Engineering of primary carbon metabolism in filamentous fungi

Tania Chroumpi [a], Miia R. Mäkelä [b] & Ronald P. de Vries [a]

[a] Fungal Physiology, Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

[b] Department of Microbiology, P.O. Box 56, Viikinkaari 9, University of Helsinki, Helsinki, Finland

Corresponding authors: Ronald P. de Vries, Email: r.devries@wi.knaw.nl & Miia R. Mäkelä, E-mail: miia.r.makela@helsinki.fi

Abstract

Filamentous fungi are important industrial cell factories used for the production of a wide range of enzymes and metabolites. Their primary metabolism is a significant source of industrially important compounds, as well as of monomeric building blocks for the production of secondary metabolites and extracellular enzymes. Therefore, large efforts have been made towards the development of suitable strains for the industrial scale production of primary metabolites. Over the last decades, metabolic engineering of primary metabolism has become a powerful tool to enhance production of both primary and secondary metabolites. This review summarises the different metabolic engineering methods that have been applied to rationally improve the production of industrially relevant primary metabolites in filamentous fungi, and discusses related challenges and future perspectives.

Keywords

Filamentous fungi; metabolic engineering; primary metabolism; TCA cycle; Pentose catabolic pathway; D-galacturonic acid pathway; glycolysis; organic acids; genome-scale models

Abbreviations

TCA: tricarboxylic acid cycle; **rTCA:** reductive tricarboxylic acid pathway; **CS:** citrate synthase; **ACO:** aconitase; **ICDH:** isocitrate dehydrogenase; **KGDH:** α-ketoglutarate dehydrogenase; **SCS:** succinyl-CoA synthetase; **SDH:** succinate dehydrogenase; **FUM:** fumarase; **MDH:** malate dehydrogenase; **ICL:** isocitrate lyase; **MS:** malate synthase; **PYC:** pyruvate carboxylase; **PDC:** pyruvate decarboxylase; **CTP:** malate-citrate antiporter; **MTT**: mitochondrial TCA transporter; **PFK:** phosphofructokinase; **PKI:** pyruvate kinase; **T6P:** trehalose-6-phosphate; **CAD:** *cis*-aconitate decarboxylase; **MFS:** itaconate plasma membrane transporter; **PEP:** phosphoenolpyruvate; **OAH:** oxaloacetate hydrolase; **GDH:** glyoxylate dehydrogenase; **GLK:** glucokinase; **HXK:** hexokinase; **PGI:** phosphoglucose isomerase; **FBA:** fructose-bisphosphate aldolase;

GPD: 3-phosphate-glyceraldehyde dehydrogenase; **PGK:** phosphoglycerate kinase; **PGM:** phosphoglycerate mutase; **ENO:** enolase; **LAB:** lactic acid bacteria; **LDH:** lactate dehydrogenase; **GOX:** glucose oxidase; **D-galUA:** D-galacturonic acid; **GAR:** D-galacturonate reductase; **LGD:** L-galactonate dehydratase; **LGA:** 2-keto-3-deoxy-L-galactonate aldolase; **GLD:** glycerol dehydrogenase; **UDH:** D-galacturonate dehydrogenase; GaaR**:** the regulator of D-galacturonic acid-responsive genes; **PCP:** pentose catabolic pathway; **XYR:** D-xylose reductase; **XDH:** xylitol dehydrogenase; **XK:** xylulokinase; **LAR:** L-arabinose reductase; **LAD:** L-arabitol dehydrogenase; **LXR:** L-xylulose reductase; **GEMs:** genome-scale metabolic models

1. Introduction

Nowadays, the environmental impetus to replace traditional chemical procedures with bio-based approaches for the production of high-value products has further increased global interest in industrial biotechnology. In this field, a broad microbial spectrum is being exploited as metabolically versatile biocatalysts for the production of e.g. pharmaceuticals, chemicals, fuels, enzymes and food ingredients, all of which are of major economic, environmental and social importance. Among these microbes, filamentous fungi hold a prominent place, having inherent characteristics that make them particularly attractive cell factories for biotechnology. Apart from their natural ability of producing a remarkable wealth of commercially interesting metabolites, characteristics such as growth at high rates and to high biomass densities, growth on a broad substrate range including plant cell wall compounds (Ferreira *et al.*, 2016), and well developed methodology for genetic modification, led them to become established as dominant industrial producers of both primary (e.g. organic acids, fatty acids, vitamins and amino acids) and secondary metabolites (e.g. antibiotics).

Primary metabolites are precursor molecules, which are produced during growth and are essential for growth and maintenance of cellular functions, while they also serve as a source of monomeric building blocks for the production of secondary metabolites and extracellular enzymes. Several fungal species are currently used as industrial workhorses for production of primary metabolites, with citric acid production by *Aspergillus niger* being one of the most eminent examples of industrial scale biotechnological processes (Show *et al.*, 2015). However, the list of commercially important primary metabolites produced by filamentous fungi is much more extensive (Goldberg *et al.*, 2006; Kubicek *et al.*, 2011; Magnuson and Lasure, 2004; Zhang, 2013). Other examples of organic acid production by filamentous fungi are gluconic and oxalic acid production by *A. niger*, itaconic acid production by *Aspergillus terreus*, kojic acid production by *Aspergillus oryzae*, and fumaric, lactic and malic acid production by *Rhizopus oryzae*. Additionally, large

scale industrial production of compounds such as arachidonic acid, mostly by *Mortierella alpina*, and vitamin B_2 by *Ashbya gossypii*, also exclusively occur by fermentation of these fungi (Stahmann, 2011).

2. Metabolic engineering of primary metabolism in filamentous fungi

In the pre-genomic era, traditional strategies, such as random mutagenesis and evolutionary engineering, were predominately employed to establish improved microbial production platforms. However, the efforts for improvement of both natural and recombinant production strains have nowadays been redirected to more systematic and rational engineering strategies, e.g. metabolic engineering. Metabolic engineering is generally referred to as a targeted and purposeful alteration of metabolic pathways of an organism, using recombinant DNA technology, in order to improve cellular properties through the modification of existing or introduction of new biochemical reactions (Lessard, 1996; Stephanopoulos, 1999). In essence, it is applying the combined knowledge from fields such as molecular biology, chemical engineering, biochemistry and computational sciences with the goal to improve product yields and reduce by-product formation, by alleviating catabolite repression, and redirecting and maximizing primary metabolic fluxes towards the synthesis of the desired products (Davy *et al.*, 2017).

In filamentous fungi, application of metabolic engineering became feasible after development of efficient methods to introduce and control gene expression. Since the first reported example of successful DNA-mediated genetic transformation in *Neurospora crassa* (Mishra and Tatum, 1973), the molecular toolbox for metabolic engineering of filamentous fungi has significantly expanded. Efficient transformation methods as well as DNA- and RNA-based technologies to rationally design metabolic fluxes have been developed for many industrially important species (Li *et al.*, 2017; Wang *et al.*, 2017). Furthermore, recent advances in e.g. next generation sequencing, omics and bioinformatics, have substantially increased our understanding and improved the reliability of our predictions of fungal growth characteristics, physiology and metabolite formation. This enables more efficient ways to engineer these organisms. In the future, combined exploitation of these tools is expected to generate new approaches for designing fungal strains with improved characteristics.

Numerous studies have been published focusing on the developments and challenges in metabolic engineering of filamentous fungi (Brandl and Andersen, 2015; Meyer, 2008; Schmid *et al.*, 2009; Shi *et al.*, 2017; Wakai *et al.*, 2017), especially concentrating on the production of secondary and extracellular metabolites. However, while secondary metabolites show an enormous diversity of chemical structures, their biosynthesis seems to be directly linked to the more uniform network of primary metabolism, as most of them are derived from a relatively small number of precursor metabolites originating from

primary metabolism. In fungi, these precursor molecules are mainly short chain carboxylic acids (e.g. acetyl-CoA and related compounds, including tricarboxylic acid cycle (TCA) intermediates) or amino acids, which are assembled and further chemically modified to form a wide variety of secondary metabolites (Nielsen and Nielsen, 2017; Thirumurugan *et al.*, 2018).

Due to the significance of primary metabolism as a source of industrially important compounds, this review mainly focusses on metabolic engineering strategies for the production of these compounds in filamentous fungi, while examples described for non-conventional yeasts, such as *Ustilago*, *Yarrowia*, *Kluyveromyces*, *Candida* and *Pichia*, are also shortly addressed. Large efforts have been made towards the development of suitable strains for the industrial scale production of primary metabolites, aiming in higher yields and product titers. Over the last decades, metabolic engineering of primary metabolism has been proven to be a powerful tool for enhanced production of both primary and secondary metabolites (Davy *et al.*, 2017). The different metabolic engineering strategies that have been applied to improve the production of industrially relevant primary metabolites in filamentous fungi are described below, subdivided based on the metabolic pathway they are associated with.

3. The tricarboxylic acid cycle

The tricarboxylic acid cycle (TCA), also known as the citric acid cycle or the Krebs cycle (Krebs, 1970), has been used for decades by industry as a source of key building block chemicals. Chemical intermediates of the TCA cycle, such as citrate, α-ketoglutarate, succinate, fumarate, malate and oxaloacetate, as well as TCA-derived metabolites, such as itaconate and oxalate, play a central role in the organic acid industry due to their broad spectrum of applications.

In fungi, the TCA cycle occurs in the mitochondria and is a key metabolic pathway that has two main functions, generation of energy and synthesis of precursors for anabolism. It consists of eight nine steps catalyzed by different enzymes (Kubicek, 1988) (Fig. 1). In the first step of the TCA cycle, catalyzed by citrate synthase (CS) (Byeong Wook *et al.*, 1997; Kubicek and Röhr, 1980; Måhléan, 1972; Ruijter *et al.*, 2000), acetyl-CoA is fused with oxaloacetate to form citrate. Citrate is then isomerized in a two-step reaction by dehydration and rehydration to isocitrate by aconitase (ACO) (Kubicek and Röhr, 1985), with *cis*-aconitate as the intermediate. Next, isocitrate dehydrogenase (ICDH) (Meixner-Monori *et al.*, 1986) catalyzes the oxidation of isocitrate to α-ketoglutarate, which is further oxidized to succinyl-CoA by α-ketoglutarate dehydrogenase (KGDH) (Delattre *et al.*, 1985; Meixner-Monori *et al.*, 1985). The sixth reaction of the TCA cycle is catalyzed by succinyl-CoA synthetase (SCS), which converts succinyl-CoA to succinate. Succinate is then oxidized to fumarate, which is further hydrated to malate by succinate

dehydrogenase (SDH) and fumarase (FUM) (Friedberg *et al.*, 1995), respectively. Finally, malate dehydrogenase (MDH) (Benveniste and Munkres, 1970; Ma *et al.*, 1981) catalyzes the oxidation of malate to oxaloacetate, completing the cycle. Each complete turn of the cycle results in the regeneration of oxaloacetate and the formation of two molecules of CO_2. In a variation of the TCA cycle, the glyoxylate cycle, isocitrate is directly converted into glyoxylate and succinate by isocitrate lyase (ICL) (Ballance and Turner, 1986; Gainey *et al.*, 1992). Glyoxylate is then combined with acetyl-CoA to produce malate in a reaction catalyzed by malate synthase (MS) (Sandeman and Hynes, 1989; Thomas *et al.*, 1988).

In filamentous fungi, a cytosolic version of the reductive tricarboxylic acid (rTCA) pathway has been shown to also be involved in the production of oxaloacetate, malate and fumarate (Wright *et al.*, 1996) (Fig. 1). In this pathway, pyruvate carboxylase (PYC) catalyzes the carboxylation of pyruvate to oxaloacetate, while the cytosol-localized MDH and FUM enzymes catalyze the conversion of oxaloacetate to malate and fumarate, respectively (Kenealy *et al.*, 1986; Osmani and Scrutton, 1985; Peleg *et al.*, 1989; Song *et al.*, 2011). Since MDH and FUM are located in both cytosol and mitochondria, the ability of various filamentous fungal species to overproduce certain carboxylic acids has been specifically attributed to the exclusive cytosolic localization of the key enzyme PYC (Goldberg *et al.*, 2006; Osmani and Scrutton, 1983, 1985).

Metabolic engineering strategies of both mitochondrial TCA cycle and cytosolic rTCA pathway have been described to improve production of industrially relevant organic acids (Yin *et al.*, 2015).

3.1 Citric acid

Citric acid (citrate) is a C6-tricarboxylic acid, which is the most widely used organic acid in food and beverages, detergents, pharmaceuticals, cosmetics and other technical applications (Ruijter *et al.*, 2002). Although many microorganisms can produce significant amounts of citrate (Berovic and Legisa, 2007), *A. niger* remains the best industrial production organism due to its high product titer (>150 g/l), ease of handling, and ability to use cheap substrates (Papagianni, 2007; Wang *et al.*, 2015).

In fungi, biosynthesis of citrate occurs in both the cytosol and the mitochondria (Cleland and Johnson, 1954; Karaffa and Kubicek, 2003). After conversion of D-glucose to two molecules of pyruvate through the cytosolic glycolytic pathway (see glycolysis section), one molecule is transported into the mitochondria and converted to acetyl-CoA, while the other enters the rTCA pathway (Fig. 1). The formed malate enters the mitochondria *via* a malate-citrate antiporter (CTP) and is further converted into citrate through the TCA cycle (Ruijter *et al.*, 2002). Citrate is then pumped out of the mitochondria by counter transport with malate, leading to accumulation of citrate (Karaffa and Kubicek, 2003). The significance of malate

accumulation as a stimulus for citrate production is stressed by the observation that a rise in the intracellular concentration of malate in *A. niger* seems to precede citrate accumulation (Röhr and Kubicek, 1981). This pathway has a maximum theoretical citrate yield of 1 mol per mol glucose when the starting pyruvate originates from glycolysis.

Over the years, various metabolic engineering strategies have been developed to improve citrate production in *A. niger* (Fig. 2). However, overexpression of rate-limiting enzymatic steps involved in glycolysis and the TCA cycle, in order to increase the metabolic flux towards citrate, had limited success due to the tight regulation of central carbon metabolism. Both individual or combined moderate overexpression of the genes encoding phosphofructokinase (PFK) and pyruvate kinase (PKI) in *A. niger* did not significantly increase citrate production (Fig. 2a and 2b), and neither the activities of other enzymes in the pathway, nor the intermediary metabolite levels were influenced. On the contrary, overexpression of *pfk* resulted in decreased activity of the enzyme due to a strong reduction of the positive allosteric regulator of PFK, fructose 2,6-bisphosphate. This shows that the cells compensated for the increased amount of PFK by decreasing the concentration of fructose 2,6-bisphosphate (Ruijter *et al.*, 1997). Up to 11-fold increased expression level of CS-encoding gene did also not improve citrate production (Ruijter *et al.*, 2000) (Fig. 2c). Likewise, no significant changes were observed in the activity of other enzymes relevant to citrate biosynthesis or in the levels of the intermediary metabolites tested. This suggests that also CS does not significantly contribute to flux control in the pathway involved in citrate biosynthesis in *A. niger*.

A kinetic model was used to show that in order to achieve a significant increase (3- to 50-fold) in citrate production rate, at least 12 simultaneous enzyme modulations are required (Alvarez-Vasquez *et al.*, 2000). More specifically, the transport processes and most of the glycolytic enzyme activities were indicated as the most relevant steps in this optimization. In another study, the glucose carrier was pointed as a suitable target for up-modulation, showing that a 2-fold up-modulation of its activity could cause a 45% increase in the citrate productivity, while an increase in the mitochondrial phosphate carrier activity could also enhance the citrate rate production (Guebel and Torres Darias, 2001). All these findings imply that, due to the tight control of central carbon metabolism in *A. niger*, direct improvements in citrate production by single enzyme modulation or by simply up-modulating any given set of two enzymes is unlikely, explaining also the previously discussed unsuccessful attempts (Ruijter *et al.*, 1997; Ruijter *et al.*, 2000).

The only successful attempts for increased metabolic flux towards citrate production have been through means, such as reduction of feedback inhibition, induction of pelleted morphology, elimination of by-product formation, change of the availability of cytosolic dicarboxylic acids or improvement of citrate transport out of the cytosol.

In particular, decreased inhibition of hexokinase by trehalose-6-phosphate (T6P), after disruption of the T6P synthase-encoding gene (*ggsA*), resulted in earlier accumulation of citrate (Arisan-Atac *et al.*, 1996) (Fig. 2d). Although the disruption of the *ggsA* gene did not increase the product yield, a significant decrease in the time required to reach the half-maximal citrate concentration was reported.

Pelleted morphology has been shown to be directly linked to increased citrate production (Clark, 1962). Induction of pelleted growth, after antisense expression of Brsa-25 (a putative amino acid transporter), resulted in about 30% increased citrate production, under normally inhibiting Mn^{2+} concentrations (Dai *et al.*, 2004). Brsa-25 was shown to be responsive to Mn^{2+}, the concentration of which influences the morphology, and as a result citrate production of *A. niger*.

Construction of the double oxaloacetate hydrolase (*oahA*) and glucose oxidase (*goxC*) deficient strain (Fig. 2e and 2f), unable to produce both by-products oxalate and gluconate, also had a positive effect in citrate production in *A. niger*. This *A. niger* strain lacking both these genes (Δ*oahA*Δ*goxC*) produced citrate from sugar substrates in a regular synthetic medium at pH 5 that is optimal for oxalate production (Kubicek *et al.*, 1988), while under these conditions production was completely insensitive to Mn^{2+} (Ruijter *et al.*, 1999). The amount of citrate produced by the Δ*oahA*Δ*goxC* mutant under these conditions was slightly higher than that observed in the traditional citrate fermentation (i.e. pH <2, no Mn^{2+}).

