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MINI-REVIEW

An overview of *Trichoderma reesei* co-cultures for the production of lignocellulolytic enzymes

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Abstract
Biorefineries are core facilities for implementing a sustainable circular bioeconomy. These facilities rely on microbial enzymes to hydrolyze lignocellulosic substrates into fermentable sugars. Fungal co-cultures mimic the process of natural biodegradation and have been shown to increase certain enzyme activities. *Trichoderma reesei* and its many mutant strains are major cellulase producers and are heavily utilized as a source of carbohydrate-active enzymes. Several reports have demonstrated that *T. reesei* co-cultures present higher enzyme activities compared with its monocultures, especially in the context of β-glucosidase activity. The performance of *T. reesei* during co-culturing has been assessed with several fungal partners, including *Aspergillus niger*, one of the most recurrent partners. Various aspects of co-cultivation still need further investigation, especially regarding the molecular interactions between fungi in controlled environments and the optimization of the resulting enzyme cocktails. Since plenty of genetic and physiological data on *T. reesei is* available, the species is an outstanding candidate for future co-culture investigations. Co-cultures are still a developing field for industrial enzyme production, and many aspects of the technique need further improvement before real applications.

Key points
- *T. reesei co-cultures are an alternative for producing lignocellulolytic enzymes.*
- *Several reports suggest an increase in certain enzyme activities in co-cultures.*
- *More in-depth investigations of co-cultures are necessary for advancing this field.*

Keywords Co-culture · *Trichoderma reesei* · Lignocellulose · Lignocellulolytic enzymes

Introduction

As awareness of environmental problems caused by the linear fossil-based economy grows, society is slowly moving towards a more circular bioeconomy. Lignocellulosic biorefineries are core facilities for this new model, utilizing lignocellulosic residues to produce new biotechnological products in a sustainable manner (Silva et al. 2017). Biorefineries rely on enzymes to decompose plant residues into fermentable sugars. Such enzymes generally come from microbial sources, such as bacteria and fungi (Adrio and Demain 2014). The fungus *Trichoderma reesei* is a major workhorse in the carbohydrate-active enzyme industry. The industrial strain *T. reesei* RUT-C30 is utilized for its outstanding production of cellulases, sometimes yielding approximately 100 g L^{-1} of protein in submerged cultures (Bischof et al. 2016).

Cellulases are a generic denomination for enzymes specialized in deconstructing cellulose, the major component of plant cell walls, and thus, the major component of lignocellulosic feedstocks (LCFs). These lignocellulosic materials are the most suitable substitutes for fossil fuels because of their versatility and sustainability. A great challenge in the new bioeconomy is the conversion of lignocellulosic biomass into fermentable sugars, which in turn can be converted into biofuels and other bioproducts. This objective is, however, often hindered by the cost of the enzymes.

Not even *T. reesei* and its many mutant strains are perfect cellulose degraders. It is well known that the production of β-glucosidase, the enzyme responsible for hydrolyzing

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cellobiose into two glucose monomers, is low in this fungus (Bischof et al. 2016; Grous et al. 1985; Okeke 2014). The imbalance between β-glucosidase and the other cellulases creates a bottleneck at the end of the hydrolysis chain due to feedback inhibition of cellobiose over other cellulases (Sørensen et al. 2013).

Several approaches can be employed to improve enzyme cocktails. At the strain level, modifications, such as random mutagenesis (Bischof et al. 2016) and genetic engineering (Chen et al. 2020), have been employed to improve *T. reesei* enzyme expression. The carbon source can also be modified by pre-treatment (Kumar and Sharma 2017) and liquefaction (Cunha et al. 2014) techniques, increasing the enzyme production and improving enzymatic access to the substrate.

Another alternative for improving enzyme cocktails is to cultivate two or more fungi with complementary enzyme activities to create a more diverse and robust enzyme pool. This technique is referred to as a co-culture. There are still very few investigations addressing fungal co-cultures; some of them show promising results. In this review, co-cultures of *T. reesei*, the major cellulase producer, have been discussed, highlighting culture partners, major findings, and future challenges of this largely unmapped field.

