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# **Yeast as a Versatile Tool in Biotechnology**

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<http://dx.doi.org/10.5772/intechopen.70130>

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## **Abstract**

Yeasts represent a very diverse group of microorganisms, and even strains that are classified as the same species often show a high level of genetic divergence. Yeasts biodiversity is closely related to their applicability. Biotechnological importance of yeast is almost immeasurable. For centuries, people have exploited its enzymatic potential to produce fermented food as bread or alcoholic beverages. Admittedly, yeasts application was initially instinctual, but with science and technology development, these microorganisms got the object of thorough scientific investigations. It must be recognized that yeast represents an excellent scientific model because of its eukaryotic origin and knowledge of genetics of yeast cells as well as metabolism examined in detail. In 1996, the genome of baker yeast *Saccharomyces cerevisiae* has been elucidated, what opened the opportunity for the global study of the expression and functioning of the eukaryotic genome. Also, currently, an international team is working on the synthesis of the 16 yeast chromosomes by synthetic biology tools, and the results are expected till the end of the year. Nowadays, yeast is regarded as a versatile tool for biotechnological purposes.

**Keywords:** fermentation, SCP, biocatalysis, molecular biology applications—fundamentals

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## **1. Introduction**

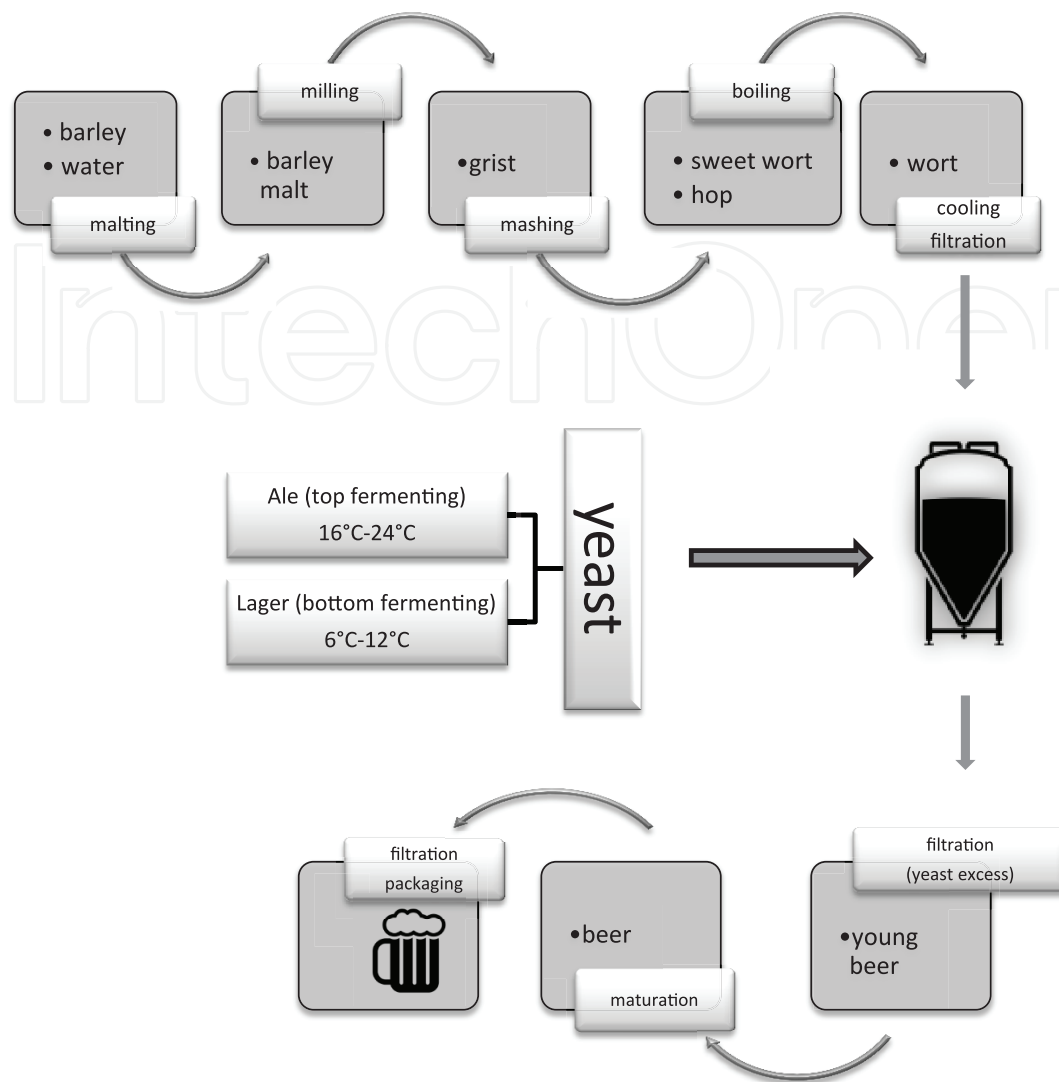
### **1.1. Yeasts: commercial applications**

Yeasts have a wide range of applications mainly in food industry (wine making, brewing, distilled spirits production, and baking) and in biomass production (single-cell protein [SCP]). More recently, yeast has also been used in the biofuel industry and for the production of heterologous compounds. Obviously, their main application arises from the

metabolic capacity to carry out the transformation of sugars into ethyl alcohol and carbon dioxide under anaerobic conditions. Moreover, a large number of secondary flavor compounds are created what implies on organoleptic attributes of particular food products. However, it would be misguided to trivialize their metabolic capacities only to fermentative activity. The main factors influencing yeast metabolism are the oxygen availability and the type of carbon source. Many yeast strains can function under both anaerobic as well as aerobic conditions of environment, switching their metabolism types easily [1]. Obviously, the courses of main metabolic pathways are conserved, but some regulative mechanisms attract the attention, denoting unusual metabolism flexibility [2]. In food industry, *Saccharomyces cerevisiae* is the genus of yeast most frequently used, whereas *Candida*, *Endomycopsis*, and *Kluyveromyces* are crucial for SCP production. Yeast strains of industrial importance are carefully selected from the immense natural biodiversity and their properties improved according to process outcome. Both classical approaches as well as modern strategies of gene manipulations are applied to generate variants relevant to work under industrial specific conditions [3, 4]. Strains alterations concern not only the route of fermentations step and its direct yield but also facilitation of product recovery procedures and finally the best quality of particular end product. These nonpathogenic strains with both genotypic and phenotypic stability should have short-generation time and low nutritional requirements. They should perform the fermentation process quickly to minimize the contamination risk. Additionally, they should be tolerant of a wide range of physiological stresses, such as low pH, high ethanol concentration, and high osmotic stress.

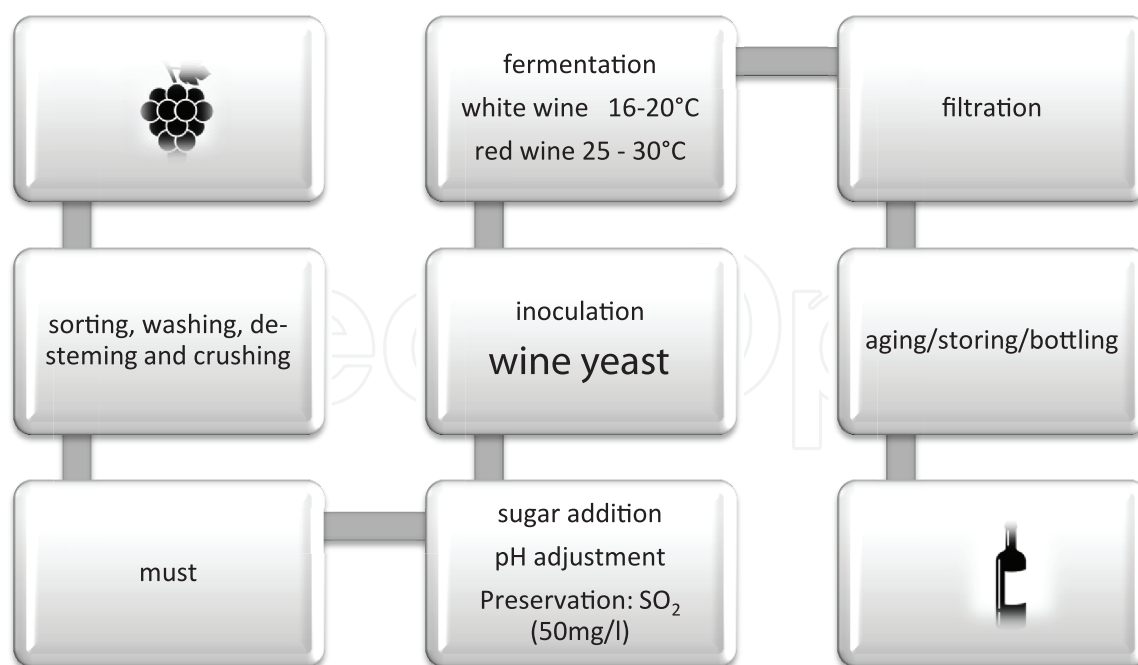
The enzymatic power of yeast is central to beer manufacturing (**Figure 1**). Industrial species are carefully selected, nonsporulated polyploides that do not perform sexual reproduction process [5].

Brewer's yeasts are divided into two separate categories: top-fermenting yeast (ale) and bottom-fermenting one (lager). Both yeast types have similar cell morphology, but they differ in some physiological and metabolic features [6], what closely corresponds to the process conditions and type of end product. Fermentation of ale yeast is carried out at room temperature and results in beers with a characteristic fruity aroma. In the case of lager yeast, the fermentation temperature is lower, and therefore, this step takes longer than fermentation with ale strains [7]. The share of aerobic respiration in yeast metabolism is higher in case of ale strains than in lager yeast. Both top-fermenting and bottom-fermenting yeast belong to the genus *Saccharomyces*. Ale-brewing yeasts are genetically more diverse and classified as *Saccharomyces cerevisiae*, whereas the taxonomy of the lager strains has undergone several changes. Initially, bottom-fermenting yeast was classified as *Saccharomyces carlsbergensis*, but due to the application of modern taxonomic approaches, the species *S. carlsbergensis* were included as a part of *Saccharomyces pastorianus* taxon. On the basis of genetic studies, *S. pastorianus* strains are now considered as allopolyploid interspecies hybrids of *S. cerevisiae* and *S. bayanus* [8]. From technological point of view, beside the high fermentative activity of yeast cells and tolerance for several environmental stresses, the aptitude for asexual aggregation known as flocculation seemed to be especially important, because this ability causes the yeast to sediment to the bottom of the fermenter at the end of fermentation step, what simplifies downstream processing [9, 10].



**Figure 1.** Beer production process.

Various yeast species constitute the predominant microbial group of natural microbiota of fruits ecosystems, what is the reason of fast and spontaneous fermentation of juices or musts resulted in wine production. Yeast colonizing various fruits belongs to the genera *Saccharomyces*, *Brettanomyces* (its sexual form (teleomorph) *Dekkera*), *Candida*, *Cryptococcus*, *Debaromyces*, *Hanseniaspora* (anamorph *Kloeckera*), *Hansenula*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Torulaspora*, *Schizosaccharomyces*, and *Zygosaccharomyces* [11]. It goes without saying that most fermented products are generated by a mixture of microbes. These microbial consortia perform various biological activities responsible for the nutritional, hygienic, and aromatic qualities of the product [12]. Doubtlessly, yeast plays a principal role in wine making both in domestic environment as well as in commercial scale (**Figure 2**). Nonconventional yeast (*non-Saccharomyces species*) dominates during early and middle stages of fermentation process, whereas the latter phase of natural process is mediated by *Saccharomyces cerevisiae*. The raw material of particular importance in global wine making is grapes that are harvested at specific stages of ripeness depending on the style of wine to be made.