By overexpressing genes encoding enzymes known to catalyze reactions involving cytosolic malate, fumarate and succinate, the impact of altered cytosolic dicarboxylic acid concentrations on citrate production was also evaluated (de Jongh and Nielsen, 2008). Several heterologous genes were expressed in *A. niger*, either individually or simultaneously, i.e. a malate dehydrogenase (*mdh2*) from *Saccharomyces cerevisiae* (Fig. 2g), two cytosolic fumarases (*fum1s* and *fumRs*) from *S. cerevisiae* and *R. oryzae* (Fig. 2h), and the cytosolic fumarate reductase (*frds1*) from *S. cerevisiae* (Fig. 2i). All the resulting transformants showed enhanced citrate yields and production, while, contrary to the wild type, they were able to produce citrate in the presence of Mn^{2+}. The *mdh2*-expressing mutant showed an increased citrate production rate only in the initial phase of the fermentation compared to the other transformants and the wild type, supporting the theory that citrate production is triggered by an increased cytosolic malate concentration (Röhr and Kubicek, 1981). The overproduction of both fumarases provided more substrate to the mitochondrial malate-citrate antiporter and led to increased citrate production. Even higher citrate production was observed after expression of the gene encoding Frds1, which catalyzes the irreversible conversion of cytosolic fumarate to succinate (Enomoto *et al.*, 2002). The connection between cytosol succinate synthesis and citrate accumulation implies that succinate can be also used for the mitochondrial antiport of citrate. Combined expression of *fumRs* and *frds1* possibly raised the concentration of succinate

in the cytosol even more, resulting in their best citrate producing strain. This strain gave a maximum citrate yield of 0.9 g/g of glucose and a maximum specific productivity of 0.025 g/gDW/h.

Finally, the transport of citrate out of the cytosol was also shown as an important citrate production limiting step (Karaffa and Kubicek, 2003). The *cexA* gene, homologous to the itaconate transporter gene from *Ustilago maydis*, was shown to encode the main citrate transporter in *A. niger* (Steiger *et al.*, 2019). The disruption of this gene resulted in oxalate accumulation, while secretion of citrate was completely abolished. Confirmation of CexA as the main citrate exporter in *A. niger* was also given by comparative transcriptomics between two alternative citrate producing conditions (Odoni *et al.*, 2018). Overexpression of *cexA* (Fig. 2j), using the *ptet-on* inducible expression system, led to 5-fold higher citrate secretion levels compared to the parental strain (Steiger *et al.*, 2019).

Metabolic engineering strategies for citrate production have been also described for the non-conventional yeast *Yarrowia lipolytica*. In this fungus, citrate production was improved by expression of the invertase *suc2* gene from *S. cerevisiae* for the utilization of sucrose and the ICL-encoding gene (*icl1*). Their best strain produced 140 g/l citrate from sucrose with a productivity of 0.73 g/l/h in fed-batch fermentation (Forster et al., 2007). Citrate production from inulin was also achieved in *Y. lipolytica* after heterologous expression of the *Kluyveromyces marxianus* inulinase encoding gene (*inu1*). This strain utilized inulin to synthesize citrate reaching a titer of 68.9 g/l, with 4.1 g/l isocitrate as a by-product (Liu et al., 2010). Additional deletion of ATP-citrate lyase encoding gene (*acl1*) and expression of *icl1* finally resulted in a strain that yielded citrate from 10% inulin at a titer of 84 g/l (Liu et al., 2013).

3.2 Itaconic acid

Itaconic acid (itaconate) is an unsaturated C5-dicarboxylic acid, which has drawn considerable interest as a bio-based building block chemical for a wide range of industrial applications, such as plastics, detergents, paper, coating and adhesives, and biomedicals (Kubicek *et al.*, 2011; Okabe *et al.*, 2009; Robert and Friebel, 2016; Willke and Vorlop, 2001). The first reported itaconate producer was *Aspergillus itaconicus* (Kinoshita, 1931), while shortly after, it was discovered that itaconate production was even higher for *A. terreus* (Calam *et al.*, 1939). Nowadays, itaconate is produced industrially *via* fermentation of *A. terreus* reaching titers of 80 g/l (Kuenz *et al.*, 2012).

Biosynthesis of itaconate occurs by the same metabolic pathway as citrate, but whereas citrate is the end-product in *A. niger*, in *A. terreus* two additional enzymatic steps lead to itaconate formation (Bentley and Thiessen, 1957) (Fig. 1). After conversion of citrate to *cis*-aconitate by ACO, *cis*-aconitate is transported back into the cytosol with the help of a mitochondrial tricarboxylic acid transporter and serves as a

precursor for itaconate production (Jaklitsch *et al.*, 1991; Scarcia *et al.*, 2019; Steiger et al., 2016; Hosseinpour Tehrani *et al.*, 2019; Wierckx *et al.*, 2020). The *cis*-aconitate is converted to itaconate by *cis*-aconitate decarboxylase (CAD), which is exclusively located in the cytosol, and is further excreted through a plasma membrane transporter (MFS) to the extracellular space (Hosseinpour Tehrani *et al.*, 2019; Wierckx *et al.*, 2020). This pathway has a maximum theoretical molar yield of 1 mol itaconate per mol glucose when the starting pyruvate originates from glycolysis.

For improved production of itaconate in both *A. terreus* and *A. niger*, overexpression of potential flux-controlling glycolytic steps to increase the pool of TCA intermediates, was proven once more a rather unsuccessful strategy. Neither the overexpression of the native *pfk* in *A. terreus* (Tevz *et al.*, 2010) (Fig. 3a), nor the overexpression of glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) in *A. niger* (Li *et al.*, 2012) were beneficial for itaconate production, even though the latter was shown to be highly expressed under itaconate production conditions (Li *et al.*, 2011). Only, the insertion of a modified *A. niger pfkA* gene into *A. terreus*, which gave a highly active and citrate inhibition-resistant shorter form of PFK, led to a significant increase in itaconate production (Tevz *et al.*, 2010) (Fig. 3a). This shows that the reason behind this enhanced productivity probably relies on the release of feedback inhibition.

In *A. terreus*, five genes were identified as potentially involved in biosynthesis of itaconate, four of which were located in the "itaconate biosynthesis gene cluster" (Li *et al.*, 2011). This cluster consists of genes encoding the *cis*-aconitate decarboxylase (CadA), a mitochondrial TCA transporter (MTT), a plasma membrane transporter (Mfs) and a transcription factor (Reg) proposed to regulate the expression of the other genes of the cluster. To further enhance itaconate productivity, all four cluster genes were separately overexpressed in an already high-performance *A. terreus* strain (Shin *et al.*, 2017). Overexpression of *reg* and *mtt* did not result in significant improvements in itaconate production (Fig. 3b), despite the higher transcription levels of both genes during the entire fermentation period. However, overexpression of *cad* and *mfs* had a positive effect on itaconate production (Fig. 3c and 3d, respectively). The highest-yielding *cad* transformant produced up to 75 g/l itaconate in shake-flask fermentations, which represents a 5% increase of the itaconate titer compared to the parental strain, while overexpression of *mfs* resulted in 7% higher itaconate production. Similar results were also obtained after using a different industrial *A. terreus* strain, where overexpression of *cadA* and *mfsA* separately increased itaconate production by 9.4% and 5.1% in shake-flask fermentations, respectively (Huang *et al.*, 2014).

Naturally, *A. niger* is not able to produce itaconate, since it lacks the essential CAD enzyme, but the ability for itaconate accumulation can be introduced in *A. niger* by insertion of the *cadA* gene from *A. terreus* (Li *et al.*, 2013; Li *et al.*, 2011). Co-expression of *cadA* and either the mitochondrial carrier (*mtt*) or the plasma

membrane carrier (*mfs*) gene from *A. terreus* led to increased itaconate production in *A. niger* (9-fold and 5.4-fold, respectively), but combined expression of *cadA, mfs* and *mtt* did not further affect production (Li *et al.*, 2013). However, overexpression of *citB*, encoding a native putative cytosolic CS, resulted in an even higher itaconate production as well as elimination of citrate by-product formation (Hossain *et al.*, 2016).

Overexpression of the codon-optimized *cadA*, *mttA* and *mfsA* genes for *A. niger* in an oxaloacetate hydrolase (OahA) and glucose oxidase (GoxC) -deficient strain, led to increased yields and itaconate production (van der Straat *et al.*, 2014). Codon optimization of the *cadA* gene for *A. niger* was reported to result in a more than 3-fold increase in itaconate production compared to the ones expressing the native *cadA* gene of *A. terreus*, while co-expression of codon optimized *cadA* and *mttA* resulted in >20-fold increased itaconate levels. In contrast, when overexpression of codon-optimized *cadA* and *mfsA* were combined, no effect on the production levels of itaconate was detected, suggesting that the produced itaconate can be efficiently secreted by an endogenous *A. niger* transporter. Co-expression of these three genes led to *A. niger* strains with over 25-fold higher levels of itaconate and a 20-fold increased yield when compared to a strain expressing only the codon optimized *cadA* gene.

Finally, targeting enzymes to the right cellular compartment was also beneficial for itaconate production in *A. niger* (Blumhoff *et al.*, 2013). Co-producing the enzymes ACO and CAD in the mitochondria, increased itaconate production 2-fold compared with strains overexpressing both enzymes in the cytosol. By selectively combining the above mentioned strategies, namely a) mitochondrial overexpression of the codon-optimized *cadA* (Blumhoff *et al.*, 2013; van der Straat *et al.*, 2014), b) overexpression of the codon-optimized *mtt* and *mfs* (van der Straat *et al.*, 2014) and c) elimination of by-product (citrate, oxalate and gluconate) formation (Hossain *et al.*, 2016; van der Straat *et al.*, 2014), even higher itaconate production titers may be possible in *A. niger*.

Itaconate production was also shown in *Y. lipolytica* after the overexpression of the *A. terreus* CAD enzyme, resulting in an initial titer of 33 mg/l (Blazeck *et al.*, 2015). Co-expression of CAD and cytosolic localized ACO enzymes further improved itaconate production to 4.6 g/l, representing an improvement of nearly 140-fold. Ustilaginaceae have also gained biotechnological interest over the last years due to their ability to naturally produce itaconate. In *Ustilago maydis*, by combining the deletion of *cyp3* encoding an itaconate oxidase that converts itaconate into 2-hydroxyparaconate, and the overexpression of the itaconate gene cluster regulator *ria1*, an itaconate hyper producer strain was obtained that produced up to 4.5–fold more itaconate compared to the wild type (Geiser *et al.*, 2016). Additional deletion of the gene clusters involved in the biosynthesis of the accumulated glycolipids (mannosylerythritol lipids and ustilagic acid), as well as of a putative diacylglycerol acyltransferase gene

(*dgat*) involved in the biosynthesis of intracellular triacylglycerols led to reduced by-product spectrum of *U. maydis* and improved metabolic flux into the targeted itaconate biosynthesis pathway (Becker *et al.*, 2020) . This resulted in a more efficient conversion of glucose into itaconate and therefore in a 21 – 27% improvement of itaconate yield, titer and rate. *Ustilago vetiveriae* was also used as new production organism for itaconate synthesis from glycerol (Zambanini *et al.*, 2017a). Overexpression of the itaconate gene cluster regulator *ria1* or the mitochondrial transporter *mtt1* from the itaconate cluster of *U. maydis* resulted in a 2-fold (*ria1*) and 1.5-fold (*mtt1*) higher itaconate titer in comparison to the wild type strain, simultaneously reducing malate by-product formation by 75 and 41%, respectively.

3.3 Succinic acid

Succinic acid (succinate), also known as butanedioic acid or amber acid, is a valuable C4-dicarboxylic acid with applications in food, pharmaceutical and surfactant/detergent markets. It can also be used as a precursor for the production of biodegradable polymers and other commodity or specialty chemicals, including 1,4-butanediol and adipic acid (Song and Lee, 2006). Currently, it is mainly chemically produced by hydrogenation of maleic anhydride derived from petroleum materials (Muzumdar *et al.*, 2004; Zhao *et al.*, 2012). However, the need for a more efficient, cost effective and environmentally friendly production method has caused an interest into the biotechnological production of succinate (Sauer *et al.*, 2013; Sauer *et al.*, 2008; Willke and Vorlop, 2004).

Several fungal, yeast and bacterial species have been considered for industrial bio-based production of succinate (Beauprez *et al.*, 2010; Jansen and van Gulik, 2014). Apart from native succinate overproducers, efforts have also been made towards metabolic engineering of bacterial (e.g. *E. coli*) and yeast (e.g. *S. cerevisiae*) strains (Ahn *et al.*, 2016). In contrast, only a few metabolic engineering studies for succinate production in filamentous fungi have been published (de Jongh and Nielsen, 2008; Meijer *et al.*, 2009a; Meijer *et al.*, 2009b; Yang *et al.*, 2016), probably due to their limited natural capacity to produce succinate compared to bacteria.

Three main biosynthetic routes for succinate production have been proposed in microbes (Cheng *et al.*, 2013): a) the TCA cycle, b) the cytosolic rTCA pathway and c) the glyoxylate cycle (Fig. 1). In *A. niger*, an attempt to redirect fluxes towards the glyoxylate pathway by overexpressing the isocitrate lyase (*icl)* gene, did not result in improved succinate production (Meijer *et al.*, 2009b). Instead, a significant increase in fumarate levels was observed, showing that the overexpression of *icl* finally led to increased flux through the oxidative part of the TCA cycle.

Overexpression of the genes involved in the cytosolic rTCA pathway had also no significant impact on succinate production in *A. niger* (de Jongh and Nielsen, 2008) (Fig. 4b and 4c), while fumarate reductase, that catalyzes the conversion of fumarate to succinate, has never been identified in any fungal species (Knuf *et al.*, 2014). This indicates that the rTCA pathway is not directly involved in succinate production in *A. niger*. Heterologous expression of a cytosolic fumarate reductase (Frds1) encoding gene from *S. cerevisiae* (Fig. 4d) improved succinate production in *A. niger*, while similar results were also obtained for *Aspergillus saccharolyticus* through heterologous expression of a fumarate reductase gene from the flagellated protist *Trypanosoma brucei* (Yang *et al.*, 2016).

In *A. niger*, deletion of a cytosolic ATP: citrate lyase (*acl*) gene, which catalyzes the conversion of citrate to oxaloacetate and acetate, directly improved the production of the total amount of organic acids, including succinate with 3-fold increase in its yield (Meijer *et al.*, 2009a) (Fig. 4e). However, to develop *A. niger* as a cell factory for succinate, further optimization is needed to reach levels of industrial importance, comparable to other succinate overproducing organisms.

Accumulation of succinate has been also reported using engineered *Y. lipolytica* by deleting or downregulating the genes encoding different subunits of SDH (Yuzbashev *et al.*, 2010, Jost *et al.*, 2015, Gao *et al.*, 2016). In particular, reduction or loss of SDH activity in *Y. lipolytica* through deleting *Ylsdh1* and *Ylsdh2* genes or exchanging the native promoter of *Ylsdh2* gene with a weak promoter resulted in accumulation of about 4–5 g/l succinate using glycerol as substrate (Yuzbashev *et al.*, 2010, Jost *et al.*, 2015). Deletion of *Yl*sdh5 also resulted in similar results (5.2 g/l) at low pH with high acetate formation as by-product (Gao *et al.*, 2016). Combination of *Yl*sdh5 deletion with overexpression of PEP carboxykinase (*pck*) from *S. cerevisiae* and the endogenous SCS beta subunit (*Ylscs2*) genes further improved succinate production by 4.3-fold, while deletion of CoA-transferase (*Ylach)* gene also eliminate acetate production (Cui *et al.*, 2017). In fed-batch fermentation, this strain produced 110.7 g/L succinic acid with a yield of 0.53 g/g glycerol without the need of pH control.

3.4 Fumaric acid

Fumaric acid (fumarate) is a C4-dicarboxylic acid that has been applied as a chemical intermediate for other platform chemicals such as succinic and maleic acids, and as monomer or building block in the polymer industry. Additionally, it is non-toxic and has been commonly used as an acidulant agent in the food, feed, beverage and pharmaceutical industry. Currently, fumarate is mainly produced *via* catalytic isomerization of petroleum-derived maleic acid (Zhang, 2013), while as an alternative to chemical production, microbial production of fumarate is facilitated using filamentous fungi.

Fumarate production occurs mainly in filamentous Mucoralean fungi, particularly those belonging to the genus *Rhizopus*, such as *R. nigricans*, *R. arrhizus*, *R. oryzae*, and *R. formosa* (Foster and Waksman, 1939). However, only *R. arrhizus* and *R. oryzae* have been extensively studied for their fumarate production potential (Rhodes *et al.*, 1959). Since the 1990s, *R. oryzae* has been the front liner in microbial fumarate production, owing to its simple nutrient requirements and high productivity (Xu *et al.*, 2012). Extensive research efforts for more efficient biological production of fumarate have mainly focused on optimization of fermentation processes and downstream methods (Das *et al.*, 2016; Zhang, 2013). In contrast, the number of metabolic engineering studies for improved production of fumarate is rather limited.

Although fumarate is an intermediate of the TCA cycle, fungal production under aerobic conditions seems to occur entirely *via* the reductive branch of the pathway (see TCA section and Fig. 1) (Friedberg *et al.*, 1995; Kenealy *et al.*, 1986; Peleg *et al.*, 1989). This has been confirmed as the primary pathway for fumarate biosynthesis with a high theoretical molar yield of 2 mol per mol glucose, when the starting compound pyruvate originates from glycolysis.

Metabolic engineering was applied in order to funnel and maximize the metabolic flux towards fumarate production in *R. oryzae* (Zhang *et al.*, 2012). In this study, the authors tried to increase the pool size of oxaloacetate by overexpression of the endogenous pyruvate carboxylase (*pyc*) gene of *R. oryzae* (Fig. 5a), involved in the first step of the rTCA pathway, and the phosphoenolpyruvate (PEP) carboxylase (*pepc*) gene from *E. coli* (Fig. 5b), which catalyzes the production of oxaloacetate from PEP with CO_2 fixation. By increasing the copy number of the endogenous *pyc* gene in *R. oryzae*, the *pyc* transformants exhibited 56%-83% increased PYC activity compared to wild type. However, this did not result in higher fumarate yields, but in a higher accumulation of malate and ethanol, attributed to the growth morphology of the *pyc* transformants (large pellets; 250-500 μm in diameter) as has been also previously shown (Zhou *et al.*, 2011). Ethanol production usually occurs under anaerobic or oxygen-limited conditions (Magnuson and Lasure, 2004), while fumarate is mainly produced under aerobic conditions. Consequently, limited oxygen supply caused by growth as large compact pellets could explain both the negligible performance of the *pyc* transformants and ethanol accumulation. In contrast, the heterologous expression of *pepc* from *E. coli* in *R. oryzae* significantly increased fumarate production (Zhang *et al.*, 2012). The *pepc* transformants exhibited significant PEPC activity (3-6 mU/mg), that was absent in the wild type, while they grew as smaller pellets (~150 μm in diameter). The highest yield of fumarate was obtained when fungi were grown as small pellets, and the lowest yield when grown as clumps (Zhou *et al.*, 2011). Fumarate production by the *pepc* transformant increased approximately 20% compared to wild type that grew as loose mycelial clumps.

