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Genetically Modified Baker's Yeast *Saccharomyces cerevisiae* in Chemical Synthesis and Biotransformations

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1. Introduction

Yeast *Saccharomyces cerevisiae* has been associated with human beings for more than 6000 years due to its use in food production, baking, wine and beer making. Potable and industrial ethanol production constitutes the majority of use of *S. cerevisiae* in biotechnological applications. However, baker's yeast also plays an important role as a model organism in the field of biochemistry, genetics and molecular biology. *S. cerevisiae* was the first eukaryotic organism to be sequenced in 1996 [Goffeau et al., 1996], and is clearly the most ideal eukaryotic microorganism for biological studies. Furthermore, the ease of genetic manipulation of yeast allows it to be used for analyzing and functionally dissecting gene products from other eukaryotes. The field of metabolic engineering, which utilizes genetic tools to manipulate microbial metabolism to enhance the production of compounds of interest has a particularly strong impact by providing new platforms for chemical production and facilitating the expansion of industrial (white) biotechnology [Nevoigt, 2008]. Baker's yeast can also be used as host organism for novel production of some industrially relevant chemicals. Our review focuses on the progress that has been achieved in the production of fine chemicals, bulk chemicals and fuels by genetic manipulation of the enzymatic activity of yeast, the combining of enzyme pathways from different microorganisms into *S. cerevisiae* and expressing genes from *S. cerevisiae* in other hosts. Attention to biotransformations catalyzed by genetically modified yeast will also be considered.

2. Bioethanol production

Bioethanol is usually obtained from the conversion of carbon based renewable feedstock and can be used as a fuel for vehicles in its pure form or as a gasoline additive to increase octane rating and improve vehicle emissions. Bioethanol is primarily produced by fermentation of sugar or the sugar components of starch. However, there has been constant research on its production from fibrous substances such as cellulose and hemicelluloses, which make up the bulk of most plant matter. Two chemical reactions take place during biomass conversion to ethanol: the hydrolysis of complex polysaccharides in the raw feedstock to simple sugars followed by their subsequent fermentation to ethanol. The second step of bioethanol production is caused by yeast or bacteria which feed upon the sugars. Therefore high ethanol

yields from lignocellulosic biomass from agricultural and agro-industrial residues are dependent upon efficient hydrolysis of sugar polymers and utilization of all the available sugars including D-glucose, D-xylose, L-arabinose and other fermentable compounds.

S. cerevisiae, which plays a traditional and major role in industrial bioethanol production, has several advantages due to its high ethanol productivity as well as its high ethanol tolerance. However, baker's yeast cannot hydrolyze cellulose and is not able to use pentoses, which constitute up to 20% of lignocellulosic biomass. Many studies regarding the use of *S. cerevisiae* in metabolic engineering for xylose utilization have been reported and several reviews have been published [Chu & Lee, 2007; Hahn-Hägerdal et al., 2007; Matsushika et al., 2009]. The first step of D-xylose metabolism is its direct isomerization to D-xylulose catalyzed by bacterial xylose isomerase XR (EC 5.3.1.5) or stepwise transformation in yeast cells, firstly to xylitol (xylose reductase XR EC 1.1.1.21) and then to D-xylulose (xylitol dehydrogenase XDH EC 1.1.1.19). After phosphorylation of D-xylulose to D-xylulose-5-phosphate (xylulokinase XK EC 2.7.1.17) further metabolism proceeds via a pentose phosphate pathway. Different strategies have been applied to engineering yeast including the introduction of initial xylose metabolism and xylose transport. However, these change the intracellular redox balance and result in over-expression of xylulokinase and further metabolism via a pentose phosphate pathway. Nevertheless they are insufficient for industrial bioprocesses mainly due to a low rate of reaction as compared with glucose fermentation [Kondo et al. 2010; Young et al., 2010].

3. Yeast cells as microbial chemical factories

Many plant secondary metabolites (i.e. small molecules, with complicated structures which are not involved in basic metabolic pathways and are not directly essential for photosynthetic or respiratory processes) have been identified as having beneficial effects on human health or nutrition, but their chemical synthesis is generally laborious and their isolation from natural sources is a difficult process with low yields. Furthermore, there is a wide range of chemicals essential in many industries as substrates in production processes or compounds necessary for appropriate process progress, that are produced by many microorganisms with low efficiency or during chemical synthesis, which is nowadays considered as non-ecological.

Baker's yeast can produce a diverse array of secondary metabolites [Pscheidt & Glieder, 2008], therefore the metabolic engineering of microorganisms is a new area of effective biosynthesis of these compounds and yeast is an important and attractive host for the heterologous and function expression of foreign genes encoding many important secondary metabolites of plants. Moreover baker's yeast possesses GRAS status, which is an advantage in the production of compounds that are intended for human consumption (e.g. the formation of antioxidants and aroma compounds during wine fermentation). A few examples in which modified *S. cerevisiae* were used as a whole-cell factories in production of chosen chemical compounds are presented in this chapter.

3.1 Alcohols (other than ethanol)

3.1.1 Butan-1-ol

Butan-1-ol can be regarded as superior biofuel to ethanol because of its greater hydrophobicity, higher energy density and the possibility of mixing with gasoline and

transporting through existing pipeline infrastructure. Microbial butanol production is performed by members of the genus *Clostridium* [Inui et al. 2008]. The butanol pathway is expressed in *S. cerevisiae* from a range of organisms (*Escherichia coli*, *Clostridium beijerinckii* and *Ralstonia eutropha*) and owing to the tolerance of baker's yeast to alcohols, the production of butanol is raised ten-fold to 2.5 mg dm⁻³. The most productive strains harbour the *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase, which uses NADH as a co-factor, and the acetoacetyl-CoA transferase from *S. cerevisiae* or *E. coli* [Steen et al., 2008].

3.1.2 Glycerol

Glycerol is considered to have a positive effect on the sensory properties of wine. Its concentration in wine varies between 1 - 15 g/dm³. Many growth and environmental factors have been reported to influence the amount of glycerol produced by yeast in wine. In *S. cerevisiae* metabolism glycerol is a byproduct of the fermentation of sugar to ethanol. It is synthesized in the cytosol from dihydroxyacetone phosphate in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (GPDH) and glycerol-3-phosphatase (GPP) respectively. The former is the key enzyme in glycerol production [Wang et al., 2001]. More recently, genetic engineering approaches have been successful in redirecting the carbon flux towards glycerol. GPDH, a limiting enzyme for glycerol formation, is encoded by *GPD1* and *GPD2* genes. Overexpression of *GPD1* in a laboratory strain of baker's yeast and in a haploid strain V5 resulted in a marked increase in glycerol production at the expense of ethanol. Up to 28 g of glycerol per liter was formed by an engineered *S. cerevisiae* strain under conditions simulating wine fermentation [Remize et al., 1999].

