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XXV Ciclo

A protein-based biorefinery for bulk chemicals production

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"Che poi l'allossana, destinata ad abbellire le labbra delle dame,
scaturisse dagli escrementi delle galline o dei pitoni,
era un pensiero che non mi turbava neanche un poco.
Il mestiere di chimico (fortificato, nel mio caso, dall'esperienza di
Auschwitz) insegna a superare, anzi ad ignorare, certi ribrezzi,
che non hanno nulla di necessario né di congenito:
la materia è materia, né nobile né vile, infinitamente trasformabile,
e non importa affatto quale sia la sua origine prossima.
L'azoto è azoto, passa mirabilmente dall'aria alle piante,
da queste agli animali, e dagli animali a noi;
quando nel nostro corpo la sua funzione è esaurita, lo eliminiamo,
ma sempre azoto resta, asettico, innocente."

Tratto da:"Il sistema periodico" di Primo Levi

"Imparare è un'esperienza, tutto il resto è informazione"

Albert Einstein

E la dedica è per te… che non puoi che essere presente…

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ABSTRACT

A biorefinery should ideally integrate biomass conversion processes to produce a range of fuels, power, materials, and chemicals from biomass. The term biorefinery is derived both from the raw material feedstock which is renewable biomass and also from the bioconversion processes often applied in the treatment and processing of the raw materials. Renewable sources are the basis of the alternative energy, fuels and compound obtained in a biorefinery-based way to meet the energy demand in a world where petrol fuels are becoming scarce and more expensive.

The current biorefinery schemes are mainly based on hexose sugars (first generation) or lignocellulosic-based material (second generation) as feedstocks and depend on microbial cell factories for obtaining the products of interest. In most cases, the substrate utilization is not optimized jet: at the end of the process in addition to the main product, by-products such as huge quantity of lignin and some C5 sugars (deriving from incomplete utilization of lignocellulose) but also proteins (mainly the exhausted biomass at the end of the production) are still available. Focusing the attention on proteins, they are nowadays mainly absorbed as animal feed, but the quantities produced exceed the demand. Considering the implication related to GMO utilization and the increasing number of biorefineries, it can be stated that proteins could represent an interesting

"no-cost" starting material to produce more biofuel, bioproducts or biopower in a biorefinery-based way.
This study has the aim to present some examples of production by using the protein side-stream of a biorefinery. In particular, we report the possible destiny of two aminoacids released from said proteins: glycine, to obtain butanol and isobutanol, and glutamate, to produce succinic acid.

Butanol represents the most feasible alternative to gasoline, but, differently from ethanol, microbial fermentations are not enough developed to sustain the market demand. The natural producers, different species of the Gram positive group of *Clostridia*, present many and different limitations which are challenging the scientific communities in finding alternative hosts.
Among the different alternative hosts, yeasts can present attractive features, being the most relevant the existence of industrial plants based on *Saccharomyces cerevisiae* fermentation for the production of ethanol. Up to now, in *S. cerevisiae* the production of butanol has been obtained by expressing heterologous genes from *Clostridia*. The best reported butanol titer is only 2.5 mg/L, probably mainly depending on problems related to the overexpression of heterologous prokaryotic enzymes. On the contrary, the isobutanol production is obtained at high level (0.63 g/L) in *S. cerevisiae* by using and optimizing an endogenous valine degradation pathway.
We present an alternative and novel way to produce butanol and isobutanol using glycine as substrate, through a new identified and

biochemically characterized pathway. Starting from 15 g/L of glycine we have obtained 92 mg/L of butanol and 58 mg/L of isobutanol. Considering that the pathway has been just discovered and no optimization has been designed or applied, the obtained results has to be considered extremely positive.

Since one of the most common stresses that microorganisms have to face during productions is the toxicity of the final product, we investigated also the possibility to use the glycine as protective agent for the cells. This idea originated from different studies that explain how the use of aminoacids, among which glycine, can help the cells to better respond to different stresses, such as high ethanol concentration and hydrogen peroxide. As never reported in literature, we have notably found that butanol causes a sort of oxidative stress, peroxidising the membrane lipids. Moreover, we have demonstrated that glycine can help the cells to better tolerate the presence of hydrogen peroxide, acetic acid, ethanol and butanol.

The succinic acid occurs naturally in humans, animals, plants, and microorganisms as TCA cycle intermediate. Its use as a precursor to produce many important commodity chemicals used to make a wide assortment of products such as solvents, fiber and polymer production make it one of the most important relevant bulk chemicals. The microbial production of succinic acid via reductive TCA cycle using engineered *Saccharomyces cerevisiae* is nowadays on the market commercialised by Reverdia (Cassano Spinola, Italy).

Here we investigated the possibility to exploiting the glutamate degradation pathway through the γ-aminobutyric acid (GABA) intermediate as an additional way of obtaining succinate when a source of glutamate is feeded to the cells. Since this pathway, also called GAD/GABA shunt, is transcriptionally controlled, we transcriptionally up-regulated it through the constitutive overexpression of the involved genes, *GAD*1, *UGA*1 and *UGA*2. Even if the pathway was clearly overexpressed, as revealed by transcriptional analysis, the obtained amount of succinic acid did not reflect this modification. Further investigating the possible reason of that, we have demonstrated that, despite the higher transcription levels, the corresponding enzymatic activities of the pathway did not increased. We can argue that post-translational regulations occur in the pathway, what at the moment impaired the initial aim of our work. However, the data add new and relevant information for the comprehension of the role and the regulation of this pathway in *S. cerevisiae*. It cannot be excluded that the production of succinic acid in yeasts might be in a future optimized by exploiting the GAD/GABA pathway, once it will be fully characterized.

We have finally investigated the possibility to produce succinic acid in a strain of *S. cerevisiae* deleted in the gene encoding the enzyme glutamate synthase, *GLT*1. During this study we have tested different growing conditions, since the target gene of deletion is located in a critical node of the nitrogen sensing and utilization, which is related to cellular plasticity and adaptability in perturbed environment. We could demonstrate that

when cells are growing in low salts concentration medium, the deleted strain compared to wild type strain is able to accumulate more reduced products, such as succinic acid, malate/fumarate, glycerol and ethanol, indicating a sort of necessity to reduce the excess of NAD(P)H generated by the *GLT*1 deletion. Regarding this preliminary study, deeper investigations have to be performed to find a fine tuned way to intentionally redirect the metabolic flux and obtain the product of interest, which in turn could be succinic acid, but also malic or fumaric acid, or again ethanol, being this last the product that has the responsibility to prove that bio-based second generation processes can effectively and positively have an impact on our future society.

RIASSUNTO

Una bioraffinera idealmente integra i processi di conversione delle biomasse per produrre carburanti, energia, materiali e composti chimici. Il termine bioraffineria deriva sia dall'uso di materie prime rinnovabili che dai processi di bioconversione che spesso sono applicati nel trattamento e nel processamento delle materie prime.

Le fonti di energia rinnovabile sono alla base della produzione di energia, carburanti e composti alternativi ottenuti tramite un approccio di bioraffineria, necessari per sopperire alla domanda globale di energia, in un mondo in cui il petrolio è scarso e diventa ogni giorno più costoso.

Gli attuali schemi di bioraffineria sono basati sull'utilizzo di zuccheri esosi (prima generazione) o derivati lignocellulosici come materia prima (seconda generazione) e dipendono dai microorganismi utilizzati come *cell factories* per ottenere i prodotti di interesse

In molti casi l'utilizzo del substrato non è stato ancora completamente caratterizzato: alla fine del processo, insieme al prodotto principale, vengono prodotti anche una serie di prodotti secondari come la lignina e zuccheri a 5 atomi di carbonio (derivanti dall'incompleto utilizzo della lignocellulosa) ma anche proteine (principalmente biomasse esauste alla fine della produzione). Riguardo alle proteine, attualmente sono usate nella mangimistica per animali, ma la quantità prodotta eccede la domanda. Considerando l'implicazione relativa all'utilizzo di organismi

geneticamente modificati a l'aumento del numero di bioraffinerie, è possibile affermare che le proteine potrebbero essere utilizzate come materiale di partenza a "costo zero" per produrre biocarburanti, bioprodotti o bioenergia in un processo basato sul concetto di bioraffineria.
Il presente lavoro di tesi ha l'obiettivo di presentare alcuni esempi di produzione utilizzando un cosidetto "*side-stream process*" all'interno di una bioraffineria. In particolare viene riportato il possibile destino di due aminoacidi provenienti dalle sudette proteine: la glicina, per ottenere butanolo e isobutanolo, e il glutammato, per produrre acido succinico.

Il butanolo rappresenta la principale alternativa alla benzina, ma, a differenza dell'etanolo, le fermentazioni microbiche non sono ancora state completamente sviluppate per sostenera la domanda di mecato. I produttori naturali, differenti specie di Gram positivi appartenenti al gruppo dei *Clostridia*, presentano molti e differenti limiti i quali hanno portato la comunità scientifica a trovare ospiti alternativi.
Tra i differenti ospiti alternativi, i lieviti rappresentano un'attraente alternative, a causa dell'esistenza di impianti industriali basati sulle fermentazioni di *Saccharomyces cerevisiae* per la produzione di etanolo. Fino a dora, in *S. cerevisiae* la produzione di butanolo è stata ottenuta mediante l'overespressione eterologa di geni di *Clostridia*. La migliore produzione di butanolo è di soli 2.5 mg/L, probabilmente a causa di problemi legati all'overespressione eterologa di enzimi procariotici. Al contrario, la produzione di isobutanolo è stata ottenuta ad alti livelli (0.63

g/L) in *S. cerevisiae* utilizzando e ottimizzando il *pathway* endogeno di degradazione della valina.

In questo lavoro viene presentato un metodo alternativo per la produzione contemporanea di butanolo e isobutanolo usando la glicina come substrato, attraverso un nuovo *pathway*, che è stato identificato e caratterizzato dal punto di vista biochimico. A partire da 15 g/L di glicina, abbiamo ottenuto 92 mg/L di butanolo e 58 mg/L di isobutanolo.

Considerando che il *pathway* è stato appena scoperto e che non sono state applicate né disegnate ingegnerizzazioni per l'ottimizzazione del *pathway*, i risultati ottenuti sono da considerarsi estremamente positivi.

Considerando che uno degli stress più comuni che i microrganismi devono affrontare durante i processi di produzione è la tossicità del prodotto finale, abbiamo deciso di studiare il possibile uso della glicina anche come agente protettivo per le cellule. Questa idea nasce dal fatto che vari studi hanno dimostrato che l'uso di aminoacidi, tra cui la glicina stessa, può aiutare le cellule a rispondere a differenti stress, come l'alta concentrazione di etanolo e di perossido di idrogeno. Abbiamo dimostrato che il butanolo causa uno stress ossidativo, attraverso la perossidazione dei lipidi di membrana. Questa è la prima osservazione sperimentale in tal senso e non esistono dati a riguardo in letteratura. D'altra parte, la glicina aiuta le cellule a tollerare meglio la presenza nel terreno di perossido di idrogeno, di acido acetico, di etanolo e di butanolo.

L'acido succinico è presenta naturalmente nell'uomo, negli animali, nelle piante e nei microorganismi come intermedio del ciclo degli acidi

tricarbossilici. Il suo utilizzao come precursore per produrre molti composti chimici utilizzati poi per produrre un vasto assortimento di prodotti come solventi, così come per la produzione di fibre e polimeri fanno si che l'acido succinico sia uno dei più importanti *bulk chemicals* prodotti. La produzione microbia dell'acido succinico attraverso il ciclo riduttivo degli acidi tricarbossilici, utilizzando cellule di *Saccharomyces cerevisiae* ingegnerizzate per tale produzione, è attualmente commercializzato dalla Reverdia (Cassano Spinola, Italy).

Abbiamo studiato la possibile produzione di acido succinico attraverso un approccio che sfrutta il processo di degradazione del glutammato tramite un *pathway* in cui si forma acido γ-aminobutirrico (GABA) come intermedio, fornendo glutammato nel terreno. Poiché è risaputo che questa via, chiamata anche *pathway* del GAD/GABA, è regolata a livello trascrizionale, abbiamo provato a deregolarla overesprimendo i geni in essa coinvolti, *GAD*1, *UGA*1 e *UGA*2. Anche se l'analisi trascrizionale dimostrava l'overespressione del *pathway*, la quantità di acido succinico ottenuta nel ceppo ingegnerizzato non riflette tale modifica. Inoltre, studiando ciò abbiamo dimostrato che, nonostante i livelli di trascrizione siano alti, le corrispondenti attività enzimatiche del *pathway* non aumentano. E' possibile dire che probabilmente sono presenti regolazioni post-translazioni, i quali al momento influenzano lo scopo del nostro lavoro, ma che aggiungono nuove e rilevanti informazioni per la comprensione del ruolo e della regolazione di questo pathway in *S. cerevisiae*.

Non è tuttavia da escludere che la produzione di acido succinico in lievito non possa essere in un futuro ottimizzata sfruttando il *pathway* GAD/GABA, una volta che esso sarà pienamente caratterizzato.

Infine abbiamo studiato la possibilità di produrre acido succinico in un ceppo di *S. cerevisiae* in cui è stato deleto il gene codificante per l'enzima glutammato sintasi, *GLT*1. In questo studio abbiamo testato differenti condizioni di crescita, dal momento che il gene in questione è costituisce un nodo critico per il sensing e l'utilizzo dell'azoto, il quale è correlato alla plastività e all'adattamento cellulare in condizioni perturbate. Potremmo dimostrare che quando le cellule crescono in presenza di basse concentrazioni di sali, il ceppo deleto comparato con il ceppo wild type è in grado di accumulare maggiormente prodotti ridotti, come l'acido succinico, il malato/fumarato, il glicerolo e l'aetanolo, indicando una sorta di necessità di ridurre l'eccesso di NAD(P)H che si genera a causa della delezione del gene *GLT*1.

Ulteriori analisi dovranno essere effettuate al fine di ridirezionare intenzionalmente il flusso metabolico per ottenere il prodotto di interesse, il quale potrebbe essere l'acido succinico ma anche l'acido malico o fumarico, o ancora l'etanolo, essendo quest'ultimo il prodotto che ha la responsabilità di provare che i processi di seconda generazione possono effettivamente e positivamente avere un impatto sulla nostra futura società.

INTRODUCTION

Production of biofuels, chemicals and energy from renewable feedstocks is necessary to meet the energy demand in a world where petrol fuels are becoming scarce and more expensive.

The United States have set the goal to produce 21 billion gallons of advanced biofuels by 2022. This target creates an urgent need to bridge the gap between promising research and commercial, large-scale production of advanced biofuels. Achieving national energy and climate goals will require a large, economically viable, and environmentally sustainable bio-industry (U.S Department of Energy; URL: www.biomass.energy.gov). A crucial step in developing bio-industry is to create biorefineries capable of efficiently converting a broad range of biomass feedstocks into low cost biofuels, biopower, and other bioproducts.

It is possible to compare the biorefineries to today's petroleum refineries with the important distinction that biorefineries are based on the use of renewable materials as feedstock whereas today’s petroleum refineries are based on the use of non-renewable materials such as fossil fuels (The National Renewable Energy Laboratory; URL: www.nrel.gov/biomass/biorefinery.html). A biorefinery transforms biomass derived from renewable raw materials into a wide range of products such as bioethanol, bioplastics, biochemicals and ingredients for

the food and feed industry, by the means of advanced biotechnological processes such as enzymatic hydrolysis (**Figure 1**). The biomass comes from a variety of sources such as trees, energy crops such as switchgrass and agricultural products such as grain, maize and waste products such as municipal waste or other residual waste coming from other process of production (Europa Bio; URL: www.bio-economy.net).

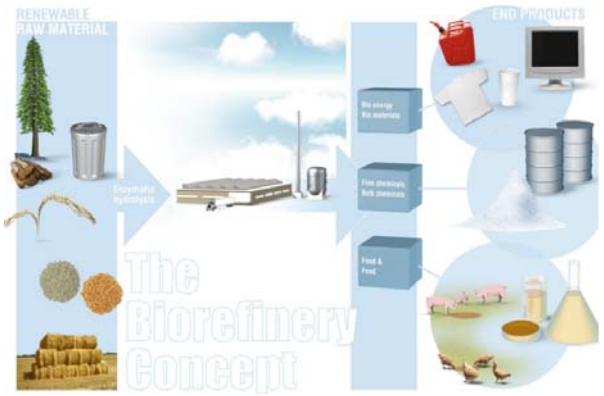

Figure 1: Schematic representation of a biorefinery (Europa Bio; URL: www.bio-economy.net)

Thus it is easy to imagine that biorefineries are more sustainable than petroleum refineries and can increase the sustainability of industrial production.

Currently biorefineries scheme are based on carbohydrates and lipids as feedstock to produce biofuels and chemicals (Wackett, 2011; Almeida et al., 2012; Hasunuma et al., 2012; Thakker et al., 2012). An example of this type of process is the production of bioethanol, one of the alternatives to conventional petroleum-based transport fuels (Balat, 2011). It can be

produced using lignocellulosic biomass as feedstock (Mussatto et al., 2010) but there are some limitations: although lignocellulosic biomass is the most promising feedstock considering its great availability and low cost, the large-scale commercial production of fuel bioethanol from lignocellulosic materials has still not been implemented (Balat, 2011). In order to be used for bioethanol fermentation the microorganisms requires the hydrolysis treatment of lignocellulosic material during which glucose, but also various monosaccharides (e.g. xylose, mannose, fructose, galactose, and arabinose) and oligosaccharides are formed (Madhavan et al., 2012; Peralta-Yahya et al., 2012). Thus to efficiently ferment these sugars for the successful industrial production of bioethanol the microorganisms have to be engineered. Currently there are many studies, also in *S. cerevisiae*, about this aspect of the entire process (Zhao et al., 2012).

However, practically all the existing schemes do not take into consideration the recycling of waste product to convert it in other interested products. For example proteins are rich in the industrial fermentation residues and thus can be used as feedstock for the production of biofuels. In particular it is possible to use algae as feedstock for the lipids-derived fuels in which a huge amount of protein by-products are accumulated, due to the fact that to extract the lipids necessary to obtain the biofuel, the algal biomass have to be disrupted (Wijffels & Barbosa, 2010). The proteins, which are a major component of fast-growing photosynthetic microorganisms, could be converted into biofuels or biochemical products (Wijffels & Barbosa, 2010; Huo et al., 2011).

Currently, these protein by-products are mainly used as animal feed but the market doesn't absorb the total protein by-products produced from the biorefinery industry (Huo et al., 2011).

Table I. Proteins accumulation during industrial ethanol production. The current annual production of ethanol in the United States is 32.9 billion pounds produced from 110 billion pounds of corn. For these processes are exploited 1.5 billion kilograms of microorganisms. The protein content of corn and of the microorganisms is equal to 9.8 and 40% and this represents a potential protein accumulation total of 11.4 billion pounds. Since the maximum theoretical yield of conversion of proteins in alcohol is 60% the potential production of biofuels is 6.84 billion pounds, which correspond to 2.26 billion gallons. (Huo et al., 2011)

Source of Biomass Feedstock	Annual Accumulation (milion metric tons)	Protein Content in Biomass (%)	Potential Protein Leftover (million metric tons)	Potential Biofuel Production (billion gallons)
Corn Ethanol	110	9.8	10.8	2.14
Fermentation	1.5	40	0.6	0.12
Total	-	-	11.4	2.26

We have to keep in mind that the objective of a biorefinery is to optimize the use of resources and minimize wastes, thereby maximizing benefits and profitability (U.S Department of Energy; URL: www.energy.com).
The present study has the aim to offer some example about the utilization of proteins as raw material in a protein-based biorefinery process. In particular, we report the utilization of aminoacids, released by proteins degradation process, as substrates to obtain biofuels and biochemical products. On the one side the production, using *Saccharomyces cerevisiae* as cell factory, of butanol and isobutanol starting from glycine and on the other side the production of succinic acid starting from glutamate as substrate.

