continuous cysteine feeding was applied in the late stage of fermentation. Thereby, a GSH concentration of 1459 ± 57 mg/l was reached by continuously feeding cysteine, which meant an increase to 253 % compared to the control without cysteine addition (577 mg/l GSH).

3.2 Introduction

The global market for yeast products reached 5.8 billion US$ in 2013 and is expected to grow to 9.2 billion US$ in 2019 (Marz, 2014). Glutathione (GSH) is an example for a yeast derived product of high industrial interest because of its broad range of applicability.

GSH, also designated as γ-glutamyl-cysteinyl-glycine, is the most abundant non-coding protein thiol in most pro- and eukaryotic cells with concentrations of 0.2-10 mM (Anderson, 1998). It is synthesized via two ATP-dependent enzymatic reactions, the first one being rate limiting (Lu, 2009). Because of its unusual γ-linkage, which prevents GSH from cleavage of most peptidases, it serves as a sulfur and cysteine source respectively via the γ-glutamyl cycle (Lu, 2013). Inside the cell, the reduced form (GSH) as well as the oxidized form (glutathione disulfide, GSSG) can be found in a ratio of 30-100:1, forming a redox couple (E0 = -240 mV (Ayer et al., 2014)) of great importance for the redox status of the cell, e.g. scavenging free radicals or heavy metals (Ask et al., 2013; Bachhawat et al., 2009; Drakulic et al., 2005; Stephen & Jamieson, 1997). Due to these properties, GSH can be used in medicine to treat diseases exhibiting GSH deficiency as well as in cosmetic industry. Moreover, GSH-enriched yeasts can be used in flavor industry (kokumi-taste (Ueda et al., 1997)) and food industry, in the latter substituting cysteine and avoiding the need of declaration and thus providing a so-called clean label (Suas, 2008).

Organisms of choice for glutathione production are Candida utilis and *Saccharomyces cerevisiae*. Thereby, titers of e.g. up to 2448 mg/l GSH were achieved with *C. utilis* (Wang, B. et al., 2010). In contrast, the use of GMOs offers an alternative to achieve unequally higher

titers, e.g. 11300 mg/l with E. coli JM109(pTrc99A-gshF)a (Wang, D. et al., 2016), which is also discussed below. A lot of research has been done on maximizing glutathione production, whereby two promising approaches are prominent: increasing biomass concentration and intracellular GSH content (Li et al., 2004).

In order to efficiently achieve high cell densities and satisfying product yields, especially the cultivation medium has to be carefully considered. Of course, the used medium type should be justified by the manufactured products concerning economical aspects: Applying a chemically defined medium (CDM) for the production of low-cost products appears to be expensive whereas the usage of CDM to produce recombinant proteins can be advantageous. Beneficial properties of CDM are high process reproducibility, robustness and a less challenging and faster scale-up from laboratory to industrial scale (Zhang & Greasham, 1999). The additional downstream processing will be facilitated because of the absence of by-products during autoclaving and the fact that ingredients do not impede product purification (Zhang & Greasham, 1999). In context of quality by design, authorities, e.g. FDA and EMA, demand a high degree of quality assurance to guarantee the value of the clinical product. One critical quality attribute is the variability of used raw materials as well as their monitoring and control (Rathore & Winkle, 2009; Read et al., 2010). Beside the advantages of complex supplements like yeast or soy extract, these natural medium additives show inconsistencies, e.g. lot-to-lot variations and a decrease of quality of one batch over a defined period of time. Therefore, processes for new biological entities will be developed with chemically defined and animal component free media. For scientific and fundamental investigations like OMICS studies, CDM is also well suited (Hahn-Hagerdal et al., 2005). Kennedy and Krouse (1999) comprehensively summarized some constraints, challenges and strategies about medium designing.

Moreover, the specific growth rate μ of the production strain should be controlled (Li et al., 2004) in order to avoid undesired by-products. Wen et al. (2006) reported that a low ethanol concentration is favorable for biomass as well as glutathione production. Therefore, specific growth rate has to be <0.3 1/h, to prevent Crabtree effect occurrence (Leuenberger, 1971). Shimizu et al. (1991) have developed a mathematical model for a specific growth rate profile for GSH production. Furthermore, as the chemostat experiences a revival (Hoskisson & Hobbs, 2005), Paalme et al. (1997) applied the so-called accelerated-stat (A-stat) fermentation strategy to show effects on growth characteristics. Thereby, the A-stat approach provides quasi infinite settings that can be tested in a short period of time (Paalme & Vilu, 1992).

To raise the intracellular GSH content, the addition of the precursor amino acids cysteine, glycine and glutamate was applied by different research groups. Alfafara, C. G. et al. (1992) and Wang, M. et al. (2012) both discovered, that cysteine is the key amino acid for glutathione production.

The aim of the present work was to investigate the influence of the specific growth rate μ on the intracellular GSH content in *S. cerevisiae* via the A-stat approach. Further, a new

fermentation medium based on WMIX (see material and methods section) was to be designed based on yeast's elemental composition to get a chemically defined medium which avoids the dependency on lots of complex nutrients but also ensures high cell densities. Additionally, it had to meet the nutritional demand of *S. cerevisiae* Sa-07346 as a model organism and thus ensure optimal growth as well as glutathione production, whereby also different kinds of supplementation of cysteine were applied.

3.3 Material and methods

3.3.1 Media

Pre-cultures were inoculated in chemically defined WMIX (WM = white molasses, version number IX), a modified version of WMVIII (Lang & Looman, 1995). Chemicals were purchased from Sigma-Aldrich, Inc. The composition was as follows (g/l): salt solution (sterilized by autoclaving): NH_4Cl 2.8, myo-Inositol 0.075, $MgCl_2 \cdot 6H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.55. Moreover 4 ml/l vitamin solution and 1 ml/l trace element solution were aseptically added (g/l): vitamin solution (solubility of vitamins can be increased by pH adjustment; vitamins were sterilized by microfiltration): biotin 0.625, pantothenic acid calcium salt 12.50, nicotinic acid 2.50, pyridoxine hydrochloride 7.60, thiamine hydrochloride 2.50; trace element solution in 0.01 M EDTA (sterilized by microfiltration): $CuSO_4 \cdot 5H_2O$ 0.10, $FeSO_4 \cdot 7H_2O$ 0.50, $MnCl_2 \cdot 4H_2O$ 0.50, $NaMoO_4 \cdot 2H_2O$ 0.10, $ZnSO_4 \cdot 7H_2O$ 0.175. Final concentrations of glucose and glutamic acid monosodium salt monohydrate were (g/l) 50 and 10, respectively. Medium pH was adjusted with 40 ml/l 0.5 M potassium phosphate buffer (pH 6.8) (K_2HPO_4, KH_2PO_4). This medium was also used as batch medium but containing 20 g/l glucose and additionally 11 g/l yeast extract (YE) (Leiber GmbH, Bramsche, Germany).

For the feed medium, salt, vitamin and trace element concentrations were 6-fold increased to ensure high biomass. 14 g/l citrate was primarily added for complexing Mg^{2+} and Ca^{2+} cations. Additionally, Wang, Y. et al. (2013) gave a hint that the addition of citrate leads to an increased intracellular ATP content, which might be beneficial for the ATP dependent generation of GSH. Potassium phosphate buffer content was doubled; glucose concentration was adjusted to 400 g/l and 15 g/l YE was used, whereas glutamic acid monosodium salt was left out.

In order to further improve the medium from an economically point of view, the nutrient requirement of yeast was calculated, based on the approximated mean values of the elemental composition of baker's yeast as described by Bronn (1986), which is a common approach for medium development (Connors, 2003; Ertola et al., 1995). Those averaged values are dependent on the medium composition as well as cell age and growth rate and thus serve as guiding values. For the calculation of media components, a final cell dry weight of 100 g/l was assumed. The following equation was used to calculate the concentration (c) of each of the elements out of the substances in the medium (YE was not considered as the exact composition was inexplicit and lot-dependent):

$$C_{element} = \frac{C_x \cdot element\ \% \cdot M_{salt}}{M_{element}} \quad (1)$$

where $c_{element}$ is the set value of the element in the medium, c_X is the assumed final biomass of 100 g/l, element % is the percentage of the element in yeast composition, and M_{salt} and $M_{element}$ are the molecular mass values for the substance in the medium and the element, respectively. Based on this estimation of yeast's nutritional needs, an adjusted feed medium was applied containing the following salts (g/l) (sterilized by autoclaving): citrate 14, $(NH_4)_2SO_4$ 70, myo-Inositol 0.45, $CaCl_2 \cdot 2H_2O$ 0.6, $MgSO_4 \cdot 7H_2O$ 3.6. Further, 24 ml/l vitamin solution, 6 ml/l trace element solution and 400 g/l glucose were aseptically added as stated above. Medium pH was adjusted with 120 ml/l 0.5 M potassium phosphate buffer (pH 6.8). Additionally, 2 ml 40 g/l Fe(II)SO_4-solution was aseptically added.

Table 3-1 shows the set values and actual values for the existing WMIX batch and feed medium and the adjusted chemically defined batch and feed medium. To evaluate different intermediates between the described WMIX medium including yeast extract and the adjusted chemically defined fermentation medium, different combinations of fermentation medium compositions were tested. For clarity, they are shown in Table 3-2.

3.3.2 Strain and pre-cultures

S. cerevisiae Sa-07346 was originally obtained from Organobalance GmbH in Berlin, Germany as a strain exhibiting high intracellular GSH concentrations. The seed train was realized as follows: 0.1 ml of a cryo-stock was inoculated into a shaking flask containing WMIX medium (see section above) and incubated for 24 h at 30 °C and 180 rpm. Thereof, a second pre-culture was set up fitting an optical density ($OD_{600\ nm}$) of 0.1, which was incubated under the same conditions.

3.3.3 A-stat

For the realization of the A-stat approach, a continuous culture was set up in a 5 l-bioreactor (Biostat® Aplus, Sartorius AG, Göttingen, Germany) at 30 °C, 30 % pO2 and pH = 5.5. WMIX containing 10 g/l glucose and without yeast extract was used as batch and feed medium. After the batch phase of about 6.5 h, a continuous feeding with a flow/dilution rate of $F = D = \mu = 0.05$ l/h was initiated and executed for 5 volume turnovers. Then, the actual A-stat was started with an acceleration (a) of 0.0035 1/h² until $F = D = \mu = 0.3$ 1/h. The flow rate was calculated as described by Paalme et al. (1997):

$$F(t) = D(t) \cdot V = V \cdot (D_0 + a \cdot t) \quad (2)$$

Samples were taken periodically from the discharge flow for the analysis of biomass, GSH content and HPLC analysis of glucose and ethanol.

3.3.4 Shake flask experiments for the determination of a suitable nitrogen source

A simplex centroid design was used to compare ammonium chloride, ammonium sulfate and urea as nitrogen sources (see Table 3-1) in regard to the medium adjustment as described above. Thereby, WMIX medium without yeast extract was used including the nitrogen sources in equimolar amounts based on nitrogen as stated in Table 3-1, respectively. From the second pre-culture, the flasks were inoculated with $1 \cdot 10^7$ cells/ml and incubated for 32 h at 30 °C on a rotary shaker at 180 rpm. At 24 h, 10 mmol/l cysteine were spiked into the flasks for GSH induction.

3.3.5 Fed-batch fermentations

Fed-batch fermentations were carried out in 5 l-bioreactors (Biostat® Aplus, Sartorius AG, Göttingen, Germany). The bioreactors were inoculated with 10 % (v/v) of the second pre-culture. The feeding was started after glucose depletion at about 6.5 h of fermentation and followed the equation as described by Liang et al. (2008):

$$F = \frac{\mu}{Y_{X/S} \cdot S} \cdot V_F \cdot C_X \cdot e^{\mu \cdot t} \qquad (3)$$

where F is the feeding rate, μ is the specific growth rate, V_F is the initial volume of medium, C_X is the concentration of biomass at feeding time 0 h, S is the residual glucose concentration, $Y_{X/S}$ is the yield coefficient of biomass on glucose and t is the fermentation time. In general, 1 l batch medium was inoculated and after the batch phase, 1 l of feeding medium was fed. During the fed-batch cultivation, pH was regulated to 5.5 ± 0.1 by 25 % H_3PO_4 and 20 % ammonia or 5 M NaOH, respectively, temperature was set to 30 ± 1 °C and DO was maintained at 30 ± 5 % (1 vvm air, regulation via stirrer speed or pure oxygen if necessary). For additional information, see the respective sections.

3.3.6 Biomass and sugar determination

Biomass and sugar determination was carried out as described elsewhere (Lorenz et al., 2015). In brief, 5 ml cell suspension were centrifuged at 4000 × g and washed with 0.9 % NaCl solution. The supernatant was taken for additional HPLC analysis (determination of low molecular sugars as described below). Cell pellet was dried for 24 h at 100 °C, cooled in a desiccator and weighed for cell dry weight (CDW) determination.

The analysis of sugars and ethanol was achieved by HPLC (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) equipped with a NUCLEOGEL® ION 300 OA column (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The samples were eluted using 0.005 mol/l H_2SO_4 at a flow rate of 0.4 ml/min.

Table 3-1 **Set and actual values for the existing WMIX batch and feed medium and the adjusted batch and feed medium.**

Element	N	P	K	S	Mg	Na	Ca	Cl
C_{set} (g/l)	7.70	1.20	1.00	1.00	0.20	0.10	0.04	0.02
$C_{actual\ WMIX}$ (g/l)	2.98	0.93	1.17	0.25	0.21	0.68	0.10	7.04
$C_{actual\ adjusted\ medium}$ (g/l)	8.03	1.24	1.56	7.99	0.20	0.68	0.10	0.17

Set values are calculated based on the approximated mean values of the elemental composition of baker's yeast.

3.3.7 Glutathione determination

Determination of reduced GSH was based on the colorimetric method via Ellman's reagent (5,5'-dithio-(bis-2-nitrobenzoic) acid, DTNB, Sigma-Aldrich) based on Tietze (1969) and carried out as described elsewhere (Tietze, 1969). In brief, the yeast cell suspension was centrifuged and washed with 0.9 % NaCl solution. Afterwards, yeast cells were lysed in 0.1 M H_3PO_4 at 80 °C for 5 min in a water bath. After a reapplied centrifugation at 4000 × g for 10 min, the supernatant was used for the GSH assay. 100 µl of the sample was pipetted into a 96-well-plate and covered and mixed with the same amount of 0.5 M sodium phosphate buffer, pH 8. Then, 5 µl of a 4 g/l stock solution of DTNB were added and the reaction took place for 10 min. Using a 96-well-plate reader (Molecular Devices GmbH, Biberach, Germany), three-fold determinations were conducted at 412 nm. Moreover, a GSH standard curve was recorded covering concentrations of 12.5 to 100 mg/l reduced GSH. The intracellular GSH content (g/l) was calculated by using this standard curve (linear regression) and afterwards related to the CDW as follows:

$$Intracellular\ reduced\ GSH\ \left(\%, \frac{w}{w}\right) = \frac{GSH\ \left(\frac{g}{L}\right)}{CDW\ \left(\frac{g}{L}\right)} \cdot 100\% \qquad (4)$$

3.3.8 Flow cytometry

The finally developed fermentation process was monitored via flow cytometry. Flow cytometric measurements were done with the CyFlow® Cube 8 (Sysmex Partec, Görlitz, Germany) using the 488 nm laser for excitation. Staining dyes were propidium iodide (PI) (Sigma-Aldrich, Inc., Steinheim, Germany) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4$ (Lu, 2009)) (Life Technologies GmbH, Darmstadt, Germany). For all assays, the cells were washed with PBS and the concentration was adjusted to about $1\text{-}3 \cdot 10^6$ cells/ml. From this cell suspension, $1 \cdot 10^5$ cells were used for each single measurement. PI and $DiBAC_4$ (Lu, 2009) was combined in one assay for the determination of cell viability. For that purpose, 10 µl PI (working solution: 1 mg/ml in water) and 10 µl $DiBAC_4$ (Lu, 2009) (0.5 mg/ml in PBS working solution)

were added to 1000 µl of cell suspension and incubated for 5 min at room temperature. Analysis of the obtained data was realized via FlowJo v10 (FlowJo LLC, Ashland, USA).

3.4 Results

3.4.1 A-stat approach to determine a favorable specific growth rate

First, the influence of the specific growth rate on GSH was investigated via the A-stat approach. It was revealed that by raising the specific growth rate the percentage intracellular GSH content declined Figure 3-1. Within the probed region from µ = 0.05-0.30 1/h, the intracellular GSH content decreased from 0.86 % to 0.46 %. The fermentation was glucose-limited at all time and no Crabtree effect could be observed, as ethanol values were ≤ 2 g/l. As the lowest dilution rate of 0.05 l/h was not feasible for the following tests from an economical point of view regarding e.g. overall process time or space time yield, further experiments were carried out applying a specific growth rate of 0.1 1/h.

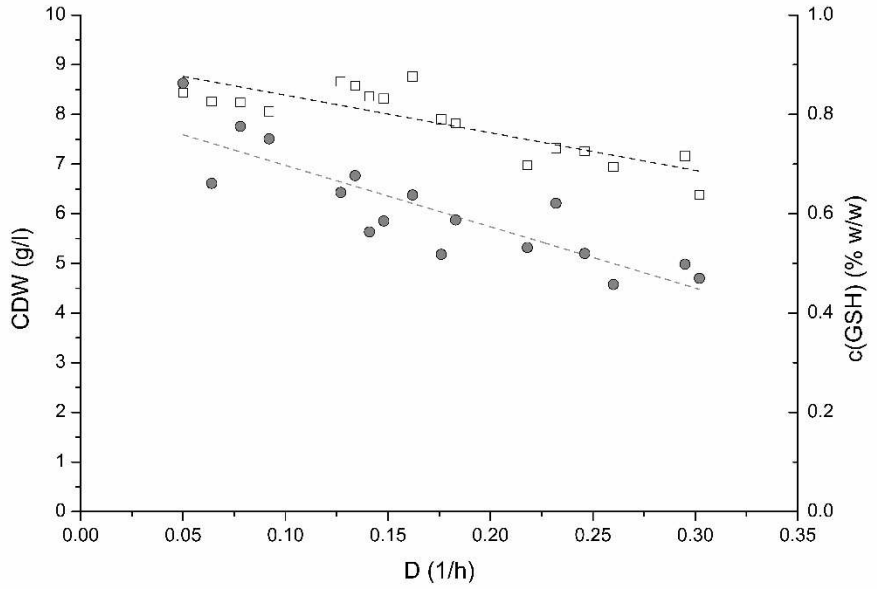

Figure 3-1 **A-stat approach to determine the influence of the specific growth rate on the intracellular GSH content (closed circles) of *S. cerevisiae* Sa-07346. The intracellular GSH content decreases with increasing growth rate µ ("y = -1.235·x + 0.82" , r2 = 0.742). The decreasing cell dry weight (CDW, open squares, "y = -7.571·x + 9.144" , r2 = 0.696) is caused by the lacking ability of the yeast to adapt fast enough to the changing conditions.**

Table 3-2 Different variations of the composition of the WMIX fermentation medium.

Medium/ Component	Variation 1		Variation 2		Variation 3		Variation 4	
	Batch -YE	Feed -YE	Batch -YE	Feed +YE	Batch +YE	Feed +YE	Batch -YE	adjusted Feed -YE
Citrate (g/l)	0	14	0	14	0	14	0	14
NH_4Cl (g/l)	2.8	16.8	2.8	16.8	2.8	16.8	2.8	0
$(NH_4)_2SO_4$ (g/l)	0	0	0	0	0	0	0	70
myo-Inositol (g/l)	0.075	0.45	0.075	0.45	0.075	0.45	0.075	0.45
$MgCl_2 \cdot 6H_2O$ (g/l)	0.25	1.5	0.25	1.5	0.25	1.5	0.25	0
$CaCl_2 \cdot 2H_2O$ (g/l)	0.1	0.6	0.1	0.6	0.1	0.6	0.1	0.6
$MgSO_4 \cdot 7H_2O$ (g/l)	0.55	3.3	0.55	3.3	0.55	3.3	0.55	3.6
Glucose (g/l)	20	400	20	400	20	400	20	400
Sodium glutamate (g/l)	10	0	10	0	10	0	10	0
KPO_4 buffer (pH 6,8; 0,5 M) (ml/l)	40	80	40	80	40	80	40	120
YE (g/l)	0	0	0	15	11	15	0	0
TE solution (ml/l)	1	6	1	6	1	6	1	6
Vitamin solution (ml/l)	4	24	4	24	4	24	4	24
$Fe(II)SO_4$ (g/l)	0	0	0	0	0	0	0	0.08

The influence of the composition of the WMIX fermentation medium on biomass and GSH production was evaluated in bioreactors. Thereby, an adjusted chemically defined medium gave the best results (see Table 3-4 as well). -YE: without yeast extract; +YE: with yeast extract.