Nowadays, the demand for glycerol is restricted due to the large quantity of glycerol generated during biodiesel production, so this direction of study is impractical.

3.1.3 Propane-1,3-diol

Propane-1,3-diol (1,3-PD) has numerous applications in production of polymers for cosmetics, foods, lubricants and medicines. Recently, there has been a strong industrial interests in a new kind of polyester, poly(trimethylene terephthalate), with 1,3-PD as a monomer. Nevertheless, its availability is restricted owing to its expensive chemical synthesis.

Non-modified *S. cerevisiae* can produce glycerol from D-glucose but cannot synthesize 1,3-propanediol. By taking advantage of genetically engineered *S. cerevisiae* 1,3-PD production is possible in two ways [Celińska, 2010]. One is to clone the *yqhD* gene from *E. coli* and *dhaB* gene from *K. pneumonia* required for the production of propane-1,3-diol from glycerol, and to integrate them into the chromosome W303-1A of *S. cerevisiae* by the *Agrobacterium tumefaciens* genetic transfer system. Both the *yqhD* and *dhaB* genes function in the engineered *S. cerevisiae* and lead to the production of 1,3-propanediol from D-glucose, but the amount of 1,3-PD is relatively small, due to low availability of glycerol in the reaction medium [Rao et al., 2008]. An alternative method of microbial 1,3-PD synthesis is described by Mendes et. al. using two recombinant microorganisms. The first step of the process is the conversion of sugar into glycerol by the metabolic engineered *Saccharomyces cerevisiae* strain HC42 adapted to high glucose concentrations (> 200 g dm⁻³). The second step, carried out in the same bioreactor, is performed by the engineered strain *Clostridium acetobutylicum* DG1 (pSPD5) which converts glycerol to propane-1,3-diol [Mendes et al., 2011].

3.2 Organic acids – Pyruvic, lactic and malic

Saccharomyces cerevisiae does not naturally produce organic acids in large amounts, but its robustness and pH tolerance make it an excellent microorganism for researches in this field [Abbott et al., 2009]. The first attempts involved blocking the ethanol formation via deletion of four structural genes for alcohol dehydrogenase. Better results were obtained by eliminating the pyruvate decarboxylase (PDC) activity. Deletion of all three PDC genes completely eliminated alcoholic fermentation. Aerobic fermentation at pH 5 yielded pyruvic acid (fig. 1) at a concentration of approximately 135 g dm⁻³ [Ischida et al., 2006]. The strategy for lactic acid (fig. 1) production consisted of two steps – deletion of one or more of three functional genes encoding pyruvate decarboxylase and the introduction of the bovine lactate dehydrogenase (L-LDH) in the genome under the control of PDC1 promoter.

Two enzymes, pyruvate carboxylase and malate dehydrogenase, produced by *S. cerevisiae* are involved in L-malic acid accumulation (Fig. 1). Overexpression of cytosolic malate dehydrogenase (MDH2) caused up to a 3.7 fold increase in L-malic acid production and an elevated accumulation of fumaric and citric acids [Pines et al., 1997]. Alternative way of L-malic production, including biotransformations, will be described in the chapter 4.4.

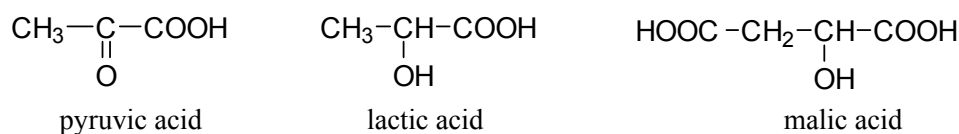


Fig. 1. Organic acids produced by genetically modified *S. cerevisiae*

3.2.1 Sugar alcohols

Sugar alcohols (polyols, Fig. 2) can be synthesized from carbohydrates as a result of carbonyl group reduction to a hydroxyl one and can be used as a sweeteners. Xylitol is the most popular one, due to its lower energy value than sucrose and the comparable sweetening power.

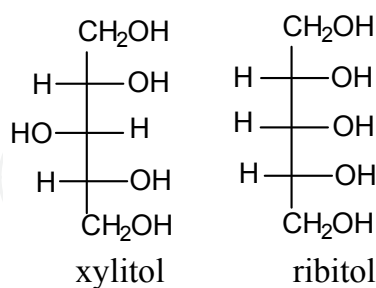


Fig. 2. Sugar alcohols

Toivari et al described recombinant *Saccharomyces cerevisiae* strains that produce xylitol and ribitol from D-glucose in a single fermentation step. 8.5-fold enhancement of the total amount of the excreted sugar alcohols was achieved via expression of the xylitol dehydrogenase-encoding gene *XYL2* of *Pichia stipitis* in the transketolase-deficient strain of *S. cerevisiae*. The additional introduction of the 2-deoxy-glucose 6-phosphate phosphatase-encoding gene *DOG1* into the transketolase-deficient strain expressing the *XYL2* gene induced further 1.6-fold increase in ribitol production [Toivari et al., 2007]

3.3 Lipid compounds

3.3.1 Fatty acids

Fatty acids are of considerable interest due to their pharmaceutical and nutritional values. These compounds are also necessary for cellular functions such as regulation of membrane fluidity. *Saccharomyces cerevisiae* are able to synthesize *de novo* only some saturated and monounsaturated fatty acids, mainly the C-16 and C-18 acids [Daum et al., 1998]. To reconstitute other long-chain and polyunsaturated fatty acids it is necessary to introduce genes of suitable enzymes such as desaturases and elongases eg. *A. thaliana* oleate desaturase gene (*FAD2*) or fatty acid desaturase from the fungus *M. alpina*. The simultaneous expression of Δ^{12} -desaturase and Δ^6 -desaturase from *M. alpina* resulted in an increase in the content of γ -linolenic acid (18:3) to 8% of total fatty acids in yeast cells [Veen & Lang, 2004].

Due to the important roles of polyunsaturated fatty acids (PUFAs) in human health and nutrition the effect of overexpression of cytochrome b5 genes on fatty acid desaturation has been explored. The modification does not affect the fatty acid synthesis very much, but significantly enhances the synthesis of PUFA at 30 °C [Yazawa et al., 2010]. A number of desaturases from different sources (e.g. *Mucor rouxii*, *M. alpina*) have been functionally expressed in *S. cerevisiae* with a view to attain PUFAs formation [Chemler et al., 2006].