Lignocellulose degradation and co-cultures

Lignocellulose is a complex network of carbohydrates and lignin and is composed of three major components: cellulose, hemicellulose, and lignin. Cellulose is composed of glucose monomers linked together by linear β-1,6 glycosidic bonds (Cosgrove 2014). Hemicellulose is a heterogeneous compound with a myriad of carbohydrate polymers, such as xylans, xyloglucans, and mannan (Moreira and Filho 2008; Scheller and Ulvskov 2010). Lignin is a complex polyphenolic network and is one of the major causes of recalcitrance of the plant cell wall owing to its non-repetitive structure (Espiñeira et al. 2011). Further, the presence of pectin, a gel-like structure composed of galacturonic acid polymers, helps keep all the aforementioned constituents together (Daher and Braybrook 2015). Due to its complexity, lignocellulose degradation is not efficiently accomplished by a single organism in nature. It is actually a long process involving many organisms across space and time. Different microorganisms are specialized in degrading different portions of the lignocellulosic material. For example, *T. reesei* is a good cellulose degrader, but lacks enzymes responsible for lignin degradation, which are present in white rot fungi (Kong et al. 2017).

Saprophytic fungi compete over space in decaying lignocellulosic substrates when multiple species are present simultaneously. While doing so, they employ several strategies, such as secretion of inhibitory volatile and diffusible organic compounds (Hiscox et al. 2018; Siddiquee 2014), to outcompete other fungi (Fig. 1). They may also increase the expression of oxidative stress-related (Igarashi et al. 2018; Ujor et al. 2018), lignin-modifying, and carbohydrate-active (Igarashi et al. 2018) enzymes. The latter strategy is of special biotechnological interest, since it could potentially improve the production of commercially important enzymes.

Since lignocellulosic degradation is a slow process, different species of fungi can colonize the same material over time. In such cases, the first colonizer modifies the substrate, creating either a suitable or an inhibitory environment for other species (Ottosson et al. 2014). This relationship creates a succession of different species over time on the same lignocellulosic substrate, depending on the microbial community present (Ottosson et al. 2014).

As stated, nature rarely utilizes a single organism to perform a certain task. This concept of co-culturing a microbial consortium can be applied to industrial microbiology and is already being used in the food and beverage industry (Bokulich et al. 2014; Hymery et al. 2014). It can also be applied to the production of lignocellulosic enzymes by co-culturing more than one fungus to obtain enzyme cocktails that differ from their monoculture counterparts (Sperandio and Filho 2019).

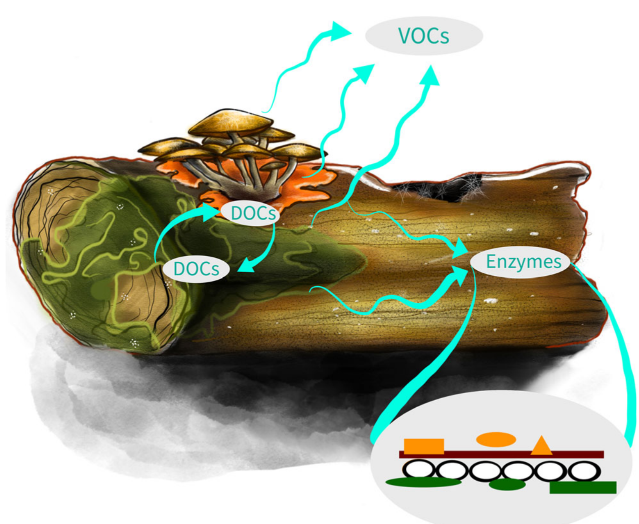

Fig. 1 a A hypothetical fungal interaction between *Trichoderma reesei* (green) and another fungus on a decaying tree trunk. During fungal interactions, the participants secrete a myriad of diffusible and volatile organic compounds (DOCs and VOCs, respectively) in order to outcompete the other species. The fungi also secrete more enzymes to better utilize the available substrate and harvest sugars to fuel their growth and combative metabolism. b The combination of fungal enzymes achieves a more complete degradation of the lignocellulosic substrate. Green and red geometric shapes represent *T. reesei* and other fungal enzymes, respectively, acting on lignocellulosic fibers. Empty circles represent the carbohydrate portion, whereas the brown straight line represents lignin

T. reesei and its co-cultures with *Aspergillus niger*

T. reesei was first isolated during World War II as a fungus known to degrade US military equipment in the South Pacific (Do Vale et al. 2014). The first strain was denominated as "QM6a," and all the hypercellulolytic mutants available today are derived from it (Bischof et al. 2016). Currently, the mutant *T. reesei* RUT-C30 is the standard strain used for cellulase production.

