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Non-Conventional Yeasts in Fermentation Processes: Potentialities and Limitations

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Abstract

Traditionally the term 'yeast' means *Saccharomyces cerevisiae* and its close relatives. This yeast is used in traditional fermentation processes, mainly for ethanol formation, baking, winemaking and beer production. The classical carbon substrates for typical yeast processes are glucose or sucrose, however, the successful expansion of industrial biotechnology drives research toward the utilization of alternative carbon sources. New technologies require very specific challenges and differ from those found in conventional fermentation processes. Most microbial habitats, especially in modern biotechnological processes, do not provide culture media rich in mono- and disaccharides. They include fermentation environments with various compositions of carbon and energy sources as well as the presence of various cytotoxic compounds which inhibit the growth of industrial yeasts. About 1500 various yeast species have been identified nowadays. Microbiologists and biotechnologists have named all non-*S. cerevisiae* yeasts as 'non-conventional' yeasts. Their features present a potential that can be used for non-conventional processes. Non-*Saccharomyces* strains provide alternative metabolic routes for substrate utilization and product formation. The diversity of these yeasts includes many species possessing useful, and sometimes uncommon, metabolic features potentially interesting for biotechnology. The selected strains of non-conventional yeasts could be used as pure or mixed cultures for improving industrial fermentations.

Keywords: non-*Saccharomyces*, yeasts, fermentation, stress resistance

1. Introduction

Yeasts belong to the most studied microorganisms. More than 1500 species of yeast have been described so far [1]. Many of them have been used in various fermentation processes [2].

Taxonomic analysis of the microflora in active spontaneous fermentations revealed variety of yeasts, but still the predominant genera is *Saccharomyces* [3, 4]. This yeast has become the model organism for research studies and valuable results for numerous eukaryotic cells have been obtained [5]. *S. cerevisiae* was also the first species whose genome was sequenced [6]. The ability of *S. cerevisiae* to conduct metabolic processes under both aerobic and anaerobic conditions, and ethanol production meant that this species has been used for many years as starter cultures for production of bread and numerous fermented beverages [7]. This yeast has also been used in the biofuel industry and for the production of heterologous proteins, human insulin, hepatitis and human papillomavirus vaccines [8].

However, new technological processes, for example, production of second-generation bioethanol, are different from those encountered in conventional fermentation processes. These new technologies pose special challenges. They include fermentations in various environments, with wide spectrum of carbon and energy sources, as well as with significant content of numerous cytotoxic compounds that may inhibit the growth of industrial microorganisms [9, 10]. Strong pressure to improve the economic viability of bioethanol production from waste plant materials makes strains of *S. cerevisiae* rather ineffective in fermentation processes with lignocellulosic hydrolysates. This fact stimulates research to use other non-*Saccharomyces* strains that exhibit broad spectrum of assimilated carbon compounds and higher resistance to cytotoxic compounds.

2. Ethanol production

Ethanol production on the industrial scale has been carried out in the conventional manner using mesophilic strains of *Saccharomyces* spp. The commonly used carbon sources are molasses, beet juice, beet sugar, corn or potato starch. However, these raw materials are expensive and their availability is usually dependent on seasonal productivity. Additionally, the use of plant food such as corn and potatoes in biofuel production is morally and socially controversial. Therefore, diversified actions have been taken to convert a variety of agricultural and forestry wastes, rich in lignocellulosic sugars, into biofuels (**Table 1**).

Biomass	Ethanol yield (litres per dry metric ton)
Hardwood	350
Softwood	420
Corn stover	275–300
Wheat straw	250–300
Sugarcane bagasse	314
Municipal solid waste	170–486

Table 1. Ethanol yields from selected waste biomass [11].

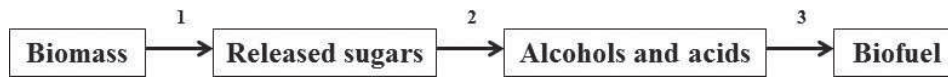


Figure 1. Flow diagram of biofuel production from plant biomass. (1) Pretreatment, (2) fermentation and (3) separation and purification.

According to the Directives 2008/98/EC and Regulation (EU) No 1357/2014, by-products generated can be used directly, without further processing, but wastes may be subjected to recovery, disposed of or liquidated. Proper management of waste biomass is an important issue for environmental protection. However, the use of production residues not only minimizes the negative impact on the environment, but it is also possible to get additional economic benefits [12]. Organic waste from the agroindustry and forestry, according to their physicochemical properties, can be used for the production of bioethanol, butanol, acetone and new chemical building blocks for advanced materials [13, 14] (**Figure 1**).

3. Starch and lignocellulosic biomass

Starch is the carbohydrate accumulated in plants, made up of long chains of glucose units joined by α -1,4 linkages and joined at branch points by α -1,6 bonds. Many microorganisms, including *S. cerevisiae*, are not able to degrade starch since they do not produce starch decomposing enzymes such as α -amylase, β -amylase, pullulanase, isoamylase and glucoamylase. To simplify the fermentation process by eliminating the separate saccharification step, numerous genetically engineered *S. cerevisiae* strains capable of secreting glucoamylase or α -amylase were constructed. However, starch decomposition abilities presented by these yeast strains are usually unsatisfactory because of the limited amounts of secreted amylolytic enzymes [15].

Lignocellulose is the most abundant renewable biomass on earth. It is composed mainly of cellulose, hemicellulose and lignin. Both the cellulose and hemicellulose fractions are polymers of sugars and thereby a potential source of fermentable carbon sources. Hence the interest in research on procedures for chemical degradation of the lignocellulosic structure and for maximization of its decomposition into glucose, xylose and phenolic compounds. The resulting carbon substances can then be assimilated by yeast, which considerably increases the efficiency of biodegradation [16, 17].

Different pretreatment technologies published in public literature are described in terms of the involved mechanisms, advantages versus disadvantages, and economic calculation. Pretreatment technologies for lignocellulosic biomass include biological, mechanical or chemical methods, and their various combinations in particular. It is not possible to define the best pretreatment method because it depends on the type of lignocellulosic biomass and desired products. The acidic (H_2SO_4) or alkali (NaOH) hydrolysis, oxidation techniques (H_2O_2), heat and enzymatic (cellulases, cellobiase and xylanase) treatments are the most frequently used

for this purpose [18, 19]. However, each of these methods leads to release of various decomposition products. When fermentable sugars are produced, special attention must be paid to the formation of fermentation inhibitors. Especially the formation of phenolic compounds from lignin degradation, as well as the formation of furfural and 5-(hydroxymethyl)-2-furfural from sugar degradation should be limited by keeping the process parameters: temperature and time as low and as short as possible. Therefore, the choice of the appropriate pretreatment method of plant biomass hydrolysis is the crucial step for effectiveness of fermentation processes [20].