406 The endogenous FUM-encoding gene (*fumR*) of *R. oryzae* was also overexpressed to investigate its effects
407 on cell growth and fumarate production in *R. oryzae* (Friedberg *et al.*, 1995) (Fig. 5c). FUM catalyzes the
408 reversible hydration of fumarate to malate and is present in both cytosolic and mitochondrial forms (see
409 TCA section). In mitochondria, FUM catalyzes the conversion of fumarate to malate, while in the
410 cytoplasm it catalyzes the conversion of malate to fumarate (Friedberg *et al.*, 1995; Peleg *et al.*, 1989).
411 However, increased FUM activity did not result in higher fumarate production (Friedberg *et al.*, 1995).
412 Instead, *fumR* transformants produced about twice as much malate compared to the wild type, suggesting
413 that the overexpressed *fumR* gene does not code for the cytosolic FUM enzyme responsible for the
414 overproduction of fumarate in *R. oryzae*.
415 Based on FUM activity detected in cell lysates, Goldberg *et al.* (2006) suggested that *R. oryzae* also harbors
416 a second *fum* gene encoding a cytosolic FUM enzyme with preference for conversion of malate to
417 fumarate. Furthermore, they suggested that this second enzyme was inhibited by 2 mM fumarate.
418 Sequence analysis of both enzymes revealed that this second FUM missed 15 amino acids from its N-
419 terminal region compared to the previously reported FUM (Song *et al.*, 2011). Additionally, this new FUM
420 had a higher affinity for malate than for fumarate and, furthermore, the conversion of fumarate to malate
421 was completely inhibited by 2 mM fumarate, which is consistent with the earlier observation (Goldberg
422 *et al.*, 2006). These results indicate that this second FUM is the cytosolic enzyme responsible for the
423 accumulation of fumarate in *R. oryzae*, but application of this knowledge for improved production of
424 fumarate has not yet been reported.
425 In summary, immobilization and pellet morphology control have resulted in better improvement of
426 fumarate production than metabolic engineering. However, more research towards genetic or metabolic
427 modifications of the *Rhizopus* species may still lead to improved and cost-effective fumarate production.
428 *Scheffersomyces stipitis* was also engineered to produce fumarate from D- xylose by overexpressing the
429 heterologous rTCA pathway from *R. oryzae* (Wei *et al.*, 2015). Furthermore, three strategies were
430 performed to improve the fumarate production, including increasing the activities of the heterologous
431 rTCA pathway enzymes (PYC, MDH and FUM) by codon optimization, blocking the conversion of fumarate
432 to malate through the TCA cycle by deleting the native FUM genes *Psfum1* and *Psfum2*, and improving the
433 fumarate transportation by overexpressing a codon optimized Mae1 transporter from
434 *Schizosaccharomyces pombe*. In this strain, the fumarate production was increase to 4.67 g/l, representing
435 an increase of 37.92-fold compared to the control strain.

3.5 Malic acid

Malic acid (malate) is a C4-dicarboxylic acid, which is predominantly used in the food and beverage industries as an acidulant, preservative and flavoring agent. It is also essential in the preparation of medical and personal care products, while having a potential market as a raw material for the production of polymalate (Gross and Kalra, 2002). Currently, malate is primarily produced through chemical synthesis *via* catalytic hydration of maleic anhydride (Lohbeck *et al.*, 2000). Another production method involves the enzyme-catalyzed conversion of fumarate to malate by immobilized bacterial cells showing high FUM activity, which is however not applied commercially (Zhang, 2013).

Fermentation of malate has been also carried out by various microorganisms (Bercovitz *et al.*, 1990; Zambanini *et al.*, 2016), with *A. flavus* being the best producer organism, accumulating 113 g/l after 8 days, with a yield of 94% (w/w) based on the consumed glucose and a high overall productivity (0.59 g/l/h) (Battat *et al.*, 1991). However, due to the accompanying production of aflatoxins, alternative malate overproducers, such as *Penicillium sclerotiorum* and *A. oryzae*, have been also considered as industrial production platform organisms (Brown *et al.*, 2013; Wang *et al.*, 2013).

Although malate is a key intermediate in the mitochondrial TCA cycle, similarly to fumarate, malate accumulation in microbial cells is attributed to the cytosolic rTCA pathway followed by its transport across the cell wall. The mechanism leading to malate production is the same as the pathway leading to fumarate, but abbreviated by one step (Fig. 1). This pathway has a maximum theoretical molar yield of 2 mol malate per mol glucose, when the starting compound pyruvate originates from glycolysis.

In order to improve malate production in *A. oryzae*, a native C4-dicarboxylate transporter (C4T318) gene, ortholog of the *S. pombe* malate transporter (Mae1), was overexpressed in *A. oryzae* (Brown *et al.*, 2013) (Fig. 6a). This resulted in a >2-fold increase in the rate of malate production in *A. oryzae*, with the highest producing C4T318 transformant achieving malate titers of 122 g/l and an overall malate production rate of 0.74 g/l/h. When overexpression of *C4T318* and of the native cytosolic *pyc* and *mdh* genes was combined (Fig. 6a, 6b and 6c), malate production was further increased by 27%. This strain was able to produce 154 g/l malate with a productivity of 0.94 g/l/h, showing >3-fold improvement in malate production rate compared to the parental strain. For both malate producing strains, small amounts of succinate, fumarate and citrate were formed as by-products. Even though citrate is not naturally produced in *A. oryzae*, similarly to *A. niger*, malate accumulation may trigger citrate accumulation through a process mediated by mitochondrial tricarboxylate transporters (de Jongh and Nielsen, 2008; Karaffa and Kubicek, 2003).

Malate production of *A. oryzae* was further improved by applying metabolic engineering strategies to rewire the rTCA and malate transport pathways (Liu *et al.*, 2017). Initially, to strengthen the metabolic flux towards malate, native *pyc* and *mdhA* genes were co-overexpressed in *A. oryzae* (Fig. 6b and 6c), improving malate production 1.6-fold compared to wild type. An anaplerotic route for oxaloacetate synthesis from PEP, in order to increase the oxaloacetate pool available for malate synthesis, was also constructed. Additional heterologous expression of *pepc* and *pck* genes from *E. coli*, both catalyzing the production of oxaloacetate from PEP with CO_2 fixation (Fig. 6d), further increased malate production 2.7-fold compared to wild type. Furthermore, to improve the transport efficiency of malate across the cytosolic membrane and reduce feedback inhibition of intracellular malate, they also overexpressed the native C4T318 dicarboxylate transporter (Fig. 6a) and the malate permease (*maeI*) gene from *S. pombe* (Fig. 6e). Overexpression of these two additional heterologous genes improved malate production 4.6-fold compared to the wild type, while additional overexpression of the 6-phosphofructokinase (*pfk*) gene (Fig. 6f) improved the malate titer even further. Overall, the final strain displayed a 4.9-fold increase in malate productivity compared to wild type in shake flask fermentations, while malate production after 5 days reached a maximum of 165 g/l with a productivity of 1.38 g/l/h in fed-batch fermentation.

As mentioned earlier (see Fumarate section), overexpression of *fumR* did not increase fumarate, but increased malate biosynthesis in *R. oryzae* (Friedberg *et al.*, 1995). Therefore, overexpression of *fumR* may be another beneficial strategy to further improve malate production in *A. oryza*e.

Ustilago trichophora RK089 has been also shown to be a good natural malate producer from glycerol (Zambanini *et al.*, 2016). This strain has undergone adaptive laboratory evolution for enhanced substrate uptake rate resulting in the *U. trichophora* TZ1 strain with a 6.6-fold increased production rate. Overexpression of pyruvate carboxylase, two malate dehydrogenases (*mdh1* and *mdh2*), and two malate transporters (*ssu1* and *ssu2*) genes in *U. trichophora* TZ1 increased the malate yield by 54% in shake flasks reaching a titer of 120 g/l (Zambanini *et al.*, 2017b).

3.6 Oxalic acid

Oxalic acid (oxalate) is a strong dicarboxylic acid, commercially used as a chelator, detergent, or tanning agent. Although, it is currently produced through chemical processes (Pernet, 1991), production of oxalate does also naturally occur by several fungi (Dutton and Evans, 1996). *A. niger* has been reported as a very efficient oxalate producer with production of 13 g/l oxalate after 70 h with a yield of 65% (w/w) based on the consumed sugar (van de Merbel *et al.*, 1994). Oxalate production of 38 g/l, which is close to the

497 solubility of sodium oxalate, has been also reported (Strasser *et al.*, 1994) in fed-batch fermentation (pH
498 6) with sucrose as the carbon source.

499 Two major pathways have been described for oxalate biosynthesis in microorganisms. One pathway
500 involves the hydrolysis of oxaloacetate by oxaloacetate hydrolase (OAH), while the other involves
501 oxidation of glyoxylate by glyoxylate dehydrogenase (GDH) (Balmforth and Thomson, 1984; Han *et al.*,
502 2007; Kubicek *et al.*, 1988). Although, it has been postulated that both pathways should be present in *A.*
503 *niger* (Cleland and Johnson, 1956), any attempts to measure glyoxylate-oxidizing enzymes in extracts of
504 oxalate producing *A. niger* were not successful (Kobayashi *et al.*, 2014; Müller, 1975). However, evidence
505 points towards oxalate production in *A. niger* by a cytosolic Mn^{2+}-dependent OAH (OahA) (Kubicek *et al.*,
506 1988) (Fig. 7). Deletion of *oahA* results in non-oxalate producing *A. niger* mutants (Pedersen *et al.*, 2000;
507 Ruijter *et al.*, 1999), which implies that OAH is the only enzyme responsible for production of oxalate in
508 *A. niger*.

509 Overexpression of *oahA* in a citrate-producing *A. niger* strain resulted in an oxalate hyper-producing strain
510 (Kobayashi *et al.*, 2014) (Fig. 7a). In this study, the amount of produced oxalate increased 1.85-fold
511 compared to the parental strain. Oxalate production of the *oahA* transformant reached 28.9 g/l after 12
512 days of cultivation, with a final yield of 96.3% (w/w) based on the consumed glucose. Increased oxalate
513 production was also observed in *A. niger* after expression of two heterologous genes encoding cytosol-
514 targeted fumarases (Fum1s and FumRs) from *S. cerevisiae* and *Rhizopus oryzae*, respectively (de Jongh
515 and Nielsen, 2008) (Fig. 7b). Insertion of a cytosolic FUM in *A. niger* seems to result in the conversion of
516 cytosolic fumarate to malate, which is then used as substrate for oxalate production. The oxalate
517 production titers obtained for the *fum1s*/*frds1* double insertion mutant were 8-fold higher than for the
518 wild type in production medium containing 100 g glucose/l and with the pH controlled to 3.5. This was
519 probably an effect of high OAH enzyme activity at pH values above 3 (Ruijter *et al.*, 2002) in combination
520 with the inserted cytosolic FUM activity.

521 A further increase of the oxaloacetate pool size, by overexpressing both *pyc* and *pepc* from *E. coli*, which
522 was previously shown to be beneficial for fumarate production (Zhang *et al.*, 2012), or by blocking the
523 cytosolic rTCA pathway after oxaloacetate, could also have a positive effect on oxalate production.

524 4. Glycolysis

525 Glycolysis, also known as glycolytic pathway or Embden-Meyerhof-Parnas pathway, is a cytosolic oxygen-
526 independent metabolic pathway in which one molecule of D-glucose, or other sugars that are funneled
527 into the pathway, is converted *via* a series of intermediate metabolites into two molecules of pyruvate.

This pathway, apart from being a source of metabolites that can serve as building blocks for the synthesis of other cellular products, also provides energy captured in the form of the high-energy molecules ATP and NADH to support cellular metabolism.

Glycolysis is a sequence of ten enzyme-catalyzed reactions (Fig. 8). D-Glucose is phosphorylated to glucose-6-phosphate by glucokinase (GLK) or hexokinase (HXK) (Panneman *et al.*, 1996; Panneman *et al.*, 1998). D-fructose can also be phosphorylated to fructose-6-phosphate by HXK and enter glycolysis. Glucose-6-phosphate is converted to fructose-6-phosphate by phosphoglucose isomerase (PGI) (Ruijter and Visser, 1999), which is further converted to D-fructose-1,6-bisphosphate by phosphofructokinase (PFK) (Ruijter *et al.*, 1997). Fructose-bisphosphate aldolase (FBA) catalyzes the conversion of D-fructose-1,6-bisphosphate to D-glyceraldehyde-3-phosphate (Nakajima *et al.*, 2000) and 3-phosphate-glyceraldehyde dehydrogenase (GPD) catalyzes the subsequent conversion to glycerate-1,3-bisphosphate (Punt *et al.*, 1988). This can then be converted to glycerate-3-phosphate and glycerate-2-phosphate by phosphoglycerate kinase (PGK) (Clements and Roberts, 1985) and phosphoglycerate mutase (PGM) (Flipphi *et al.*, 2009), respectively. Glycerate-2-phosphate is then converted to phosphoenol pyruvate by an enolase (ENO) (Machida *et al.*, 1996), followed by conversion to the end-product of glycolysis, pyruvate, by pyruvate kinase (PKI) (Ruijter *et al.*, 1997).

Depending on oxygen availability, pyruvate can cross the mitochondrial membrane, be converted into acetyl-CoA and, in aerobic metabolism, enter the TCA cycle (see above) or, in anaerobic metabolism, be fermented into interesting products such as lactic acid. Metabolic engineering strategies for the production of lactic acid, but also of products such as gluconic acid, whose production is indirectly linked to glycolysis, are described below.

4.1 Lactic acid

Lactic acid (lactate) is a carboxylic acid that has broad applications in the food, pharmaceutical, cosmetics, leather and chemical industries (Vijayakumar *et al.*, 2008). Its role as the building block of the biodegradable polymer poly-lactate (Jem *et al.*, 2010) and the eco-friendly solvent ethyl lactate (Watkins, 2002) has recently increased the demand for lactate. Nevertheless, more efficient and cost-effective methods for its large-scale production still need to be explored. The industrial production of lactate traditionally involves the biological conversion of D-glucose into lactate either by lactic acid bacteria (LAB) or *Rhizopus* species. Fermentations with *Rhizopus* oryzae are often preferred to LAB, due to the outstanding ability of this filamentous fungus to directly produce almost optically pure L-lactate with a low nutritional requirement from cheap abundant carbon sources (Soccol *et al.*, 1994; Zhang *et al.*, 2007).

559 However, this process still requires near neutral pH conditions and lactate yields are compromised due to
560 formation of ethanol, fumarate and glycerol as by-products.

561 Lactate is a natural end-product of primary metabolism and is produced from pyruvate by an NAD-
562 dependent lactate dehydrogenase (LDH) (Fig. 8). Until now, any engineering efforts to improve lactate
563 production in *R. oryzae* have been mainly focused in increasing LDH activity, so lactate fermentation could
564 more effectively compete for the available pyruvate (Skory, 2001, 2004; Skory and Ibrahim, 2007).
565 Overexpression of the *R. oryzae ldhA* gene (Fig. 8a), which was previously identified as the gene primarily
566 responsible for the conversion of pyruvate to lactate (Skory, 2000), resulted in accumulation of 60 g/l
567 lactate after 3 days of fermentation, with a yield of 60% (w/w) based on the consumed glucose (Skory,
568 2001). This means that lactate production in this recombinant *R. oryzae* strain was improved 1.3-fold
569 compared to the non-transformed control strain as a result of increased LDH activity, followed by a
570 concurrent decrease in ethanol, fumarate and glycerol formation by 1.4-fold, 3.4-fold and 1.3-fold,
571 respectively.

572 Although Aspergilli do not naturally produce lactate and a functional LDH has not been reported in these
573 fungi, their low nutrient requirements and their ability to endure the weak acid stress caused by organic
574 acid accumulation, also make them promising hosts for lactate production. Recently, three different
575 *Aspergillus* species were engineered to produce lactate, where the formed pyruvate was directed to
576 lactate by each time expressing a suitable *ldh* gene (Dave and Punekar, 2015; Liaud *et al.*, 2015; Wakai *et*
577 *al.*, 2014) (Fig. 8a).

578 In *A. oryzae*, expression of a codon-optimized bovine LDH resulted in accumulation of 38 g/l optically pure
579 lactate after 10 days, with a yield of 38% (w/w) based on the consumed glucose (Wakai *et al.*, 2014).
580 Additional disruption of the native *ldh* gene in *A. oryzae*, which was suspected to hinder product
581 accumulation by catalyzing the reverse reaction from lactate to pyruvate, further improved lactate
582 production 1.3-fold. In *Aspergillus brasiliensis*, the heterologous overexpression of *ldh* from *R. oryzae* also
583 resulted in lactate production with lactate titers of 13.9 g/l after 138 h, and a conversion yield of 27%
584 (w/w) based on the consumed glucose (Liaud *et al.*, 2015). After efficient pH regulation by using $NaNO_3$
585 as nitrogen source and optimization of the culture feed, an additional 1.8-fold increase in lactate
586 conversion yield was obtained. Finally, an *A. niger* strain capable of producing 7.7 g/l of lactate after 3
587 days in non-neutralizing medium, with a yield of 12.8% (w/w) based on the consumed glucose, was
588 reported after heterologous expression of mouse LDH under the control of a strong constitutive *A. niger*
589 CS promoter (Dave and Punekar, 2015).