WHY BUTANOL ANS ISOBUTANOL?

Like petrol (gasoline), an ideal biofuel should drop into today's infrastructure and carry enough juice to get any vehicle where it has to go. Although many studies have been done to produce ethanol and make it the biofuel of the future, ethanol has some limitation (Connor & Liao, 2009). Compared with petroleum-based fuels, it's much less dense in energy: a litre of ethanol takes a car only about 70% as far as a litre of gasoline, and ethanol cannot provide enough power for heavy trucks or aircraft (Savage, 2011). Moreover, ethanol is also corrosive and so cannot easily be used in today's engines or be delivered cheaply through existing pipelines.

Table II: Properties of fuel alcohols and gasoline. Adapted from (Lee et al., 2008)

	Methanol CH_3OH	Ethanol C_2H_5OH	Butanol C_4H_9OH	Gasoline
Energy content (BTUs/gallon)	63 k	84 k	110 k	115 k
Motor octane	91	92	94	96
Air:fuel ratio	6.6	9	11–12	12–15
Vapor pressure (psi@100°F)	4.6	2	0.33	4.5

To overcome these limitations, researchers are trying to turn biomass into substances that can be either placed directly in the fuel tank or slotted into the processing chain in existing biorefineries. A said "higher alcohol", a molecule with more carbon and hydrogen atoms than ethanol such as butanol and isobutanol, seem to comes closer to the ideal biofuel (Savage,

2011). Since the carbon–hydrogen bonds are where the useful energy is stored, breaking these bonds through combustion releases energy. With its four carbons butanol and isobutanol for example has more energy than ethanol (**Figure 2**) and are also less corrosive respect to the ethanol. Moreover, today's petrol blends generally don't exceed 10% ethanol, but it's easy to imagine a fuel blend containing 50-70% of butanol which has higher energy and is less corrosive than ethanol. Ultimately, butanol might also be cheaper. When ethanol is produced in a batch, the alcohol comes out mixed with water. To obtain the pure fuel, the water has to be boiled off which means using additional energy. Butanol (or isobutanol) and water don't mix, so they can be separated by less energy-intensive processes (Dürre, 2008). In addition, isobutanol can be readily used as a precursor for a number of valuable chemical syntheses (Atsumi et al., 2008b).

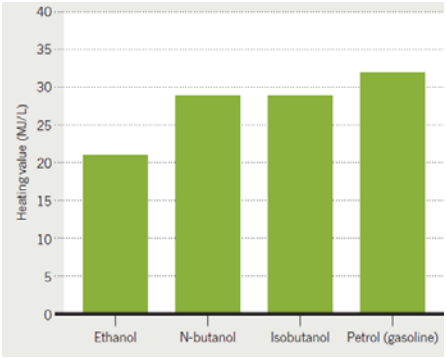

Figure 2. The biofuels are different for their energetic density. The higher alcohol such as butanol and isobutanol can be used alternatively to ethanol, since their higher energetic density respect to ethanol. Adapted from (Savage, 2011).

Thanks to the described characteristics higher alcohols, and in particular butanol and isobutanol, are actually among the most promising and interesting molecules that might replace gasoline as fossil fuel and ethanol as biofuel. Different companies are actually involved in financing projects to promote the butanol and isobutanol production starting from renewable biomasses (Savage, 2011). The Green Biologic, Butamax Advanced Biofuels and Gevo are some examples.

WHY SUCCINIC ACID?

Organic acids constitute a key group among the building-block chemicals because thanks to their functional groups are extremely useful as starting materials for the chemical industry (Sauer et al., 2008). Succinic acid is considered among the most versatile building-block because different chemical compound can be formed starting from it (**Figure 3**). Butanediol, tetrahydrofuran and γ-butyrolactone, for example, are substances used in the chemical industry and can be obtained from succinic acid by chemical conversion (McKinlay et al., 2007). These important derived-compounds are used for fiber and polymer production. Moreover, the succinic acid can also be directly polymerized to form the biodegradable aliphatic polyester bionolle (Showa Denko, URL: www.showa-denko.com and as reviewed by (Sauer et al., 2008). Finally, The succinic acid itself can be used as it is as a surfactant, detergent or foaming agent, as an ion chelator, and also in the food industry (as an acidulant, flavouring agent or anti-microbial agent) as well as in health-related products (such pharmaceuticals and antibiotics) (Sauer et al.,

2008). Thus, the potential market volume for succinic acid is high, fuelling substantial efforts to establish a microbial process for succinic acid production to replace the today's petrol-based process (McKinlay et al., 2007) with a renewable-based process.

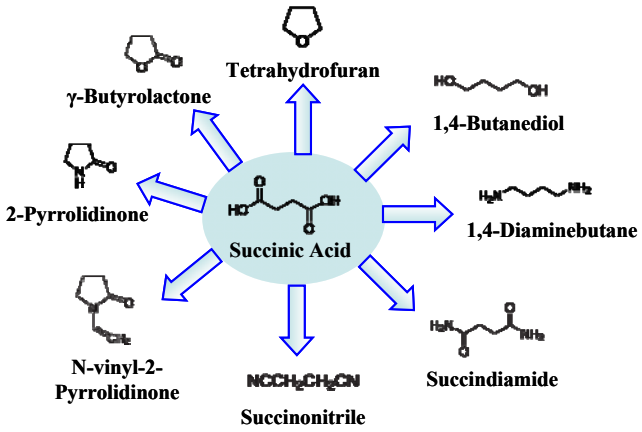

Figure 3. Various substances that can be derived from succinic acid by chemical conversion. Adapted from (Sauer et al., 2008).

WHY *Saccharomyces cerevisiae*?

The yeast *Saccharomyces cerevisiae* is considered one of the most important yeast because of its industrial significance (Branduardi et al., 2008). It is recognized by the American Food and Drug Administration (FDA) as an organism generally regarded as safe (GRAS) (FDA, URL: www.fda.gov/default.html), and since it does not produce toxins it has been used safely for centuries in the brewing and baking industries.

Because of its importance both in basic research and biotechnology, *S. cerevisiae* was the first eukaryote to have its genome completely sequenced (Goffeau et al., 1996). *Saccharomyces cerevisiae* is one of the

most widely used microorganism for the production of many compound of biotechnological interest (Branduardi et al., 2008). For example, *S. cerevisiae* is regarded as an industrial working horse for bioethanol production because it can produce ethanol in high titre using hexose sugars and have high ethanol tolerance (Zhang & Geng, 2012).

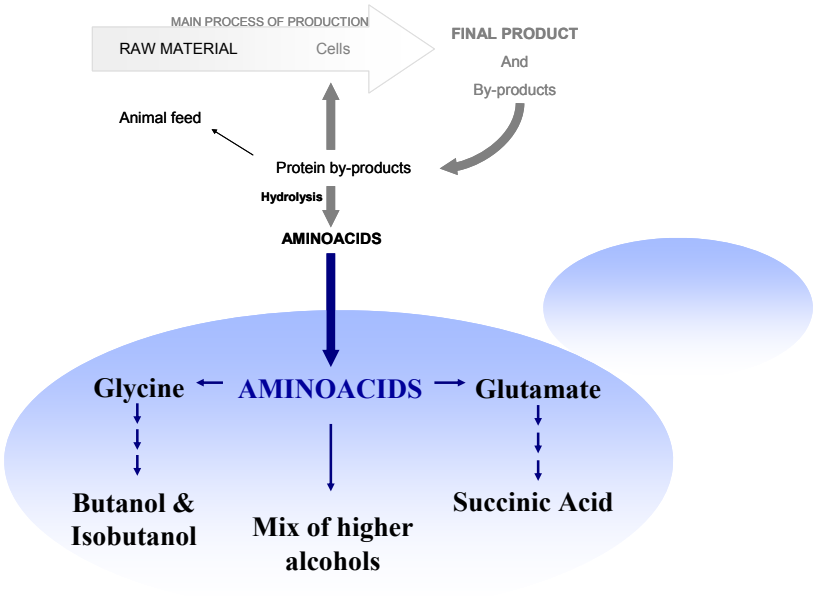

Figure 4. Simplified illustration of protein-based biorefinery scheme for the production of butanol (and isobutanol) or succinic acid using the yeast *S. cerevisiae*. In grey are represented the general existing processes of production scheme. In blue is reported the protein by-products side stream discussed in the present study.

Although naturally do not produce lactic acid engineered yeasts are also candidate to enter in the market for the production of lactic acid due to

their low pH tolerance and grow on mineral media which allow the purification of the acid (Sauer et al., 2010).

In respect to what said up to now the yeast cells could be the best compromise to produce both biofuels and biochemicals. In particular, considering a protein-based biorefinery approach the aminoacids utilization system has to be redirected to produce butanol and isobutanol or succinic acid (**Figure 4**).

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CHAPTER 1

BUTANOL AND ISOBUTANOL PRODUCTION IN *Saccharomyces cerevisiae* THROUGH THE GLYCINE DEGRADATION PATHWAY

Manuscript in preparation for "Biotechnology for Biofuels" journal

Chapter 1

Introduction

The production of biofuels from renewable biomasses is one of the answers to help solving the problems associated with limited fossil resources and climate change. Butanol has superior liquid-fuel characteristics in respect to ethanol, with similar properties to gasoline and thus it has the potential to be used as a substitute for gasoline in currently running engine (Savage, 2011).

Clostridia are recognized as good butanol producers and are employed in the industrial-scale production of solvents. Due to the complex metabolic characteristics and to the difficulty of performing genetic manipulations on *Clostridia*, in recent years the *Clostridia* butanol pathway was expressed in other microrganisms such as *E. coli* (Atsumi et al., 2008a) and *S. cerevisiae* (Steen et al., 2008) but the results obtained were not so promising. Alternative pathways for the butanol production have been characterized and discovered (Peralta-Yahya et al., 2012). *Liao et al* proposed that proteins, and thus the aminoacids released from proteins-degradation process, can also be used as a raw material for biorefining and biofuels production, since amazingly proteins are abundantly present as final waste of lignocellulose processing (Huo et al., 2011).

Saccharomyces cerevisiae is able to use different aminoacids as nitrogen source among which glutamine, glutamate, proline and glycine (Zaman et al., 2008). The cells have one or more carrier system specific for each aminoacid even if they are not all currently known. Among them, the general aminoacids permease, encoded by *GAP*1 gene, is involved in glycine transport (Hofman-Bang, 1999). In the cytosol, glycine can be catabolised in different ways, according to nutritional requirements. For

example, it can be converted into serine through serine hydroxymethyltransferase enzyme (SHM) (McNeil et al., 1994) or into CO_2 and NH_3 through the enzymatic complex of glycine decarboxylase enzyme (GDC) (Sinclair & Dawes, 1995).

More recently and thanks to the development of metabolic models, Villas-Bôas and co-workers (Villas-Bôas et al., 2005) have *in silico* demonstrated the generation of glyoxylate from glycine deamination. Moreover, the authors described the formation of α-ketovalerate and α-isoketovalerate as subsequent intermediates of the same pathway through not identified reactions.

Starting from this indication and knowing from literature that α-ketovalerate can be converted into butanol (Shen & Liao, 2008) and that α-isoketovalerate can be converted into isobutanol (Atsumi et al., 2008b), we wondered if it would be possible to connect glycine with the production of this fusel alcohol. By deeply investigating the present literature, we have first hypothesized and then demonstrated the butanol and isobutanol production through the glycine degradation pathway via glyoxylate and α-ketoacids formation. The proposed pathway, which has to be considered as novel, was biochemically confirmed step by step. We obtained 92 mg/L of butanol and 58 mg/L of isobutanol, starting from 15 g/L of glycine. Even if the obtained amount of butanol is very little, it is important to consider that the best butanol production reported for engineered *S. cerevisiae* was only 2.5 mg/L (Steen et al., 2008). Diversely, we can not simply compare the titer of isobutanol accumulation that we have obtained with those reported in literature,

0.63 g/L, by engineered *S. cerevisiae* (Brat et al., 2012). In fact, it does not represent the final main product in the proposed glycine degradation pathway, but it derives from the conversion of a α-ketovalerate fraction.

Chapter 1

Materials And Methods

STRAINS AND GROWTH CONDITIONS

The *S. cerevisiae* strains used in this study were CEN.PK 102-5B. (*MATa,ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2* - Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany) (van Dijken JP et al., 2000) and BY4741 *(MATa, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1)* (EUROSCARF collection, Heidelberg, Germany). The strains BY4741Δ*MLS*1 (*Mat a; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; YNL117w::kanMX4*) and BY4741Δ*DAL*7 (*Mat a; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; YIR031c::kanMX4*) are provided by EUROSCARF deleted strain collection (EUROSCARF collection, Heidelberg, Germany). The strain deleted in all three isoform of pyruvate decarboxylase (*ΔPDC1, 5, 6*) is CEN.PK RWB837 (*MAT*a *pdc1*::*loxP pdc5*::*loxP pdc6*::*loxP ura3-52*) (van Maris et al., 2004). Strains designed with "c" correspond to the respective parental strain transformed with empty plasmids (see below) to render them prototrophic. Strains designed with "*goxB* opt" are the corresponding parental strain transformed with plasmid pYX212 (see below) with the *B. subtilis goxB* coding sequence optimized for the *S. cerevisiae* codone usage. *GoxB* opt gene was expressed under the control of the *S. cerevisiae TPI*1 promoter. Yeast transformations were performed basically according to the LiAc/PEG/ss-DNA protocol (Gietz & Woods, 2002). All the parental strains are reported in table below.

Parental strain	
Strain	**Genotype**
CEN.PK 102-5B	*MATa,ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*
CEN.PK RWB837	*MAT*a *pdc1 ::loxP pdc5 ::loxP pdc6 ::loxP ura3 -52*
BY4741	*MATa, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1*
BY4741Δ*MLS1*	*Mat a; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; YNL117w::kanMX4*
BY4741Δ*DAL7*	*Mat a; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; YIR031c::kanMX4*

Transformed strain		
Strain	**Plasmids**	**Obtained strain**
CEN.PK 102-5B	pYX212, pYX022, pYX242	CEN.PKc
BY4741	pYX212, pYX022, pYX242	BY4741c
BY4741	pYX212goxB opt, pYX022, pYX242	BY4741c *goxB* opt
BY4741Δ*MLS1*	pYX212, pYX022, pYX242	BY4741Δ*MLS1* c
BY4741Δ*MLS1*	pYX212goxB opt, pYX022, pYX242	BY4741Δ*MLS1* c *goxB opt*
BY4741Δ*DAL7*	pYX212, pYX022, pYX242	BY4741Δ*DAL7* c

CELL GROWTH AND BIOCONVERSION MEDIA COMPOSITION FOR BUTANOL AND ISOBUTANOL PRODUCTION

Independent transformants and the respective control strains (at least three for each transformation) were cultivated in shake flasks with ratio of flask volume/medium of 5/1 in minimal synthetic medium with 20 g/L of glucose and supplemented with glycine, glyoxylate, α-ketovalerate or α-isoketovalerate, as specifically indicated in the experiments. All strains were grown at 30°C on orbital shaker at 160 r.p.m for 72 hours.

Kinetic experiment

The butanol and isobutanol production starting from glycine was performed by kinetic experiment using Verduyn medium (Verduyn et al., 1992) with 20 g/L of glucose and glycine 15 g/L as substrate.

Bioconversion experiment

The bioconversion experiments were performed in two phases: 1) cells growth in YPD medium until the stationary growth phase, 2) after

centrifugation for 10 min at 4000 rpm the cells were inoculated in appropriate medium to perform the bioconversion phase. The medium for glyoxylate bioconversion (Figure 3A) experiment was minimal synthetic medium with 20 g/L of glucose and 5 g/L of glyoxylate at pH 2.5. The medium for α-ketovalerate (or α-isoketovalerate) bioconversion (Figure 5C) experiment was minimal synthetic medium with 20 g/L of glucose and 1.1612 g/L of α-ketovalerate (or α-isoketovalerate).

GENE AMPLIFICATION AND PLASMIDS CONSTRUCTION

The *B. subtilis goxB* gene was amplified with codone usage optimized for *S. cerevisiae* by Eurofins MWG Operon.

The amplified fragments were sub-cloned into the *Escherichia coli* vector pSTBlue (Novagen) obtaining the plasmid pSTBlue*goxB*. The complete sequence of *goxB* synthesized with codone usage adaptation for *S. cerevisiae* is reported in appendix 1. *GoxB* opt gene was expressed under the control of the *S. cerevisiae TPI*1 promoter using multicopy plasmid pYX212*goxB* opt, bearing the auxotrophic *URA*3 gene marker (R&D Systems, Inc., Wiesbaden, D).

For the construction of the plasmid pYX212*goxB* opt, the recipient vector was *EcoR*I cut, blunted and dephosphorylated, while the insert was *EcoR*I blunt excised from the Eurofins plasmid. DNA manipulation, transformation and cultivation of *E. coli* (Novablue, Novagen) were performed following standard protocols (Sambrook et al., 1989). All the restriction and modification enzymes utilised are from NEB (New England Biolabs, UK) or from Roche Diagnostics.

CELL GROWTH AND METABOLITES DETERMINATION

The cellular growth was spectrophotometrically monitored at 660nm and was reported as variation in the optical density (OD) as a function of time (h). The amount of extracellular glucose, butanol, isobutanol, glyoxylate and α-ketovalerate were determined by HPLC based method using H_2SO_4 5 mM as mobile phase and Aminex HPX-87P column, 300 x 7.8 mm with a polystyrene divinylbenzene-based matrix (BioRad). The glycine quantification was performed using a previously described assay (Stella et al., 2000).

DETERMINATION OF ENZYMATIC ACTIVITIES

Cells from exponential cultures were harvested by centrifugation at 4000 rpm for 10 min and washed with cold deionised water. The cell pellet was then re-suspended in 25 mM Tris-HCl pH 8.0 with protease inhibitor cocktail (Roche diagnostics, Cat. No. 04906837001) and 1 mM of phenylmethylsulfonyl fluoride (PMSF) and mechanically disrupted using glass microbeads (600 μm, Sigma-Aldrich). Cells debris was removed by centrifugation at 14000 rpm for 10 min at 4°C and the clarified crude extract was used for enzymatic analysis. The protein concentration in cell-free extracts was estimated according to Bradford (Bradford, 1976) using bovine serum albumin as reference.

Enzyme activities were measured on cell-free extracts by spectrophotometric assays. Activities were expressed as Units/mg of total proteins.

Glycine oxidase activity

Glycine oxidase activity was assayed spectrophotometrically via determination of H_2O_2 with an enzyme-coupled assay using horseradish peroxidase and *o*-dianisidine, as previously described with some modifications (Job et al., 2002). The assay was performed on a final volume of 1 ml in Tris-HCl 100 mM pH 8, phosphoric acid 10 mM, glycine 50 mM, *o*-dianisidine 1 mM, FAD 0.198 μM, horseradish peroxidase 14.72 U/mL, cell-free extract 8-10 mg/mL. The reaction was incubated at 37°C for 90 minutes and yellow colour, developed by *o*-dianisidine oxidation, was monitored at 530 nm. The glycine oxidase activity was expressed as U/mg of total proteins using the following equation:

Activity (U/mg prot tot) = (((OD 530nm/min)/ε)*dilution factor)/mg prot tot

Were $\varepsilon = 8.3\ mM^{-1}\ cm^{-1}$

One glycine oxidase unit is defined as the amount of enzyme that converts 1 mol of substrate (glycine) per minute at 25 °C.