4. *S. cerevisiae* or non-conventional yeasts?

All yeasts are capable of assimilating glucose, almost all such as fructose and mannose, while galactose can also be assimilated by many species. Among the disaccharides, sucrose is the most commonly used. However, in ethanol production, second generation classical yeast *Saccharomyces* spp. are not useful because they are not able to ferment pentoses, exhibit low tolerance to alcohols, acids and solvents. Additionally, they are characterized by high sensitivity to pH changes and cytotoxic compounds: furfural, 5-(hydroxymethyl)-2-furfural and other organic compounds produced during hydrolysis. Limitations of *S. cerevisiae* make the course of new industrial fermentation processes very difficult.

The fuel ethanol production from lignocellulosic materials requires co-fermentation of both hexoses and pentoses, mainly D-xylose and L-arabinose. *S. cerevisiae* cannot utilize pentoses because of the lack of specific metabolic pathways and transport systems. Genomic resources from a variety of microorganisms as well as biological systems combined with mutagenesis have been used to engineer yeast with pentose fermentation abilities [21]. By expressing heterologous D-xylose or L-arabinose pathways, *S. cerevisiae* could obtain the metabolic capacity but this efficiency still needs to be improved [22, 23].

The main strategies for constructing D-xylose-utilizing *S. cerevisiae* include two paths. The first one is XR-XDH pathway, containing D-xylose reductase (XR) and xylitol dehydrogenase (XDH), and converts D-xylose to xylulose. Due to the cofactor imbalance in this pathway, the accumulation of byproduct is the main problem, which needs to be solved. Another one is XI pathway, which only needs to introduce one D-xylose isomerase (XI) that directly converts D-xylose to xylulose. However, the activity of XI still needs to be increased. The xylulose from both pathways could be phosphorylated to xylulose-5-P by endogenous xylulokinase. Subsequently xylulose-5-P can be further entered into the endogenous pentose phosphate pathway (PPP) to produce ethanol [23].

There are also two main L-arabinose metabolic pathways which are both candidates for constructing L-arabinose-metabolic yeasts. L-Arabinose could be converted to D-xylulose-5-phosphate that then enters into PPP. This pathway needs five important enzymes, including aldose reductase, L-arabinitol-4-dehydrogenase, L-xylulose reductase, D-xylulose reductase and xylulokinase. In addition, this pathway contained two reduction reactions which utilize NADPH, two oxidation reactions which generate NADH, and a kinase reaction [23].

Therefore, the construction of stable *S. cerevisiae* strains able to ferment xylose and/or arabinose is not easy. The co-utilization of D-xylose and L-arabinose was obtained in engineered *S. cerevisiae* strain with a high ethanol yield 0.43 g/g of total sugar [24]. Also selected strains of other yeast belonging to *Pichia stipitis* were shown to ferment hydrolysates with ethanol yields of 0.45 g/g of sugar, so commercialization seems feasible for some applications [25].

An additional problem for the simultaneous consumption of pentoses and hexoses is the inhibition of pentose uptake by D-glucose. Researchers have engineered xylose metabolism in *S. cerevisiae* by over-expressing genes for aldose (xylose) reductase, xylitol dehydrogenase and moderate levels of xylulokinase-enabled xylose assimilation and fermentation. The results obtained by Subtil and Boles suggested that co-fermentation of pentoses in the presence of D-glucose can significantly be improved by the overexpression of pentose transporters, especially if they are not inhibited by D-glucose [26]. However, a balanced proportion of NAD(P) and NAD(P)H must be maintained to avoid xylitol production. It was noted that respiration is critical for growth on xylose by both native and recombinant xylose-fermenting yeasts. Reducing the respiration capacity of xylose-metabolizing yeasts increases ethanol production. In studies conducted by Jeffries and Jin, *S. cerevisiae* was engineered for D-xylose utilization through the heterologous expression of genes for aldose reductase, xylitol dehydrogenase and D-xylulokinase and produced only limited amounts of ethanol in xylose medium. It was observed that levels for glycolytic, fermentative and pentose phosphate enzymes did not influence significantly on glucose or xylose under aeration or oxygen limitation. However, expression of genes encoding the tricarboxylic acid cycle and respiration enzymes increased significantly when cells were cultivated on xylose, and the genes for respiration were even more elevated under oxygen limitation. These results suggest that recombinant *S. cerevisiae* does not recognize xylose as a fermentable carbon source. However, the petite respiration-deficient engineered strain produced more ethanol and accumulated less xylitol from xylose [25, 27].

The results obtained by Wang et al. for co-utilization of D-glucose, D-xylose and L-arabinose in engineered *S. cerevisiae* showed that the pentose metabolic capacity is prominently lower than that of D-glucose due to D-glucose-inhibition effect. To alleviate the phenomenon, the pentose metabolic flux can be improved and a pentose specific transporter without inhibition by D-glucose might also be needed [23].

The progress in fermentation of pentose sugars has gone on slow pace as there are few microorganisms known, which are capable of pentose metabolism. While numerous metabolic engineering strategies have been developed in laboratory yeast strains, only a few approaches have been realized in industrial strains. Ethanol yields of more than 0.4 g of ethanol/g of sugar have been achieved with several xylose-fermenting industrial strains with the heterologous xylose utilization pathway consisting of xylose reductase and xylitol dehydrogenase, which demonstrates the potential of pentose fermentation in lignocellulosic ethanol production [28]. In the future, desired perspective is to find organisms that would be able to ferment high density hydrolysates without purification. The genetic and metabolic engineering routes also should be continued. Also a direct or a sequential fermentation system using mixed populations of yeasts needs to be worked out [29].

The interest of microbiologists has been also directed to the use of yeasts belonging to other genera than genus *Saccharomyces* or *Schizosaccharomyces*, commonly called 'non-conventional' yeasts. Due to the information collected on abilities of some of these yeasts, as well as their applications in many fields, their 'unconventional' status may change in the future. Some of the 'non-conventional' yeasts of today will be the 'conventional' yeasts of tomorrow [30]. The similar thesis was given by Sibirny and Scheffers [31]. They highlighted that, since an increasing number of non-conventional yeasts and increasing importance in both fundamental and applied sciences, the term 'non-conventional' is gradually losing significance and usefulness.

