Perspectives for the application of Ustilaginaceae as biotech cell factories

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Abstract

Basidiomycete fungi of the family Ustilaginaceae are mainly known as plant pathogens causing smut disease on crops and grasses. However, they are also natural producers of value-added substances like glycolipids, organic acids, polyols, and harbor secretory enzymes with promising hydrolytic activities. These attributes recently evoked increasing interest in their biotechnological exploitation. The corn smut fungus *Ustilago maydis* is the best characterized member of the Ustilaginaceae. After decades of research in the fields of genetics and plant pathology, a broad method portfolio and detailed knowledge on its biology and biochemistry are available. As a consequence, *U. maydis* has developed into a versatile model organism not only for fundamental research but also for applied biotechnology. Novel genetic, synthetic biology, and process development approaches have been implemented to engineer yields and product specificity as well as for the expansion of the repertoire of produced substances. Furthermore, research on *U. maydis* also substantially promoted the interest in other members of the Ustilaginaceae, for which the available tools can be adapted. Here, we review the latest developments in applied research on Ustilaginaceae towards their establishment as future biotech cell factories.

Keywords

Ustilaginaceae, *Ustilago maydis*, polyol, glycolipid, online monitoring, organic acid, unconventional secretion

Abbreviations

CBP	Consolidated bioprocessing
MEL	Mannosylerythritol lipids
MTP	Microtiter plate
RAMOS	Respiration Activity Online Monitoring System
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
STR	Stirred tank reactor
UA	Ustilagic acid
U. maydis	*Ustilago maydis*

Introduction

Ustilaginaceae belong to the basidiomycete fungi and comprise a family of mostly plant pathogenic microorganisms that infect various crops and grasses, causing smut disease (1). Well known family members are *Ustilago maydis* causing corn smut (2), *U. hordei* eliciting covered smut disease in barley (3), or *Sporisorium reilianum*, the infectious agent of maize and sorghum head smut (4). Representatives without known pathogenic lifestyles are for example members of the anamorph genus *Pseudozyma* like the biocontrol agent *Pseudozyma flocculosa* (5).

U. maydis in particular has been studied in the laboratory since decades and has thus developed into a valuable fungal model. Initial work focused on homologous recombination and DNA repair as well as on plant-pathogen interaction (6-8). Nowadays, research on *U. maydis* has largely expanded to fields like RNA and cell biology, effector biology and molecular plant pathology, signal transduction, protein secretion and the unfolded protein response, or even to the elucidation of basic principles of the primary metabolism (9-15). Applied research aspects progressively came into focus in the past years because *U. maydis*, like the other family members, is a natural producer of valuable small molecules like glycolipids, itaconic acid, triacylglycerols, polyols and organic acids (16, 17). In addition, it harbors the potential for production of interesting hydrolytic secretory enzymes (16, 18).

The biology of *U. maydis* is very well understood. The dimorphic fungus grows as a saprobe in a yeast-like, haploid form that proliferates by budding. For infection of maize plants, compatible yeast cells mate and proliferate as dikaryotic hyphae within the plant, where tumors with haploid teliospores develop (19). Of note, *U. maydis* is not harmful to humans and infected corn cobs are even edible (Textbox 1).

Textbox 1:

A delicious pest: While known as a plant pathogen, *U. maydis* is not harmful to humans. Rather, it has a long history of safe use as a delicacy ("Huitlacoche") in Mexico and Central America dating back to the heydays of the Aztec empire (20) and Switzerland has placed *Ustilago maydis* on its official list of edible mushrooms (Verordnung über Speisepilze; https://www.fedlex.admin.ch/eli/oc/2002/145/de; last accessed 02/10/2021). Plant infection strictly relies on mating of compatible cells and the formation of a dikaryon while the different haploid yeast strains are apathogenic. Moreover, the fungus can easily be trapped in its non-infectious form by disruption of the cognate signal transduction pathways (21, 22). These attributes qualify it as a very safe host for biotechnological applications.

Corn smut infection by *U. maydis.*

In its haploid yeast stage *U. maydis* is genetically tractable and simple to handle in the laboratory (23). The doubling time during exponential growth on glucose is about two hours and thus, comparable to *S. cerevisiae* and other established yeasts. A manually curated high-quality genome sequence of reference strain UM521 (24) is available (19, 25) and sequences of several other isolates are published at the National Center for Biotechnology Information (NCBI) (26). Based on decades of fundamental research, a comprehensive toolset for genetic manipulation has been compiled. Modifications, mainly introduced by homologous recombination, generate genetically defined, stable *U. maydis* strains (23). These developments fostered the discovery of several biosynthetic routes for molecules of biotechnological interest (27-31). In addition, the recent establishment of a GC-MS/MS based metabolomics platform for absolute quantification of primary metabolites allows for identification of underlying networks for metabolic engineering (32).