**Figure 2.** Wine production process.

Once again yeast enzymatic power is crucial for the product chemical profile—except for ethanol biosynthesis, the creation of flavor compounds and fragrances from substrates abundant in fruits implies the organoleptic features of particular wine product. During the degradation of grape sugars, amino acids, fatty acids, terpenes, and thiols, some by-products like glycerol, carboxylic acids, aldehydes, higher alcohols, esters, and sulfides are formed, and their synthesis is largely dependent on the peculiarities of the strains used [13, 14]. In 1965, the first two commercially active dried wine yeasts called Montrachet and Pasteur Champagne were produced for a large Californian winery [15]. Nowadays, modern winegrowers routinely use selected yeast starters in practice. These microorganisms dominate native yeast species and give desired direction of chemical transformations occurring in musts and allow to obtain product of predictable quality [16]. Presently exploited commercially available starters have been created as a result of naturally occurring phenomenon called “genome renewal” as well as planned processes of genetic improvement [17] followed by a careful selection for their good fermentation performance. Genome renewal hypothesis in the standard version assumes that infrequent sexual cycles, characterized by a high degree of selfing, can help to purge deleterious alleles and fix beneficial alleles, thus helping to facilitate adaptation in yeast [18]. This hypothesis had to be re-evaluated [19] due to the fact that in the case of many environmental isolates, very high levels of genomic heterozygosity had been observed [20, 21]. Presently, the majority of commercial wine yeast comprises strains of *Saccharomyces cerevisiae*, including those described by enologists as *S. bayanus*, which has been re-identified in most cases as *S. cerevisiae* [22, 23]. The growing demand for more diversified wines or for specific characteristics has led to the exploration of new species for wine making [24–26]. This non-conventional yeast may contribute to the wine's organoleptic characteristic by producing a broad range of unique secondary metabolites and secreting particular enzymes or exhibiting

others substantial features (release of mannoproteins, contributions to color stability etc.) [27]. The wine industry currently proposes starters of a few nonconventional yeast (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Lachancea thermotolerans*, etc.) [28]. Yeast variants used as starters must qualify for commercial application. The list of desired properties is very long and includes both fermentation characteristics as well as flavor characteristics (e.g., low sulphide/dimethyl sulphide/thiol formation, liberation of glycosylated flavor precursors, low higher alcohol production, etc.), metabolic properties with health implications (low formation of sulphite, biogenic amines, or urea) and technological properties (e.g., high sulphite tolerance, low foam formation, flocculation properties, etc.) [29].

Another example of *Saccharomyces cerevisiae* strains of commercial interest encompasses distiller's yeasts, applied in industry where alcohol fermentation is followed by distillation. In this industry type, spontaneous fermentations are not practiced, and specific yeast is inoculated into fermenter. Starters used for distilled beverages (aquavit, gin, vodka, whisky, rum, tequila, cognac, brandy, and kirsch) commercial production should exhibit exquisitely intensive anaerobic metabolism, what should result not only in high ethanol productivity but also in the high level of cellular tolerance to this product. Additionally, thermostable variants tolerant to acids and increased osmotic pressure are in greater demand for distillers [30]. Since many years, there has been a rising interest in new variants that are able to degrade starch. Many attempts have been made to produce ethanol from starch using recombinant haploid strains that express amylolytic enzymes, due to the simplicity of genetic manipulation of these strains. However, it is difficult to use laboratory haploid strains in practice because their fermentation characteristics are not as good as those of industrial polyploidy strains [31, 32].

In addition to alcoholic beverages production, enzymatic power of yeasts is also essential for baking industry, where concentrated yeast biomass is used as a starter in dough fermentation in order to produce bread and other bakery products. Commercially available baker's yeast forms include fresh compressed biomass, dehydrated cells (dry yeast), and lyophilized cells (instant). Fermentation of dough substrates leads to ethanol production as well as number of volatile and nonvolatile compounds that have an important contribution to the flavor of bread [33]. As a result of carbohydrates (maltose mainly), fermentation carbon dioxide is generated what increases the dough volume and is responsible for crumb texture. Baker's yeast is simply brewery yeast produced *via* submerged fermentation process carried out in the presence of oxygen (**Figure 3**). Aerobic conditions favor yeast cells production, which is not of interest to ethanol producers, but is important when large amount of cells mass must be produced.

The main ingredient of industrial production medium used in yeast production factories are beet or cane molasses, mainly because of the low cost of this waste products and high sucrose content. In most cases, the industrial production is a multistage process carried out under batch or fed-batch conditions with sequential stages differing in fermenter size, performed under controlled intense aeration [34]. Aeration is generally considered as the most important single factor to increase yeast yield and numerous studies have been carried out to investigate the optimization of particular technological solutions [35]. It should be underlined, the particular uniqueness of yeast metabolism—baker's yeast must exhibit efficient respiratory metabolism during yeast manufacturing, which determines biomass yield, but at the same time, cells must



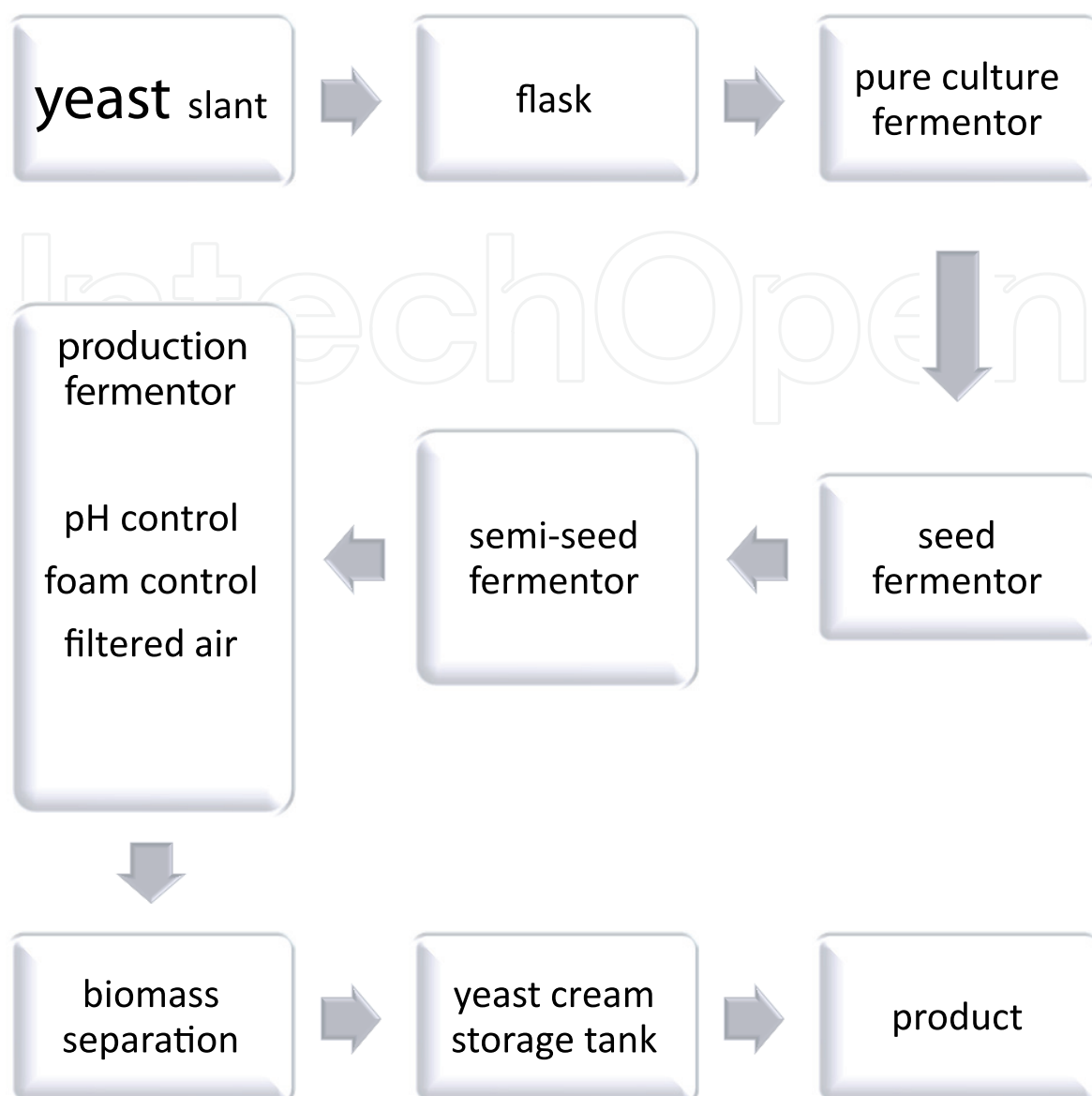


Figure 3. Baker's yeast production process.

possess strong fermentative potential in order to produce excellent bakery products. During the fermentation of dough yeast cells are exposed to numerous environmental stresses (*baking associated stresses*) such as freeze thaw, high sugar concentrations, air drying, and oxidative stresses [36]. Nor should it be surprising that industrial starters should be characterized by appropriate stress tolerance. Yeasts certainly have evolved some mechanisms of adaptation, but if the level of stress will increase too much, their enzymatic potential will be restricted. It has been demonstrated that two molecules: trehalose and proline are extremely important for yeast stress tolerance, so the engineering of their metabolism is a promising approach to the development of stress-tolerant yeast strains relevant to industrial use [37].

Next long-standing industrial processes involving yeast are the production of single-cell protein (SCP)—alternative source of high nutritional value proteins used as a food or feed supplements. Idea of such protein concentrate production was born in response to growing

human population in the world and worldwide protein deficiency [38]. SCP manufacture includes simply the cell mass obtaining by way of the application of cheap, waste raw materials to cultivate various nonpathogenic microorganisms (bacteria, fungi, algae) under conditions of submerged (rarely solid-state) fermentation. Besides its high protein content (about 60–82% of dry matter), SCP also contains fats, carbohydrates, nucleic acids, vitamins, and minerals [39]. In practice, several technologies were evaluated, products commercialized and currently obtained SCP found the application primarily in animal feeding. However, baker's yeast mentioned above can be considered also as an example of particular SCP preparation. Many fungal species are used as protein-rich food. Most popular among them are the yeast species *Candida*, *Hansenula*, *Pichia*, *Torulopsis*, and *Saccharomyces* [40], but also, marine yeast is considered now as a valuable source of protein [41, 42]. Yeast-derived SCP has desired nutritional value and contains essential amino acids—above all sulfur containing amino acids. It is also significant that people accept yeast as a food source, because of its historical impact. The main disadvantage of this preparation limiting the utilization as food is the nucleic acid content what is associated with additional purification step in downstream processing [43] as well as with external mannoprotein layer of yeast cell wall what impedes the digestion.