T. reesei is a reasonable choice for using in a co-culture. It has an outstanding ability to produce cellulases and has a low β-glucosidase activity (BGA), the disadvantage that has to be mitigated (Rana et al. 2014). Among the studies already conducted with *T. reesei*, one of the most common partners in the co-culture is *A. niger*.

A. niger is an ascomycete belonging to the black *Aspergilli* group (Abarca et al. 2004). This species is commonly found in soil and is also a frequent food contaminant (D'hooge et al. 2019). *A. niger* has a worldwide distribution, and some specimens have even been isolated from the International Space Station (Abarca et al. 2004; Romsdahl et al. 2018). Several industrial applications, such as the production of organic acids, foods, pharmaceuticals, and enzymes, rely on *A. niger* strains (Abarca et al. 2004; D'hooge et al. 2019; Papagianni 2007), many of which possess the *G*enerally *R*egarded *A*s *S*afe (GRAS) title.

A. niger is a rational co-culture choice for *T. reesei* considering its secretion of β-glucosidases (Ahamed and Vermette 2008; van Munster et al. 2014). Theoretically, *A. niger* could provide the final enzyme cocktail with higher BGA, while avoiding many other costs, such as double fermentation structure, downstream processing of enzymes, and mixing of two independent cocktails. Many positive results in this regard have already been obtained and have been compiled in Table 1. However, loss in the activity of certain enzymes, such as reduction in endoglucanase activity (EGA) by 49% (Rabello et al. 2014) and 30% (Kolasa et al. 2014), has also been reported.

Reduction in the activity of certain enzymes is one of the known challenges of co-cultivation. This can occur due to different culture conditions that may not be suitable for all the organisms involved. In the *T. reesei*/*A. niger co-culture*, the latter strongly acidifies the medium when inoculated

Table 1 Summary of co-culture reports utilizing *Trichoderma reesei* in combination with *Aspergillus niger*, including type of culture and carbon source

T. reesei strain	*A. niger* strain	Type of culture and C-source	Enzyme activity[a]	Reference
Recombinant *T. reesei* RUT-C30	*A. niger* NL02	SmF with steam-exploded corn stover	+ 34% FPA + 10% CBA + 40% EGA + 739% BGA	Zhao et al. (2018)
T. reesei RUT-C30	*A. niger*	SsF with wheat bran	− 49% EGA + 1550% BGA + 129% XA	Rabello et al. (2014)
T. reesei RUT-C30	*A. niger*	SsF with wheat bran	+ 1650% BGA − 3.2% CBA − 30% EGA	Kolasa et al. (2014)
T. reesei RUT-C30	*A. niger* NL02	SmF with steam-exploded corn stover	+ 30% FPA + 300% BGA	Fang et al. (2013)
T. reesei LM-UC4	*A. niger* ATCC 10864	SmF with lactose	+ 200% FPA + 250% protein secreted	Gutiérrez-correa and Villena (2012)
T. reesei RUT-C30	*A. niger* BC-1	SsF with rice straw	+ 9.5% FPA + 27.2% EGA + 78.2% BGA + 65.1% XA	Dhillon et al. (2011)
T. reesei M (QM 9414 mutant)	*A. niger*	SsF with wheat bran	+ 169% FPA + 200% BGA	Deshpande et al. (2008)
T. reesei Qm-9123	*A. niger*	SsF with paper-mill sludge	+ 178% substrate utilization + 500% EGA + 600% BGA	Maheshwari et al. (1994)

Some of the calculations were extracted from Sperandio and Filho (2019)

SmF submerged fermentation, *SsF* solid-state fermentation, *BGA* β-glucosidase activity, *CBA* cellobiohydrolase activity, *EGA* endoglucanase activity, *FPA* filter paper activity, *LA* laccase activity, *LiP* lignin peroxidase activity, *MnP* manganese peroxidase activity, *XA* xylanase activity

[a] Approximate percentage of increase/decrease in enzyme activity, protein secreted, or substrate utilization compared to *T. reesei* monoculture

before the former, thus, creating suboptimal conditions for the former (Maheshwari et al. 1994). To better understand such relationships, studies regarding the order and ratios of inoculation for each participant are extremely relevant (Kolasa et al. 2014; Ma and Ruan 2015).