Here, we review the latest relevant methodological improvements and provide an overview on the most promising value-added substances produced in Ustilaginaceae. Based on the superb methodological foundation developed for *U. maydis*, the Ustilaginaceae provide prime candidates for industrial applications in the near future.

Strain and bioprocess engineering

In industrial biotechnology, it is important to "begin with the end in mind" by developing strain and bioprocess engineering in a closely integrated fashion (33). Chassis engineering can adapt the organism towards process demands, while bioprocess engineering tools can be used for scale-up to economically favorable conditions.

Tools for strain engineering

Due to its early use in fundamental research, molecular genetic tools are highly developed in *U. maydis*. A versatile vector set for genetic manipulation via homologous recombination developed in 2004 (34, 35) has recently been adapted to the Golden Gate cloning technology where Type IIS restriction enzymes are used for directional assembly (36). Besides simple gene

deletions, also transcriptional and translational fusions can be easily generated. Furthermore, a set of integrative vectors for gene insertion at specific loci has been developed, even promoting the generation of multiple insertions as a means to enhance gene expression (37, 38). The use of 2A peptides was successfully established and enables the creation of eukaryotic polycistronic mRNAs (39). Resistance marker recycling allows for re-use of available resistance cassettes, but also for the generation of marker-free strains (36, 40) with multiple genetic modifications (40-43). Moreover, the development of CrispR/Cas9 tools (44) enables precise, scar-free genomic engineering (43). The initial protocol was substantially improved by application of the high-fidelity endonuclease Cas9HF1 which minimizes off-target effects (45).

Importantly, studies in other Ustilaginaceae largely benefit from this toolset. Methods can readily be transferred as demonstrated e.g. for *U. bromivora* (46), *U. trichophora* (47) or *S. reilianum* (48). Notably, modifications and verifications are needed (and not always successful) on the genetic level, such as the adaptation of the targeted integration sequence in the *ip* locus or the activity verification of common *U. maydis* promoters like P_{otef} (21, 49). The interest in other Ustilaginaceae is currently further fostered by the rising availability of genome sequences (50, 51). By now, at least 20 full genomes including representatives of the genera *Anthracocystis*, *Ustilago*, *Sporisorium*, *Pseudozyma*, *Kalmanozyma* and *Moesziomyces* are available at NCBI (52)) and/or at EnsemblFungi (25).

Cultivation modes and scales

To continuously expand the available bioprocess engineering toolbox, cultivation approaches in different scales for various members of the Ustilaginaceae have been in the focus of intense research in the recent decade (Fig. 1).

In general, three major cultivation categories can be defined ranging from small to large scale: Microtiter plates (MTP), shake flasks and bioreactors. To screen for new production hosts for desired natural products, MTPs have been successfully applied (17, 78). These scaled-down cultivation methods are also very useful for investigation of oxygen-sensitive processes such as itaconic acid production, provided that they accurately represent larger-scale cultures (84). Even a brief interruption in shaking can have a significant metabolic impact (60) and parallel scaled-down cultures enable sacrificial sampling with minimal interruption of aeration. Besides conventional shake flasks as the currently most common system, different reactor types like jar fermenters have been used for cultivation of *U. scitaminea* (85) and *Pseudozyma* species (recently renamed as *Moesziomyces*) (76, 77, 86). With increasing fermentation volumes, stirred tank bioreactors (STR) have been mainly applied for cultivation. Scale-up of itaconic acid production to STRs has been demonstrated with *U. rabenhorstiana* (69), *U. maydis* (63, 71) and *U. cynodontis* (70). *In-situ* product removal of itaconic acid has been realized in continuous mode by addition of a membrane module for cell retention with *U. maydis* (68, 73). In a different approach, calcium-itaconate has been precipitated to enable product titers of over 200 g/l (43, 71).

