Chapter

Biotechnological Applications of Nonconventional Yeasts

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Abstract

Yeasts not belonging to species of the *Saccharomyces* genus, called nonconventional yeasts, have gained prominence recently in the biotechnological scenario. For many years, they have been generally characterized as undesirable contaminants in fermentative processes. However, several studies pointed them as useful for many biotechnological applications. This chapter will cover some of these applications, highlighting the most widely employed nonconventional yeasts. The use of non-*Saccharomyces* strains in (I) xylose fermentation for the production of ethanol and xylitol, (II) brewing industry, (III) improvement of coffee and cocoa fermentation, and (IV) plant growth promotion will be presented.

Keywords: nonconventional yeasts, xylose fermentation, brewing industry, coffee/cocoa fermentation, plant growth promotion

1. Introduction

Yeasts have been extensively exploited by humanity for the production of bread, alcoholic beverages, bioethanol, biomass (as human or animal protein supplement), and glycerol [1–4]. They are suitable models for studies of molecular genetics, biotechnology, evolutionary biology, genomics, and eukaryotic cell biology due to their ease of culture, simple and relatively fast life cycles, and small genomes [5]. The most known species of yeasts belong to the subphylum Saccharomycotina, including *Saccharomyces cerevisiae* (eukaryotic model system), *Candida albicans* (common human commensal and opportunistic pathogen), and over 1000 other species. These eukaryotic single-celled microorganisms are found in every biome and continent and are more genetically diverse than chordates or angiosperms [5].

The different applications of yeasts represent one of the oldest uses of biotechnology by mankind. Although *S. cerevisiae* is the most domesticated and widely used industrial yeast, many other yeast species that evolved over half-a-billion years have been overshadowed, but could also show a high biotechnological potential [5–7]. A variety of other yeast genera and species than *Saccharomyces*—called nonconventional yeasts—may support experimental studies and favor the biotechnological generation of value-added products [8, 9].

The wide variety of nonconventional yeasts has gained attention for numerous applications in the areas of biocatalysis (pharmaceuticals, chemical intermediates, and biotransformations), biofuels, alcoholic beverages (enhancement of desirable flavors), fundamental biological research (molecular and cellular biology, genomics, functional genomics, pathway engineering, and system biology mechanisms), biomedical research (drug discovery, drug resistance and metabolism, and elucidation of disease mechanism), biocontrol (crop protection, food and feed safety, and probiotics), environmental biotechnology (bioremediation and pollutant degradation), and heterologous protein production (protein pharmaceuticals, enzymes, hormones, vaccines, and toxins) [10–14].

In this chapter, we mined the literature in order to present some applications of nonconventional yeasts. First, a metabolism not present in *S. cerevisiae*, the fermentation of the pentose sugar xylose, is addressed whose process can generate both ethanol and xylitol as the final products. The metabolic pathway, the main nonconventional yeast species able to perform this activity, and the challenges of the process are discussed. Then, the potential of nonconventional yeasts for the production of new flavors and aroma attributes in the production of craft beer, coffee, and chocolate is described. The chapter is finished with a review regarding an alternative biotechnological application of yeasts, the use of nonconventional strains able to perform many activities associated with plant growth promotion.

2. Some applications of nonconventional yeasts

2.1 Xylose fermentation

The world energy scenario urgently needs alternative sources for replacing fossil-based fuels, mainly because of the damage that they cause to the environment [15]. Petroleum-based fuels are the most widely used, a scenario that involves high costs and climate concerns [16, 17]. The burning of fuels such as gasoline and diesel contributes to the release of most of the gases causing the greenhouse effect [17, 18]. For these reasons, biofuels are attractive alternatives to the finite fossil fuels [19]. Bioethanol is a promising example of clean and renewable energy source. It is the most widely used biofuel worldwide, both in pure form or as a gasoline additive [20].

Brazil is the world's second largest ethanol producer, whereas USA is the leader using corn starch as the fermenting substrate [21]. However, the main raw materials used for obtaining fermentable substrates, *i.e.* corn, sugarcane, and sugar beet, also show food destinations that raise a recurrent discussion about new alternatives for ethanol production [22]. In addition, the conventional production of biofuels faces several obstacles to achieve the desired levels, encouraging the use of advanced technologies, such as ethanol produced from lignocellulosic biomass, also known as second-generation ethanol [23].

Lignocellulose has a great biotechnological value and comprises most part of the plant dry weight, composed of 35–50% of cellulose, 20–35% of hemicellulose, and 10–25% of lignin. It is the largest source of renewable organic material, highly generated through agricultural and forestry practices [24, 25]. The polysaccharide components of the lignocellulosic biomass (cellulose and hemicellulose) can be processed through a hydrolysis reaction to release sugar monomers, such as hexoses and pentoses (especially glucose and xylose, respectively) that can be used as substrates in fermentation processes [26, 27]. Xylose has a high biotechnological potential; it is the most abundant sugar of the biosphere after glucose. D-xylose can be used by nonconventional yeasts for the bioconversion to ethanol or xylitol [28, 29], while *Saccharomyces cerevisiae*—the most widely employed yeast species in

fermentative processes—is not able to metabolize it [30–32]. In this context, exploring the biodiversity of yeast strains not belonging to *Saccharomyces cerevisiae* species for xylose fermentation is of fundamental importance.