There is an enormous biodiversity of non-conventional yeasts. Currently 1500 species have been described although this is only thought to be 1% of yeast that may exist on Earth. These yeasts are phylogenetically diverse and thus may probably harbor industrially relevant traits to augment the currently used *S. cerevisiae*. In addition, due to the carbon substrates utilization range, as well as a poor stress tolerance drawback, there is need to search for novel traits in other yeasts. Therefore, biodiversity is an alternative approach to genetically improved yeasts [32]. Due to the progress in identification and characteristic of a new species found in nature, it is possible to increase the diversity and number of yeasts used in industrial purposes. It is indisputable that the exploration for new species will lead to additional novel technologies, including fermentation of pentoses to ethanol.

A lot of genera different than *Saccharomyces* may also be interesting for their use in specific technological applications. In fact, some species have already attracted researchers in the last years on different aspects: *Kluyveromyces lactis* as a possible utilizer of the residual whey in dairy industries; some methylotrophic yeasts for the production of heterologous proteins; *Yarrowia lipolytica* for its ability to grow on particular substrates and its high protein excretion capacity. As it was mentioned above, transport of carbohydrates into cells is the very important step in yeast metabolism, except in those cases in which di- or trisaccharides are hydrolyzed outside the cell. Transport of monosaccharides such as glucose, fructose or mannose in *S. cerevisiae* is a facilitated diffusion process; however, the situation may be different in other yeasts. For example, in *K. lactis* glucose transport appears to proceed by facilitated diffusion. In *Candida utilis*, the popular 'fodder yeast', glucose appears to be transported by a proton symport when the organism is grown at low glucose concentration [33].

The non-conventional yeasts may overcome many problems related with narrow spectrum of carbon sources assimilation presented by conventional *S. cerevisiae* [15]. Some non-conventional yeasts show many uncommon, metabolic features potentially interesting to biotechnology. Non-conventional yeasts represent the vast majority of genera and species so far described. Several yeast species are diverged by evolution from *S. cerevisiae* and possess several unique genes and growth characteristics to withstand different stress conditions [34]. These exceptional strains are able to utilize various sources of carbon such as starch, cellulose, raffinose, arabinose, xylose and sugar alcohols (xylitol, sorbitol, mannitol, etc.) [8, 35].

At least 22 yeast strains have been shown to produce some ethanol from D-xylose. However, only six strains such as *Brettanomyces naardenensis*, *C. shehatae*, *C. tenuis*, *Pachysolen tannophilus*, *P. segobiensis* and *P. stipitis* are able to produce significant amounts of ethanol, and of these, only three: *C. shehatae*, *P. tannophilus* and *P. stipitis* have been studied extensively [36, 37].

The production systems exploiting some non-*Saccharomyces* yeasts have one important advantage—they are not pathogenic organisms received the 'generally recognized as safe' (GRAS) designation from the Food and Drug Administration (FDA) [38–40].

The non-conventional yeast systems may have several beneficial traits like ethanol tolerance, thermotolerance, inhibitor tolerance, genetic diversity, etc. However, not all non-conventional yeasts possess these important characteristics. Currently, studies on non-conventional yeasts concern limited number of species like *Hansenula polymorpha*, *K. lactis*, *P. pastoris* and *Y. lipolytica* [21, 40]. However, more non-conventional yeasts are worth the special attention. For example, amylases from non-conventional yeasts were found to have the ability to hydrolyze starch. These interesting enzymes are α -amylase and glucoamylase from *Debaryomyces (Schwanniomycetes) castelli*, glucoamylase from *Saccharomycopsis fibuligera* and *C. antarctica*, α -amylase from *Cryptococcus* sp., *C. lusitaniae*, *C. famata*, amylopullulanase from *Clavispora lusitaniae* and pullulanase from *Aureobasidium pullulans*. There is a number of reviews on bacterial and fungal amylases and their applications. They clearly indicate that α -amylases and pullulanase from yeasts are one of the most popular and important forms of industrial amylases. Non-conventional yeasts were studied as both free and immobilized cells for production of amylolytic enzymes [41–44] (**Photo 1**).

Fermentation trials with immobilized conventional *S. cerevisiae* and non-conventional cells *D. occidentalis* showed that both tested yeasts are able to adapt to the specific conditions inside carrier materials. Nevertheless, the mechanical endurance of alginate carriers, commonly used in yeast immobilization, shows better applications in industrial fermentation especially with non-conventional yeasts. In the case of fermentative yeast, *S. cerevisiae* alginate beads may be destroyed, as a result of intense CO₂ formation [44]. Furthermore, *Debaryomyces* spp. ability to tolerate and decompose both phenols and polyphenols at concentrations that are highly toxic to bacteria and other yeast species, demonstrated that these yeasts may be an attractive system for biofuel production from renewable starch sources [15]. It is worth to note that methylotrophic yeasts belonging to *Hansenula*, *Candida*, *Pichia* and *Torulopsis* genera are able to metabolize monocarbonic compounds like methanol and formaldehyde [45]. As

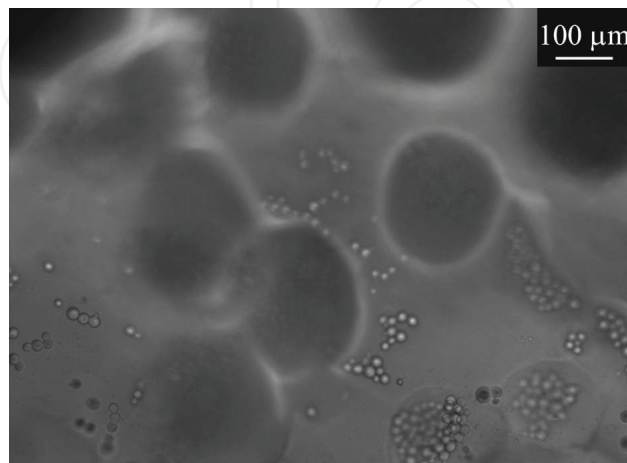


Photo 1. Amylolytic non-conventional yeast *D. occidentalis* immobilized in alginate (author: Dorota Kregiel).

a result, the use of non-conventional yeasts allows for the utilization of various waste plant biomass, and—which is worth emphasizing—to receive the post-fermentation yeast biomass rich in protein and amino acids [39, 46, 47].

Non-conventional yeasts are large but not fully known, diverse group of microorganisms. Yeast species other than *Saccharomyces* spp., in addition to the previously mentioned ability to use the complex substrates, exhibit features particularly important in the industrial processes—thermotolerance and tolerance to the presence of chemical inhibitors. The majority of non-conventional yeasts have been isolated and characterized as microflora of spoiled food and beverages [48, 49]. It can be assumed that some non-conventional yeasts have developed specific mechanisms to survive in various natural environmental conditions. Therefore, it is believed that most species of non-conventional yeasts have acquired specific mechanisms that are not included in the classical model yeast *S. cerevisiae* [49–51]. It is worth to explore the molecular basis of their tolerance to numerous environmental stress such as increased osmotic pressure, high concentrations of ethanol, high temperature or the presence of toxic compounds.