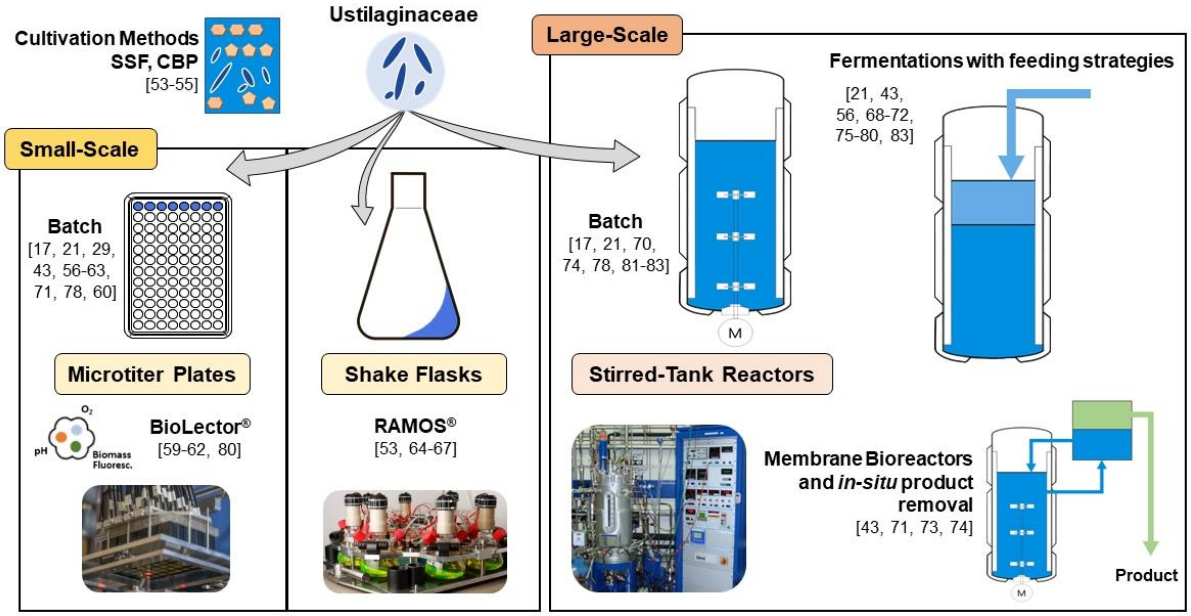

Figure 1. Bioprocess engineering tools applied for Ustilaginaceae in the past decade. Cultivations in non-monitored batch-operated shake flasks are considered as standard procedures and are not quoted separately. Abbreviations: RAMOS® = Respiration Activity Monitoring System, SSF = Simultaneous saccharification and fermentation, CBP = Consolidated bioprocessing.

For application in large scale STRs, microorganisms need to be robust against hydromechanical stress induced by the impellers. In this regard, the yeast-like morphology of *U. maydis* is a distinctive feature, which makes it superior to well-known industrial fungi like *Aspergillus terreus* (29, 87). In addition, issues like local oxygen limitation in the centre of large pellets do not occur for yeast-like microbes (64). Thus, *U. maydis* and other Ustilaginaceae showing unicellular growth can easily be cultivated in stirred, submerged cultures (59, 74). Even high-cell density cultivations are possible, as oxygenation of the fermentation broth with yeast-like growing microbes can be maintained at desired levels by an increased stirring rate without negative impacts of hydromechanical stress. This has been documented for example for *U. trichophora* (79). For *U. cynodontis*, which shows yeast-like growth in shaken cultures, but filamentous growth in stirred tank reactors (17), suppression of filamentous growth has been achieved by genetic engineering of the underlying regulatory signalling cascade (21). Although wild type *U. maydis* is generally considered to grow as a yeast, engineered strains subjected to stress by metabolite overproduction can also switch to filamentous growth, which can be suppressed with similar genetic means (43, 71) This knowledge is very likely to be transferable also to other Ustilaginaceae to facilitate their handling and enable reliable use in STR applications.

Online cultivation monitoring

STRs are commonly equipped with online measurement tools for pH, temperature and dissolved oxygen tension. In addition, biomass determination and off-gas analytics can be implemented. Therefore, crucial information on the culture status is continuously accessible. This enables maintenance of the pH value, like for example during itaconic acid production

(56, 79, 88). In addition, more complex monitoring of substrate consumption and product formation is possible (53, 70). In contrast to STRs, small-scale experiments usually depend on the analysis of offline samples. Novel parallelized small-scale online monitoring technologies have the power to bridge fundamental research and industrial application as they significantly reduce the number of required experiments (89-91). Different online monitoring techniques have been applied to Ustilaginaceae in the past years. The BioLector® technology for example, which enables online measurement of biomass, pH, dissolved oxygen tension and (biogenic) fluorescence in microtiter plates, has been successfully exploited for characterization of *U. maydis* (60, 62) and *S. scitamineum* variants (80). In addition, screening experiments of several Ustilaginaceae species to identify promising MEL producers were reported (59). This technology is especially powerful when combined with intracellular fluorescent biosensors for the monitoring of, e.g. gene expression (62) or biochemical parameters like NADH/NAD^+ ratio (60). The Respiration Activity Monitoring System (RAMOS®) is suited for online determination of the respiration activity of a culture in shake flasks (92), providing the unique possibility of constantly visualizing the physiologic state of a culture. The technique has for example been implemented to characterize *U. maydis* expression strains for heterologous proteins lacking different extracellular proteases (42). More recently, also non-invasive estimation of enzyme activity and residual substrate concentration based on total oxygen consumption was demonstrated in the framework of biomass valorisation approaches (see below) (66, 67). Moreover, knowledge about the oxygen transfer rate is useful for scale-up from shake flasks to stirred-tank reactors (93).