Yeast can also be considered as an alternative source of lipids. Some species are capable of synthesis and accumulation of over 20% of biomass in form of neutral lipids and for that reason are called “oleaginous.” Under optimal growth conditions and/or as a result of genetic improvement, the level of lipid accumulation can reach even 70%. Oleaginous yeast includes species of *Candida*, *Cryptococcus*, *Pichia* (*Hansenula*), *Lipomyces*, *Pseudozyma*, *Rhodospiridium*, *Rhodotorula*, *Trichosporon*, *Trigonopsis*, *Yarrowia*, and *Saccharomycopsis* [44]. The ability of these yeast species to accumulate high quantities of lipids offers the commercial potential for production of single-cell oil (SCO). Microbial oils can serve both as alternative edible oils for food industry as well as substrates used in synthesis of the oleochemicals such as fuels, soaps, plastics, paints, detergents, textiles, rubber, surfactants, lubricants, additives for the food and cosmetic industry, and many other chemicals [45]. Microorganisms regarded as an alternative oils source cannot presently compete directly with plants, but their use has many advantages like shorter process cycle, independence of season and climate, facility for genetic improvement and the possibility to manufacture lipid of unique structure, and nonsynthesized by plants. It should be mentioned that the composition of yeast oils is similar to vegetable products, and predominant fraction of them is made of triacylglycerols (TGA) rich in polyunsaturated fatty acids [46]. Lipids are stored intracellularly mainly as granular forms called “lipid body,” and their content and profile of fatty acids differs between species [47]. The main fatty acids formed by oleaginous yeast are the myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), palmitoleic (C16:1), and linoleic (C18:2) acids [48]. Two different pathways are involved in lipid accumulation by oleaginous yeast: *de novo* lipid synthesis and *ex novo* lipid accumulation. Fundamental differences at the biochemical level exist between *de novo* lipid accumulation from hydrophilic substrates and *ex novo* lipid accumulation from hydrophobic substrates. In contrast, *ex novo* lipid production is a growth-associated process that occurs simultaneously with cell growth and is entirely independent of nitrogen exhaustion of the culture medium. The synthesis of *ex novo* lipids is the modification of fats and oils by oleaginous microorganisms [49]. The production of microbial oils became more important in the light of biodiesel production. The oleaginous yeast is considered as potential candidate for the production of “2nd generation” biodiesel deriving from lipid

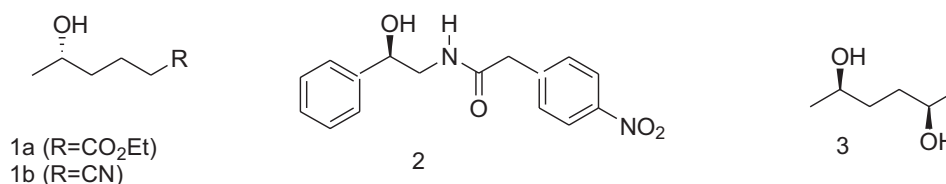


produced by oleaginous microorganisms growing on wastes or agro-industrial residues like sewage sludge, hemicelluloses, hydrolysates, waste glycerol, cheese whey, etc. [50, 51]. SCO may be produced *via* submerged fermentation performed under aerobic conditions through the mode of batch, fed batch, or continuous operation. To achieve both sufficient biomass formation and proper lipid accumulation level, yeast must be cultivated under carefully evaluated conditions. Many factors have been described as influencing lipid accumulation process and proper medium composition seemed to be one of the most important ones [52]. After biomass had been formed, to promote lipid accumulation process, stress conditions must be induced. Lipid storage is triggered by a nutrient limitation combined with an excess of carbon. Mostly nitrogen limitation is used to trigger lipid accumulation, but also other nutrients as phosphorus and sulphur have been shown to induce lipid storage. In spite of the applicative potential, the commercialization of microbial products is limited to oils containing polyunsaturated fatty acids like docosahexanoic acid (DHA), arachidonic acid (ARA), and eicosapentaenoic acid (EPA) [53, 54]. Producing other microbial oils either for human consumption, industrial use, or for biofuel production is still cost inhibitory—currently, the production of microbial oils is more expensive than that of vegetable oils [55]. To extend the industrialization of yeast as alternative source of oils, all efforts should be focused on genetic improvement of cell factories combined with optimization of nutrient supply and cultivation conditions and modifications of downstream technology [56].

## 2. Yeasts as whole-cell biocatalysts

Different genera of yeasts are convenient biocatalysts applied in many fields of chemistry, especially for the synthesis of chiral building blocks and fine chemicals. They are interesting catalytic tools, not only for their varied enzymatic activities but also for their microbiological features such as simplicity of cultivation, low nutritional requirements, and adaptive capacities. These capacities result from their flexible metabolism, which responds to the environmental impacts, so the direction (also stereoselectivity) and the effectiveness of the biotransformations can be driven by the physical chemical parameters of the process. What is also important, they are susceptible to the engineering of the reaction media (e.g., water, organic) and to the biocatalysts form (e.g., permeabilization, immobilization). This significantly broadens the field of their application by overcoming the limitations such as low solubility of the bioconversion substrates. It can be said that yeasts are used for decades and are one of the first whole-cell biocatalysts applied in industrial processes.

Literature data proved that the core applications of yeasts are connected with their extraordinary reductive abilities. Since it was proven that whole-cell biocatalysis is (as enzymatic one) chemoselective, regioselective, and stereoselective and able to regenerate dehydrogenases cofactors under biocatalytic conditions, yeasts were extensively examined as reductive catalysts for chiral building blocks synthesis—especially chiral alcohols of defined absolute configuration. The activity of a number of yeasts genera has been tested toward structurally different ketones, and in the most cases desired, alcohols have been obtained as pure enantiomers [57]. Alcohols drawn below (**Figure 4**) represent both, simple, and more complicated—unusual structures obtained thanks to the whole-cell biocatalysis driven by yeasts.



**Figure 4.** Alcohols synthesized using whole yeasts cells.

*Pichia methanolica* is one of the most important biocatalysts employed in industry because of its reductive ability and low substrate specificity. Thus, *Pichia methanolica* mediated reduction of ethyl-5-oxo-hexanoate and 5-oxohexanenitrile led to S-alcohols: 1a and 1b (**Figure 4**) obtained with conversion degree up to 90 and 97% of *ee* (enantiomeric excess). What is also important, bioreduction of compound 1a (**Figure 4**) was performed successfully on the gram scale with the similar yield. *Pichia methanolica* is employed for the reduction purposes by Bristol-Mayers Squibb Company (USA). Alcohol 2 (**Figure 4**) is important for pharmaceutical industry, this molecule is considered as supporting drug in the diabetes type 2 therapy by elevating the rate of metabolisms. Application of *Candida sorbophila* MY 1833 allowed receiving pure product on the large scale level with up to 60% of conversion degree and 98% of *ee*. Optically pure diols are also important, mainly as chiral building blocks for pharmaceuticals and fine chemicals synthesis. As an example—compound 3 (**Figure 4**)—2*S*, 5*S*-hexanediol can be received by diketone reduction performed with *Saccharomyces cerevisiae* on preparative scale, with the complete substrate reduction and with the *de* (distereomeric excess) of 96%. Except to mentioned diol, such procedure can be applied also for 5-hydroxyhexane-2-on formation, which is a substrate for chiral tetrahydrofuranes synthesis—chemicals of significant meaning for obtaining of the biodegradable polymers, drugs, and perfumes. Among different chiral compounds with hydroxyl functionalities, there are some of special interest—they are building platforms for the synthesis of series compounds. Such importance can be attributed to the (S)-4-chloro-3-hydroxybutanoate ester (S)-CHBE (**Figure 5**) [58]. A number of fungal catalysts, e.g., *Geotrichum* sp., *Candida* sp., *Aureobasidium* sp. are active toward appropriate ketone, but the reaction proceeds with an average stereoselectivity. Among others, *Pichia stipites* CBS 6054 was found as one allowed obtaining chiral product with *ee* of 80%. The other products of yeast-mediated enantioselective bioreduction are chiral bicyclic alcohols, e.g., synthons of (S)-pramipexole (anti-Parkinson drug) (**Figure 6**) and also its (R) isomer—considered as an anti-amyotrophic lateral sclerosis (ALS) agent [59]. Chirality is introduced into these molecules by the prochiral bicyclic ketone (**Figure 6**) reduction mediated by *Saccharomyces cerevisiae*, and this is the crucial step of the sequences of the reaction leading to the final enantiomers of pramipexole. Discussed objective is also an example of the yeast biotransformation of the compounds with heteroatoms in their structures—here, sulphur atom is an element of the bicyclic.

Reductive activity of *Saccharomyces cerevisiae* is also applied for some nontypical reactions such as geraniol into citronellol hydrogenation achieved with resting yeast cells on preparative level (**Figure 7**) [60]. Process productivity reaches 2.38 g/L for the reaction carried out in the continuous-closed-gas-loop bioreactor.

Entirely a different activity of yeasts as biocatalysts found some practical application, lately. Successful experiments with *Saccharomyces cerevisiae* were performed according to **Figure 8**

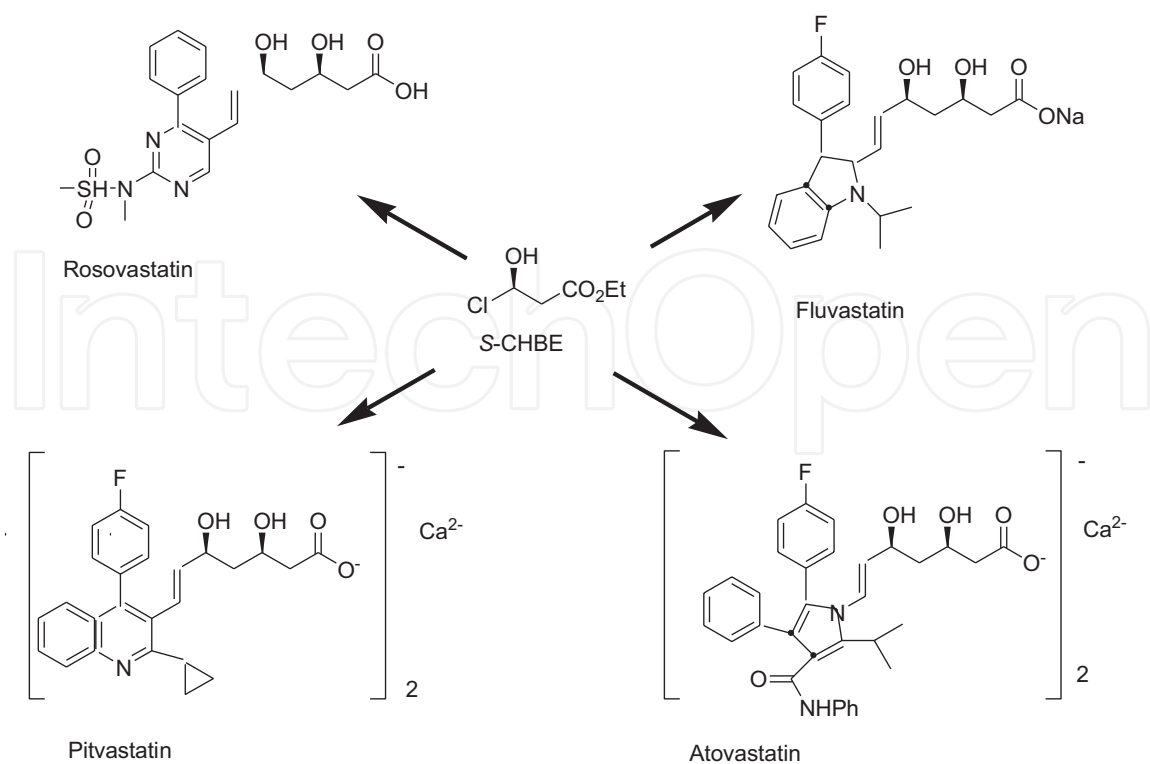
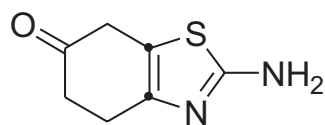
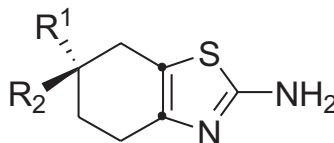


Figure 5. (S)-CHBE as chiral building platform.

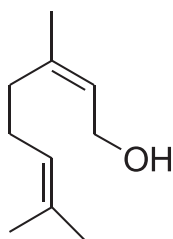


prochiral substrate

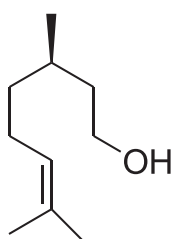


(*R*)-pramipexole.  $R^1=H$ ,  $R^2=NHCH_2CH_2CH_3$   
 (*S*)-pramipexole.  $R^1=NHCH_2CH_2CH_3$ ,  $R^2=H$

Figure 6. Pramipexole (2-amino-6-propylamino-4,5,6,7-tetrahydrobenzothiazole).