Xylitol has a sweetening power similar to sucrose and is found in nature, fruits, and vegetables. It is effective in sucrose substitution, since its metabolism is independent of insulin, which allows its utilization in the treatment of diabetes [33–36]. Due to its physical and chemical properties, xylitol is a compound of high added value, attracting high interest of the pharmaceutical, food, and dentifrice industries. Moreover, its proven efficiency in reducing the incidence of tooth decay promoted application in the oral health field [37–39]. The concentration of xylitol in plants is relatively low; thus, it is not economically feasible to extract it. On the other hand, bacteria, filamentous fungi, and non-*Saccharomyces* yeast species are microorganisms capable of performing an efficient bioconversion of D-xylose to xylitol. Yeasts belonging to the genus *Candida* are especially known for the capacity to perform this bioconversion. *C. guilliermondii* and *C. maltosa* are species that have been identified with high rates of D-xylose consumption and xylitol production in the fermentation under microaerophilic conditions [40, 41].

The discovery of D-xylose-fermenting yeasts began in the 1980s [42], and since this decade, we can list some species of nonconventional yeasts studied for ethanol production: *Pachysolen tannophilus* [43], *Kluyveromyces cellobiovorus* [44], *Scheffersomyces* (*Candida*) *shehatae* [45], and *Scheffersomyces* (*Pichia*) *stipitis* [45–47]. Most recently, *Spathaspora arborariae* [48, 49], *Sp. passalidarum* [50–53], and *Sp. piracicabensis* are other species described as able to perform this activity [54]. *Spathaspora passalidarum*, *Sp. arborariae*, *Sp. piracicabensis*, *Sp. gorwiae*, *and Sp. hagerdaliae* produce ethanol mostly from D-xylose, while the remaining species within this clade are considered xylitol producers. Among the set of *Spathaspora* species able to ferment D-xylose, *Sp. passalidarum* is the highest ethanol producer under oxygen-limited or anaerobic conditions, showing rapid D-xylose consumption and also the ability to ferment glucose, xylose, and cellobiose simultaneously. *Sp. passalidarum* is a potential candidate for use in the fermentation of sugars from lignocellulosic biomass [55].

Many other *Candida* species are also reported as capable of fermenting xylose for ethanol and/or xylitol bioconversion: *C. tenuis* (*Yamadazyma tenuis*), *C. tropicalis*, *C. utilis* (*Cyberlindnera jadinii*), *C. blankii*, *C. friedrichii*, *C. solani*, and *C. parapsilosis*. Also described as xylose fermenters are species of the genera *Debaryomyces*, *Brettanomyces*, *Clavispora*, and *Schizosaccharomyces* [56–58].

The hydrolysis reaction of the lignocellulose biomass is conducted first through a pretreatment that can be mechanical, chemical, or heat-based. It is performed in order to reduce the amount of lignin and enable the following enzymatic hydrolysis of the polysaccharides [59]. Although there are many studies with particular species able to ferment D-xylose, the conversion of this pentose into ethanol still faces a high number of challenges, such as (I) metabolic repression due to a fermentation involving a mix of pentose and hexose sugars present in hydrolyzed substrate, (II) the presence of toxic substances generated by the pretreatment and hydrolysis reaction (furfural and acetic acid), (III) the unbalance between coenzymes required by the enzymes associated to the early stages of xylose metabolism, and (IV) the need of an oxygen supply for ethanol production [32]. Therefore, ethanol production from D-xylose has not yet reached commercial levels, which requires deeper studies aiming to achieve an appropriate conversion process [60]. The exploitation of yeasts for D-xylose fermentation is also limited by the low tolerance of cells to ethanol, slow fermentation rates, and also the difficulty in controlling the rate of oxygen supply at an optimum level during fermentation. The combined presence of sugars (hexose and pentose) present in the hydrolysate also introduces difficulties as

mentioned above [61], because hexoses can inhibit D-xylose metabolism by repression and inactivation of the D-xylose transport systems or catabolic enzymes [62].

Throughout the biosphere, microbial diversity is poorly known and represents a huge potential source for the discovery of new species [63, 64]. This untapped biodiversity probably contains species able to ferment D-xylose and other sugars efficiently. Bioprospection and identification of new yeasts capable of fermenting sugars from lignocellulosic biomass are of particular interest for the development of technologies aiming to produce biofuels in a useful and viable way. D-xylose fermenting yeasts have been isolated for example from the gut of wood-boring insects such as passalid beetles (*S. stipitis, S. shehatae, Sp. passalidarum, C. jeffriesii,* and *Sp. allomyrina*) [51, 65, 66], rotting wood (*Sp. arborariae* and *Sp. piracicabensis*) [50, 54], mushrooms, bromeliads (*S. shehatae*) [67, 68], larval gut of the silkworm (*Blastobotrys bombycis*) [69], and peat in a tropical swamp forest in Surat Thani province of Thailand (*C. kantuleensis*) [70]. The prospection of species in Brazilian ecosystems has focused mainly on Atlantic Rainforest, and other few studies have been conducted on Cerrado ecosystem and Amazonian Forest sites [71]. The draft or complete genomes of the D-xylose fermenting yeasts *S. stipitis*, *Sp. arborariae*, and *Sp. xylofermentans* were already sequenced and have been studied accurately. The genes involved in the mechanisms of regulation of xylose metabolism are very important for engineering xylose metabolism in *S. cerevisiae* [72–74].

