

Yeast as a production platform in biorefineries: conversion of agricultural residues into value-added products

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Abstract. In contrast to a petroleum-based economy, which relies on the unlimited presence of fossil fuels, a biobased economy utilizes a broad spectrum of natural crops and biomass as raw substrates for the production of valuable materials. Biorefineries represent a promising approach for the co-production of bioenergy (biofuels, biogas) and value-added products (biochemicals, biomaterials, food). Within Europe, wheat straw represents the major crop residue and has been extensively considered as a promising feedstock in the biorefining process. Firstly, wheat straw is hydrolysed to obtain a sugar solution that is further converted into the desired product in a biocatalytic manner. Microbial fermentation is the core component of biorefineries and yeast, as for instance *Candida guilliermondii*, is an effective production platform for both, biofuels and biochemicals. One limiting aspect in using yeast in the biorefinery approach is the presence of inhibitors in lignocellulosic hydrolysates, such as acetic acid or furfural, influencing cellular growth and diverse metabolic processes. In order to overcome this problem, several genetic engineering approaches are used to increase yeast resistance towards these inhibitors and to enhance the overall production. In this paper, we summarized: 1) the pretreatment technologies for wheat straw bioconversion; 2) the *Candida guilliermondii* genetic engineering technologies and their biotechnological potential. In conclusion, biorefineries are a crucial factor in the transition towards a biobased and circular economy, and the implementation of yeast into this system offers a great opportunity to develop innovative strategies for a sustainable production in an environmentally friendly and economically feasible manner.

Key words: biorefinery, wheat straw, genetic engineering, yeast, *Candida guilliermondii*.

INTRODUCTION

Climate change, depletion of natural resources and an increased worldwide population are three actual and future challenges faced by political leaders. The replacement of a petroleum-based economy by a global biological-based economy (bioeconomy) has become of great importance for European countries in the last decades and is currently accepted as an economically feasible option to address these worldwide issues. In order to achieve long term environmental and economical sustainability, the European Commission set up a goal to implement a low carbon economy by 2050 (Scarlat et al., 2015). Bioeconomy is described as the part of an economy where renewable biological sources are used for the production of vital compounds, biofuels and bioenergy. During the last decades, many organizations contributed to raise public

acceptance of these products and it is expected that by 2025, 15% of the chemicals produced worldwide will derive from biological sources (Savvanidou et al., 2010; Scarlat et al., 2015; Amin et al., 2017). The development of cost-effective technologies for biomass processing and conversion into value-added products are key factors in the implementation of a bio-based economy. Biorefinery systems, which are successfully being used for the co-production of biofuels and biochemicals, are a great example of the potential contribution of biotechnological processes to worldwide waste management and a reduced energy demand (Almeida et al., 2012).

Within lignocellulosic feedstocks, wheat straw, corn fiber, corn stover, switchgrass and barley straw are examples of biomass that has been successfully used as substrates for the production of biochemicals and biofuels (Qureshi et al., 2013). In European countries, wheat straw represents the major crop residue with an overall production of 170×10^6 tons per year, and can therefore serve as an easy accessible raw material for its bioconversion in biorefineries (Dias et al., 2010). Additionally, its high carbohydrate/sugar content makes it a promising substrate for microbial growth. The three main components of lignocellulosic residues are hemicellulose (heteropolysaccharide composed of glucose, galactose, xylose and mannose), cellulose (polysaccharide from glucose) and lignin (aromatic polymer composed of three different phenyl propane monomers: coumaryl alcohol, coniferyl alcohol and syringyl alcohol) (Marcos et al., 2013). The common process to convert wheat straw into valuable compounds consists of four major steps: biomass pretreatment, enzymatic hydrolysis, fermentation and purification of the product (downstream processing) (Fig. 1) (Silveira et al., 2015; A. Kumar et al., 2016). In order to get an efficient biomass (e.g. wheat straw) reutilization, innovative technologies combining two key processes have to be developed: 1) Biomass pretreatment technologies and 2) Optimization of yeast as a whole-cell biocatalyst.