3.4.2 Influence of the media composition on GSH production

In this section the influence of yeast extract of the usually used WMIX batch or feed fermentation medium in combination with the previously evaluated growth rate (0.1 1/h) on CDW and GSH concentrations should be investigated. Further, an adjusted chemically defined feed medium based on the explained calculations in the material and methods section was applied. The duration of fed-batch cultivations was 48 h. In parallel, a shake flask experiment was done to evaluate different nitrogen sources as described in the material and methods section.

The simplex centroid design revealed that high amounts of ammonium sulfate gave the best results for CDW and GSH production Figure 3-2. Nevertheless, the model showed that the optimum is reached, when also ammonium chloride is included in the medium. Therefore, ammonium chloride was chosen in the batch medium, whereas ammonium sulfate was used in the adjusted feed medium for fed-batch trials.

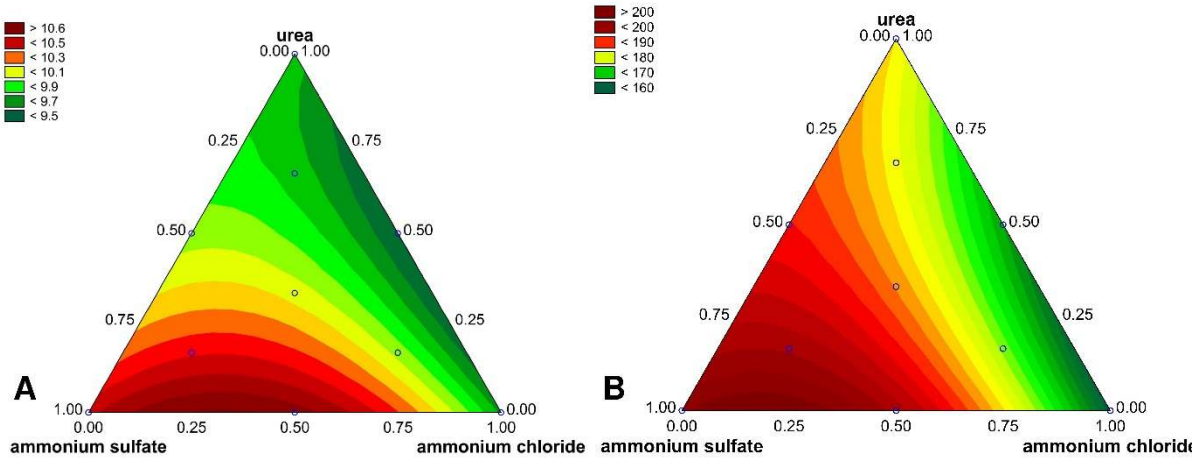

Figure 3-2 **Influence of different nitrogen sources on (A) CDW (g/l) and (B) GSH (mg/l) production. Ammonium chloride, ammonium sulfate and urea were tested as nitrogen sources in the WMIX medium. It was shown that ammonium chloride gave the best results. A quadratic model ($p < 0.05$) was chosen for data fitting in case of both CDW and GSH. The adjusted r-squares were 0.927 and 0.971 for CDW and GSH, respectively. The lack of fit value was above an α-value of 0.05 for both A and B indicating that the model fit the data. Further, a significant interaction between ammonium sulfate and ammonium chloride could be elucidated and is also displayed in this figure.**

The results of the fed-batch fermentations with the different medium variations (Table 3-4) indicated that yeast extract is a main ingredient of the WMIX fermentation medium, because biomass and GSH concentrations were negatively affected, when yeast extract was left out.

Moreover, due to certain limitations, glucose was accumulated in the culture broth and the Crabtree effect was initiated in variation 1 and 2, which consequently resulted in reduced biomass yields. In contrast, the usually used WMIX including yeast extract in batch and feed medium led to a maximum CDW of 78.34 g/l and a volumetric GSH concentration of 300.46 mg/l within 48 h. Interestingly, when applying the adjusted chemically defined feed medium, an even higher biomass of 92.64 g/l as well as a more than doubled GSH concentration of 711.22 mg/l was achieved after 48 h of fermentation. These results indicate that even the formerly used WMIX fermentation medium including yeast extract combined with the evaluated growth rate had some deficiencies. Therefore, the adjusted chemically defined feed medium in combination with WMIX batch fermentation medium without yeast extract was used for further experiments.

3.4.3 Application of a continuous cysteine feeding

In a following set of fed-batches, different cysteine concentrations were applied as a spike to study the efficiency of the cysteine incorporation into GSH using the adjusted chemically defined medium. The cysteine spike was conducted at 36 h of cultivation. This set-up resulted in unsatisfactory outcomes regarding CDW and GSH concentrations (data not shown). It was consequently assumed from these fed-batches, that the cysteine addition should be carried out at higher biomasses to improve the efficiency. Therefore, the exponential feeding was applied via the equation shown in the material and methods section. After 32 h of cultivation, the feeding scheme was changed from exponential to linear (0.034 l/h). In parallel to the growth feed and beginning with 48 h, a continuous cysteine feeding (17.5 mmol cysteine in 0.01 M H_3PO_4) was started. Duration of cysteine addition was 6 h, as described in a previous work (Lorenz et al., 2015). The overall fermentation duration was 60 h. Additionally, one fermentation served as a control without cysteine addition (Figure 3-3 B).

Within these fermentations, again high biomasses were achieved. The control yielded 91.78 g/l CDW (Figure 3-1). The fermentations accompanied by the feeding of cysteine resulted in biomasses of 90.09 ± 5.82 g/l CDW and a maximum GSH concentration of 1459 ± 57 mg/l corresponding to 1.75 ± 0.16 % GSH with an efficiency of 0.39 ± 0.04 mol GSH/mol cysteine and a $Y_{X/S}$ of 0.39 ± 0.01. The viability remained high during the whole fermentation process with a minimum of 87.70 ± 2.48 % viable cells. In conclusion, the described GSH production process using a chemically defined medium led to high cell densities as well as high GSH concentrations without affecting cells' viability.

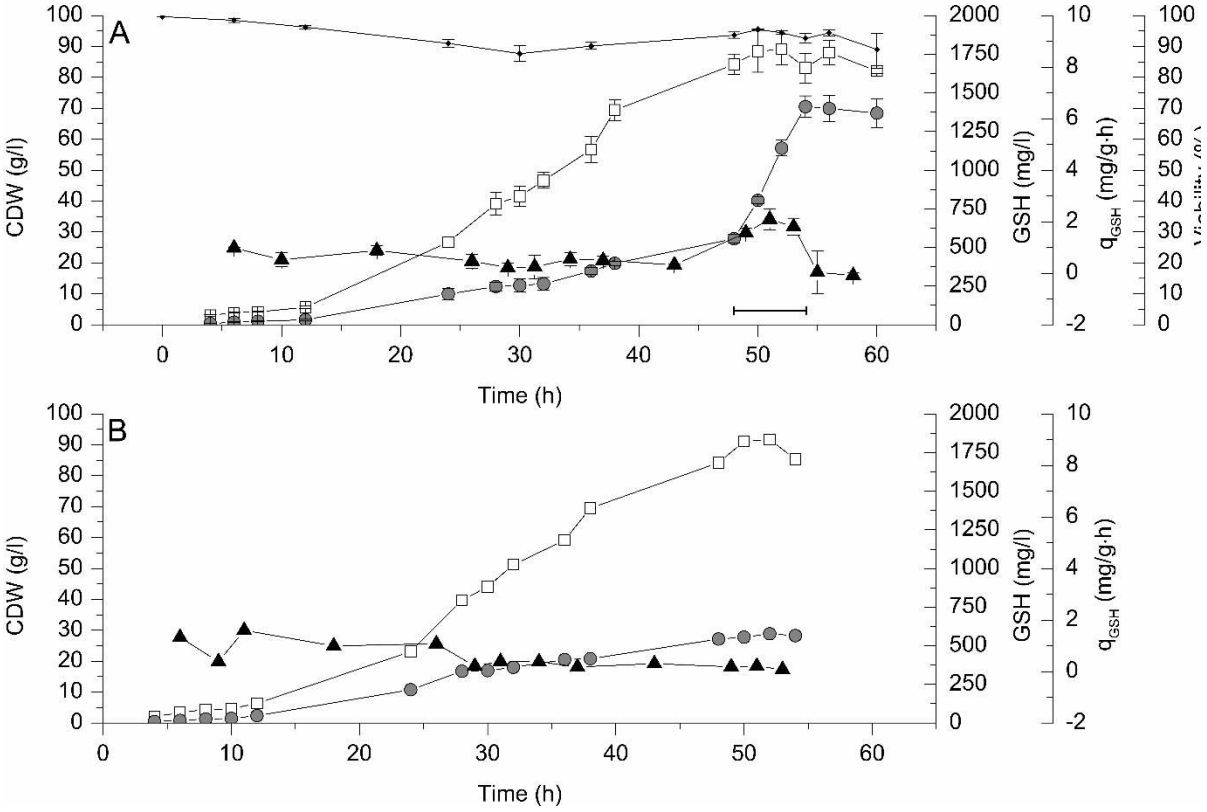

Figure 3-3 **Fed-batch fermentations applying the adjusted fermentation medium and cysteine feeding in 5-l bioreactors. A: Progress of cell dry weight (CDW) (open squares), volumetric GSH concentration (closed circles) and instantaneous specific GSH production rate (closed triangles) of the fed-batches with continuous cysteine addition. To gain physiological insights, viability (closed diamonds) was measured via flow cytometry. Curves show mean values ± SD, n = 3. 17.5 mmol cysteine were applied as continuous feed at 48 h of fermentation for 6 h (as indicated by the bar). B: Control run without cysteine addition.**

3.5 Discussion

3.5.1 A-stat

During the A-stat approach, which has the advantage of providing quasi infinite settings (Paalme & Vilu, 1992), the optimal specific growth rate for maximum GSH production was determined to be the better the lower it was. This is in accordance to Patzschke et al. (2015) who also stated that the growth is inversely correlated to the intracellular GSH concentration which indicates that this finding is not medium dependent.

Table 3-3 **Simplex centroid experimental design for the investigation of ammonium chloride, ammonium sulfate and urea as nitrogen source in the WMIX fermentation medium in shake flasks.**

X_1- ammonium sulfate	X_2- ammonium chloride	X_3- urea	X_1- ammonium chloride	X_2- ammonium sulfate	X_3- urea	CDW	GSH
coded values			g/l			g/l	mg/l
0.5	0.5	0	1.73	1.40	0.00	10.72	195.42
0.5	0	0.5	1.73	0.00	0.79	9.96	189.27
0.167	0.667	0.167	0.58	1.87	0.26	9.94	176.59
0.333	0.333	0.333	1.15	0.93	0.52	9.96	183.73
0.167	0.167	0.667	0.58	0.47	1.05	9.92	179.47
0	1	0	0.00	2.80	0.00	9.68	158.84
0	0	1	0.00	0.00	1.57	9.68	182.64
0	0.5	0.5	0.00	1.40	0.79	9.51	164.66
1	0	0	3.46	0.00	0.00	10.46	202.03
0.667	0.167	0.167	2.31	0.47	0.26	10.40	197.41
0.333	0.333	0.333	1.15	0.93	0.52	10.03	187.51
0.333	0.333	0.333	1.15	0.93	0.52	9.98	189.12

The coded values as well as the actual concentrations of the nitrogen sources in the medium are given. CDW = cell dry weight; GSH = glutathione.

The observed slight decrease in biomass was due to the deviation between chemostat and A-stat, which means that the yeast cells were not able to instantly adapt to the constantly changing dilution rate (van der Sluis et al., 2001). Therefore, $\frac{dC_X}{dt} = (\mu - D) \cdot C_X$ becomes negative, which explains the decrease in biomass (van der Sluis et al., 2001).

In order to avoid this decrease caused by the adaption of the yeast cells, van der Sluis et al. (2001) suggest an acceleration rate of 0.001 $1/h^2$. In contrast, Kasemets et al. (2003) note that chemostat conditions are rare in industrial processes, which is why experiments under constantly changing growth conditions are more relevant for industrial applications. However, they say that high acceleration rates may promote the accumulation of inhibitory by-products and therefore recommend acceleration rates of 0.01-0.04·μmax. As the used *S. cerevisiae* Sa-07346 has a μ_{max} of 0.38 (data not shown), the applied acceleration rate of 0.0035 $1/h^2$ was in a similar range.

3.5.2 Medium composition

The fermentation medium was improved in a way that yeast extract as a complex ingredient could have been left out of the medium. This is beneficial because yeast extract is expensive compared to the other used salts and its quality is lot-dependent. However, the state of the art for baker's yeast production is still the usage of complex media. Nevertheless, in general, the herein developed medium could be applied for further research on metabolic activities in different kinds of yeast cells as it also provides high cell densities which is relevant for industrial applications.

According to Table 3-1, the WMIX fermentation medium lacks especially in nitrogen and sulfur. There was no additional sulfur source except from yeast extract in the WMIX medium. Presumably, there was not enough sulfur, which is necessary e.g. for the production of the amino acids cysteine and methionine or the vitamins thiamine and biotin (Taskin, 2013), to obtain high biomasses. Hence, GSH was used as a sulfur source via the γ-glutamyl cycle (Elskens et al., 1991). This could be an explanation why the variations without yeast extract had only low GSH levels at the end of the fermentation (Table 3-2). In contrast, nitrogen was supplied via 20 % ammonium for pH-control. To overcome the demand on nitrogen and sulfur simultaneously, the feed medium was adjusted by an exchange of ammonium chloride by ammonium sulfate. Liu et al. (1999) reported, that ammonium sulfate was advantageous for biomass generation in comparison to ammonium chloride during shaking flask experiments with *S. cerevisiae* CCRC 21727 (41). Moreover, Khan et al. (1995) stated that ammonium sulfate was also superior to peptone or urea regarding biomass yield. This was also confirmed in this study via the experimental design in shake flasks (Figure 3-2). In conclusion, ammonium sulfate, which serves both as a nitrogen and sulfur source, was beneficial for both biomass as well as GSH production and is preferred in laboratory as well as in industrial production (Ertola et al., 1995).

Furthermore, the formerly used WMIX fermentation medium had a high content of chloride as ammonium chloride was the nitrogen source. However, chloride is only necessary in small amounts for microbial growth, but even at low concentrations, it can inhibit growth (Taskin, 2013). This underlines the usefulness of the change from ammonium chloride to ammonium sulfate and also justifies the removal of magnesium chloride and its substitution by magnesium sulfate in the adjusted feed medium. Further, as high ammonia concentrations can be toxic and inhibit cell growth (Hess et al., 2006), pH-control was realized via NaOH in the runs with the adjusted medium, which also simplifies handling in a way that no ammonia gas is involved.

Finally, it was seen in medium variations 1 and 2 that ethanol was formed caused by the Crabtree effect as glucose could not fully be metabolized due to the stated limitations. However, in the final runs the relatively low feeding rates (μ = 0.1 1/h) assured the absence of glucose overflow. Because of the resulting C-limitation, no ethanol was formed and therefore an optimal growth was guaranteed.

3.5.1 Cysteine addition

In the first fermentations, cysteine was added in the exponential phase, which led to an impediment in cell growth (data not shown). This was mainly due to the entrance into the Crabtree effect, because by the generation of ethanol, the tricarboxylic acid cycle is down-regulated, which also influences the supply of glutamate and cysteine (Bachhawat et al., 2009; Blank & Sauer, 2004; Frick & Wittmann, 2005). Wang, B. et al. (2010) and Wei et al. (2008) showed that addition of cysteine in the stationary phase was beneficial for glutathione outcome. That is why in the following experiments in this work cysteine was added in the late stage of fermentation.

Furthermore, it was seen that continuous cysteine feeding in the late stage of the fermentation was superior to cysteine spiking, which was already seen in an earlier study, where also a more detailed explanation was given (Lorenz et al., 2015). In brief, it was assumed that excess cysteine, which is toxic to the cells, is partially converted to the non-toxic GSH and H_2S. The continuous cysteine feeding ensures that moderate cysteine concentrations that are not leading to H_2S production are reached. However, the mechanism is not fully understood, yet.

The investigated process in this work without yeast extract and the adapted new feed and feeding rate led to comparable results as with semi-complex WMIX (Lorenz et al., 2015).

Table 3-4 Fermentation parameters of the fed-batch fermentations with different medium compositions.

Run/ Parameter	Variation 1 Batch -YE Feed -YE	Variation 2 Batch -YE Feed +YE	Variation 3 Batch +YE Feed +YE	Variation 4 Batch -YE adjusted Feed	Final fermentations with adjusted feed and cysteine supply (n = 3)	Control with adjusted feed medium
max. CDW (g/l)	36.68	48.74	78.34	92.64	90.09 ± 5.82	91.78
final CDW (g/l)	25.32	34.26	70.22	92.64	82.08 ± 0.90	85.34
max. GSH (mg/l)	239.52	225.45	300.46	711.22	1458.96 ± 56.74	577.24
max. GSH (%, w/w)	0.81	0.69	0.69	0.77	1.75 ± 0.16	0.93
final GSH (%, w/w)	0.18	0.10	0.23	0.77	1.67 ± 0.10	0.66
max. specific GSH productivity (mg/g·h)	1.92	1.15	2.19	1.60	2.18 ± 0.26	1.60
$Y_{X/S}$ (g/g)	0.13	0.17	0.35	0.46	0.39 ± 0.01	0.43
max. volumetric GSH productivity (mg/l·h)	8.75	8.74	10.73	14.82	26.69 ± 0.88	11.98

Batch and feeding phases were investigated at a growth rate of 0.1 1/h. For better comparison, also the control run with the adjusted feed medium and without cysteine addition, as well as the final fermentations with the adjusted feed medium and the continuous cysteine feeding are shown.
-YE: without yeast extract; +YE: with yeast extract.

Whereas slightly lower biomass and $Y_{X/S}$ values were achieved with the chemically defined medium (90.09 ± 5.8 g/l vs. 99.5 ± 5.9 g/l and 0.39 ±0.01 vs. 0.50 ± 0.03, respectively), the percentage GSH content per biomass was higher during the whole process leading to the same maximum (1.76 ± 0.08 % in semi-complex WMIX vs. 1.75 ± 0.16 % in chemically defined medium). As stated above, it is assumed that this is due to the higher sulfur content in the adjusted chemically defined medium. This also explains why the $Y_{GSH/cysteine}$ is lower in the adjusted chemically defined medium compared to the semi-continuous WMIX (0.39 ± 0.04 (Table 3-4) vs. 0.54 ± 0.01) as cysteine is not needed to overcome the general sulfur deficiency.

A comprehensive overview of current research approaches and GSH production titers is given by Lorenz et al. (2016). The most recent studies using fed-batch strategies were conducted with genetically modified *E. coli* strains and reached titers between 5870 and 15210 mg/l GSH (Wang, C. et al., 2015; Wang, D. et al., 2016; Zhang et al., 2016). However, the use of GMOs is generally not accepted by consumers in food products, which is a large field of application for GSH. Shang et al. (2008), Wang, M. et al. (2012) and Xiong et al. (2010) used fed-batch strategies in combination with strains of *S. cerevisiae* and complex fermentation media and were able to reach titers between 2100 and 2280 mg/l GSH. In contrast, Ubiyvovk et al. (2011) and Wei et al. (2008) applied synthetic media. Thereby, titers of 1312 mg/l GSH were achieved with the natural *C. utilis* WSH02-08 and 2257 mg/l GSH were synthesized with a genetically modified *H. polymorpha* MOXp-GSH2 (52, 46). The 1459 ± 57 mg/l GSH that were achieved in this study with the natural strain *S. cerevisiae* Sa-07346 are in a comparable range to Ubiyvovk et al. (2011) who also used the natural strain. It becomes clear, that the usage of GMOs allows high GSH titers, however, problems concerning consumer acceptance can occur. The process strategy shown in this study resulted in industry-relevant high cell densities and high intracellular GSH concentrations as well applying a synthetic medium. Therefore, it has the potential to be useful for metabolic studies resulting in optimized GSH production pathways.

To conclude, an adjusted chemically defined medium based on the elemental composition of *S. cerevisiae* and its nutritional requirements was successfully used for GSH production. This medium was combined with a robust and reproducible fed-batch strategy that ensured high cell density cultivations up to 90 g/l CDW with variabilities below 6.5 %. Furthermore, biotransformation of cysteine into glutathione with a high efficiency could be realized. Moreover, it was shown that the applied continuous addition of cysteine at the late stage of fermentation was beneficial for GSH production with *S. cerevisiae* Sa-07346.

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Kapitel 4

Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in Saccharomyces spp.

Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in Saccharomyces spp.

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4 Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in Saccharomyces spp.