The uptake of D-xylose by yeast cells occurs through membrane-carrying proteins. D-xylose transport occurs by a facilitated diffusion mechanism; thus, the only driving force is the sugar gradient. It also occurs by an active transport system (co-transport of H^+ and sugar) [32, 75]. The metabolism of D-xylose in yeast occurs according to the descriptive scheme of **Figure 1** [29, 42, 61, 76, 77]:

1. D-xylose is initially transported into the cell;

2. With the presence of coenzymes NADH and NADPH, the enzyme xylose reductase (XR) converts xylose to xylitol, releasing NAD^+ and $NADP^+$, respectively;

3. The xylitol formed can be either excreted in the medium or oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH) in the strict presence of the NAD^+ coenzyme, releasing NADH;

4. The produced xylulose is phosphorylated by the enzyme xylulokinase (XK) and forms xylulose-5-phosphate;

5. Xylulose-5-phosphate is metabolized by pentose phosphate pathway (PPP);

6. The pentose phosphate pathway (PPP) has two phases: the oxidative phase and the nonoxidative phase (regeneration phase);

7. Metabolites resulting from the pentose phosphate pathway (fructose-6P and glyceraldehyde-3P) are metabolized in glycolysis (Embden-Meyerhof route) and converted to pyruvate;

8. Pyruvate can be oxidized by the Krebs cycle and recovers the coenzymes through the respiratory chain, or it can also be fermented into ethanol by the action of pyruvate decarboxylase and alcohol dehydrogenase enzymes (in this process NADH is reoxidized as a result of glyceraldehyde-3P oxidation).

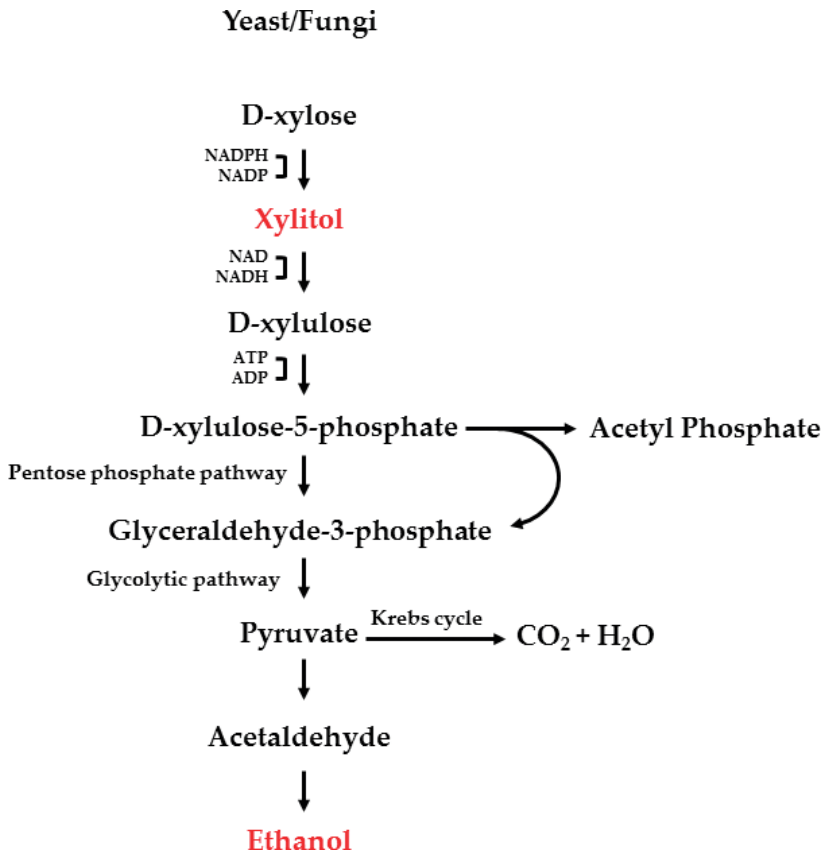

Figure 1.
Schematic diagram of D-xylose metabolism.

Understanding xylose metabolism is important for obtaining high yield fermentation. In some yeast strains, the enzyme xylose reductase (XR) is only dependent on the NADPH coenzyme, releasing $NADP^+$. In others, the XR enzyme can use both coenzymes (NADPH and NADH), releasing $NADP^+$ and NAD^+, respectively. In the following step, xylitol dehydrogenase (XDH) is strictly dependent on the NAD^+ coenzyme, causing the unbalance of coenzymes, which limits the fermentation process of D-xylose to ethanol, since this specific dependence can cause xylitol accumulation [78].

The coenzymes used by the main enzymes involved, xylose reductase (XR) and xylitol dehydrogenase (XDH), proceed with even more unbalance of coenzymes under anaerobic conditions, since the coenzyme $NADP^+$ can be reduced via fructose-6-phosphate, and the coenzyme NADH cannot be oxidized in the absence of oxygen. In this way, xylitol accumulates, and hence, ethanol production decreases [77]. Therefore, oxygenation control at the optimal level is one of the most important physiological factors. Aeration determines the division of xylose carbon flux between cell growth and bioconversion. The enzyme XR that has activity in the presence of both coenzymes can favor the production of ethanol under anaerobic conditions [79, 80].

Industrial xylitol is produced by chemical reduction of xylose. However, its biological production would be more attractive due to the low costs associated to its production and better organoleptic characteristics. The chemical production requires more energy supply, which increases the price of the product and makes it less competitive compared to other sweeteners. The biotechnological process is carried out under conditions of moderate temperature and pressure, and therefore

has the lowest energy requirements. This bioconversion is highly specific, resulting in higher yields and lower costs of product separation and purification, as well as cleaner effluents [37, 81, 82]. The biotechnological production of xylitol is associated with the ability of microorganisms to synthesize the enzyme xylose reductase (XR). As mentioned above, XR catalyzes the reduction of xylose to xylitol with the participation of the NADPH or NADH. Xylitol can be either excreted from the cell or oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH), whose activity requires the NAD^+ cofactor. Briefly, the production of xylitol depends on a high activity of the XR enzyme or a low activity of the enzyme XDH. The degree of activity of these enzymes is a criterion used to identify the best producers [78, 82–86].