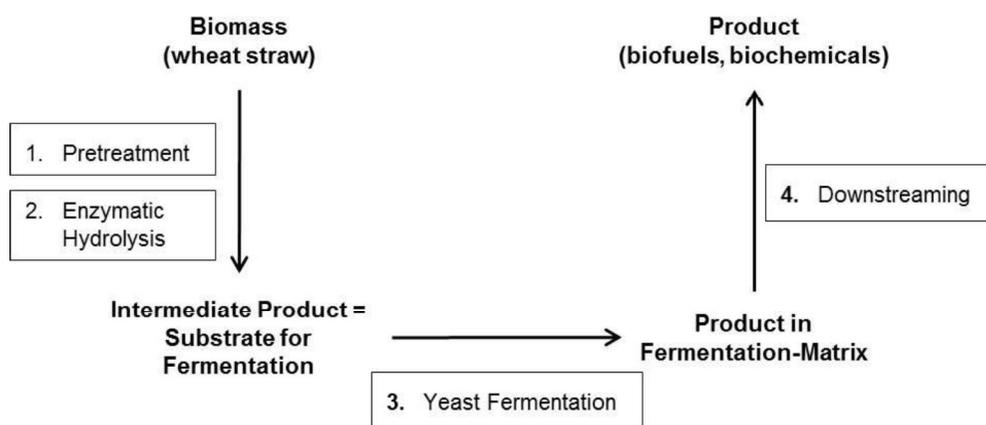


Figure 1. Biorefinery circular system. Wheat straw as a substrate for the production of biochemicals and biofuels in a four steps process that involves biomass pretreatment, enzymatic hydrolysis, fermentation (by mean of yeast, e.g. *C. guilliermondii*) and product purification.

The goal of the first two steps is to obtain a sugar solution, by pretreating wheat straw and hydrolysing it enzymatically, which can then be utilized during the third step as a fermentable substrate for yeast cells. In the last step, the synthesized product is recovered and purified via downstream processing. During this cycle, several by-products are released, (e.g. glycerol during biodiesel production) and can be re-introduced into this circular system to a certain extent, providing an excellent opportunity to generate a cost-effective system for continuous microbial growth.

During evolution, yeast has been an essential component of human societies, as it is used for human activities, such as the making of bread, wine, beer or other distilled beverages. Additionally, it is also a research model organism used in many laboratories worldwide to elucidate the molecular mechanisms behind cellular processes relevant to biotechnology (Mortimer, 2000). In the actual bioeconomy era, biotechnological processes associated with yeast are believed to be a key factor for the establishment of a circular economy, due to its potential to generate industrially relevant compounds from natural sources and waste streams in a cost-effective and environmentally friendly manner (Moore et al., 2017). Thus, exploiting yeast genetic diversity offers a great opportunity to search for additional ways of increasing production yields, as well as the spectrum of biochemicals and biofuels produced in biorefineries. For this aim, researchers have developed numerous technologies to elucidate the molecular basis of cellular metabolic processes in these microorganisms and applied them for the bioprocessing of agricultural residues (Campbell et al., 2017).

Saccharomyces cerevisiae is the most commonly used yeast for biotechnological applications and it is regularly used at industrial scale for the production of biochemicals and biofuels (Mattanovich et al., 2014; Kavšček et al., 2015; Kwak & Jin, 2017). Other non-conventional yeasts represent a great biocatalytic alternative to be used as an economically feasible whole cell production platform, as for instance *Candida guilliermondii* for the production of xylitol, the oleaginous yeast *Yarrowia lipolytica* for the production of lipids and *Ashbya gossypii* for the production of riboflavin (Vitamin B₂) (Rodrigues et al., 2006; Revuelta et al., 2017; Niehus et al., 2018). Recent advances in genetic engineering (GE) technologies, such as the discovery of the CRISPR-Cas9 system and 'omics' approaches, offer a great opportunity to explore the biotechnological potential these non-conventional yeasts (Raschmanová et al., 2018).

In the first part of this paper, we summarize the main pretreatment methods applied to wheat straw, as an example of a potential biomass substrate that can be used in biorefineries. Then, specific GE approaches designed for *C. guilliermondii* are discussed, as well as examples of potential industrially relevant compounds synthesized by this yeast.

WHEAT STRAW PRETREATMENT TECHNOLOGIES

The first step in using wheat straw as a biomass source for the production of industrially relevant compounds is the extraction of its contained sugars, which will then be further utilized during microbial fermentation. The optimization of the different pretreatment technologies is the main challenge for biorefineries, due to their high energy demands and costs, as well as the negative effects of inhibitors released during this process on microbial growth (Cardona & Sánchez, 2007).

Regarding the pretreatment of lignocellulose, four different technologies are mostly used: biological, physical, physico-chemical and chemical (Fig. 2). Independently of the chosen pretreatment technology, wheat straw is chopped to increase the yield of the subsequent steps (Eisenhuber et al., 2013). Overall, the described methods aim to increase the enzymes' accessibility to the hemicellulosic and cellulosic polymers.