4.1 Abstract

Economical yeast based glutathione (GSH) production is a process that is influenced by several factors like raw material and production costs, biomass production and efficient biotransformation of adequate precursors into the final product GSH. Nowadays the usage of cysteine for the microbial conversion into GSH is industrial state of practice. In the following study, the potential of different inducers to increase the GSH content was evaluated by means of design of experiments methodology. Investigations were executed in three natural *Saccharomyces* strains, *S. cerevisiae*, *S. bayanus* and *S. boulardii*, in a well suited 50 ml shake tube system. Results of shake tube experiments were confirmed in traditional baffled shake flasks and finally via batch cultivation in lab-scale bioreactors under controlled conditions. Comprehensive studies showed that the usage of cysteine ethyl ester (CEE) for the batch-wise biotransformation into GSH led up to a more than 2.2 times higher yield compared to cysteine as inducer. Additionally, the intracellular GSH content could be significantly increased for all strains in terms of 2.29 ± 0.29% for cysteine to 3.65 ± 0.23% for CEE, respectively, in bioreactors. Thus, the usage of CEE provides a highly attractive inducing strategy for the GSH overproduction.

4.2 Introduction

The worldwide annual turnover of yeast-derived products is supposed to exceed the 9 billon dollar threshold by the year 2019 (Marz, 2014). Glutathione (GSH) containing yeasts and purified GSH are part of this market.

GSH (γ-glutamyl-cysteinyl-glycine) consists of the three amino acids glutamate, cysteine and glycine. This non-coding tripeptide thiol is found in most pro- and eukaryotic cells with concentrations of 0.2-10 mM and is synthesized via two ATP-dependent enzymatic reactions (Anderson, 1998). Thereby, the first step in GSH biosynthesis is the rate limiting step [3] due to a feedback inhibition of glutamate-cysteine ligase (GSH1, L-glutamate + L-cysteine) by GSH. The second and final reaction is catalyzed by glutathione synthetase (GSH2, γ-glutamyl-L-cysteine + glycine) to glutathione. The redox couple of glutathione (E0 = -240 mV [4]; GSH = reduced; GSSG = oxidized) is located in cytoplasm as well as mitochondria membrane where it maintains the redox status of the organism by e.g. detoxifying reactive oxygen species (ROS) or scavenging free radicals or heavy metals (Ayer et al., 2014; Meister & Anderson, 1983).

Based on the described properties of GSH, the substance itself but also GSH enriched yeasts can be used as food additive (dough modifier or flavor enhancer) in food industries (Gänzle et al., 2008; Ueda et al., 1997). Other applications are the usage of GSH as skin whitening agent in cosmetics or as supporting medication in cancer therapies (Bachhawat et al., 2009; Townsend et al., 2003).

The organisms of choice for an efficient production of GSH are yeasts, especially species like *Candida utilis* and *Saccharomyces cerevisiae* (Li et al., 2004). These organisms provide advantages such as the ability to grow fast to high cell densities or in low-price media (Bachhawat et al., 2009). In the early past, genetic modified bacteria, e.g. Escherichia coli, were also successfully used (Wang, C. et al., 2015). However, consumers usually do not accept the use of GMOs particularly in food.

Concerning the development of a production process with high intracellular GSH content, several approaches in the area of biotransformation, genetic-, evolutionary- and bioprocess engineering were done, triggered by high industrial interest. The most common process strategy to produce GSH in an industrial scale is the fed-batch mode. However, production via repeated fed-batch or continuous (chemostat) processes are also content of current research (Lorenz et al., 2015; Schmacht et al., 2015). Li et al. (2004) and Xiong et al. (2015) summarized different successful production processes and control strategies for fed-batch fermentations (Li et al., 2004; Xiong et al., 2015). Besides process strategies, media design and selection of an efficient production strain are key approaches for optimization. Based on the current knowledge, the most frequently used inducer for glutathione overproduction is cysteine, which is often combined with the other two precursor amino acids glutamate and glycine (Wang, B. et al., 2010). However, combinations of other inducing amino acids as methionine and serine are also published (Wang, Y. et al., 2013; Wen, S. H. et al., 2004). In Table 4-7, there is given an overview of current research approaches for an efficient GSH production, wherein also the results of this study were included for comparison.

In the present work, a full factorial screening of known and unknown GSH inducing substances was executed. Subsequently, selected inducers were combined with different concentrations of yeast extract and investigated via response surface model (RSM). Experiments were performed with an affordable screening platform (50 ml shake tubes) adapted from cell culture technique (De Jesus et al., 2004). To underline the reliability of the investigated induction processes, studies were carried out with three different Saccharomyces strains (baker's yeast, *S. cerevisiae*; brewer's yeast, *S. bayanus*; probiotic yeast, *S. boulardii*) and finally verified under controlled fermentation conditions in 5-l-stirred tank bioreactors.

4.3 Material and methods

4.3.1 Media

For pre-cultures chemically defined WMIX medium (WM = white molasses), a modified version of WMVIII (Lang & Looman, 1995), was used. All chemicals were purchased from Sigma-Aldrich, Inc. The composition was as follows (g/l): salt solution (sterilized by autoclaving): NH_4Cl 2.8, myo-Inositol 0.075, $MgCl_2 \cdot 6H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.55. Moreover 4 ml/l vitamin solution and 1 ml/l trace element solution were aseptically added: vitamin solution (solubility of vitamins can be increased by pH adjustment;

vitamins were sterilized by microfiltration) (g/l): biotin 0.625, pantothenic acid calcium salt 12.50, nicotinic acid 2.50, pyridoxine hydrochloride 7.60, thiamine hydrochloride 2.50; trace element solution in 0.01 M EDTA (sterilized by microfiltration) (g/l): $CuSO_4 \cdot 5H_2O$ 0.10, $FeSO_4 \cdot 7H_2O$ 0.50, $MnCl_2 \cdot 4H_2O$ 0.50, $NaMoO_4 \cdot 2H_2O$ 0.10, $ZnSO_4 \cdot 7H_2O$ 0.175. Final concentrations of glucose and glutamic acid monosodium salt monohydrate were (g/l) 50 and 10, respectively. Medium pH was adjusted with 40 ml/l 0.5 M potassium phosphate buffer (pH 6.8) (K_2HPO_4, KH_2PO_4). This medium was also used as batch-medium containing 50 g/l glucose and additionally 10 g/l yeast extract (Leiber GmbH, Bramsche, Germany).

4.3.2 Strains and pre-cultures

For investigations, three different *Saccharomyces* strains were used. Thereby *S. cerevisiae* Sa-07346 was originally obtained from Organobalance GmbH in Berlin, Germany. The other strains, *S. bayanus* Sa-00645 and *S. boulardii* Sa-07145, were selected from yeast strain collection of the Research and Teaching Institute for Brewing in Berlin (VLB). Pre-culture one was executed by inoculation of 0.1 ml of a cryo-stock into a shaking flask and incubated in WMIX for 24 h at 30 °C and 180 rpm. The second pre-culture was seeded with an optical density (OD_{600nm}) of 0.1 and incubated under the same conditions mentioned above.

4.3.3 Shake tube and shake flask cultivation

In cell culture, orbital shaking technology is one of the most used techniques for early stage process development especially concerning strain or media screening. This technology has been characterized very well (De Jesus et al., 2004; Xie et al., 2011). In this study, it was adapted for scheduled screening trials of yeast cultivation. Shake tubes (50 ml) were sealed with AirOtop® Enhanced Seals, pore size 0.22 µm (Thomson Instrument Company, USA) to ensure a suitable aeration and sterility. Cultivations were executed in vertical arrangement at 30 °C with 25 mm offset at 240 rpm. Initial cell concentration was adjusted to approximately 1 - 2×10^7 cells/ml. Working volume was always 10 ml of yeast suspension in WMIX medium. The orbital shaker was equipped with a special rack (Sartorius AG, Göttingen, Germany) for 50 ml reaction tubes.

Concerning shake flask cultivation, cells were cultivated in 250 ml shake flasks (DURAN Group GmbH, Wertheim, Germany) equipped with four baffles on the bottom for increased power input. The working volume, shaking speed and cultivation temperature were always 25 ml WMIX medium, 180 rpm and 30 ± 0.1 °C, respectively. The induction of glutathione overproduction was for both systems (tubes and flasks) executed by spiking of inducer at 24 h. Analysis of biomass and reduced glutathione (see section 2.5 and 2.7) was done at 32 h of cultivation.

4.3.4 Batch fermentations

Batch fermentations were carried out in 5 l-bioreactors (Biostat®A_{plus}, Sartorius AG, Göttingen, Germany). 2 l of WMIX medium containing 50 g/l glucose as carbon source were

used. The bioreactor was inoculated with 10 % of the second pre-culture. During the cultivation, pH was regulated to 5.5 ± 0.1 by 25 % H_3PO_4 and 20 % ammonia, respectively. Furthermore, temperature was set to 30 ± 1 °C and DO was maintained at 30 ± 5 % (via stirrer speed and constant air flow of 0.5 vvm).

4.3.5 Biomass determination

5 ml cell suspension was centrifuged at 4000 × *g* and washed with 0.9 % NaCl solution. The supernatant was taken for additional HPLC analysis (determination of low molecular sugars as described below). Cell pellet was dried for 24 h at 100 °C and cooled in a desiccator. The cell dry weight (CDW) was periodically calculated.

4.3.6 Determination of low molecular sugars

Sugar analysis was realized by HPLC (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) applying a NUCLEOGEL® ION 300 OA column (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The samples were eluted with 0.005 mol/l H_2SO_4 at a flow rate of 0.4 ml/min.

4.3.7 Glutathione determination

Determination of GSH was based on the colorimetric method via Ellman's reagent (5,5'-dithio-(bis-2-nitrobenzoic) acid, DTNB, Sigma-Aldrich) based on Tietze (1969). In contrast to Tietze (1969), glutathione-disulfide reductase was not used in this assay. That is why only the reduced form (GSH), which shows 30-100 times higher concentrations than GSSG (Ask et al., 2013; Bachhawat et al., 2009; Drakulic et al., 2005), was detected. Yeast cells were washed with 0.9 % NaCl solution, suspended in 0.1 M H_3PO_4 and incubated at 80 °C for 5 min in a water bath. After centrifugation at 4000 × *g* for 10 min, the supernatant was taken for the GSH assay. 100 µl of the sample was pipetted into a 96-well-plate and covered and mixed with 100 µl 0.5 M sodium phosphate buffer, pH 8. Afterwards 5 µl DTNB (4 g/l stock solution) were added and incubated for 10 min. Measurements in triplicates were conducted with a 96-well-plate reader (Molecular Devices GmbH, Biberach, Germany) at 412 nm. Additionally, a GSH standard curve was recorded ranging from concentrations of 12.5 to 100 mg/l reduced GSH. The intracellular GSH (g/l) content was calculated by using a standard curve (linear regression) and related to the CDW as follows:

$$\text{intracellular reduced GSH } (\%,\text{w/w}) = \frac{\text{GSH (g/l)}}{\text{CDW (g/l)}} \cdot 100\% \qquad (1)$$

4.3.8 Experimental design & data analysis

In the beginning, four different substances were investigated with regard to their glutathione inducing properties. A glutathione inducer was defined as a substance that can efficiently be bio-transformed into GSH. Therefore, a full factorial design (2^k-design) was chosen. Variables were the amino acids: X_1-methionine, X_2-cysteine and its chemical derivatives X_3-cysteine ethyl

ester (CEE) and X_4-N-acetyl-cysteine. The concentration of the substances varied between 0 mmol/l (*level -1*) and 5 mmol/l (*level +1*). All factor combinations were performed as single trials with exception of center points, resulting in 22 randomized runs (see Table 4-1).

Table 4-1 **Scheme of experimental full factorial design for the investigation of the GSH inducing potential of four different substances in shake tubes. 22 randomized trials including six center points to perform statistical tests of curvature or non-linearity in the relationship of the factors and the dependent variable were executed. The intracellular GSH content (%, w/w) was used as process output variable.**

X_1-methionine	X_2-cysteine	X_3-CEE	X_4-NAC	intracell. GSH
	(mmol/l)			(%)
2.5	2.5	2.5	2.5	1.28
0.0	5.0	0.0	5.0	1.45
5.0	0.0	0.0	5.0	0.81
0.0	5.0	0.0	0.0	1.40
0.0	0.0	5.0	5.0	1.34
0.0	0.0	0.0	5.0	0.66
2.5	2.5	2.5	2.5	1.27
0.0	0.0	5.0	0.0	1.30
2.5	2.5	2.5	2.5	1.24
2.5	2.5	2.5	2.5	1.36
0.0	5.0	5.0	0.0	1.65
0.0	5.0	5.0	5.0	1.52
2.5	2.5	2.5	2.5	1.22
5.0	0.0	0.0	0.0	0.75
5.0	5.0	5.0	5.0	1.58
0.0	0.0	0.0	0.0	0.60
5.0	5.0	5.0	0.0	1.79
5.0	5.0	0.0	5.0	1.24
5.0	0.0	5.0	5.0	2.03
5.0	5.0	0.0	0.0	1.08
2.5	2.5	2.5	2.5	1.33

X_1-methionine	X_2-cysteine	X_3-CEE	X_4-NAC	intracell. GSH
(mmol/l)				(%)
5.0	0.0	5.0	0.0	1.70

Further, the initial investigations were carried out with *S. cerevisiae* Sa-07346. After identification of potential high inducers, a two factor response surface central composite design (RSCCD, face centered) was chosen to investigate the influence of yeast extract combined with the identified inducer on growth and intracellular GSH content. In sum, six different setups comprising two factors (yeast extract and inducer) multiplied with the three yeast strains (*S. cerevisiae, S. bayanus, S. boulardii*) were executed. Tested concentrations were 0, 1 and 2 % of yeast extract and 0, 5 and 10 mmol/l of the inducer. In Table 4-2, the experimental setup of RSCCD is displayed. For planning of the experimental design and evaluation of the obtained data, Statistica 10.0 (Statsoft Inc. USA) software was employed. Second-degree polynomial model equation was used to describe the obtained results from RSCCD:

$$y=\beta_0+\sum_{i=1}^{k}(\beta_i \cdot x_i)+\sum_{i=1}^{k}(\beta_{i,i} \cdot x_i^2)+\sum_{i=1}^{k}\sum_{j=i+1}^{k}(\beta_{i,j} \cdot x_i \cdot x_j)+\varepsilon \quad (2)$$

Beside regression and variance analysis (ANOVA) was used to identify significant factors and their effect sizes. For better interpretation of results, additional parameters were calculated and listed in the corresponding result-table.

4.4 Results

4.4.1 Identification of potential GSH inducing agents

Four substances, methionine (X_1), cysteine (X_2), cysteine ethyl ester (CEE, X_3) and N-acetylcysteine (NAC, X_4) were screened in a 2-level full-factorial design with regard to their potential properties to increase the intracellular GSH content in *S. cerevisiae* Sa-07346. Cysteine was used as a kind of control agent because it is already known as GSH inducer (Alfafara, C. G. et al., 1992; Lorenz et al., 2015; Wang, M. et al., 2012; Wang, Y. et al., 2012). As mentioned in section 4.3.8, all four inducers were used in concentrations of 0 (*level -1*) and 5 mmol/l (*level +1*). The center point concentrations were set for every used substance to 2.5 mmol/l. All 22 trials of experimental design were executed in 50 ml shake tubes containing WMIX without yeast extract.

Table 4-2 **Set-up and results of the two factor response surface central composite design investigating the relation of inducer and yeast extract on obtainable intracellular GSH concentration in the three yeast strains in shake tubes. Data represent the mean values ± standard deviation (n = 3 for each factor combination; n = 6 for center points).**

X_1-cysteine/ CEE	X_2-yeast extract	Sa-07346				Sa-00645				Sa-07145			
		CEE		cysteine		CEE		cysteine		CEE		cysteine	
		intracell. GSH	SD	intracell. GSH	SD	intracell. GSH	SD	intracell. GSH	SD	intracell. GSH	SD	intracell. GSH	SD
(mmol/l)	(%)	(%)	(-)	(%)	(-)	(%)	(-)	(%)	(-)	(%)	(-)	(%)	(-)
0	0	0.93	0.07	0.93	0.07	0.71	0.18	0.71	0.18	0.66	0.04	0.66	0.04
0	1	0.70	0.08	0.70	0.08	0.49	0.06	0.49	0.06	0.59	0.08	0.59	0.08
0	2	0.60	0.07	0.60	0.07	0.61	0.09	0.61	0.09	0.55	0.06	0.55	0.06
5	0	2.33	0.31	2.28	0.23	1.99	0.11	1.98	0.07	1.53	0.06	1.60	0.01
5	1	2.23	0.30	1.89	0.19	2.06	0.17	1.66	0.11	2.05	0.20	1.33	0.27
5	2	1.70	0.14	1.49	0.09	1.67	0.01	1.32	0.37	1.62	0.09	1.05	0.02
10	0	3.71	0.49	2.32	0.23	3.93	0.22	2.69	0.03	4.19	0.41	1.73	0.14
10	1	4.00	0.89	2.05	0.18	3.91	0.63	2.06	0.41	4.34	0.57	1.65	0.34
10	2	3.49	0.05	1.78	0.09	3.50	0.29	1.41	0.06	3.02	0.30	1.29	0.10

The average of CDW of all fermentations was 8.99 ± 0.4 g/l. An acceptable correlation and predictability denoted as adjusted and predicted R-square of 0.948 and 0.866 was calculated for the relationship of dependent and independent variables, respectively. In Figure 4-1, standardized effects are visualized. Bars representing effects that crossed the vertical dashed line can be considered as statistically significant. Additionally, Table 4-3 lists all main factors of the ANOVA and their significant interactions (non-significant interactions were rejected). However, a closer look into the data indicates that all listed interactions are significant, due to the high influence of cysteine or CEE. Therefore, to avoid false-positive data interpretation, only the main effects should be taken into consideration. That means that only two of the four tested substances showed a real biologically significant effect on intracellular content: cysteine and CEE. Although methionine showed a significant effect, it was far behind the benchmark of cysteine. Therefore, cysteine and CEE were used for further investigations. However, the effect of CEE (23.1) was almost twice as large as the effect of cysteine (11.9).

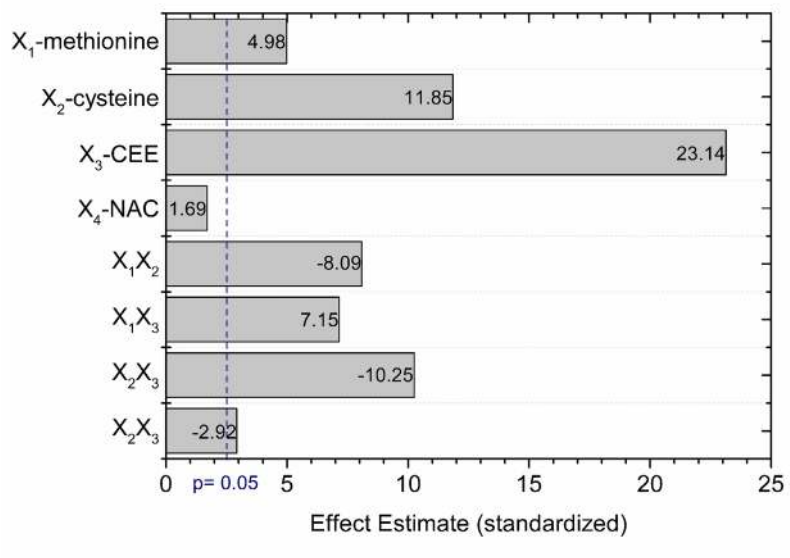

Figure 4-1 **Standardized effects of the full factorial design to identify effective GSH inducers in shake tubes. The blue vertical dashed line indicates the minimum magnitude of statistically significant effect at p < 0.05.**

4.4.2 Evaluation of identified inducer

For a detailed view of the identified inducers cysteine and CEE and their associated effects on the intracellular GSH, a two factor response surface central composite design (RSCCD, face centered) was used. The inducers (X_1; 0, 5, 10 mmol/l) were tested separately, as well as combined with different yeast extract concentrations (X_2; 0, 1, 2 %) for all three *Saccharomyces* strains. Yeast extract contains high concentrations of amino acids, trace elements, salts and unknown growth factors. The choice of the applied yeast extract was based on previous studies, where 13 different tryptone, peptone and yeast extract formulations were characterized concerning growth performance (data not shown). Experiments were executed in 50 ml shake tubes under the conditions described above. In Figure 4-2 row 1 the 3-D response surface graphs for cysteine/yeast extract of three *Saccharomyces* strains are displayed.