The enzyme xylose isomerase (XI) is able to transform xylose directly into xylulose. The xylose isomerase (XI) pathway does not produce xylitol and is performed by some prokaryotes, fungi, and plants. However, such natural ability in yeasts has not yet been described. There are genetically modified strains with this enzymatic capacity [87]. Research also reports the ability of genetically modified *S. cerevisiae* strains to ferment xylose into ethanol, but these studies also raise questions about the fermentation viability [30, 32].

2.2 Beer fermentation

The most widely used yeasts for beer fermentation are of the genus *Saccharomyces*. Yeast strains belonging to the species *S. cerevisiae* and *S. pastorianus* are used for the production of the two main families of beers: ale and lager, respectively. The selection and use of *Saccharomyces* spp. (mainly *S. cerevisiae*) for controlled fermentations over time is related to several attractive traits, such as high fermentation performance, tolerance to ethanol and other stressors, production of desirable flavor and aroma compounds, and the safe use for fermented foods and beverages, *i.e.*, the absence of production of toxic metabolites [88].

Despite the widespread use of such traditional starter yeasts for brewing, the use of nonconventional yeasts, especially non-*Saccharomyces* species, has been gaining attention with the increasing demand for product innovation and diversity [89]. The growth of the craft beer segment has boosted the search for strategies to bring differentiation to the beverages, and the role of different yeasts in defining the beer traits has been exploited and gained special attention [90].

Generally, non-*Saccharomyces* yeasts present lower fermentative efficiency when compared to *Saccharomyces* spp., exhibiting reduced ethanol yield [91], and they usually have been seen as contaminant agents of the brewing process due to associated problems and negative influence on beer quality [92]. However, these yeasts may display distinct physiologic and metabolic activities, which can all contribute to secondary metabolite production, impacting on the sensorial complexity of the beer [89, 91].

The use of non-*Saccharomyces* yeast strains in the production of beers has the potential to provide distinctive flavor and aroma attributes [93]. In food and beverages, the production or enhancement of flavor compounds via (micro) biological systems is referred to as bioflavoring [94], and several non-*Saccharomyces* yeasts species have been pointed out as feasible agents to enhance, improve, and diversify the beer sensorial characteristics [95]. Besides bioflavoring, other applications have been highlighted, such as the production of low-/no-alcohol beers and light (low calorie) beers [89, 93, 95]. Non-*Saccharomyces* yeasts are commonly encountered in spontaneous and uncontrolled fermentation processes, e.*g.,* in the production of sour beers, such as Belgian lambics and American coolship ales [92]. During fermentation, a succession of autochthonous yeasts and bacteria are observed along the process, which lasts for 1–3 years in oak barrels. Among non-*Saccharomyces,* primarily *Debaryomyces* has been detected in lambic, and *Rhodotorula* in coolship ale

at the first month of fermentation. Both yeasts are replaced by *Saccharomyces* spp., which dominate the main alcoholic fermentation for 3–4 months. Subsequently, the latter is gradually outcompeted by *Brettanomyces* (teleomorph *Dekkera*), mainly *B. bruxellensis*, which presents high tolerance to ethanol and remains the predominant non-*Saccharomyces* yeast until the end of fermentation (along the acidification-maturation phase) [96, 97]. During this phase, *Dekkera/Brettanomyces* contributes to a higher attenuation of the wort, due to the consumption of oligosaccharides that *Saccharomyces* is not able to metabolize, and to the production of several aroma compounds, such as caprylic and capric fatty acids and their ethyl esters, ethyl phenol, ethyl guaiacol, isovaleric acid, acetic acid, and ethyl acetate, which together characterize those particular beer styles [88, 93, 95]. Other non-*Saccharomyces* yeasts of the genera *Kluyveromyces, Torulaspora, Candida, Hanseniaspora, Pichia, Meyerozyma, Wickerhamomyces, Cryptococcus*, and *Priceomyces* have already been found in such spontaneous fermentations [92, 97], but not many of them have been evaluated to be used under controlled beer fermentations [98].

Nowadays, the most commonly used nonconventional yeast in craft and specialty beers is *Dekkera/Brettanomyces* [99]. The ability to produce a large diversity of aroma compounds, most notably phenolic compounds, ethyl esters, and (fatty) acids, has made this yeast an interesting bioflavoring agent to bring specific sensory characteristics to the beers [88, 99]. A lot of aromatic descriptors, both positive and negative, have been associated to *Dekkera/Brettanomyces* spp. ferments, including floral, fruity, citrus, spicy, clove, leather, barnyard, smoky, plastic, mousy, phenolic, medical, and/or "band-aid", which are usually called "Brett flavor" [100]. Some common beer styles in which those species can contribute to with their specific flavors include Lambic, Gueuze, and American Kettle sour (marked by sour character); Kriek, Berliner Weisse, and Brett IPA (fruity character); Saison Farmhouse ale, Belgian Trappist ale, and Old English ale (phenolic character); and Cask Brett barley wine, Old stout, and Flanders Red ale (woody character) [99].