3.3.2 Isoprenoids

Isoprenoids (terpenoids) are ubiquitous in nature. They are a structurally diverse group of compounds and range from essential cell components to unique secondary metabolites. Terpenoids are based on combinations of isoprene units (C_5H_8), and their carbon skeleton is multiples of five carbon atoms e.g. monoterpene (10-carbon), sesquiterpene (15-carbon), diterpene (20-carbon) and carotenoids (40-carbon). Biosynthesis of these compounds proceeds via formation of isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Fig. 3).

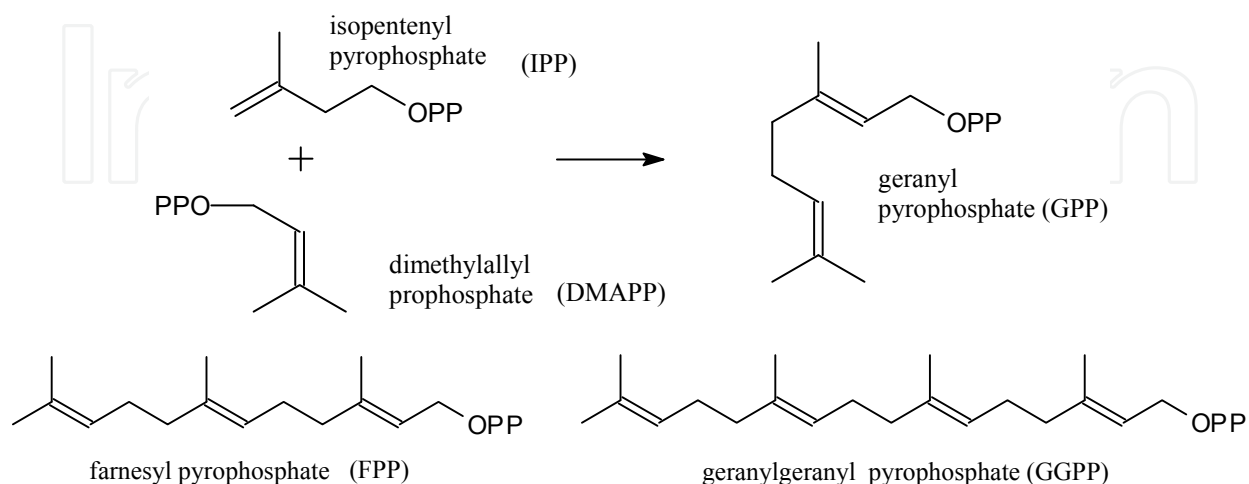


Fig. 3. Some terpene alcohols pyrophosphates – precursors of other terpenoids (OPP-pyrophosphate group)

Isoprenoids exist in low concentrations in their host organisms. They are complex molecules with a specified stereochemical structure, which makes them difficult to synthesize by chemical methods. Therefore, these compounds are of particular interest for microbial production. *E. coli*, *A. thaliana* and *S. cerevisiae* are the organisms with the most advanced genetic tools and are applied as heterologous hosts for terpenoids production, but they usually have very limited isoprenoid secondary metabolism minimizing competing metabolic fluxes.

Phosphorylated derivatives of geraniol (monoterpene) and geranylgeraniol (diterpene) are important molecules in the synthesis of various isoprenoids. (*E,E,E*)-geranylgeranyl pyrophosphate (GGPP) is an intermediate in carotenoids and ubiquinone biosynthesis [Tokuhiko et al., 2009] and was synthesized in engineered *S. cerevisiae* as a result of enhancement of the mevalonate pathway and redirection of carbon flux through overexpression of several key enzymes. The production achieved was 70.9 mg g⁻¹ of dry cell weight (3.31 g dm⁻³), which is very high in comparison to the production of other isoprenoids reported in the literature (e.g. 5.9 mg g⁻¹ for carotene) [Verwaal et al., 2007].

Commercially important terpenoids are artemisinin and paclitaxel, so much work has focused on the introduction of exogenic metabolism in yeast to synthesize the precursors of these compounds [Chang & Keasling, 2006; Huang et al., 2008].

Artemisinin, an endoperoxide sesquiterpene lactone (Fig 4), was originally isolated from the chinese plant *Artemisia annua*. Its derivatives are now a group of drugs used worldwide for the treatment of malaria by means of artemisinin-combination therapies (ACTs). Engineered yeast systems are able to produce artemisinin or its precursor (artemisinic acid), which can be converted to artemisinin via chemical reactions (Fig. 4) [Shiba et al., 2007; Arsenault et al., 2008].

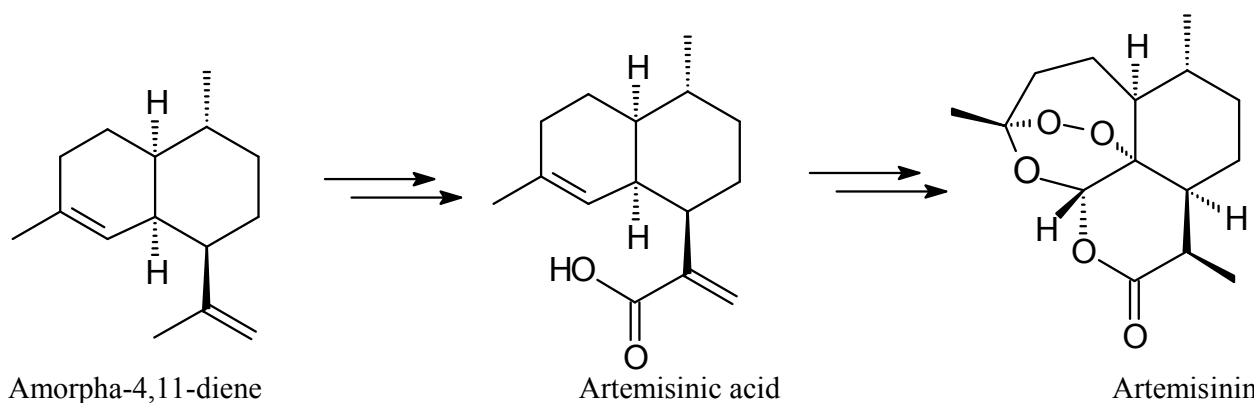


Fig. 4. Synthesis of artemisinin [Ro et al., 2006]

Ro *et al.* cloned a gene from *A. annua*, which codes a cytochrome P 450, enabling the transformation of amorpha-4,11-diene to artemisinic acid, which in turn was converted to artemisinin by chemical synthesis [Ro et al., 2006]. In order to achieve a higher yield of amorpha-4,11-diene (Fig. 4), which is synthesized by cyclization of farnesyl pyrophosphate Shiba *et al.* engineered the pyruvate dehydrogenase bypass in *S. cerevisiae* [Shiba et al., 2007].