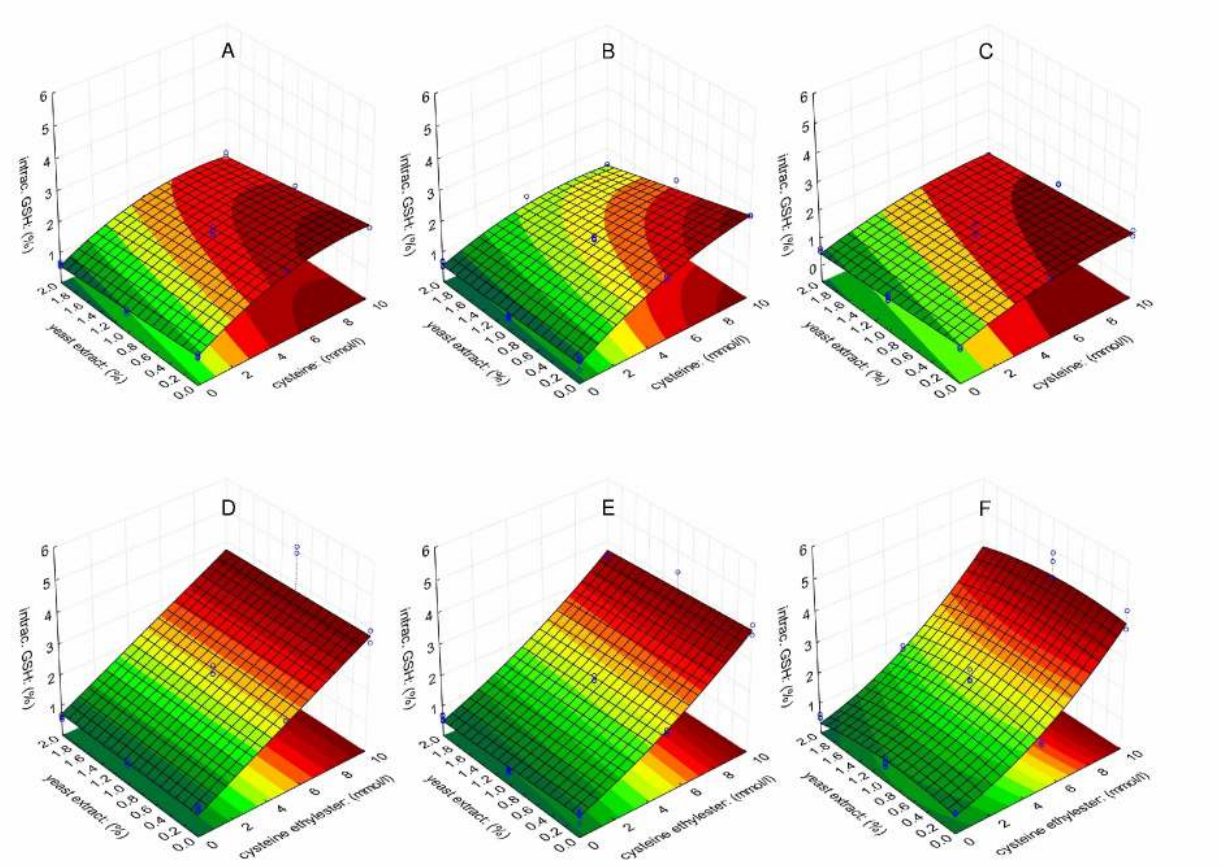

Figure 4-2 **Response surface models for the evaluation of the influence of inducer and YE concentration on intracellular GSH content of the different Saccharomyces strains in shake tubes. Row 1: Effect of cysteine and YE concentration on GSH in *S. cerevisiae* Sa-07346 (A), *S. bayanus* Sa-00645 (B) and *S. boulardii* Sa-07145 (C). Row 2: Effect of CEE and YE concentration on GSH in *S. cerevisiae* Sa-07346 (D), *S. bayanus* Sa-00645 (E) and *S. boulardii* Sa-07145 (F). Model relevant parameters are stated in table 4 and 5.**

All plots illustrate the expected positive effect of cysteine on intracellular GSH content. Corresponding equations (3, 4, 5) for regression for each related strain were as below:

$$\text{Sa-07346: } y=0.9365+0.3320\cdot X_1-0.1882\cdot X_2-0.1856\cdot X_1^2-0.0149\cdot X_1\cdot X_2 \quad (3)$$

$$\text{Sa-00645: } y=0.6425+0.3366\cdot X_1-0.0501\cdot X_2-0.0132\cdot X_1^2-0.0584\cdot X_1\cdot X_2 \quad (4)$$

$$\text{Sa-07145: } y=0.6790+0.2138\cdot X_1-0.0868\cdot X_2-0.0098\cdot X_1^2-0.0183\cdot X_1\cdot X_2 \quad (5)$$

Surprisingly, a negative effect was determined for the main factor yeast extract and weak but significant interaction between cysteine and yeast extract. Detailed GSH values of all significant factor combinations and the ANOVA table including high probability values (p-values) and correlation quotients (r-squared) are listed in Table 4-2, Table 4-4 and Table 4-5. The

application of 10 mmol/l cysteine for glutathione production delivered maximal values of intracellular GSH for all strains in a range between 2.69 to 1.29 %. Thereby, strain Sa-00645 showed the highest GSH content of all tested strains of about 2.69 ± 0.03 % (Sa-07145, 1.73 ± 0.14 % and Sa-07346, 2.32 ± 0.23 %).

Table 4-3 ANOVA data table showing F- and probability (p) values for main factors and their interaction of the full factorial design to identify effective GSH inducers in shake tubes. A p-value below 0.05 indicates a significant effect.

Factor / Parameter	Sa-07346	
	F-value	p-value
model	48.76	<<0.05
X_1-methionine	24.84	<<0.05
X_2-cysteine	140.41	<<0.05
X_3-CEE	535.22	<<0.05
X_4-NAC	2.87	0.151
X_1X_2	65.41	<<0.05
X_1X_3	51.08	<<0.05
X_2X_3	105.08	<<0.05
X_2X_4	8.50	0.033
lack of fit	3.26	0.104
r-squared	0.968	
adj. r-squared	0.948	
predicted r-squared	0.866	

In Figure 4-2 row 2, the associated graphs of the combination of CEE and yeast extract are illustrated. Corresponding equations (6, 7, 8) for regression for each related strain were as below:

$$\text{Sa-07346: } y=0.708+0.3025\cdot X_1 \quad (6)$$

$$\text{Sa-00645: } y=0.6200+0.2183\cdot X_1-0.0996\cdot X_2+0.0141\cdot X_1^2 \quad (7)$$

$$\text{Sa-07145: } y=0.591+0.1453\cdot X_1+0.3840\cdot X_2+0.0021\cdot X_1^2-0.2590\cdot X_2^2 \quad (8)$$

The obtained results indicate that an increase of inducer concentration leads to increased GSH content. Further, calculations showed a weak impact for yeast extract on glutathione content indicated by p-values (see Table 4-5). Interactions could not be detected for the predictors. The highest GSH concentration was also associated with the highest variation. Especially, for the factor combination of 10 mmol/l CEE and 1 % yeast extract an average value of intracellular

GSH of more than 4.08 ± 0.70 % was detected. Due to the observed variation, the predictability of this design point is slightly inaccurate despite the high predicted R-square values for the models. Nevertheless, through the application of 10 mmol/l CEE (with and without yeast extract) a range between 4.34 to 3.02 % GSH for all strains could be reached. The average of all GSH values for 10 mmol/l CEE or cysteine is 3.79 ± 0.41 and 1.89 ± 0.44, respectively. This is an increase of nearly two times. Thereby, strain Sa-07145 showed the highest GSH content of all tested strains of about 4.34 ± 0.57 % (Sa-00645, 3.93 ± 0.22 % and Sa-07346, 4.00 ± 0.89 %). Even though yeast extract has a slightly negative effect on intracellular GSH content when using cysteine as inducer, it is prerequisite to ensure adequate biomass concentration (Figure 4-3 A). Analysis of variances of all strains and their biomasses indicated a significant positive influence of yeast extract on growth. Figure 4-3 A displays the best performing yeast extract concentration of about 1 % for suitable biomass growth for all three *Saccharomyces* strains. Also visualized in Figure 4-3 B are the significant higher biomasses measured for the strains Sa-07145 and Sa-00645 compared to strain Sa-07346. One requirement to achieve a satisfactory GSH concentrations is high biomass (Li et al., 2004). Therefore 1 % of yeast extract in the fermentation medium was used in further trials to achieve higher absolute GSH-levels.

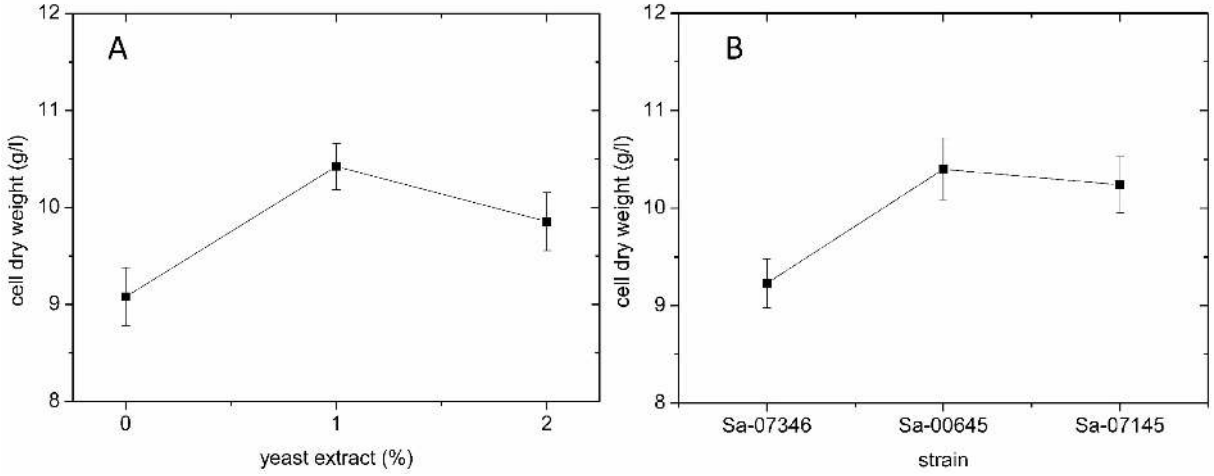

Figure 4-3 **Effect of yeast extract on cell dry weight calculated out of all shake tube experiments for all strains (A) and dependency of growth related to the used strains (B). Data are given as mean values. Error bars denote confidence interval of 95 %.**

Due to the already mentioned deviations for CEE, a transition into shake flasks was performed to confirm the data obtained from shake tubes. Finally, it is obvious that the intracellular GSH concentration can be increased by cysteine and CEE in all used *Saccharomyces* strains.

Table 4-4 **ANOVA table including probability values (p-values) and correlation quotients (r-squared) of all factor combinations of the two factor response surface central composite design investigating the relation of cysteine and yeast extract on obtainable intracellular GSH concentration in the three yeast strains in shake tubes.**

Factor / Parameter	Sa-07346 A		Sa-00645 B		Sa-07145 C	
	F-value	p-value	F-value	p-value	F-value	p-value
model	140.91	<<0.05	147.31	<<0.05	59.880	<<0.05
X_1-cysteine	379.45	<<0.05	383.24	<<0.05	177.820	<<0.05
X_2-yeast extract	50.960	<<0.05	73.010	<<0.05	17.810	<<0.05
X_1X_1	65.890	<<0.05	26.400	<<0.05	14.130	<<0.05
X_2X_2	rejected		rejected		rejected	
X_1X_2	3.230	0.089	41.360	<<0.05	3.513	0.072
lack of fit	1.350	0.299	0.124	0.323	0.840	0.513
r-squared	0.969		0.956		0.898	
adj. r-squared	0.962		0.950		0.884	
pred. r-squared	0.948		0.939		0.859	

4.4.3 Confirmation of shake tube results

For evaluation and confirmation of the achieved knowledge from shake tubes, shake flask experiments were performed. As explained above, yeast extract is needed to generate sufficient biomass. Therefore, 1 % yeast extract combined with 10 mmol/l of inducer (cysteine or CEE, respectively) were used to verify the effects of the inducers. Cultivation conditions as well as induction of GSH production is described in section 4.3.3. Figure 4-4 shows the intracellular concentrations of GSH (Figure 4-4 A) and the corresponding biomasses (Figure 4-4 B) for all three strains. Briefly, the results of the experiments in shake tubes were confirmed. Thereby, the range of measured GSH values for CEE and cysteine of all three strains are slightly decreased in shake flask experiments. Based on results achieved from shake tube experiments, a factor of 2.1 (4.08 ± 0.23 % GSH via CEE compared to 2.25 ± 0.48 % GSH via cysteine) compared to the data of shake flasks with a factor of 1.6 (3.48± 0.40 % GSH via CEE compared to 2.37± 0.38 % GSH via cysteine) was determined. The superior growth of strain Sa-00645 and Sa-07145 in comparison to Sa-07346 was also confirmed (Figure 4-4 B). Taken together, the achieved results in this section verified the results from the response surface design with regard to the intracellular GSH content and biomass. Variations seen at 1 % yeast extract and 10 mmol/l CEE in shake tubes could not be observed in shake flask cultivation. That may indicate that the observed variations are caused by the cultivation system.

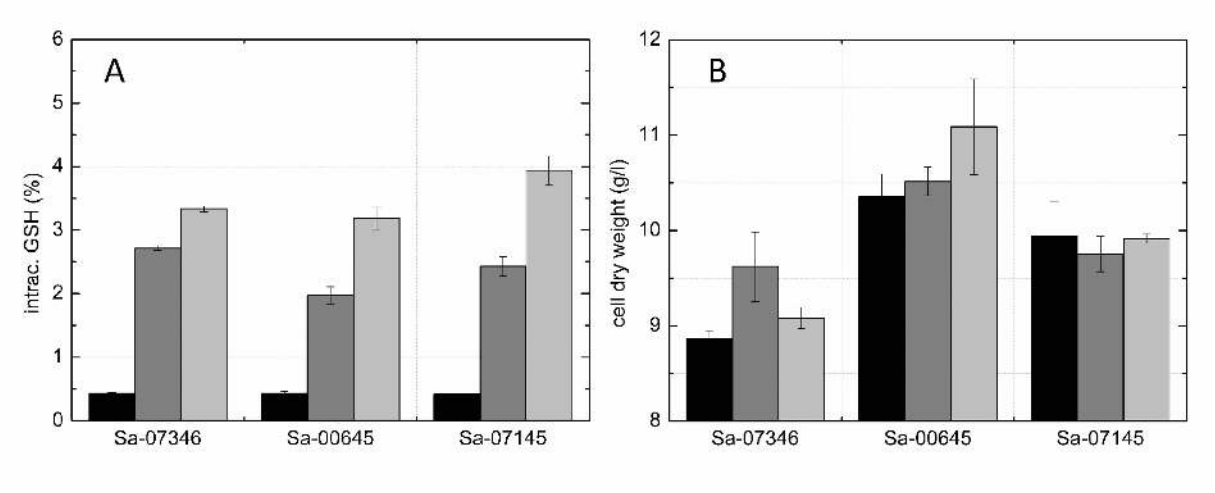

Figure 4-4 **Influence of GSH inducing agents on maximum intracellular GSH concentration (A) and CDW (B) of the tested Saccharomyces strains in shake flasks. Black: control without inducing agent; gray: 10 mmol/l cysteine were spiked at 24 h of fermentation; light gray: 10 mmol/l CEE were spiked at 24 h of fermentation. Mean values and standard deviations were obtained from triplicates.**

However, in previous studies the system was considered as valid because achieved data for reproducibility showed a variation quotient of lower than 10 % (data not shown). In addition, described variations were only detected for the factor combination mentioned above. For further affirmation, results were transferred and verified using a lab-scale bioreactor cultivation system.

Table 4-5 **ANOVA table including probability values (p-values) and correlation quotients (r-squared) of all factor combinations of the two factor response surface central composite design investigating the relation of CEE and yeast extract on obtainable intracellular GSH concentration in the three yeast strains in shake tubes.**

Factor / Parameter	Sa-07346 D		Sa-00645 E		Sa-07145 F	
	F-value	p-value	F-value	p-value	F-value	p-value
model	227.59	<<0.05	355.44	<<0.05	154.78	<<0.05
X_1-CEE	227.59	<<0.05	985.89	<<0.05	601.00	<<0.05
X_2-yeast extract	rejected		4.130	0.052	2.630	0.117
X_1X_1	rejected		8.810	<<0.05	16.080	<<0.05
X_2X_2	rejected		rejected		5.310	0.029
X_1X_2	rejected		rejected		rejected	

Factor / Parameter	Sa-07346 D		Sa-00645 E		Sa-07145 F	
	F-value	p-value	F-value	p-value	F-value	p-value
lack of fit	0.660	0.700	1.880	0.140	5.240	<0.05
r-squared	0.898		0.976		0.958	
adj. r-squared	0.894		0.974		0.952	
pred. r-squared	0.879		0.964		0.933	

4.4.4 Batch fermentations in bioreactors

The evaluation of CEE as potential and efficient inducer for GSH overproduction was conducted under defined controlled conditions in a lab-scale bioreactor (described in section 2.4).

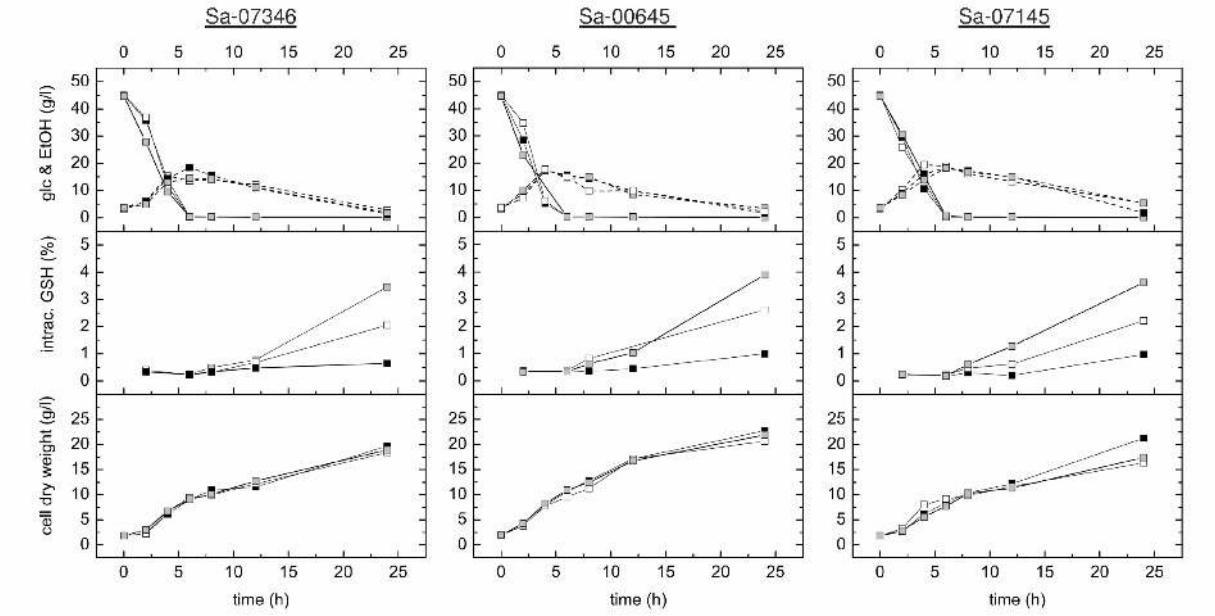

Figure 4-5 **Influence of GSH inducing agents on intracellular GSH concentration in the three different Saccharomyces strains in 5 l lab-scale bioreactors. For better process understanding, progress of cell dry weight as well as glucose and ethanol concentrations during the fermentation are also shown. GSH inducing agents were CEE (grey squares), cysteine (open squares), and water as control (black squares). The substances were spiked at time 6 h at a concentration of 10 mmol/l.**

Due to the expected enhanced growth in bioreactor compared to shake tubes or flasks, the spiking of 10 mmol/l of inducer was performed at 6 h instead of 24 h. In sum, 9 different batches (control, cysteine and CEE) with 3 different strains were executed. Figure 4-5 shows the progresses of CDW, glucose concentration, ethanol concentration and glutathione content, for every single run. The growth behavior of all fermentations was linked to the availability of carbon source type. During the first 6 h, glucose was fully metabolized to biomass and ethanol.

Table 4-6 Overview of batch fermentations applying different inducers for GSH overproduction in different Saccharomyces strains in 5 l lab-scale bioreactors.

Parameter	Inducer	Sa-07346	Sa-00645	Sa-07145
GSH (mg/l)	control	127.49	224.95	207.71
	cysteine	378.02	537.34	361.88
	CEE	655.71	852.74	629.27
intracell. GSH (%)	control	0.65	0.99	0.98
	cysteine	2.05	2.61	2.21
	CEE	3.46	3.89	3.62
efficiency (%)	control	n/a	n/a	n/a
	cysteine	8.15	10.16	5.02
	CEE	17.19	20.43	13.72
$Y_{X/S}$ (g/g)		0.38 ± 0.01	0.43 ± 0.02	0.37 ± 0.05
CDW (g/l)		19.04 ± 0.64	21.74 ± 1.05	18.35 ± 2.59

Between 6 h and 8 h of cultivation, yeast metabolism changed from respiro-fermentative to purely oxidative and ethanol was used as carbon source. At the end of cultivation, ethanol had almost been consumed and strain specific biomass yield coefficients (g/g) were between 0.37 – 0.43 (Table 4-6) which was much higher compared to shake tubes and flasks. The often seen negative influence on biomass triggered by spiking of inducers could not be detected. Progress of intracellular GSH of used strains and corresponding inducers confirmed the data obtained from shake tubes and flasks. Further, for the used *Saccharomyces* strains, CEE as inducer for GSH production was also superior to cysteine in bioreactor conditions (3.66 ± 0.22 % vs. 2.29 ± 0.29 %, respectively). In addition, the detected incorporation yields (listed in Table 4-6 as efficiency) were in mean 2.2 times higher for CEE compared to cysteine.