Regarding the production of volatile phenolic compounds, the interest in some *Dekkera/Brettanomyces* spp. for use in craft and specialty beers also relies on their ability to produce ethyl phenols [99]. Their syntheses are related to hydroxycinnamic acid (ferulic, p-coumaric, and caffeic acids derived from cereal grains used for mashing) metabolization, in which two consecutive reactions catalyzed by specific enzymes are involved, phenylacrylic acid decarboxylase and vinylphenol reductase, the last one being exclusive to *Dekkera/Brettanomyces* [100, 101]. Some strains of *D/B anomala* and *D/B bruxellensis* have showed efficient conversion of ferulic acid into 4-ethyl guaiacol, making them suitable for beers in which spicy, clove-like and vanilla flavors are desired [102]. Despite the ability to metabolize hydroxycinnamic acids is strain specific, many strains suitable for brewing prefer to consume ferulic acid among other hydroxycinnamic acids [103].

Another common property of *Dekkera/Brettanomyces* is its ability to metabolize complex sugars, such as dextrins, which are not assimilable by *Saccharomyces* and account for the main residual sugars in the beer. This metabolization capacity enables its use in the production of super-attenuated and low-calorie beers [100]. These yeasts were also shown to present β-glucosidase activity, a strain dependent feature that enables the hydrolysis of glycosides present in hops, fruits, flowers, and woods, releasing more aromatic molecules, *e.g.* terpenes derived from hops [95, 99]. Furthermore, one *D. bruxellensis* expressing high β-glucosidase activity was shown to be able to produce resveratrol, a molecule with antioxidant and antiaging actions [104]. Future researches could consider such ability for the production of functional beers, which can provide health benefits for consumers.

Besides *Dekkera/Brettanomyces, Torulaspora delbrueckii* (anamorph *Candida colliculosa*) has been pointed out as a feasible agent to enhance beer bioflavor,

displaying a great contribution to produce specialty beers with different flavors and aromas [89, 95, 102, 105]. These microorganisms have shown ability to produce esters and higher alcohol compounds both in pure and mixed fermentations with *Saccharomyces* spp., positively impacting on the overall analytical and sensory profile of beer [106–108]. Its co-inoculation with *S. cerevisiae* seems to raise the concentration of esters, such as both ethyl decanoate and ethyl dodecanoate as well as citronellyl acetate [105], phenyl ethyl acetate, ethyl hexanoate and ethyl octanoate [106], and ethyl acetate and isoamyl acetate [107], which can overall contribute to fruit-like flavors. Increased levels of higher alcohols, mainly amylic and isoamylic alcohol, in *S. cerevisiae/T. delbrueckii* mixed fermentations [107], and 2-phenylethanol and amyl alcohol in some *T. delbrueckii* pure fermentations [108] have also been described. The ability of some strains to augment the concentration of 4-vinyl guaiacol (clove-like descriptor), a desirable flavor in some wheat and blond beer styles, has been stood out in fermentations sequentially inoculated with *S. cerevisiae* [102] and, furthermore, pure culture of some *T. delbrueckii* has already been used to produce Hefeweizen beers (a German wheat beer style), imprinting rose, bubblegum, banana, and clove-like aromas [109]. Also, its ability to convert some monoterpenoids of hops to linalool, terpineol, and geraniol, which can stamp floral and fresh aromas to beers [110], has been reported.

Pichia kluyveri is another yeast species that may increase the levels of fruity acetate esters in beer [102]. In sequential fermentations with *S. cerevisiae*, a considerable enhancement was observed in the isoamyl acetate (banana-like flavor) concentration in a wheat beer production [111]. Another yeast species, *Wickerhamomyces anomalus*, whose characteristics have been further studied for winemaking, seems to have potential to be used for beer fermentations. Several strains were shown to present a wide range of enzymatic activities, including β-glucosidase activity, and they were shown to be good producers of esters, mainly ethyl acetate, and other fruity acetate esters, such as isoamyl acetate, contributing to improve aromatic complexity of fermented wort, likewise in co- or sequential inoculations with *S. cerevisiae* [112].

Regarding the efficiency in producing lactic acid and ethanol during wort fermentation, and the sensory characteristics, some isolates of the species *Hanseniaspora vineae, Lachancea fermentati, Lachancea thermotolerans, Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus* have been pointed out to be used for the production of sour beers in a single fermentation step, without the need of lactic acid bacteria for souring [113, 114]. This process was called primary souring, and the resulting beers showed both lactic tartness and fruity aromatic and flavor notes [114].

Several strains of nonconventional yeast species have been demonstrated to be useful in producing beers with reduced ethanol levels, besides contributing on improved sensory profile. The inability to consume the main brewery wort fermentable sugars, maltose and maltotriose, makes them less efficient at producing ethanol, enabling the production of low-alcohol (0.5–1.2% v/v) and alcohol-free (<0.5% v/v) beers [89, 115]. Pure cultures of several strains of *T. delbrueckii* were shown to ferment beers with an ethanol content varying from 0.9 to 2.6% (v/v) and characterized by rich fruity flavors [106, 108]. In the same way, strains of the species *Saccharomycodes ludwigii* stand out as suitable candidates, producing beers containing low alcohol concentration and higher amount of esters, besides lower diacetyl levels, contributing to mask the wort-like flavor, which is commonly identified in these kind of beers [115, 116]. Moreover, this yeast is already being used commercially to produce alcohol-free beers with increased fruity notes [115]. On the other hand, in a study aiming at screening basidiomycetous yeasts of *Mrakia* spp. for low alcohol beers, one strain of *M. gelida,* a psychrophilic one, was revealed to produce a

more aromatic beer, judged to be fruitier with apricot, grape, and litchi descriptors, when compared with that produced by a commercial starter of *S. ludwigii* [117].