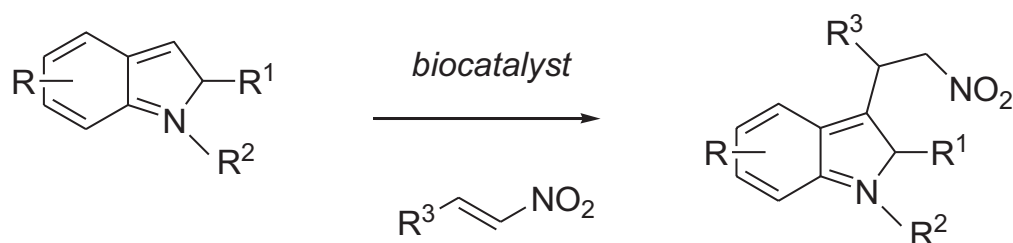


Geraniol

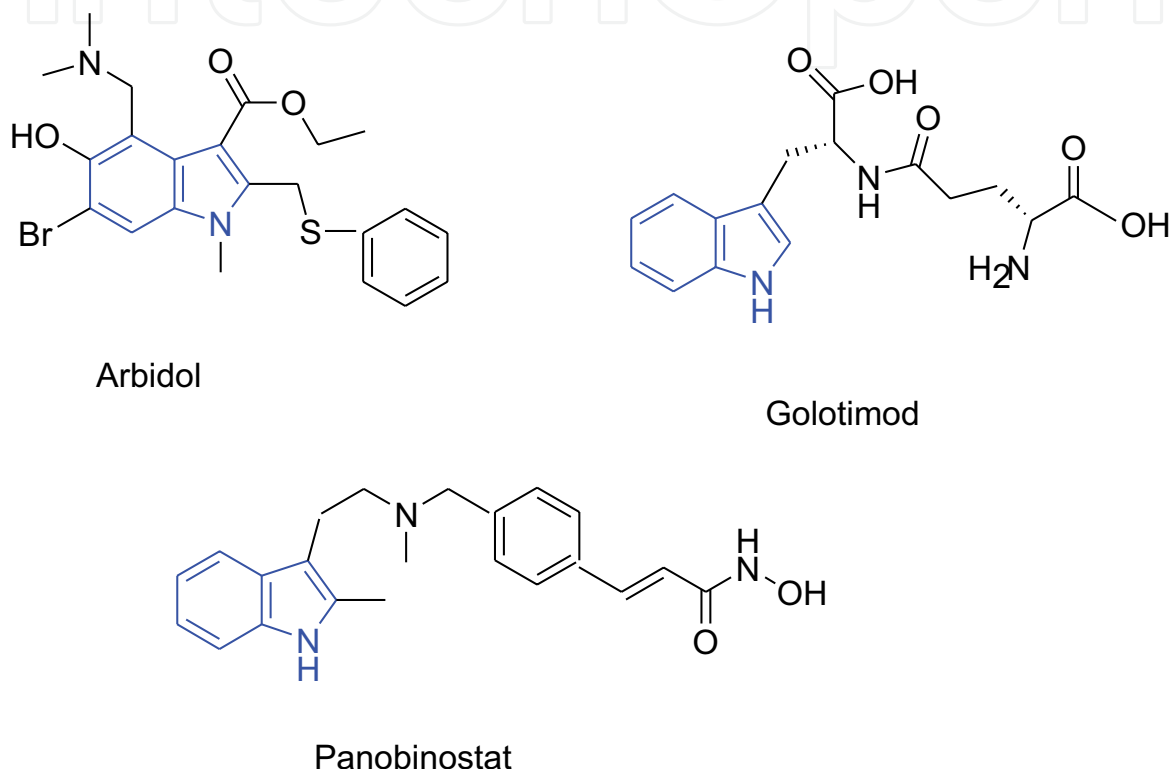


*R*-(+)-β-citronellol

Figure 7. Structures of geraniol and citronellol.



**Figure 8.** Biocatalyzed addition of indoles to nitroolefins.



**Figure 9.** Compounds with the indol-motif.

[61], which illustrates the addition of diverse 3-substituted indol derivatives to nitroolefines. Chemical synthesis of such substituted indols requires some hazardous organic solvents, while biological synthesis significantly reduces this problem and offers easy operated and effective system (yield of the reaction range between 72 and 90%).

Such indol motifs are crucial part of the biologically active compounds such as arbidol (influenza A and B virus treatment and prophylactic); golotimod (immunostimulating, antimicrobial, and antineoplastic agent); and panobinostat (acute myeloid leukemia treatment; **Figure 9**).

### 3. Yeast's enzymes applications

Biocatalysis includes both biotransformations (e.g., the conversion of xenobiotics using whole cells or resting cell systems) and enzyme catalysis (e.g., the conversion of xenobiotics using

cell-free extracts or purified enzymes) [62]. Although both whole cells and isolated enzymes can be used as biocatalysts, whole cells are very often preferable because they are more stable and cheap sources than purified enzymes, without the need for purification and coenzyme addition. However, in the case of single-step biotransformation, isolated enzymes can be considered as a better choice and can be used as a free or immobilized biocatalyst either in aqueous or organic media [63]. Yeasts, especially *Saccharomyces* species, are primarily known from whole cell reductive activity [64, 65] and are used in the food industry for the production of alcoholic beverages as well as for bread fermentation [3]. However, yeasts are a source of enzymes such as: lipases, dehydrogenases, or invertase.

### 3.1. Yeast's lipases

Lipases are widely distributed in nature and are produced by plants, animals, and microorganisms. Microbial enzymes are more useful than the other ones because of the diversity of catalytic activities, simplicity of manipulations, and low cost production (extracellularly during rapid growth on inexpensive media) [66]. Additionally, microbial enzymes are free from problems associated with contamination with hormones, viruses, and can be used in food processing and pharmaceuticals productions for vegetarian or kosher diets [67]. Microbial lipases (EC 3.1.1.3) are suitable enzymes for organic synthesis because they are active toward broad range of nonphysiological substrates and are stable in biphasic systems or pure organic media. Lipases can be applied for either of lipid modifications and synthesis of special compounds: pharmaceuticals, polymers, biodiesels, and biosurfactants [68]. Under physiological conditions, lipases catalyze hydrolysis of ester bond in triacylglycerol to glycerol and free fatty acids. Under nonaqueous conditions, they catalyze the reverse process—esterification. The term transesterification refers to exchange the group between an ester and acid, ester and alcohol, or at least between two esters (Figure 10) [69].

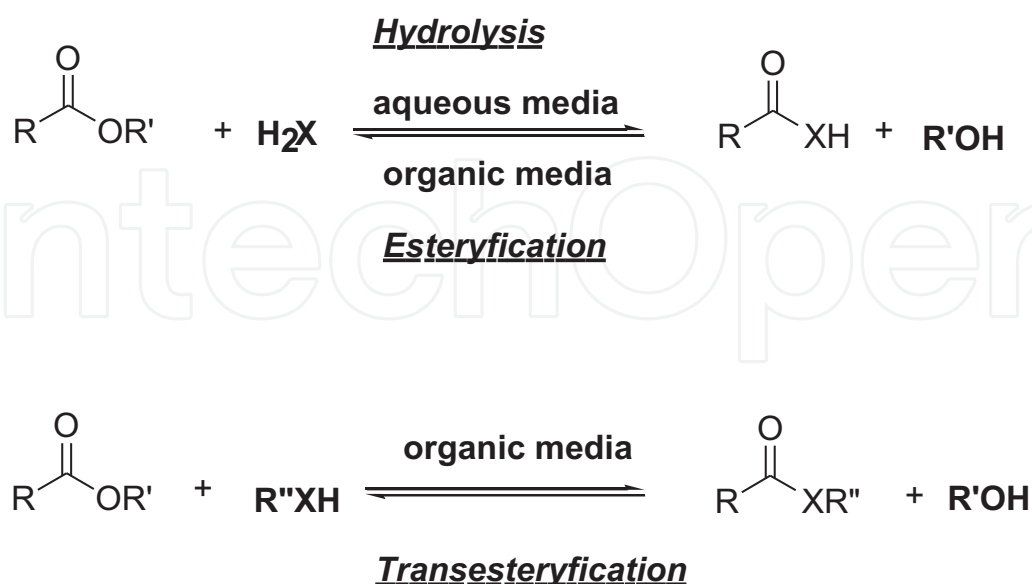


Figure 10. Types of reactions carried out by lipases.



Mentioned features make them significant biocatalyst for various applications. There are a certain number of yeast species able to produce lipases, most of them belong to *Candida* genus: *Candida utilis*, *C. rugosa* (cylindracea), *C. antarctica*, *C. viswanathii*, and additionally, *Yarrowia lipolytica* [70].

### 3.1.1. Hydrolysis

For pharmaceutical industry, lipases are used to resolve racemic mixtures of alcohols or carboxylic acids through asymmetric hydrolysis of acyl derivatives. *Candida antarctica* lipase, isoform B (CAL-B) is one of the most employed psychrophilic lipases for many different applications (kinetic resolutions, desymmetrization, aminolysis, etc.) [71]. Commercial CAL-B is available either in free, lyophilized, and immobilized forms (onto Lewatit VP OC 1600 (poly(methyl methacrylate-co-divinylbenzene)—Novozyme 435, Chirazyme L2-C2). Novozym 435 is a suitable catalyst for both small organic molecules [72, 73] and for polymerization reactions [74, 75]. Also, the immobilization of CAL-B onto different supports may result in different activity and enantioselectivity (Table 1) and may be a tool of control of selectivity of the hydrolysis. This feature was used for the resolution of racemic mixture of 2-O-butyryl-2-phenylacetic acid—precursor of both enantiomers of mandelic acid (Figure 11) and for the enantioselective hydrolysis of 3-phenylglutaric dimethyl diester—precursor in the drug synthesis (e.g., HIV inhibitor) [76]. As it is shown in the Table 1, it is possible to change the enantioselectivity of biocatalyst just by simple replacement of one support material to another one (Figure 11).

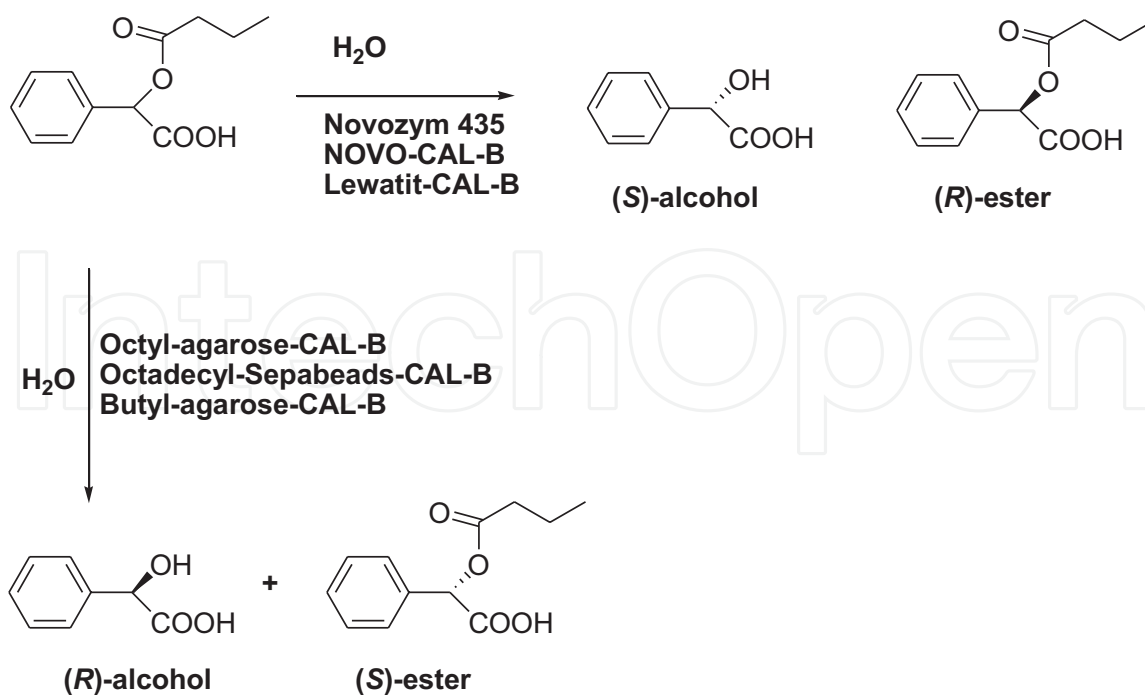
Another possible way to change the enantioselectivity of hydrolysis is the addition of the organic co-solvent to reaction medium. In organic media, the conformation of enzyme appears to be more rigid which may influence the enantioselectivity of the reaction. For the *Candida rugosa*, lipase-catalyzed hydrolysis of various substituted phenoxypropionates, the addition of 30–70% dimethyl sulfoxide (DMSO) or sodium dodecyl sulfate (SDS) improved the enantioselectivity, ( $E = 4$  to  $>100$ ) [77, 78]. For the same lipase (*C. rugosa*), complete

Biocatalyst	Activity $\mu\text{mol g}_{\text{cat}}^{-1} \text{ h}^{-1}$	$ee_p$ (%) <sup>1</sup>	Stereochemical preference	$E^2$
Novozym 435	0.75	>99	S	>100
NOVO-CAL-B	0.56	>99	S	>100
Lewatit-CAL-B	0.55	>99	S	>100
Octyl-agarose-CAL-B	0.19	95	R	49
Octadecyl-sepabeads-CAL-B	0.35	90	R	23
Butyl-agarose-CALB-B	0.16	72	R	7.5

Optical purity of product.