Table 4-7 Overview of current research on GSH production.

Process	Strain	Max. c(GSH) (mg/l)	Max. c(CDW) (g/l)	Yield GSH/CDW (g/g)	Added precursor (mmol/l)		Yield: GSH/precursor (mol/mol)		Reference
					cysteine	CEE	cysteine	CEE	
Batch	*S. cerevisiae* Sa-07346	378.0	18.4	0.021	10.0	-	0.082 **	-	This study
Batch	*S. cerevisiae* Sa-07346	655.7	19.0	0.035	-	10.0		0.172 **	This study
Batch	*S. bayanus* Sa-00645	537.3	20.6	0.026	10.0	-	0.102 **	-	This study
Batch	*S. bayanus* Sa-00645	852.7	21.9	0.039	-	10.0		0.204 **	This study
Batch	*S. boulardii* Sa-07145	361.9	16.4	0.022	10.0	-	0.050 **	-	This study
Batch	*S. boulardii* Sa-07145	629.3	17.4	0.036	-	10.0		0.137 **	This study
Fed-Batch	*E. coli* JM109(pTrc99A-gshF)[a]	11300.0	28.0	0.404	75.0	-	0.456 *	-	(Wang, D. et al., 2016)
Batch	*S. cerevisiae* YSF2-19[a]	319.0	11.3	0.028	-	-	-	-	(Yin et al., 2016)
Fed-Batch	*E. coli* ZJ12345[a]	5870.0	6.8	0.870	25.0	-	0.634 *	-	(Zhang et al., 2016)
Fed-Batch	*S. cerevisiae* Sa-07346	1650.7	99.5	0.017	17.9	-	0.228 **	-	(Lorenz et al., 2015)
Batch	*S. cerevisiae* A4-19[b]	320.0	8.2	0.039	-	-	-	-	(Patzschke et al., 2015)
Batch	*S. cerevisiae* A4-19-13[b]	270.0	4.6	0.059	-	-	-	-	(Patzschke et al., 2015)
Fed-Batch	*E. coli* BL21(pUC18-gshF)[a]	15210.0	31.9	0.476	75.0	-	0.573 *	-	(Wang, C. et al., 2015)
Batch	*S. cerevisiae* ATCC 7754	236.1	25.0	0.009	4.0	-	0.151 *	-	(Anschau et al., 2013)

Process	Strain	Max. c(GSH) (mg/l)	Max. c(CDW) (g/l)	Yield GSH/CDW (g/g)	Added precursor (mmol/l)		Yield: GSH/precursor (mol/mol)		Reference
					cysteine	CEE	cysteine	CEE	
Batch	*S. cerevisiae*	271.0	17.1	0.016	34.9	-	0.014 **	-	(Taskin, 2013)
Batch	*C. utilis* CCTCC M 209298	669.3	15.5	0.043	6.0	-	0.201 **	-	(Wang, Y. et al., 2012)
Fed-Batch	*S. cerevisiae* 26-2	2250.0	105.0	0.021	6.0	-	0.651 *	-	(Wang, M. et al., 2012)
Fed-Batch	*H. polymorpha* MOXp-GSH2[a]	2257.0	70.0	0.032	-	-	-	-	(Ubiyvovk et al., 2011)
Fed-Batch	*C. utilis* WSH02-08	2448.0	112.3	0.022	36.0	-	0.133 **	-	(Wang, B. et al., 2010)
Fed-Batch	*S. cerevisiae* T65	2100.0	126.0	0.017	5.0	-	0.391 **	-	(Xiong et al., 2010)
Fed-Batch	*S. cerevisiae* GE-2	2280.0	110.0	0.021	10.0	-	0.462 *	-	(Shang et al., 2008)
Fed-Batch	*C. utilis* WSH02-08	1312.5	83.5	0.016	20.0	-	0.088 **	-	(Wei et al., 2008)
Batch	*S. cerevisiae* ATCC 7754	154.5	9.1	0.017	-	-	-	-	(Santos et al., 2007)
Fed-Batch	*S. cerevisiae* G-14	2020.0	132.0	0.015	4.0	-	0.325 **	-	(Wang, Z. et al., 2007)
Fed-Batch	*S. cerevisiae* T65	2190.0	133.3	0.016	12.1	-	0.125 **	-	(Wen, S. et al., 2006)
Batch	*S. cerevisiae* T65	329.3	19.8	0.017	5.4	-	0.097 **	-	(Wen, S. H. et al., 2005)

* GSH concentration before and after cysteine addition; ** Calculation based on control fermentation; [a] GMO; [b] Evolutionary engineered

4.5 Discussion

4.5.1 Shake tube cultivation system

In this study, three different cultivation systems were used to investigate the influence of various potential glutathione inducers. Screening of key factors and the followed effect evaluation was executed in shake tubes equipped with special membranes for an adequate gas exchange. However, it can be assumed that the k_La values in the shake tubes are somewhat lower than in baffled shake flasks. Nevertheless, the yields for GSH/CDW by use of CEE showed for all strains only marginal differences between shake tubes and bioreactors (e.g.: Sa-07346, 3.33 %/3.46 %; Sa-00645, 3.18 %/3.89 %; Sa-07145, 3.94 %/3.62 %). This confirms that the used shake tube technique is an affordable screening system for the first steps in bioprocess development as e.g. strain, substance or media screenings. Its main advantage is the high reproducibility and the increased level of parallelism and throughput achievement, which is often needed in early development stage. The introduced shake tube technique lacks controlled pH and DO but there are fluorescence-based sensors for 50 ml shake tubes. However, the mentioned advantages often compensate the high acquisition and consumption costs of the sensors. Further, the application of fed-batch fermentations in shake tubes is technically challenging, but there are platforms to mimic this conditions as e.g. EnBase® (BioSilta Ltd., UK) (Panula-Perala et al., 2008) or Feed Plate® (PS Biotech, Germany), which could be a feasible add-on. Finally, both applied shaking systems provided very similar values with respect to the intracellular GSH content, which was the main process output variable. Although shake tube and shake flask platforms are generally understood as up-scalable, the proof of concept for bioprocess development has to be verified under fully controlled conditions in the bioreactor.

4.5.2 Final effect verification

In fact, bioreactor cultivations were carried out under controlled conditions to verify the impact of CEE on intracellular glutathione concentration. Basically, the significantly higher influence of CEE compared to cysteine on GSH production (in mean of factor 1.6) was shown and confirmed the data obtained from shaking systems. Thereby, it is noteworthy that the slope of GSH production was very low for all strains (Figure 4-5). However, final values of intracellular GSH were comparable with shake flasks. One reason for this smooth increase in GSH could be the degradation of ethanol instead of glucose. The primary carbon source glucose was metabolized under respiro-fermentative conditions to biomass, carbon dioxide and ethanol plus minor amounts of other by-products (e.g. glycerol and acetate). Afterwards, metabolism changed from respiro-fermentative to purely oxidative conditions and ethanol served as carbon source. This explains the reduced growth rate because it is determined by respiratory capacity of the organism (Sonnleitner & Kappeli, 1986). It can be assumed that the increased demand of energy used for ethanol degradation to acetaldehyde and finally to acetyl-CoA is responsible for the slower growth as well as GSH biosynthesis. Nevertheless, it seems that the efficiency of

biotransformation of cysteine or CEE into GSH is not affected by ethanol concentrations lower than 2 %. In addition, the achieved biomass yields for batch cultivations were in an acceptable range of 0.37 to 0.43 g/g.

4.5.3 Selection and evaluation of GSH inducer

In literature and industrial practice, the most common inducer for glutathione overproduction is cysteine (Alfafara et al., 1992; Wang, M. et al., 2012; Wang, Y. et al., 2012), whether as pure substance or bound at proteins and later extracted by hydrolysis with enzymes or acids. In order to gain a cheap source of cysteine, Taskin (2013) pursued an alternative and sustainable approach. He hydrolyzed inexpensive chicken feathers and obtained a peptone that comprised high contents of minerals, ash and of course free cysteine and glycine. However, the induction of biotransformation was always initiated by cysteine itself. In this study, cysteine served as benchmark to evaluate the potential of alternative inducers.

It is assumed that the transport of cysteine from medium into *S. cerevisiae* cells is mediated by at least nine different permeases, none of which show high specificity and performance at enhanced cysteine concentrations (Kaur & Bachhawat, 2007). Thus, the investigation of alternative GSH inducers seemed to be a promising approach to promote the biotransformation process and was examined within this study.

Due to the closely related biosynthesis pathway from methionine and cysteine as well as the promising results from Sadhu et al. (2014), the thiol-containing amino acid methionine was chosen as first potential inducer. The second examined inducer was N-acetylcysteine chosen by Yang et al. (2012) for reduction of ROS formation. In that study, measurements of ROS were performed by the cell-permeable dye CM-H_2DCFDA via flow cytometry analysis. In combination with the findings obtained from Jakubowski and Bartosz (2000) with derivatives of H_2DCF it can be assumed that N-acetylcysteine can increase the intracellular GSH content, which results in decreased measurement of ROS in yeast cells. The third investigated inducer, a further derivative of cysteine, was CEE. It is understood that an esterification of a carboxylic group of cysteine to CEE increases the lipophilicity of cysteine. In studies of Laffleur et al. (2015), the linkage of CEE to hyaluronic acid was used to enhance its permeation in Caco-2 cells, showing the potential to increase the bioavailability. Giustarini et al. (2012) and co-workers compared the properties of N-acetylcysteine and its derivative N-acetylcysteine ethyl ester in a pharmacologic study with rats. They showed that N-acetylcysteine ethyl ester rapidly enters the cell and is transformed into GSH. Thereby, an improved reduction of oxidative stress level in brain triggered by enhanced GSH concentrations could be observed. Similar approaches were pursued by Joshi et al. (2007), who could illustrate the improved GSH inducing properties of γ-glutamyl cysteine ethyl ester (GCEE). It was assumed that the ethyl ester moiety of γ–glutamyl cysteine on one side increases the efficacy of GCEE to cross the plasma membrane and on the other side avoids feedback inhibition, compared to cysteine (Drake et al., 2002). The described up-regulated GSH biosynthesis is in accordance with the presented results of

enhanced GSH concentrations mediated by CEE in this study. This becomes obvious acknowledging the comparison of different strains and the inducers cysteine and CEE. In fact, the application of CEE resulted in an increase of intracellular GSH content of more than 60 %, compared to cysteine in bioreactors.

For comparison, there is an overview of the current research on GSH production given in Table 4-7. From that data, it becomes clear that in the very recent past a lot of research was done on genetically modified microorganisms as well as evolutionary engineering or a combination of both (Patzschke et al., 2015; Ubiyvovk et al., 2011; Wang, D. et al., 2016; Yin et al., 2016; Zhang et al., 2016). However, these approaches are more likely to be applied for GSH production itself than for GSH enriched yeasts for food applications, as consumers usually do not accept GMOs in food. Concerning the yield of GSH/CDW regarding the wild-type strains (Anschau et al., 2013; Lorenz et al., 2015; Santos et al., 2007; Shang et al., 2008; Taskin, 2013; Ubiyvovk et al., 2011; Wang, B. et al., 2010; Wang, Y. et al., 2012; Wang, Z. et al., 2007; Wei et al., 2008; Wen, S. et al., 2006; Wen, S. H. et al., 2005; Xiong et al., 2010), only the work of Chen et al. (2012); Wang, Y. et al. (2012) showed a higher yield (0.043) than was achieved in this work via CEE in *S. bayanus* (0.039), which underlines the usefulness of CEE as GSH inducing agent. In contrast, the yield of GSH/precursor is very fluctuating. This is probably due to the use of different media and other precursor amino acids. In some cases GSH might be used by the cells as sulfur source via the γ-glutamyl-cycle due to sulfur limitations in the medium which negatively affects this yield (Lu, 2013). There is still potential to find a medium that is best suited for GSH production.

From an economical point of view, the application of cysteine as precursor for biotransformation, as a low-cost bulk chemical with a world market price of around 20 $/kg, is very attractive. Nevertheless, due to the esterification of amino acids being a low-priced common chemical process that does not require high technical effort, also CEE could be a valuable inducer for an improved production of GSH enriched yeast.

4.5.4 Applicability of the process on different strains

Three different *Saccharomyces* strains were selected to display the broad applicability of the investigated procedure to enhance the GSH content. It is very attractive for food industry to use GSH enriched yeast as a baking modifier. GSH cleaves the disulfide bonds between the dough proteins. This results in an improved extensibility and machinability as well as a decreased mixing time. Another advantage is the providing of a so-called clean label by GSH enriched yeasts compared to cysteine because no declaration is needed (Anonymous, 2006; Suas, 2008). Already in 1997, Ueda and co-workers described the potential of different γ-glutamylcysteine derivatives, e.g. GSH, as a kind of flavor enhancer (kokumi-taste) (Ueda et al., 1997). Also in wine making GSH as well as inactive GSH enriched yeast are used to improve the flavor stability during wine aging (Dubourdieu & Lavigne-Cruege; Rodríguez-Bencomo et al., 2014).

In the section above, the effect of CEE was pointed out. Especially, the properties of the ethyl ester moiety in mammalian models and cells were discussed. Musatti et al. (2014) investigated the absorption and bioavailability of GSH or GSH enriched yeast in an in vitro gastrointestinal model. Thereby, it could be demonstrated that less than 20 % of GSH was absorbed. However, if cells (Caco-2 and HT29-MTX) were treated with H_2O_2, GSH protected the intestinal cells against oxidative stress (Musatti et al., 2014). It is known that *S. boulardii* has probiotic properties and is successfully used for therapy of gastrointestinal disorders (Kelesidis & Pothoulakis, 2012). Based on the above discussed results of Musatti et al. (2014) and shown data in this study, it could be an interesting approach to combine the protective role of GSH against oxidative stress with the probiotic properties of *S. boulardii* for treatment of gastrointestinal disorders.

For all the named applications, an efficient GSH production is the key for an efficient commercial process. The application of GSH inducing substances as e.g. cysteine or CEE can in general be considered as hindering as this increases costs (Hara et al., 2012) and also may impede the downstream processing as an additional component. However, for the production of GSH enriched yeasts for food applications this may be the only way to achieve high intracellular GSH content as usually no GMOs are accepted in this field. Here, CEE can significantly increase the intracellular GSH content. To improve downstream processing, research is done on GSH production and following secretion (Sasaki et al., 2016). Nevertheless, such approaches are more interesting for GSH production itself. As also genetically modified organisms are provided with precursor amino acids (Wang, D. et al., 2016; Yin et al., 2016; Zhang et al., 2016), it would be valuable to investigate whether also here the application of CEE can improve the overall performance.

4.6 Conclusion

The demand of GSH and GSH enriched yeasts for industrial application is still high. As the production process should be as efficient as possible, the highest possible biomass containing high GSH content in a short period of time combined with a high incorporation yield of inducer into GSH should be achieved. The significantly higher influence of CEE compared to the most commonly applied inducer cysteine was evaluated and verified in different cultivation systems. Its successful application led to a 2.2-times higher incorporation yield. Based on literature, it is assumed that the determined higher GSH values by the application of CEE within this study are caused by the increased lipophilicity and membrane permeability triggered by the ethyl ester moiety. Moreover, the positive effect of CEE in *S. cerevisiae* is reproducible in the brewer's yeast *S. bayanus* Sa-00645 and the probiotic yeast *S. boulardii* Sa-07145.

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Kapitel 5

Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*

Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*

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5 Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*

5.1 Abstract

In the following work a high cell density fed-batch process with *Saccharomyces cerevisiae* coupled with a high efficient incorporation of cysteine for glutathione (GSH) overproduction was developed. Therefore, a feeding strategy based on the respiratory quotient (RQ) was applied to ensure high biomass (96.1 g/l). Furthermore, the optimal cysteine concentration and time of cysteine addition were investigated. Low concentrations of cysteine at late fermentation phases resulted in relatively high incorporation yields of about 0.40 mol/mol and maintained the physiology of cultivated yeast. By changing the cysteine feeding from standard single shot to continuous addition, an often observed cell specific toxicity, triggered by high cysteine concentrations, could be prevented and the cysteine incorporation yield (0.54 ± 0.01 mol/mol) and GSH content (1650.7 ± 42.8 mg/l; 1.76 ± 0.08%) were maximized, respectively. The developed process was transferred from laboratory into pilot plant scale. Further, the reduced cell specific toxicity enabled the development of a repeated fed-batch procedure with a suitable performance concerning cysteine incorporation yield (0.40 ± 0.1 mol/mol), biomass (84.2 ± 1.2 g/l) and GSH content (1304.7 ± 61.4 mg/l).

5.2 Introduction

The global market for yeast products reached $5.8 billion in 2013. This market is expected to grow to $9.2 billion in 2019 (Marz, 2014). Important yeast derived products are autolysates as fermentation starters, flavor enhancers and spreads. These products are often applied in biotechnology and pharmaceutical processes to ensure excellent growth behavior of microorganisms and cell line cultures. Further, yeast extracts are also used as a kind of flavor enhancer (umami-taste and kokumi-taste) in food industry. Kokumi-taste is affected by different γ-glutamylcysteine derivatives, for instance GSH (Ueda et al., 1997). Intact yeast cells enriched with GSH is a very attractive application in food industry, where it can be used as a baking modifier in order to improve extensibility, dough flow and machinability and decrease mixing time. GSH enriched yeast is traded with 4 $/kg (Quantz, 2014). In contrast to cysteine, it has, as a natural product, the advantage of no need for declaration, thus being convenient for a so-called clean label (Anonymous, 2006; Suas, 2008). In pharmaceutical and cosmetic industry, GSH is taken as skin whitening agent or scavenger for free radicals or heavy metals (Bachhawat et al., 2009; Stephen & Jamieson, 1997; Wen, S. et al., 2006).

The tripeptide GSH is an important antioxidant occurring in most of eukaryotic and prokaryotic organisms. Major function of GSH is to maintain the cellular redox potential in cooperation with glutathione disulfide (GSSG) and to prevent damage to important cellular components caused by reactive oxygen species (ROS) (Ayer et al., 2014). Further functions of GSH range

from detoxifying heavy metals by generating GSH conjugates to protecting of proteins during stress conditions by S-glutathionylation (Ayer et al., 2014; Herrero et al., 2008). It is synthesized via two enzymatic ATP dependent steps and consists of the amino acids L-glutamate, L-cysteine und glycine, why it is also called γ-glutamyl-cysteinyl-glycine (Meister & Anderson, 1983). At first, the GSH synthesis is catalyzed by glutamate-cysteine ligase (GSH1, L-glutamate + L-cysteine) followed by final reaction of glutathione synthetase (GSH2, γ-glutamyl-L-cysteine + glycine).

In wild type yeast strains the concentration varies between 0.1 % - 1 % of biomass with a ratio of reduced (GSH) to oxidized (GSSG) form of 30-100:1 (Ask et al., 2013; Bachhawat et al., 2009; Drakulic et al., 2005).

The common method of choice for GSH production is fermentation of yeast such as *Candida utilis* and *Saccharomyces cerevisiae*, providing advantages such as the ability to grow fast to high cell densities or cheap growth media (Bachhawat et al., 2009). However, recently a genetic modified *E.coli* strain was used to produce GSH concentrations of about 15 g/l (Wang, C. et al., 2015).

Driven by a high industrial interest, various approaches in the fields of genetic and evolutionary engineering, biotransformation and bioengineering were developed to increase the intracellular GSH content of the producing cells. Especially concerning developed GSH overproducing yeast strains, classical mutagenesis by UV radiation, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) or Dimethylsulfate (DMS) was applied. Those, randomly mutagenized yeast cells were plated and cultivated on solid culture medium consisting different selection pressure substances. As reported by (Li et al., 2004), with these proceedings, yeast strains with GSH contents higher than 2 % could be reached. However, the strains obtained by the mentioned strategy could be negatively affected in growth behavior (Cakar et al., 2005). Preventing this disadvantage, another strategy to receive high glutathione producing strains is evolutionary engineering. Thereby, yeast strains with a GSH content of more than 3.9 % were achieved (Patzschke et al., 2015). Genetic engineering tools represent a different approach and possibility to produce yeast strains with high intracellular GSH concentrations. But there are authority issues to apply these target-engineered strains in food industry (Cakar et al., 2012).

Enhancing the production of GSH via upstream bioprocessing approaches, many research groups optimized the cultivation medium with regard to the source of nitrogen (N), carbon (C), phosphate (PO_4), sulfate (SO_4), magnesium (Mg) and zinc (Zn) (Liu et al., 1999; Rollini & Manzoni, 2006; Zhang et al., 2007).