Other yeast strains of the species *Pichia kluyveri,* able to consume only glucose of brewing wort, were also shown to produce low-alcohol and alcohol-free beers with enhanced amounts of ester compounds and with a flavor profile similar to commercial standard beers of around 4% alcohol (v/v) [118]. One strain of *Williopsis saturnus* var. *mrakii* was demonstrated to have application in fermenting extra-fruity low-alcohol beers, due to the ability to increase the levels of acetate esters and to retain the terpenes and terpenoids of hopped wort, positively stamping fruity and floral flavors, besides preserving the hop aromas [119]. *Candida shehatae, C. tropicalis*, and *Zygosaccharomyces rouxii* are other species that have already been considered for the purpose of producing beers with low or no alcohol [89, 115].

The use of nonconventional yeasts in the brewing processes, especially considering the growth in the craft beer sector, stands out as natural and innovative choices to improve and bring differentiation to the beers. The potential of different yeast species to enrich and diversify the flavors and aromas, bringing sensory complexity to the beverages, recently comes out and should be more explored and studied as these abilities can result in peculiar beers, with unique features.

2.3 Coffee and cocoa fermentation

For satisfactory conduction of industrial fermentative processes, yeasts are applied in order to (I) reduce fermentation time, (II) generate end-products with high productivity, and (III) standardize processes. Concerning coffee and cocoa, yeast fermentation has not been well established. Recent studies pointed out that yeast fermentation can control cocoa and coffee fermentations and produce beans with improved quality. However, to reach a uniform level of high quality, similar to those obtained by fermentation processes of wines, beers, dairy products, and meat, the prerequisite is to know the different yeast groups that actively participate in these fermentation processes to draw a correlation of the final quality. The focus of this section is to describe the main nonconventional yeasts associated with the fermentation of coffee and cocoa beans and their role for each process.

During fermentation, depulped coffee beans are exposed to a diversity of microorganisms, such as yeasts, filamentous fungi, and bacteria that find favorable conditions for their development. The microbial activity generates a range of metabolites that can influence the final quality of the beverage [120, 121]. Yeasts are considered to be important due to pectin degradation and formation of flavor metabolites such as ethanol, organic acids, and esters. Due to these characteristics, many yeasts have been used as starter cultures for cocoa and coffee [122–124]. Different studies have demonstrated a high diversity of nonconventional yeasts during the coffee processing stage, including *Pichia kluyveri, P. anomala, P. guilliermondii, Kloeckera apiculata, Hanseniaspora uvarum, S. marxianus* (*Kluyveromyces marxianus*), *S. bayanus, Debaryomyces hansenii, D. polymorphus, Torulaspora delbrueckii, Torulopsis famata, Candida guilliermondii, C. parapsilosis, C. pelliculosa, C. famata, C. tropicalis, C. fermentati, C. membranifaciens, Rhodotorula mucilaginosa, Arxula adeninivorans, Cryptococcus albidus*, *Schizosaccharomyces* sp. and *Kloeckera* sp. [120–122, 125–128].

During coffee processing, the use of pectinolytic microorganisms is made to remove the mucilage layer from the coffee beans. This activity can significantly reduce fermentation time from 80 to 20 hours by enzymatic treatment. Thus, the production of pectinases has been the main attribution of yeasts and bacteria during coffee processing. Nonconventional yeasts, such as *P. anomala, P. kluyveri, P. Caribbean, P. guilliermondii*, and *H. uvarum,* have been associated with the production of pectinases during coffee fermentation [126]. The ability of yeasts

belonging to the species *P. anomala, P. kluyveri*, and *H. uvarum* to produce pectic enzymes suggests that these species may act in the degradation of pectin during fruit fermentation [129].

Yeasts as starter cultures for the fermentation industry have been widespread by the ability to generate uniform and safe products, as well as the ability to modify various constituents related to organoleptic properties and nutritional, chemical, and microbiological characteristics. Most research on starter cultures for coffee fermentation has focused on selection of pectinolytic yeasts. Nonconventional yeasts, such as *P. guilliermondii* and *C. parapsilosis*, have revealed important pectinolytic potential [130]. In addition, yeasts such as *C. parapsilosis* and *S. cerevisiae* are coffee starters due to differentiated aroma production [131].

Researches with nonconventional yeasts in coffee fermentation have revealed the production of different volatile organic compounds. Different yeast species isolated from the dry and semi-dry processes of coffee produce significant amounts of aroma compounds (*e.g.*, acetoin, furfural, butyric acid, 2-phenyl-ethanone, 1,2-propanediol, hexanoic acid, decanoic acid, and nonanoic acid, among others), suggesting the strains *P. guilliermondii* UFLACN731 and *C. parapsilosis* UFLACN448 as promising candidates for coffee fermentation [130].