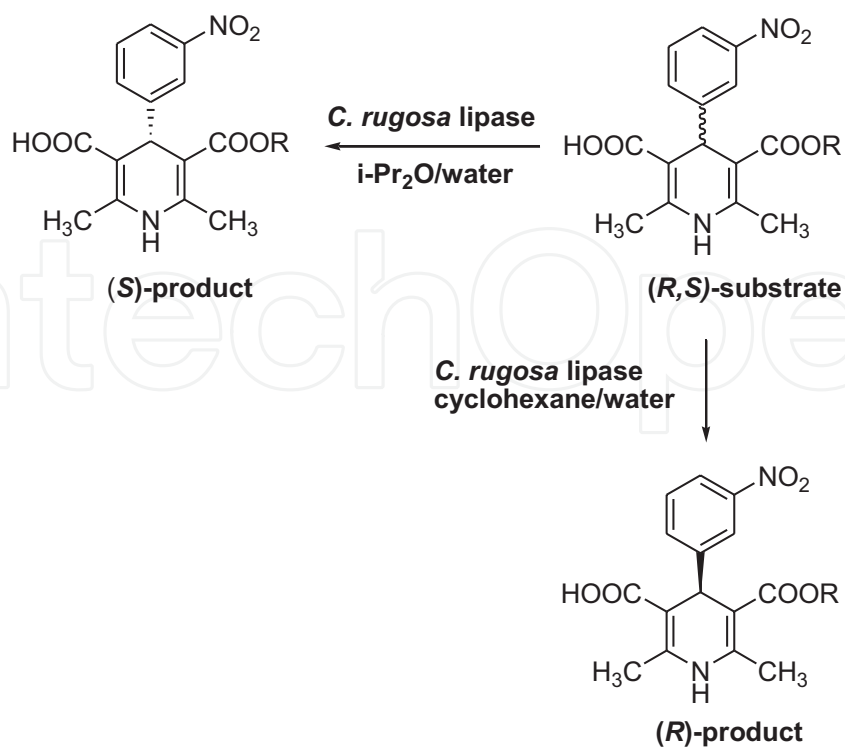
Enantioselectivity of the enzyme towards substrate.

**Table 1.** The influence of different methods of immobilization on the activity and enantioselectivity of CAL-B applied for hydrolytic resolution of 2-O-butyryl-2-phenylacetic acid.



**Figure 11.** Kinetic resolution of 2-O-butryl-2-phenylacetic acid by immobilized CAL-B on different supports.

reversal of enantioselectivity of hydrolysis of 1,4-dihydropyridines was observed in different organic solvents saturated with water [79], this allowed to obtain both enantiomers of 1,4-dihydropyridine (**Figure 12**).



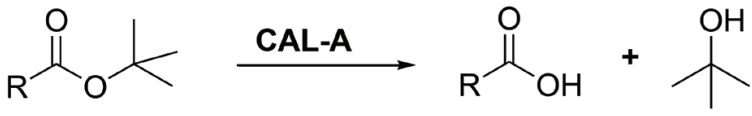
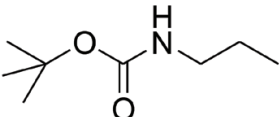
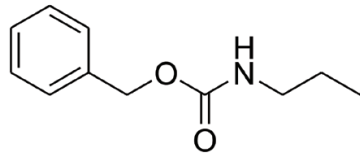
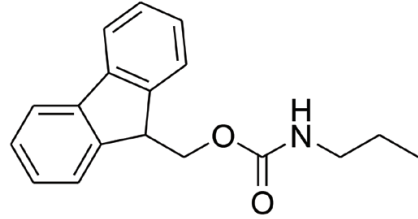
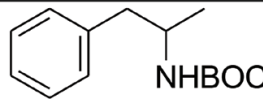
**Figure 12.** Reversal of enantioselectivity in hydrolysis catalyzed by *Candida rugosa* lipase in organic solvent saturated with water [79].

*Candida antarctica* produces two isoforms of lipases (A and B). However, more attention has been directed to the application of CAL-B, but in the last few years, also CAL-A has found remarkable applications. The most surprising biochemical property of CAL-A is its high thermostability of over 90°C [71, 80]. It is quite strange that rather psychrophilic microorganism produces thermostable enzyme. This feature allows using the CAL-A under unique reaction conditions [81] and towards unusual substrates, especially sterically hindered tertiary alcohols and their derivatives or bulky cyclic compounds. Bioconversion of tertiary alcohols can be useful for the removal of *tert*-butyl protecting group even under high temperature (**Table 2**) [82]. Several examples of such applications are summarized in **Table 2**.

Another interesting application of CAL-A is the regioselective hydrolysis of cyclic diacetates, useful building blocks for the synthesis of vitamin D<sub>3</sub> derivatives [83], or hydrolysis of different sterically hindered carboxylic acids [84].

### 3.1.2. Esterification and transesterification

The most important application of lipases in organic synthesis is esterification important for the resolution of racemic mixtures of secondary alcohols and carboxylic acids. Chiral secondary alcohols serve as intermediates for pharmaceutical synthesis [85–87]. Lipase-catalyzed

	
R	Yield (%)
	50
	80
	40
	68

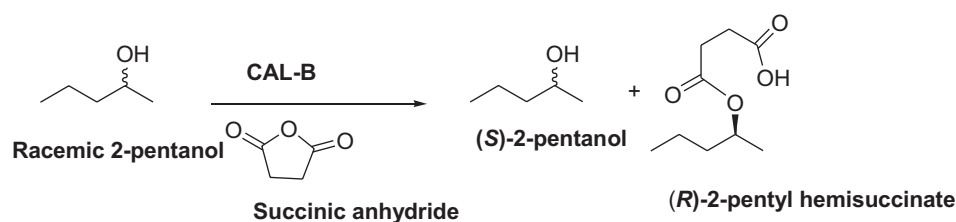
**Table 2.** Examples of substrate structures accepted by CAL-A–protecting group removal.

methods available for the preparation of enantiopure compounds are kinetic resolution (KR), dynamic kinetic resolution (DKR), and desymmetrization. Enzymatic kinetic resolution is based on the difference between the reaction rates of the enantiomers of a racemate at the presence of chiral catalyst—enzyme. Dynamic kinetic resolution combines kinetic resolution with the *in-situ* racemization of the unreacted enantiomer. Racemization can be performed chemically or enzymatically. The kinetic resolution of secondary alcohols and esters is carried out in organic solvents with lipase catalyzed acylation and alcoholysis. It leads to the formation of one enantiomer obtained as an alcohol and the other one as an ester. The maximum theoretical yield for each enantiomer is 50%. *C. antarctica* enzymes are often used for the resolution of secondary alcohols, mostly with bulky group [88, 89] but also for resolution of aliphatic compounds [90]. As an example—CAL-B was applied for the resolution of racemic mixture of 2-pentanol in heptane—media (yield (49,6%) and *ee* >99%) (**Figure 13**). *S*-(+)-2-pentanol is a key chiral intermediate for synthesis of anti-Alzheimer drugs, which inhibit  $\beta$ -amyloid peptide release and/or its synthesis [91, 92].

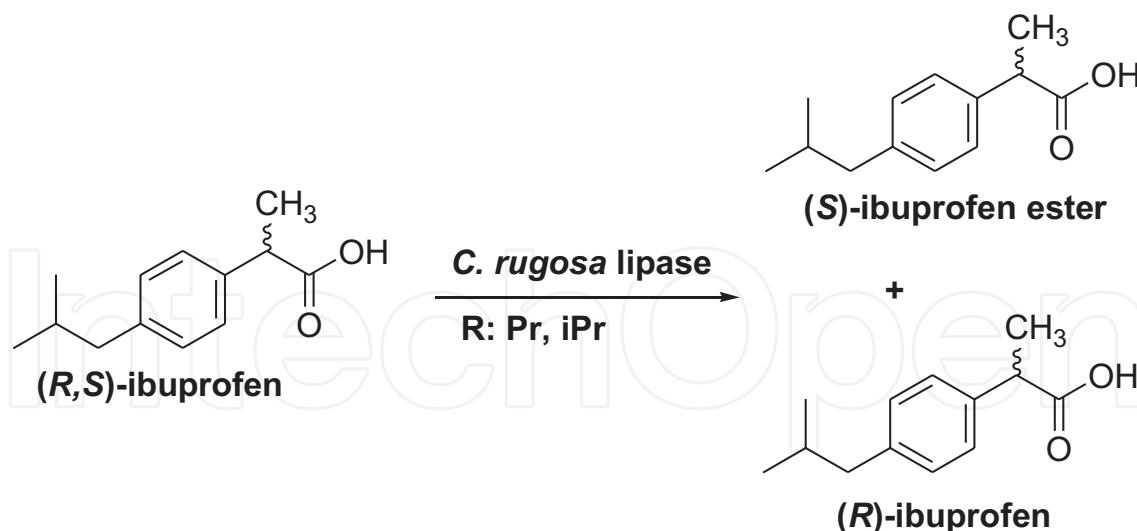
*C. rugosa* is the producer of lipase employed for the resolution of profens (2-aryl propionic acids) in enantioselective transesterification process. Profens are an important group of nonsteroidal anti-inflammatory drugs, and their biological activity depends on the optical purity of the compounds, mainly (*S*)-enantiomer [93]. For instance, (*S*)-ibuprofen ((*S*)-2(4-isobutylphenyl) propionic acid) is 160 times more effective than (*R*)- isomer in the inhibition of prostaglandins synthesis. Optically, pure profens can be synthesized by asymmetric chemical synthesis, catalytic kinetic resolution, and chiral chromatography [69], but enzymatic enantioselective esterification seems to be the best method (**Figure 14**). Discussed reaction was carried out in saturated cyclohexane with 1-propanol or 2-propanol as acyl agents and completed with good conversion degree and excellent enantiomeric excess of (*S*)-ibuprofen [94].

Also, enantiomerically pure amines constitute a class of compounds with possible biological properties and industrial applications [95]. *Candida antarctica* lipase B is one of the most effective catalyst in the preparation of enantiomerically pure nitrogenated compounds (e.g., amines, amides, amino acids, amino alcohols, etc.). This is achieved by enantioselective acetylation [96]. For example, resolution of aminoalkylpyridines was most effective (conversion 50%, time 4h, *ee* of product, and substrate >99%) with the use CAL-B and ethyl acetate as an acyl donor in the *tert*-butyl methyl ether (TBME-medium) (**Figure 15**) [97].