There are various strategies for the fermentation process of GSH production, the most common being fed-batch procedure with the yeast *S. cerevisiae*. The feeding processes are mostly controlled via respiration quotient (RQ), dissolved oxygen (DO), ethanol (EtOH) production or growth rate (μ), aiming to avoid byproducts and ensure high productivity and yields (Nisamedtinov et al., 2010; Wang, Z. et al., 2007; Wen, S. et al., 2006; Xiong et al., 2010). A

popular strategy to induce the GSH overproduction is the single shot addition of GSH precursor amino acids. Thereby, a lot of strain specific precursor supplementation approaches were investigated to achieve an increase of GSH (Alfafara, C. G. et al., 1992; Liang, G. et al., 2008; Wang, Z. et al., 2007; Wen, S. et al., 2006; Wen, S. H. et al., 2005). Thereby, Alfafara and coworkers analyzed the effect of different concentrations of the precursors glutamate, glycine and cysteine its consequences to the specific growth rate μ. In accordance to Alfafara, C. G. et al. (1992), Wang, M. et al. (2012) and Wang, Y. et al. (2012) pointed out, that cysteine is the key amino acid for GSH production.

One process strategy that can influence the yield of GSH/cysteine is a low oxygen saturation prior to the amino acid addition to reduce the oxidation of cysteine to L-cystine (Liang, G.-B. et al., 2008). The reduction of the temperature and/or the pH-value after cysteine addition are other possibilities to push the yield towards GSH (Wei, G. et al., 2003; Wei, G. Y. et al., 2008).

Although there is extensive scientific discourse in the field of GSH overproduction, neither the yield of GSH/cysteine was considered in the past nor corresponding investigations due to internal industrial circumstances and restrictions. Here we present a suitable feeding strategy via RQ-control to ensure commercially relevant high biomass concentration with the GSH accumulating strain *S. cerevisiae* Sa-07346 using a 5-l bioreactor. Based on this HCD process we optimized the supplementation time, concentration and type of addition of cysteine for GSH induction, leading to an efficient addition strategy with both increase of the GSH/cysteine yield and high biomass and GSH production. The fermentation process was successfully transferred into a pilot plant scale to mimic industrial process conditions. Based on the knowledge, that was obtained during this work, a so-called repeated fed-batch process was demonstrated showing an outlook for further process optimization from an economic point of view.

5.3 Material and methods

5.3.1 Media

For pre-cultures chemically defined WMIX, a modified version of WMVIII (Lang & Looman, 1995), was used. Chemicals were purchased from Sigma-Aldrich, Inc. The composition was as follows (g/l): salt solution (sterilized by autoclaving): NH_4Cl 2.8, myo-Inositol 0.075, $MgCl_2 \cdot 6H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.55. Moreover 4 ml/l vitamin solution and 1 ml/l trace element solution were aseptically added (g/l): vitamin solution (sterilized by microfiltration): biotin 0.625, pantothenic acid calcium salt 12.50, nicotinic acid 2.50, pyridoxine hydrochloride 7.60, thiamine hydrochloride 2.50; trace element solution in 0.01 M EDTA (sterilized by microfiltration): $CuSO_4 \cdot 5H_2O$ 0.10, $FeSO_4 \cdot 7H_2O$ 0.50, $MnCl_2 \cdot 4H_2O$ 0.50, $NaMoO_4 \cdot 2H_2O$ 0.10, $ZnSO_4 \cdot 7H_2O$ 0.175. Final concentrations of glucose and glutamic acid monosodium salt monohydrate were (g/l) 50 and 10, respectively. Medium pH was adjusted with 40 ml/l 0.5 M potassium phosphate buffer (pH 6.8) (K_2HPO_4, KH_2PO_4).

This medium was also used as batch-medium but containing 20 g/l glucose and additionally 11 g/l yeast extract (Leiber GmbH, Bramsche, Germany).

For the feed medium, salt, vitamin and trace element concentrations were 6-fold increased to ensure high biomass. Potassium phosphate buffer content was doubled, glucose and yeast extract concentration were adjusted to 400 g/l and 15 g/l, respectively. Glutamic acid monosodium salt was left out.

5.3.2 Strains and pre-cultures

S. cerevisiae Sa-07346 was obtained from Organobalance GmbH in Berlin, Germany. For the preparation of the first pre-culture 0.1 ml of a cryo-stock was transferred into a shaking flask and incubated in WMIX for 24 h at 30 °C and 180 rpm. The second pre-culture was seeded with an optical density (OD_{600nm}) of 0.1 and was incubated under the same conditions.

5.3.3 Fed-batch fermentations

Fed-batch fermentations were carried out in 5 l bioreactors (Biostat®Aplus, Sartorius AG, Göttingen, Germany). The applied feeding scheme for fed-batch cultivations was developed by using a RQ (respiration quotient) based closed loop feedback control. In accordance to Alfafara, C. G. et al. (1992), Shang et al. (2006)and Tippmann et al. (2016), the RQ-value was set to 1 ± 0.1 to prevent the formation of by-products like glycerol, ethanol or acetate known as Crabtree effect and to guarantee high biomass. Consumed feed was monitored and recorded by scale. Feeding data from three independent RQ-based fermentations were fitted with the help of a logistic function conducted by the software OriginLab 8.0. The obtained feeding strategy ensured high-cell-density cultivation and was used in all present fed-batch cultivations independent of and without off-gas analyzer. The feeding was started at glucose depletion (corresponding to OD_{600nm} 15-20). During the 48 h of fed-batch cultivation, pH was regulated to 5.5 ± 0.1 by 25 % H_3PO_4 and 20 % ammonia, respectively, temperature was set to 30 ± 1 °C and DO was set to 30 ± 5 %, firstly regulated via stirrer speed and secondly, in case of additional need, via supply of pure oxygen.

5.3.4 Repeated fed-batch fermentation

Based on the fed-batch fermentations, batch-phase was conducted till OD_{600nm} 15-20 and then feeding was started. At 30 h of fermentation, a cysteine feed was initiated, obtaining a final concentration of 8.7 mmol/l cysteine after a time of 6 h. Attaining 38 h of cultivation, fermentation broth was reduced to 50 ml and a new batch-phase was started. This procedure was repeated twice.

5.3.5 Pilot plant fermentation

For the scale up of the best performing fed-batch process, this run was performed in a pilot plant (150 l-reactor, Bioengineering AG, Wald, Switzerland). 40 l of batch medium were inoculated

with 4 l of pre-culture and after the batch-phase, 40 l of feed medium were fed. After 32 h, the continuous cysteine feed was applied, obtaining a final concentration of 8.7 mmol/l cysteine after a duration of 6 h.

5.3.6 Biomass determination

5 ml cell suspension was centrifuged at 4000 × g and washed with 0.9 % NaCl solution. The supernatant was taken for additionally HPLC analysis. Cell pellet was dried for 24 h at 100 °C and cooled in a desiccator. The biomass was periodically calculated.

5.3.7 Determination of low molecular sugars

Sugar analysis was realized by HPLC (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) applying a NUCLEOGEL® ION 300 OA column (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The samples were eluted with 0.005 mol/l H_2SO_4 at a flow rate of 0.4 ml/min.

5.3.8 Glutathione determination

HPLC comprises high sensitivity but due to the long duration of sample preparation and elution, there is a risk of oxidation of GSH to GSSG (Rahman, 2006). Therefore, determination of GSH was based on the colorimetric method via Ellman's reagent (5,5'-dithio-(bis-2-nitrobenzoic) acid, DTNB, Sigma-Aldrich) based on Tietze (1969). In contrast to Tietze (1969), glutathione-disulfide reductase was not used in this assay. That is why only the reduced form (GSH), which, as stated above, shows 30-100 times higher concentrations than GSSG, was detected. Yeast cells were washed with 0.9 % NaCl solution, suspended in 0.1 M H_3PO_4 and incubated at 80 °C for 5 min in a water bath. After centrifugation at 4000 × g for 10 min, the supernatant was taken for the GSH assay. 100 µl of the sample was pipetted into a 96-well-plate and overlayed with 100 µl 0.5 M sodium phosphate buffer, pH 8. Afterwards 5 µl DTNB (4 g/l stock solution) were added and incubated for 10 min. Measurements in triplicates were conducted with a 96-well-plate reader (Molecular Devices GmbH, Biberach, Germany) at 412 nm. Additionally, a GSH standard curve was recorded ranging from concentrations of 12.5 to 100 mg/l reduced GSH. The intracellular GSH (g/l) content was calculated by using a standard curve (linear regression) and related to the cell dry weight concentration as follows:

$$\text{intracellular reduced GSH } (\%,\text{w/w}) = \frac{\text{GSH (g/l)}}{\text{CDW (g/l)}} \cdot 100\%$$

5.4 Results

5.4.1 Determination of an optimal feeding profile

A high cell density process is required for an efficient GSH production via *S. cerevisiae*. This was realized by fed-batch strategy. Avoiding accompanied adverse effects of a non-optimal

feeding, e. g. the generation of undesired by-products and Crabtree effect, a feeding process was developed via off-gas analyzer, explained in detail in section 5.3.3. The resulting feeding profile gave a biomass of 96.1 g/l as shown in the control run (Table 5-1)

5.4.2 Investigation of the optimal time of cysteine spike

Secondly, the induction of the GSH overproduction is essential. Using cysteine as inducer, the optimal cysteine supplementation time (24, 28, 32 h; cysteine concentration: 0.17 ± 0.1 mmol/g cell) in the fed-batch fermentation process was investigated (Table 5-1). With regard to GSH production, the addition at 28 h led to 15 % higher volumetric concentration (1204 mg/l) compared to early addition (24 h, 1048 mg/l). However, the averaged intracellular GSH content of 1.61 ± 0.06 % as well as the averaged specific productivity of 5.4 ± 0.5 mg/g·h revealed that the impact of the different applied spiking times were almost equal. This stable maximal GSH level might be strain specific and was further investigated (Table 5-1). Moreover, it could be shown that adding cysteine in the late phase of cultivation (32 h) had no effect on growth while early addition (24 h) resulted in an inhibitory effect on biomass generation (Table 5-1).

5.4.3 Influence of the cysteine concentration on GSH production

In order to exclude strain specific dependencies, different concentrations of cysteine (0.04 - 0.17 mmol/g cell) were added at 24 h to determine growth inhibition caused by cysteine and the optimal cysteine incorporation into GSH (Table 5-1). A comparison of the biomasses showed a moderate increase (13 %) at low cysteine concentrations combined with a distinct increase of cysteine incorporation into GSH up to 0.40 mol/mol. Interestingly, no biologically significant increase in biomass and GSH concentration for cysteine addition of 15.7 mmol and 11.9 mmol was observed, indicating that higher cysteine spiking concentrations are inefficient and finally uneconomic. The results exhibit that low amounts of cysteine led to improved cysteine incorporation yield (see Table 5-1) and confirmed the obtained data from section 5.4.2 with regards to biomass generation.

5.4.4 Application of multiple cysteine spikes

To avoid negative effects due to the concentration-dependent cysteine toxicity on biomass generation and to increase the incorporation yield, its addition was divided into four small steps (2.9 mmol/l cysteine each). These cysteine addition steps were performed every 4 h beginning at a cultivation time of 24 h (Figure 5-1, black arrows). This gradual addition resulted in a GSH concentration up to 95 0 ± 12 mg/l. A recurring increase of the instant specific GSH productivity initiated by each of the spikes could be observed. These increases were all approximately at the same level of 2.0 ± 0.3 mg/g·h and obtained during the first 2 h after each of the spikes. Interestingly, the results are in accordance with Mast-Gerlach (1994). Nevertheless, the last step of cysteine addition at 36 h gave no rise of GSH, indicating the ineffectiveness of this step.

Table 5-1 Overview of fed-batch fermentations with defined cysteine spiking times and concentrations as well as control run without cysteine addition.

Run / Parameter	Control	Trials on spiking time			Trials on L-cysteine concentration		
Strategy	n/a	Spike 24 h	Spike 28 h	Spike 32 h	Spike 24 h	Spike 24 h	Spike 24 h
Max. CDW (g/l)	96.1	67.8	79.3	82.4	63.3	68.7	71.6
Max. GSH (mg/l)	394.3	1048.0	1204.1	1158.7	992.4	781.5	570.6
Max. GSH (%, w/w)	0.42	1.67	1.61	1.54	1.61	1.14	0.80
Max. specific GSH productivity (mg/g·h)	0.3	5.9	5.0	5.1	4.2	3.4	2.0
$Y_{X/S}$ (g/g)	0.48	0.42	0.43	0.40	0.41	0.46	0.47
Max. volumetric GSH productivity (mg/l·h)	13.5	37.8	37.6	32.2	35.4	27.9	20.4
Sum of used L-cysteine in the process (mmol)*	n/a	15.7	18.1	20.5	11.9	7.9	4.0
$Y_{GSH/cysteine}$ (mol/mol)**	n/a	0.30	0.31	0.27	0.33	0.36	0.40

*Amount of cysteine was dependent on biomass as explained in the text

** $Y_{GSH/cysteine}$ was calculated by dividing the maximum GSH concentration by used cysteine concentration

By negligence of the fourth spiking point an increase of the incorporation yield of about 0.36 ± 0.01 mol/mol could be achieved. However, the biomass (CDW: 73.8 ± 1.4 g/l) and the volumetric GSH concentration were not in a satisfying range (see Table 5-2).

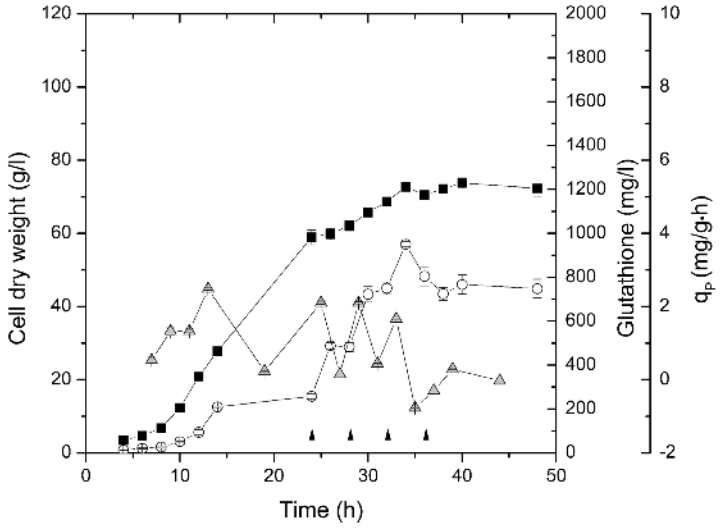

Figure 5-1 **Cell dry weight (■), instantaneous specific GSH productivity (▲) and intracellular GSH concentration (○) during the fed-batch fermentation applying repeated cysteine spikes. The arrows indicate the points in time of supplementation of 4.0 mmol/L cysteine. Data are mean values ± SD from triplicate fermentations.**

5.4.5 Continuous cysteine feeding

Several scientific experts promote the single shot or two shot strategy with positive results regarding biomass and GSH concentration often indicating a suboptimal cysteine incorporation yield (see Table 5-3). Therefore, in our work, a change in the addition strategy from shot wise to continuous cysteine addition was conducted. In fact the same amount of cysteine compared to the shot-wise approach was fed over a period of 12 h, beginning at 24 h of cultivation time. To prevent an oxidation to the non-functional dipeptide L-cystine, the cysteine feeding solution was protonated by acidification via 0.01 M H_3PO_4. The results of these modifications are shown in Figure 5-2. The comparison of the continuous strategy (CDW: 95.2 ± 1.6 g/l) with the previously described four step strategy (CDW: 73.8 ± 1.4 g/l) revealed a 29 % higher biomass at 48 h combined with a successful increase in the volumetric GSH concentration of 35 % to 1281 ± 42 mg/l. Through the continuous smooth addition of the GSH production boosting substance cysteine, the whole production process was improved and the incorporation yield enhanced to 0.41 mol/mol (Table 5-2).

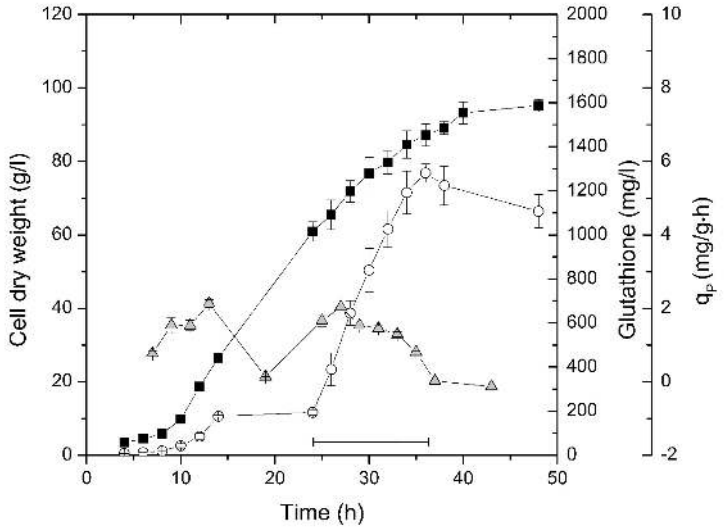

Figure 5-2 **Cell dry weight (■), instantaneous specific GSH productivity (▲) and intracellular GSH concentration (○) during the fed-batch fermentation applying a continuous L cysteine feed for 12 h. The bar indicates the time of continuous cysteine feeding (24 h – 36 h). Data are mean values ± SD from triplicate fermentations.**

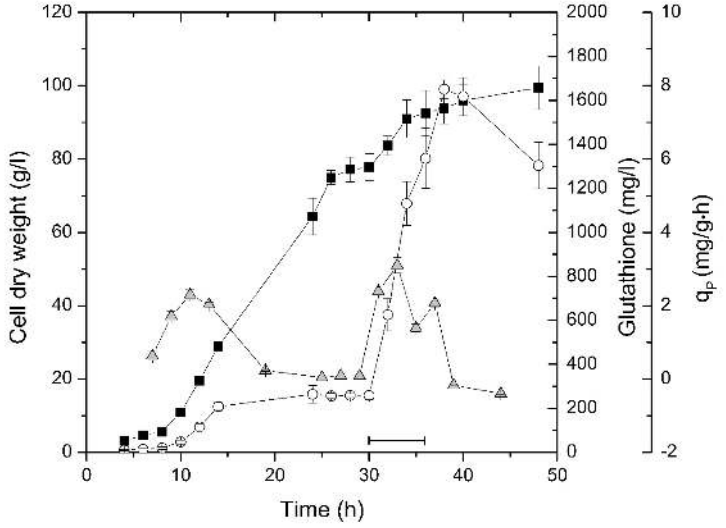

Figure 5-3 **Cell dry weight (■), instantaneous specific GSH productivity (▲) and intracellular GSH concentration (○) during the fed-batch fermentation of *S. cerevisiae* applying a continuous cysteine feed for 6 h. The bar indicates the time of continuous cysteine feeding (30 h - 36 h). Data are mean values ± SD from triplicate fermentations.**

For the next step in process development, a delayed addition of cysteine was combined with an accelerated continuous cysteine addition. This was to achieve high biomasses and an improved

volumetric GSH concentration as well as incorporation yield. The cysteine feeding solution was protonated by acidification again. In Figure 5-3 the growth behavior and the final biomass concentration of 99.5 ± 5.9 g/l after 48 h are visualized. This growth was not affected by any of the conducted changes. In contrast to biomass, the maximal volumetric GSH concentration could be increased significantly to 1650.7 ± 43 mg/l or in comparison to previous runs to 129 %. The intracellular GSH content was further improved to 1.76 ± 0.08 % (see Table 5-2). Caused by the mentioned strategy, the incorporation yield was doubled and maximized to 0.54 mol/mol comparable with the initial developmental runs. Besides, for all fermentations with applied continuous cysteine feeding, no hydrogen sulfide scent was detected. This could be a further indication for an efficient utilization and incorporation of cysteine.

5.4.6 Fermentation in pilot plant

Mimicking production conditions, a scale-up to 150 l pilot plant bioreactor was done. Progresses of main parameters including CDW, volumetric GSH concentration and specific GSH productivity are presented in Figure 5-4. Both the biomass and GSH developed satisfyingly and were in the expected range. Reaching a maximum of biomass concentration of 86 g/l, this was only 13 % less than the mean values of the best performing runs. The second highest value of volumetric GSH concentration of 1340 mg/l was achieved in this fermentation and resulted in an intracellular GSH content of 1.55 %. The incorporation yield of 0.41 mol/mol was slightly lower than in the best performing lab-scale runs (0.54 mol/mol).