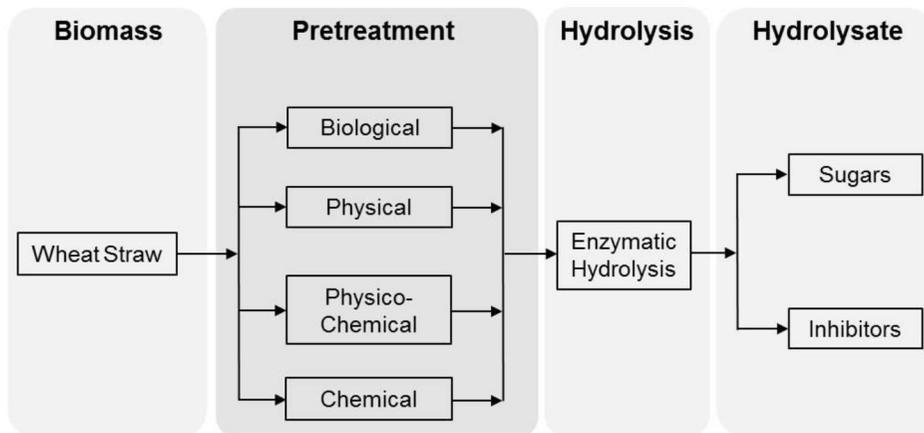


Figure 2. Pretreatment methods used to facilitate wheat straw sugar polymers hydrolysis during enzymatic hydrolysis and production of a fermentable hydrolysate solution.

Biological – Biological pretreatment is done by different fungi, as for instance the white rot fungus from the *Trametes* species (Knežević et al., 2016). The special characteristic of this fungi is its ability to produce a lignin-degrading enzyme system, containing a laccase, a lignin-peroxidase and a Mn-dependent peroxidase (Dias et al., 2010). Biological pretreatment has been proven to be a cost-effective manner to delignify wheat straw, thus representing a promising environmentally friendly method to be used in biorefineries. Due to its high lignin and low nitrogen content, the decomposition of wheat straw is a rather slow process (Dias et al., 2010). Lignin is a recalcitrant polymer that offers mechanical resistance during its disruption and makes the subsequent enzymatic hydrolysis step harder.

Physical – Mechanical processing of wheat straw, such as milling, grinding, ultrasound or chipping, is a commonly used pretreatment method that confers a cheap and rapid way to increase wheat straw surface area and facilitate the enzymes accessibility to the substrate (Eisenhuber et al., 2013; Silveira et al., 2015). Due to its inability to completely remove the lignin and hemicellulose, it is mostly used in combination with the other pretreatment methods, which then increases enzymatic hydrolysis efficiency.

Physico-Chemical – Physico-Chemical pretreatments combine chemical and physical techniques to disrupt the structure of the lignocellulosic materials, as for instance combination of water at high temperatures and high pressures (Liquid Hot Water - LHW) or combination of liquid anhydrous ammonia, high pressure and moderate temperature (Ammonia Fiber Explosion – AFEX) (Brodeur et al., 2011). Another pretreatment technique is Steam Explosion (SE), which has been successfully applied to wheat straw and other agricultural crops and wood residues (Guerrero et al., 2017).

During this process, wheat straw is incubated at high steam temperatures and high pressures during a defined time period. The rapid release of pressure provokes a decompression of the steam and induce structural changes and partial disruption of wheat straw fibers, but also transforms the lignin into other chemicals, e.g. acetic acid, formic acid, furfural and 5-hydroxymethylfurfural (HMF), that can inhibit microbial growth (Auxenfans et al., 2017). Optimization of SE parameters (temperature/time) was shown to be an effective way to recover the maximum amount of the desired sugar (Marcos et al., 2013; Alvira et al., 2016). Additionally, during this process different liquid and solid fractions are recovered and can be used in the subsequent steps, depending on the desired outcome (Alvira et al., 2016). On the one hand, SE is an attractive option requiring no chemicals and having a low energy demand (García-Aparicio et al., 2006). During this process, different inhibitory compounds influencing microbial growth are formed and released (Palmqvist & Hahn-Hägerdal, 2000). Further optimization of this process, for example using N₂ gas instead of compressed air, showed greater results in the overall glucose recovery from wheat straw biomass (Raud et al., 2016; Tutt et al., 2016)

Chemical – The alkaline pretreatment process aims to remove the lignin and partially degrade the hemicellulose to facilitate the enzymatic access to cellulose and hemicellulose. This can be achieved for instance by using sodium hydroxide (NaOH). It was shown that a higher concentration of this base positively influences the enzymatic hydrolysis step (Han et al., 2012). Acidic pretreatment is performed at temperatures from 140 °C to 200 °C and involves the addition of acids, such as hydrochloric acid (HCl) or sulfuric acid (H₂SO₄), in a diluted or undiluted form, which hydrolyze and remove the hemicellulose (Silveira et al., 2015). The main disadvantages of this method are the requirement of special equipment, due to the high corrosive power of the acids, and the production of a high concentration of microbial growth inhibitors (Sun & Cheng, 2005).