A partial biosynthesis of paclitaxel (trade name taxol, Fig. 5) – terpenoid, which is widely used in cancer therapy and produced as a secondary metabolite of yew trees (*Taxus sp.*), was

constructed in *S. cerevisiae*. Dejong reported the functional expression of eight taxoid biosynthetic genes from *Taxus brevifolia* in yeast, elevating the accumulation of intermediate taxadiene [Dejong et al., 2006].

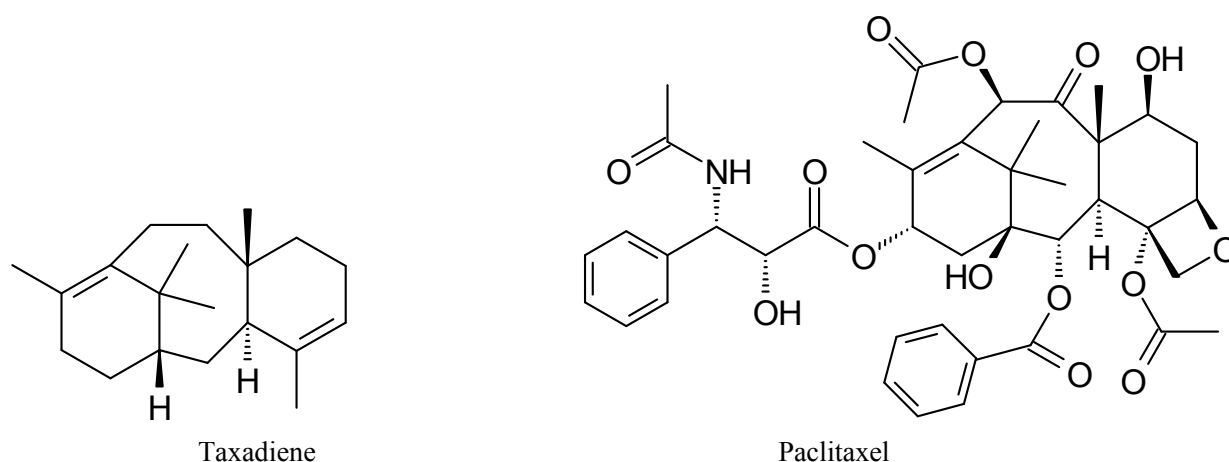


Fig. 5. Taxadiene and paclitaxel [Dejong et al., 2006]

Another way of synthesis of taxol building blocks is the biotransformation catalyzed by modified whole cell *S. cerevisiae* catalysts. This will be described in chapter 4.1.

3.3.3 Steroids

Steroids are the other group of compounds originating from the terpenoids precursors dimethylallyl pyrophosphate and isoprenylpyrophosphate (Fig. 3). One of them, hydrocortisone (Fig. 6), is an important starting material for synthesis of drugs with anti-inflammatory effect.

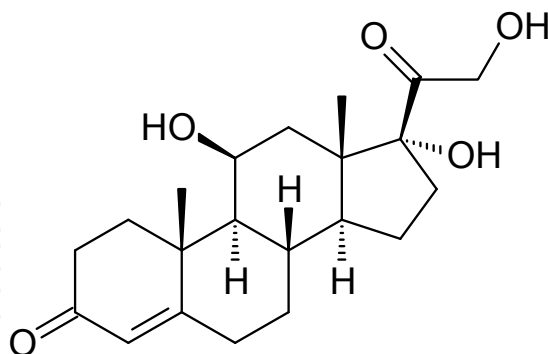


Fig. 6. Hydrocortisone

Hydrocortisone can be synthesized in *Saccharomyces cerevisiae* cells from simple carbon sources [Szczebara et al., 2003]. To optimize its yield the natural yeast pathway for sterols synthesis was rerouted after expressing genes from one plant enzyme and eight mammalian proteins including four of the P450 superfamily oxidases, three electron carriers and 3β -hydroxysterol oxidase/isomerase (3β -HSD). Under optimum conditions, a 70% yield of hydrocortisone was produced from the total steroid formation from glucose [Dumas et al., 2006].

3.4 Flavonoids

Flavonoids are a diverse class of plant secondary metabolites derived from the phenylpropanoid pathway. There has been increasing interest in flavonoids because many of them exhibit antioxidative activity due to free-radical scavenging. This is an attractive feature for drugs in coronary heart disease prevention and anticancer activity.

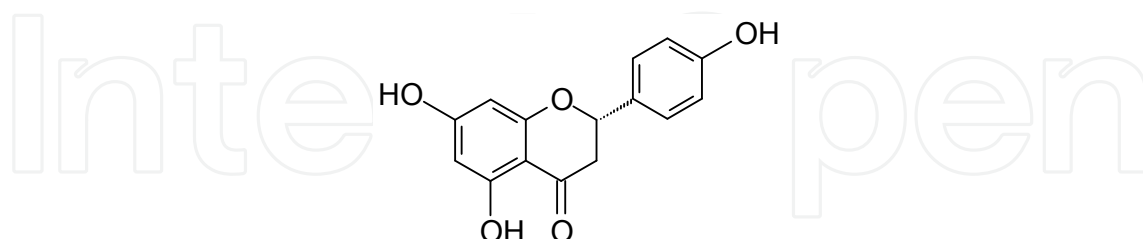


Fig. 7. Naringenin – flavanone

The biosynthesis of flavanone naringenin (Fig. 7), the central precursor of many flavonoids, is accomplished by introducing the phenylpropanoid pathway into *Saccharomyces cerevisiae* strains. It is achieved with the genes for phenylalanine ammonia lyase (PAL) from *Rhodospiridium toruloides*, 4-coumarate:coenzyme A (CoA) ligase (4CL) from *Arabidopsis thaliana* and chalcone synthase (CHS) from *Hypericum androsaemum* [Jiang et al., 2005] or with plant genes from heterologous origin such as 4-coumarate:coenzyme A ligase 4CL from *Petroselinum crispum*, chalcone synthetases CHS from *Medicago sativa* and *Petunia x hybrida* and chalcone reductase CHR and chalcone isomerase from *M. sativa* [Yan et al., 2005].