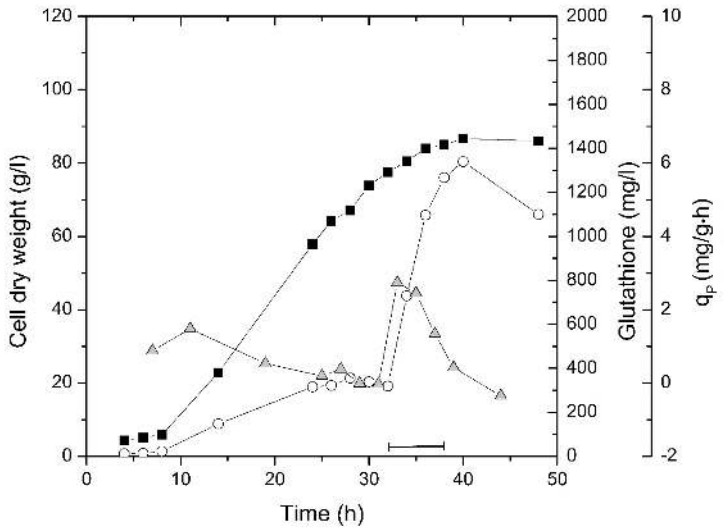

Figure 5-4 **Cell dry weight (■), instantaneous specific GSH productivity (▲) and intracellular GSH concentration (○) during the pilot scale fed-batch fermentation of *S. cerevisiae* Sa-07346. Cysteine feeding was applied for 6 h after 32 h of fermentation time (bar).**

Table 5-2 **The effect of stepwise spiking in contrast to continuous cysteine addition as well as the final approach in pilot scale (150 l) is shown. The repeated fed-batch represents an economically relevant alternative to usually used fed-batch formats.**

Run / Parameter	Stepwise spiking	Continuous		Pilot plant	Repeated fed-batch
	24, 28, 32, 36 h	24 h	30 h	32 h	30 h each
Strategy	Spike	Feeding for 12 h	Feeding for 6 h	Feeding for 6 h	Feeding for 6 h each
Max. CDW (g/l)	73.8 ± 1.4	95.2 ± 1.6	99.5 ± 5.9	86.7	84.2 ± 1.2
Max. GSH (mg/l)	950.0 ± 12.0	1281.1 ± 41.6	1650.7 ± 42.8	1340.0	1304.7 ± 61.4
Max. GSH (%, w/w)	1.31 ± 0.02	1.47 ± 0.05	1.76 ± 0.08	1.55	1.65 ± 0.09
Max. specific GSH productivity (mg/g·h)	2.14 ± 0.08	1.70 ± 0.13	2.76 ± 0.23	2.42	3.21 ± 0.29
$Y_{X/S}$ (g/g)	0.36 ± 0.03	0.48 ± 0.01	0.50 ± 0.03	0.43	0.40 ± 0.02
Max. volumetric GSH productivity (mg/l·h)	27.9 ± 0.04	35.1 ± 2.9	43.4 ± 1.1	33.5	36.0 ± 1.8
Sum of used L-cysteine in the process (mmol)*	17.9	17.9	17.9	716.0	17.9
$Y_{GSH/cysteine}$ (mol/mol)**	0.36 ± 0.01	0.41 ± 0.01	0.54 ± 0.01	0.41	0.39 ± 0.01

*Amount of cysteine was dependent on biomass as explained in the text

** $Y_{GSH/cysteine}$ was calculated by dividing the maximum GSH concentration by used cysteine concentration

The slightly lower GSH concentration could be due to the fact, that the percentage sampling volume was much higher in the development runs compared to pilot plant bioreactor. This means there was a decreased cysteine feeding rate in the pilot plant with regards to the fermentation volume, because the cysteine feed was more diluted than in lab-scale runs. Reduced biomass combined with a lower feeding rate might be the influencing factors that should be considered at further fermentation processes.

5.4.7 Repeated fed-batch fermentation

To improve the economic efficiency, we examined an alternative process strategy in form of a repeated fed-batch. As shown in Figure 5-5, this fermentation strategy resulted in a maximal biomass concentration of 84.2 ± 1.2 g/l for each phase. Maximal volumetric GSH concentration and intracellular GSH content were 1304.7 ± 61.4 mg/l and 1.65 ± 0.09 %, respectively. These data clarify the advantages of a continuous cysteine addition to prevent inhibitory effects triggered by high cysteine concentrations. In contrast to previous described fed-batches, at least 10 h of overall fermentation and handling time could be saved, thus providing an economically relevant alternative. Assuming a further optimization of this strategy for industrial production plants, time and money could be saved.

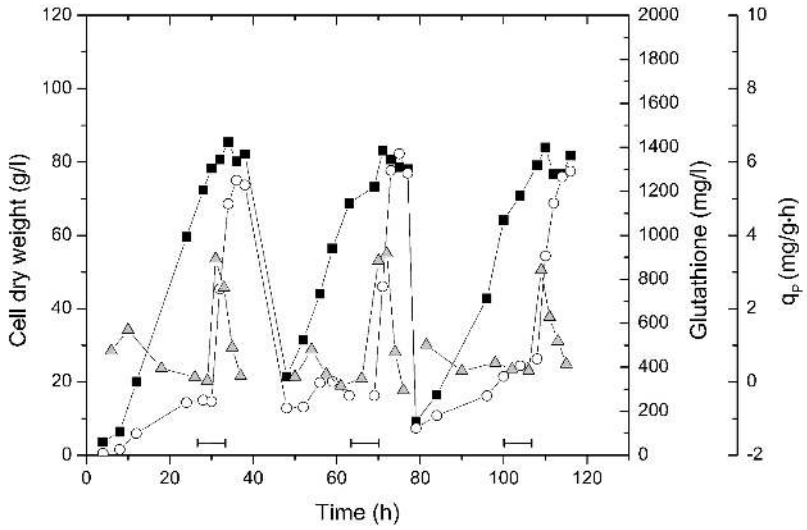

Figure 5-5 **Cell dry weight (■), instantaneous specific GSH productivity (▲) and intracellular GSH concentration (○) during the repeated fed-batch of *S. cerevisiae* Sa-07346. Fermentation broth was reduced to 50 ml after 38 h and 77 h, respectively. In each case a new feeding was started after 6 h. The bars indicate the start of cysteine feeding for 6 h after 30 h of each phase.**

Table 5-3 Compilation of different GSH production processes in yeast.

Process	Strain	Max. GSH conc. (mg/l)	Max. biomass conc. (g/l)	Added cysteine (mmol/l)	Yield: GSH/cysteine (mol/mol)	Reference
Batch	*S. cerevisiae*	271.0	17.1	34.9	0.014 **	Taskin (2013)
Batch	*C. utilis CCTCC M 209298*	669.3	15.5	6.0	0.201 **	Wang, Y. et al. (2012)
Fed-Batch	*S. cerevisiae* 26-2	2250.0	105.0	6.0	0.651 *	Wang, M. et al. (2012)
Fed-Batch	*C. utilis WSH02-08*	2448.0	112.3	36.0	0.133 **	Wang, B. et al. (2010)
Fed-Batch	*S. cerevisiae* LYCC7048	-	-	-	0.400 ***	Nisamedtinov et al. (2010)
Fed-Batch	*S. cerevisiae* T65	2100.0	126.0	5.0	0.391 **	Xiong et al. (2010)
Fed-Batch	*S. cerevisiae* GE-2	2280.0	110.0	10.0	0.462 *	Shang et al. (2008)
Fed-Batch	*C. utilis WSH02-08*	1312.5	83.5	20.0	0.088 **	Wei, G. Y. et al. (2008)
Fed-Batch	*S. cerevisiae* G-14	2020.0	132.0	4.0	0.325 **	Wang, Z. et al. (2007)
Fed-Batch	*S. cerevisiae* T65	2190.0	133.3	12.1	0.125 **	Wen, S. et al. (2006)
Batch	*S. cerevisiae* T65	329.3	19.8	5.4	0.097 **	Wen, S. H. et al. (2005)

* GSH concentration before and after cysteine addition; ** Calculation based on control fermentation; *** Total yield

5.5 Discussion

In the past scientists intensively focused on improving the intracellular or volumetric GSH concentration by genetic engineering, media development or bioprocessing methods as summarized by Li et al. (2004). In contrast, the incorporation yield from cysteine into GSH is rarely considered. However, for an economical production process it is important that the biotransformation of GSH from cysteine is efficient. For a better estimation and comparison of published studies, different GSH production processes and cysteine incorporation yields are listed in Table 5-3.

In this work different procedural approaches for an efficient GSH production via *S. cerevisiae* Sa-07346 were investigated. Based on the little acceptance of genetic modified organisms (GMOs), we used a non-modified food grade strain. An alternative would be so-called evolutionary engineered strains that show high potential in GSH production (Patzschke et al., 2015). As a requirement for high biomass production in the fed-batch fermentation process, an adequate feeding profile was developed by means of off-gas analyzer to control the feeding via RQ.

Further investigations revealed an optimal point in time for cysteine supplementation. Our results suggest that cysteine addition should be carried out in the stationary phase of fermentation. This is in accordance with Wang, B. et al. (2010) and Wei, G. Y. et al. (2008), who spiked cysteine in the stationary phase to prevent the inhibitory effects of cysteine on final biomass. Nevertheless, both intracellular GSH content and incorporation yield were approximately at the same level and independent of the time of cysteine addition. To enhance the precursor usage and thus increase the economy of the process, cysteine addition was gradually reduced. It was shown that comparable amounts of GSH could be achieved by a reduction of the cysteine concentration of about 25 % (from 15.7 to 11.9 mmol/l). This procedure is generally suitable to estimate the potential of a production strain in the initial phase of process development. Another effect of stepwise reduction was the increase in the incorporation yield leading to values of 0.40 mol GSH per mol cysteine for the addition of the smallest cysteine concentration.

This proceeding was verified and improved by means of low concentrated multiple cysteine additions. Wen, S. et al. (2006) used four cysteine shots to overproduce GSH. In our procedures with the four-shots-of-cysteine addition strategy, no improvement of the process could be observed. In consequence, we changed the proceeding of cysteine addition from multiple single shots to continuous cysteine feeding. This strategy is contradicting to the studies of most scientists, who promote the single shot method (see compilation in Table 5-3). However, the focus of these investigations was usually on maximizing GSH concentration, whereby the efficiency of the biotransformation and the profitability were mostly neglected.

Nisamedtinov et al. (2010) investigated the GSH and cysteine biosynthesis and the expression of related genes in ethanol-controlled fed-batches. These approaches were realized by different cysteine-enriched feeding media. They pointed out that high concentrations of cysteine led to lower incorporation yields (Table 5-3). These findings are in accordance with our results of continuous cysteine addition, which showed that growth and incorporation yield was retained at high level, when cysteine was added continuously. In fact, a lower concentration of cysteine in the fermentation broth was guaranteed that way. Finally the combination of a delayed and accelerated continuous cysteine addition resulted in the highest intracellular and volumetric GSH concentration. The incorporation yield was almost doubled by this procedure compared to single shot method to 0.54 mol/mol, too. In order to enhance the industrial relevance of the illustrated results, a transfer from lab-scale into 150 l pilot plant bioreactor was successfully performed. Despite slightly lower values for biomass and GSH concentration, the feasibility of this strategy has been proven.

The repeated fed-batch process demonstrated an alternative cost effective prospect of GSH production. This process strategy reduces the bioreactor set-up and –down time including CIP/SIP as well as seed-train processes and enables to save up to 10 % of variable costs (Hermann, 2003). Repeated fed-batch processes were especially applied to produce low-cost products e. g. amino acids (Hermann, 2003), xylitol (Bae et al., 2004), ethanol (Lu et al., 2003) or lipids (Qu et al., 2013). Even though the maximum number of repetitions of our process was not investigated in this work, the used strain was stable and other studies show promising potential. Yang and Tsao (1995) developed an acetone–butanol production process using *Clostridium acetobutylicum* and applying nine cycles and Xue et al. (2010) achieved a five times repeated fed-batch using *Klebsiella pneumoniae* for 1,3-propanediol production. Basic requirement for GSH production via repeated fed-batch is to keep the cells repitchable for as many periods as possible. The implementation of the described proceedings ensured good growth performances and high GSH concentrations and emphasized the advantage of the continuous cysteine addition. To sum up, the moderate stress levels caused by low cysteine concentrations during smooth continuous cysteine feeding are very suitable for this process, whereas long regeneration times should be avoided.

This assumption is supported by results from Kumar et al. (2006) and Trotter and Grant (2002). Investigations of reductive stress showed an increase in both KAR2 gene expression, a marker for stress in the endoplasmic reticulum (ER) (Kumar et al., 2006; Normington et al., 1989) and unfolded protein response (UPR). Cellular stress is triggered by high toxic cysteine concentrations resulting in growth inhibition. Enhanced hydrogen sulfide production was also observed under high cysteine conditions (Nisamedtinov et al., 2010). However, there are two major possibilities to produce hydrogen sulfide, firstly via sulfur assimilation pathway and secondly by catabolism of cysteine (Stipanuk, 2004; Thomas & Surdin-Kerjan, 1997). Cysteine is used as the preferred sulfur source compared to inorganic sulfate. Under enriched cysteine concentrations, the MET-regulon, which is responsible for synthesis of the sulfur-containing

amino acids methionine and cysteine, is repressed (Sadhu et al., 2014). Taking account of the repression of the Met4-transcription factor via increased cytosolic cysteine concentration, the yeast could attempt to incorporate the excess of cysteine into the non-toxic thiol-compound GSH. However, the capacity of GSH as sulfur storage is limited (Menant et al., 2006; Winter et al., 2014). Therefore the catabolism of cysteine takes place and hydrogen sulfide is produced. As mentioned above, this scenario can be avoided by our developed continuous cysteine addition strategy. Nevertheless, a low level of hydrogen sulfide explains only conditionally the overall higher values of GSH concentration and cysteine incorporation yields with regard to competitive feedback inhibition of γ-glutamylcysteine synthetase encoded by GSH1.

Recent studies revealed that both extremes, oxidative and reductive stress, result in generation of reactive oxygen species (ROS) (Cortassa et al., 2014; Korge et al., 2015). As already known, ROS in turn induce a stress response triggered by stress-responsive transcription factors (Ayer et al., 2014). We assume there is an unknown cross-link between sulfur assimilation pathway and ROS generation caused by reductive stress, initiated under non-physiological cysteine concentrations. With regard to our results and findings from published literature we assume a complex and so far undiscovered regulation of GSH biosynthesis, sulfur amino acid biosynthesis as well as sulfur assimilation pathways, especially under conditions of sudden excess and smooth continuous addition of cysteine.

5.6 Conclusion

After more than 30 years, the industrial interest in the production of GSH, as a pure substance or accumulated in yeast, is still prominent. A lot of research was done to provide efficient production procedures. Since cysteine is significantly cheaper than GSH the enhancement of GSH/cysteine incorporation yield is of high economic interest. During various development stages in our work, a continuous improvement concerning biomass, GSH concentration as well as incorporation yield was achieved, resulting in a highly efficient fed-batch process for GSH production with the yeast *Saccharomyces cerevisiae* Sa-07346. Moreover, the feasibility of this approach in pilot scale was shown. Furthermore, as an economically relevant alternative for the production of low cost products, a repeated fed-batch procedure was also successfully established. The stated results in combination with latest scientific literature revealed that mechanisms behind sulfur assimilation and the regulation of GSH accumulation in the yeast, especially under production conditions, are still partially uncovered.

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5.8 Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jbiotec.2015.10. 016.

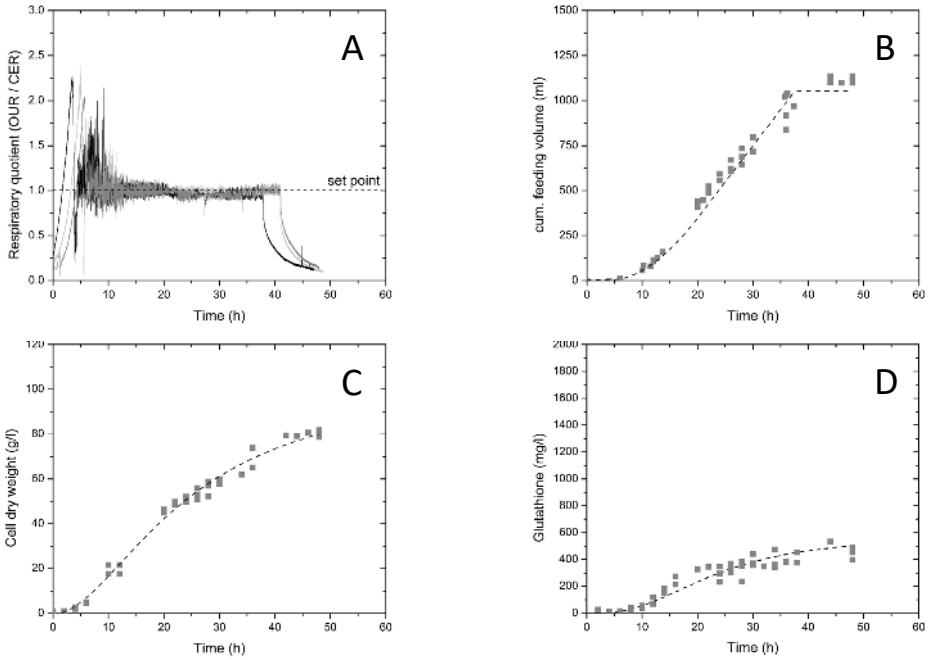

Figure 5-6 **The developed RQ-feeding profile was experimentally confirmed in three independent fed-batch fermentations with *S. cerevisiae* Sa-07346. A: Actual progress of RQ during the fermentation with a set-point of RQ=1±0.1; B: Cumulative volume of the feed solution (the dashed line shows the set-values of the feeding profile); C: Cell dry weight behavior during the fed-batches (the dashed line shows the mean of n=3); D: Volumetric glutathione concentration during the fed-batches (the dashed line shows the mean of n=3).**

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6 Diskussion & Ausblick

In den vorangegangenen 5 Kapiteln wurde umfangreich auf unterschiedlichste Ansätze zur Optimierung der GSH-Produktion eingegangen und erzielte Ergebnisse gänzlich diskutiert. In diesem letzten Kapitel werden die erzielten Ergebnisse und Erkenntnisse im Kontext einer wirtschaftlichen Herstellung betrachtet. Es werden die Hauptkostentreiber einer Hefeproduktion diskutiert, Einblicke in die Aufarbeitung der GSH-Hefen und deren spätere Produkte bzw. Applikationen gegeben sowie die größten GSH-Hersteller aufgeführt.

In marktwirtschaftlichen Analysen werden als Produzenten meist Kyowa Hakko Bio Co. Ltd., Kohjin Life Sciences Shenzhen GSH Bio-tech, Kaiping Genuine Biochemical Pharmaceutical und Shandong Jincheng Biological Pharmaceutical genannt. Heutzutage werden mehr als 200 Tonnen an kristallinen GSH hergestellt (Orumets et al., 2012). Der Preis von reinem GSH in entsprechender Qualität liegt bei ca. 200-400 USD/kg (www.alibaba.com, Suchbegriff: USP/EP Glutathione, abgerufen 07/2019) bzw. für Hefeextrakte mit hohem GSH-Gehalt bei ca. 150 USD/kg (Orumets, 2012). Traditionell wird GSH in *Saccharomyces* oder *Candida* produziert. Verfügbar sind auch andere Organismen wie gentechnisch veränderte und optimierte *E.coli* Stämme. Jedoch wird der größte Anteil an GSH mit den o.g. Hefengattungen hergestellt. Marktdaten zu GSH zeigen, dass 2017 der globale Umsatz mehr als 0,8 Milliarden USD betrug und zukünftig bei einem prognostizierten jährlichen Wachstum von 12%, also 2027 bei geschätzt 2,5 Milliarden USD liegen wird (Research-Nester, 2018). Ein großer Anteil am Wachstum ist auf den steigenden Bedarf im Kosmetiksektor zurückzuführen. Andere Abnehmer sind, wie bereits in Kapitel 2 erwähnt, der Pharmazie-, Lebensmittel- und Gesundheitssektor. Der wachsende Bedarf geht einher mit der Herausforderung wie zunehmend steigenden Rohstoffkosten. Diese zählen neben den Prozesswasser-, Energie-, und den Personalkosten zu den Hauptkostentreibern. Für Forschungszwecke sind CD-Medien wie in Kapitel 3 beschrieben vorteilhaft, weil diese nur geringen Qualitätsschwankungen unterworfen sind und zu reproduzierenden Ergebnissen führen. Die Entwicklung solcher Medien nimmt jedoch viel Zeit und Ressourcen in Anspruch. Aus wirtschaftlichen Gründen werden daher zur Hefeproduktion oft Komplexbestandteile verwendet. Die meist verwendete Kohlenstoffquelle ist hierbei die Melasse. Um 1925 herum hat sich Melasse als Standard-Rohmaterial zur Herstellung von Backhefe etabliert (Gélinas, 2009). Die Substanz/Sie ist ein Nebenprodukt, das während der Zuckerherstellung im letzten Kristallisationsschritt und nach der Zentrifugation anfällt. Dabei wird unterschieden in Zuckerrohr- und Zuckerrübenmelasse. Diese enthält nicht nur durchschnittlich 53% bzw. 62% fermentierbare Zucker, sondern auch Spurenelemente und Vitamine (Olbrich, republiziert 2006). Nichtsdestotrotz müssen aus Melasse als Fermentationsgrundlage Salze und Spurenelemente wie Sulfat, Phosphat, Zink und Magnesium sowie Wuchsstoffe wie Vitamine supplementiert werden (Bronn, 1986), um adäquate Biomassen zu erreichen. Da der assimilierbare Stickstoff (ca. 2%) in der Melasse ebenfalls nicht ausreicht, wird in der Praxis zudem Ammonium addiert. Dies wird über die Zugabe von

Ammoniakwasser, welches gleichzeitig als pH-Korrekturmittel dient, realisiert (Walker & Smith, 1999).