U. maydis has also served as a model microorganism to implement and describe novel online measurement techniques (94). In MTPs, it was applied to validate an impedance spectrometry measurement to monitor viable biomass (61). For STRs, a low-field NMR measurement for non-invasive online-monitoring of substrates and products was validated (82). These examples underline the potential and the versatile exploitation of Ustilaginaceae for technological development in the field of process engineering.

Hydrolytic enzymes and biomass valorisation

Ustilaginaceae are promising microbes for biomass depolymerization because they encode a number of hydrolytic enzymes, most of these acting on carbohydrates (reviewed in (16)). Examples from *U. maydis* include an α-L-arabinofuranosidase acting on wheat arabinoxylan with a specific activity of 9 U/mg protein (95) or a CalB-type lipase with novel esterase activities reaching 53 U/mg on Tween 80 (96). Another esterase from *Pseudozyma antarctica* (PaE) has been described to decompose biodegradable plastics like poly(butylene succinate) or polycarolactone (97). Secretion of this cutinase-like enzyme is tied to MEL production (98) and it may therefore play a role in propagation on the plant leaf surface (99). Of note, most intrinsic hydrolytic enzymes of interest encoded in the genome of *U. maydis* are expressed in the pathogenic hyphal form that cannot be handled efficiently in the laboratory. A similar phenomenon is observed for sugar uptake transporters. *U. maydis* possesses a high affinity sucrose transporter named Srt1 that may be interesting for the conversion of this sugar derived from sugar cane or beet (100), but the respective gene is only expressed *in planta* (101).

Interestingly, for *Pseudozyma* species this limitation does not seem to occur, befitting its presumed non-pathogenic lifestyle (98, 102). However, for *U. maydis*, a recently developed strategy for genetic activation in the yeast stage by insertion of strong constitutive promoters has also overcome this problem and enabled enzyme secretion in the biotechnologically relevant yeast-like growth form (57, 66, 67). Further progress towards depolymerization of complex substrates has been achieved by genetic complementation with genes for powerful hydrolytic heterologous enzymes of fungal and bacterial origin (67). Co-cultivation of engineered Ustilaginaceae strains secreting enzymes with combinatorial activities improved substrate hydrolysis. These strategies resulted in successful expansion of the substrate spectrum including complex polysaccharides like cellobiose, xylan (57) and polygalacturonic acid (67).

With the presence of biomass-degrading enzymes, Ustilaginaceae are promising candidates for valorisation approaches, covering the entire process from biomass to desirable products in one bioreactor (57, 66, 67). This has been demonstrated in a simultaneous saccharification and fermentation (SSF) (103, 104) with *Pseudozyma* spp. (*Moesziomyces*) to produce MEL from cellulosic materials and xylan (54, 55). Another powerful tool is the co-cultivation of Ustilaginaceae with other microorganisms to expand the enzymatic repertoire. A consolidated bioprocess (103, 104), has been successfully applied with *U. maydis* and the filamentous Ascomycete *Trichoderma reesei* to produce itaconic acid from cellulose (53).

Synthesis of small molecules

Ustilaginaceae are natural producers of several small molecules with potential industrial application. Here, we provide an update on the developments focusing on the most promising molecules.

Organic acids

Organic acids find widespread use in polymer, food, and pharmaceutical applications (105, 106). Their production is a common trait in the Ustilaginaceae and almost all tested strains synthetize malate and succinate (17, 107). In contrast, the ability to produce itaconate seems to be relatively rare (17, 78, 107, 108). The main biotechnological focus has so far been on malate and itaconate. Their production is connected via cytoplasmic anaplerosis, which is paramount for high-yield production. Malate can be produced via several possible pathways, the most efficient of which proceeds via pyruvate carboxylase (49). Malate production is especially efficient on glycerol using *U. trichophora*. This carbon source, when combined with high CO_2 levels generated by the presence of $CaCO_3$ as buffering agent, enables titers above 200 g/l with high rate and yield (58, 79).