Pichia pastoris has been also engineered to produce lactate by heterologous expression of the bovine *ldh* gene (de Lima *et al.*, 2016). Increased lactate secretion by additional overexpression of a putative lactate transporter further resulted in a higher lactate yield of 0.67 g/g of glycerol, representing an increase of 46 % in lactate yield compared to the control strain. Similar results were also obtained after combination of *ldh* overexpression with the deletion of a pyruvate decarboxylase (PDC)- encoding gene in *P. pastoris*, in order to funnel pyruvate into the lactate production pathway (Melo *et al.*, 2018). Overexpression of the bovine LDH and deletion of PDC in *Kluyveromyces lactis* also resulted in a lactate yield of 0.58 g/g of glucose, suggesting that a large fraction of the glucose consumed was not converted into pyruvate (Porro *et al.*, 1999). In a different attempt to redirect pyruvate flux toward homolactic fermentation, *K. lactis* LDH transformant strains lacking both PDH and PDC activities showed even higher yield levels of as high as 0.85 g/g of glucose (Bianchi *et al.*, 2001).

4.2 Gluconic acid

Gluconic acid (gluconate) is a weak carboxylic acid that has applications in the food, feed, beverage, detergent, textile, pharmaceutical and building industries (Cañete-Rodríguez *et al.*, 2016; Kubicek *et al.*, 2011; Ramachandran *et al.*, 2006). Although there are different methods available for the production of gluconate (Ramachandran *et al.*, 2006), submerged fermentation by *A. niger* using D-glucose is almost exclusively applied for its commercial production (Blom *et al.*, 1952). However, the high fermentation production costs of gluconate and its derivatives (Singh and Kumar, 2007) demand further optimization of its production process. Design of improved production strains for continuous fermentation of gluconate, in combination with exploitation of cheaper alternative carbon sources, could potentially lead to cost-effective production of gluconate.

In fungi, gluconate is formed in a two-step process that involves the oxidation of D-glucose to D-glucono-δ-lactone by glucose oxidase (GOX) and the subsequent hydrolysis of the lactone to gluconate (Fig. 8). The second conversion can either occur spontaneously or be catalyzed by a lactonase (Roehr *et al.*, 2008). In *A. niger*, both GOX and lactonase have been shown to be located outside the cell, indicating that formation of gluconate occurs extracellularly (Witteveen *et al.*, 1992). As intracellular glycolysis competes with extracellular gluconate formation for D-glucose, fine-tuning of the glycolytic flux may have a positive effect on gluconate production.

In the past, most attempts to create selective mutants of *A. niger* and *Penicillium* sp. with improved gluconate producing capacity involved random mutagenesis and subsequent selection of mutants with increased production (Singh and Kumar, 2007). However, since repeated mutagenic treatments increase

621 the frequency of undesired mutations, which can ultimately affect overall fitness and stability of the
622 production strains, metabolic engineering could be a more promising strategy.

623 So far, actual metabolic engineering strategies for improved production of gluconate have not been
624 reported. Since production of gluconate is directly linked to GOX activity, GOX-overproducing strains may
625 be employed to improve fermentation efficiency. Overexpression of *goxC*, the gene encoding GOX in *A.*
626 *niger*, resulted in a significant increase of GOX activity compared to the reference strain (Witteveen *et al.*,
627 1993) (Fig. 8b). Overexpression of *goxC* in a constitutive GOX-overproducing (Δ*goxB*) background strain
628 resulted in even higher (6-fold) GOX activity. However, growth of these transformants was poor, probably
629 due to the high H_2O_2 production.

630 Co-overexpression of catalase, an enzyme involved in the conversion of H_2O_2 to molecular oxygen and
631 water, could potentially reduce the toxic effect of H_2O_2 accumulation and improve the stability of the
632 GOX-overproducing strain. Fowler *et al.* suggested that the *catR* gene may encode a catalase localized
633 extracellularly together with GOX (Fowler *et al.*, 1993). Increased expression of *catR* under the control of
634 the strong glucoamylase (*glaA*) promoter, resulted in transformants that produced up to 10-times higher
635 levels of catalase than their reference strain. However, the effect of *catR*-overexpression in gluconate
636 production still needs to be assessed.

637 Since the catalytic efficiency for the conversion of D-glucose to gluconate is highly dependent on GOX
638 stability, strategies to increase its oxidative and thermal stability have been successful. A single mutation
639 of a methionine (M561S) located close to the GOX active site resulted in 2.5-times increased half-life of
640 GOX in the presence of H_2O_2 (Kovačević *et al.*, 2019). In addition, three amino acid substitutions
641 (Q90R/Y509E/T554M) resulted in 3-fold higher GOX residual activity after heating (60^{o}C for 45 min)
642 compared to their reference strain (Marín-Navarro *et al.*, 2015), while five amino acid substitutions
643 (R145N/A36M/T10K/G274S/E374Q), predicted *via* computational design, resulted in 2-fold higher GOX
644 residual activity after similar heat treatment (Mu *et al.*, 2019). In the last study, the improved
645 thermostability of GOX resulted in a 2-fold increase in gluconate production.

646 5. D-Galacturonic acid pathway

647 Filamentous fungi are particularly interesting as industrial workhorses due to their inherent ability to
648 efficiently degrade and utilize plant biomass materials as carbon source. Pectin, one of the
649 major polysaccharide constituents of plant cell walls, consists mainly of D-galacturonic acid (D-galUA)
650 residues (Mohnen, 2008). As a result, the fungal D-galUA pathway is of fundamental importance for the
651 utilization of pectin and its conversion to useful and more valuable products. Intermediates of the

pathway are of high industrial interest (e.g. L-galactonate and its keto-deoxy derivative sugar), while they can also serve as precursors for the production of other important metabolites (e.g. L-ascorbic acid and mucic acid). In the following sections, metabolic engineering strategies applied for the construction of fungal strains producing these compounds are described in detail.

The D-galUA catabolic pathway has been described for three filamentous fungi, *T. reesei*, *A. niger* and *Botrytis cinerea* (Richard and Hilditch, 2009; Zhang *et al.*, 2011), but identification of a D-galUA transporter (GatA) has been only described for *A. niger* (Sloothaak *et al.*, 2014). All three proposed pathways comprise four enzymatic steps, involving the conversion of D-galUA to pyruvate and glycerol *via* L-galactonate, 2-keto-3-deoxy-L-galactonate and L-glyceraldehyde (Fig. 9). The conversion of D-galUA to L-galactonate is catalyzed by D-galacturonate reductase (GAR; Gar1 in *T. reesei*, GaaA in *A. niger*, and BcGar1 and BcGar2 in *B. cinerea*) (Kuorelahti *et al.*, 2005; Martens-Uzunova and Schaap, 2008; Zhang *et al.*, 2011), while in the second step L-galactonate is converted to 2-keto-3-deoxy-L-galactonate by L-galactonate dehydratase (LGD; Lgd1 in *T. reesei*, GaaB in *A. niger* and BcLgd1 in *B. cinerea*) (Kuorelahti *et al.*, 2006; Martens-Uzunova and Schaap, 2008; Zhang *et al.*, 2011). The third step is catalyzed by 2-keto-3-deoxy-L-galactonate aldolase (LGA; Lga1 in *T. reesei*, GaaC in *A. niger* and BcLga1 in *B. cinerea*), where 2-keto-3-deoxy-L-galactonate is metabolized into pyruvate and L-glyceraldehyde (Hilditch *et al.*, 2007; Zhang *et al.*, 2011). Finally, in the fourth step of the D-galUA pathway, L-glyceraldehyde is converted to glycerol by glycerol dehydrogenase (GLD; Gld1 in *T. reesei*, GaaD in *A. niger* and BcGlr1 in *B. cinerea*) (Liepins *et al.*, 2006; Martens-Uzunova and Schaap, 2008; Zhang *et al.*, 2011). Disruption of different steps of the fungal D-galUA metabolism has resulted in strains performing alternative D-galUA conversions (Kuivanen *et al.*, 2012; Kuivanen *et al.*, 2016; Mojzita *et al.*, 2010c; Wiebe *et al.*, 2010).

5.1 L-Galactonate

L-Galactonate, a 6-carbon carboxylic acid with similar physicochemical properties to gluconate, is a compound with potential to be used in food, pharmaceutical, cosmetic, and other industries (e.g., dyes, detergents, solvents, and paints). L-Galactonate is also a precursor for L-ascorbic acid synthesis. Specifically, L-galactono-1,4-lactone, which is formed upon acidification of L-galactonate, can be converted to L-ascorbic acid either chemically (Csiba *et al.*, 1993) or through microbial fermentation (Onofri *et al.*, 1997; Roland *et al.*, 1983). Additionally, production of polymers derived from L-galactonate has been considered (Romero Zaliz and Varela, 2003, 2005). However, L-galactonate and its lactone are currently expensive specialty chemicals and are not widely used or produced on a large scale. Microbial production could be the solution for a more efficient and cost-effective production process.

As mentioned above, L-galactonate is an intermediate metabolite in the fungal D-galUA catabolic pathway (Fig. 9). Deletion of the genes coding for L-galactonate dehydratase (Fig. 10a), in both *T. reesei* (*lgd1*) and *A. niger* (*gaaB*), resulted in strains unable to grow on D-galUA (Kuivanen *et al.*, 2012). These strains converted D-galUA to L-galactonate, which was excreted into the culture medium. In this study, yields from 0.6 to 0.9 g of L-galactonate per g of consumed D-galUA were reported. However, intracellular accumulation of L-galactonate in both strains suggested that export may be a bottleneck in extracellular production. In *A. niger* Δ*gaaB*, induction of both *gaaA* and the putative D-galUA transporter gene (*gatA*) was negatively affected, while overexpression of *gaaA* significantly improved the initial production rates (Fig. 10b).

In a later study, the pathway intermediate 2-keto-3-deoxy-L-galactonate was shown to promote the induction of genes required for D-galUA utilization in *A. niger* (Alazi *et al.*, 2017), which explains the previous results. They proposed that intracellular accumulation of 2-keto-3-deoxy-L-galactonate in the Δ*gaaC* deletion strain would increase the concentration of active GaaR, which is needed for the expression of genes required for pectin degradation and transport and catabolism of D-galUA in *A. niger*. Overexpression of *gaaR* resulted in an increased transcription of the genes encoding pectinases, D-galUA transporters, and catabolic pathway enzymes even under non-inducing conditions (Alazi *et al.*, 2018). Although overexpression of *gaaA* significantly improved the initial production rates of L-galactonate in *A. niger*, additional overexpression of *gaaR* would probably remove the bottleneck and as a result promote even higher L-galactonate production.

5.2 2-Keto-3-deoxy-L-galactonate

Applications for 2-keto-3-deoxy-L-galactonate, also known as 3-deoxy-L-*threo*-hex-2-ulosonic acid, have not yet been clearly described. However, the potential of keto-deoxy sugars as precursors for the synthesis of various sugar derivatives that are of great interest to biochemical and pharmaceutical research (Hanessian, 1967; Tanimura *et al.*, 2003; Wiebe *et al.*, 2010), has attracted interest towards their production. In fungi, 2-keto-3-deoxy-L-galactonate is an intermediate in the metabolism of D-galUA (Richard and Hilditch, 2009), where removal of water from L-galactonate leads to the formation of 2-keto-3-deoxy-L-galactonate.

Production of 2-keto-3-deoxy-L-galactonate in both *T. reesei* and *A. niger* was achieved by simply deleting the gene coding for the 2-keto-3-deoxy- L-galactonate aldolase (*lga1* and *gaaC*, respectively) (Wiebe *et al.*, 2010) (Fig. 11). Both *A. niger* Δ*gaaC* and *T. reesei* Δ*lga1* deletion strains were able to convert D-galUA to 2-keto-3-deoxy-L-galactonate, which was accumulated extracellularly when they were cultivated in the

presence of D-galUA. Production rates for both *A. niger* Δ*gaaC* and *T. reesei* Δ*lga1* mutants were further improved (0.8- and 0.7-fold, respectively), when D-xylose was provided as an energy source in order to replenish the NADPH pool required for the conversion of D-galUA. However, *A. niger* Δ*gaaC* was more efficient than *T. reesei* Δ*lga1* in producing 2-keto-3-deoxy-L-galactonate, showing a production rate of 0.33 g/l/h (0.12 g/gDW/h) and a yield of 0.85 g/g of D-galUA, while it was also able to produce keto-deoxy-L-galactonate directly from pectin or polygalacturonic acid at similar or higher (0.54 g/l/h) rates. The authors suggest that optimization of the provision of co-substrate for biomass and energy production may have a positive effect in productivity. However, the high intracellular accumulation of 2-keto-3-deoxy-L-galactonate suggests that its export from the cell may also be a bottleneck for the *A. niger* Δ*gaaC* overproduction strain. Since only concentrations above 12 g/l were exported to the culture medium, to further enhance production of 2-keto-3-deoxy-L-galactonate, improvement of its export rate should also be considered.

5.3 Galactaric acid

Galactaric acid (galactarate), also known as meso-galactaric acid or most commonly as mucic acid, is a dicarboxylic 6-carbon-containing organic acid, which is applied in food, cosmetic and pharmaceutical industries. It is mainly used as a chelator and in skin care products, while it can also find applications in polymer synthesis and as a platform chemical (Kiely *et al.*, 2000; Lewkowski, 2003; Mehtiö *et al.*, 2016). Nowadays, galactarate is produced either by oxidation of D-galactose or galactose-containing compounds using nitric acid (Kiely and Hash, 2010), or by electrolytic oxidation of D-galUA (Fauvarque *et al.*, 1994).

However, it has been shown that galactarate could also be produced by biotechnological means, using filamentous fungi as cell factories (Kuivanen *et al.*, 2016; Mojzita *et al.*, 2010c). Galactarate is not an intermediate in the reductive fungal D-galUA catabolic pathway, but it is the first intermediate in the oxidative pathway for D-galUA catabolism in some bacteria, such as *Agrobacterium* and *Pseudomonas*. In these organisms, the oxidation of D-galUA to galactarate is catalyzed by a NAD-dependent D-galacturonate dehydrogenase (UDH). Introduction of the bacterial *udh* gene into *A. niger* and *T. reesei* strains with a disrupted D-galUA metabolism resulted in galactarate production (Mojzita *et al.*, 2010c) (Fig. 12a and 12b). Both strains lacking the D-galacturonate reductase (Δ*gaaA* and Δ*gar1*, respectively) were unable to grow on D-galUA, while the strains additionally expressing the bacterial Udh-encoding gene (Δ*gaaA-udh* and Δ*gar1-udh*, respectively) converted D-galUA to galactarate. Unlike in *T. reesei*, expression of Udh in *A. niger* Δ*gaaA* deletion strain restored its ability to grow on D-galUA, implying that this organism can use the resulting galactarate as a carbon source. This strain produced only a small

amount of galactarate, while the conversion of D-galUA to galactarate in *T. reesei* was very efficient, reaching yields close to the theoretical maximum.

Since *A. niger* could potentially be a more suitable organism than *T. reesei* for conversion of pectin-rich biomass due to its higher pectinase content, improved production of galactarate was attempted by identification and disruption of its galactarate catabolism (Kuivanen *et al.*, 2016). Candidate genes were identified using RNA sequencing and deleted from the genome of *A. niger*. Deletion of one of these genes (protein ID 39114, JGI MycoCosm) abolished growth on galactarate (Fig. 12c). Deletion of this gene in the *A. niger* Δ*gaaA-udh* strain resulted in a strain converting D-galUA to galactarate at an equimolar ratio. However, during production of galactarate directly from pectin-rich biomass in a consolidated process using the Δ*gaaA*-Δ*39114-udh* strain, significantly reduced consumption of D-galUA was observed. The authors suggest that these modifications may have changed the co-factor balance, which now seems to be insufficient to maintain the complete conversion of the available D-galUA (Kuivanen *et al.*, 2016).

5.4 L-Ascorbic acid

L-Ascorbic acid (ascorbate), vitamin C, is a six-carbon organic compound with several applications in food, beverage, pharmaceutical, cosmetics, animal feed/agricultural and various other industries (Bauernfeind, 1982). It is naturally produced in many animal and plant cells, having biological properties as an antioxidant agent and enzyme cofactor (Linster and Van Schaftingen, 2007; Valpuesta and Botella, 2004). Currently, the industrial production of ascorbate relies on an efficient multi-step process, which involves two sequential bacterial fermentation steps followed by several chemical conversion steps (Pappenberger and Hohmann, 2014; Yang and Xu, 2016). In this two-step fermentation process, after hydrogenation of D-glucose to D-sorbitol, oxidation of D-sorbitol to L-sorbose is commonly carried out using *Gluconobacter oxydans*. The resulting L-sorbose is subsequently converted to 2-keto-L-gulonic acid in a mixed fermentation, consisting of *Ketogulonicigenium vulgare* and *Bacillus* spp., which is finally converted to ascorbate after several chemical steps.

Engineered *A. niger* strains for direct conversion of D-galUA or pectin-rich biomass to ascorbate have been described (Kuivanen *et al.*, 2015). In these strains, the native fungal D-galUA metabolism was disrupted, by knocking out the *gaaB* gene (Fig. 13a), and a plant ascorbate biosynthetic pathway originating from D-galUA was introduced. More specifically, an algal and plant gene coding for L-galactono-1,4-lactone lactonase from *Euglena gracilis* (*EgALase*) (Fig. 13b) and L-galactonolactone dehydrogenase from the plant *Malpighia glabra* (*MgGALDH*) (Fig. 13c), respectively, were introduced into the *A. niger* Δ*gaaB* strain, under the control of both constitutive and D-galUA inducible promoters. Alternatively, an unspecific L-

776 gulono-1,4-lactone lactonase encoding *smp30* gene (Fig. 13b), involved in the mammalian ascorbate
777 biosynthetic pathway, was introduced instead of *EgALase*. Even though lactonase (EgALase or smp30)
778 enzyme activity was not observed in any of the resulting strains, increased production of ascorbate was
779 achieved for all strains overexpressing *MgGALDH*, regardless of the presence or absence of *EgALase* or
780 *smp30*. This indicates that conversion of L-galactonate to L-galactono-1,4-lactone may be spontaneous
781 and overexpression of only *MgGALDH* is sufficient to initiate production of ascorbate in *A. niger*. Although
782 ascorbate production was delayed compared to the constitutive expression, inducible expression of the
783 plant-derived pathway led to higher production levels from pure D-galUA. Significantly higher product
784 titers, up to 170 mg/l, were achieved when the same strain was cultivated in a consolidated bioprocess
785 with pectin-rich biomass as a substrate. As suggested by the authors, this is probably due to higher NADPH
786 levels available for GaaA, involved in the first reaction of fungal D-galUA catabolic pathway.
787 Also for this process, overexpression of *gaaA*, as described by Alazi *et al.* (2018), may improve the
788 production rates of ascorbate in *A. niger* by increasing the available L-galactonate pool. Additional
789 overexpression of *gaaR* could also increase the transcription of the genes encoding pectinases and D-
790 galUA transporters, which could result in production of ascorbate in earlier stages.