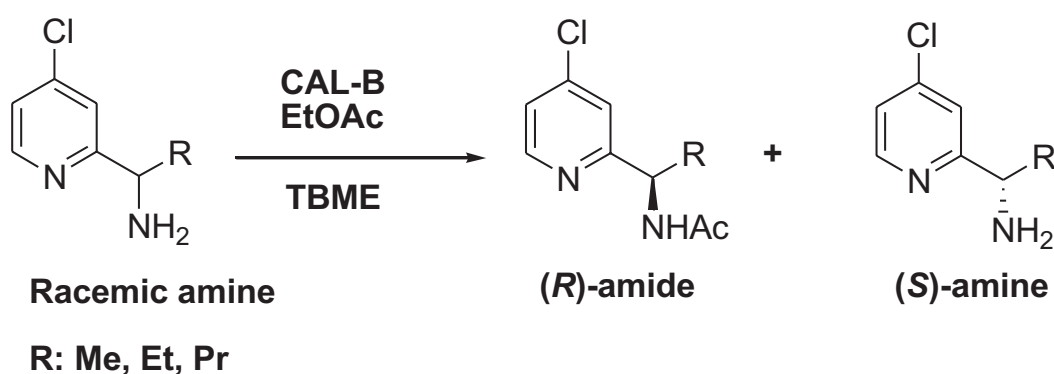
CAL-A isoform of *C. antarctica* lipase is able to selectively acylate cyclic, sterically hindered structures via kinetic resolution (**Figure 16**) alicyclic  $\beta$ -aminocarboxylic acids esters—



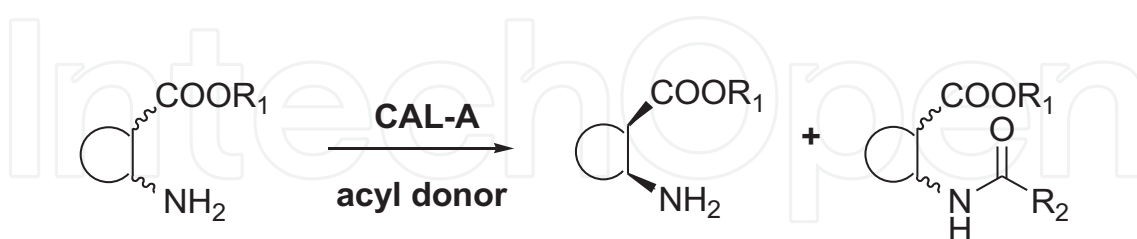
**Figure 13.** Kinetic resolution of 2-pentanol by esterification.



**Figure 14.** Enantioselective esterification of *(R,S)*-ibuprofen.



**Figure 15.** Kinetic resolution chloro-aminopyridines.



**Figure 16.** Kinetic resolution of alicyclic amino acids.

building blocks for the synthesis of various pharmaceutical important heterocycles [81] are synthesized this way. The best activity and enantioselectivity were observed in diethyl ether or diisopropyl ether with 2,2,2-trifluoroethyl hexanoate as an acyl donor.

CAL-A is also active towards sterically hindered tertiary alcohols. This feature is quite unique among hydrolases. The first example of enantioselective kinetic resolution of racemic mixture



of tertiary alcohol was acylation of 2-phenylbut-3-yn-2-ol. The reaction was quite enantioselective, but the yield was rather moderate (25%) because of the steric hindrance. Another interesting application of CAL-A is selective acylation of sterols [98], furyl substituted allyl alcohol [99], or cyanohydrins [100, 101].

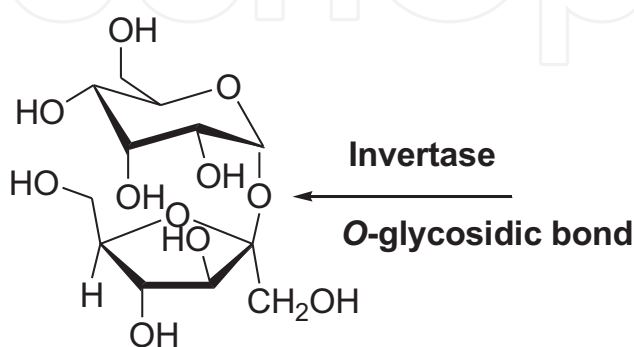
### 3.2. Yeast's invertase

Invertase ( $\beta$ -fructofuranosidase- EC 3.2.1.26) catalyses hydrolysis of the glycoside bond from the terminal nonreducing beta-fructofuranoside side in disaccharide [102]. It is also widely distributed in the environment, mainly in plants and microorganisms. The most important application of invertase is production of invert syrup—equimolar mixture of fructose and glucose, released from sucrose (**Figure 17**), which is used in food and beverage industries. Monosaccharides mixture is sweeter than sucrose and hygroscopic, it mainly is used for production of soft-centered candies and fondants. Invertase is also applied for the manufacture of artificial honey, plasticizing agents for cosmetics, pharmaceutical and paper industries, and enzyme electrodes for the detection of sucrose [103, 104]. Additionally, it can be applied for the synthesis of probiotic oligosaccharides like non-digestible oligosaccharides (NDO), e.g., lactosucrose [105]. Commercially invertase is produced mainly by *Saccharomyces cerevisiae* (Baker's yeast) or *Saccharomyces carlsbergensis*. In yeast cells, invertase is produced either in intracellular or extracellular form [106].

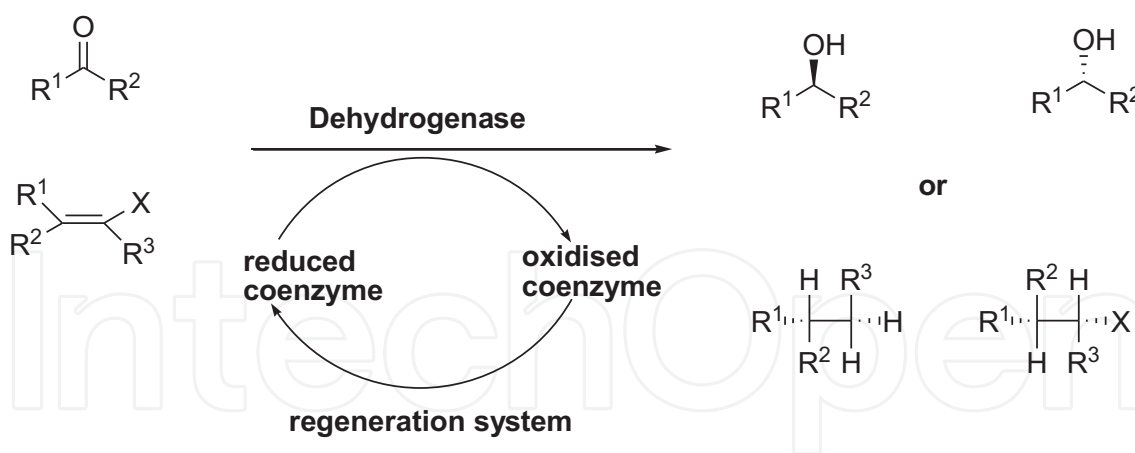
### 3.3. Yeast's oxidoreductases

Enantiometrically pure alcohols including  $\alpha$ - and  $\beta$ -hydroxyesters are important and valuable intermediates in the synthesis of pharmaceuticals and other fine chemicals [107]. Enantioselective ketone reductions are one of the most common methods applied for optically pure alcohols productions. Because reactions catalyzed by dehydrogenases/reductases require cofactors (NADH or NADPH), the use of whole cells rather than isolated enzymes is preferred, to decrease the cost of enzyme purification and cofactor regeneration. However, isolated dehydrogenases employment decreased product purification problems (**Figure 18**).

Generally,  $\alpha$ -ketoesters are reduced with lower enantioselectivities by whole yeast cells, so pair of purified reductases are selected to produce both enantiomers of (S)-ethyl-3-hydroxybutyrate



**Figure 17.** Hydrolysis of sucrose by invertase.

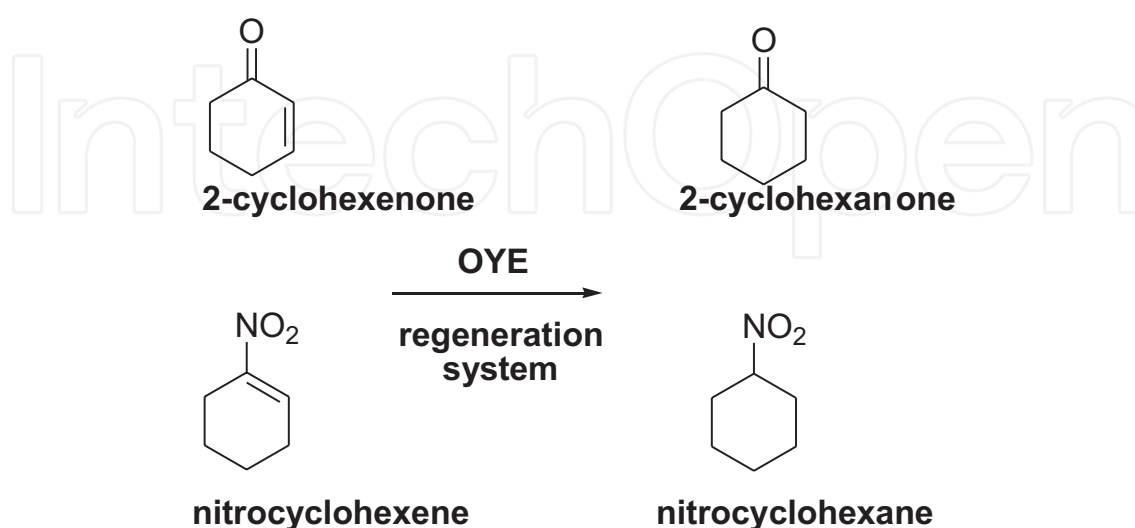


**Figure 18.** Reduction reactions catalyzed by dehydrogenases. Cofactor regeneration circle.

(pharmaceutical building block) in optically pure forms on preparative scale [108]. Reduction of  $\beta$ -ketoesters depends on both structure of substrate and specificity of the enzyme and usually yields in desired enantiomer of high optical purity [109].

*Saccharomyces* are also known as a producers of old yellow enzyme (OYE), the first discovered and characterized flavoprotein [110], which can be used for double bond reduction (**Figure 19**) or for dismutation reactions toward cyclic substrates (**Figure 20**) [111] and also for the reduction of nitrate esters [112] with the addition of coenzyme—NADPH.

Other example of reductase is selective carbonyl reductase from *Candida magnolia*, active toward the structurally different ketones, reduced to the corresponding optically pure (*R*)- aryl and aliphatic alcohols. This enzyme also catalysis reduction of ketones with anti-Prelog enantioselectivity which is an unusual feature of bioreductions (**Figure 21**) [107]. Configuration of obtained aryl alcohols is mostly *R*—enantiomers but also strongly dependent on R group structure (**Figure 21**).



**Figure 19.** Example of reduction of double bonds catalysed by OYE.

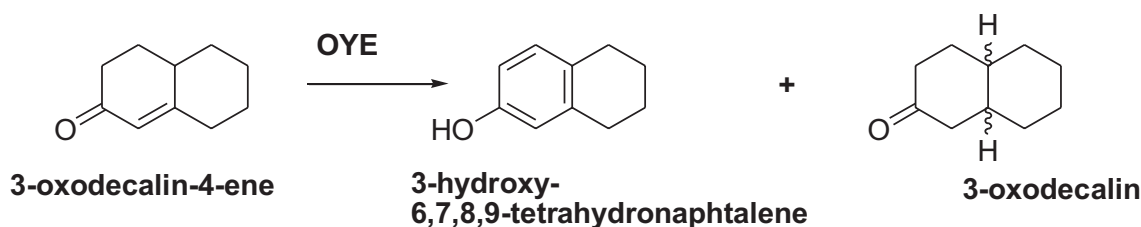


Figure 20. Dismutation reaction catalysed by OYE.