The importance of fermentation in the contribution of chocolate quality has been recognized for more than 90 years. Several studies and research have been conducted in different countries to determine the species of microorganisms associated with this process. A succession between yeast, lactic acid bacteria, and acetic acid bacteria is generally observed. This succession begins when high concentrations of sugars, low pH, and oxygen tension favor the growth of yeasts that convert the carbohydrates of the pulp into ethanol, dominating the process for approximately 48 hours. Lactic acid bacteria also ferment the sugars and utilize citric acid from the pulp; its growth is favored by the scarcity of oxygen and slight elevations of pH and temperature. With disintegration of the mucilaginous pulp surrounding the cocoa beans and together with the pH and aeration of mass, the citric acid present in the pulp is reduced by action of the yeasts, which favors the growth of acetic bacteria. These bacteria promote the oxidation of ethanol, initially produced by the yeast, to acetic acid in an extremely exothermic reaction, raising the temperature of the fermentative mass to levels of 45–50°C. The high temperature is important to enzymatic reactions, necessary for the development of the aroma and flavor of chocolate. Acetic acid, when penetrating cotyledonous tissues, promotes the death of the bean embryo (48–72 hours), and, together with ethanol, they act synergistically causing the diffusion of polyphenols in cotyledonary tissues. These reactions are important in the generation of well-fermented cocoa beans. Other microbial groups, such as *Bacillus* and filamentous fungi, develop in the final stages of fermentation, but the role of these microorganisms has not yet been fully elucidated [132–135].

To date, cocoa and coffee fermentations have been carried out with wild microorganisms present in the raw material and equipment used. Some studies have been carried out for the selection of cocoa and coffee cultures, where the main objective is to develop a faster fermentation process through the use of microorganisms producing pectinolytic enzymes. A study carried out cocoa fermentation inoculated with *Saccharomyces chevalieri* (now classified as *Saccharomyces cerevisiae*), *Candida zeylanoides*, and *Kluyveromyces fragilis* (now classified as *Kluyveromyces marxianus*) [136]. These yeasts were selected as being part of the natural flora of the cocoa fermentation environment and for producing pectinolytic enzymes. The fermentations inoculated with these yeasts were faster and produced chocolates with similar sensory characteristics in relation to spontaneously conducted fermentations [136].

The use of aromatic nonconventional yeasts, *P. kluyveri* and *K. marxianus,* during the cocoa fermentation process is able to alter the flavor profile of the chocolates

compared to a spontaneously fermented control. The chocolates obtained after being submitted to this fermentation process can obtain higher grades in relation to the fruity flavor, cocoa aroma, and general taste. Some patents have been developed with the objective of establishing methods to optimize the cocoa fermentation process [137]. A method consisted of the addition of aromatic substances during the cocoa fermentation process with the aim of obtaining modified cocoa almonds. These substances comprise fruit pulp, aromatic leaves, and wood parts, among others [138]. *Pichia kluyveri* strain is a starter culture during cocoa fermentation adjusting the contents of isobutyl acetate and isoamyl acetate in obtained cocoa nibs. In addition, *P. kluyveri* demonstrated the ability to liquefy the cocoa pulp completely, due to its high pectinolytic activity [139].

2.4 Plant growth promotion

Throughout this chapter, the biotechnological potential of yeasts from other genera than *Saccharomyces* for activities and products commonly performed and generated by *Saccharomyces* spp. was discussed and exhibited. These activities are mainly associated with the industrial utilization of yeasts for the production of food, bioethanol, and alcoholic beverages through the fermentative metabolism. Nonconventional yeasts were initially shown to be able to generate high levels of a compound conventionally produced by *S. cerevisiae* (ethanol), but fermenting an alternative sugar (xylose) whose *Saccharomyces* spp. are naturally unable attracts interest in the bioenergy industry. Many of these strains are also able to generate a compound of industrial interest that *Saccharomyces* spp. cannot produce, *i.e.*, xylitol. Then, several strains of nonconventional yeasts able to replace *Saccharomyces* spp. in the industry of beer, coffee and chocolate were presented, which can improve aroma and flavors of these products. Yeasts are being unintentionally used by mankind for such purposes since the primitive beginning of microbial biotechnology. Therefore, food and bioenergy are the traditional fields of application of these unicellular fungi. However, the application of nonconventional yeasts is beyond the fields where *Saccharomyces* spp. are traditionally used. In this section, the potential application of nonconventional yeasts in a nonconventional field: agricultural biotechnology will be shown.

Many strains of nonconventional yeast species have been demonstrated as plant growth-promoting (PGP) microorganisms. PGP microorganisms live in the soil surrounding plant roots (rhizosphere), inside plant roots, stems and leaves (endophytes), or externally attached to plant surfaces (epiphytes) [140, 141]. The set of microbes inhabiting these many plant compartments is organized in microbial communities comprising the plant microbiome [140, 141]. The rhizosphere harbors a microbiome with higher diversity, abundance, and activity than the other plant compartments and is enriched in PGP microbes [142]. PGP microbes perform many activities that support plant growth and health, helping plants against biotic and abiotic stressors, besides directly acting in plant nutrition and growth regulation [142].

Inoculation of PGP microbes has the potential to replace the agrochemicals normally used in cropping systems, which can be expensive and environmentally harmful, and from nonrenewable sources [143]. The studies and applications of plant growth-promoting yeasts have lagged behind those of bacteria and other fungi [144, 145]. However, some studies showing beneficial activities of yeasts for plant productivity indicate that they can be as important as other microbes in agricultural biotechnology [146–148]. Some PGP features, as well as the main nonconventional yeast species able to perform these activities are discussed below.

Among the PGP activities performed by yeasts, biosynthesis of indole-3-acetic acid (IAA) is the most reported in the literature, found in many distinct yeast species and genera [144, 145, 147, 149–155]. IAA is a plant hormone of the auxin class, associated

with phytostimulation by increasing root growth [156, 157]. In the *Rhodotorula* genus, two species were identified as able to produce IAA in many studies. *R. graminis* is a yeast species isolated from the internal tissues of poplar tree and able to produce high amounts of IAA [149]. This capacity results in the growth promotion of distinct plants, such as poplar itself, as well as pepper and maize [150–152]. A genome survey did not detect the genes involved in the conventional pathways of IAA biosynthesis and suggested putative genes for alternative pathways in this species [154]. Strains of the species *R. mucilaginosa*, isolated from poplar stems or from soils cropped with legumes, were also found as high IAA producers [149, 155]. Experiments using this species indicated its growth promotion activity in poplar and tomato [150–152].