***Candida guilliermondii* – GENETIC ENGINEERING APPROACHES AND BIOTECHNOLOGICAL POTENTIAL**

During metabolic engineering approaches, the knowledge of different disciplines, such as systems biology, synthetic biology and ‘omics’ (genomics, proteomics, transcriptomics and metabolomics) technologies are combined to enable the production of a desired compound. Enzymatic reactions are improved by modulating specific activities, or expanded by integrating heterologous genes using synthetic biology tools. In this case, where a compound is not naturally produced by a host organism, synthetic biology tools significantly reduced the time to generate genetically optimized production strains. Recent advances in GE technologies, such as the discovery of the CRISPR-Cas9 system, provided an essential tool to rapidly integrate genetic modifications in non-conventional yeast strains, as opposed to the genetic modifications performed using the cellular DNA repair system (Raschmanová et al., 2018).

Using the inherent DNA repair mechanisms, researchers developed tools to integrate foreign DNA into the yeast genome by using the natural DNA homologous recombination (HR) and non-homologous end joining systems (NHEJ). Although different yeast species usually prefer one of the systems, both of them have been successfully used to genetically modify *Candida guilliermondii* (Papon et al., 2015). Additionally, the novel CRISPR-Cas9 technology has been applied to perform genetic

modifications on other *Candida* species, such as *Candida albicans* and *Candida glabrata* (Vyas et al., 2015; Enkler et al., 2016).

Candida guilliermondii (teleomorph *Meyerozyma guilliermondii*) is an ascomycetous yeast found in a wide number of environmental sources. During the last decades, researchers studied the biotechnological potential of this non-conventional yeast, including its ability to convert xylose to xylitol and efficiently utilize hemicellulosic hydrolysates as an energy source (Canilha et al., 2003; Rodrigues et al., 2006; Papon et al., 2013).

The complete sequenced genome of the *C. guilliermondii* reference strain ATCC6260 allowed a more detailed study of *Candida*'s biology (Butler et al., 2009). In contrast to other species from the subphylum *Saccharomycotina*, *C. guilliermondii* belongs to the CTG fungal clade, where the CTG codon encodes serine instead of leucine. Therefore, specific efforts have been made in order to design a standardized and versatile genetic toolbox for genetic engineering of yeasts from this clade (Papon et al., 2012; Defosse et al., 2018). This includes different drug-resistance cassettes, as for instance nourseothricin- and hygromycin B-resistance markers, which enable the genetic transformation of wild type strains (Millerioux et al., 2011a; Foureau, et al., 2013b). Additionally, a *C. guilliermondii* mutant strain, named NP566U (*ura5*), was selected during a screening to identify uracil auxotrophic strains and enabled the development of a URA5 integrative cassette which can be used in combination with the so-called URA5 blaster system (Millerioux, et al., 2011b). In this method, the cassette is introduced at a desired position in the genome via homologous recombination and allows the generation of mutant strains in which specific genes are overexpressed, knocked out or proteins are tagged with fluorescence markers such as GFP (Courdavault et al., 2011). Using this system, an optimized recipient strain (KU141F1) derived from the reference strain ATCC6260, which presents an increased homologous recombination rate, has been successfully used to knock out genes and elucidate factors responsible of *C. guilliermondii* virulence in mouse models or for the production of long-chain α,ω -dicarboxylic acids (Foureau, et al., 2013a; Navarro-Arias et al., 2016; Werner et al., 2017).