Malate synthase activity

The malate synthase activity was performed as described in Sigma-Aldrich protocol [URL: http://www.sigmaaldrich.com], using acetyl-CoA (or butyryl-CoA) + glyoxylate. The assay take into consideration that the glyoxylate condensation with acetyl-CoA (or butyryl-CoA) produce malate (or β-ethylmalate) and CoA. The free CoA can react with the Ellman reagent DTNB (5,5'-Dithio-bis(2-Nitrobenzoic Acid)) which

reacts with free thiol groups, producing CoA-derivative and TNB (5-Thio-2-Nitrobenzoic Acid) (Ellman, 1959). The TNB produced quantity is in stoichiometric ratio of 1:1 with free thiol groups and was monitored spectrophotometrically at 412 nm.

β-isopropylmalate dehydrogenase activity using glyoxylate and butyryl-CoA as substrates

The β-isopropylmalate dehydrogenase enzyme catalyzes the NAD-dependent oxidation of the substrate with simultaneously conversion of NAD^+ to NADH. The activity was spectrophotometrically determined at 340 nm. The assay was performed on a final volume of 1 ml in cuvette with imidazole 50 mM pH 27 mM, $MgCl_2$ 10 mM, butyryl-CoA 0.125 mM, glyoxylate 0.5 mM, NAD^+ 1.575 mM. After incubation at 30°C for 10 min 60 µL of cell-free extract 8-10 mg/mL were added and increasing of absorbance at 340 nm was monitored for 10 min.

The β-isopropylmalate dehydrogenase activity was expressed as U/mg total proteins using the following equation:

Activity (U/mg prot tot) = (OD 340nm/min*dilution factor)/ε*Ev

Were ε is the millimolar extinction coefficient of NADH at 340 nm (6.22 mM^{-1} cm^{-1}) and Ev is the volume of cell extract used (expressed in millilitres).

Pyruvate decarboxylase activity using glyoxylate and butyryl-CoA as substrates

The pyruvate decarboxylase enzyme catalyzes the decarboxylation of ketoacid to form the derived aldehyde which is reduced by alcohol-dehydrogenase NADH-dependent activity. The conversion of NADH to NAD^+ is spectrophotometrically revealed at 340 nm.

The assay was performed based on pyruvate decarboxylase assay protocol of Sigma-Aldrich (URL: http://www.sigmaaldrich.com) with some modifications: imidazole buffer 34.25 mM, $MgCl_2$ 10 mM, butyryl-CoA 0.125 mM, glyoxylate 0.5 mM and NADH 0.16 mM were added in cuvette in a final volume of 1 mL. After incubation at 30°C for 10 min 20 µL of alcohol dehydrogenase enzyme solution and 20 µL cell-free extract 8-10 mg/mL were added. The decrease of absorbance at 340 nm was monitored for 15 min.

The activity was expressed as U/mg total proteins using the following equation: Activity (U/mg prot tot) = (OD 340nm/min*dilution factor)/ε*Ev

Were ε is the millimolar extinction coefficient of NADH at 340 nm (6.22 mM^{-1} cm^{-1}) and Ev is the volume of cell extract used (expressed in millilitres).

Chapter 1

Results

FINDING THE NATURAL WAY THAT YEASTS USE TO PRODUCE BUTANOL AND ISOBUTANOL FROM GLYCINE

In this session we describe how we hypothesized and subsequently demonstrated that butanol and isobutanol can be produced in *S. cerevisiae* through the glycine degradation pathway (**Figure 1**).

The first step is characterized by glycine conversion into glyoxylate, reaction described by Villas-Bôas et al. through *in silico* study (Villas-Bôas et al., 2005). The authors hypothesized that this reaction occurs to the glycine deaminase activity but up to date no genes annotation are present in *S. cerevisiae* which encodes for this enzyme. For the second step we hypothesized the glyoxylate condensation with butyryl-CoA to form the β-ethylmalate intermediate, similarly to what happens in *Pseudomonas aeruginosa* (Rabin et al., 1963). For the next step, once again, looking at metabolic reactions in other microorganisms, we found that the in *E. coli* the β-ethylmalate could be converted into α-ketovalerate through the β-isopropylmalate dehydrogenase enzyme (Shen & Liao, 2008). The last step has been well reported in literature because the Ehrlich pathway describes the α-ketoacid conversion into corresponding alcohol through the reactions catalyzed by α-ketoacid decarboxylase and alcohol dehydrogenase. Thus, the α-ketovalerate is converted into butanol through the reductive decarboxylation reaction (Hazelwood et al., 2008).

Summarizing, the single steps have been already described in literature, but until now they were not linked together in a pathway justifying butanol production from glycine. Here we describe and demonstrate that

not only there is a correlation between glycine and butanol production but additionally we indicate at least one possible gene encoding for the enzymatic reaction responsible of any single step of the novel pathway.

The hypothesized pathway results to be present independently from the genetic background, as we could prove using different *S. cerevisiae* strains, despite the production levels resulted to be different.

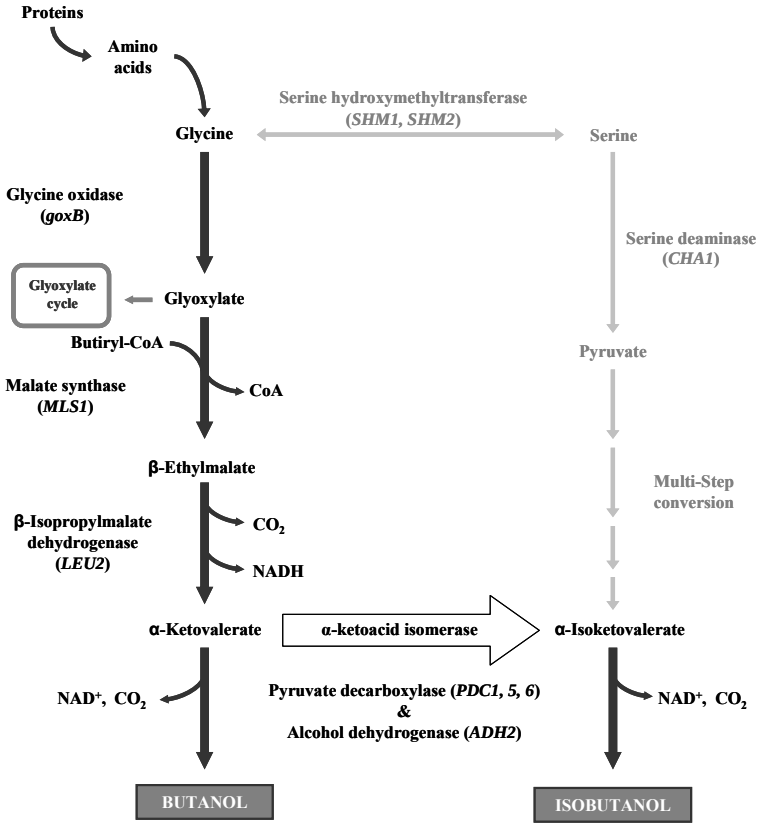


Figure 1. Glycine degradation pathway for the butanol and isobutanol production. Metabolic pathway for butanol and isobutanol production from

glycine in *S. cerevisiae* through the glyoxylate, β-ethylmalate and α-ketoacids intermediates. The enzymatic activities involved and the associated gene(s) are also represented

More in detail, the production levels obtained in the BY4741 genetic background were always lower than that obtained with the CEN.PK yeast strains, but still significant and reproducible. For this reason all the kinetic experiments to evaluate butanol and isobutanol titer reported relates only to the CEN.PK yeast genetic background. However, all the enzymatic activities experiment were performed using the BY4741 yeast strain due to the convenience of using for comparison and/or demonstration the single gene deletion mutants available from the Euroscarf collection.

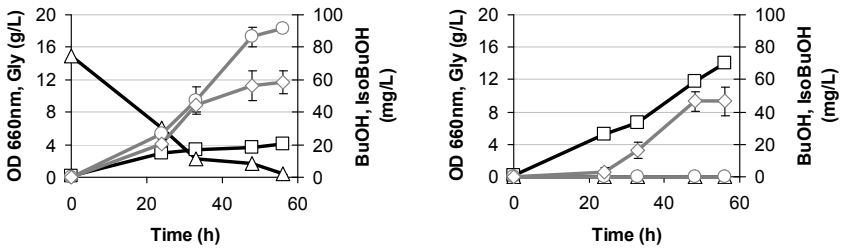


Figure 2. Butanol and isobutanol accumulation from glycine. Yeasts (CEN.PK background) were grown in Verduyn medium in presence of glycine 15 g/L as sole nitrogen source (left panel) and in presence of glycine 15 g/L and ammonium sulphate 5 g/L (right panel). Butanol (circle) and isobutanol (diamond) titer was monitored by HPLC at different time point from the inoculum. Biomass accumulation (square) and glycine consumption (triangle) are also reported. The data presented here are representative of three independent experiments. The standard deviations have a maximum variation of 10%.

Yeast cells were grown in Verduyn medium using glycine 15 g/L as sole nitrogen source to promote the glycine uptake, since the glycine is considered a poor nitrogen source respect to ammonium sulphate or glutamine or glutamate (Piper et al., 2002); butanol and isobutanol accumulation was evaluated at different time point after the inoculum (**Figure 2**). The cell growth was monitored as optical density at 660 nm and the metabolites were determined by HPLC method, while the glycine consumption was monitored through the ninhydrin-based colorimetric assay (Stella et al., 2000).

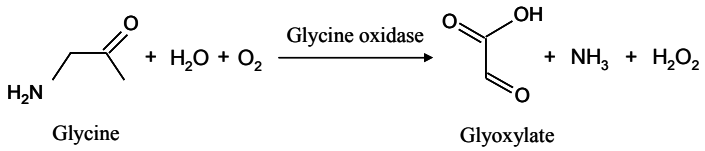
Using glycine as substrate, we obtained butanol and isobutanol accumulation, 92 mg/L and 58 mg/L respectively (Figure 2, panel left). As control experiment we verified the alcohols accumulation in presence ammonium sulphate 5 g/L (**Figure 2, right panel**). In this case almost 50 mg/L of isobutanol was obtained, indicating that: a) the butanol production requires the glycine as substrate, b) the isobutanol obtained through the glycine degradation pathway in part derives, as expected, from other pathway such as serine degradation. In fact, if serine (20 g/L) was used as substrate, almost 80 mg/L of isobutanol were present in the supernatant after 24 hour after the inoculum (data not shown) and no butanol was detected.

The following sections describe the pathway step by step. In particular, the substrates used are glycine, glyoxylate, α-ketovalerate and α-isoketovalerate. Unfortunately the intermediate β-ethylmalate is not commercially available and for this reason this step was characterized by coupling two reactions and using glyoxylate as substrate.

THE FIRST REACTION OF THE PATHWAY: FROM GLYCINE TO GLYOXYLATE

The first step of glycine degradation is its conversion into glyoxylate, by a glycine deaminase activity (Villas-Bôas et al., 2005), which has not been annotated yet. Being the identification of a putative gene encoding for the desired function not trivial, we searched a similar activity in other microrganisms, finding out that in *Bacillus subtilis* the glycine conversion in glyoxylate is catalyzed by the glycine oxidase enzyme, encoded by *goxB* gene (**Figure 3A**). This enzyme catalyzes the primary amines oxidative deamination to form the corresponding α-ketoacids, with concomitant ammonium and hydrogen peroxide production.

A



B **C**

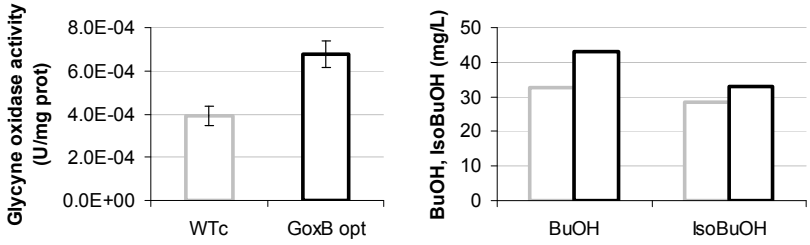


Figure 3. Yeast and bacterial glycine oxidase: reaction, enzymatic assay and butanol and isobutanol production. (A) Reaction catalyzed by glycine

oxidase. (B) Glycine oxidase activity for the WTc (grey) and modified (black) yeast strain, overexpressing the bacterial *goxB* gene optimized for the yeast codon usage. The activity is reported as U/mg total proteins. The data presented here are representative of two independent experiments. (C) Butanol and isobutanol accumulation by WT (grey) and modified (black) yeast strain.

The *B. subtilis* glycine oxidase is homotetrameric flavoprotein which effectively catalyzes the oxidation of sarcosine (N-methylglycine), N-ethylglycine and glycine. Lower activities on D-alanine, D-valine, and D-proline were detected although no activities were shown on L-amino acids and other D-amino acids (Nishiya & Imanaka, 1998). The *B. subtilis goxB* gene was synthesized with optimized codone usage for *S. cerevisiae* (*goxB* opt) and it has been expressed in BY4741c yeast strain. The heterologous enzymatic activity was tested using *in vitro* assay for glycine oxidase, as described in material and methods section (Job et al., 2002) (**Figure 3B**). As expected, both strains (the control, transformed with empty vector and the *goxB* opt overexpressing strain) show the desired activity, and this turned out to be 1.5 fold higher in the recombinant yeast in respect to the control strain. It has to be mentioned that, according to literature data (Pedotti et al., 2009), the measured activity resulted to be quite low. Nevertheless, also the activity measured in our positive control (total protein extracted from *E. coli* BL21 strain overexpressing the *B. subtilis goxB* gene) was low (~ 4.E-03 U/mg proteins, data not shown), suggesting that the activity assay protocol might be still optimized.

We preliminary tested the effect of glycine oxidase overexpression on butanol and isobutanol accumulation, using two different genetic

backgrounds: CEN.PK and BY4741 yeast strains both transformed with plasmid containing the *B. subtilis goxB* opt gene (data not shown).

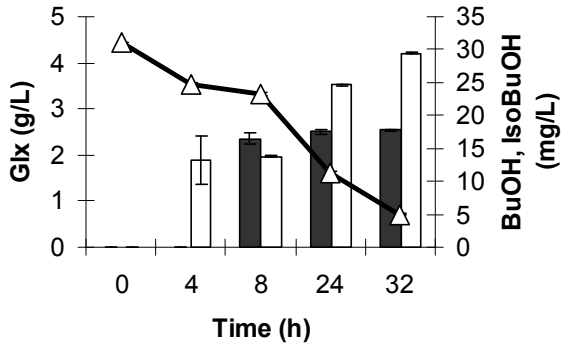
Both *goxB* overexpressing and the control strains were grown in Verduyn medium using glycine 15 g/L as sole nitrogen source. The best alcohols accumulation was obtained after 56 h after the inoculum: the butanol and isobutanol titer obtained with modified yeast strain was higher respect to those obtained with the control strain (**Figure 3C**). The butanol accumulation was 43.2 mg/L *versus* 32.7 mg/L while the isobutanol accumulation was 33 mg/L *versus* 28.6 mg/L for the modified and the control strain, respectively. This data, despite preliminary, suggested that the overexpression of a glycine oxidase can have a positive effect, at least on butanol accumulation. More importantly, with the described experiment we could prove that an activity responsible of glycine conversion into glyoxylate is the first step leading to butanol accumulation, and we also show a first example of how to improve said activity, with a direct positive effect on product accumulation. Despite the levels of butanol can appear quite low, they are much higher than the one obtained in recombinant yeasts up to now (Steen et al., 2008).

THE CHARACTERIZATION OF GLYOXYLATE - β-ETHYLMALATE STEP

If our hypothesized pathway is correct, also glyoxylate can be converted into butanol and isobutanol by yeasts. Yeast cells were grown in minimal medium in the presence of different glyoxylate concentration (0, 0.5, 1, 5 g/L) and pH (2.5 and 5.5) to determine the best growth and production

conditions (data not shown). Here we reported the butanol and isobutanol accumulation obtained with glyoxylate 5 g/L at pH 2.5 (**Figure 4A**), value near to glyoxylic acid pKa to promote the diffusion of glyoxylate inside the cells, since no carrier for this metabolite is reported. In this condition almost the total amount of glyoxylate was consumed, as reported in the figure (see line with triangle). The cells were able to accumulate butanol and isobutanol during the time with titer respectively of ~ 18 and 30 mg/L. This is important evidence that confirms the involvement of glyoxylate intermediate in the formation of desired alcohols, considering only the endogenous capacity of the cells.

A



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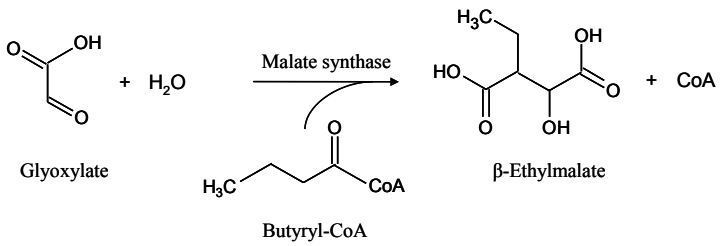

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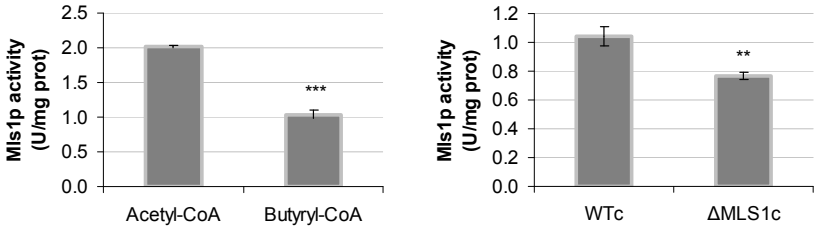


Figure 4. Glyoxylate bioconversion into butanol and isobutanol and malate synthase activity involvement in alcohols production. (A) Butanol (dark grey) and isobutanol (white) production as well as glyoxylate consumption (triangle) was reported at different time point during the bioconversion phase. The data are representative of two independent experiments. (B) Glyoxylate conversion reaction performed by malate synthase enzyme. (C) Malate synthase activity for the glyoxylate conversion in β-ethylmalate intermediate. (Left panel) The malate synthase activity was tested using acetyl-CoA and butyryl-CoA as donor group. (Right panel) The *MLS*1 deletion effect on the enzymatic activity was also reported. The data presented here are representative of three independent experiments. $p \leq 0,05$ = *; $p \leq 0,01$ = **; $p \leq 0,001$ = ***; $p > 0,05$ = n.s.

In yeast no activity able to catalyze the condensation of glyoxylate with butyryl-CoA to form β-ethylmalate has been described yet. However, the malate synthase enzyme catalyzes a similar reaction: the glyoxylate condensation with acetyl-CoA to form malate (Hartig et al., 1992). Two isoforms of malate synthase activity are described, encoded respectively by *MLS*1 and *DAL*7 genes (Hartig et al., 1992). The first encodes for an enzyme which is expressed when non fermentable carbon sources, such as fatty acid, ethanol or acetate, are present in the medium and its expression is repressed in the presence of glucose. The second encodes for an enzyme which participates in nitrogen metabolism, to recycle the

glyoxylate generated during allantoin degradation. *DAL*7 expression is controlled by nitrogen source present in the medium: it is repressed in the presence of rich nitrogen sources, such as asparagine and ammonium, and derepressed in the presence of poor nitrogen sources such as proline (Hartig et al., 1992).