Transporters play a key role in driving organic acid production and product specificity (Fig. 2). The latter is especially important in the context of downstream processing and purification, where separation of two organic acids is often cost-prohibitive (109). Cytosolic malate can either be secreted via the putative malate exporters Ssu1 or Ssu2 (49), or it can be imported into the mitochondria. In the latter case, it is reduced back to oxaloacetate to enable synthesis of citrate and subsequently *cis*-aconitate. In *U. maydis*, this *cis*-aconitate is exported from the mitochondria by the mitochondrial tricarboxylate transporter Mtt1. This transporter

is the driving force for itaconate production and deletion of *mtt1* reduces itaconate production (56, 71). In turn, overexpression of *mtt1* greatly increases production (21, 56, 108). Mtt1 is an antiporter, preferentially exchanging cytosolic malate for mitochondrial *cis*-aconitate (110). Interestingly, the substrate specificity of the MttA antiporter from *Aspergillus terreus* is different in that 2-oxoglutarate and oxaloacetate are preferentially transported (110, 111). Mtt1 not only balances the mitochondrial exchange of metabolites for efficient anaplerosis, it also plays a role in the determination of the specificity of malate and itaconate production (78). From a biochemical perspective, there is likely also competition between mitochondrial antiporters for the production of itaconate and MEL, UA, or triglycerides. Assuming that these lipidic products rely on fatty acids synthesized in the cytoplasm (112), a high flux towards cytosolic acetyl-CoA is necessary, which is likely provided via Ctp1-mediated exchange of malate and citrate, followed by citrate lyase-mediated formation of acetyl-CoA (Fig. 2) (113, 114). Transporters further play a role in the determination of specificity of production of itaconate and its derivatives 2-hydroxyparaconate and itatartarate (115), and a similar effect can be expected for secretion of malate and succinate (Fig. 2).

The large diversity of interesting metabolites secreted by *U. maydis* make it an interesting biotechnological workhorse, but also hampers efficient production of individual chemicals. This drawback can be circumvented by the metabolic engineering of chassis strains, in which the genes encoding the production pathways of competing metabolites are deleted to reduce by-product formation (63). This, combined with the abovementioned morphological engineering and overexpression of bottleneck enzymes in the metabolic pathway of the desired product, can enable very high yield production of a single product (43).

Sugar alcohols

Sugar alcohols are interesting non-caloric sweeteners with promising food and pharma applications (116, 117). Ustilaginaceae have been shown to produce mannitol and erythritol (107), with main research focus on the latter. *Pseudozyma tsukubaensis* strain KN75 reaches an impressive erythritol titer of 241 g/l at a rate of 2.8 g/l/h and yield of 0.61 g/g at 50 m^3 scale (118). Erythritol production is induced by high osmotic pressure (>300 g/l sugars), and maintaining this pressure in a specific range without inhibiting the cells, i.e. by fed-batch cultivation, is key in achieving a high rate (118). Other, more distantly related Basidiomycetes like *Moliniella* are also efficient erythritol producers. Interestingly, despite the fact that fermentative erythritol production is an established biotechnological process, knowledge of the underlying biochemistry is fragmented and incomplete. An erythritol degradation pathway via erythrulose has been identified (119, 120). The pentose phosphate pathway is the main supplier of erythrose-4-phosphate (E4P) as key precursor and several studies have identified an erythrose reductase as rate-limiting step, but other key factors such as the E4P phosphatase or the erythritol transporter are thus far poorly understood (116, 117).