Candida tropicalis is another important yeast species isolated from the rhizosphere of rice and maize, whose ability to synthesize IAA besides other PGP activities was shown to promote growth of these mentioned crops [145, 153]. Other examples of IAA biosynthesizing yeast species are *Aureobasidium pullulans*, *Cryptococcus flavus*, *Hannaella sinensis*, *Rhodosporidium paludigenum*, *Torulaspora globosa*, and *Williopsis saturnus* [144, 147, 155].

Another important PGP activity performed by some yeast species is phosphate solubilization and interaction with mycorrhizal fungi able to mobilize phosphate to plants [153, 158, 159]. Phosphorus (P) is a macroelement required by plants in high levels. Plants cropped in soils with low availability of phosphate are highly benefited from the activity of P-solubilizing microbes for their nutrition. These PGP microbes produce organic acids that release the phosphate attached to mineral surfaces, making this nutrient available for root absorption [160].

The yeast species *Yarrowia lipolytica*, *Torulaspora globosa*, and *Candida tropicalis* were shown as able to solubilize inorganic phosphate in *in vitro* tests [147, 153, 160]. Some strains of the species *Candida railenensis* and *Cryptococcus flavus* were not able to directly solubilize phosphate, but exhibited a microbial interaction with arbuscular mycorrhizal fungi resulting in higher P-solubilization and promotion of maize growth [159]. On the other hand, another study observed that yeast strains from the genera *Rhodotorula* and *Cryptococcus* were able to both interact with the mycorrhizal fungus *Glomus mosseae* and solubilize phosphate [158].

The agricultural productivity of many crops is highly impaired by plant diseases, mainly caused by fungal pathogens. Use of PGP microbes that perform biocontrol of these fungi is a promising alternative to the use of expensive and bioaccumulative fungicides [143]. The capacity of pathogen biocontrol is present in many nonconventional yeast species that perform different types of antagonism, including competition for space and nutrients, antibiosis, fungal cell wall degradation, mycoparasitism and induction of host resistance [146].

Strains of the species *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from the sugar beet rhizosphere, were shown to control the fungus *Rhizoctonia solani* that causes root damping-off, promoting growth and health of this plant [161]. Another strain of *R. glutinis* was isolated from the inner tissues of the apple fruit and was able to promote growth of apple tree by controlling the pathogen *Botrytis cinerea* [162]. The species of the genus *Rhodotorula* commonly produce a siderophore called rhodoturulic acid, which inhibits *B. cinerea* spore germination. Utilization of *R. glutinis* in combination with the application of rhodoturulic acid resulted in a more efficient control of *B. cinerea* [162]. Other yeast species like *Hannaella sinensis* and *Rhodosporidium paludigenum* produce different siderophores, but the analyzed strains of these species showed no antifungal activity [147].

An epiphyte yeast strain of the species *Torulaspora globosa* isolated from rice leaf surface is antagonistic to many fungi causing plant diseases, including *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani* [147]. In addition to efficient biocontrol, this strain synthesizes IAA, indicating its high potential to be used

as an agricultural inoculant [147]. Strains of the species *Aureobasidium pullulans* and *Rhodotorula mucilaginosa*, isolated from soils cropped with legumes, showed antagonism to the fungi *Phytophthora infestans* and *Fusarium graminearum*, respectively [155].

In addition to the three mentioned PGP activities previously shown, *i.e.* IAA biosynthesis, P-solubilization and biocontrol, many others are present in nonconventional yeasts. For example, yeasts can help plants against several types of stress. The compound 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor in ethylene biosynthesis. High ethylene levels are induced in plants facing many types of stress [163]. PGP microbes that produce the enzyme ACC deaminase decrease the levels of ethylene, alleviating plant stress and indirectly promoting their growth [163]. Despite this activity is more present in bacteria, the yeast species *Candida tropicalis*, also capable of P-solubilization and IAA biosynthesis, was shown to produce ACC deaminase, which possibly contributed to rice growth promotion in pot experiments [145]. The biotechnological potential of the strain *C. tropicalis* HY was validated with the inclusion of this strain in the commercial biofertilizer product BioGro, which improves paddy rice yield [145].

Other type of common stress in plants is the oxidative. Reactive oxygen species such as hydrogen peroxide (H_2O_2) can damage plant tissues. The accumulation of these compounds can be mitigated by the activity of the enzyme catalase. Although this PGP function is also more observed in bacteria, some yeasts that use methanol as carbon and energy sources contains catalases, since H_2O_2 is a by-product of that metabolism [147]. Strains of *Cryptococcus flavus*, *Hannaella sinensis*, *Rhodosporidium paludigenum*, and *Torulaspora globosa* produce catalase and thus can potentially help plants against the oxidative stress, in addition to the PGP activities already described for these species [147].

The biotechnological utilization of PGP microbes in agriculture is in its infancy, since much knowledge and technology must be developed in order to efficiently replace agrochemicals. Nonconventional yeasts were shown to perform many beneficial activities to plants and need to be explored more to increase their utilization together with PGP bacteria and filamentous fungi for a more sustainable agriculture.

Conflict of interest

The authors declare that they have no competing interests.

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