Results of co-cultures may vary greatly even when the same species are utilized, as can be seen in Table 1. In case of *A. niger*, apart from the culture conditions, one of the possible factors contributing to this variation is the misidentification of the microorganism. Many *Aspergilli* in the section *Nigri* are morphologically similar; thus, solely relying on morphological identification can be misleading (D'hooge et al. 2019). Molecular identification of strains is highly recommended, when possible, to confirm the identity of *A. niger*.

Proteomic analysis is another crucial tool to understand the interaction between *A. niger* and *T. reesei*. Florencio et al. (2016) investigated the secretome of both fungi and monocultures, under submerged and sequential cultivation, using sugarcane bagasse as a carbon source. They found that only 27% and 29% of total proteins are common between the two cultivation methods for *T. reesei* and *A. niger*, respectively. If changing the cultivation method has such an impact on the secretome content, it is very likely that co-cultivation will also have the same impact. The secretome of both fungi has been studied elsewhere (Borin et al. 2015; di Cologna et al. 2018), but never as co-cultures. The data already available for *A. niger* and *T. reesei* secretomes will undoubtedly facilitate future comparisons, once co-culture studies of this nature are published.

T. reesei co-cultures with other fungi

Apart from *A. niger*, other *Aspergilli* have been co-cultured with *T. reesei*. Brijwani et al. (2010) co-cultured *T. reesei* with *A. oryzae* using solid-state fermentation (SsF), with soybean hulls and wheat bran as carbon sources. They obtained a 64.6% increase in filter paper activity (FPA) and a 70% higher BGA in comparison to *T. reesei* monocultures. Kolasa et al. (2014) found an impressive increase of more than 1000% in BGA upon co-cultivating *T. reesei* RUT-C30 and *A. saccharolyticus* under SsF, with wheat bran as the carbon source. Influence of the order of inoculation of the participants on the final enzyme activity was also observed in the study. Simultaneous inoculation of both *T. reesei* RUT-C30 and *A. saccharolyticus* resulted in an almost 2-fold increase in BGA compared to cultures with a 48-h delay in *Aspergillus* inoculation. However, for EGA, the same 48-h delay yielded almost three times better results. Findings of the

Table 2 Summary of co-culture reports utilizing *Trichoderma reesei* as a participant, including type of culture and carbon source

T. reesei strain	Fungal partner(s)	Type of culture and C-source	Enzyme activity[a]	Reference
T. reesei	*Monascus purpureus*	SsF with wheat straw	+ 20% FPA + 20% EGA Same XA	Fatma et al. (2020)
T. reesei QM 9414	*Aspergillus fumigatus* M51	SmF with sugarcane straw	− 33% XA − 90% FPA	Campioni et al. (2020)
T. reesei	*Coprinus comatus*	SmF with corn Stover, corn cobs and wheat bran	Same EGA − 44% XA + 21% LA[b]	Ma and Ruan (2015)
T. reesei RUT-C30	*Aspergillus saccharolyticus*	SsF with wheat bran	+ 1025% BGA + 29% CBA − 15% EGA	Kolasa et al. (2014)
T. reesei RUT-C30	*Phanerochaete chrysosporium* Burdsall	SmF with pumpkin residues	+ 92% BGA + 66% EGA + 110% CBA + 37% LiP[b] + 110% MnP[b]	Yang et al. (2013)
T. reesei LM-UC4	*Aspergillus phoenicis* QM329	SmF with lactose	+ 136% FPA + 150% protein secreted	Gutiérrez-correa and Villena (2012)
T. reesei (ATCC 26921)	*Aspergillus oryzae* (ATCC 12892)	SsF with soybean hulls and wheat bran	+ 64.6% FPA + 70% BGA + 67.3% EGA − 2.1% XA	Brijwani et al. (2010)