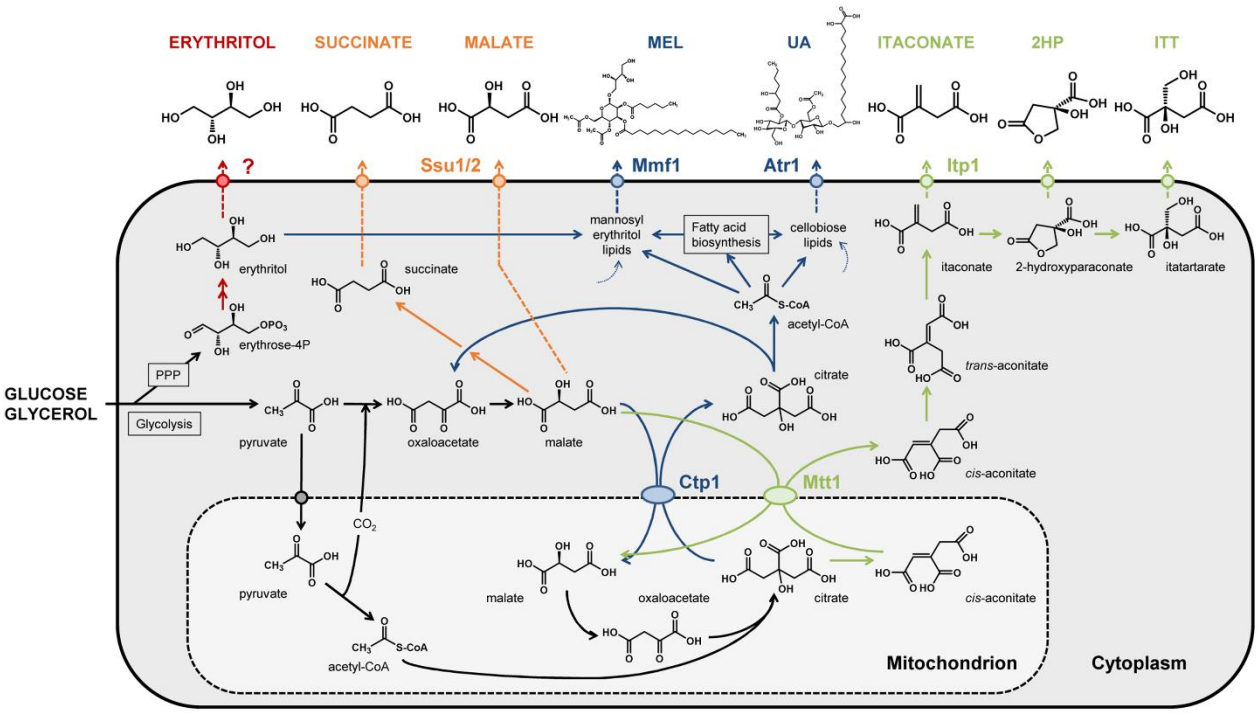

Figure 2. Interconnection between biochemical pathways for the main biotechnological products of Ustilaginaceae. Both mitochondrial antiporters and plasma membrane exporters (indicated in bold colors: red, erythritol; green, itaconate; orange, C4 dicarboxylates; blue, glycolipids) play a major role in the determination of product specificity. Small dotted arrows indicate input of further metabolites. PPP, pentose phosphate pathway; MEL, mannosylerythritol lipids; UA: ustilagic acid; 2HP, 2-hydroxyparaconate; ITT, itatartarate.

Glycolipids

Members of the Ustilaginaceae family produce two glycolipids, cellobiose lipids and mannosylerythritol lipids (MEL) (17, 121). These amphiphilic compounds with hydrophilic head groups and hydrophobic medium- to long-chain fatty acid tails reduce the surface tension. Thus, they represent environmentally friendly biosurfactants with potential industrial applications as ingredients for example in cosmetics, food, paint, washing agents or bioremediation. Additionally, pharmaceutical applications have been discussed (122-124). While production of MEL was observed in all tested Ustilaginaceae and other smuts of the Ustilaginales order, cellobiose lipids seem to be restricted to a few members (17).

Glycolipid biosynthesis is coupled to nitrogen limiting conditions and well understood. Genes encoding the respective enzymes are organized in gene clusters. In addition, clusters encode putative glycolipid transporters (Fig. 2).

MEL are composed of a mannosylerythritol sugar moiety, acylated with fatty acids of varying lengths. Acylation occurs in peroxisomes and is essential for production of the natural MEL spectrum. This also prevents competition between MEL and cellobiose lipid synthesis (125). Furthermore, MEL are characterized by differing degrees of acetylation (122). Studies of the putative MEL transporter Mmf1 are somewhat contradictory. An *mmf1* deletion in *P. tsukubaensis* did not abolish MEL secretion (126), but its disruption in *U. maydis* does (31). The spectrum of produced MEL variants is very broad between the different species (124) and

novel tailor-made variants can be engineered by replacing biosynthetic enzymes with homologs from other fungi (127). *Pseudozyma sp.* are the yet most promising MEL producers with titers of up to 165 g/L (128). While some species can produce MEL using glucose as carbon source, others rely on hydrophobic substrates (122). Highest production titres were achieved on plant oils (124).

The best characterized cellobiose lipids originate from *P. flocculosa* (flocculosin) and *U. maydis* (ustilagic acid; Fig. 2) (27, 28, 129, 130). In these molecules the disaccharide cellobiose is connected to a hydroxy palmitic acid. Eventually, the sugar moiety is further decorated with acetyl groups and a short-chain β-hydroxy fatty acid (122, 129). Product titres for cellobiose lipids are generally lower than for MELs and range at about 20 g/L (80, 131).