791 6. Pentose catabolic pathway

792 D-Xylose and L-arabinose are the most abundant monosaccharides in nature after D-glucose, being major
793 constituents of the hemicelluloses xylan and xyloglucan, and of pectin. In fungi, L-arabinose and D-xylose
794 are metabolized through the pentose catabolic pathway (PCP), comprised of two interconnected oxido-
795 reductive pathway branches (Seiboth and Metz, 2011; Witteveen *et al.*, 1989). Both pentose sugars go
796 through oxidation, reduction and phosphorylation reactions to form D-xylulose-5-phosphate.
797 Intermediates of the pathway include polyols, such as xylitol and L-arabitol, which are industrially
798 important compounds. As a result, study and exploitation of the PCP for the production of important
799 chemicals from lignocellulosic materials has attracted significant interest.
800 Xylitol is the first common intermediate of the interconnected D-xylose and L-arabinose fungal
801 metabolism (Fig. 14). In most fungi, D-xylose is reduced to xylitol by either NADH- or NADPH-dependent D-
802 xylose reductase (XYR; XyrA in *A. niger* and Xyl1 in *T. reesei*) (Hasper *et al.*, 2000; Seiboth *et al.*, 2007),
803 while the resulting xylitol is further oxidized to D-xylulose by NAD^+-dependent xylitol dehydrogenase
804 (XDH; XdhA in *A. niger* and Xdh1 in *T. reesei*) (Seiboth and Metz, 2011). In a final irreversible step, D-
805 xylulose is converted to D-xylulose-5-phosphate by xylulokinase (XK; XkiA in *A. niger* and Xkl1 in *T. reesei*)
806 (vanKuyk *et al.*, 2001), which can then enter the pentose phosphate pathway (Seiboth and Metz, 2011).

These last two reactions of the fungal D-xylose pathway are shared with the L-arabinose catabolic pathway.

In *A. niger*, L-arabinose catabolism initiates with reduction of L-arabinose to L-arabitol by NADPH-dependent L-arabinose reductase (LarA) (de Groot *et al.*, 2005; Mojzita *et al.*, 2010a) (Fig. 14). Specifically, LarA converts L-arabinose and also D-xylose to their corresponding sugar polyols with a higher affinity for L-arabinose. However, in *T. reesei*, the conversion of L-arabinose to L-arabitol is catalyzed again by Xyl1, although, with lower specificity compared to D-xylose (Akel *et al.*, 2009; Seiboth *et al.*, 2007). L-Arabitol is then converted to L-xylulose by NAD^+-dependent L-arabitol dehydrogenase (LAD; LadA in Aspergilli and Lad1 in *T. reesei*) (de Vries *et al.*, 1994; Richard *et al.*, 2001; Seiboth and Metz, 2011). In *T. reesei*, it has been shown that Lad1 can also partially compensate the loss of Xdh1 by converting xylitol to D-xylulose (Seiboth *et al.*, 2003). Finally, L-xylulose is reduced to xylitol by L-xylulose reductase (LXR; LxrA in *A. niger* and Lxr3 in *T. reesei*) (Metz *et al.*, 2013; Mojzita *et al.*, 2010b).

6.1 Xylitol

Xylitol is a 5-carbon sugar alcohol, which can be naturally found in low concentrations in various fruits and vegetables. Due to its low calorigenic and non-cariogenic properties, xylitol has attracted significant interest as an alternative sweetener in food, pharmaceutical and cosmetic industries (Mussatto, 2012; Ur-Rehman *et al.*, 2015). It also has applications as building block for the production of various organic chemicals, such as glycerol, hydroxyl furan, glycol and xylaric acid (Delgado *et al.*, 2018; Granström *et al.*, 2007). Large scale production of xylitol occurs mainly by chemical reduction of D-xylose from biomass hydrolysates (Melaja and Hämäläinen, 1977; Nigam and Singh, 1995). However, this chemical process has several drawbacks, such as high cost and energy investments.

Biotechnological approaches for industrial xylitol production, including fermentation by microorganisms and/or enzymatic approaches, have been considered as alternative to the chemical method (Winkelhausen and Kuzmanova, 1998). In nature, several microorganisms are able to produce xylitol, including filamentous fungi, yeast and bacteria. Among them, yeasts are the best xylitol producers, particularly those belonging to the genus *Candida* (Guo *et al.*, 2006). Metabolic engineering of *Candida tropicalis*, by disruption of the XDH-encoding gene (*xyl2*) resulted in xylitol production with a volumetric productivity of 3.23 g/l/h, a specific productivity of 0.76 g/g/h, and a xylitol yield of 98% (Ko *et al.*, 2006). In another non-conventional yeast, namely *S. stipitis*, disruption of the *xyl3* gene coding for D-xylulokinase resulted in a strain produced 26 g/l of xylitol with a volumetric productivity of 0.22 g/l/h from D-xylose (Jin *et al.*, 2004). *P. pastoris* was also engineered for conversion of glucose to xylitol by expressing the D-xylulose-forming D-arabitol dehydrogenase (DalD) gene from *Klebsiella pneumoniae* and the *xdh* gene from *G. oxydans* (Cheng *et al.*, 2014). The Recombinant *P. pastoris*

strain with both *dalD* and *xdh* genes could produce xylitol from glucose with the highest yield of 0.078 g/g of glucose and productivity of 0.29 g/l/h. However, since yeasts can only use biomass hydrolysates as substrate for xylitol production, use of filamentous fungi would be more advantageous due to their ability to directly use plant biomass.

Metabolic engineering attempts for construction of xylitol overproducing filamentous fungal strains has showed promising results, as deleting or overexpressing individual steps of fungal pentose metabolism resulted in increased xylitol production (Fig. 15). In *T. reesei*, antisense inhibition of *xdh1* expression resulted in xylitol accumulation in the liquid medium (Wang *et al.*, 2005) (Fig. 15a). Although XDH activity of their highest xylitol-producing strain only reduced to approx. 48% compared to the parental strain, xylitol accumulation reached a maximum of 2.37 mg/ml in a 2% D-xylose medium, which was about five times higher than the parental strain. Increased xylitol production was also achieved by disrupting the *xdhA* gene in *A. oryzae* (Mahmud *et al.*, 2013) (Fig. 15a). In this study, *A. oryzae* Δ*xdhA* strain showed a 4- and 10-fold increase in xylitol volumetric productivity compared to the parental strain, when D-xylose and oat spelt xylan, respectively, were used as substrate in the production medium.

Antisense silencing of D-xylulokinase gene *xkl1* expression also promoted xylitol accumulation in *T. reesei* (Fig. 15b), as did overexpression of the D-xylose reductase gene *xyl1* in a *T. reesei* Δ*xdh1* strain (Hong *et al.*, 2014) (Fig. 15a and 15c). However, knocking down the *xkl1* gene in *T. reesei* was more efficient than overexpressing *xyl1* showing over 8- and 1.08-times higher xylitol production as compared to their parent strains, respectively. More specifically, when expression of the *xkl1* gene was silenced (approx. 49% reduction) (Fig. 15b), xylitol production increased from 0 to 8.6 mM, while higher *xyl1* expression only marginally increased xylitol production from 22.8 mM to 24.8 mM.

Although these attempts could be considered as proof of principle for production of xylitol using filamentous fungi, more combinatorial approaches should be applied in order to obtain strains with competitive xylitol production levels. In *T. reesei*, it has been shown that the loss of XDH can be partially compensated by L-arabinitol-4-dehydrogenase (Seiboth *et al.*, 2003), which also applies for Aspergilli (our unpublished data). In *A. oryzae*, individual deletion of the *ladA* gene did not show any significant impact on D-xylose metabolism when *xdhA* was intact (Mahmud *et al.*, 2013). On the contrary, the *A. oryzae* Δ*ladA* mutant showed even lower xylitol production than the parental strain. Therefore, in order to evaluate the effect of a *ladA* deletion on xylitol accumulation, a double deletion mutant lacking both *xdhA* and *ladA* genes would need to be examined. Additionally, overexpression of *xyrA* and *larA* genes in this double deletion mutant could further increase the flux towards xylitol production, since L-arabinose reductase can compensate for D-xylose reductase activity.

As suggested by Hong *et al.* (2014), the moderate increase in xylitol production may also be due to limited NADPH available for the conversion of D-xylose into xylitol (Hong *et al.*, 2014). Ahmad *et al.* (2012) showed that overexpression of two enzymes involved in the pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, led to an increase in xylitol production in *Candida tropicalis* (Ahmad *et al.*, 2012). Finally, increase of substrate availability by overexpressing a D-xylose transporter could also improve xylitol production.

7. Conclusions and future prospects

Filamentous fungi are organisms with high industrial potential due to their inherent ability to directly convert biomass substrates to valuable metabolites. A large effort has been put into sequencing and annotation of filamentous fungal genomes, while sophisticated analytical and genome editing tools have also been developed for their analysis and manipulation at the genomic level, including the powerful CRISPR/Cas9 technology. However, to design better and more versatile industrial fungal cell factories, a thorough and holistic understanding of their physiology is essential.

While the primary metabolism of filamentous fungi has been a topic of study for many decades, the gaps in our knowledge regarding the metabolic enzymes involved in individual steps and the regulation of the pathways could be considered as actual bottlenecks in the development of more effective metabolic engineering strategies. The public availability of genome sequence information offers the opportunity to fill in these gaps and expand the scope of metabolic engineering to the entire metabolic network.

Reconstruction of genome-scale metabolic models (GEMs) is an important step in this direction. Modeling of genome-scale networks requires the integration of multi-omics data, such as transcriptomics, proteomics, metabolomics and fluxomics. Therefore, such a network reconstruction represents a biochemical, genetic, and genomic knowledgebase that contains detailed information about an organism in a more structured format (Thiele and Palsson, 2010). Thus, genome-scale metabolic modeling currently stands out as one of the most promising approaches to obtain an *in silico* prediction of cellular function in terms of physiology, providing a multi-level depiction of the metabolism and its regulation (Borodina and Nielsen, 2005).

In filamentous fungi, GEMs have been reported for *Aspergillus* and *Penicillium* species (Agren *et al.*, 2013; Andersen *et al.*, 2008; Thykaer *et al.*, 2009), both used as industrial cell factories. However, at the moment, most of these models do not contain full experimental validation of the function of the genes and are partially based on similarity to characterized genes in other species. For *A. niger*, a first GEM focusing on primary metabolism provided a tentative connection between genome sequences, genome-scale

expression data, and literature studies (Andersen *et al.*, 2008). This GEM relies on constraint-based analysis, which uses physicochemical constraints such as mass balance, energy balance, thermodynamics and flux limitations to describe the potential behavior of an organism. Such a model, however, ignores much of the dynamic nature of the system, and its ability to highlight changes in the cellular metabolism in order to be used as a reliable and accurate predictive tool in metabolic engineering is limited.

Although, the *S. cerevisiae* GEM can be considered as a reference model for eukaryotic microorganisms as one of the best described models with high level of curation and experimental validation, its transferability to filamentous fungi is debatable. This can basically be attributed to the differences in complexity (in particular the number of secreted products, growth morphology and more limited metabolism with respect to the sugars it can convert) and level of characterization of the individual organisms. To improve the efficiency of metabolic engineering, accurate metabolic models that can help predict which steps should be manipulated are required. Dynamic extensions of the constraint-based modelling have been developed to overcome this limitation (Sanchez *et al.*, 2014). These aim to create parametrized algorithms that link the steady-state intracellular metabolic flux distribution with dynamic changes in the environment to provide prediction of microbial growth, substrate utilization and product formation dynamics. Such dynamic follow-up GEMs of primary metabolism of *A. niger* has been recently published, in which the genes involved in the various steps were verified using transcriptome data (Aguilar-Pontes *et al.*, 2018; Brandl *et al.*, 2018). However, while these novel models significantly improve our understanding of metabolism, they still have several uncertainties that need to be addressed.

One problem is the presence of paralogous genes for many of the metabolic reactions in fungal primary metabolism. Comparative genome and transcriptome analysis and detailed phylogeny can help in predicting whether such a paralog is likely to perform the same function as the original enzyme, but often it is difficult to draw firm conclusions without experimental validation. Secondly, several enzymes may have side-activities in addition to their primary physiological role, which can compensate for the absence of another enzyme. Finally, the existence of back-up pathways that have so far not been identified cannot be excluded. Therefore, experimental validation of such models in order to address these issues is essential before being able to use their full potential as accurate predictive tools in metabolic engineering.

For all these reasons, metabolic pathway optimization can generally be very challenging because of the metabolic complexity that cells have evolved to maintain robustness. The involvement of several genes in the same step of the pathway may complicate metabolic engineering, as it gets harder to tune that step by modifying multiple target genes. Application of metabolic engineering in order to redirection the metabolic flow towards specific products could then involve reduction of the number of genes involved

in the target step to a single one and then only manipulate that one based on the desired efficiency or affinity of the encoded enzymes.

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Figures

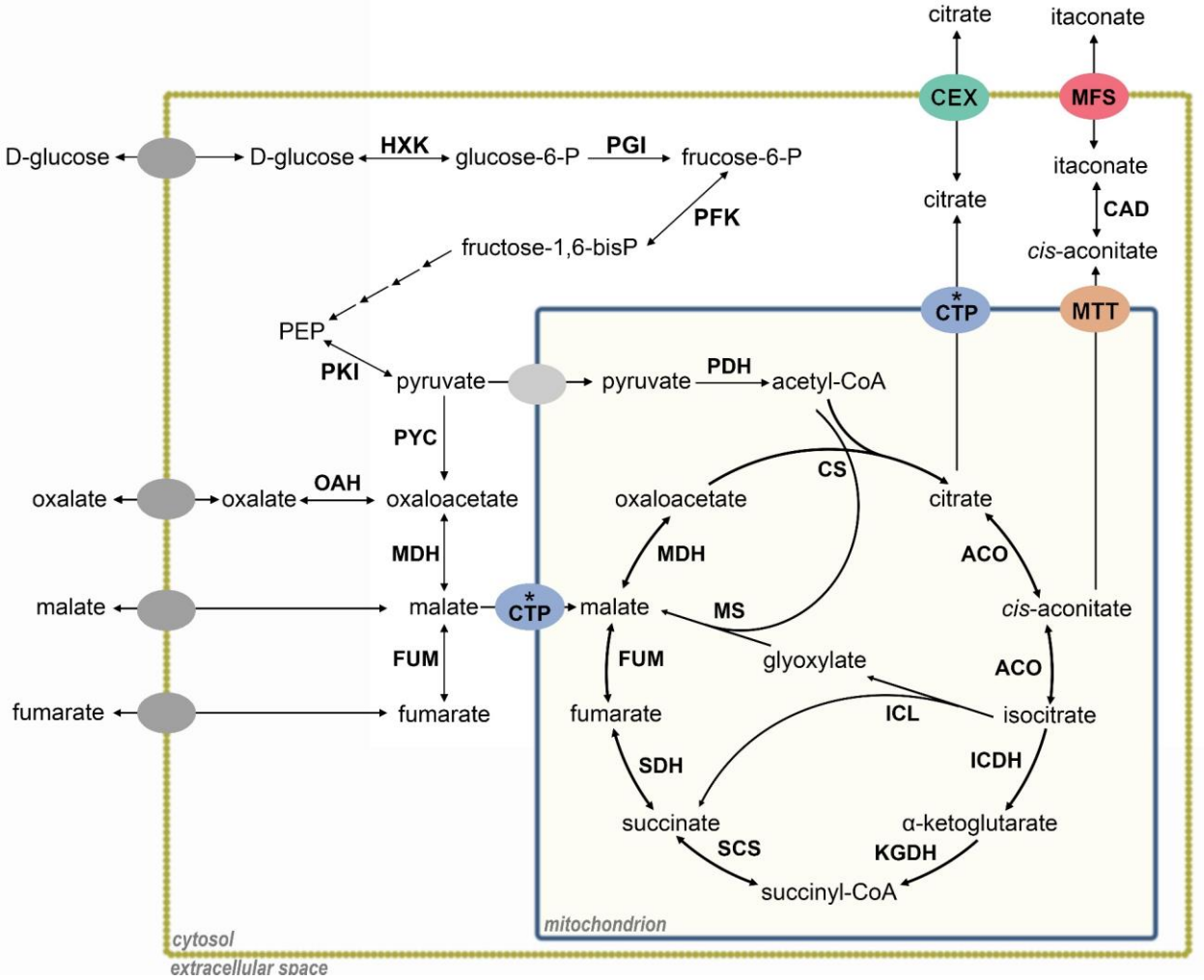


Figure 1. Schematic presentation of the tricarboxylic acid cycle in filamentous fungi.