Some of the calculations were extracted from Sperandio and Filho (2019)

SmF submerged fermentation, *SsF* solid-state fermentation, *BGA* β-glucosidase activity, *CBA* cellobiohydrolase activity, *EGA* endoglucanase activity, *FPA* filter paper activity, *LA* laccase activity, *LiP* lignin peroxidase activity, *MnP* manganese peroxidase activity, *XA* xylanase activity

[a] Approximate percentage of increase/decrease in enzyme activity or protein secreted compared to the *T. reesei* monoculture [b] Approximate percentage of increase/decrease in enzyme activity compared to the other fungus

aforementioned studies and more examples of co-cultures between *T. reesei* and other fungi have been compiled in Table 2.

Cases where the monocultures performed better than their co-cultures have also been reported. Campioni et al. (2020) co-cultured *T. reesei* QM 9414 with *T. harzianum* and two strains of *A. fumigatus* in submerged fermentation (SmF), using sugarcane straw as the carbon source, and all combinations had lower xylanase and cellulase activities compared to *T. reesei* QM 9414 cultured alone (Table 2). *T. reesei* QM 9414 and *A. fumigatus* M51 co-culture resulted in 90% and 33% reduction in FPA and xylanase activity, respectively, compared to the monoculture of the former (Campioni et al. 2020).

Another recurrent strategy is to co-culture *T. reesei* strains with white-rot fungi, basidiomycetes capable of secreting lignin-degrading enzymes (Alfaro et al. 2014). As a defense mechanism, white-rot fungi can secrete more lignin-degrading enzymes, such as laccases and manganese peroxidase, when cultivated with other fungi compared to their monocultures (Igarashi et al. 2018; Mali et al. 2017). Mixing the capacity to produce such enzymes with the cellulolytic capabilities of *T. reesei* could, theoretically, create an enzyme cocktail that fully degrades all lignocellulose components. Yang et al. (2013) co-cultured *T. reesei* RUT-C30 with *Phanerochaete chrysosporium*, a white-rot fungus, under SmF, utilizing pumpkin residues as the carbon source. The study reported an increase in the activity of all carbohydrate-active enzymes analyzed in the co-culture compared to *T. reesei* monoculture, as well as higher lignin-degrading activities compared to *P. chrysosporium* monoculture (Table 2).

As reported by Ma and Ruan (2015), co-cultivation of *T. reesei* with *Coprinus comatus* under SmF, with a complex lignocellulosic mixture as the carbon source (Table 2), resulted in the same EGA as *T. reesei* alone. There was also a 44% decrease in xylanase activity compared to *T. reesei* monocultures. However, this co-culture achieved a 21% increase in laccase activity in relation to *C. comatus* monoculture.

Conclusion

Natural biodegradation of lignocellulosic residues is achieved in nature by microbial co-cultures. From a biotechnological perspective, *T. reesei* co-cultures with both *A. niger* and other fungi have shown an improvement in the activity of certain enzymes, especially β-glucosidase, which *T. reesei* is deficient in. Thus, the technique is a promising alternative for producing new enzymatic cocktails for biorefineries, expanding the frontiers of the new bioeconomy.

To date, co-culturing is a trial-and-error exercise, with limited predictability. Lack of information about the molecular interactions between the participants hinders any rational design of co-cultures. This is a challenge for all co-cultures, and not just for those utilizing *T. reesei*. Future studies must focus on utilizing omics approaches to unveil the molecular interactions between fungi in industrial cultivation environments. In addition, the evaluation of co-cultures in large-scale experiments is necessary before this technique can be effectively employed in real biorefineries.

Co-culturing filamentous fungi for enzyme production still poses many challenges and unanswered questions that must be addressed in order to fully harness the potential of this technique. It is very likely that major advances in co-cultures, especially those utilizing *T. reesei*, will be achieved in the coming years. Co-cultures show promising results as an inexpensive and effortless way of obtaining enzymes and should be further investigated as a new source of enzymes for biorefineries.

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Declarations

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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