Since the malate synthase activity catalyzes the condensation of glyoxylate with acetyl-CoA to form malate during the glyoxylate cycle, we evaluated if also butyryl-CoA could be a substrate of malate synthase enzyme. Yeasts were grown in YPD medium and malate synthase activities were detected in the presence of both acetyl-CoA and butyryl-CoA as acyl-CoA donor, using a modified TNB-based assay as described in Materials and Methods section (**Figure 4C, left panel**) (Sigma-Aldrich protocols, URL: http://www.sigmaaldrich.com). The YPD medium was chosen to have a cultural condition in which both isoforms Mls1 and Dal7 were present at similar level, as reported in literature (Hartig et al., 1992). Remarkably, the malate synthase activity was detected even when butyryl-CoA was added as donor, even if at lower value if compared to the activity measure in the presence of acetyl-CoA (1 U/mg proteins versus 2 U/mg proteins, respectively). This data has to be considered as the first experimental evidence that the yeast malate synthase enzyme can also accept butyryl-CoA as substrate. The deletion of *MLS*1 gene reduces the activity of 25% (**Figure 4C, right panel**) and the same activity impairment is caused also by *DAL*7 gene deletion (data not shown), suggesting that the single deletion is not sufficient to nullify the malate synthase activity, or in other words that the two enzymes might similarly

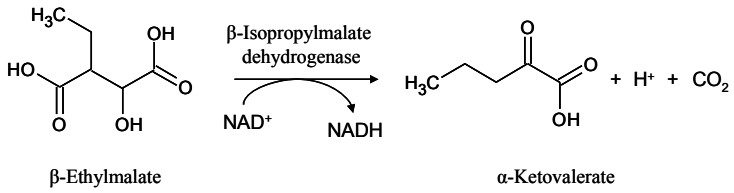
contribute to the reaction of interest. It has to be said that with this specific experiment we could not prove that β-ethylmalate was generated, but this will appear evident in the next paragraph.

THE CHARACTERIZATION OF B-ETHYLMALATE – α-KETOVALERATE STEP

In *E. coli* the β-isopropylmalate dehydrogenase, encoded by *LeuB* gene, is able to catalyze the conversion of β-isopropylmalate into α-ketoisocaproate. Shen and Liao (2008) have demonstrated the possibility to use this enzyme to additionally catalyze the conversion of β-ethylmalate into α-ketovalerate in *E. coli* (**Figure 5A**) (Shen & Liao, 2008). The homologous gene identified in *S. cerevisiae* is the *LEU*2 gene (Kohlhaw, 1988). We thus tested the Leu2p activity by using the strain BY4741 auxotrophic for leucine because deleted in the *LEU*2 gene and the same BY4741 strain in which *LEU*2 was overexpressed by transforming it with a multicopy plasmid bearing *LEU*2 as selection marker (**Figure 5B**). The activity assay was performed using two coupled reactions since β-ethylmalate is not commercially available. In the first reaction glyoxylate and butyryl-CoA should be converted into β-ethylmalate by the malate synthase activity, as described in the previous paragraph. The second coupled reaction catalysed by the Leu2p activity utilizes the β-ethylmalate produced to generate the α-ketovalerate. Said activity is spectrophotometrically determined due to NADH produced during the reaction.

In the strain overexpressing the *LEU*2 gene, the activity was ~5 fold higher if compared to the *LEU*2 deleted strain, 0.1 and 0.02 U/mg proteins, respectively. This also could indirectly demonstrate that β- ethylmalate is produced by the previous reaction.

A



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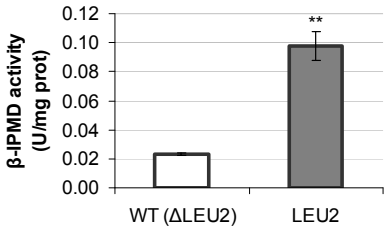


Figure 5. The β-isopropylmalate dehydrogenase involvement in the glycine degradation pathway. The BY4741 yeast cells were grown in YPD medium to extract total proteins. The activity was tested using glyoxylate as substrate instead of β-ethylmalate (not commercial available), as described in Materials and Methods section. (A) Reaction catalyzed by the β-Isopropylmalate dehydrogenase enzyme. (B) Activity measured in yeast strain auxotrophic for the *LEU*2 gene (white) and overexpressing *LEU*2 (grey) was reported as Units per milligram of total proteins. The data presented here are representative of three independent experiments. $p \leq 0,05$ = *; $p \leq 0,01$ = **; $p \leq 0,001$ = ***; $p > 0,05$ = n.s.

Interestingly, when *LEU*2 gene is deleted, a residual activity was detected, suggesting that other(s) activity(ies) could be responsible for this conversion.

The β-isopropylmalate dehydrogenase activity was only detected if either Mls1p (or Dal7p) and Leu2p are present, as reported in table 1. In fact, if Mls1p (or Dal7p) is deleted while Leu2p is present, the activity significantly decreased as well as in the case in which Mls1p (and/or Dal7p) is present and Leu2p is deleted.

Table I. Effect of *MLS*1 or *DAL*7 gene deletion coupled or not with *LEU*2 overexpression on β-Isopropylmalate dehydrogenase activity measured with assay that couples both reactions.

Gene			β-IPMD
MLS1	*DAL7*	*LEU2*	Activity (U/mg prot)
+	+	-	0.023 ± 0.001
+	+	++	0.098 ± 0.010
-	+	-	0.018 ± 0.002
-	+	++	0.021 ± 0.002
+	-	-	0.016 ± 0.002
+	-	++	0.019 ± 0.002

The data presented here are representative of three independent experiments.

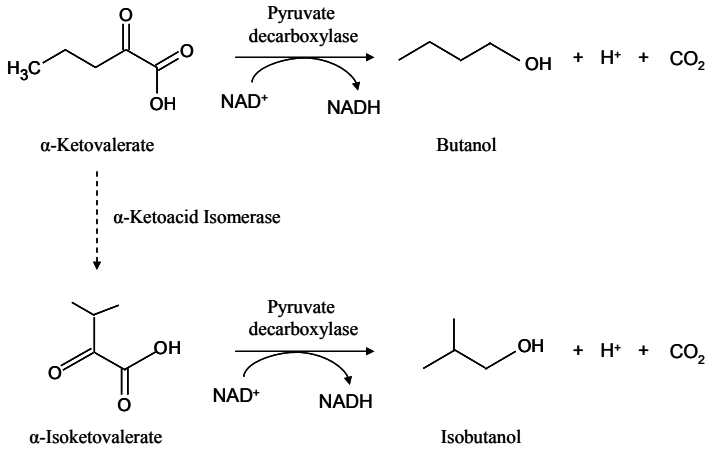
This data suggest that the presence of *LEU*2 gene coupled with malate synthase activity is necessary for glyoxylate conversion into α-ketovalerate.

THE CHARACTERIZATION OF LAST STEP: α-KETOACIDS CONVERSION INTO BUTANOL AND ISOBUTANOL

The last step in the glycine degradation to produce the desired alcohols is the reductive decarboxylation of α-ketovalerate in butanol and of α-isoketovalerate in isobutanol (**Figure 6A**). In literature the conversion of α-ketovalerate into α-isoketovalerate occurs through dehydratation reaction, catalyzed by dihydroxyacid dehydratase enzyme, (Villas-Bôas et al., 2005) but until now no experimental evidence are present. Moreover, by looking at the chemical structure of the two ketoacids, in our opinion this reaction can not occur via dehydratation, but might require an isomerisation reaction.

The conversion of α-ketovalerate into butanol requires two reactions: in the first one the aldehyde is formed after α-ketovalerate decarboxylation, in the second one the aldehyde is reduced to butanol. In literature is reported that the pyruvate decarboxylase (Pdc) is able to decarboxylase the α-ketoacids involved in the production of higher alcohols such as α-isoketovalerate, α-keto-β-methylvalerate and α-ketoisocaproate (ter Schure et al., 1998) but no data are available about the activity of Pdc on α-ketovalerate. The Pdc involvement was reported by Brat et al. for the isobutanol production, starting from valine through the α-isoketovalerate intermediate formation (Brat et al., 2012). The three Pdc enzymes are mainly involved in pyruvate decarboxylase in the ethanol fermentation pathway and for avoiding this, the authors replaced the *PDC* genes with decarboxylase encoded by *ARO*10 gene, which has no activity on pyruvate (Brat et al., 2012).

A



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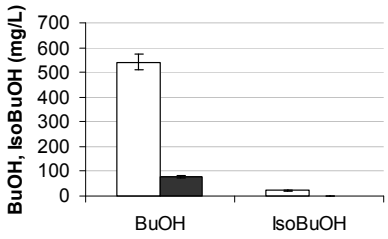

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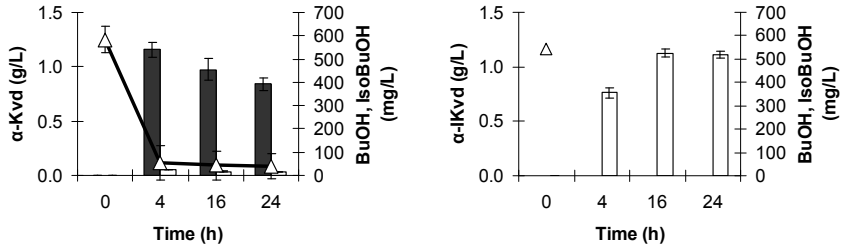

Figure 6. α-Ketoacids bioconversion step and pyruvate decarboxylase activity involvement in alcohols production. The yeast cells were grown in minimal medium using α-ketovalerate and α-isoketovalerate 1.1612 g/L (10

mM) as substrate for the bioconversion. (A) α-ketovalerate and α-isoketovalerate conversion reaction performed by pyruvate decarboxylase enzyme. (B) Butanol (dark grey) and isobutanol (white) production as well as α-ketovalerate consumption (left panel, triangle) was reported at different time point during the bioconversion phase. The α-isoketovalerate consumption (right panel, triangle) was not reported because the compound was undetectable. (C) Pyruvate decarboxylase deletion effect on butanol and isobutanol accumulation. Wild type (white) and *PCD*1, 5, 6 deleted strains (grey). The data are representative of three independent experiments.

We first tested if the yeast pyruvate decarboxylase activity is able to accept α-ketovalerate as substrate by using CEN.PKc yeast strain. The cells were grown in YPD medium and total protein extract was used to evaluate the decarboxylase activity. We used pyruvate, α-ketovalerate and α-isoketovalerate as substrates and the activities measured were 722, 1.75 and 0.3 U/mg proteins, respectively.

The involvement of pyruvate decarboxylase in the last reaction was also demonstrated by measuring the butanol and isobutanol production in a *PDC*1, 5, 6 deleted strain (**Figure 6B**). The cells were grown in YPD medium until the stationary phase and then they were collected, and incubated at 25 OD in minimal medium in the presence of α-ketovalerate (or α-isoketovalerate, data not shown) 1.1612 g/L (10 mM) as substrate, to perform the bioconversion reaction. The deletion of all three isoforms of pyruvate decarboxylase significantly decreases the butanol and isobutanol production. The butanol titer obtained was 5 times lower respect to those obtained in the wild type strain, 118 mg/L versus 583 mg/L respectively.

It is important to underline that in the presence of α-ketovalerate as substrate both butanol and isobutanol are produced (**Figure 6C, left**

panel) in yeast wild type strain. This data confirms that α-isoketovalerate can be generated starting from α-ketovalerate and that this reaction is probably irreversible, as in the presence of α-isoketovalerate only isobutanol accumulation was observed (**Figure 6C, right panel**).

THE GLYOXYLATE CONVERSION INTO BUTANOL AND ISOBUTANOL REQUIRES Mls1p, Leu2 AND Pdc(S) ACTIVITIES

According to the proposed pathway, based on literature and to the data presented up to here, during the conversion of glyoxylate into butanol and isobutanol two intermediates could be formed, β-ethylmalate and α-ketovalerate. We performed an *in vitro* assay coupling all the reactions to further demonstrate the linked reactions in the proposed pathway.

We tested the effect of the deletion/overexpression of *LEU*2 gene, coupled or not with the deletion of *MLS*1 gene on the pyruvate decarboxylase activity, using glyoxylate and butyryl-CoA as substrates, as described in Materials and Methods section (**Figure 7**). In this way all three reactions are coupled in the same assay in which the pyruvate decarboxylase activity was measured as increment of OD340 nm, since NADH is produced during the last reaction.

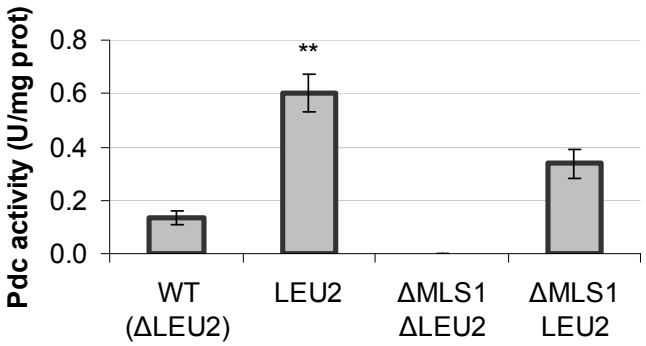

Figure 7. The evidence of glyoxylate degradation through the Mls, β-IPMD and Pdc(s) activities. The BY4741 yeast background cells were grown in YPD medium to extract the total proteins. The WT auxotrophic for *LEU*2 gene was used as control strain while the strain indicated as *LEU*2 is the *LEU*2 gene overexpressing yeast strain. The *MLS*1 deletion effect was evaluated coupled with the *LEU*2 deletion or overexpression (Respectively indicated as *ΔMLS*1*ΔLEU*2 and *ΔMLS*1 *LEU*2). The data presented here are representative of three independent experiments. $p \leq 0,05$ = *; $p \leq 0,01$ = **; $p \leq 0,001$ = ***; $p > 0,05$ = n.s.

The pyruvate decarboxylase activity was detected only in the presence of *LEU*2, indicating its important role in the pathway. Infact if *LEU*2 gene was deleted, the activity was 4.3 times lower respect to the activity levels detected when *LEU*2 was overexpressed. The double deletions of *MLS*1 and *LEU*2 have a strong negative effect on Pdc activity measured in the presence of glyoxylate and butyryl-CoA as substrates. The presence of *LEU*2 gene in strain harbouring single deletion of *MLS*1 (or *DAL*7, data not shown) results in higher Pdc activity than the wild type strain in which *LEU*2 gene is deleted, due to the presence of other enzymatic isoform. Infact when *MLS*1 is deleted, Dal7p is able to replace its function and vice versa (Hartig et al., 1992).

Chapter 1

Conclusions

This study described a possible novel pathway to produce butanol and isobutanol in yeast *S. cerevisiae* through the glycine degradation pathway. We characterized the entire pathway identifying for each step at least one enzymatic reaction with relative gene. This data has to be considered as prior art (Patent Application currently under deposit).

Even if the obtained amount of butanol is very little (92 mg/L), it is important to consider that the best butanol production reported for engineered *S. cerevisiae* was only 2.5 mg/L (Steen et al., 2008). Moreover, in our case the final product was obtained through endogenous activities which in general are involved in other reactions. Thus, it is possible to imagine optimize the entire pathway overexpressing the enzyme involved in butanol and isobutanol production as well as other enzymes with best reaction specificity to obtain high alcohols production. Nevertheless since the production of higher alcohols starting from glycine it is not sustainable process we could exploit the glycine degradation pathway for the production of biofuels from protein hydrolysates. Infact proteins could be used as a "no-cost" raw material since they are waste products derived from the traditional production of biofuels from sugar. During this process of production a considerable fraction of proteins are accumulated and currently is not fully absorbed by the market (Huo et al., 2011). The proteins hydrolysis can be a huge source of aminoacids including the glycine, the substrate of our pathway, but also others such as alanine, cysteine, serine and threonine which can be converted into pyruvate and then in glycine with appropriate metabolic engineering modifications (e.g., *GLY*1 overexpression) or can be used to produce a

mix of higher alcohols (Huo et al., 2011). In order to increase the yields of production of interested alcohols it is important to consider also the possibility to expand the starting substrates. Also in this case appropriate metabolic engineering modifications are required to increase the carbon flow toward glycine accumulation and consequently in the butanol and isobutanol through the glycine degradation pathway.

APPENDIX 1

Sequence of synthesized *goxB* gene with codone usage optimized for *Saccharomyces cerevisiae*

ATGAAGAAACACTACGACACTGCAGTTATAGGTGGAGGGATCATTGGTTGTG
CGATATCGTACGAATTGGCCAAAACTCAACAGAAGGTTGTCCTGCTAGAAGCTGGAGAA
GTAGGTAGAAAGACTACTAGTGCTGCTGCTGGAATGCTTGGAGCTCATGCCGAATGCGA
AAACAGGGATGCTTTCTTTGACTTTGCCATGCACTCACAAAGGCTTTATGAACCAGCAG
GGCAAGAATTGGAAGAAGCATGTGGTATTGATATTAGACGTCATAATGGCGGAATGTTG
AAGTTAGCCTATACGGAAGAGGATATTGCCTGTTTAAGAAAGATGGATGATTTACCTAG
CGTTACCTGGTTGTCTGCTGAAGATGCATTGGAGAAGGAACCTTATGCATCGAAAGACA
TACTAGGTGCATCCTTTATAAAAGATGATGTGCACGTAGAACCGTATTATGTCTGCAAA
GCCTACGCTAAAGGGGCTAGGAGATATGGTGCTGACATTTACGAACACACACAAGTCAC
CTCAGTGAAAAGAATGAACGGAGAGTATTGCATCACAACATCAGGTGGAGATGTTTATG
CCGACAAGGTTGCAGTTGCTTCTGGTGTATGGTCTGGTCGTTTCTTTTCCCAGTTAGGT
TTAGGTCAACCATTCTTTCCAGTAAAAGGCGAGTGTTTGAGTGTTTGGAATGACGATAC
CCCATTAACCAAGACTCTTTACCATGACCATTGTTACGTGGTTCCAAGAAAGTCCGGCA
GATTGGTCATTGGTGCCACTATGAAACATGGTGATTGGTCTGATACACCTGACATTGGT
GGCATTGAAGCTGTGATTGGTAAGGCGAAAACGATGCTACCAGCAATTGAGCACATGAA
AATCGATAGATTTTGGGCGGGTTTAAGACCGGGAACAAGAGATGGCAAACCCTTCATTG
GGAGACATCCCGAAGATAGCGGCATAATCTTTGCAGCCGGTCATTTCAGAAATGGCATA
CTGCTGGCTCCTGCAACAGCTGAAATGGTCAGAGACATGATCTTGGAACGTCAGATAAA
ACAAGAGTGGGAAGAGGCATTTAGGATCGATAGAAAAGAGGCGGTTCATATCTAA

Chapter 1

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CHAPTER 2

DIFFERENT WAYS OF APPROACHING STRESSES RELATED TO BUTANOL PRODUCTION IN THE YEAST *Saccharomyces cerevisiae*

Chapter 2

INTRODUCTION

The toxicity of the final product is one of the most common stresses that microorganisms have to face during productions. This is particularly true for low added value products, which has to reach very high concentrations, rendering the cellular responses mechanisms insufficient (Sauer et al., 2008; Porro et al., 2011). Biofuels are a prominent example of low added value products which result toxic to the cells and according to the molecule and to the microorganism used for its production the detrimental effect occurs at very different final concentrations. Ethanol, which is the most abundantly biofuel produced by microbial fermentation (about 80 million tons/year (Porro et al., 2011)), can be efficiently and naturally produced by different microorganisms, and among bacteria *Zymomonas mobilis* is a prominent example (Arcuri, 1982). However the sole microorganism used nowadays for industrial ethanol production is *S. cerevisiae*, because of its high ethanol tolerance and its capability to produce high concentrations (over 100 g/L) with yields so high to be close to the theoretical values (Porro et al., 2011).