PDH: pyruvate dehydrogenase; **CS**: citrate synthase; **ACO**: aconitase; **ICDH**: isocitrate dehydrogenase; **KGDH**: α-ketoglutarate dehydrogenase; **SCS**: succinyl-CoA synthetase; **SDH**: succinate dehydrogenase; **FUM**: fumarase; **MDH**: malate dehydrogenase; **ICL**: isocitrate lyase; **MS:** malate synthase; **CTP**: malate-citrate antiporter; **MTT**: mitochondrial TCA transporter; **CAD**: *cis*-aconitate decarboxylase; **MFS**: itaconate plasma membrane transporter; **PYC**: pyruvate carboxylase; **OAH**: oxaloacetate hydrolase; **MDH**: malate

dehydrogenase; **HXK**: hexokinase; **PGI**: phosphoglucose isomerase; **PFK**: phosphofructokinase; **PKI**: pyruvate kinase; **PEP**: phosphoenolpyruvate. ***** The import of citrate and export of malate by CTP are coordinated in the same transporter protein, but have been depicted separately to reduce the complexity of the figure.

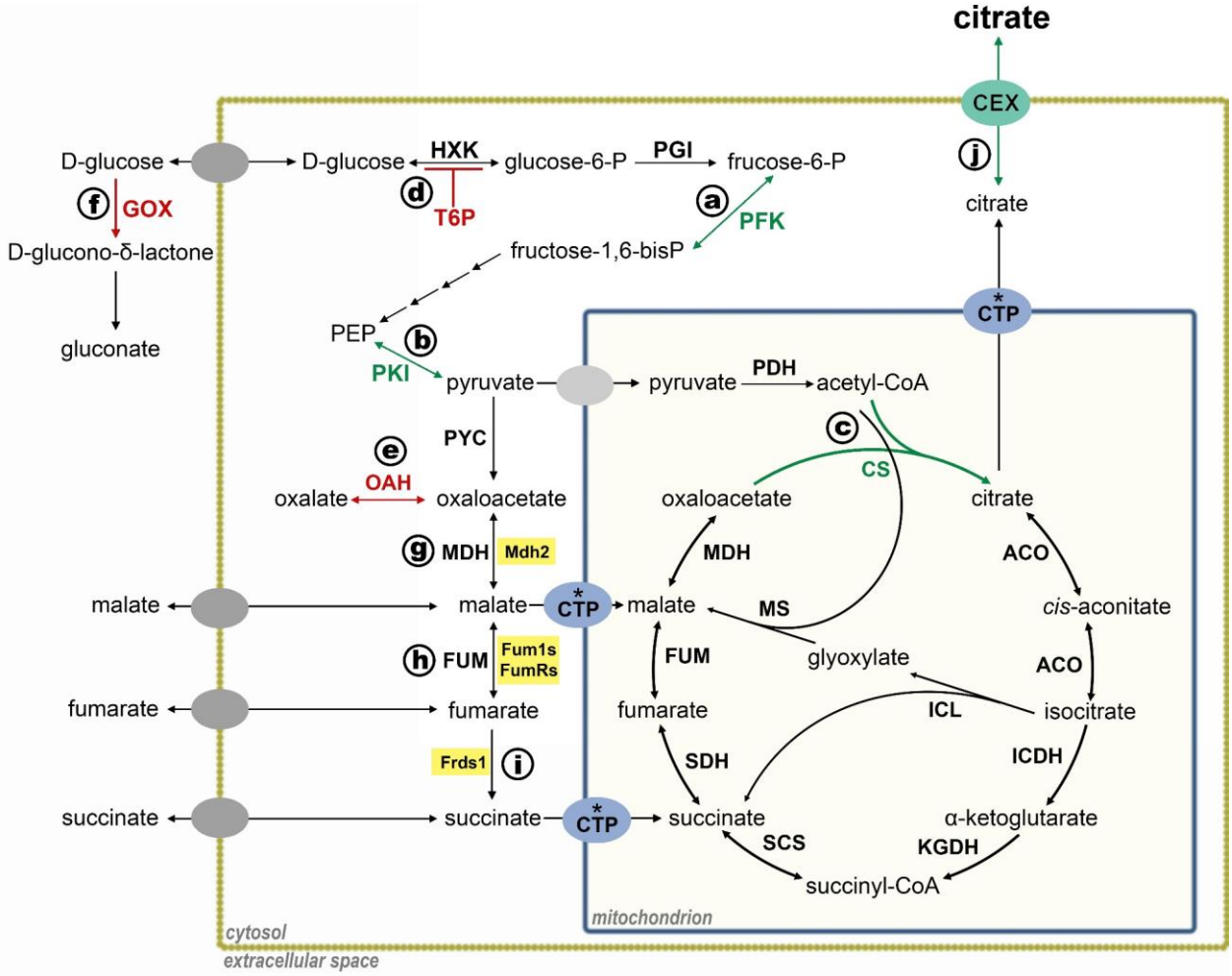


Figure 2. Metabolic engineering strategies for the improvement of citrate production in *A. niger*. The MDH from *S. cerevisiae* (Mdh2), two cytosolic FUMs from *S. cerevisiae* and *R. oryzae* (Fum1s and FumRs, respectively), and the cytosolic fumarate reductase (Frds1) from *S. cerevisiae* are highlighted in yellow. Green arrows: overexpression of *A. niger* native gene, Red arrows: deletion of *A. niger* native gene. The circled letters in the figure refer to the related text in the manuscript. Description of the enzyme abbreviations can be found in Fig. 1.

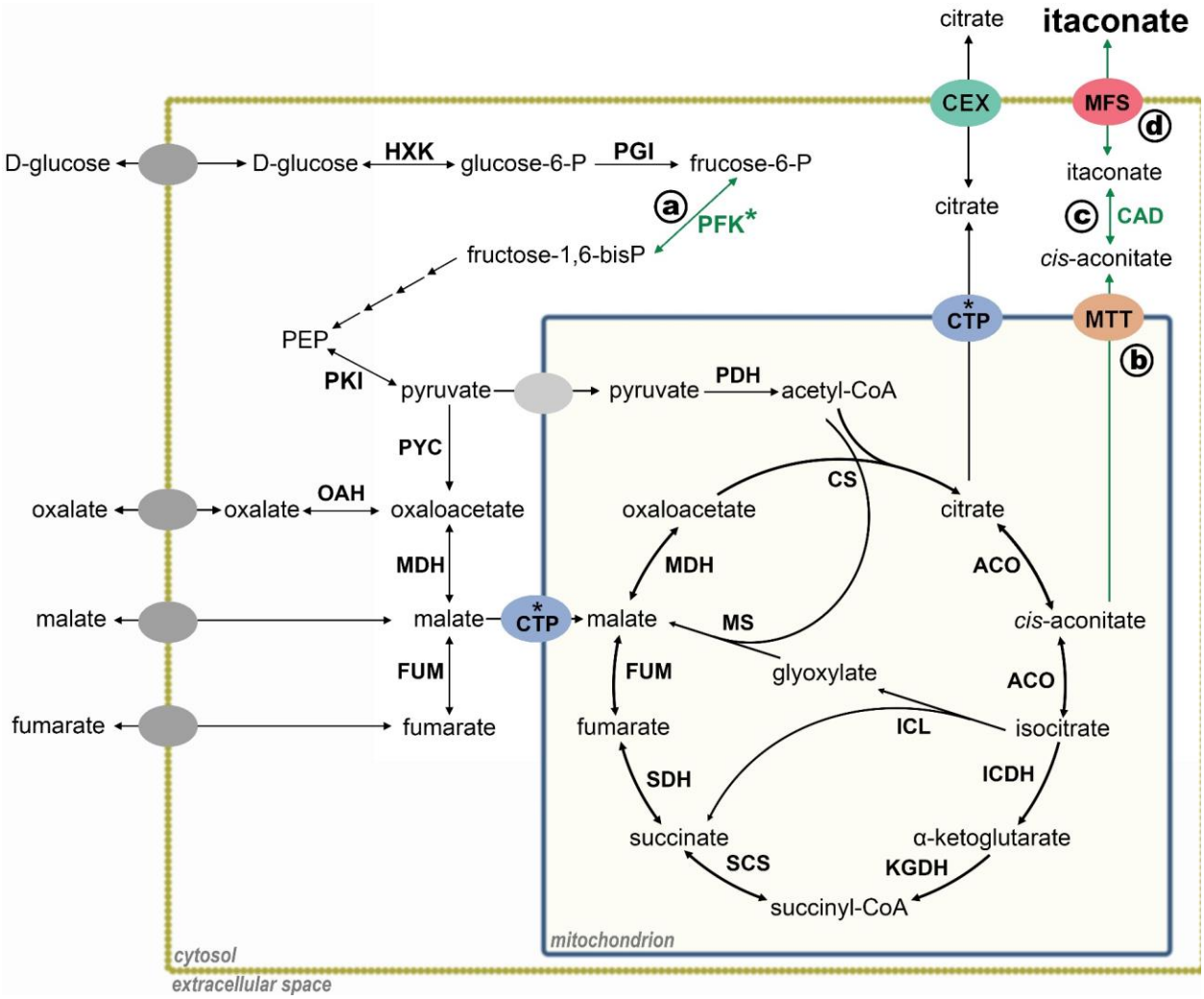
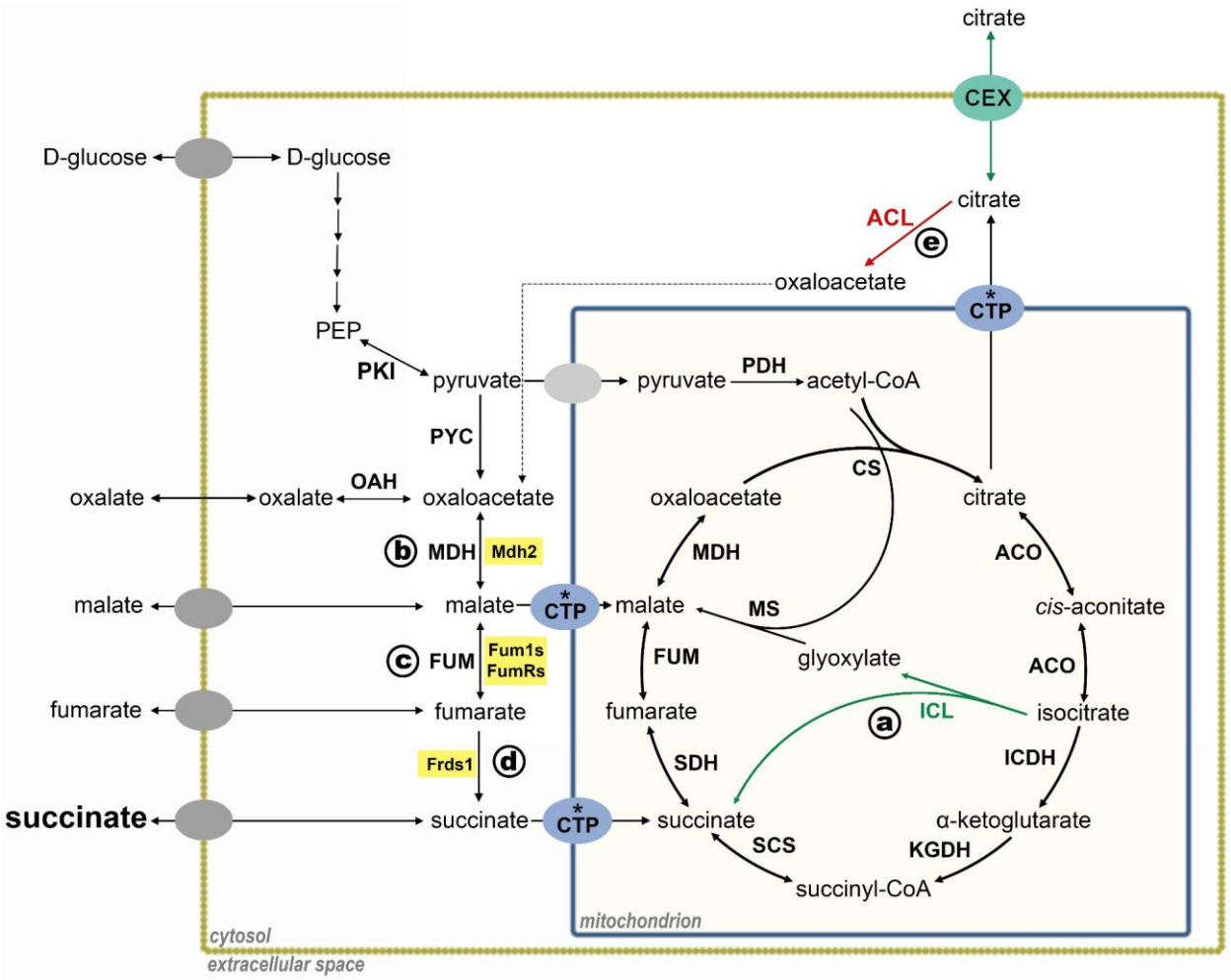


Figure 3. Metabolic engineering strategies for the improvement of itaconate production in *A. terreus*. **PFK***: highly active and citrate inhibition-resistant shorter form of *A. niger* PFK. Green arrows: overexpression of *A. terreus* native gene. The circled letters in the figure refer to the related text in the manuscript. Description of the enzyme abbreviations can be found in Fig. 1.



1533 Figure 4. Metabolic engineering strategies for the improvement of succinate production in *A. niger*.

1534 The MDH from *S. cerevisiae* (Mdh2), two cytosolic FUMs from *S. cerevisiae* and *R. oryzae* (Fum1s and

1535 FumRs, respectively), and the cytosolic fumarate reductase (Frds1) from *S. cerevisiae* are highlighted in

1536 yellow. **ACL:** ATP: citrate lyase. Green arrows: overexpression of *A. niger* native gene; red arrow: deletion

1537 of *A. niger* native gene. The circled letters in the figure refer to the related text in the manuscript.

1538 Description of the enzyme abbreviations can be found in Fig. 1.

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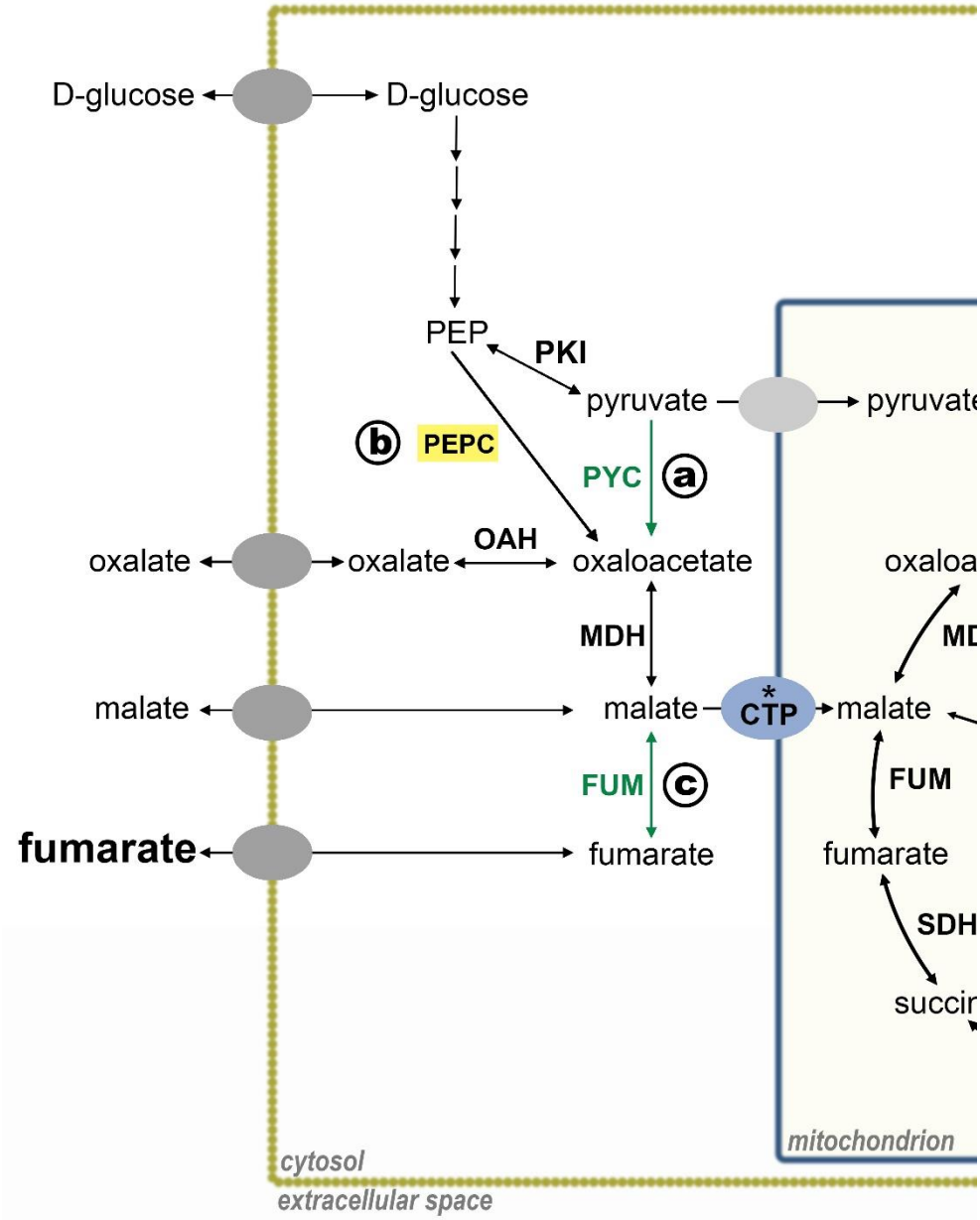

1540

1541 Figure 5. Metabolic engineering strategies for the improvement of fumarate production in *R. oryzae*.

1542 The PEP carboxylase (**PEPC**) from *E. coli* is highlighted in yellow. Green arrows: overexpression of *R. oryzae*

1543 native gene. The circled letters in the figure refer to the related text in the manuscript. Description of the

1544 enzyme abbreviations can be found in Fig. 1.