Unconventional secretion of heterologous proteins

Fungi are known for their superb abilities for protein secretion and already used for industrial production, mainly of homologous hydrolytic enzymes. By contrast, heterologous proteins are rather difficult to express. This is likely due to bottlenecks in the secretory system and the sensitivity of foreign proteins towards the high number of secreted proteases (132, 133). Hence, efficient fungal platforms for heterologous protein expression are still scarce and alternative strategies are highly demanded.

In *U. maydis*, a yet unique option for production of heterologous proteins has been developed by exploitation of an unconventional secretion pathway (Textbox 2). Two such pathways exist. Sterol carrier protein 2 (Scp2) is likely released via peroxisomes and an important pathogenicity factor (134).

Textbox 2:

Unconventional secretion: In eukaryotes, the majority of proteins is secreted via the endomembrane system. N-terminal signal sequences target proteins into the endoplasmic reticulum. By vesicular trafficking the cargo is transported via the Golgi apparatus to the plasma membrane, where the proteins are released by fusion of the membranes. Post-translational modifications like *N*-glycosylation take place in the endomembrane system and contribute to protein folding, stability and quality control (135, 136). However, a rising number of proteins has been described, which do not follow this classical route, but are secreted by alternative mechanisms. These pathways are diverse and only in rare cases well understood. Fibroblast growth factor 2 (FGF2) for example is secreted via self-sustained translocation at the plasma membrane of human cells (137). Acyl-binding protein 1 (Acb1) uses specialized compartments of unconventional secretion for cell exit (138).

In an alternative pathway chitinase Cts1 shows a cell cycle dependent accumulation in the fragmentation zone between dividing yeast cells, from where it is most likely released (Fig. 3) (13, 139, 140). Here, the enzyme contributes to the separation of mother and daughter cell. Several factors essential for unconventional Cts1 secretion have been identified, supporting a model of lock-type secretion (Fig. 3). The fragmentation zone is formed by the consecutive insertion of two septa, and the assembly of the secondary septum at the daughter cell side is mediated by kinase Don3 and GEF Don1 (140). Anchoring factor Jps1 is required for accumulation of Cts1 in the fragmentation zone (141).