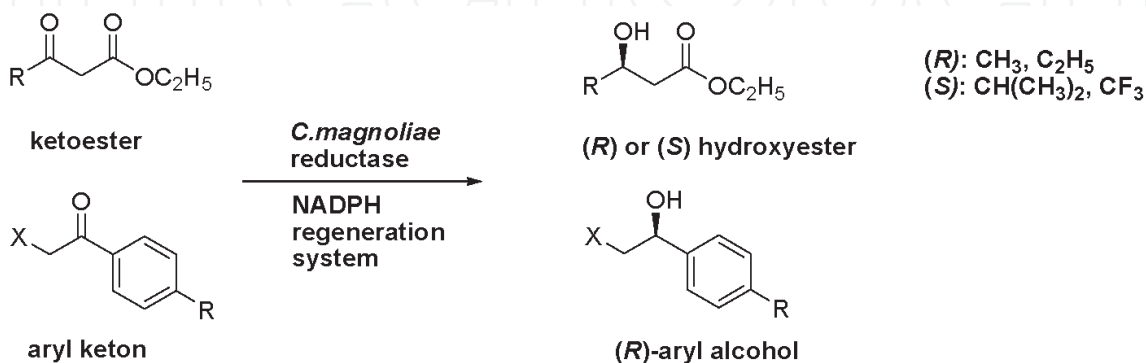


Figure 21. Anti-Prelog reduction of ketones by *C. magnoliae* reductase.

#### 4. Yeast's applications in molecular biology

Yeasts of the *Saccharomyces* genus, in particular *S. cerevisiae*, are one of the fundamental models for eukaryotic organisms, commonly used in genetic and molecular biology studies. *S. cerevisiae* is a unicellular organism that can be grown on defined media, which gives the complete control over its chemical and physical environment. Culturing yeast is simple, economical, and rapid and can be conducted under aerobic and anaerobic conditions. As a nonpathogenic and nontoxic organism, they are safe for laboratory work, without any special precautions. Big accessibility as well as easy culturing on both liquid and solid medium makes yeast cheap and handy organism with significant biotechnological capabilities.

Although yeast and humans have been evolving along separate paths for 1 billion years, still a substantial amount of yeast genes exhibit high homology to mammalian ones. Since the basic cellular mechanics of replication, recombination, cell division, and metabolism are generally conserved between yeast and larger eukaryotes, they constitute a good model for studying different processes such as aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders, and many more [113]. Furthermore, its protein expression systems have more in common with higher organisms than with prokaryotic ones, mainly due to the posttranscriptional and posttranslational processing, which makes it a great candidate for acquiring a number of industrially or medically significant biomolecules, such as recombinant proteins for pharmaceutical purposes [114].

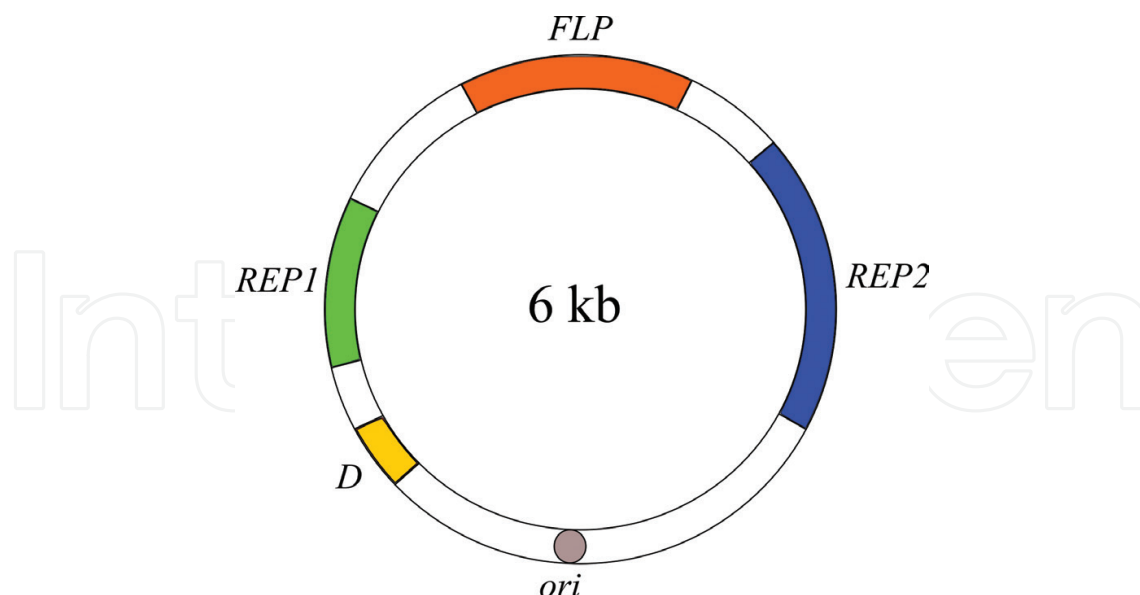
Life cycle of *Saccharomyces cerevisiae* strains include haploid and diploid phase, both of which typically grow asexually by budding. The cell cycle consists of four distinct phases (G1, S, G2, and M) and is regulated in a similar way to that of the cell cycle in larger eukaryotes [115]. Haploid yeast cells can be either mating type *a* or *α* and under normal condition can mate together to generate *a/α* diploids. The diploid cells cannot mate but can reproduce asexually by budding like haploids. However, under specific circumstances, like unfavorable environment conditions (lack of nutrients), diploid cell can undergo meiosis to produce haploid spores. Subsequently, the newly produced haploid nuclei are packaged into four spores that contain modified cell walls, resulting in structures that are very resistant to environmental stress [116]. Each single haploidic spore from tetrad arising after meiosis can be isolated and analyzed by various micromanipulation methods. It provides a unique opportunity to study the coupling between genes among many others. Haploid states cell can be also used for recessive mutation studies, while diploid strains can be exploited for complementation tests.

*S. cerevisiae* have also been first eukaryotes whose genome has been fully sequenced and published in 1996. Its nucleus genome constitutes of 12,068 kb organized into the haploid set of 16 chromosomes ranging in size from 200 to 1600 kb. The characteristic feature is that yeast genome is much more compact in comparison to other eukaryotic relatives, with genes representing 70% of total sequence. It possesses around 5885 potential protein-encoding genes, approximately 140 genes specifying ribosomal RNA, 40 genes for small nuclear RNA molecules, and 275 transfer RNA genes. Currently, an international, multidisciplinary team is involved in the production of 16 chromosomes of *S. cerevisiae* by synthetic biology tools, and the results are expected at the end of the year [117]. Another highly unique and unusual, as for eukaryotes, feature of *S. cerevisiae* genome is the presence of DNA plasmids that enables a variety of genetic manipulations and are of great importance for modern molecular biology [118].

Techniques used for yeast transformation and specific selection have been well described. For this purpose, shuttle vectors are commonly used due to the fact that they can transform both yeast and bacteria, such as *Escherichia coli*. Various yeast strains carry different auxotrophic markers that can be generated by genetic engineering methods, for instance, by gene deletion in amino acid biosynthesis pathways. Scientists have developed a number of bifunctional vectors that are easy to isolate and can autonomously replicate in each yeast and bacteria cells.

#### 4.1. Yeast's plasmid vectors

*Saccharomyces cerevisiae* is a very important microorganism in modern biotechnology, not only for its contribution to brewing and bread-making industry, but also for showing great potential in the field of molecular biology and biomedicine due to its unique form of genetic material and protein expression systems. *S. cerevisiae* is one of very few eukaryotic organisms that contain circular DNA in the form of plasmids. Almost every strain of this yeast has the 2-μm plasmid, which can constitute the outstanding basis for cloning vectors (**Figure 22**), as it is 6 kb in size and is equipped with four following elements: origin of replication; genes *REP1* and *REP2* that code for proteins involved in replication process; *FLP* gene that is utilized by the plasmid to switch between isoforms, and gene *D* which role is not established yet [119]. In order for



**Figure 22.** Genetic structure of 2  $\mu$ m yeast plasmid.

2- $\mu$ m plasmid to work as a fully functional cloning vector, there has to be an incorporated element, called a selective marker, which allows for the transformed cells to be identified after cloning experiment.

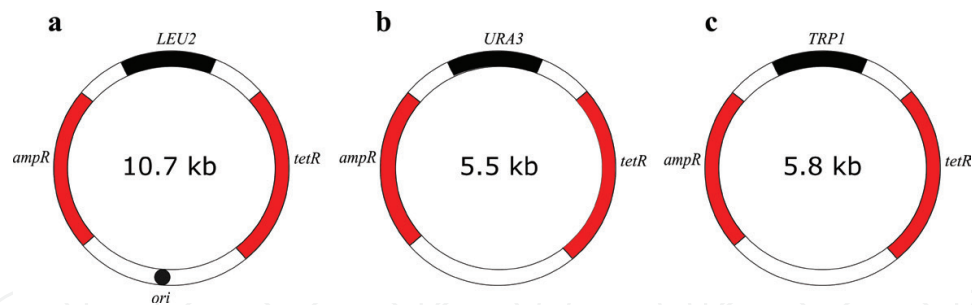
Most of the bacterial vectors are provided with genes-encoding resistance to various kinds of antibiotics, such as ampicillin (*ampR*) or tetracycline (*tetR*). Therefore, upon culturing transformed cells in the medium with the addition of such antibiotic, the colonies that were unable to grow did not carry the resistance gene (hence, the uptake of the vector did not occur); thus, the only cells that survive are the transformed ones carrying the properly inserted vector.

Cloning techniques with yeast differ mostly in the strategic approach of the selective markers. In this case, usually a special kind of organism is required as the host, namely an auxotrophic mutant that is unable to obtain or synthesize a pivotal compound of one of its metabolic pathways. A good example is *leu2<sup>-</sup>* yeast that has an inactive form of *LEU2* gene; hence, it cannot synthesize leucine and can only grow in a medium that is supplied with this amino acid. To properly use that organism as the host, a vector with *LEU2* active gene has to be prepared. The cells are then transformed with the plasmid and cultured in minimal medium that lacks leucine. This way the only colonies that will grow will be the transformed cells [120].

There are few kinds of yeast cloning vectors, but all of them are so-called shuttle vectors, which means that they can replicate and be selected in both bacteria and yeast. Shuttle vectors were developed mostly because plasmid preparation from yeast only is highly ineffective; hence, the large-scale DNA propagation and convenient genetic manipulation are performed in bacterial organism, such as *Escherichia coli*.

Yeast cloning vectors based on 2- $\mu$ m plasmid are called yeast episomal plasmids (YEp) (**Figure 23(a)**). Depending on the kind of YEp, they can either contain most of the 2- $\mu$ m plasmid or just the origin of replication, their backbone is usually constructed from *E. coli* vectors, such





**Figure 23.** Genetic structure of exemplary yeast vectors. *AmpR* and *tetR* are the antibiotic resistance genes from *E. coli* pBR322 plasmid, whereas *LEU2*, *URA3*, *TRP1* fragments represent yeast chromosomal DNA: (a) yeast episomal plasmid YEp13, (b) yeast integrative plasmid YIp5, (c) yeast replicative plasmid YRp7.

as pBR322 that contains genes encoding resistance for antibiotics, like ampicillin or tetracycline. As the name suggests, YEps can replicate independently, or they can be integrated into one of the yeast chromosomes. The most common reason for YEps integration is that they carry selective marker gene, which is very similar to the mutant chromosomal DNA of the host organism; for example, the already mentioned *LEU2* gene or others, such as *URA3*, *HIS3*, *TRP1*, or *LYS2* all involved in biosynthesis pathways of pyrimidine nucleotides, histidine, tryptophan, or lysine, respectively. YEps are considered as high copy number vectors, yielding up to 200 copies per cell with the transformation frequency between 10,000 and 100,000 transformed cells per  $\mu\text{g}$ . Unfortunately, the major drawback is that their recombinants are highly unstable, which makes it very difficult and time consuming to achieve conclusive and reliable results when working with YEps.