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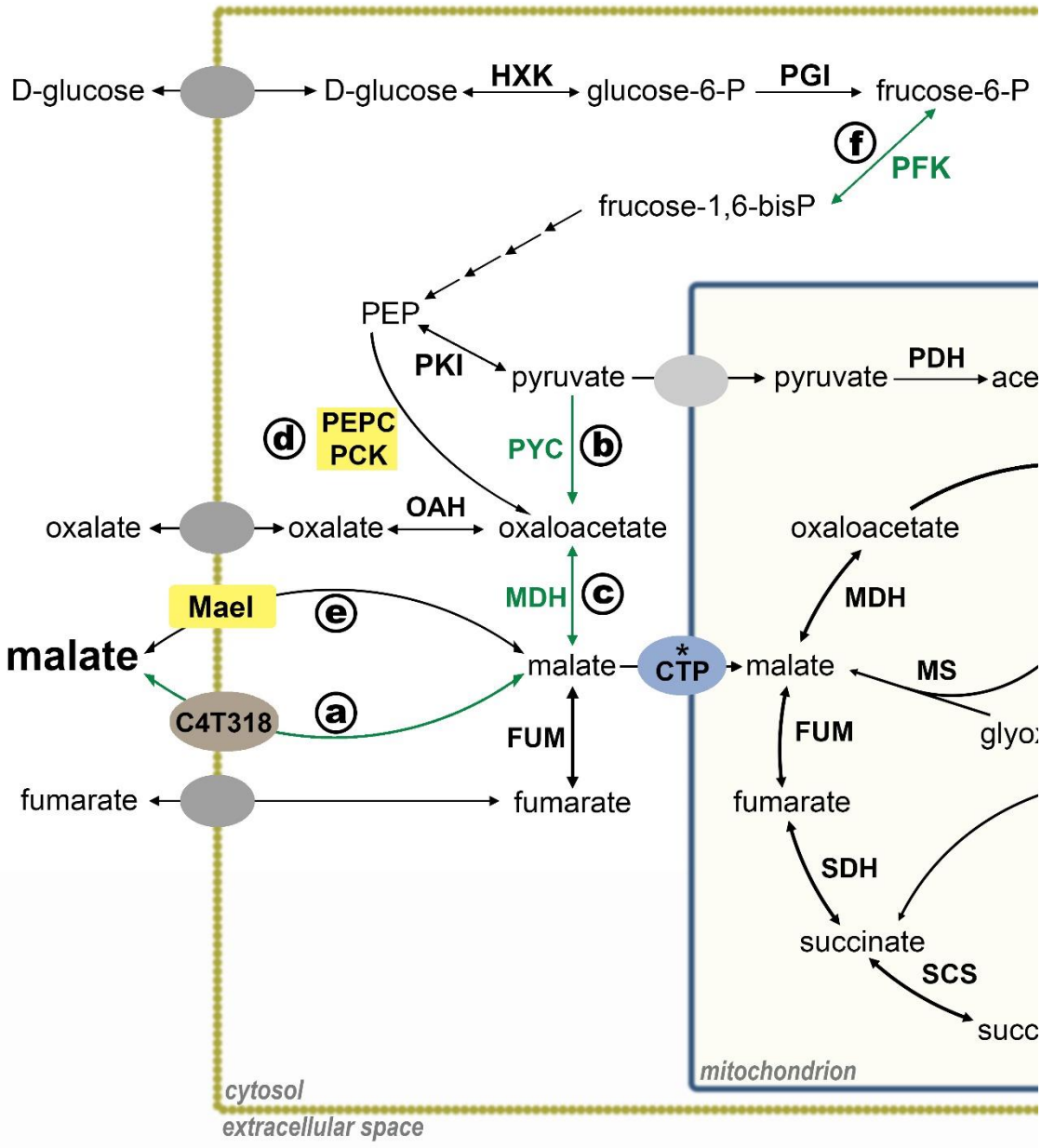

1548

1549 Figure 6. Metabolic engineering strategies for the improvement of malate production in *A. oryzae*.

1550 The PEP carboxylase (**PEPC**) and PEP carboxykinase (**PCK**) from *E. coli* are highlighted in yellow. Green

1551 arrows: overexpression of *A. oryzae* native gene. The circled letters in the figure refer to the related text

1552 in the manuscript. Description of the enzyme abbreviations can be found in Fig. 1.

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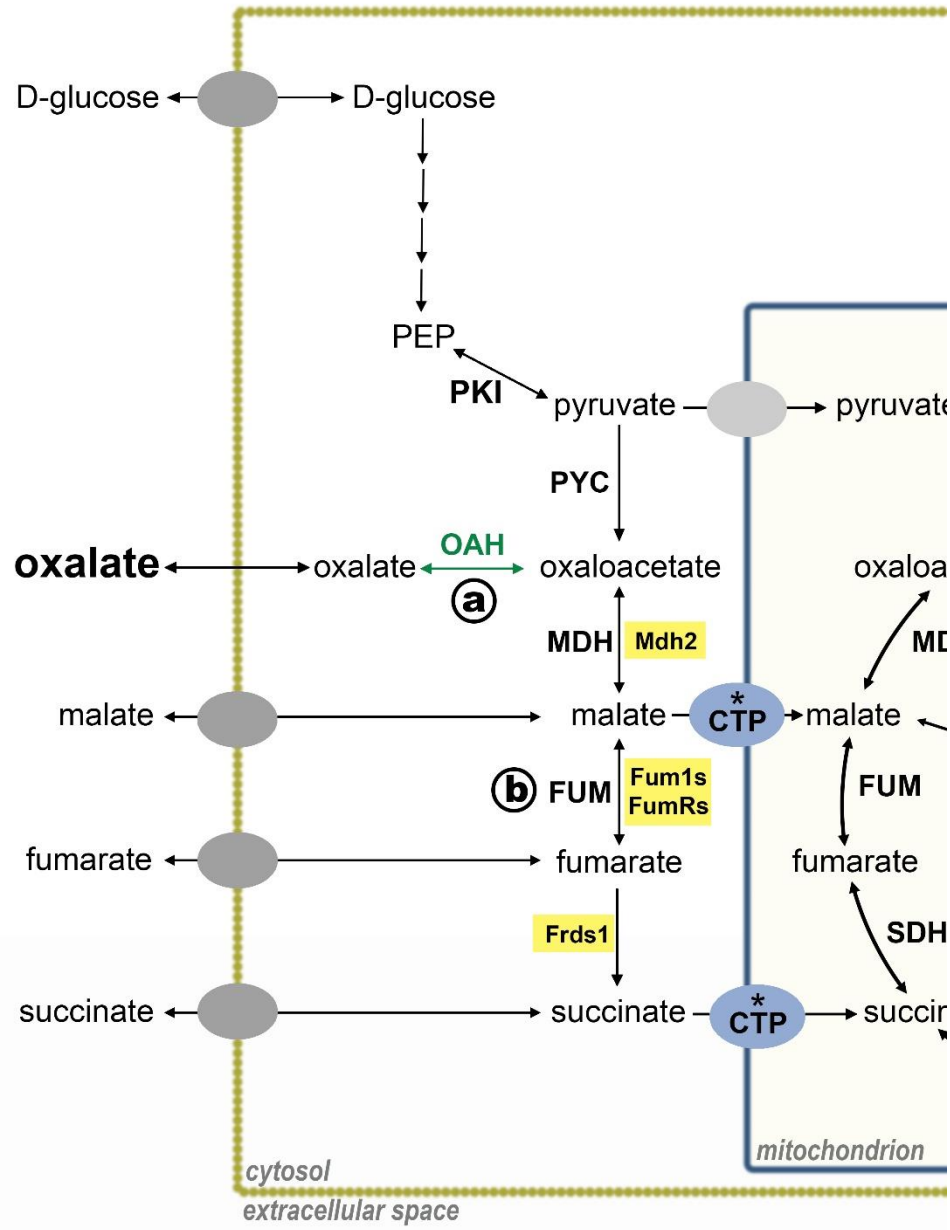

1556 Figure 7. Metabolic engineering strategies for the improvement of oxalate production in *A. niger*.
1557 The MDH from *S. cerevisiae* (Mdh2), two cytosolic FUMs from *S. cerevisiae* and *R. oryzae* (Fum1s and
1558 FumRs, respectively), and the cytosolic fumarate reductase (Frds1) from *S. cerevisiae* are highlighted in
1559 yellow. Green arrow: overexpression of *A. niger* native gene. The circled letters in the figure refer to the
1560 related text in the manuscript. Description of the enzyme abbreviations can be found in Fig. 1.
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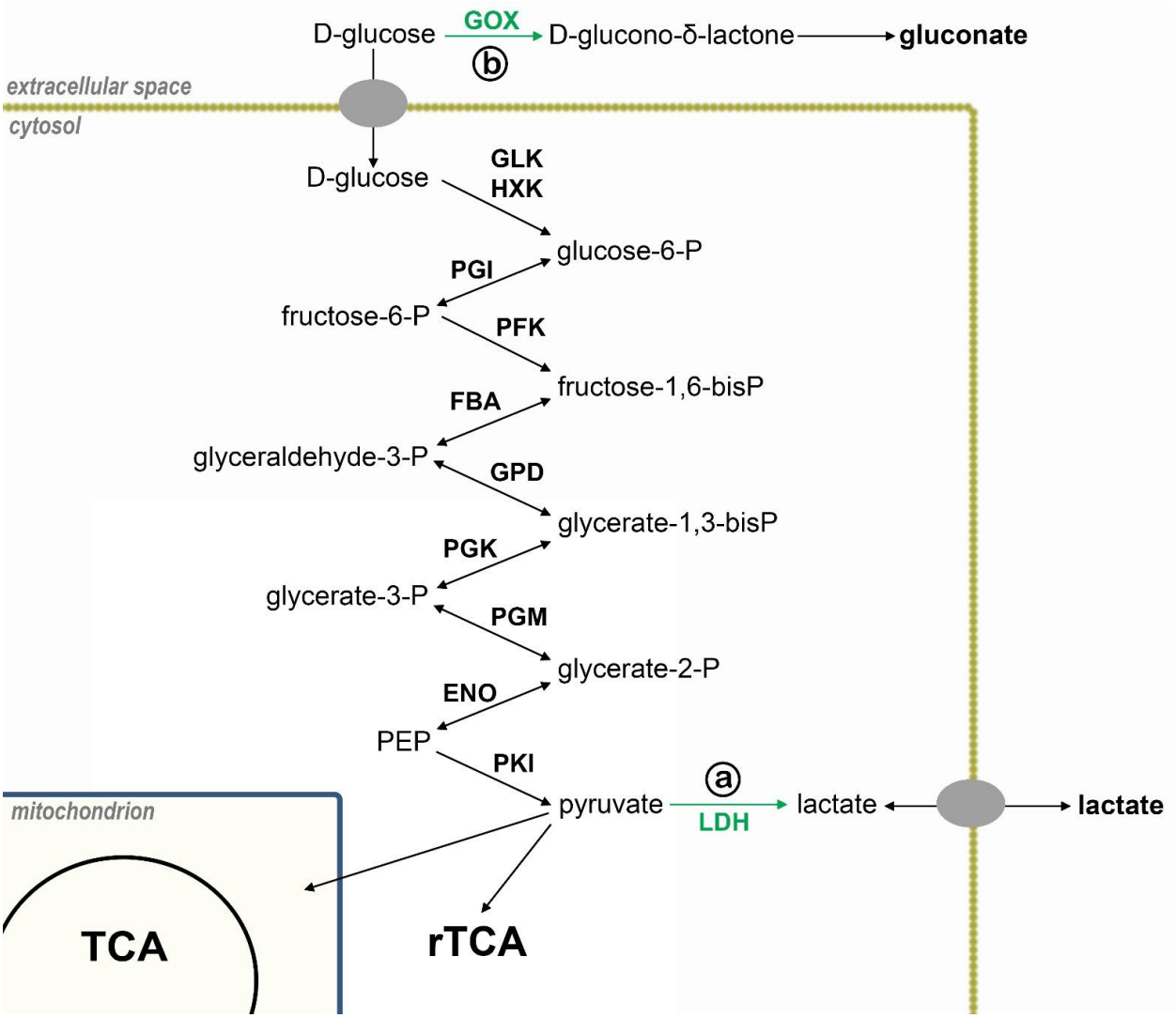

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1565 Figure 8. Metabolic engineering strategies for the improvement of lactate and gluconate production in
1566 filamentous fungi.

1567 **GLK:** glucokinase; **HXK:** hexokinase; **PGI:** phosphoglucose isomerase; **FBA:** fructose-bisphosphate
1568 aldolase; **GPD:** 3-phosphate-glyceraldehyde dehydrogenase; **PGK:** phosphoglycerate kinase; **PGM:**
1569 phosphoglycerate mutase; **ENO:** enolase; **PFK:** phosphofructokinase; **PKI:** pyruvate kinase; **LDH:** lactate
1570 dehydrogenase; **GOX:** glucose oxidase. The circled letters in the figure refer to the related text in the
1571 manuscript.

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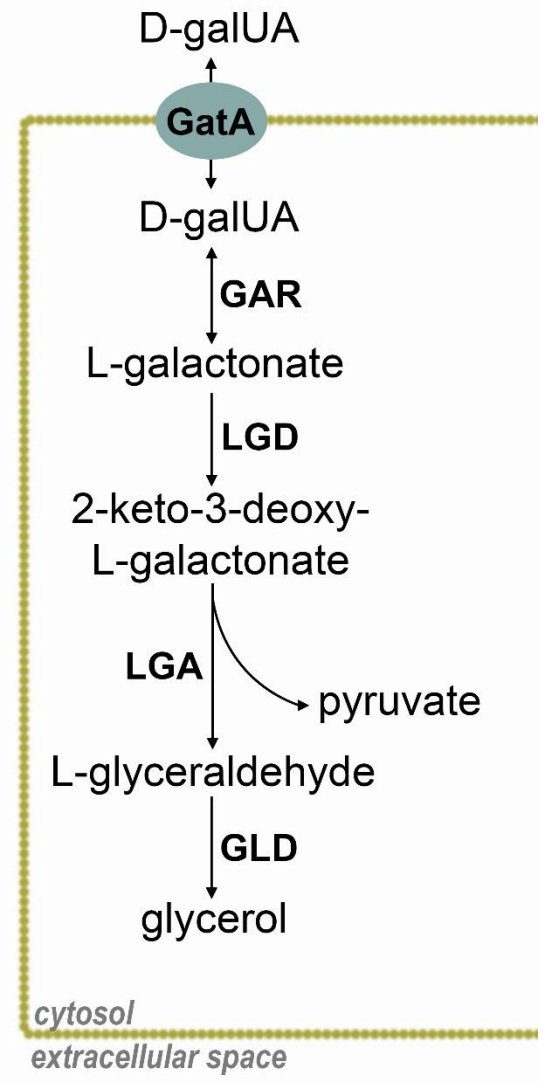


Figure 9. Schematic presentation of the D-galacturonic acid (D-galUA) catabolic pathway in filamentous fungi.

GAR: D-galacturonate reductase; **LGD:** L-galactonate dehydratase; **LGA:** 2-keto-3-deoxy-L-galactonate aldolase; **GLD:** glycerol dehydrogenase; **GatA:** D-galUA transporter described for *A. niger*.