The eukaryotic microorganism most studied for its tolerance is still *S. cerevisiae*. However, this yeast is rather sensitive and not able to adapt to ‘non-regular’ conditions. For example, some species like *D. hansenii* or *Hortaea werneckii* have been isolated from natural hypersaline environments. Therefore, these non-conventional yeasts are more suitable model organisms to study halotolerance in eukaryotes than *S. cerevisiae* [52]. It should be also noted that non-conventional yeasts show a high growth rate in fermentation processes, and are capable of producing important enzymes. For example, *D. occidentalis* is capable of secretion of α -amylase and glucoamylase, and *K. marxianus* of producing intracellular lactase, intra and extracellular pectinase and intra and extracellular inulinase [42, 44, 53–55]. These examples show the important potential of non-conventional yeasts that can be utilized in use of various waste materials.

5. Osmotolerance

Yeast cells are exposed to osmotic stress during industrial fermentations. The processes carried out in media with significant levels of saccharides above 300 g/L, need osmotolerant yeasts in particular [56–58]. Accordingly, there is a growing interest among microbiologists and technicians to obtain yeast strains that are able to grow in environments with high concentrations of salts or saccharides. The molecular mechanisms for responsibility of osmotolerant *S. cerevisiae* strains have been described extensively in available literature [59, 60]. *S. cerevisiae* remains the model organism to study the molecular basis of important physiological features, but researchers have isolated and identified non-conventional osmotolerant yeasts belonging to *Zygosaccharomyces rouxii* [48, 61].

Z. rouxii is known for its high tolerance to osmotic stress, which is thought to be caused by sets of specific genes. Important differences were found for salt tolerance and assimilation of glycerol in comparison to *S. cerevisiae*. *Zygosaccharomyces* strains show a higher resistance to salts, higher glycerol production and are able to assimilate glycerol. Under conditions of osmotic stress, the glycerol production in *Z. rouxii* strains may be much lower

than in *S. cerevisiae*, which suggests the presence of a system that efficiently retains glycerol inside *Z. rouxii* cells [48, 61, 62].

D. hansenii is also one of the most halotolerant species. This yeast was isolated from saline environments sea water, concentrated brines and salty food. It can grow in media containing as high as 4 M NaCl, while the growth of *S. cerevisiae* is limited in media with more than 1.7 M NaCl [63].

The adaptation of yeast cells to osmotic stress is a complex mechanism that combines network regulatory genes and signaling pathways that may vary depending on the species and osmotic agent in the surrounding environment [64]. Generally, the behavior of *Z. rouxii* cells resembles the activity of *S. cerevisiae* in the transport of Na⁺ ions from yeast cell, while halotolerant yeast *D. hansenii* accumulates sodium ions inside its cells.

The results obtained by Gonzalez-Hernandez et al. confirmed that *D. hansenii* grows better in the presence of moderate concentrations (0.6 M) of NaCl and KCl than in the absence or at higher salt concentration. Therefore *D. hansenii* can be considered moderate halophile yeast [65]. This ability is associated with the accumulation of high concentrations of K⁺ or Na⁺. For this reason *D. hansenii* has been called a 'sodium-includer' [66, 67]. The mechanism of the adaptation is probably an intrinsic resistance to the toxic effects of cations, not observed in other yeasts, particularly *S. cerevisiae* [65]. The problems, how yeasts regulate the intracellular ion concentration, and how ions are tolerated by enzymes promoting survival, remains controversial [67, 68]. In *D. hansenii*, the vacuolar concentration of Na⁺ was described to be equal to the one of cytoplasm, while in *S. cerevisiae* the differences between these concentrations were described [67].

Z. rouxii integrates general and osmoticum-specific adaptive responses under sugar and salts stresses, including regulation of Na⁺ and K⁺-fluxes across the plasma membrane, modulation of cell wall properties, compatible osmolyte production and accumulation and stress signaling pathways [69, 70]. According to Leandro et al., *Z. rouxii* is capable of growing in osmolarity of 3 M NaCl and glucose concentrations of 90%, due to the presence of unique transporters in plasma membrane which is higher than *S. cerevisiae* [62]. Dakal et al. described internal reactions that occur in yeast cells under different osmotic agents. They suggested that sugars and polyols modify the osmotic pressure, while salts induce changes in both osmotic pressure and ionic homeostasis [70].

According to Pribylova et al., the less osmotolerant yeasts strain possesses a more rigid cell wall than the more osmotolerant ones. They suggested that the differences in the osmotolerance are related to resistance to the lysing enzymes—lyticase and zymolyase, cell-wall polymer content and cell wall micromorphology [69].

Availability of genome sequence of osmotolerant and halotolerant strains may open up new perspectives in this direction [71].

6. Thermotolerance

Thermotolerance of yeast cells is a highly desirable feature for fermentation processes. Efficient process for bioethanol production from lignocellulosic substrates requires relatively high temperatures

(~50°C) for conducting the enzymatic hydrolysis of biomass before fermentation [72]. Moreover, fermentations carried out at high temperatures significantly reduce the costs of cooling, as well as the risk of microbial contamination [73]. A limited temperature tolerance in yeast *S. cerevisiae*, with the optimal range of 25–37°C, increases the overall cost of ethanol production [74, 75]. Therefore, in order to achieve efficient fermentation at high temperatures, thermotolerant microorganisms may be used. These strains are not only able to survive, but also to produce ethanol efficiently [75, 76]. Non-conventional strains of *K. marxianus* show ability to ferment carbon sources at the temperature of 45°C. Thermotolerance, a broad enzymatic activity and fermentation ability in high concentration of saccharides makes the yeast *K. marxianus* a good material to conduct various fermentation processes [77]. Also other non-conventional yeast species-like *Ogataea polymorpha* (syn. *H. polymorpha*) have been found to ferment xylose at 45°C [78].

Yeast thermotolerance is the result of many factors, including trehalose, heat shock proteins, ATPase, the ubiquitin-proteasome pathway, gene expression responses and heat-induced antioxidant defenses [79]. Some processes may be specific to basal thermotolerance, others may be induced during acquired thermotolerance, and many may be involved in both. High temperatures are known to affect membrane-linked processes due to alterations in membrane fluidity and permeability. Enzymes are also sensitive to higher temperatures. Heat-induced protein denaturation can lead to imbalance in metabolic pathways or to complete enzyme inactivation. These changes lead to the production of active oxygen species and, consequently, heat-induced oxidative stress [80].