In recent years a "higher alcohol", a molecule with more carbon and hydrogen atoms than ethanol, is receiving increasing attentions, since the useful energy to be released during combustion is stored in the carbon–hydrogen bonds. With its four carbons, butanol has more energy than ethanol and it is also less corrosive in respect to ethanol. Due to its physical properties, the four-carbon alcohol could better replace the gasoline than ethanol (Savage, 2011).

Clostridia are the natural producers and the first microorganisms that have been used for laboratory (Dürre, 2008) and industrial scale butanol

production (Dong et al., 2012). A number of plants have been built in China for butanol production with the ABE process (Dong et al., 2012). These Gram-positive anaerobes are able to produce butanol with other co-produced metabolites such as butyric acid, acetone, ethanol, lowering the butanol yield (Jones & Woods, 1986).

Although there are organisms that naturally produce butanol (e.g., *Clostridia*) and higher alcohols, most of them make those alcohols in tiny quantities due to their low alcohol tolerance. *Clostridia*, for example, are very sensitive to the butanol accumulation over a concentration of 1-1.5% (Jones & Woods, 1986). The engineered *Clostridia* strains tolerate butanol to a maximum of almost 2% (Tomas et al., 2003) but it is important to consider that butanol-tolerant mutants do not necessarily produce more butanol than the lesser tolerant strains (Ezeji et al., 2004; Papoutsakis, 2008; Zhu et al., 2011; Liu et al., 2012).

The low butanol tolerance of *Clostridia* species, even when engineered, is among the prominent reasons for using other microorganisms. Because of their higher tolerance, the butanol production from yeasts seems to offer interesting opportunities, despite they are not natural producers. The *S. cerevisiae* is able to naturally tolerate high alcohol concentrations (Ding et al., 2009). Moreover, since yeast cells are robust against adverse fermentation conditions such as high sugar concentrations, the hypothetical butanol process of production might be much easier compared to other process using bacteria as cell factory, which have not these desiderated characteristics (Hong & Nielsen, 2012).

There are several comprehensive reviews on the microbial solvent tolerance, and an overview of these mechanisms is also has been reviewed (Isken & de Bont, 1998; Nicolaou et al., 2010; Rutherford et al., 2010; Dunlop, 2011; Dunlop et al., 2011;).

The accumulation of solvents in the membrane has several consequences (**Figure 1**): it increases the permeability of the membrane, diminishes energy transduction, interferes with membrane protein function, and increases fluidity. Moreover the increase in membrane permeability can allow the release of ATP, ions, phospholipids, RNA, and proteins.

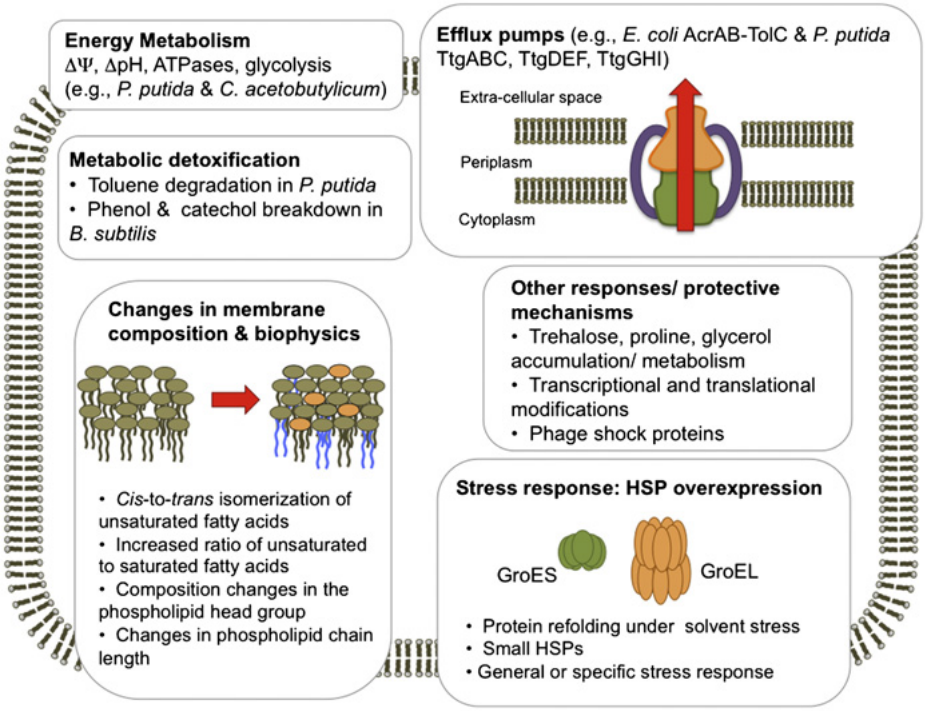

Figure 1. Illustration of the main cellular responses that might provide tolerance to prokaryotes (Gram-negative and/or Gram-positive) and/or yeast cells. Toxic

solvents induce numerous responses to combat stress and enhance survivability (Nicolaou et al., 2010).

Solvents in the cell membrane can also negatively affect membrane protein function, further interfering with essential cellular processes such as nutrient transport. Finally, the increase in membrane fluidity changes the stability and structure of the cell membrane.

The butanol tolerance of *S. cerevisiae* is at least 2% (Liu & Qureshi, 2009), data confirmed also in our laboratory in previous work (Nadia Berterame Master thesis). However, little is known about the mechanisms of said tolerance, rendering the design of further improvement quite difficult. Lorenz et al., using a screen for butanol insensitive *S. cerevisiae* mutants, have identified proteins involved in the polarized growth, mitochondrial function, and a transcriptional regulation. They also found that butanol stimulates filamentous growth in haploid cells and induces cell elongation and changes in budding pattern, leading to a pseudohyphal morphology (Lorenz et al., 2000). It was also reported that in the presence of alcohols, shifts in lipid composition to more saturated and unbranched fatty acid chains were observed.

To get good butanol production, cells need to be healthy, robust to the process of production and to the final desired product. For this reason an increased butanol tolerance of *S. cerevisiae* have to be pursued. To increase the butanol tolerance in *S. cerevisiae*, we concentrated our study on the possible oxidative stress caused by butanol, similarly to what ethanol causes to the cells (Jamieson, 1998).

In literature it has been reported that the glycine somministration in hepatotoxic rats reduces the oxidative stress in terms of lipids peroxidation and increases the levels of antioxidants such as reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase (Senthilkumar et al., 2004). When the glycine was somministrated in human intestinal cells before the oxidative stress treatment, the cells resulted protect from oxidative damages induced by tert-butylhydroperoxide, reducing the intracellular concentration of reactive oxygen species (Howard et al., 2010).

About yeasts, Thomas et al (1994) have demonstrated that in the yeast *S. cerevisiae* the addition of glycine during high glucose concentration fermentation determines a better sugars utilization, a higher growth rate and cellular viability along the time (Thomas et al., 1994). It was also found that the ethanol tolerance increases when yeast cells were exposed to ethanol solution containing three amino acids: isoleucine, methionine, and phenylalanine (Hu et al., 2005). Moreover, it has been reported that a significant increase in the yeast ethanol tolerance was due to the incorporation of the supplementary methionine, isoleucine and phenylalanine amino acids into the plasma membranes that subsequently led to the enhanced ability for plasma membranes to efficiently counteract the fluidizing effect of ethanol when subjected to this environmental stress (Ding et al., 2009). Similarly, another study identified that also L-proline accumulation can improve yeast ethanol tolerance (Takagi et al., 2005). Being an osmoprotectant, L-proline protects yeast cells from damage caused by freezing, desiccation, or oxidative stress.

Recently it has been reported the involvement of metallothioneins (MTs) proteins in the butanol tolerance. These proteins are broadly defined as a class of low-molecular-weight cysteine-rich proteins which bind heavy metals, widely distributed throughout living organisms, transcriptionally induced in response to the treatment of cells with appropriate metals. The large diversity of inducing factors for MTs biosynthesis implicates these proteins in a variety of cellular functions, from metal tolerance and homeostasis to protective role against apoptosis (Thirumoorthy et al., 2007). The MTs role in the butanol tolerance has been demonstrated in engineered *E. coli* overexpressing a *Tilapia* MT protein, resulting in better grow in presence of butanol (Lin et al., 2011).

In *S. cerevisiae* two copper-activated MT proteins (Cup1 and Crs5) are able to bind and sequester Cu^{2+} ions, providing the principal method of removing this metal ion from the cell.

The Cup1p transcription in yeast is specifically induced by the copper-dependent transcription activator Ace1p in response to high levels of Cu^{2+} ions. Moreover, MTs genes are transcriptionally regulated in response to heat shock, glucose starvation and oxidation stress. The last one, for example, can result not only from pollutants, but also from chemical or physical stressors such as some alcohols and organic acids (Lin et al., 2011).

In this section we report our preliminary studies about the butanol tolerance in *S. cerevisiae.* We investigated the effect of glycine on yeast cells tolerance in presence of butanol and other stressor agents such as

hydrogen peroxide, ethanol, isobutanol and acetic acid. We also studied the possible role of yeast MTs proteins, Cup1p and Crs5p, in the butanol and isobutanol tolerance.

Chapter 2

Materials And Methods

STRAINS AND GROWTH CONDITIONS

The *S. cerevisiae* strains used in this study BY4741 *(MATa, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1)* BY4741Δ*CRS*5 (BY4741; *Mat a; his3D1; leu2D0; met15D0; ura3D0; YOR031w::kanMX4*) and BY4741Δ*ACE*1 (BY4741; *Mat a; his3D1; leu2D0; met15D0; ura3D0; YGL166w::kanMX4*) are provided by EUROSCARF deleted strain collection (EUROSCARF collection, Heidelberg, Germany). Cells were grown in rich YPD medium in shake flasks at 30°C (agitated at 160 r.p.m), with ratio of flask volume/medium of 5/1. When required, the stressor agents, butanol 1.25% (v/v), isobutanol 2% (v/v), ethanol 7% (v/v), hydrogen peroxide 3 mM or acetic acid 3.5 g/L pH3, were added to the medium at indicated concentration (see Results section).

SPOT TEST ASSAY

The yeast cells were grown in minimal synthetic medium in presence or absence of glycine 15 g/L. After 24 hours of growth, 5 μL of 1 OD cells 1:10 serial diluted were spotted on YPD (or minimal glucose synthetic medium) with or without different stressor agents, as reported in the experiment. Plates were incubated at 30°C until the appearance of growth, which was monitored at least for two days.

DETERMINATION OF LIPIDS PEROXIDATION WITH TBARS ASSAY

Determination of Thio-Barbituric Acid Reactive Substances TBARS to evaluate lipids peroxidation was carried out following the procedure previously described (Lefèvre et al., 1998). Cells exponentially growing on either YPD or YPD + stressor agent were harvested, mechanically disrupted and the crude extract mixed with a solution containing 15 % (w/v) TCA, 0.375 % (w/v) thiobarbituric acid and 0.25 % (v/v) HCl. After boiling and cooling, samples were centrifuged and the presence in the supernatant of malondialdehyde (MDA), a product of lipid peroxidation, was monitored spectrophotometrically at 532 nm. Results were expressed as μM MDA/g biomass.

Chapter 2

RESULTS

THE GLYCINE EFFECT ON ALCOHOLS TOLERANCE

As said before, in literature it has been reported how in mammalian and in yeast cells some aminoacids prevent and/or increase the cells robustness.

In the previous section we identified an alternative pathway to produce butanol and isobutanol starting from glycine. Could be the glycine both substrate and protective agent for the cells?

The BY4741 cells were grown for 24 hours in minimal medium in the presence or absence of glycine 15 g/L. After growth, cells were spotted in presence of different stressor agents that are often present during the process of production such as hydrogen peroxide (to mimic oxidative stress), acetic acid, ethanol, butanol and isobutanol (**Figure 2**). Different concentrations were first tested to find those that do not entirely inhibit the cellular function (data not shown).

The glycine pre-treated cells are able to grow better in presence of all stressor agents tested, even if in presence of butanol and isobutanol the differences in cell growth are less appreciable respect to the other stressor agents.

This represents the first experimental evidence that glycine can increase the tolerance to different stressor agents in *S. cerevisiae*. More have to be elucidated about this phenomenon, for example if it is due to the presence of glycine alone or if it is a more general response to the presence of aminoacids in the medium.

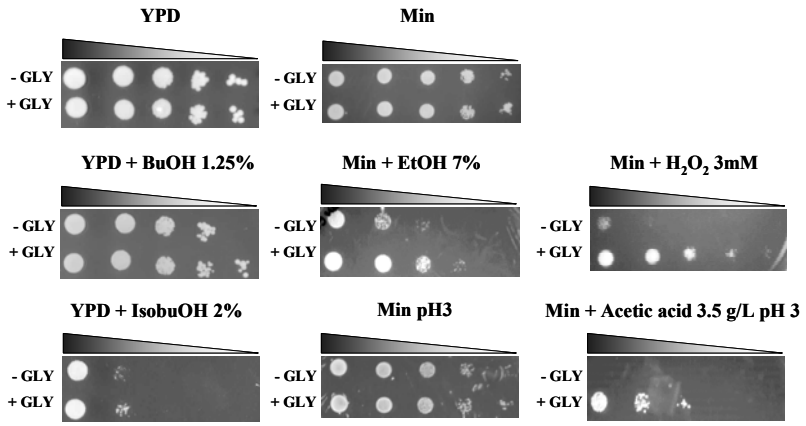

Figure 2. Spot test assay: effect of glycine pre-treatment on cells prompted with different stressing agents. Five microliter of 1:10 serial dilutions of cells pre-treated or not with glycine 15 g/L were plated on YPD or minimal medium containing different stressing agents. Butanol 1.25 % (v/v), isobutanol 2% (v/v), ethanol 7% (v/v), hydrogen peroxide 3 mM, pH3 and acetic acid 3.5 g/L pH3 stress were reported. Plates were incubated three days at 30°C.

BUTANOL CAUSES OXIDATIVE STRESS AT MEMBRANE LEVEL

The oxidative damages are so detrimental because they impact on the main cellular macromolecules: proteins, DNA and lipids, and yeasts are not an exception (Nicolaou et al., 2010). In particular, the membrane oxidative damages are due to the oxidation of polyunsaturated fatty acids, causing lipids peroxidation (Gupta et al., 1994). As result of this, the lipids undergo a process of fragmentation, generating some products very reactive such as aldehydes and alkanes which in turn generate DNA and proteins damages (Costa & Moradas-Ferreira, 2001).

Because of the strict correlation among the presence of alcohol, membrane damages and oxidative stress, we preliminary investigated if in

S. cerevisiae also the presence of butanol could cause lipids peroxidation, which has never been reported.

The malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation and for this reason it is commonly used to monitor the lipid peroxidation (Lefèvre et al., 1998). The BY4741 cells were exposed at different concentration of butanol (1.5%, 2%, 3 % (v/v)) and MDA concentration was detected using a specific assay on the total cell extract. Here we reported the MDA concentration measured in the presence of 2.5% (v/v) of butanol (**Figure 3**).

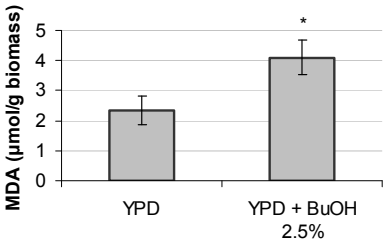

Figure 3. Malondialdehyde assay. The BY474 yeast background was grown in YPD medium in the presence or not of butanol 2.5% (v/v). The data presented here are representative of three independent experiments. $p \leq 0,05$ = *; $p \leq 0,01$ = **; $p \leq 0,001$ = ***; $p > 0,05$ = n.s.

The malondialdehyde concentration was 1.7 fold higher in the presence of butanol respect to the control medium. A similar difference was observed at the other butanol tested concentrations (data not shown).

These data show that butanol causes lipids peroxidation in *S. cerevisiae*, indicating that the membrane damages are not only due to insertion of superior alcohol in the phospholipid bilayer but also to oxidative stress.

THE BUTANOL AND ISOBUTANOL TOLERANCE REQUIRES MULTIPLE PARTNERS: THE METALLOTHIONEINS INVOLVEMENT

In literature it has been reported that in presence of lipids peroxidation the levels of MTs increase to protect the cells (Adamo et al., 2012), and we have just demonstrated that butanol causes lipids peroxidation in yeast cells. We thus investigated the MTs defensive role against cell damage specifically caused by butanol and isobutanol. The BY4741 wild type strain was treated with $CuSO_4$ 1 g/L to induce MTs transcription (Adamo et al., 2012). After induction the cells were plated on YPD medium containing 0-2.5% (v/v) of butanol and isobutanol (**Figure 4, left panel**). It is possible to see that for the wild type strain the copper induction allows the yeast growth in presence of alcohols, in respect of non-treated control strain.

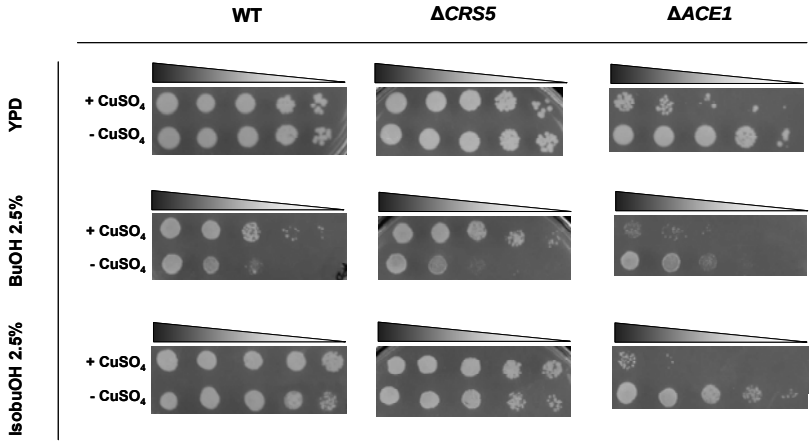

Figure 4. Spot test assay in presence of butanol and isobutanol as stress agents and in presence or absence of copper sulphate. Five microliter of 1:10 serial dilutions of cells pre-treated or not with copper sulphate 1 g/L were plated on YPD medium in presence of butanol 2.5 % (v/v) or isobutanol 2.5% (v/v). Plates were incubated three days at 30°C.

The correlation between tolerance and MTs was verified by using two additional strains: one deleted in *CRS*5 and the other deleted in *ACE*1, a copper-binding transcription factor which activates transcription of the MT genes *CUP*1-1 and *CUP*1-2 in response to elevated copper concentrations.

The *CRS*5 deletion has a significant negative effect on cells growth in the presence of butanol and isobutanol (**Figure 4, central panel**) but this can be partially rescued by cells pre-treatment with $CuSO_4$. The *ACE*1 deletion has a drastic growth effect when cells are treated either with copper and alcohols, impairing the positive effect of copper addition on increasing alcohol tolerance (**Figure 4, right panel**).

Chapter 2

Conclusions

We tested the glycine effect on the butanol and other common stressor agent that microorganisms have to face during productions. We found that when cells are pre-adapted with glycine enriched medium they are more tolerant to hydrogen peroxide, acetic acid, ethanol and butanol. It is important to underline that the glycine is the precursor of glutathione which have an important role during the oxidative stress response in yeast (Izawa et al., 1995; Grant et al., 1996) and this could be the way in which the glycine helps the cells. In favour to this hypothesis in literature has been reported how the presence of glycine can increase the production of intracellular glutathione (Wang et al., 2012). Moreover, the glycine has osmoprotective role in *S. cerevisiae* because it is the precursor of glycine betaine, as reported for some bacteria, which protects the cells against osmotic stress. Glycine betaine was also found in yeast extract (Thomas et al., 1994) but until now its biosynthesis has not been demonstrated jet.