Another method to produce flavonoid or isoflavonoid compounds in engineered yeast is to clone five soybean (*Glycine max*) chalcone isomerases (CHIs), key enzymes in the phenylpropanoid pathway [Ralston et al., 2005].

4. Biotransformations catalyzed by yeast cells

Baker's yeast may be considered as a producer of wide range of chemical compounds, a kind of microbial cell factory. However, *Saccharomyces cerevisiae* is a commonly applied to whole-cell biocatalysis in biotransformation, reactions based on enzymatic transformations of chemical compounds. Biotransformations, known as a branch of "white" biotechnology, provide efficient procedures in organic synthesis owing to the high chemo- and stereoselectivity of enzymes and offer a viable alternatives to chemical methods. Today, biotransformation is a commonly accepted method for generating optically pure substances and for developing efficient synthesis of target compounds. In general, biotransformations are performed by the hydrolases or oxidoreductases. The remaining classes (transferases, isomerases, liases and ligases) are of lower, but increasing utility [Faber 2004].

Biotransformations may be carried out using isolated enzymes or microorganisms cells producing enzymes (whole-cell biocatalysis). The application of isolated and purified enzymes is profitable since the formation of undesired byproducts is avoided, whereas in cellular biotransformation systems undesirable product formation is possible due to the presence of other enzymes or simultaneous catalysis of several reactions. However the whole-cell biocatalysis has two important advantages: it is particularly beneficial when

the regeneration of the cofactor is necessary (e.g. in redox reactions) and is favorable due to the cost effectiveness.

Baker's yeast has a great potential as a catalysts in organic chemistry owing to ease of handling, broad substrate acceptability and production of enzymes belonging to different classes. *S. cerevisiae* may be used in dry and pressed form, as raw yeast or lyophilized biomass and is capable of catalyzing many reactions in water or in organic media. These features are very important for chemists, because chemical laboratories are not usually equipped with the microbiological apparatus required for yeast cultivation [Csuk & Glanzer 1991; Servi, 1990; Białecka-Florjańczyk & Majewska, 2006].

The full sequencing of the *S. cerevisiae* genome, accompanied with the achievements in genetic engineering, have allowed new strains of yeast to be designed with specific high conversion yields and reaction selectivity. There is a growing interest in application of modified yeast in biotransformation reactions. Modern directions to improve catalytic abilities of baker's yeast include the use of surface display technology for enzymes and optimization or increase in availability of cofactor (required for bio-reduction reactions) or gene knock-out, to eliminate the activity of enzymes with conflicting and unwanted stereoselectivities. As commonly used technique is overexpression of the desired protein or expression of heterologous enzymes in yeast cells.

4.1 Reduction of carbonyl compounds

Baker's yeast is considered to produce over 20 different reductases. At present most of the characterized carbonyl (and dicarbonyl)-reducing enzymes of *S. cerevisiae* are grouped into two distinct protein categories - the aldo-keto reductase (AKR) and the short-chain dehydrogenase/reductase (SDR) superfamilies. They have been shown to reduce a broad array of ketones and ketoesters with different enantioselectivity and to have overlapping activities [Katz et al., 2003]. Reductions catalyzed with baker's yeast require the presence of nicotinoamide cofactors (mostly NADPH) [Johanson et al., 2005], which can be regenerated during the growth of microorganisms.

Enantioselectivity (or diastereoselectivity) of carbonyl compounds reduction is connected with the formation of an asymmetric center in the product (alcohol) molecule (Fig. 8). According to Csuk *et al.* and Servi, reduction of aliphatic methyl ketones using baker's yeast leads to synthesis of secondary alcohols with the (*S*) configuration [Csuk, Glanzer 1991; Servi, 1990].

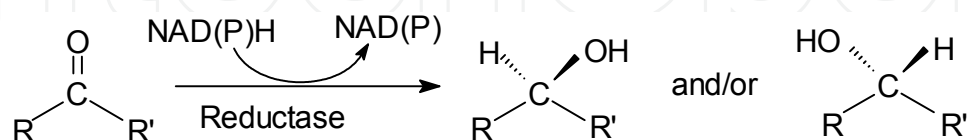


Fig. 8. Two enantiomeric alcohols formed during the reduction of carbonyl compound

Practical difficulties associated with the use of yeast as a chiral reducing agent arise from the presence of multiple enzymes overlapping substrate characteristics, but with differing enantio- and diastereoselectivities. Nakamura *et al.* isolated and characterized four oxidoreductases of raw baker's yeast in the reduction of 4-chloro-3-oxobutanoate [Nakamura et al., 1999]. The enantioselectivity of all of the enzymes examined was >99%,

but two of them produced (*R*) and the others (*S*) isomers. Thus the different specificity of enzymes produced by *S. cerevisiae* is the reason for the low specificity of unmodified cells in this biotransformation. A recombinant *Saccharomyces cerevisiae* strain over-expressing the fatty acid synthase of *S. cerevisiae* (FAS) and the glucose dehydrogenase of *Bacillus subtilis* was applied by Engelking *et al.* for enantioselective reduction of ethyl 4-chloro-3-oxobutanoate to ethyl (*S*)-4-chloro-3-hydroxybutanoate (Fig. 9) as well as the reduction of ethyl benzoylacetate to ethyl (*S*)-3-hydroxy-3-phenylpropanoate. The enantiomeric excess (ee) was 90% in the case of the former and >97% in the case of the latter product [Engelking *et al.*, 2006].

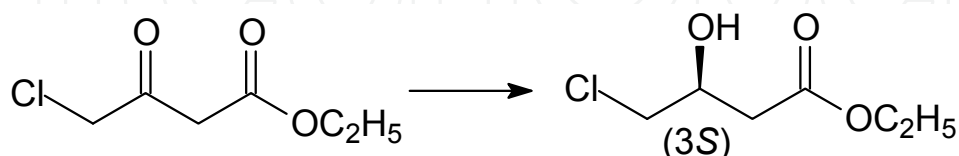


Fig. 9. Bioreduction of ethyl 4-chloroacetylacetate with *S. cerevisiae* yeast [Engelking *et al.*, 2006]

A method performed by Rodriguez *et al.* involved modified baker's yeast for β -ketoesters reduction by using recombinant DNA techniques such as overexpression of carbonyl reductase with desirable stereoselectivities. Manipulating the levels of three proteins, fatty acid synthase, (Fasp); aldo-ketoreductase, (Ypr1p); α -acetoxy ketone reductase, (Gre2p) the stereoselectivity of β -ketoester reduction was improved and reached > 85 % in all cases [Rodriguez *et al.*, 2001]. Conversely, the strain lacking aldo-ketoreductase produced hydroxyester with reversed stereoselectivity from unmodified baker yeast [Rodriguez *et al.*, 1999].