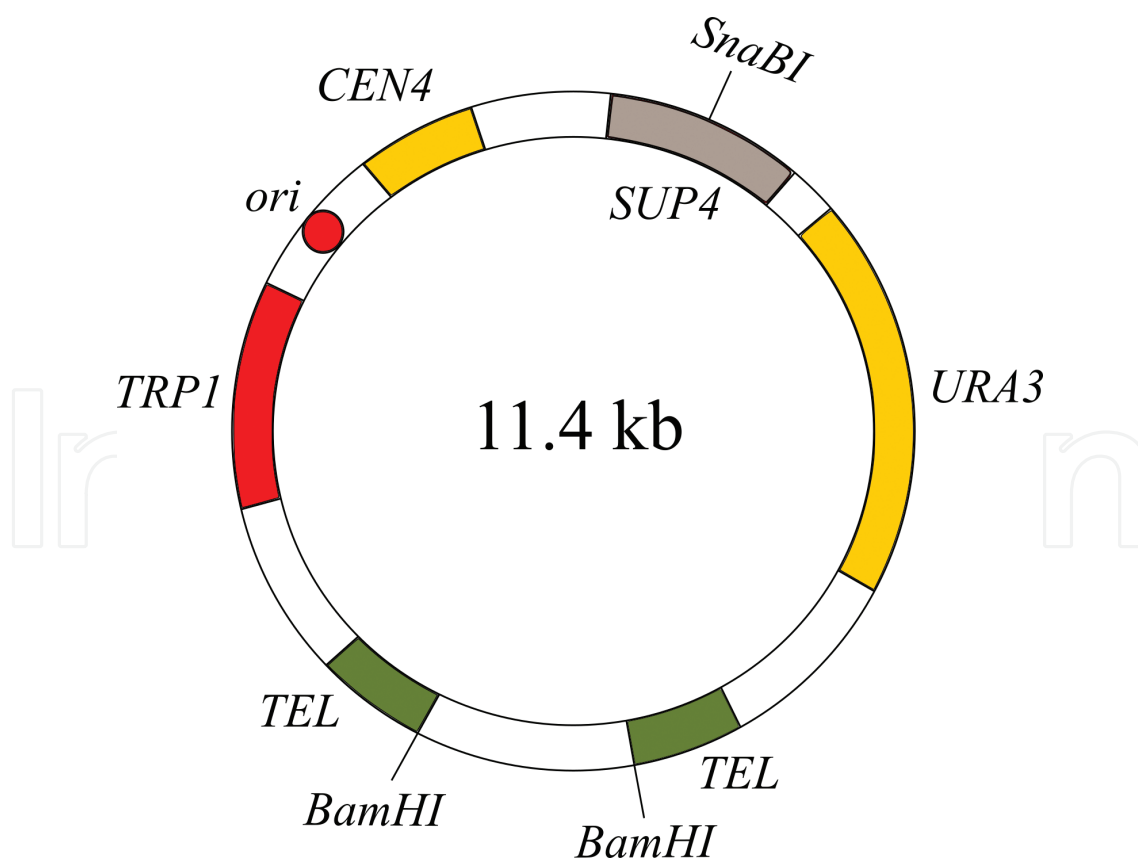
The other important type of yeast vectors are yeast integrative plasmids (YIps) (**Figure 23(b)**). They mainly consist of *E. coli* plasmid, such as pBR322 and a selective marker (usually one of the mentioned above). What is important is they do not contain yeast origin of replication; therefore, they cannot replicate in any other way than through the process of integration with chromosomal DNA. In terms of transformation frequency, YIps come at the very last place with the number significantly lower than 1000 transformed cells per  $\mu\text{g}$  and usually only one copy per cell. On the other hand, their recombinants are very stable, usually making them the top pick for the experimental purposes.

Yeast replicative plasmids (YRps) (**Figure 23(c)**) are another type of yeast cloning vectors. They contain a backbone from *E. coli* vector, the yeast origin of replication in close proximity to the selective marker. Such structure suggests that YRps can replicate independently with relatively high transformation frequency ranging from 1000 to 10,000 transformed cells per  $\mu\text{g}$  and a number of copies between 5 and 100 per cell. YRps recombinants are as unstable as the YEps ones, making them one of the last choice for laboratory work.

Along with the time, there was a growing demand for much larger pieces of DNA to be manipulated through the techniques of genetic engineering. It was at this point that the last type of yeast cloning vectors was developed, namely the yeast artificial chromosomes (YACs). The general idea behind those constructs was that yeast chromosomes usually carry several hundred kilobases of genetic material, so why not imitate the native DNA? YACs were thought to contain the three key elements of a chromosome:

- centromeres required for the proper chromosome positioning during the cell division;
- origins of replication, which are the places on chromosome where the replication of genetic material starts;
- telomers as the defenders of the chromosomes against exonucleases.

Several types of YACs have been developed over the years, they usually consist mostly of a *E. coli* pBR322 plasmid with some yeast genes, such as selective markers (usually at least two located oppositely), origin of replication, gene *CEN4* coding for centromere region, the two telomeres fragments *TEL*, and an additional selective marker with a restriction enzyme site, such as *SUP4* gene that compensates for a mutation-causing accumulation of a red pigment (**Figure 24**). YACs are equipped with a restriction enzyme site between two *TEL* sites, so that upon cleavage, an “artificial” linear chromosome is created, subsequently *SUP4* is cut, and the chromosome is divided into two arms, between which a DNA fragment to be cloned is ligated, thus recreating the single-line chromosome structure. The next step is to transform double auxotrophic mutant organisms that will not be able to survive in the minimum medium without the properly received YAC. Additional experimental control can be then tested by simple optical inspection—colonies with disrupted *SUP4* gene will appear as white, meaning they are transformed, any other color means that the colony has not been properly transformed [121].



**Figure 24.** Genetic structure of yeast artificial chromosome pYAC3.

## 4.2. Yeast expression system

Recombinant proteins are the biomolecules of great importance, because among other things, they are able to mimic the functions of native proteins; hence, they are extensively studied in biotechnology and biopharmaceutical research. The critical point of target protein production is the choice of efficient expression system which enables obtaining functional product with high yield.

Yeast expression system constitutes a good alternative for widely used bacterial and higher eukaryote expression systems. They are genetically well defined and are known to perform many posttranslational modifications, including proper protein folding, disulfide bond formation, and glycosylation [122]. The culturing of yeast is also easy, rapid, and cheap, which is their big advantage over the insect or mammalian cells. They easily undergo genetical manipulation and adapt to fermentation processes; therefore, using yeasts as a cell factory is convenient and enables to obtain a fair amount of target protein. In contrast to bacteria, recombinant proteins obtained in yeast expression systems are free of endotoxins that make this system safer, especially in terms of medical and food application [114]. In fact, about 20% of all biopharmaceuticals are produced by *S. cerevisiae*. Among them, the most dominant are insulin, human serum albumin, hepatitis vaccines, and virus-like particles used for vaccination against human papillomavirus [123].

However, yeast cells are limited in the production of human-like glycoproteins by their inability to produce complex *N*-linked glycans. In addition, *S. cerevisiae* produce the hypermannosylated *N*-linked glycans with the mannose residues being attached to the chitobiose core (a dimer of  $\beta$ -1,4-linked glucosamine units). Hypermannosylation also results in a short half life *in vivo* and thereby compromises the efficacy of most therapeutic glycoproteins [124]. To overcome this issue, great deal of effort has been put into altering the glycosylation pathways in *P. pastoris* to produce strains possessing human-like *N*-glycosylation patterns [125, 126]. This achievement has contributed to the increased usefulness of yeast in industry for the production of stable and recombinant glycoproteins.

There is no ultimate procedure for yeast expression system that could work equally well for the production of all kinds of proteins. Optimization of whole process is the critical step to obtain sufficient amounts of pure, properly folded and secreted protein of interest. While small and simple in structure proteins are easy to obtain, the big and multi-domain protein could require certain chaperones to facilitate the folding process [127]. The advantage of yeast expression system is that it allows extracellular secretion of produced protein when proper signaling sequence has been attached to the structural gene [114]. It significantly facilitates the recombinant protein purification process from the culturing medium and allows to optimize the culturing conditions. In order to increase protein secretion level, a few strategies have been developed. One of them is protein engineering of a desired product, for instance by modifying protein coding sequences and signaling sequences [128–130]. Since this methodology is highly specific against each protein, the conditions optimized for one protein do not always work for another. Different approach is to engineer the host strains and tune-up folding and secretory machinery by overexpression or deletion genes that are critical for the

protein secretion [131, 132]. Additionally, it has been shown that expression in low temperatures enhances the level of secretion [133].

There are numerous varieties of expression vectors available for producing heterologous proteins in yeast, and these are the derivatives of YIp, YEp, and YRp plasmids described previously. The DNA coding for the protein of interest is inserted into the vector. The type of selective marker and promoter strength are key factors that determine the plasmid copy number and the mRNA level of the recombinant protein. Varieties of inducible and constitutive promoters have been applied for gene expression in yeasts in the past. The first of these allow the controllable gene expression. Most of inducible promoters are responsive to catabolite repression or react to other environmental conditions, like in-cell iron concentration, stress, or lack of essential amino acids. *GAL* promoter, which is induced by adding galactose, provides a straightforward system for expression regulation of the cloned foreign gene [134]. Another good example can be *CUP1* promoter, which is induced by copper [135] or heat shock factor promoter, induced by heat stress at 39°C [136]. There are also some other groups of promoters that initiate strong and constitutive expression. *TEF1* promoter, as an example of *S. cerevisiae*, is a widely used representative of this group, as it can drive high gene expression in both high and low glucose conditions [137]. Selection of a suitable promoter depends on specific process requirements and the properties of the target protein to be produced.

Additionally, yeasts are recognized as a generally recognized as safe (GRAS) organism, which only strengthens its position as the most frequently used microbial eukaryote for recombinant protein synthesis.

#### 4.3. Yeast's two hybrid system

Ever since the Field and Song discovery described in 1989, a new approach toward the examination of protein-protein interactions emerged, it was named as the yeast two-hybrid system. It allows to detect the interaction of two proteins in the yeast cell, and it can be used to select an interacting partner of a known protein. This technique takes the advantage of the fact that majority of eukaryotic transcriptional factors, such as Gal4p, consist of two independent, functional DNA domains: binding domain (BD) and transcription activation domain (AD). While the two domains are normally on the same polypeptide chain, the transcription factor also functions when these two domains are brought together by noncovalent protein-protein interactions. In yeast hybrid system, each of these domains is connected to the one from the studied protein. As a result, two fusion proteins are created: one combined to DNA-binding domain (BD) and the other joined to activation domain (AD) [138]. The genes coding for both fusion proteins are carried by different plasmids, but each plasmid undergoes expression in the same yeast cell. If the interaction between studied proteins occurs, BD and AD domains are close enough to activate transcription of a reporter gene that is regulated by the transcription factors.

General idea of the yeast two-hybrid system can be represented by an example of transcriptional factors and a gene coding for  $\beta$ -galactosidase, wherein the interaction between studied proteins may potentially lead to the expression of the reporter gene coding for  $\beta$ -galactosidase in *E. coli lacZ* reporter gene. The presence of this enzyme can later be verified by simple reaction with X-gal, which yields a blueish insoluble product, thereby confirming or denying the

association of studied proteins. Since 1989, yeast two-hybrid system has been studied extensively and further developed to find countless new applications, some of which are summarized and generally described in Ref. [139].

## 5. Conclusions

Discussed unique yeasts features, which are fundamental for their versatile applications are still examined and after finishing the “Synthetic Yeast Genome Project (Sc2.0)” the new perspectives of the applying them will be opened as well as in the molecular biology and in the industrial applications.

## Acknowledgements

The project was supported by the Wrocław Center of Biotechnology program, the Leading National Research Center (KNOW) for the years 2014–2018.

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