We also found that the MTs proteins seem to have an effect on butanol and isobutanol tolerance in yeast. The *ACE*1 deletion has a significant effect on growth when cells are pre-treated with $CuSO_4$ and then placed in presence of butanol and isobutanol (**Figure 4**). Since Ace1p specifically induce the Cup1p transcription, this could candidate the Cup1p (and not Crs5p) as a protein involved in alcohols tolerance.

In conclusion, we found that butanol, similarly to what reported for ethanol (Gupta et al., 1994), causes a sort of oxidative stress since the lipids peroxidation was higher in presence of butanol respect to control medium. More have to be elucidated about this aspect since up to date it

is not known if butanol can also cause the oxidative stress inside the cells, for example in terms of Reactive Oxygen Species (ROS) production or in terms of proteins carbonylation. Regarding this aspect it could be interesting to study the effect of MTs and glycine not only on lipids peroxidation but also on other stress defensive mechanisms.

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CHAPTER 3

UP REGULATION OF THE GABA PATHWAY AS A POSSIBLE ADDITIONAL WAY FOR SUCCINIC ACID PRODUCTION IN *Saccharomyces cerevisiae*

Manuscript in preparation for "Bioresearch Technology" Journal

Chapter 3

Introduction

The succinic acid occurs naturally in all living organisms as TCA cycle intermediate and plays a significant role in biological metabolism (Zeikus & Elankovan, 1999). Also known as butanedioic acid or amber acid, it can be used as a precursor to produce many important commodity chemicals, including 1,4-butanediol, tetrahydrofuran, γ-butyrolactone and other bulk chemicals (McKinlay et al., 2007) that are in turn employed to make a wide assortment of products such as solvents, as well as fibers and polymers (Werpy & Petersen, 2004). Succinic acid can also be directly polymerized to form the biodegradable aliphatic polyester bionolle (Showa Denko, URL: www.showa-denko.com). The industrial potential for succinic acid fermentations was recognized as early as 1980 (as reviewed by Sauer et al., 2008 and Yu et al., 2011a).

Currently, succinic acid mainly derives from chemical processes that use fossil sources as a raw material resulting in the production of many serious pollutants. To solve this problem, and to render the production independent from an exhaustible source, the microbial succinic acid processes from renewable biomasses started to be developed and pursued (McKinlay et al., 2007), using both natural and engineered succinic acid producing strains (Oh et al., 2009; McKinlay et al., 2010; Raab & Lang, 2011; Litsanov et al., 2012; Thakker et al., 2012). The microbial production of succinic acid via reductive TCA cycle using engineered *Saccharomyces cerevisiae* is nowadays on the market, commercialised by Reverdia, with 10.000 t capacity plant located on the Roquette site in Cassano Spinola in Italy (www.roquette.com).

As bulk chemical, to have a competitive cost and replace entirely the actual petrol-based production, the succinic acid production have to be performed at maximum conversion yield and/or using side stream processes which help the cost calculation of the main stream process of production.

With this purpose, we have studied the possibility to use an alternative route, different from TCA cycle, to produce succinic acid using the glutamate degradation pathway via γ-aminobutyric acid (GABA) intermediate (Kumar & Punekar, 1997).

GABA is a ubiquitous non-protein amino acid generated from glutamate decarboxylation by the enzyme glutamate decarboxylase. The GABA pathway bypasses two steps of the tricarboxylic acid (TCA) cycle for the conversion of α-ketoglutarate into succinate (**Figure 1**). It involves three enzymes: a glutamate decarboxylase (GAD; EC 4.1.1.15), which catalyzes the decarboxylation of glutamate to GABA, a GABA transaminase (GABAT; EC 2.6.1.19), which converts GABA to succinate semialdehyde, and a succinate semialdehyde dehydrogenase (SSADH; EC 1.2.1.16), which catalyzes the oxidation of succinate semialdehyde to succinate (Ramos et al., 1985). This pathway, also called GAD/GABA pathway, is present in all living organisms and is conserved from bacteria through yeasts to animals and plants.

The role of GAD/GABA pathway has been mostly studied in mammalian cells because the γ-aminobutyric acid formed after glutamate decarboxylation represents an important neurotransmitter in the central nervous system (Bowery et al., 2004). The deficiency in the intracellular

GABA level results in epilepsy and Parkinson's diseases (Wei & Wu, 2008). In the last years, the GABA shunt received more attention in plants due to its involvement in various aspects of development, metabolism and responses to biotic and abiotic stresses, including pathogen infection, thermal shock, hypoxia and water stress (Bouché & Fromm, 2004). In *S. cerevisiae* the GAD/GABA pathway was also found (Kumar & Punekar, 1997), but its possible role is still poorly understood.

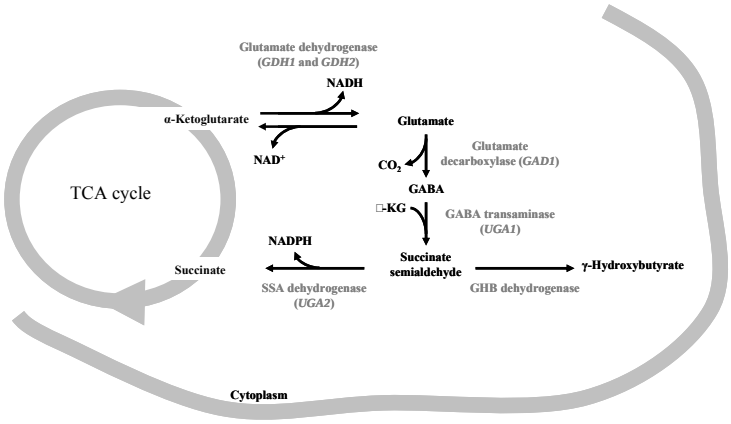


Figure 1. The GAD/GABA pathway in yeast. The enzymatic steps for the conversion of glutamate to succinate through GABA formation in *S. cerevisiae* are represented, with the corresponding genes.

The GABA can be internalized by the yeast cells from the environment by a specific transporter encoded by *UGA*4 gene (André et al., 1993), but it might also be mediated by the general amino acid permease Gap1p (Grenson et al., 1970) and by the proline-specific permease Put4p (Jauniaux et al., 1987; Bach et al., 2009). The regulation of GABA transport and degradation is controlled by the nitrogen catabolite

repression. In the presence of preferred nitrogen source, such as ammonium or glutamate the *UGA* genes (*UGA*1, *UGA*2 and *UGA*4) expression is repressed to low levels, while in the presence of GABA their expression is up-regulated (Vissers et al., 1989; Bach et al., 2009). This clearly indicates that the *UGA* genes are responsible for the use of GABA as nitrogen source.

What remains less clear is on the one hand the possible physiological function and role of this metabolite in yeast, or at least the metabolic reason of GABA generation, and on the other hand the role of the glutamate degradation through the GAD/GABA pathway to produce succinate. In literature few works are reported about the regulation of the GAD/GABA pathway and its role.

Coleman et al (2001) reported the involvement of glutamate metabolic pathway in oxidative tolerance, with particular attention at glutamate decarboxylase enzyme, encoded by *GAD*1 gene. The *gad1*Δ strain was unable to grow on plates containing 3 mM H_2O_2. The ability to grow in this condition was restored by inserting one or more copies of *GAD*1 gene in the deleted strain and the oxidant resistance resulted proportional to the *GAD*1 gene copy number (Coleman et al., 2001). Moreover, they demonstrated that in wild type cells the *GAD*1 and *UGA*1 genes were upregulated under oxidative stress conditions, but no further explanation of the possible role of intermediates is given to support the observed protective effect.

The transcriptional analysis revealed that the expression of *UGA*1 gene is related to the TOR signaling pathway, which mediates the response to

carbon and nitrogen signals in yeast: in the presence of rapamycin the *UGA*1 presents a 5.6 fold induction (Cardenas et al., 1999). In wine yeast strains, during wine fermentation, all genes involved in management of the glutamate pool such as *GDH*2*, GLN*1*, GAD*1*, UGA*1 are upregulated during the stationary phase, in conditions of nitrogen starvation (Rossignol et al., 2003). In the presence of high sugar levels, all the three genes of GAD/GABA pathway are upregulated: for *GAD*1, *UGA*1 and *UGA*2 gene the fold changes are respectively 5.5, 6.5 and 3.2, (Erasmus et al., 2003).

Recently, Kamei and co-workers demonstrated that the GAD/GABA pathway has a role in cell aging and replicative lifespan: more precisely, the deletion of *GAD*1 or *UGA*1 lengthened the lifespan while the *UGA*2 or *UGA*4 deletions did not (Kamei et al., 2011). Unexpectedly, intracellular GABA levels in mutant cells did not differ from those of wild-type cells and the addition of GABA to the culture media, which induces transcription of the *UGA* structural genes, had no effect on replicative lifespan of wild-type cells.

The GAD/GABA pathway was also investigated to demonstrate the γ-hydroxybutyric acid (GHB) production through the glutamate degradation (Bach et al., 2009). In this alternative route, the succinic semialdehyde intermediate undergoes to the reaction catalyzed by GHB dehydrogenase to form GHB.

The aim of the present study was to investigate the possibility to use the GABA shunt for succinic acid accumulation in the presence of glutamate as nitrogen source.

The literature described up to now have shown that there are different transcriptional controls active on the GAD/GABA pathway. According to different nutritional or environmental changes, it is reported that cells can regulate the pathway up or down regulating the transcription of the genes involved.

We have studied the effect of the pathway deregulation through the overexpression of GAD/GABA pathway genes *GAD*1, *UGA*1 and *UGA*2 for producing succinic acid. We found that the accumulation of succinate in the medium was higher when glutamate was used as nitrogen source, in agreement with literature data (Albers et al., 1998), but with no differences between transformed and control strains.

Here we demonstrate that the pathway was indeed up-regulated at transcriptional level, but not at enzymatic level, suggesting possible post-transcriptional or translational regulation that up to now were never investigated and even not hypothesized.

Chapter 3

Materials And Methods

STRAINS AND GROWTH CONDITIONS

The *S. cerevisiae* strains used in this study was CEN.PK 102-5B. (*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2* - Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany) (van Dijken JP et al., 2000). CEN.PK GUUc is the CEN.PK 102-5B strain transformed with integrative plasmids used to express the GAD/GABA pathway genes, as described in next paragraph. CEN.PK OOOc is the CEN.PK 102-5B strain transformed with empty vectors and used as control strain. All genes were expressed under control of the *S. cerevisiae TPI*1 promoter. Yeast transformations were performed basically according to the LiAc/PEG/ss-DNA protocol (Gietz & Woods, 2002).

Independent transformants and the respective control strains (at least three for each transformation) were cultivated in shake flasks in minimal synthetic medium with 50 g/L of glucose and different nitrogen sources. The nitrogen source used was ammonium sulphate 10 g/L in combination or not with glutamate 14 g/L, or sole glutamate 14 g/L. All strains were grown in shake flasks at 30°C at 160 r.p.m for 72 hours, with ratio of flask volume/medium of 5/1.

GENES AMPLIFICATION AND PLASMIDS CONSTRUCTION

The *S. cerevisiae GAD*1, *UGA*1 and *UGA*2 genes were PCR amplified using as a template the genomic DNA extracted from CEN.PK 102-5B strain by standard methods (Sambrook et al., 1989). Pwo DNA

polymerase (Roche catalogue no. 11 644 955001) was used on a GeneAmp PCR System 9700 (PE Applied Biosystem, Inc.). Standard conditions used were 0.2 mM primers, 1.5 U of Pwo and 50 ng of genomic DNA. The program used for amplification of genes was as follows: after 5 min at 94°C, 30 cycles (each cycle consisting of 15 s at 94°C, 30 s at 57.5°C and 1 min 30 s at 72°C) were carried out, followed by 7 min at 72°C. Oligonucleotides pairs for all amplified genes were listed in the table below.

Gene amplification	Orientation	Oligonucleotide sequence 5' → 3'
GAD1	Fwd	ACAAGGAATAATGTTACACAGGC
	Rev	ACCTTTTCAACTCAACATGTTCC
UGA1	Fwd	ATATAACTAAGAACAATGTCTATTTGT
	Rev	TCGCTAATATACAATCATAATTCATTAAC
UGA2	Fwd	AACTACTATTTCAACATGACTTTGAG
	Rev	ATTACTATTGCTTTAAATGCTGTTTGG

The amplified fragments were sub-cloned into the *Escherichia coli* vector pSTBlue (Novagen) obtaining, respectively, the plasmids pSTBlue*GAD*1, pSTBlue*UGA*1 and pSTBlue*UGA*2. The inserts were sequenced and resulted identical to the deposited *S. cerevisiae* corresponding sequences (*GAD*1 GeneID: 855291, *UGA*1 GeneID: 852902 *UGA*2 GeneID: 852291). These coding sequences were used for the construction of the integrative expression plasmids p042*GAD*1, p012*UGA*1 and pYX022*UGA*2, respectively, utilizing the basic *S. cerevisiae* integrative expression plasmids series (R&D Systems, Inc., Wiesbaden, D).

For the construction of the plasmid p042*GAD*1, the recipient vector was *Eco*RI cut, blunted and dephosphorylated, while the insert was *Mlu*I blunt/*Xho*I blunt excised from the pSTBlue*GAD*1 plasmid. For the construction of the plasmid p012*UGA*1, the recipient vector was *Eco*RI cut, blunted and dephosphorylated, while the insert was *Mlu*I blunt/*Xho*I blunt excised from the pSTBlue*UGA*1 plasmid. For the construction of the plasmid p022*UGA*2, the recipient vector was *Eco*RI cut, blunted and dephosphorylated, while the insert was *Mlu*I blunt/*Sal*I blunt excised from the pSTBlue*UGA*2 plasmid. DNA manipulation, transformation and cultivation of *E. coli* (Novablue, Novagen) were performed following standard protocols (Sambrook et al., 1989). All the restriction and modification enzymes utilised are from NEB (New England Biolabs, UK) or from Roche Diagnostics.

CELL GROWTH AND METABOLITES DETERMINATION

The cellular growth was monitored using a spectrophotometer at 660nm and was reported as variation in the optical density (OD) as a function of time (h). The amount of extracellular glucose and succinate were determined by HPLC based method using H_2SO_4 5 mM as mobile phase and Aminex HPX-87P column, 300 x 7.8 mm with a polystyrene divinylbenzene-based matrix (BioRad). The glutamate quantification was performed using a commercial kit following manufacturer's instructions (Megazyme, Cat. No K-GLUT).

RNA EXTRACTION AND cDNA PRODUCTION

Total RNA for gene expression analysis was extracted from cells growing in minimal medium using different nitrogen source: ammonium sulphate with or without glutamate or in presence of sole glutamate. The Aurum™ Total RNA mini kit (BioRad, Cat. No 732-6820) was used: extracted RNA was checked on 1.5% (w/v) agarose gel and RNA was quantified by spectrophotometer at 260 nm. A dilution of 0.5 µg/µL of RNA was used to produce the corresponding cDNA using the iScript cDNA synthesis kit (BioRad, Cat. No 170-8890) according to the following mix: 5 µL RNase-free water, 10 µL RNA and 4 µL of 5x iScript reaction mix. Retrotranscription was performed adding 1 µL of iScript reverse transcriptase followed by incubation at 42°C for 30 minutes and 85°C for 5 minutes. The obtained cDNA was diluited by adding 180 µL RNase-free water, and 5 µL of the dilution were used for gene expression analysis.

GENE EXPRESSION ANALYSIS

Relative quantifications were performed by quantitative real time PCR (RT-qPCR) to determine *GAD*1, *UGA*1 and *UGA*2 gene expression. RT-qPCR was performed using the IQ5 Multicolor real time PCR detection system (BioRad).

For gene expression analysis, cDNA gene sequences of *GAD*1, *UGA*1, *UGA*2 and *ACT*1 were retrieved from the *Saccharomyces Genome Database* (www.yeastgenome.org) and the internet-based interface

Primer-3 (Rozen & Skaletsky, 2000) was used to design PCR oligonucleotides (**Table below**).

Gene amplification	Orientation	Oligonucleotide sequence 5' → 3'
GAD1	Fwd	ATTCCGCCCCTAAGTCAGTT
	Rev	ATCCGTTGCCTTTGGTAGTG
UGA1	Fwd	CTTTGATCAAGGCAGCACAA
	Rev	GCAGCCTTGAACGCTAATTC
UGA2	Fwd	CTTTCACATGCCAACACACC
	Rev	TCCAAGGCTTGATCCAAATC
ACT1	Fwd	TGTCACCAACTGGGACGATA
	Rev	GGCTTGGATGGAAACGTAGA

Individual RT-qPCR mixtures were prepared in a 48 well plate (Bio-Rad), each 20- µL reaction containing 5 µL cDNA and 15 µL SYBR green master mix [SYBR Green® Supermix (BioRad), 0.4 pmol/ µL forward and reverse oligonucleotides and water]. Amplification conditions were: 3 min at 95°C followed by 40 cycles at 95°C for 10 sec and at 58°C for 30 sec and 81 cycles at 55°C for 8 sec. The gene expression was evaluated using the Livak method or $2^{-\Delta\Delta CT}$ method. The values were normalized against the housekeeping gene *ACT*1. For standardization, the results were expressed as ratio of the amount of the target genes (*GAD*1, *UGA*1 and *UGA*2) in GAD/GABA expressing yeast strain (GUUc) and control strain (OOOc).

DETERMINATION OF ENZYMATIC ACTIVITIES

Cells from exponential cultures were harvested by centrifugation at 4000 rpm for 10 min and washed with cold deionised water. The cell pellet was then re-suspended in 25 mM Tris-HCl pH 8.0 with protease inhibitor cocktail (Roche diagnostics, Cat. No. 04906837001) and 1 mM of phenylmethylsulfonyl fluoride (PMSF) and mechanically disrupted using glass micro beads. Cells debris was removed by centrifugation at 14000 rpm for 10 min at 4°C and the clarified crude extract was used for enzymatic analysis. The protein concentration in cell-free extracts was estimated according to Bradford (Bradford, 1976) using bovine serum albumin as the reference.

Enzyme activities were measured on cell-free extracts by spectrophotometric assays and the activities were expressed as Units/mg proteins.

Glutamate decarboxylase activity was determined using a colorimetric method as previously described with some modifications (Yu et al., 2011b). The reaction mix consisted of 200 µL sodium acetate buffer 20 mM pH 4.8, 500 µL PLP 20 mM prepared in sodium acetate 20 mM pH 4.8, 50 µL bromocresol green 1.4 mM, cell extract 50 µL (0.5 mg/mL). After incubation at 25°C for 10 min glutamate 10 mM prepared in sodium acetate 20 mM pH 4.8 were added and the increase in the absorbance at 620 nm was monitored. Data were collected every 30 sec for 10 min. Activities were calculated from the slopes measured in the linear portion of the curve. The initial rates were calculated from the average dA/dt using Eq. 1, where $\Delta\varepsilon_{620}$ = 23700 M^{-1} cm^{-1} and l = 1 cm. The activity

(μmol/min/mL) was calculated after taking into account the protein total amount of protein in each cuvette.

(1) ν (μmol/min/mL) = (dA/dt) x Q x reaction volume x 10^6

were $Q = (C_B/C_{In}) / (\Delta \varepsilon_{620} \times l)$ and C_B and C_{In} are the total molar concentration of buffer and indicator.