The best-characterized aspect of acquired thermotolerance is the production of heat shock proteins (HSPs) consisting of a helix-turn-helix class DNA binding domain, a leucine zipper domain required for trimerization, and a carboxy-terminal transcriptional activation domain. In *S. cerevisiae*, heat shock factor (HSF) is encoded by a single, essential gene, *HSF1*. It was documented that Hsf1p protein from *S. cerevisiae* and HSF from yeast *K. lactis* both contain a unique transcriptional activation domain amino-terminal to the DNA binding domain. Hsf1p appears to be primarily responsible for production of protein chaperones during heat shock [81]. At higher temperatures, organisms induce massive transcription and translation of HSPs. These proteins are proposed to act as molecular chaperones to protect cellular proteins against irreversible heat-induced denaturation and to facilitate refolding of heat-damaged proteins. Genetic evidence established that the Hsp100 family proteins are essential for the acquisition of thermotolerance [82].

The major role for the pathway in heat shock response is mediated by expression of genes required for the synthesis and degradation of the disaccharide trehalose. Originally thought to function as a storage carbohydrate, trehalose accumulates to extremely high levels in stationary phase cells. Logarithmic-phase cells have very low levels of trehalose, which are rapidly increased upon stress exposure. This acts as cytoprotectant, blocking thermally induced protein aggregation. Importantly, trehalose-stabilized proteins are maintained in a partially folded state, ready for reactivation by protein chaperones. Accordingly, the continued presence of trehalose inhibits protein refolding. Stress recovery therefore requires reduction of cellular trehalose levels. Trehalose can thus be considered a chemical chaperone for protein folding with properties remarkably similar to the chaperone Hsp104p – the ability to stabilize unfolded proteins and prevent aggregation [83].

It was documented that temperature affects both growth and ethanol tolerance. Decreasing temperature decreases membrane fluidity; increasing temperature increases membrane fluidity. Yeasts are able to adapt to low temperatures by increasing the proportion of cis-unsaturated fatty-acyl groups in lipids forming cell membranes. Physical principles suggest that fluidity would decrease as the ratio of saturated to unsaturated fatty acids increases because desaturation introduces a bend in the fatty acid chain. However, the majority of fatty acids in the membranes of *S. cerevisiae* are unsaturated, so other factors may be more important. It was found that the unsaturation level of *S. cerevisiae* cellular fatty acids increases at both sublethal or supraoptimal temperatures. On the other side, it was noted that the high content of unsaturated fatty acids is rather result from activation of oxygen-consuming desaturase activity. Membrane fluidity is also affected by the ratios of cell lipids and proteins. These vary with the yeast strain and the conditions under which it is cultivated [37, 84, 85].

Ethanol also affects membrane fluidity, but through different mechanisms. The presence of alcohols, results in the decrease of the temperature required for maximal activation of heat-shock genes, and the concentration of alcohol needed decreases with alcohol chain length. Ethanol is thought to alter membrane organization and permeability by entering the hydrophobic interior and increasing the polarity of this region [37].

The plasma membrane proton pump (H^+ -ATPase) of yeast couples ATP hydrolysis to proton extrusion, thereby providing the means for solute uptake by secondary transporters and for regulating cytoplasmic pH. By pumping protons out of the cytoplasm, the H^+ -ATPase acidifies the external medium, and makes the cytoplasm relatively alkaline. *S. cerevisiae* possesses two isoforms of this enzyme Pma1 and Pma2. They are 89% identical at the protein level, but they exhibit different activation, kinetic and regulatory properties, which may suggest their different functions. The specific activity of Pma1 increases with growth temperature. However, the increase in activity following stress is not attributable to synthesis of new protein, but rather to activation of the existing enzyme. Additionally, in *S. cerevisiae*, protein Hsp30 is a stress-inducible regulator of ATPase activity. Hsp30 is induced by heat shock, ethanol exposure, severe osmotic stress, weak organic acid exposure and glucose limitation. Hsp30 induction downregulates stimulation of H^+ -ATPase caused by stress. There were also extensive studies of ATPase activity in non-conventional yeast *P. stipitis*. The enzyme from this yeast attained its highest activity at 35°C. It is unclear whether ATPase activity in *P. stipitis* involves one protein or two, as in the case of *S. cerevisiae*. Plasma membrane ATPase activity is essential for basal heat resistance. Moreover, thermotolerance is enhanced by prior exposure to stress. Pre-stressed cells are able to protect the proton gradient longer than cells that have not adapted to heat [86].

High-temperature stress causes multiple changes in the cell that ultimately affect protein structures and function, leading to inhibition of cell growth or cell death. The denatured or aggregated proteins in live cells may be degraded via the ubiquitin proteasome pathway (UPP). It is the one of main defense strategies to ensure survival in stress conditions [87]. This is ATP-dependent process, and timely destruction is vital for controlled cell division, as well as proteins unable to fold properly within the endoplasmic reticulum. The UPP is carried out by three classes of enzymes. A 'ubiquitin activating enzyme' (E1) forms of a thio-ester bond with ubiquitin that is a highly conserved 76-amino acid protein. The next reaction allows

binding of ubiquitin to a 'ubiquitin conjugating enzyme' (E2), followed by the formation of the isopeptide bond between C-terminus of ubiquitin and the lysine rest by 'ubiquitin ligase' (E3) action. The UPP selectively eliminates abnormally folded or damaged proteins that have arisen by missense or nonsense mutations, biosynthetic errors, or damage by oxygen radicals or by denaturation, especially at high temperatures [88].

The mechanisms of yeast thermotolerance are largely controlled through the activation and regulation of specific stress-related genes involved in the synthesis of specific compounds that protect the organism from high-temperature stress. Elucidation of the function of these genes and/or proteins will give insight into the various mechanisms underlying yeast response to high-temperature stress, providing useful information to improve bioethanol production at higher temperatures.

Genetic data indicate that different genes contribute to heat tolerance at different stages of the plant life cycle and that different genes may be essential for basal and acquired thermotolerance [82]. Studies conducted by Gibney et al. have shown that gene deletions may also lead to higher thermosensitivity. Functional analysis of some identified genes confirmed that metabolism, cellular signaling and chromatin regulation play key role in controlling of yeast thermotolerance. However, the molecular mechanism of these actions remains still imprecise. They suggest that survival after heat shock depends on a small number of genes that function in assessing the metabolic health of the cell and/or regulate its growth in a changing environment [89]. To understand the mechanism of thermoadaptation, Shui et al. performed proteomic analysis for both parental and evolved strains of *S. cerevisiae*. They showed that some proteins were differentially regulated at heat-stress conditions in the parental and evolved strains. Additionally, the proteomic response of the industrial strains adapted to stress conditions was substantially different in comparison to the response of laboratory yeast to unexpected heat stress [90].