One example of efficient genetic modification concerns the asymmetric reduction of the bicyclic diketone (Fig. 10) bicyclo[2.2.2]octane-2,6-dione (1) to (1*R*,4*S*,6*S*)-6-hydroxybicyclo[2.2.2]octane-2-one ((-)-2), which is used as an intermediate in the synthesis of transition metal based chiral chemical catalysts [Sarvary *et al.*, 2001; Katz *et al.*, 2002]. When carrying out the experiment with baker's yeast, ketoalcohol (-)-2 was obtained in 92–97% enantiomeric excess with 92% yield [Almqvist *et al.*, 1993], but the diastereoselectivity of reduction of bicyclo[2.2.2]octane-2,6-dione catalyzed by whole cells of a modified strain of *S. cerevisiae* TMB4110 overexpressing the reductase gene YDR368w, was significantly improved. The product was achieved with 97% diastereomeric excess (de) and >99 enantiomeric excess (ee) [Johanson *et al.*, 2008; Parachin *et al.*, 2009].

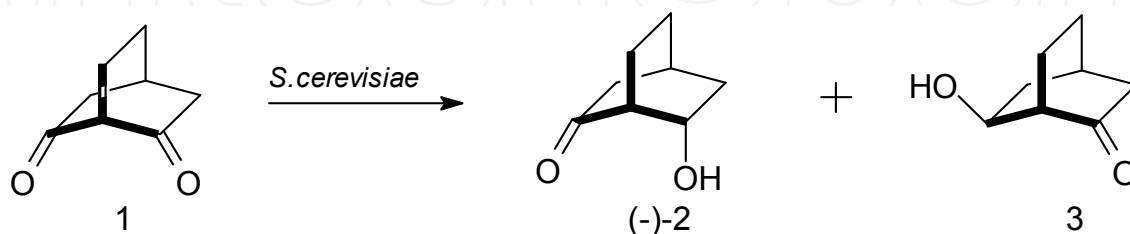


Fig. 10. Reduction of bicyclo[2.2.2]octane-2,6-dione (1) [Katz *et al.*, 2003]

Another practical solution of this problem was uncovered by expression of appropriate endogenous enzymes in host cells, which do not produce these proteins. For example, expression of GCY1 and GRE3 genes from *S. cerevisiae* in *E. coli* cells achieved chiral β -

hydroxyesters with high optical purities with over 98% enantiomeric excess [Rodriguez et al., 2000].

Genetically modified cells of *S. cerevisiae* can catalyze biotransformation reaction, producing chiral building blocks important in the pharmaceutical industry (especially those obtained by reduction of α - and β -oxoesters or amides). Optically pure alcohols are important chiral blocks in the synthesis of valuable pharmaceuticals such as carnitine (required for the transport of fatty acids from the cytosol into the mitochondria during the breakdown of lipids) or paclitaxel [Stewart, 2000]. Using the structural analogy between substrate and molecules of β -oxothioesters participating in biosynthesis of fatty acids, the *S. cerevisiae* strain with punctual mutation in the FAS-2 gene (fatty acid synthase) catalyzed the stereoselective reduction of the ketone group in the β -lactam molecule (Fig. 11), eliminating *trans*-isomer [Kayser et al., 1999]. This reduction was applied in the synthesis of the paclitaxel (after coupling with readily available 10-deacetylbaaccatin III) with a C₁₃ side chain - (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine.

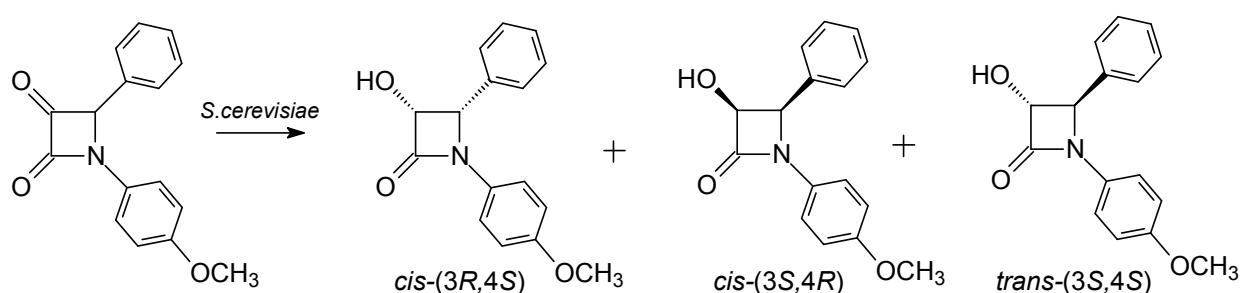


Fig. 11. Baker's yeast reduction of β -keto- α -lactam [Kayser et al., 1999]

Application of pure enzymatic preparations in organic chemistry generates a demand for coenzymes, which have to be added to the reaction medium. The use of baker's yeast in oxidoreductions is interesting, due to the ability of *S. cerevisiae* cells to synthesize not only the molecules of the specific enzymes, but also an essential coenzymes [Faber, 2004].

Although improvement of enantiomeric excess is critical for the product yield, regeneration of the co-factor is also important. This can be improved by genetic engineering. *S. cerevisiae* dicarbonyl reductases are NADPH-dependent and the reduced co-factor NADPH requires regeneration through the assimilation of a co-product, usually glucose, sucrose or ethanol. Glucose can be used as a co-substrate under both aerobic and anaerobic conditions. Unfortunately yeast generates large amounts of by-products from glucose, which may cause problems during downstream processing, with large amounts of glucose required to reduce small amounts of substrate. Ethanol is an interesting alternative to glucose as a co-substrate since it has a much lower utilisation rate and generate less CO₂. However, ethanol is toxic to the cells at high concentrations and NADPH cannot be regenerated from ethanol under anaerobic conditions.