GABA transaminase activity was determined using α-ketoglutarate as amino group acceptor according to the method previously described (Jakoby, 1962) with some modification. The reaction mixture containing 86 mM potassium pyrophosphate buffer pH 8.6, 3.3 mM 2-mercaptoehtanol, 1.2 mM β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), 6 mM γ-amino-n-butyric acid, 5 mM α-ketoglutarate, 0.5 mM potassium phosphate buffer with 0.17% (v/v) glycerol pH 7.2 at 25°C. After incubation at 25°C for 10 min to equilibrate the mixture the crude cell extract was added and immediately after mix the increase in A_{340nm} was monitored every 30 sec for 10 min. The activities were calculated using the Eq. 2, were ΔA_{340nm}/min was obtained using the maximum linear portion of the curve.

(2) U/mg protein = $[(\Delta A_{340nm}/min \times Tv \times df) / (\varepsilon_{\beta\text{-NADP}} \times Ev)]$ / mg protein total

where Tv is the total volume of assay (expressed in milliliters), df is the dilution factor, $\varepsilon_{\beta\text{-NADP}}$ is the millimolar extinction coefficient of β-NADP at 340 nm (6.22), Ev is the volume of cell extract used (expressed in millilitres).

Chapter 3

RESULTS

GROWTH CURVES AND SUCCINIC ACID ACCUMULATION PROFILE OF *Saccharomyces cerevisiae* STRAINS OVEREXPRESSING *GAD*1, *UGA*1 AND *UGA*2

The *S. cerevisiae* CEN.PK yeast strain transformed with plasmids bearing *GAD*1, *UGA*1 and *UGA*2 genes under the control of the glycolytic TPI promoter (GUUc) and the control strain (transformed with empty vectors, OOOc) were grown in minimal medium in the presence of glucose 50 g/L and different nitrogen sources: ammonium sulphate 10 g/L in combination or not with glutamate 14 g/L or sole glutamate 14 g/L. At different time point after the inoculum cellular growth, glucose and glutamate consumption as well as succinate production were monitored for both wild type and engineered yeast strains (**Figure 2**). It is clearly visible that the succinate productions obtained from the strains are perfectly overlapped (**Figure 2A**), indicating that both strains accumulate the same succinate titer, and with a similar kinetics, in all the tested conditions.

In literature it has been reported that in the presence of glutamate as nitrogen source *S. cerevisiae* accumulates more succinic acid than in the presence of ammonium sulphate, in anaerobic condition (Camarasa et al., 2003). This data goes in the same direction of our data, where in the presence of glutamate 0.56 g/L of succinate were obtained, while in the presence of sole ammonium sulphate the succinate titer accumulation was 0.05 g/L after 48 hours from the inoculum, a difference of one order of magnitude. The increased succinate accumulation could be explained with an increased biomass accumulation, as clearly visible in the figure 2,

panel B. However, the ratio of succinic acid produced per unit of biomass (expressed as optical density variation) was higher in presence of glutamate than in presence of ammonium sulphate as nitrogen source, 0.03 g/L versus 0.005 g/L succinic/OD biomass.

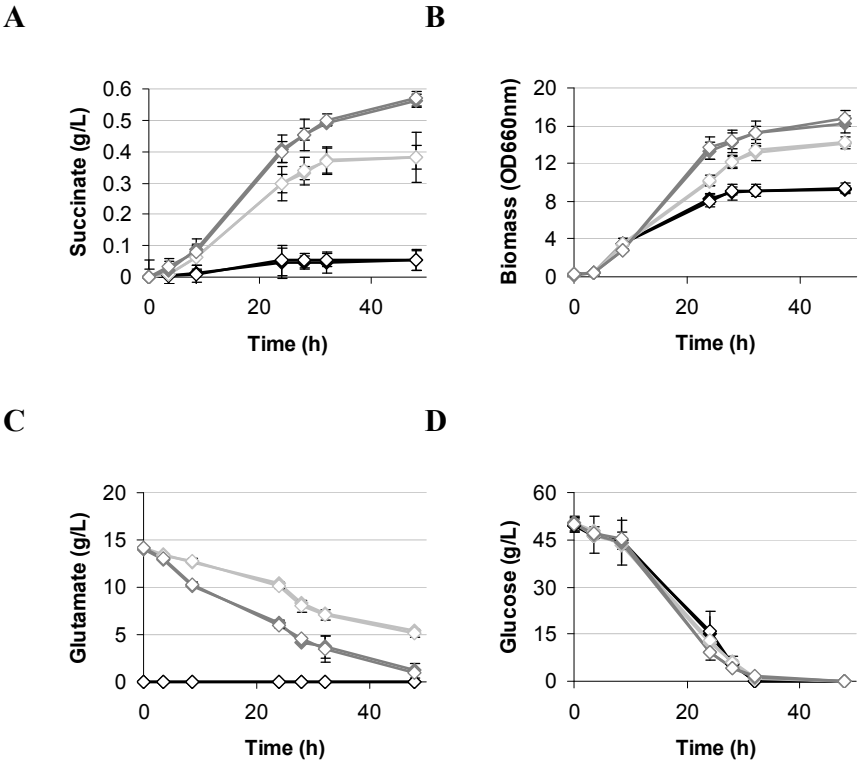


Figure 2. Metabolic profiles of *GAD*1, *UGA*1 and *UGA*2 overexpressing strain grown in minimal glucose medium in the presence of different nitrogen source. The kinetic of succinate production (panel A) was monitored in the presence of ammonium sulphate (black), ammonium sulphate plus glutamate (light grey) and glutamate (grey) as nitrogen source. Cell growth (panel B), glucose (panel C) and glutamate (panel D) consumption are reported. Engineered strain GUUc: closed symbol; control strain: open symbol. The data presented here are representative of three independent experiments.

As a consequence, also other metabolites are accumulated in higher quantity in the presence of glutamate rather than in ammonium sulphate, such as ethanol and glycerol (data not shown). However, also in this case no differences between engineered and parental strain were observed.
According to the presented data, the designed constitutive activation of GAD/GABA pathway does not lead to a higher specific productivity of the final product of interest, indicating there is no redirection of glutamate flux through the succinate production.

ANALYSIS OF THE EXPRESSION LEVELS OF *GAD*1, *UGA*1 AND *UGA*2 GENES IN WILD TYPE AND ENGINEERED YEASTS

We investigated whether the GAD/GABA pathway was effectively up-regulated during the described experiments by analyzing the expression of *GAD*1, *UGA*1 and *UGA*2. Changes in mRNA levels were determined by quantitative relative Real Time PCR on minimal medium in the presence or absence of glutamate as nitrogen source, at different time points after the inoculum (**Figure 3**).
In all the tested conditions the three genes, *GAD*1, *UGA*1 and *UGA*2 under the control of *S. cerevisiae TPI* promoter, are effectively overexpressed during all over the time of the kinetics, irrespective of the nitrogen source used (Figure 3 A-C, column Fold change); in particular the expression of *UGA*1 is always lower respect to the other two genes.

A

Gene	Nitrogen source	Gene expression after 3.5h		
		Fold change	OOOc*	GUUc*
GAD1	$(NH_4)_2SO_4$	589 ± 168	0.024 ± 0.008	13.31 ± 1.44
	$(NH_4)_2SO_4$ + Glu	530 ± 172	0.012 ± 0.002	6.01 ± 1.23
	Glu	153 ± 55	0.051 ± 0.018	7.14 ± 1.39
UGA1	$(NH_4)_2SO_4$	63 ± 22	0.005 ± 0.002	0.34 ± 0.16
	$(NH_4)_2SO_4$ + Glu	92 ± 56	0.002 ± 0.001	0.19 ± 0.07
	Glu	5 ± 3	0.053 ± 0.027	0.19 ± 0.01
UGA2	$(NH_4)_2SO_4$	408 ± 81	0.001 ± 0.00	0.45 ± 0.12
	$(NH_4)_2SO_4$ + Glu	426 ± 136	0.001 ± 0.00	0.46 ± 0.14
	Glu	6 ± 2	0.034 ± 0.010	0.19 ± 0.01

B

Gene	Nitrogen source	Gene expression after 8.5h		
		Fold change	OOOc*	GUUc*
GAD1	$(NH_4)_2SO_4$	327 ± 50	0.042 ± 0.023	19.86 ± 7.70
	$(NH_4)_2SO_4$ + Glu	194 ± 43	0.022 ± 0.007	4.14 ± 0.70
	Glu	3 ± 1	0.032 ± 0.018	0.07 ± 0.01
UGA1	$(NH_4)_2SO_4$	36 ± 18	0.005 ± 0.003	0.25 ± 0.10
	$(NH_4)_2SO_4$ + Glu	47 ± 19	0.003 ± 0.001	0.12 ± 0.05
	Glu	2 ± 1	0.015 ± 0.002	0.03 ± 0.01
UGA2	$(NH_4)_2SO_4$	223 ± 72	0.002 ± 0.001	0.60 ± 0.27
	$(NH_4)_2SO_4$ + Glu	139 ± 43	0.001 ± 0.00	0.16 ± 0.05
	Glu	2 ± 2	0.016 ± 0.003	0.04 ± 0.03

C

Gene	Nitrogen source	Gene expression after 24h		
		Fold change	OOOc*	GUUc*
GAD1	$(NH_4)_2SO_4$	143 ± 28	0.03 ± 0.01	4.16 ± 1.43
	$(NH_4)_2SO_4$ + Glu	40 ± 12	0.54 ± 0.11	21.73 ± 9.28
	Glu	25 ± 12	0.93 ± 0.13	21.91 ± 15.44
UGA1	$(NH_4)_2SO_4$	11 ± 7	0.04 ± 0.02	0.34 ± 0.01
	$(NH_4)_2SO_4$ + Glu	16 ± 7	0.04 ± 0.01	0.55 ± 0.09
	Glu	4 ± 2	0.18 ± 0.03	0.75 ± 0.51
UGA2	$(NH_4)_2SO_4$	8 ± 3	0.04 ± 0.02	0.30 ± 0.04
	$(NH_4)_2SO_4$ + Glu	10 ± 7	0.06 ± 0.04	0.54 ± 0.22
	Glu	15 ± 4	0.07 ± 0.05	1.02 ± 0.69

Figure 3. Gene expression of *GAD*1, *UGA*1 and *UGA*2. Gene expression is represented as relative expression of *GAD*1, *UGA*1 and *UGA*2 gene, in the presence of different nitrogen source, ammonium sulphate ($(NH_4)_2SO_4$), ammonium sulphate plus glutamate ($(NH_4)_2SO_4$ + Glu) or glutamate (Glu). The gene expression was evaluated at 3.5 h (A), 8.5 h (B) and 24 h (C) time point after the inoculum. The fold change was calculated as $2^{-\Delta\Delta CT}$. *$\Delta CT = 2^{CT\ ref - CT\ test}$, were "ref" is reference gene (*ACT1*) and "test" is the gene of interest (*GAD*1, *UGA*1 or *UGA*2). The data presented are representative of three independent experiments.

Along the time, the fold change decrease and this could be explained by the increasing of ΔCT of wild type strain, indicating that the basal expression rate of all genes monitored increases during the time course of the kinetic Moreover, it can be noticed that the fold change is not the same for *GAD*1, *UGA*1 and *UGA*2 In wild type strain the three genes are transcriptionally regulated by the nitrogen source (**Figure 3 A-C, column OOOc**). In the presence of glutamate the transcript levels of GAD/GABA pathway genes were higher than in ammonium sulphate, confirming what reported in literature (Rossignol et al., 2003; Bach et al., 2009). The transcript levels of *GAD1*, *UGA1* and *UGA2* in engineered yeast strain also change according to the nitrogen source used (**Figure 3 A-C, column GUUc**). For example, in the presence of ammonium sulphate the ΔCt of *GAD*1 gene in the engineered strain is almost 13 in respect to the 0.02 value obtained in the control strain, 3.5 hours after the inoculum. In the presence of glutamate, the ΔCt of *GAD*1 gene in the engineered strain is almost 7 respect to the 0.05 value obtained in the control strain (**Figure 3, panel A**). This data were not expected and their interpretation remains to be better investigated in the future.

Summarizing, according to the results it is possible to say that the pathway is generally deregulated at transcript level in the engineered strain, and in particular it results up-regulated. However, since the metabolic analyses did not shown an improvement in succinic acid accumulation, this deregulation might be not sufficient to redirect the flux of glutamate through the GAD/GABA pathway.

ACTIVITY OF ENZYMES INVOLVED IN GAD/GABA PATHWAY

To better investigate the functionality of the GAD/GABA pathway we tested the activity of the enzymes involved in the glutamate degradation.

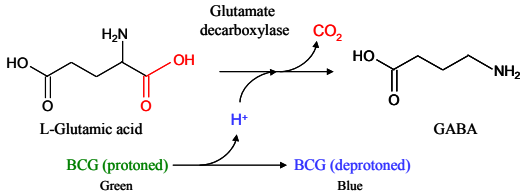
Glutamate decarboxylase activity.

The wild type and GAD/GABA pathway overexpressed strains were tested for the glutamate decarboxylase activity, at different time points (as previously done for transcription analyses), **Figure 4**. The glutamate decarboxylase activity was measured using an assay based on the detection of a pH increase that occurs since the reaction determines protons consumption (**Figure 4, panel A**). The pH increase is reflected by the colour change of the bromocresol green used as pH indicator, whose colour profile falls into the pH change of enzymatic activity (Yu et al., 2011b). No differences in the glutamate decarboxylase activity were detected when the glutamate or both ammonium sulphate and glutamate were used as nitrogen source. (**Figure 4, panel B**) Moreover, no significant differences were highlighted between the GAD/GABA

overexpressing strain and the control strain. This data revealed that even if the transcription of *GAD*1 has been up-regulated, other regulatory factors might occur at post-transcriptional/translational levels. There is little information about possible post-translational modification of this enzyme and only one report is related to yeasts, suggesting a possible regulation of Gad1p linked to calcium levels as the protein is able to bind calmodulin (Coleman et al., 2001).

For mammalian cells it was reported that the glutamate decarboxylase enzyme undergoes to various post-translational modifications such as phosphorylation, palmitoylation and activity-dependent cleavage (Wei & Wu, 2008) and we could imagine that also in yeast the Gad1p undergoes some regulation.

A



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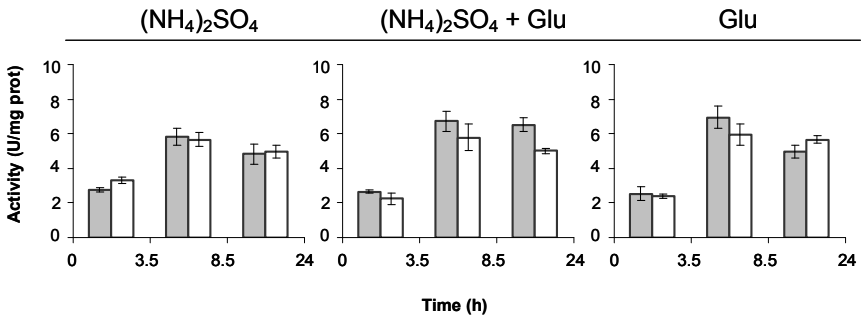
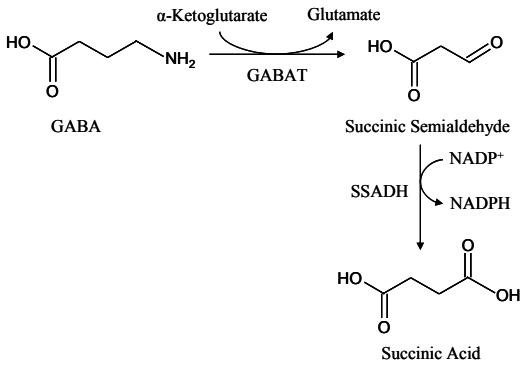


Figure 4. Glutamate decarboxylase activity. Activity of glutamate decarboxylase enzyme, based on schematic representation of reactions (A) was tested in presence of ammonium sulphate, ammonium sulphate plus glutamate and sole glutamate as nitrogen source (B). GUUc (grey) and control strain (white). The data presented here are representative of three independent experiments.

GABA transaminase activity

S. cerevisiae wild type and engineered strains were also tested for the γ-aminobutyric acid transaminase activity, which catalyzes the transamination of GABA into succinic semialdehyde using α-ketoglutarate as acceptor of amino group. The Uga1p activity was measured at different time points, as described by Jakoby et al. (1962) In this assay the succinic semialdehyde formed during the reaction is used as substrate for a second coupled reaction, catalyzed by the succinic semialdehyde dehydrogenase, which uses the β-$NADP^+$ as cofactor. The enzyme activity was measured according to the spectrophotometric assay based on the detection of β-NADPH at 340 nm (**Figure 5, panel A**).

A



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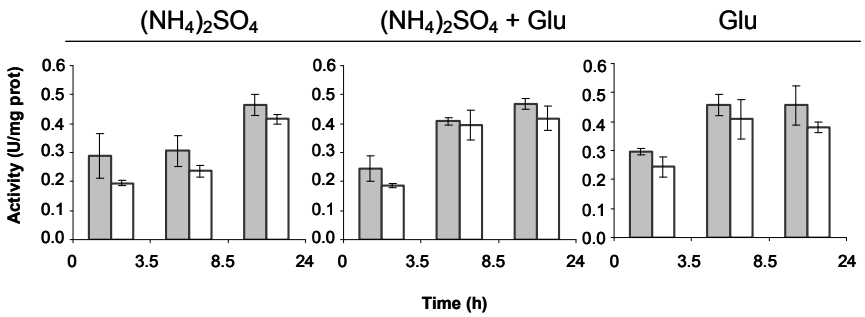

Figure 5. **GABA transaminase activity**. Activity of GABA transaminase enzyme, based on schematic representation of reactions (A) was tested in the presence of ammonium sulphate, ammonium sulphate plus glutamate and glutamate as nitrogen source (B). GUUc (grey) and control strain (white). The data presented here are representative of three independent experiments.

The activity was tested culturing the cells in minimal medium using ammonium sulphate, ammonium sulphate plus glutamate or glutamate as nitrogen source (**Figure 5, panel B**). No differences in the GABA transaminase activity were detected in presence of different nitrogen sources and no significant differences were highlighted between the GAD/GABA overexpressing strain and the control strain.

Regarding the assay it is important to underline that it was performed on free-cell total extract and no additional enzymatic activities were added. Thus, it's difficult to estimate the real contribute of GABA transaminase activity in the pathway. Infact, since in this assay no succinic semialdehyde dehydrogenase was used in excess, we can't exclude that the low production of NADPH is due to the limiting activity of this second enzyme instead of the first one.

Chapter 3

CONCLUSIONS

All literature data suggested that the GAD/GABA pathway in the budding yeast is transcriptionally controlled. As consequence of this it is possible to imagine that its up-regulation could lead to the succinic acid accumulation but this is in contrast with our obtained data. Infact, the overexpression of *GAD*1, *UGA*1 and *UGA*2 genes, despite resulting in an effective up-regulation at the transcriptional levels, does not increase the succinate specific productivity of the cells, whatever is the used nitrogen source.

What we found is that this pathway could also be post-translational regulated, since in our tested conditions we did not detect differences in the enzymatic activities between modified and wild type strain. This could be explained considering that in literature the transcriptional control of the pathway has been reported in perturbed conditions. Possibly, such transcriptional control comprises also the expression of the elements controlling the post-translational regulation of the entire pathway. Since in our tested conditions no stress is present and the perturbation-induced transcriptional control of the pathway may be absent, our imposed transcriptional de-regulation could be nullified.