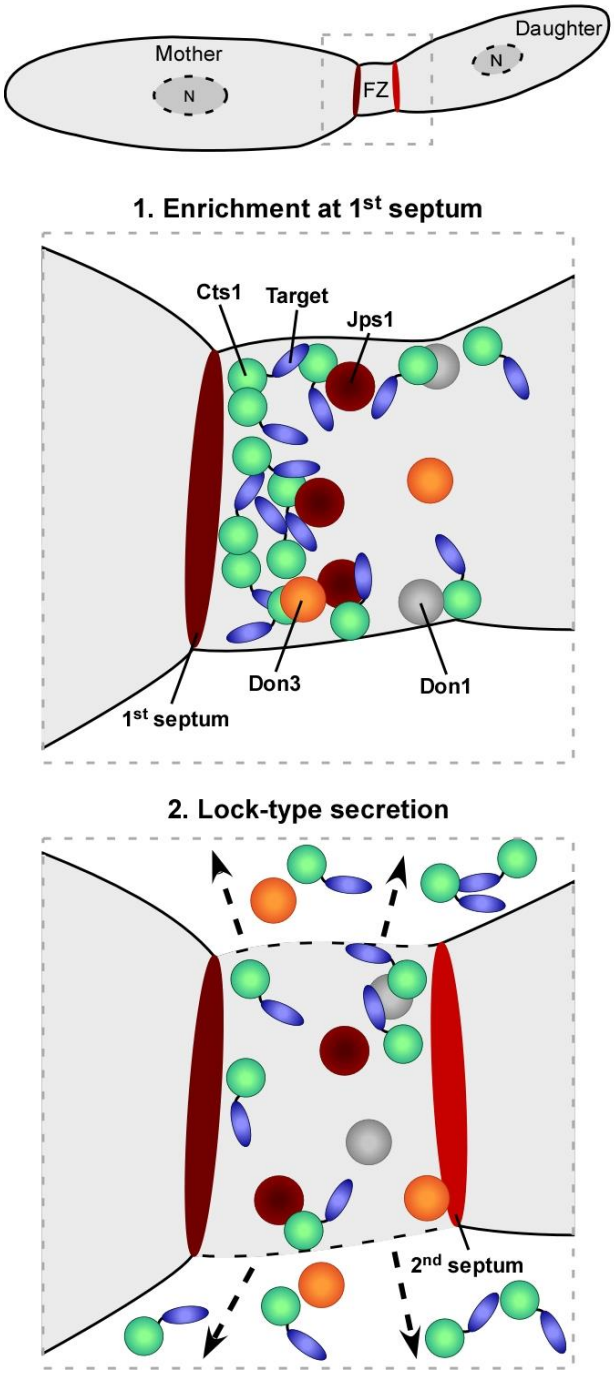

Figure 3. Exploiting lock-type unconventional secretion in *U. maydis* for heterologous protein production. Unconventional secretion of chitinase Cts1 is connected to cytokinesis. During budding, yeast like cells insert two consecutive septa at the mother and daughter cell side (dark red, primary septum; red, secondary septum), delimiting the so-called fragmentation zone (FZ). Cts1 first accumulates at the primary septum at the mother-daughter cell neck (1.). Upon insertion of the secondary septum it localizes to the fragmentation zone where it contributes to cell separation (2.). Septation factors Don1 and Don3 are essential for secondary septum formation and thus for assembly of a functional fragmentation zone. The septation factors and the potential anchoring factor Jps1 are crucial for Cts1 secretion. For heterologous protein production, proteins of interest (target) are fused to chitinase Cts1 acting as a carrier for export. N, nucleus.

Importantly, the unconventional secretion pathway can be exploited for co-export of heterologous proteins using Cts1 as a carrier protein. The key advantages of hitchhiking unconventional secretion for protein export are i) no apparent size limitation of the exported product and ii) no post-translational modifications, which potentially interfere with product activity or application (37). Thus, even bacterial enzymes can be secreted in a functional form via this pathway (67, 141), while detrimental sugar moieties might be attached due to the presence of eukaryotic *N*-glycosylation sites when forcing the enzymes through the conventional pathway. Functionality of the system was demonstrated by several proof-of-

principle studies using the examples of hydrolytic enzymes like β-glucuronidase, β-galactosidase, polygalacturonases or antibody fragments (37, 41, 67, 141, 142). Recently, first inducible systems and an autoinduction protocol were implemented, allowing to efficiently separate protein production from its secretion and protecting the product against extracellular proteases (38).

Currently, the titers of the unconventional secretion system are only in the mg/L range (142). Thus, the system is currently engineered on different levels, including genetic and cultivation aspects (Fig. 1), towards a competitive protein expression platform. While the idea of exploiting unconventional secretion for biotechnological application is not new (143), the lock-type pathway in *U. maydis* is the first verified example demonstrating the future potential of its industrial exploitation.

Conclusions and future directions

In the past decade, Ustilaginaceae have shown a rising potential for industrial exploitation as novel biotech cell factories. Relevant fields of application involve the synthesis of diverse small molecules and proteins. Deep fundamental research on *U. maydis* has been a strong driver to exploit the biodiversity of substrates and products. As shown by first successful consolidated bioprocessing strategies, Ustilaginaceae even possess the desired microbial skills for direct conversion of complex substrates into valuable products. These now need to be expanded and implemented in industrial scales.

Basidiomycete fungi thus continue to establish themselves as suitable production hosts for an increasing number of compounds. Mannan has for example been discussed as an additional homologous substance with medical relevance produced in *Pseudozyma* species (144). Moreover, the advent of modern genetic tools and synthetic biology enables novel possibilities to also produce heterologous molecules. A recent example is the synthesis of sesquiterpenes by metabolically engineered *U. maydis* (145). We expect that in the long run, more native and heterologous molecules will be added to the list, including for example bioactive substances from higher eukaryotes, which might be hard to obtain with long established Ascomycete work horses like the yeast *S. cerevisiae* (146, 147). The exquisite capacity to use plant biomass components as substrates makes *U. maydis* a perfect system for consolidated bioprocessing.

Summary

- Ustilaginaceae are natural producers of a broad variety of valuable substances
- *Ustilago maydis* represents a fungal model with huge potential as an industrial chassis
- Detailed biochemical and genetic knowledge of biosynthetic routes enable efficient pathway optimization for homologous products
- Insights into fungal biology and establishment of synthetic biological approaches foster production of heterologous molecules

- Future directions involve novel genetic strategies and co-cultivation for development of further consolidated bioprocessing processes

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Author contributions

K.M., N.W., N.I. and K.S. contributed to conceptualization of text and figures, wrote the manuscript and generated figures. K.P.H. prepared a figure. J.B. provided editorial support and contributed to writing. K.S. directed compilation of the manuscript.

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