These new technologies offer an excellent opportunity to further explore the biotechnological potential of *C. guilliermondii*. GE approaches, as well as growth optimization processes for wild type strains, are strategies that are exploited by the research community to optimize the production of valuable compounds within this yeast, as for example:

Xylitol – Xylitol is a five-carbon polyol industrially produced by a chemical hydrogenation reaction from the five-carbon sugar xylose, a main component of lignocellulosic feedstocks. However, it is also synthesized naturally by some microorganisms (Pal et al., 2016). This rare sugar alcohol is used by the food industry as a sugar substitute for people suffering from diabetes, and it has shown beneficial properties for human health (Granström et al., 2007). Over the last decades, biotechnology research on *C. guilliermondii* was performed aiming to increase the levels of xylitol produced by this yeast. For this, optimization of fermentation parameters or influencing cell permeabilization were the strategies used to efficiently bioconvert xylose to xylitol by wild type strains (De Albuquerque et al., 2014; Cortez et al., 2016). Interestingly, microbial synthesis of xylitol was optimized using metabolic engineering approaches on other yeasts belonging to the *Candida* genus. For instance, in *Candida*

tropicalis, xylitol metabolism was modulated by knocking out the gene encoding a xylitol dehydrogenase (XDH), an enzyme catalysing the conversion of xylitol to D-xylulose, or expressing *C. parapilopsis* xylitol reductase (XR) in *C. tropicalis*. This increased xylitol productivity by 25% and 33%, respectively (Lee et al., 2003; Ko et al., 2006).

Riboflavin – Among other yeast species, *C. guilliermondii* belongs to the group of flavinogenic yeasts, which are capable of overproducing riboflavin (vitamin B2) under iron limitation conditions (Tanner et al., 1945). Interestingly, it was shown that this yeast is capable of producing riboflavin when grown on media with xylose as a sole carbon source. By using an insertion mechanism approach, the genes responsible for the accumulation of this compound were recently identified (Leathers & Gupta, 1997; Boretsky et al., 2011).

Ethanol – *S. cerevisiae* is the most commonly used yeast strain in the bioethanol industry for the production of ethanol (Meijnen et al., 2016). Within the non-conventional yeasts, an osmotolerant *C. guilliermondii* strain was isolated and showed the ability to obtain in 48 hours an ethanol yield of 0.46 g ethanol g⁻¹ of sugar, using soybean hull hydrolysates as feedstock (Schirmer-Michel et al., 2008).

Biodiesel – due to their ability to accumulate high concentrations of lipids within the cell, oleaginous yeasts are the most efficient microorganisms used for biodiesel production. In a recent study, using a high-throughput method to identify strains with a high-lipid content, a novel *C. guilliermondii* oleaginous strain (BI281A) was identified and characterized (Ramírez-Castrillón et al., 2017). Similarly to other oleaginous yeast, such as *Yarrowia lipolytica*, this new strain is able to utilize raw glycerol, derived from a biodiesel refinery, as a carbon source and represents a new possibility for biodiesel production.

Citric acid and α-amylase – taking advantage of its ability to utilize glucose and galactose, citric acid was produced by a *C. guilliermondii* UV mutagenized strain grown on hydrolysed whey permeate as a substrate (Tisnadjaja et al., 1996). Additionally, date wastes, containing high levels of starch, have been described as a possible growth substrate for the production of α-amylase by this yeast (Acourene & Ammouche, 2012).

Inulinase – Inulin is a storage carbohydrate widely found in plants, consisting of a mixture of fructose oligo- and polysaccharides of different lengths. The enzyme hydrolysing these complex carbohydrates into fructose is called inulinase. Interestingly, a *C. guilliermondii* strain isolated from the surface of marine algae showed the ability to produce 60 U mL⁻¹ of inulinase and was further optimized by UV-mutagenesis to increase the production to 115 U mL⁻¹ of inulinase (Sirisansaneeyakul et al., 2007; Guo et al., 2009).

CONCLUSION

Biorefineries are a promising alternative for the synthesis of chemicals and fuels in an environmentally friendly manner. Agricultural crops, e.g. wheat straw, are used as a biomass substrate and are converted into value-added products. To achieve this, several biomass pretreatment technologies are used, as for instance steam explosion. Future development of less energy intensive methods, including the combination of different biomass processing technologies, will contribute to an economically more feasible production within biorefineries.

Yeast has been used over many years for the synthesis of products used by humans on a daily basis. Exploration of yeast diversity offers a great chance to search and increase the spectrum of industrially relevant compounds produced by microorganisms, such as yeast. Recent advances in ‘omics’ technologies, such as proteomics and lipidomics, might facilitate the rapid and efficient analysis of biological samples. Additionally, new technologies for genome analysis and DNA editing greatly facilitated the genetic optimization of production strains.

The yeast *Candida guilliermondii*, which was mostly studied for its ability to produce xylitol, has been successfully modified genetically using drug-resistance cassettes. Thus, the development and implementation of this yeast by a marker-free genetic modification method, such as CRIPSR-Cas9, might contribute to broaden the range of compounds that can be produced by this yeast and exploit its biotechnological potential.

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