Strain engineering for the design of efficient biocatalysts using glucose as a co-substrate has two potential objectives, to redirect the carbon flow towards NADPH-regenerating pathways and to slow down the rate of co-substrate utilisation in order to balance the reduction rate. In the *S. cerevisiae* strain the activity of phosphoglucose isomerase (PGI) was decreased and the baker's yeast strain alcohol dehydrogenase activity was deleted. In both cases the glucose consumption was limited without loss of reductase activity [Johanson et

al., 2005]. The yeast phosphoglucose isomerase activity was decreased, and the short-chain dehydrogenase/reductase encoded by YMR226c was overexpressed in the genetically engineered *Saccharomyces cerevisiae* strain TMB4100 (1% PGI, YMR226c).

The whole cell biocatalyst was used for the kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione (*rac*-1, Fig. 12). This framework is a commonly occurring motif amongst natural products, displaying a wide scope of biological activities. Genetic modification reduced the demand for the glucose to regenerate NADPH, resulted in a greater reaction rate and produced higher selectivity towards the (+)-1 stereoisomer, reaching an enantiomeric excess of 100% after 75% conversion of the isomer (Fig.12) [Carlquist et al., 2008].

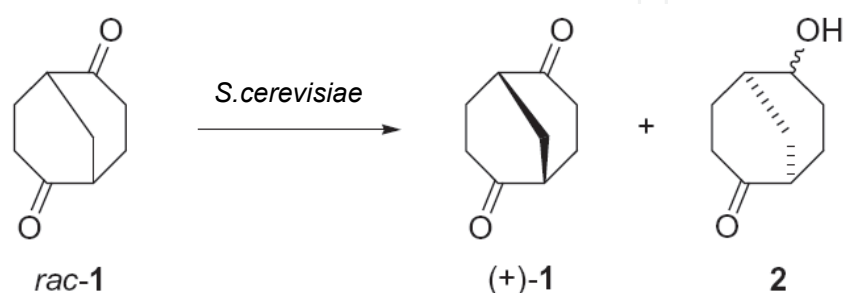


Fig. 12. Kinetic resolution of bicyclo[3.3.1]nonane-2,6-dione by genetically modified baker's yeast [Carlquist et al., 2008]

In addition (+)-5,6-epoxybicyclo[2.2.1]heptane-2-one, ((+)-1, Fig. 12), and *endo*-(-)-5,6-epoxybicyclo [2.2.1]heptane-2-ol, *endo*-(-)-2, were obtained by asymmetric bioreduction catalyzed by the same *S. cerevisiae* yeast strain. *Rac*-1 was kinetically resolved to give (+)-1 with 95% enantiomeric excess and *endo*-(-)-2 with 74% enantiomeric excess (Fig. 13) [Carlquist et al., 2009].

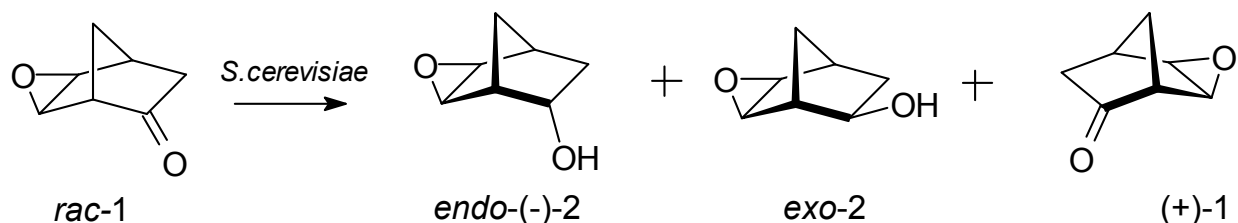


Fig. 13. Kinetic resolution of 5,6-epoxybicyclo[2.2.1]heptan-2-one [Carlquist et al., 2009]

4.2 Baeyer–Villiger oxidation

“Designer yeast” is a new catalyst for Baeyer–Villiger (BV) oxidation, an organic reaction in which a ketone is oxidized to an ester by treatment with peroxy acids (Fig. 14).

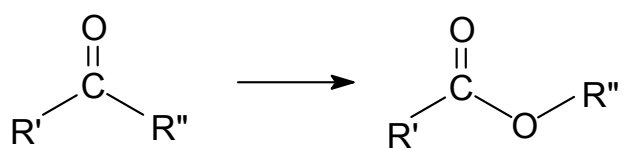


Fig. 14. Baeyer-Villiger reaction

The enzymatic Baeyer–Villiger oxidation represents an efficient approach to the asymmetric synthesis of chiral lactones and can be performed by biocatalysis with flavoenzymes called Baeyer–Villiger monoxygenases (BVMOs). BVMOs catalyze nucleophilic oxidation of ketones as well as electrophilic oxidation of heteroatoms such as boron, sulfur, selenium, nitrogen or phosphorus in organic compounds. Depending on the type of cofactor necessary for running the reaction BVMOs are distinguished between FAD- and NADPH-dependent enzymes and FMN- and NADH-dependent enzymes. Baeyer–Villiger monoxygenases are produced by the bacteria *Acinetobacter* sp. and *Pseudomonas* sp. and fungi such as *Aspergillus* sp. [Alphand et al., 2003]. Although a number of Baeyer–Villiger monoxygenases (EC 1.14.13.22) have been isolated from a variety of organisms, the NADPH-dependent cyclohexanone monoxygenase from the bacterium *Acinetobacter* sp. NCIB 9871 has been studied in the most detail. This enzyme catalyzes the second step in a catabolic pathway that allows the cells to utilize cyclohexanol as their sole source of carbon and energy. The ability of the purified enzyme or whole *Acinetobacter* cells to oxidize a diverse array of cyclic ketones to the corresponding lactones with high stereoselectivity is well established [Stewart et al., 1998].

NADPH-dependent cyclohexanone monoxygenase from *Acinetobacter* sp. has been expressed in baker's yeast. These genetically modified yeast cells can be used as biocatalysts in 4-alkylcyclohexanone oxidation to produce caprolactones (Fig. 15) with high enantiomeric excess [Stewart et al., 1998].

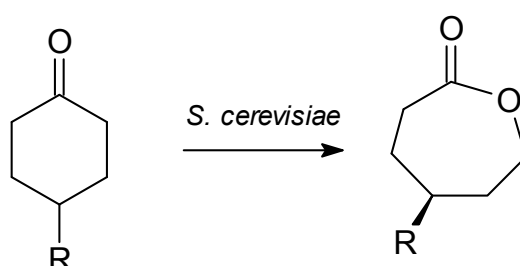


Fig. 15. 4-alkylcyclohexanone oxidation with baker's yeast [Stewart et al., 1998]

The same recombinant yeast can completely convert 2- and 3-substituted cyclopentanones and 2 and 3- substituted cyclohexanones. These reactions are found to be highly enantioselective, allowing the possibility to synthesize different stereoisomers depending upon the size of the ring and the sort of alkyl group. As a result of these modifications, reduction of the carbonyl group is minimized [Kayser et al., 1999; Stewart et al., 1998]. Chiral lactones are useful building blocks in the pharmaceutical industry since bicyclic and polycyclic lactones have received considerable attention as antitumor compounds, cardiac sarcoplasmic reticulum Ca^{2+} -pumping ATPase activators and as useful intermediates in the synthesis of potent drugs for the treatment of glaucoma and hypertension [Alphand et al., 2003].