Es wird geschätzt, dass 2018/2019 die globale Produktion, also Zuckerrüben- und Zuckerrohrmelasse zusammen, bei ca. 65 Millionen Tonnen liegen wird (www.suedzucker.de, 2018/2019 Annual Report, abgerufen 07/2019). Der Preis für Zuckerrohrmelasse bewegte sich von 01-07/2019 an der „Getreide und Futtermittelbörse“ in Bremen zwischen 141-162 USD/t (www.proplanta.de, Suchbegriff: Zuckerrohrmelasse, abgerufen 07/2019). Die Kosten für Melasse machen, bei einer industriellen Backhefeproduktion, ca. 80% des Rohstoffpreises aus. Die Preise für Weißzucker bzw. Dextrose, welche alternativ als Kohlenstoffquellen verwendet werden können, unterliegen starken Schwankungen. Der Preis für Weißzucker in kristalliner Form z.B. variierte zwischen 2017-2019 innerhalb der EU von 358-610 USD/t (www.suedzucker.de, 2018/2019 Annual Report, abgerufen 07/2019). Für eine Tonne Glucose werden laut Milling & Baking News zwischen 870-948 USD/t aufgerufen (Milling&BakingNews, 2018). Zu Fermentationszwecken werden meist Sirupe der o.g. Zucker verwendet, welche etwas geringer im Preis ausfallen. Beleg fehlt hier? Für Maisquellwasser und Hefeextrakt fallen Kosten in Höhe von ca. 200 USD/t bzw. bei hoher Nachfrage bis zu 1000 USD/t an (Tan et al., 2016).

Die Induktion der GSH-Synthese erfolgt in der industriellen Praxis durch die Zugabe der Präkusoren L-Glutamat, Glycin und L-Cystein, wobei sich die Preise der Aminosäuren stark unterscheiden. Mit einem Marktvolumen von mehr als 15000 Tonnen und einem Umsatz von 570 Millionen USD jährlich (Keeper, 2019) ist Cystein der kostenintensivste Präkusor. In Kapitel 4 wurden vielversprechende Derivate von Cystein untersucht. Es konnte gezeigt werden, dass durch Verwendung von Cystein-Ethyl-Ester (CEE) der intrazelluläre GSH-Gehalt im Vergleich zu Cystein signifikant erhöht werden kann. Jedoch ist CEE ca. 2-3 Mal teurer als Cystein. Ein Einsatz wäre nur dann wirtschaftlich, wenn das Produkt hochpreisiger wäre. Dies wäre bspw. bei der Hefe *Saccharomyces boulardii der Fall,* welche als probiotisch eingestuft wird und bei gastrointestinalen Störungen oder während Antibiotikakuren zum Schutz der Darmflora verabreicht wird (Bekatorou et al., 2006). Hier wäre es vorstellbar, dass der erhöhte GSH-Gehalt der Hefen den zellulären Stress im Darm reduziert und so zu einem verbesserten Heilungsverlauf führt (Musatti et al., 2014).

Schlussendlich bewegt sich der Preis für das Medium unter Berücksichtigung der meisten Substanzen inkl. der o.g. Aminosäuren zwischen 200-250 USD/t. Es konnte jedoch gezeigt werden, dass auch ohne Präkusoren, aber mit einem speziellen Hefestamm und einer entsprechenden Prozessführung, GSH in ausreichender Konzentration im industriell relevanten Maßstab (120 m^3) produziert werden kann (Sakato & Tanaka, 1992). Ein ebenfalls vielversprechender Hefestamm, der ohne Induktor hohe GSH-Konzentrationen von bis zu 5,9% intrazellulär akkumulieren kann, wurde von Patzschke et al. (2015) in Zusammenarbeit mit Orangobalance isoliert. Der mit Acrolenin isolierte Stamm wurde jedoch nur in einer

Batchkultivierung im Labormaßstab getestet. Es bleibt abzuwarten, ob dieser Hefestamm im Fed-Batch-Verfahren und im größeren Maßstab die Produktivität aufrechterhalten kann.

Wie bereits oben erwähnt sind die Energiekosten ebenfalls ein Kostentreiber und sollten bei Kalkulationen berücksichtigt werden, wie z.B. Kosten für die Wärme- bzw. Dampferzeugung und zur Belüftung der Reaktoren. Der durchschnittliche Energiebedarf pro Tonne Hefe (Trockenmasse; TS) wird laut Fischer & Rahn (2004) auf ca. 2000 kWh (ca. 0,17-0,19 USD/kWh; https://de.statista.com, Suchbegriff: Industrie - Strompreis in Deutschland bis 2017, abgerufen 07/2019) beziffert, exklusive Dampf. Dieser kann in Tonnen oder in kWh berechnet werden und liegt preislich bei ca. 40-60 USD/t Dampf und kann in Abhängigkeit des Öl- bzw. Gaspreises variieren. Dabei wird mit ca. 3 Tonnen Dampf/t oder mit 1500kWh/t Hefe (TS) kalkuliert.

Beim Prozesswasser, welches meist aus Brunnen gewonnen wird, liegt der Preis bei ca. 2,02 USD/m³ (www.bwb.de/de/204.php, abgerufen 05/2019). Gemäß der Schätzungen von Fischer & Rahn (2004) werden ca. 65 m³ Frischwasser/t Hefe (TS) benötigt. Dazu kommen noch die Kosten für das Prozessabwasser, welches in Westeuropa in genossenschaftlichen bzw. kommunalen Klärwerken eingeleitet wird. Des Weiteren belaufen sich in Deutschland die Personalkosten auf ungefähr 10% vom Umsatz. Diese Kosten können jedoch aufgrund von regionalen Unterschieden stark schwanken (z.B. zwischen Westeuropa und dem asiatischen Raum). Gleiches gilt natürlich auch für den Energiepreis.

Geht man nun von einer konventionellen Hefeanlage mit 4-6 Bioreaktoren mit je 100 m³ Arbeitsvolumen aus, können pro Reaktor und je nach Prozessführung zwischen 50-60 kg/m³ Biomasse also 5-6 Tonnen Hefe produziert werden (siehe Enfors & Häggström (2000)). Natürlich können auch höhere Biomassen erzeugt werden, doch meist ist das Belüftungssystem und die damit verbundene Sauerstoffversorgung der Hefe der limitierende Faktor. Bei der GSH-Produktion mit *Saccharomyces* oder *Candida* werden im industriellen Großmaßstab vergleichbare Biomassen erreicht, jedoch ist die Prozesszeit im Vergleich zur Backhefeproduktion (15-20 h) um eineinhalb bis zwei Mal länger, was in der unterschiedlichen Prozessführung begründet ist. Während es bei der Backhefeproduktion ausschließlich um die Erzeugung von Biomasse geht, folgt bei der GSH-Produktion zusätzlich noch die Induktionsphase, also die GSH-Biosynthese, welche durch Zugabe der Präkusor(en) gestartet wird. Auch bringt die erhöhte Prozesszeit natürlich auch Risiken hinsichtlich der Keimbelastung mit sich (Presshefe: $\geq 10^6$ Keime/g Hefe). In Kapitel 5 wurden unterschiedliche Cysteinkonzentrationen, Zugabemodi sowie verschiedene Induktionszeitpunkte zur GSH-Biosynthese untersucht. Hierbei erwies sich eine späte Zugabe (Ende der exponentiellen Wachstumsphase) des Präkusors Cystein als vorteilhaft. Auch die kontinuierliche Zugabe von Cystein in geringen Menge führte schlussendlich zu einer Optimierung des GSH/Cystein Ratios. Auch konnte gezeigt werde, dass durch eine optimierte Prozessführung wie den Repeated-Fed-Batch die Ausbeute gesteigert werden kann. Im Gegensatz zur klassischen

Presshefe, welche ca. 0,70-1,00 USD/kg kostet, können GSH-angereicherte inaktive Trockenhefen zu einem Preis von mehr als 4,00 USD/kg verkauft werden.

Die produzierten Hefen werden in Abhängigkeit ihrer späteren Verwendung unterschiedlich prozessiert. In viele Arbeiten wird die spätere Aufarbeitung der produzierten Glutathionhefen nicht thematisiert. Ein Grund hierfür könnte die industrielle bzw. kommerzielle Relevanz sein (Xiong et al., 2009). Als Aufarbeitungsmethoden sind die Walzentrocknung oder das Extrudieren mit anschließender Wirbelschichttrocknung vorstellbar, aber auch die Sprühtrocknung wird eingesetzt. Die so prozessierten Hefen sind meist inaktiv mit Ausnahme der sprühgetrockneten Hefen. Typische Produktformen sind rieselfähiges Pulver, Pellets, Granulat oder Flakes. Beispiele solcher Spezialhefen sind u.a. die inaktivierten Hefen von Lesaffre und Lallemand, Saf-Pro Relax 190/200 bzw. LBI 2130 und Fermaid Super Relax. Diese werden in Lebensmittelbetrieben, meist industriellen Großbäckereien, eingesetzt, um die Teigrheologie, Krustenbildung und den Geschmack gezielt zu beeinflussen und das Produkt weiter aufzuwerten. Die verwendete Menge an GSH-Hefen beträgt ca. 0.1-0.3% vom eingesetzten Mehl. Im Vergleich dazu werden als Triebmittel ca. 1-4% Presshefe verwendet. Interessant ist ebenfalls die Verwendung und Aufarbeitung von sogenannten Brauereiabfallhefen. Bereits Selman & Myers (1945) patentierten hierzu ein Verfahren, um den GSH-Gehalt in der Brauereihefe zu sichern und das Produkt später zur Modifikation der Teigrheologie einzusetzen (siehe auch Gélinas (2009)).

Verheyen bestimmte in einer Studie 2015 den GSH-Gehalt von verschiedensten aktiven Hefen mit einem GSH-Gehalt von ≤81 mg GSH/g Hefe und zeigte, wie sich die Teigrheologie in Abhängigkeit des GSH-Gehalts der Hefe verändert (Verheyen et al., 2015). Auch Ozturk et al. (2017) verwendeten in ihren Studien zur Herstellung von Gebäck sowohl reines GSH als auch GSH angereicherte inaktive Hefe. Neben der verbesserten Teigrheologie konnte dabei auch der Proteingehalt signifikant erhöht werden, was die Funktionalität des Produkts nochmals erhöht. Allgemein gibt es zur Verwendung von inaktivierten GSH-angereicherten Hefen in Bäckereiprodukten nur wenig wissenschaftlich veröffentlichte Studien. Jedoch wirbt fast jeder Hersteller solcher Hefen mit entsprechenden Applikationen auf den eigenen Interseiten dazu. Aber auch zur Weinherstellung werden GSH-Hefen, z.B. von Lallemand (OptiWhite, OptiMUM White und Booster Blanc) angeboten, um die Farbe, das Mundgefühl und das Aroma des Weines zu schützen bzw. zu unterstützen (Gabrielli et al., 2017). Auch finden GSH-Hefepräparate Anwendung als Nahrungsergänzungsmittel. Beispiele hierfür sind Engevita-GSH und Lalmin-Detox von Lallemand, Hithion von Kohjin oder Glutathione-YE von Angel Yeast, um nur einige zu nennen. Andere Hefeextrakte werden nicht nur als Nahrungsergänzungsmittel, sondern auch in der Lebensmittelindustrie eingesetzt. Ueda beschreibt in seiner Studie, wie GSH den Geschmack beeinflusst, und spricht vom „kokumi“ Geschmack (Ueda et al., 1997). Zusammen mit L-Glutamat, was in Hefeextrakt ebenfalls bis zu 20% vorhanden ist, können so gezielt die Eigenschaften eines Produktes unterstützt bzw. manipuliert werden.

Die FDA bestätigte 2009 den von Kohjin angefragten GRAS Status von Glutathion zur Verwendung in Lebensmitteln (FDA GRAS Notice No. GRN 000293), wobei in diesem Fall ausschließlich reines GSH thematisiert wurde.

Die Aufreinigung von reinem GSH erfolgt in mehreren Schritten. Als Erstes erfolgt die Extraktion und Lyse der Hefezellen durch eine Heißwasserextraktion. Durch Zugabe von Säure wird die Perforation der Hefezellwand unterstützt und dabei gleichzeitig das GSH vor Oxidation geschützt (Satyanarayana & Kunze, 2009). Die Extraktion kann auch per Ethanol ohne Zelllyse realisiert werden (Wang, 2018; Xiong et al., 2009). Nach der Extraktion erfolgt die Separation des Feststoffanteils vom Extrakt. Den größten Anteil am Feststoff haben die Hefezellwandbestandteile und die präzipitierten Proteine, welche ebenfalls weiter prozessiert und verkauft werden können (siehe Kapitel 1). Der Extrakt kann anschließend filtriert werden, um weitere Verunreinigungen zu entfernen.

Bei der Kupferpräzipitations-Methode wird Kupferoxid in den Extrakt gegeben. Dabei bildet sich das Kupfersalz des GSHs und fällt aus. Der entscheidende Nachteil dieser Methode ist, dass das Kupfer wieder abgetrennt werden muss. Hierfür wird viel Schwefelwasserstoff (H_2S) benötigt, welches toxisch ist. Das sich gebildete Kupfersulfid wird per Filtration oder Separation entfernt und das gereinigte GSH liegt gelöst in der wässrigen Phase vor (Satyanarayana & Kunze, 2009). Aus ökologischer und gesundheitlicher Sicht sind hierbei allerdings die großen Mengen an benötigtem Kupfer sowie H_2S kritisch zu bewerten (Wu et al., 2010).

Eine andere Methode ist die Adsorption von GSH an Ionenaustauschern in mehreren Schritten. Die Elution des GSHs erfolgt dann meist durch Zugabe von wässrig-sauren Puffern (Michio & Itaru, 1994; Zhang et al., 2011). Danach erfolgt die Kristallisation mit organischen Lösungsmitteln und Vakuumtrocknung. Diese Methode ist insbesondere für die spätere pharmazeutische Nutzung interessant, da die notwendige Reinheit von über 98% ermöglicht wird. Weitere Methoden sind die wässrige Zwei-Phasen Extraktion und die Ultra-/Nanofiltration (Wang, 2018; Wu et al., 2010). Hochreines GSH findet zudem Anwendung in der Kosmetikindustrie, meist in Cremes zur Beeinflussung der Melanoyzten bzw. deren Melaninkonzentration (als sog. Skin Whitener; (Dilokthornsakul et al., 2019). Pures GSH wird ebenfalls als Sport- und Nahrungsergänzungspräparat zur allgemeinen Leistungsteigerung und für erhöhte Zellregeneration angeboten. Beispiele hierfür sind von Kohjin OPITAC oder von Kyowa Hakko, Setria.

In klinischen Studien wurde und wird die Wirkung von GSH zur Behandlung diverser Krankheiten wie cystischer Fibrose, Schizophrenie, Depression, HIV, Parodontose und MS untersucht. Aber auch die Aktivierung der Immunabwehr bzw. der Einfluss von GSH auf den allg. Gesundheitszustand ist Teil von Studien (U.S. National Library of Medicine, www.clinicaltrials.gov, Suchbegriff: Glutathione, abgerufen 07/2019). In der Pharmaindustrie

wird GSH bereits als Exzipient in hochreiner Form verwendet, um das Produkt vor Reduktion bzw. Oxidation zu schützen.

Schlussendlich wird der globale Bedarf an GSH und GSH-Produkten in den nächsten Jahren in allen genannten Industriezweigen (wie prognostiziert) weiter ansteigen. Parallel dazu steigen die Rohstoffkosten ebenfalls. Damit rückt eine effiziente und bioökonomische Produktion in den Fokus, was einen gezielten und effizienten Einsatz von Ressourcen und Technologien notwendig macht. Diese Arbeit liefert dazu potentielle Lösungsansätze und zeigt auf, welche Faktoren integrale Bestandteile zur Optimierung der GSH-Produktion sind.

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7 Im Zuge dieser Dissertation entstandene wissenschaftliche Beiträge

7.1 Eigenanteil von E. Lorenz an den Publikationen der vorliegenden kumulativen Schrift („Peer-reviewed journals“)

- **Lorenz, E**. Schmacht, M., Stahl, U., Senz, M., (2015) Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*;

  Journal of Biotechnology 216, 131-139,
  http://dx.doi.org/10.1016/j.jbiotec.2015.10.016

Konzept:	Durchführung/Methoden:	Verfassen der Publikation:
80%	70%	60%

- **Lorenz, E**. Schmacht, M., Senz, M., (2016) Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in *Saccharomyces spp*.;

  Enzyme and Microbial Technology 93-94, 122-131,
  https://doi.org/10.1016/j.enzmictec.2016.08.004

Konzept:	Durchführung/Methoden:	Verfassen der Publikation:
80%	60%	60%

- Schmacht, M., **Lorenz, E.** Senz, M., (2016) Medium adjustment based on yeast's elemental composition for glutathione production in *Saccharomyces cerevisiae*;

  Journal of Bioscience and Bioengineering, 123, 555-561,
  https://doi.org/10.1016/j.jbiosc.2016.12.011

Konzept:	Durchführung/Methoden:	Verfassen der Publikation:
40%	40%	40%

- **Lorenz, E.**, Schmacht, M., Senz, M., (2017) Microbial production of glutathione;

  World Journal of Microbiology and Biotechnology, 33, 106,
  https://doi.org/10.1007/s11274-017-2277-7

Konzept:	Durchführung/Methoden:	Verfassen der Publikation:
50%	50%	40%

7.2 Weitere wissenschaftliche Beiträge

- Lorenz, E., Neumann, K., Riedel, S.L., Stahl, U., Bader, J. (2012) DoE based screening system for development of upscalable bioreactor cultivation for glutathione-enriched yeasts; Posterbeitrag

  15th European Congress on Biotechnology; Abstract im New Biotechnology, 29

- Lorenz, E., Stahl, U., Bader, J. (2013) Combination of an affordable screening system and process development for GSH enriched yeast; Fachvortrag
  - 26. VH-Conference, Berlin
- Lorenz, E., Senz, M., Stahl, U., (2014) Process development of production of glutathione enriched yeasts; Fachvortrag
  - 4th conference of DGHM and VAAM, 5-8.10.2014, Dresden
- Schmacht, M., Lorenz, E., Senz, M. (2016) Alternative bioprocessing strategies for glutathione production in *Saccharomyces spp*.; Posterbeitrag
  - BioProcessingDays, 22.-24.2.2016, Recklinghausen
- Schmacht, M., Lorenz, E., Stahl, U., Senz, M., (2015) Glutathione overproduction in high cell density continuous cultivation of *Saccharomyces spp*.; Posterbeitrag
  - VH-Conference, Berlin
- Schmacht, M., Lorenz, E., Stahl, U., Senz, M. (2015) New fermentation strategy for glutathione production in *Saccharomyces spp*.; Fachartikel
  - Brauerei Forum International Edition (1) 16-17.
- Schmacht, M., Lorenz, E., Stahl, U., Senz, M. (2015) Neue Fermentationsstrategien zur Glutathionproduktion mit *Saccharomyces spp*.; Fachartikel
  - Brauerei Forum (8) 12-13.

7.3 Assoziierte Abschlussarbeiten

- Männig J. (2011); Studien-Arbeit; Technische Universität Berlin

  Etablierung einer Screening-Plattform-Technologie für Hefen

- Schäfer T. (2013); Bachelor-Thesis; Hochschule Zittau/Görlitz

  Synthese von intrazellulären Glutathion mit der Brauabfallhefe *Saccharomyces carlsbergensis* und die Etablierung einer Hefefermentation mit Melasse zur Produktion von Glutathion

- Ehrhardt F. (2013); Master-Thesis; Ernst-Abbe-Fachhochschule

  Etablierung einer Selektionsplattform im 96-Well-Format zur Erzeugung von *Saccharomyces cerevisiae* Mutanten mittel EMS und MNNG mit anschließender Charakterisierung hinsichtlich Glutathion und oxidativem Stress

- Müller, J. (2014); Bachelor-Thesis; Beuth-Hochschule für Technik

Charakterisierung einer Stressinduktion in *Saccharomyces cerevisiae* mittels Durchflusszytometrie: Einfluss auf den intrazellulären GSH Gehalt

- Schmacht M. (2014); Master-Thesis; Beuth-Hochschule für Technik

  Untersuchung des Einflusses unterschiedlicher Prozessführungen auf die Glutathionbildungsrate in einer kontinuierlichen Hefefermentation

- Wolf, S. (2015); Praxisarbeit; Beuth-Hochschule für Technik

  Identifikation von potenziellen Substanzen zur Erhöhung der Glutathionbildung bei Hefen

8 Appendix

Tabellenverzeichnis

Abbildungsverzeichnis