7. Fermentation activity and ethanol tolerance

Oxygen is one of key factors in regulation of fermentation in yeast. According to the role of oxygen in their metabolism, yeasts can be classified as: (a) obligatory aerobic, with only respiratory metabolism; (b) facultative fermentative or respiro-fermentative, displaying both respiratory and fermentative metabolism and (c) obligatory fermentative [91]. Although the majority of yeast species described so far is able to ferment sugars into ethanol and carbon dioxide, most of the respire-fermentative yeasts do not grow well under strictly anaerobic conditions [92].

Van Dijken and Scheffers explained the central role of two redox couples NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ in the metabolism of sugars by yeasts. NADH is preferentially used in dissimilatory metabolism, whereas NADPH is generally required for assimilatory reactions. In *S. cerevisiae*, *C. utilis* and probably in the yeasts in general, NADH and NADPH cannot be interconverted owing to the absence of a transhydrogenase activity [93].

Barnett [94] described 678 yeast species, and around 60% are considered to be fermentative on the basis of taxonomic tests such as gas production (Durham tubes) in laboratory conditions. However, this number is even higher since, under certain conditions, some of those

species considered as non-fermentative are also able to ferment glucose. The ability to ferment glucose under oxygen limitation turns out to be a common feature of the different yeast species, but the capability of growth under anaerobic conditions is not widespread among these microorganisms. In fact, only very few yeast species are capable of fast growth under those conditions and *S. cerevisiae* stands out as the yeast generally acknowledged as a facultative anaerobe. Anaerobic growth is associated with a low energy yield compared with that observed under complete oxidative processes [94].

The Pasteur and the Crabtree effects are the examples of special competition between respiration and fermentation of glucose [93]. The Pasteur effect refers to an activation of anaerobic glycolysis in order to meet cellular ATP demands owing to the lower efficiency of ATP production by fermentation compared with respiration. The Crabtree effect is currently defined as the occurrence of alcoholic fermentation under aerobic conditions. These two regulatory effects are very important in industrial fermentation [95, 96].

S. cerevisiae utilizes glucose by fermentative pathway (Crabtree positive) and some non-conventional yeasts like *K. lactis*, *P. pastoris* and *Y. lipolytica* are predominantly oxidative (Crabtree negative). However, among non-conventional yeasts are also Crabtree-positive ones. *S. cerevisiae* shows tolerance and good adaptation to high concentrations of ethanol. It was found that *S. cerevisiae* cells grown in the presence of ethanol appear to increase the amount of monounsaturated fatty acids in cellular lipids [97]. However, several non-conventional yeasts such as *Dekkera bruxellensis*, *P. kudriavzevii*, *Torulaspora delbrueckii* or *Wickerhamomyces anomalus* show quite good fermentation abilities and similar levels of ethanol tolerance in comparison to *S. cerevisiae* [98–103]. Especially Crabtree-positive *D. bruxellensis* strains are able to remain viable in fermentation media containing up to 16% ethanol. It has been shown that the yield of ethanol formation by *D. bruxellensis* in batch culture under anaerobic conditions is comparable with conventional yeasts. Additionally, *D. bruxellensis* shows the ability to 'compete' with conventional yeasts in industrial conditions, presumably due to the predominance of *S. cerevisiae* in the assimilation of nitrates [101, 102].

Several attempts were initiated to increase ability of yeast fermentation or to convert Crabtree-negative yeasts into Crabtree-positive for improving ethanol fermentation efficiency. Schifferdecker et al. created a metabolically engineered strain *D. bruxellensis* by increasing its fermentation capability. The gene encoding for alcohol dehydrogenase was overexpressed under the control of highly active *TEF1* promoter. As result, the improved strain produced 1.4–1.7 times more ethanol than the parental yeast [104]. Other unconventional strain of *K. lactis* was constructed as a mutant in the single gene encoding for a mitochondrial alternative internal dehydrogenase. This strain showed unaffected rate of exogenous NADH oxidation, but this mutation shifted the metabolism from respiration to fermentation. As a consequence, the mutant of *K. lactis* showed the increased rate of ethanol production [105].

Cost-effective fermentation depends on, among other factors, rapid and high yielding conversion of carbohydrates to ethanol, which in itself depends on improvements in the survival and performance of yeast cells under industrial conditions. Conventional *S. cerevisiae* is responsible for industrial alcoholic fermentation. On the other hand, most non-conventional yeasts that do not show such regulatory effect, which does not allow for efficient ethanol production

in industrial conditions. Therefore, in traditional fermentation processes (beer production and winemaking), the non-*Saccharomyces* yeasts, initially present in fermentation medium at high numbers (ranging from 10^3 to 10^5 cells/ml), grow only during the early stages (up to 4–5% v/v of ethanol) and they are soon overtaken by strongly fermentative *S. cerevisiae* strains that complete the fermentation process [92].

Ethanol is well known as an inhibitor of microbial growth. Large concentrations of ethanol can be toxic to yeasts. Ethanol in low amounts inhibits cell division, decreases cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death [106]. Ethanol also influences cell metabolism and macromolecular biosynthesis. The main results of these changes are production of heat shock-like proteins, low rate of RNA and protein accumulation, numerous petite mutants, denaturation of intracellular proteins and reduction of glycolytic enzymes activity. The response of yeasts to ethanol stress is complex, involving various aspects of cell sensing, signal transduction, transcriptional control, protein-targeting, accumulation of protectants and increased activity of repair functions. The efficiency of these processes in a given yeast strain determines its worth in industrial processes [107].

8. Furan and acetate tolerance

The use of hydrolysates obtained from plant biomass for the production of second generation bioethanol may be very problematic. In the pre-treatment processes, a lot of by-products toxic to the yeast cells may be formed [10]. The composition and concentration of inhibitory compounds is variable and depends on the type of lignocellulosic raw material and the method of its pretreatment [108]. Generally, after the pre-treatment and enzymatic hydrolysis of the hemicellulose fraction, hexoses: D-glucose, D-galactose, D-mannose and D-rhamnose and pentoses: D-xylose and L-arabinose are obtained [109, 110]. However, under high temperature and pressure, hexoses and pentoses may be degraded to 5-(hydroxymethyl)-2-furfural and furfural. The harmful effects of these compounds, even at low concentration, have been confirmed. RNA, DNA, proteins and membranes of yeast cells are particularly sensitive [111, 112].