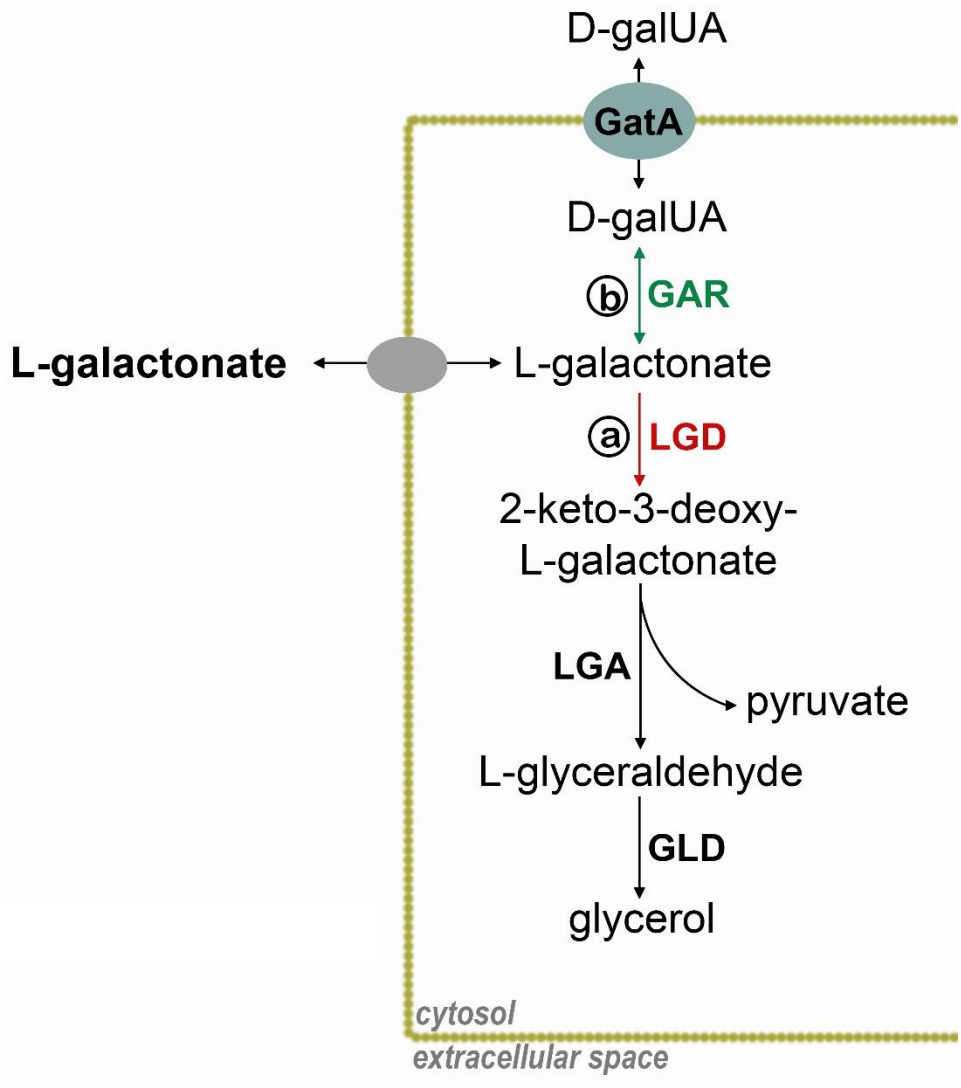
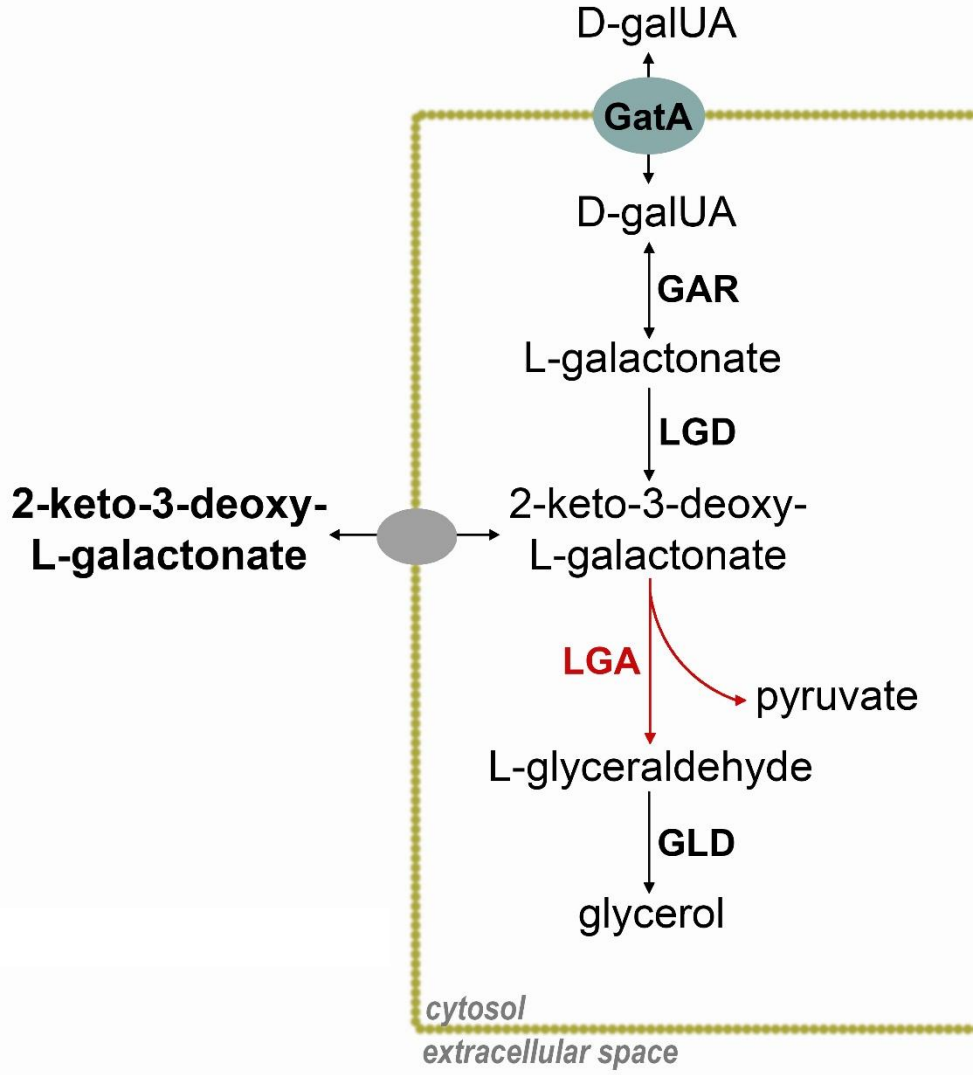


Figure 10. Metabolic engineering strategies for the improvement of L-galactonate production in filamentous fungi.

Green arrow: overexpression of native gene, Red arrow: deletion of native gene. The circled letters in the figure refer to the related text in the manuscript. Description of the enzyme abbreviations can be found in Fig. 9.



1597 Figure 11. Metabolic engineering strategies for the improvement of 2-keto-3-deoxy-L-galactonate
1598 production in filamentous fungi.
1599 Red arrow: deletion of native gene. Description of the enzyme abbreviations can be found in Fig. 9.
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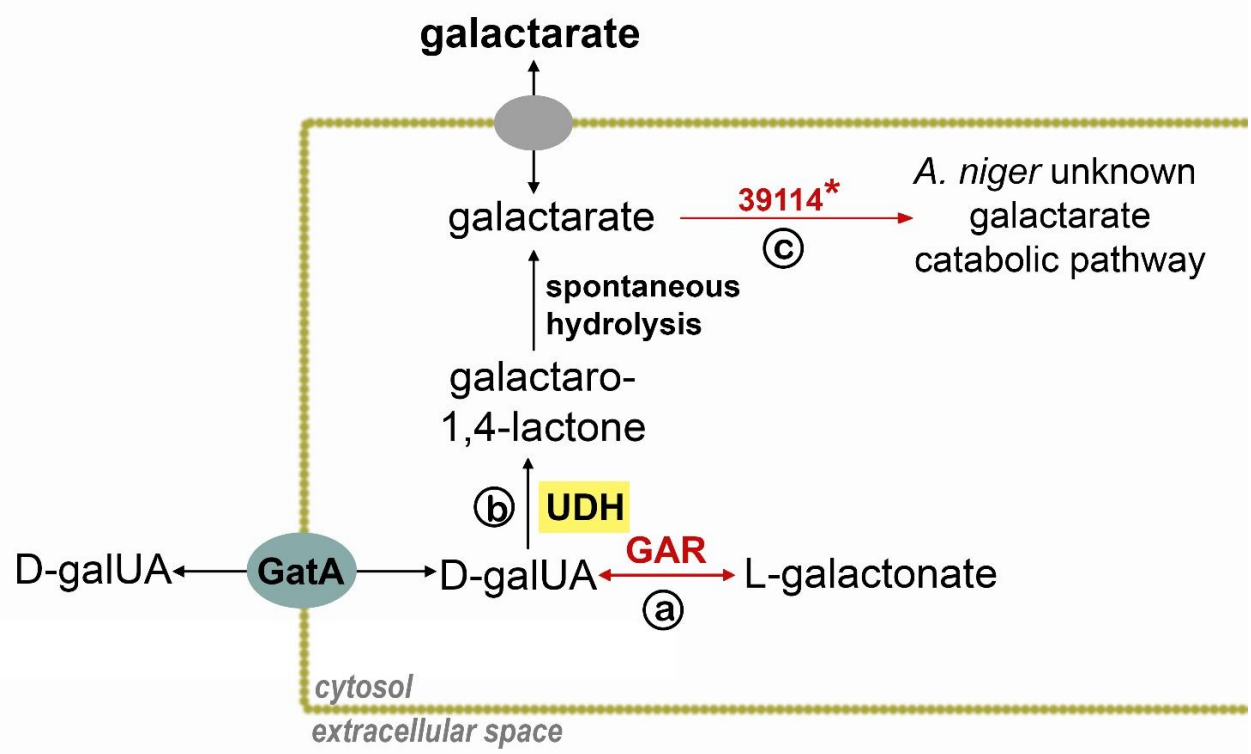

1608 Figure 12. Metabolic engineering strategies for galactarate production in filamentous fungi.

1609 The D-galacturonate dehydrogenase (**UDH**) from *Agrobacterium tumefaciens* is highlighted in yellow.

1610 **39114***: enzyme involved in *A. niger* unknown galactarate catabolic pathway. Red arrows: deletion of

1611 native gene. Description of the enzyme abbreviations can be found in Fig. 9.

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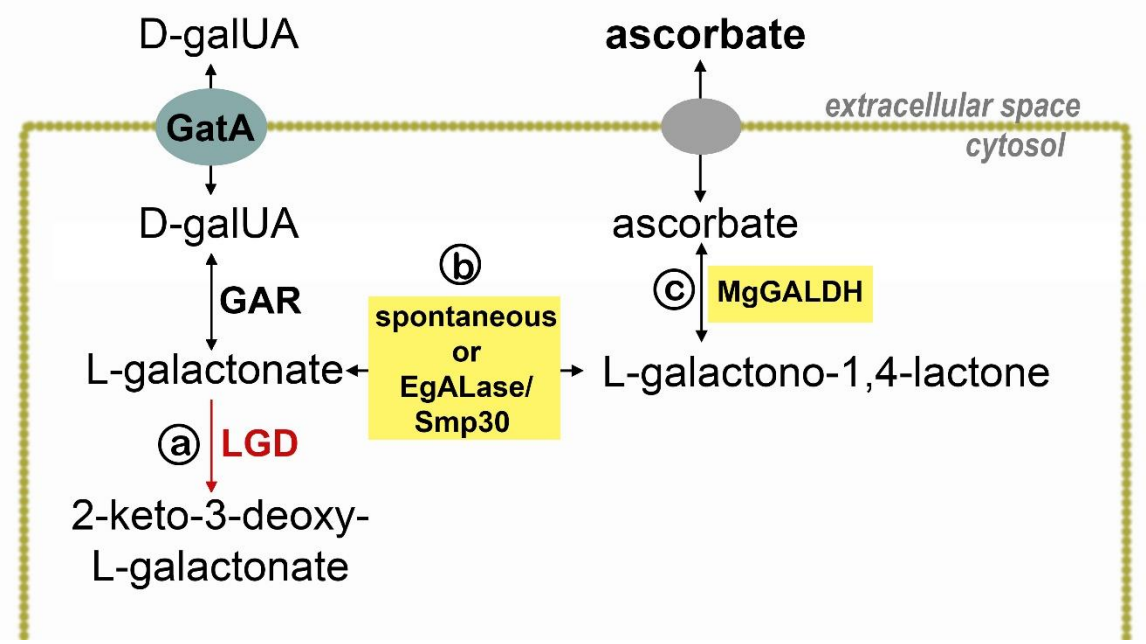

1614

1615 Figure 13. Metabolic engineering strategies for ascorbate production in *A. niger*.

1616 The L-galactono-1,4-lactone lactonase from *Euglena gracilis* (EgALase), a mammalian unspecific L-gulono-
1617 1,4-lactone lactonase (Smp30) and the L-galactonolactone dehydrogenase from *Malpighia glabra*
1618 (MgGALDH) are highlighted in yellow. Red arrow: deletion of native gene. Description of the enzyme
1619 abbreviations can be found in Fig. 9.

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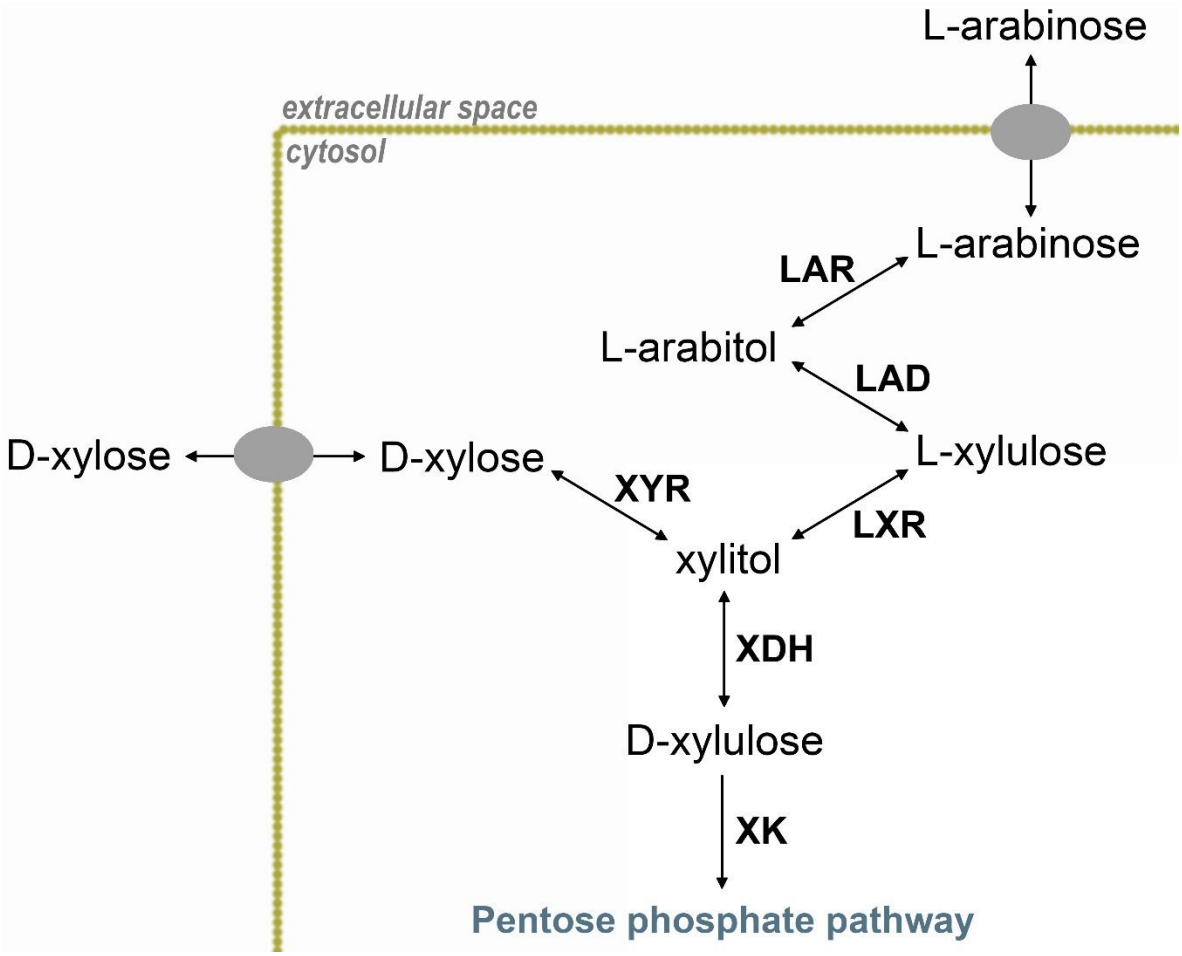

1622 Figure 14. Schematic presentation of the pentose catabolic pathway in filamentous fungi.

1623 **XYR:** D-xylose reductase; **XDH:** xylitol dehydrogenase; **XK:** xylulokinase; **LAR:** L-arabinose reductase; **LAD:**

1624 L-arabitol dehydrogenase; **LXR:** L-xylulose reductase.

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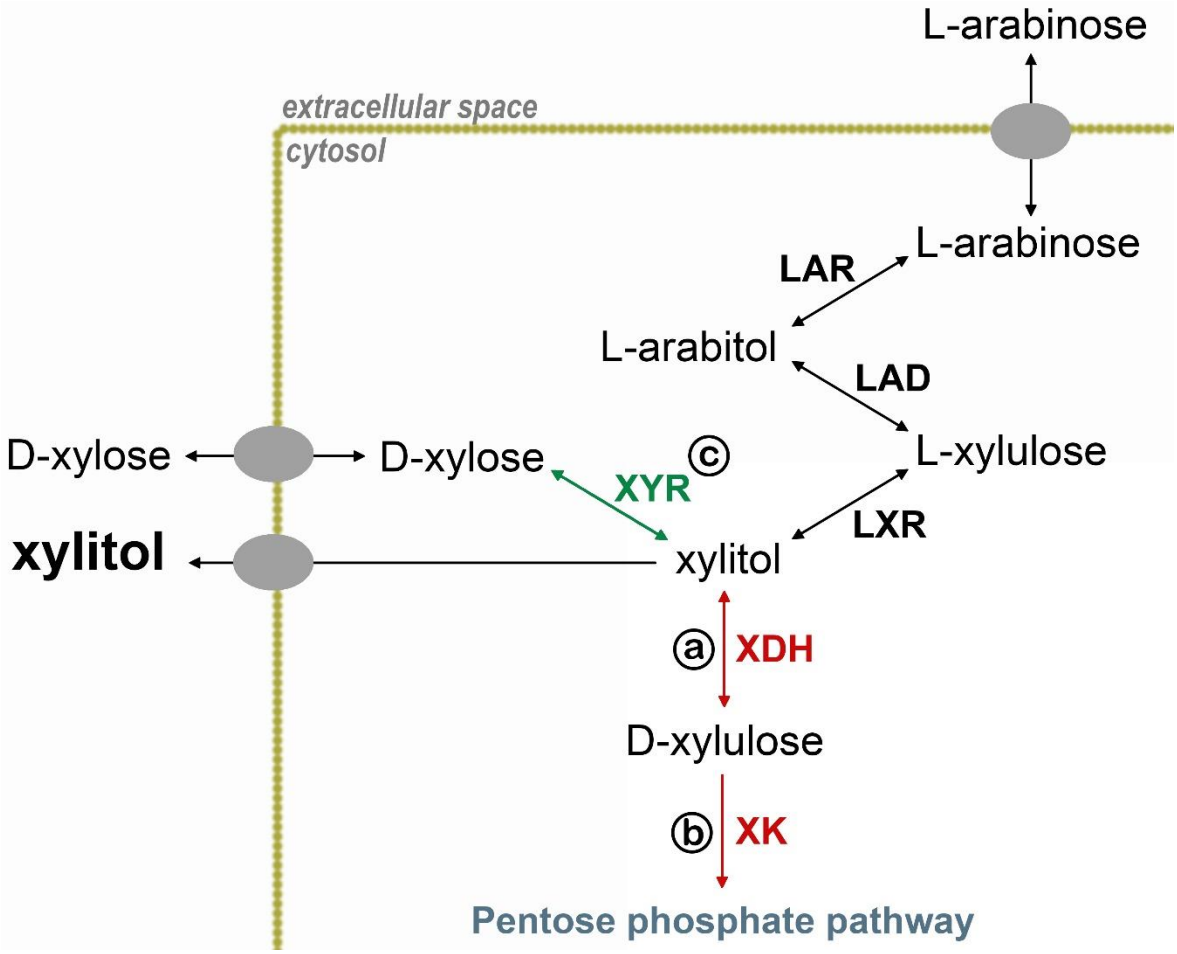

1626 Figure 15. Metabolic engineering strategies for xylitol production in filamentous fungi.

1627 Green arrow: overexpression of native gene, Red arrows: deletion of native gene. The circled letters in

1628 the figure refer to the related text in the manuscript. Description of the enzyme abbreviations can be

1629 found in Fig. 14.