Currently, the proposed aim to produce succinic acid through the GAD/GABA pathway was not achieved, but this study can be considered as additional point to study the role and the regulation of this pathway in *S. cerevisiae*. More has to be investigated, such as post-translational modifications and competitive reactions that could nullify the introduced engineering. As reported in literature, for example, the presence of γ-hydroxybutyric acid dehydrogenase can compete with succinic

semialdehyde dehydrogenase for the succinic semialdehyde intermediate (**Figure 1**), reducing the accumulation of succinic acid through this pathway (Bach et al., 2009). It cannot be excluded that the production of succinic acid in yeasts might be in a future optimized by exploiting the GAD/GABA pathway, once it will be fully characterized.

Chapter 3

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CHAPTER 4

THE EFFECT OF *GLT*1 DELETION ON THE SUCCINIC ACID PRODUCTION IN THE YEAST *Saccharomyces cerevisiae*

Chapter 4

Introduction

Saccharomyces cerevisiae is able to use a variety of nitrogen-containing compounds as the sole source of all cellular nitrogen (Hofman-Bang, 1999). Once inside the cell, ammonia reacts with α-ketoglutarate to produce glutamate and can react with glutamate to produce glutamine (**Figure 1**, reactions 1 and 2) (Magasanik, 2003). Thus, the yeast cells convert these molecules into ammonia, glutamate and glutamine, which act as amino donor in many other biosynthetic reactions. These metabolites are the partners involved in the Central Nitrogen Metabolism (CNM) (Magasanik & Kaiser, 2002).

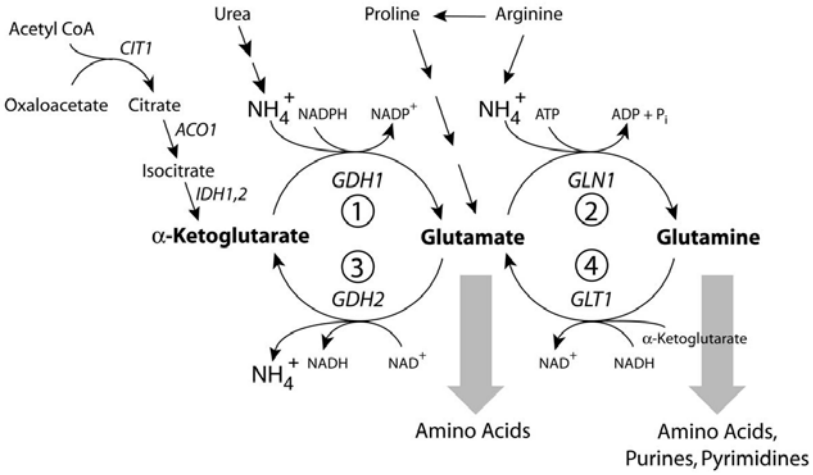

Figure 1. Central pathways for nitrogen metabolism. Schematic representation of nitrogenous compounds synthesized from either glutamate or glutamine. The pathways for utilization of a variety of nitrogen sources, including urea, proline and arginine, are shown. The *S. cerevisiae* gene for each of the enzymatic steps is designated in italics (Magasanik & Kaiser, 2002)

The aminoacid glutamate can be used as sole nitrogen source and its amino group serves as the source of 85% of the total cellular nitrogen

while the amide group of glutamine is the source of the remaining 15% (Miller & Magasanik, 1990). When glutamate is used as sole nitrogen source, the NAD-linked glutamate dehydrogenase, the product of *GDH*2, is responsible for the release of ammonia required for the synthesis of glutamine from glutamate (**Figure 1,** reaction 3) (Miller & Magasanik, 1990). This mitochondrial reaction can support the formation of succinic acid through the TCA cycle starting from glutamate. In fact, once inside the cell the glutamate can enter into mitochondria through the Agc1p carrier (Cavero et al., 2003). Here the glutamate dehydrogenase converts glutamate into α-ketoglutarate, which can enter in the TCA cycle (DeLuna et al., 2001).

The glutamate can also be used as precursor of γ-aminobutyric acid through the reaction catalyzed by glutamate dehydrogenase encoded by *GAD*1 gene (Coleman et al., 2001). As reported in the previous section, the GAD/GABA pathway could lead to the formation of succinate as final product. Since the overexpression of the GABA pathway genes did not resulted in succinate accumulation, we studied the effect of different deletions of genes acting at the same nitrogen node to investigate their possible contribution for increasing succinate accumulation.

The presence of glutamate as nitrogen source requires the combined activities of glutamine synthetase (GS) and glutamate synthase (GOGAT), encoded by *GLN1* and *GLT*1, respectively (Cogoni et al., 1995). Gln1p catalyzes amination of glutamate to form glutamine and Glt1p then transfers the amide group of glutamine to α-ketoglutarate, generating two molecules of glutamate (**Figure 1,** reactions 2 and 4).

These two reactions are fine regulated to ensure the correct balance of glutamate and glutamine inside the cells. Glutamine synthetase is the only way by which glutamine can be synthesized and cells lacking this enzyme require glutamine for growth (Zaman et al., 2008).

As said before GOGAT is NADH-dependent enzyme in *S. cerevisiae* and it is able to convert one molecule of glutamine and one molecule of α-ketoglutarate into two molecules of glutamate (Guillamón et al., 2001). It has been reported that strains of *S. cerevisiae* deleted in *GLT*1 grow well in the presence of glutamine as sole nitrogen source (Soberón & González, 1987; Magasanik & Kaiser, 2002), indicating the presence of glutaminase enzyme (GDA), which degrades glutamine into glutamate and ammonia. It has been also reported that the putative genes encoding for this activity are *SNO*1 and *SNZ*1 (Dong et al., 2004). Although many studies have been reported with both wild type and mutant strain impaired in GOGAT activity (as reported in Guillamón et al., 2001), the function of this enzyme in *S. cerevisiae* is still unclear, as well as its localization.

It has generally been accepted that GOGAT plays an important physiological role in yeast (Folch et al., 1989; Valenzuela et al., 1998). The deletion in *GLT*1 causes an incomplete metabolism of the nitrogen contained in glutamine, as reported by Guillamòn et al. (2001), probably due to the unbalanced redox state and this is also supported by proven correlation between the central nitrogen metabolism and the intracellular redox balance (Boles et al., 1993).

As the GOGAT uses NADH as cofactor, it is possible to imagine that when *GLT*1 is overexpressed the NADH/NAD^+ ratio decreases and thus

such modification could be designed when a process of production causes a NADH excess to the cells. This is what Cao et al. (2007) have done to rebalance the redox state to increase the ethanol production in a strain in which *GPD* and *FPS*1 gene were previously deleted to impair the glycerol accumulation (Cao et al., 2007).

On the contrary, when the GOGAT activity is impaired the $NADH/NAD^+$ ratio is expected to increase. For this reason we investigated the effect of the deletion of *GLT*1 gene in yeast *S. cerevisiae* on metabolites redirection in presence of glutamate as nitrogen source.

Chapter 4

Materials And Methods

STRAINS AND GROWTH CONDITIONS

The *S. cerevisiae* strains used in this study were BY4741 *(MATa, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1)* (EUROSCARF collection, Heidelberg, Germany) and BY4741Δ*GLT1* (*Mat a; his3D1; leu2D0; met15D0; ura3D0; YDL171c::kanMX4*) provided by EUROSCARF strain collection (EUROSCARF collection, Heidelberg, Germany). The strains BY4741c and BY4741Δ*GLT1*c are parental strains transformed with the integrative empty plasmids to make it prototrophic.

Yeast transformations were performed basically according to the LiAc/PEG/ss-DNA protocol (Gietz & Woods, 2002).

Independent transformants and the respective control strains (at least three for each transformation) were cultivated in shake flasks in modified Verduyn medium with 150 g/L of glucose and different nitrogen sources. The nitrogen source used was ammonium sulphate 5 g/L in combination or not with glutamate 50 g/L, or sole glutamate 50 g/L. All strains were grown in shake flasks at 30°C (agitated at 160 r.p.m) for 72 hours, with ratio of flask volume/medium of 5/1.

The modified Verduyn medium was based on Verduyn medium (Verduyn et al., 1992) with some modifications: the salt concentration of $MgCl_2$, EDTA and KH_2PO_4 were provided 50 fold low concentrated.

CELL GROWTH AND METABOLITES DETERMINATION

The cellular growth was monitored using a spectrophotometer at 660nm and was reported as variation in the optical density (OD) as a function of time (h). The amount of extracellular glucose, succinate, malate/fumarate,

glycerol and ethanol were determined by HPLC based method using H_2SO_4 5 mM as mobile phase and Aminex HPX-87P column, 300 x 7.8 mm with a polystyrene divinylbenzene-based matrix (BioRad). The α-ketoglutarate quantification was performed using a commercial kit as indicated by manufacturer (BioVision, Cat. No K677-100). To determine the intracellular α-ketoglutarate amount at different time point, 20 OD cells were collected and mechanically disrupted using glass micro beads (600 μm, Sigma-Aldrich) in 5% TCA buffer. After 10 min centrifugation at 14000 rpm at 4°C the supernatant was used to perform the assay.

Chapter 4

Results

GLT1 DELETION CAUSES CHANGE IN METABOLITES PROFILES DURING GROWTH ON GLUTAMATE AS NITROGEN SOURCE

The BY4741 *GLT1* deleted strain and the respective control strains were grown in modified Verduyn medium with high glucose concentration (150 g/L) and glutamate (50 g/L) as nitrogen source. Cell growth and metabolites profiles were monitored at different time point after the inoculum (**Figure 2**). The medium low salt content was chosen based on our previous studies.

In the modified Verduyn medium, when ammonium sulphate was used (as control medium) instead of glutamate the cell growth was almost completely inhibited (data not shown), no matter if *GLT*1 is deleted or not.

When glutamate is the nitrogen source, the growth is similar for both strains (**Figure 2, panel A**) but the glucose consumption rate is higher in *ΔGLT*1 strain respect to the control strain (**Figure 2, panel B**). This consumption reflects other changes in the metabolites profile. The succinate, malate/fumarate (not distinguishable with the utilized HPLC protocol), glycerol and ethanol profiles are different. In the deleted strain, a higher accumulation of reduced metabolites respect to the control strain has been observed. The succinate accumulation was up to 50% higher when *GLT*1 is deleted, 0.7 g/L versus 0.3 g/L of the wild type strain (**Figure 2, panel C**). The malate/fumarate accumulation was almost 4

times higher, while the ethanol accumulation was 2 times higher in the deleted strain (**Figure 2, panel D, F**).

The *GLT*1 deletion seems to have an effect on metabolic flux mediated by NADH accumulation.

THE *GLT1* DELETION AND THE α-KETOGLUTARATE CORRELATION

When glutamate is used as sole nitrogen source, the glutamate dehydrogenase enzyme catalyzes the deamination of glutamate to form α-ketoglutarate and ammonium. The α-ketoglutarate is also used to form glutamate during the reaction catalyzed by glutamate synthase, encoded by *GLT*1. Since α-ketoglutarate is essential for the interaction of carbon and nitrogen metabolism, we the α-ketoglutarate inside the cell and in the medium, in presence of different nitrogen source. The deleted and control strains were grown in modified Verduyn medium with glucose 150 g/L and ammonium 5 g/L or ammonium sulphate 5 g/L plus glutamate 50 g/L or sole glutamate 50 g/L. After growth, at different time points the cells and supernatant were collected and α-ketoglutarate was monitored (**Figure 3**).

In the *GLT*1 deleted strain the α-ketoglutarate concentration is higher both inside the cells and in the medium, especially when glutamate is used as nitrogen source (**Figure 3, right panel**). In the presence of ammonium sulphate as nitrogen source the low concentration of α-ketoglutarate, compared with those obtained in presence of glutamate, can be explained with the very poor growth (data not shown) (**Figure 3, left panel**).

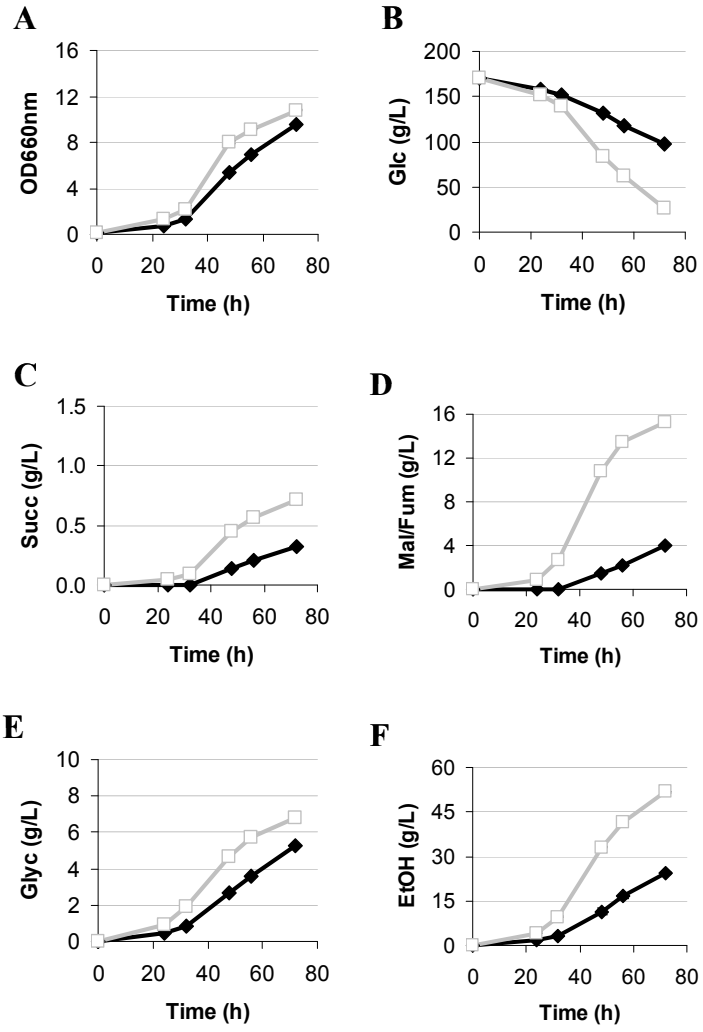

Figure 2. Metabolites profiles of *GLT1* deleted strain in presence of glutamate as nitrogen source. The BY474 yeast background was grown in modified Verduyn medium in presence of glutamate 50 g/L as sole nitrogen source. Biomass accumulation (panel A), glucose (panel B), succinate (panel C), malate/fumarate (panel D), glycerol (panel E) and ethanol (panel F) are reported for WT (black) and *GLT*1 deleted strain (grey). The data presented here are representative of three independent experiments.

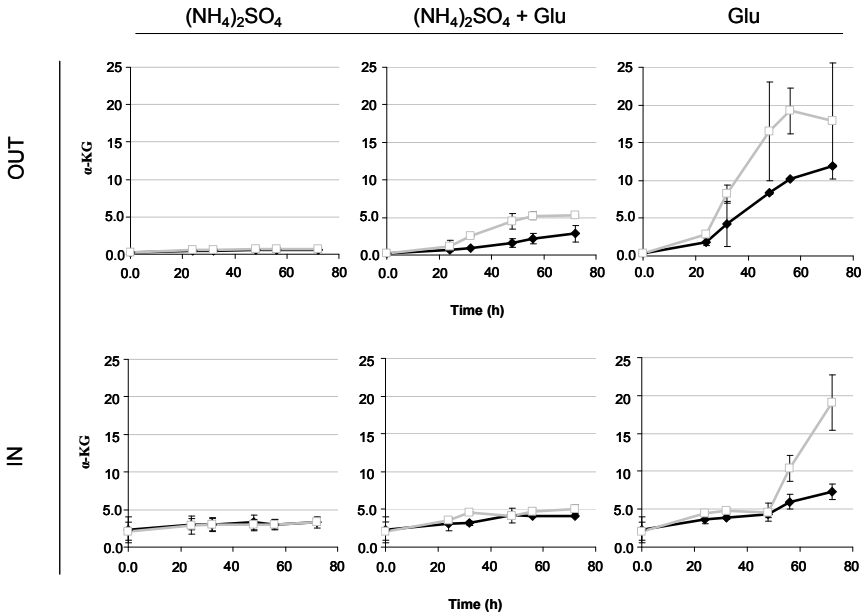

Figure 3. Intra and extra cellular α-ketoglutarate measurement. The BY474 yeast background was grown in Verduyn medium in presence of glutamate 50 g/L as sole nitrogen source. Intra and extra cellular α-ketoglutarate measurement was performed for WT (black) and *GLT*1 deleted strain (grey), in presence of three different nitrogen source. The data presented here are representative of three independent experiments.

The accumulation of α-ketoglutarate is intermediate when glutamate and ammonium sulphate are added as nitrogen sources, being still significantly higher in the supernatant of the deleted strain but similar to that of the wild type strain for the intracellular measurement. (**Figure 3, central panel**).

Chapter 4

Conclusions

This preliminary study confirmed the important role that *GLT*1 plays in cellular metabolism, shedding light on unpredictable consequences of its deletion, resulting in accumulation of reduced metabolites. Remarkably, said consequences became clear when glutamate is used as the sole nitrogen source and using a medium with low salt concentrations. This particular medium was formulated in our previous studies and revealed that in this condition yeast cells grow slowly in the presence of glutamate as nitrogen source, and even slower in the presence of ammonium sulphate. In literature the effect of low phosphorous concentration has been well described using steady-state cultures of *S. cerevisiae* (Boer et al., 2010). When phosphorous is limited, the yeast cells are characterized by high intracellular amino acid content and low nucleotide levels and this causes a redirection of metabolites fluxes having as a consequence the slowdown of anabolic reactions.

Our data are perfectly in line in confirming the key role of nutrient sensing. In modified Verduyn medium (low salt concentration) the *GLT*1 deleted strain accumulates higher concentration of succinate, glycerol, ethanol and malate/fumarate (reduced compounds) if compared to the control strain. Interestingly, when Verduyn or minimal YNB synthetic medium (in which the salt concentration is defined for supporting yeast normal growth) are used, only malate/fumarate result to be higher accumulated in the deleted strain (data not shown).

Said that GOGAT and Gdh2 are physiologically strictly connected, we performed similar experiments on the single *GDH*2 strain as well as on the double *GDH*2 *GLT*1 strain, but both manipulations resulted to have

similar effects of *GLT*1 deletion on metabolites accumulation (data not shown). In particular, we expected the double deleted to perform similarly to the parental strain, to clarify the correlation with α-ketoglutarate and TCA intermediates accumulation.

Since this preliminary data were not sufficient to better clarify the metabolic network, it appears crucial to obtain more information about the intracellular concentrations of key metabolites, with particular attention to ATP, α-ketoglutarate and NADH/NAD+, possibly discriminating between cytoplasm and mitochondrial compartments.

Notably, in this work we demonstrate that in *S. cerevisiae* (BY4741 background) growing in minimal medium, the *GLT*1 deletion causes accumulation of malate/fumarate, independently from the nitrogen source as well as from the salt and glucose concentration in the medium.

Chapter 4

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Grazie…perché non lo so neanche dire quanto Grazie ti devo, quanto grazie c'è in questa parola per te e cosa rappresenta…sarebbe una lista troppo lunga di cose, una lista assurda che solo io e te (e forse neanche io e te) potremmo capire…perché questo grazie è diverso da tutti gli altri e ha quel qualcosa di

segreto che in fondo non voglio svelare…perché posso dire a gran voce che se sono qui oggi è per te!

…"Il più grande spettacolo dopo il Big Bang siamo noi, io e te"

Grazie…perché un grazie speciale lo dedico alla mia famiglia… Numerosa, bella, accogliente, unita, speciale per davvero. Non sono mai stata così brava a dirvi che vi voglio bene, uno ad uno. Un grazie doveroso perché senza ognuno di voi non sarei diventata la Valeria che sono adesso. Un grazie davvero importante perché siete il centro della mia vita, il mio punto di riferimento. Un grazie unico alla Nonna…con la "N" maiuscola…come lei nessuno mai.

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