The removal of toxic compounds from the fermentation medium is usually very expensive. Therefore, in order to improve the fermentation processes, the use of furan-tolerant yeast strains is more practical. Scientists recognize the molecular basis of cell tolerance to furan and its derivatives for model yeast *S. cerevisiae*. It has been found that *SIZ1* gene encoding the ligase E3, can bring the significant increase in tolerance to furfural. Some non-conventional yeast species, namely *W. anomalus*, *P. kudriavzevii*, *C. stellata*, *C. ethanolica*, *P. fermentans* and *Z. bailii*, show good tolerance to furfural and its derivatives. For example, the resistance of *P. kudriavzevii* to hydroxymethyl reaches up to 7 g/L [103].

The tolerance to weak acids is essential in the second generation bioethanol production. During the pretreatment of the lignocellulosic feedstock, released hemicellulose acetyl groups form acetic acid in the concentration of 5–10 g/L [113, 114]. It is known that weak acids exhibit cytotoxic effects. These compounds are transported through the cell membrane into the yeast

cells by passive diffusion in non-dissociated form. In the yeast cells they are subject to dissociation, and protons are accumulated in the cytoplasm, causing acidification of cytosol [115–118]. In this case the cell metabolism slows down significantly by inhibiting glycolytic enzymes and NADH dehydrogenase [119–122]. Low intracellular pH inhibits the growth of yeasts, the adaptive phase increases and consequently, the efficiency of ethanol production decreases [123, 124]. Therefore, the use of yeast strains resistant to weak acids is essential for industrial production of bioethanol. Non-conventional yeast *Z. bailii* has been described as the most resistant to acetic acid. This yeast can grow at the concentration as high as 24 g/L, while conventional *S. cerevisiae* shows sensitivity at 9 g/L of acetate [125].

9. Mixed populations and biocontrol

S. cerevisiae are able to produce high concentrations of ethanol reaching approximately 20% (v/v) but in conventional media. This yeast shows high fermentation rates, whereas they are unexpectedly less tolerant to high concentrations of ethanol and other toxic compounds. That is the reason why several ethanol-tolerant yeasts are used in industrial fermentations.

The profusion of selected starter cultures has allowed the more widespread use of inoculated fermentations, with consequent improvements to the control of the fermentation process, and the use of new biotechnological processes. Mixed fermentations using controlled inoculation of *S. cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a feasible way toward improving the complexity and enhancing the particular and specific characteristics of fermentation products [126–128].

Mixed cultures with different yeasts also provide an advantage in bioethanol production. In starchy media, using raw unhydrolysed starch in a single-step fermentation, ethanol production by a co-culture of *S. diastaticus* and *S. cerevisiae* was 24.8 g/L. This was 48% higher than the yield obtained with the monoculture of *S. diastaticus* (16.8 g/L). In another coculture fermentation with *Endomycopsis capsularis* and *S. cerevisiae*, maximum ethanol yield was 16.0 g/L, higher than *E. capsularis* the yield with the monoculture [129].

In second-generation ethanol production, xylose and arabinose are the significant fraction of lignocellulosic biomass. Therefore, their utilization is essential for a feasible bioethanol production process. The selection of yeast strains for the fermentation of pentoses has a large effect on ethanol yield [130]. The naturally xylose-fermenting non-conventional yeasts such as *C. shehatae* and *P. stipitis* have been widely studied because of their ability to ferment xylose into ethanol [131]. *P. stipitis* is considered as a promising strain because it can ferment a wide range of sugars, including cellobiose. *Candida* species have been shown to ferment D-xylose to ethanol as the major product. Strain improvement by mutation is one of the best methods to increase the ethanol yield, and in this case, two strains capable of producing significantly higher ethanol yields than the parental strains were obtained [132].

The influence of non-*Saccharomyces* yeasts on fermentation processes was studied and their biotechnological potential was evaluated. The industrial yeast market, which was historically

focused on *S. cerevisiae*, now offers *S. cerevisiae*/non-*Saccharomyces* multi-starters. However, the development of these mixed populations requires knowledge about possible interactions between yeast strains. Considering the use of mixed populations, the special attention should be paid not only to the selection of the proper assimilation-competent strains, their inoculation, culture media, but also to the interactions between these yeast monocultures. The interesting results were obtained by Yamaoka et al. [128]. This research was carried out to investigate the influence of non-*Saccharomyces* yeast, *K. lactis*, on metabolite formation and the ethanol tolerance of *S. cerevisiae* in mixed cultures in synthetic minimal medium containing 20% glucose. It was noted that co-cultivation of *K. lactis* seems to prompt *S. cerevisiae* to be ethanol tolerant by forming protective metabolites such as glycerol.

In turn, studies on mixed cultures *S. cerevisiae*/*T. delbrueckii* showed that physical contact between yeast cells induced rapid death of *T. delbrueckii*. This phenomenon was previously described as a cell-cell contact mechanism. However, when these yeast cultures were physically separated from each other, the sensitive strain of *Torulopsis* sp. kept its viability [133].

The mixed yeast populations have been explored not only for improvement of ethanol yield but also as biological control—an alternative to the use of synthetic chemicals for prevention of microbial spoilage. The possibility of using the selected antagonistic yeasts against undesirable spoilage microorganisms is the subject of interest for both scientists and technologists. The presence of undesired microflora may lead to significant reduction in the efficiency of biotechnological processes. Non-conventional yeasts, characterized by antagonistic activity against spoilage microflora include genera *Pichia*, *Candida*, *Aureobasidium*, *Metschnikowia* and *Debaryomyces*. The interactions between microorganisms have been described in numerous scientific studies [126, 127, 134–136]. Industrial yeast strains, due to the high reproductive potential and rich enzymatic equipment, have the ability to colonize fermentation environments rapidly. The presence of microbial contamination not only reduces available nutrients for industrial microorganisms, but also reduces the potential living space. Low nutrient availability is one of the most important mechanisms of competition between yeast strains.

The killer phenomenon was first observed in yeast *S. cerevisiae* in the 1960s of the last century. However, the killer features have also been found in representatives of non-conventional yeasts belonging to genera *Debaryomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Cryptococcus*, *Ustilago*, *Rhodotorula*, *Williopsis*, *Torulopsis*, *Zygosaccharomyces*, *Hansenula* and *Hanseniaspora*. Killer protein toxins show specific activity spectra dependent on pH level, temperature and aeration conditions. These toxins differ in resistance to proteolytic enzymes, chemicals, pH, and they are mutually antagonistic. The impact of killer yeasts to sensitive yeast cells include killer protein receptors on the cell wall of sensitive cells. The consequences of killer toxin binding to cell wall are physiological changes that lead to death of the sensitive cells. Initially, there is a breakdown of amino acids and proton gradient, leakage of potassium ions from ATP, reduction of metabolite levels and the destruction of the pH gradient. All these processes lead to a gradual death of sensitive yeast cells [4, 137, 138].

It has been found that yeast strains *Metschnikowia pulcherrima* have a great potential to be a leading natural and biological control against a broad spectrum of pathogens [139–141].

M. pulcherrima forms pulcherimic acid, which is accumulated in growth medium and forms red pulcherrimin—a chelate complex with Fe(III) ions (**Photo 2**).

It has been shown that the antibacterial and antifungal activity of yeast depends on pulcherrimin formation [139]. Therefore, strains that produce large amounts of pulcherrimin are of great interest to engineers and microbiologists, as biocontrol agents inhibiting growth of pathogenic bacteria, yeasts and molds. This substance may be an alternative to antibiotics and fungicides. Oro et al. evaluated *M. pulcherrima* for the antimicrobial activity against numerous yeast strains belonging to *Pichia*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Saccharomyces*, *Torulaspora*, *Brettanomyces* and *Saccharomyces* genera [141]. *M. pulcherrima* displayed a broad and effective antimicrobial action on undesired wild spoilage yeasts (*Brettanomyces/Dekkera* spp., *Hanseniaspora* spp., *Pichia* spp.). Interestingly, the antimicrobial activity of *M. pulcherrima* did not have any influence on the growth of *S. cerevisiae*. The oxygen availability strongly influences population dynamics in mixed populations of conventional and non-conventional yeasts. Additionally, in the presence of non-*Saccharomyces* yeasts, species-specific chemical volatile profiles were noted, in particular increases in some higher alcohols and medium chain fatty acids. This data show the potential use of selected *M. pulcherrima* strains in controlled multi-starter fermentations with *S. cerevisiae* starter cultures [142, 143].

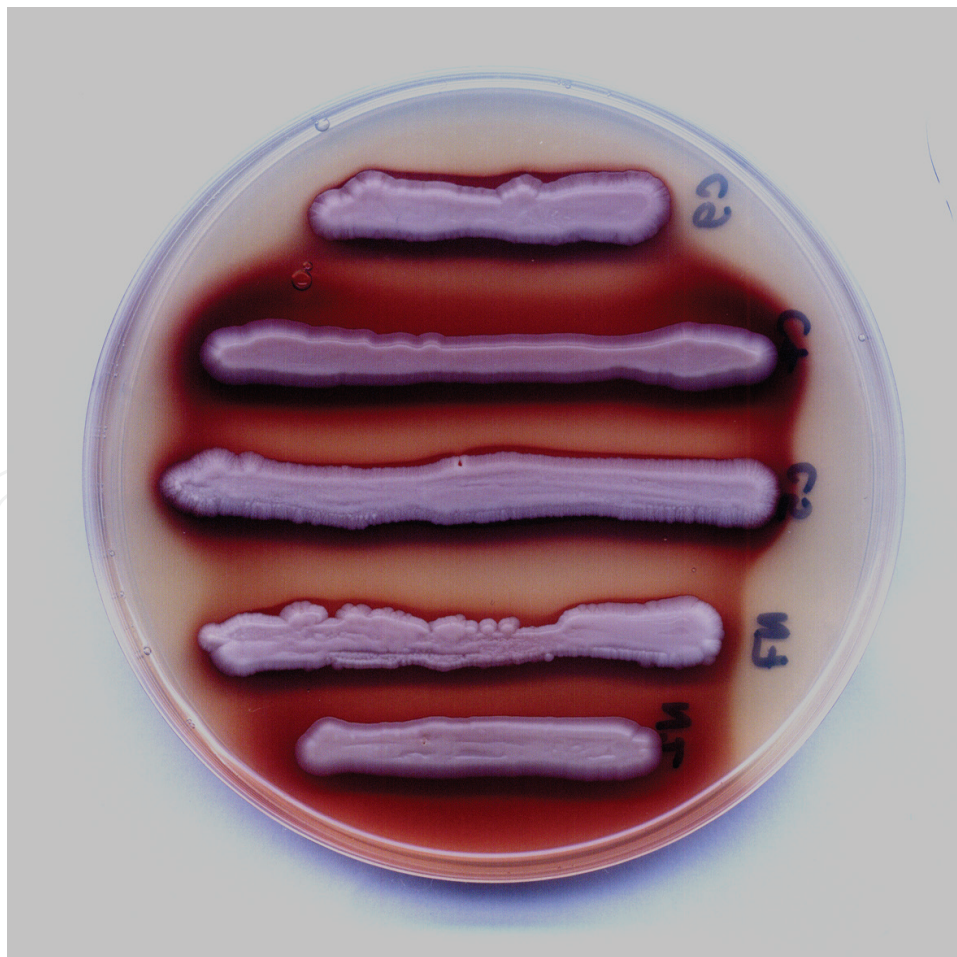


Photo 2. Pulcherrimin formation by *M. pulcherrima* on YPD agar with Fe(III) ions (author: Ewelina Pawlikowska).

10. Conclusion

The conventional yeast *S. cerevisiae* is the best known species used in numerous industrial high-tech processes. However, in new technologies, including second generation ethanol production, the use of this yeast may encounter a number of difficulties. Research studies suggest that it is possible and even necessary to use selected non-conventional yeast strains to increase the use of carbon sources as well as to improve the economic effects of ethanol production from plant waste materials. It is worth paying attention to one more aspect—many species of non-conventional yeasts produce unique biocontrol compounds, which can be seen as an additional valuable feature for conducting fermentation processes. These yeasts may find use as monocultures or mixed complementary populations. Although the exploration of existing natural biodiversity of non-conventional yeasts is attractive, the major bottleneck is that industrially applicable traits are not commonly found in nature. However, there are multiples of classical approaches to develop strains with improved phenotypes such as mutagenesis, sexual hybridization, genetic modification, adaptive evolution and other emerging tools. Among them, non-genetic modification, adaptive evolution, is preferable; as the use of strains developed using genetic methods in the food industry remains controversial. In addition, such a traditional phenotype improvement based on random appearance of adaptive mutations based on selective regimes requires no prior knowledge of the genetic background of the strains is under development. This is important, as the current limitation in applications of non-conventional yeasts is that they are less studied and their genetic architectures and pathways are less understood. Therefore, we can conclude that era of research on non-conventional yeasts has just begun.

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