4.3 Hydrolysis and esterification

About two thirds of reported practical biotransformations may be categorized as hydrolytic reactions involving ester and amide bonds using proteases, esterases or lipases [Loughlin, 2000]. Lipases, in addition to their biological significance, hold tremendous potential for exploitation in biotechnology. They display exquisite chemo-, regio-, and stereoselectivity, do not usually require cofactors and are readily available from microbial organisms [Jaeger &

Eggert, 2002]. The crystal structure of many lipases has been solved, facilitating considerably the design of rational engineering strategy. For these reasons lipases are commonly used in ester synthesis, acylglycerols modifications and in biodiesel production. Consequently, lipase genes are often integrated with host cell genomes of other microorganisms.

“True” lipases are defined as carboxylesterases, catalyzing the bioconversion of long-chain acylglycerols. The essential difference between lipases and esterases is the former act at the water-lipid interface, requiring a micelle formation by a water-insoluble substrate [Gill & Parish, 1997]. Although *S. cerevisiae* produces several hydrolytic enzymes such as esterases and lipases [Bialecka-Florjańczyk et al., 2010] (some of which have been isolated), it is generally used as a host organism to express other microbial lipases of special catalytic activity.

4.3.1 Cell surface engineered baker’s yeast in esterification reactions

The whole-cell catalysed reactions are often limited by barrier functions of the cell wall or membrane and thus the localization of enzyme, (extracellular, intracellular and membrane bound) plays an important role in lipase activity [Deive et al., 2009]. By the means of molecular engineering, the cell surface properties can be designed by displaying various functional proteins, especially enzymes. This technique provides to avoid mass transport problems of substrate and/or product across the cell membrane as the enzyme, necessary for catalysis, is displayed on the cells surface. Besides the anchoring to the cell surface usually results in increased biocatalyst stability. A cell surface engineering system of yeast *Saccharomyces cerevisiae* has been established and novel yeasts displaying lipases in their active form on the cell surface were constructed [Ueda & Tanaka, 2000]. Lipase-displaying whole-cell yeast biocatalysts have recently attracted attention for their use in biodiesel synthesis.

Biodiesel, a fuel for diesel engines, represents an alternative environmentally-friendly source of energy obtained from renewable materials. It is produced via transesterification of vegetable oils with alcohols (methanol or ethanol) and comprises fatty acid methyl (FAME, Fig. 16) or ethyl esters (FAEE). For ecological reasons, the enzymatic transesterification is becoming of increasing interest, yet the high cost of enzymes (lipases) obstructs its full industrial application [Akoh et al., 2007, Ribeiro et al., 2011]. Much research has focused on methods which allow increased whole-cell biocatalytic activity and stability through changes in microorganism culture conditions, their immobilization and application of genetic engineering techniques [Kucharski et al., 2009].

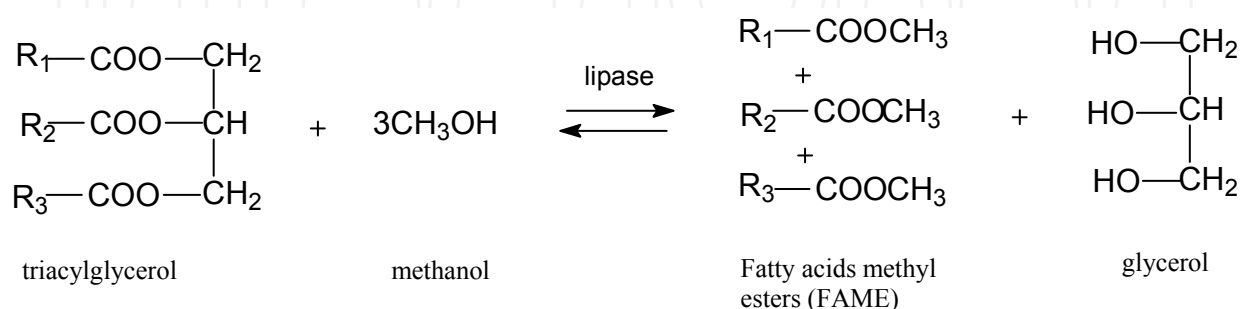


Fig. 16. Biodiesel production scheme

It has been shown that the application of recombinant baker’s yeast reduces the number of operations required in biodiesel production and simplifies the removal of glycerol [Fukuda

et al., 2008; Takahashi et al., 1998, 1999]. The possibilities of heterologous expression of nonspecific lipases such as *Candida rugosa*, *Pseudomonas cepacia* and *Pseudomonas fluorescens* has been reported, all of which exhibit relatively high conversion rates and methanol tolerance [Fukuda et al., 2008]. Whole-cell yeast biocatalysts, which intracellularly overproduce a recombinant lipase with a pro-sequence from *Rhizopus oryzae* (rProROL) were constructed and the content of active lipase in *S. cerevisiae* cells was maximized by optimizing the cultivation procedure. Lipase from *Rhizopus oryzae* (ROL) was chosen because its secretory production has been accomplished in *S. cerevisiae*. Overproducing ROL lipase baker's yeast strain MT8-1 was permeabilized and used for the synthesis of FAME with 71 % efficiency [Takahashi et al., 1999].

The cell surface engineering of yeasts by which functional proteins are displayed on the cell surface has enabled the development of a novel strategy for whole-cell biocatalysts. To enhance lipase activity and preserving the conformation of the active site near the C-terminal portion a linker peptide (spacer) consisting of the Gly/Ser repeated sequence was inserted at the C-terminal position. The display of active lipase from *Rhizopus oryzae* on the *S. cerevisiae* cell surface resulted in production of 2,3-dimercaptopropan-1-ol tributyl ester and insoluble triolein [Washida et al., 2001]. This eliminates the cell disintegration step, allowing the production of intracellular enzyme and subsequent ease of separation of the products from the catalysts. Both these factors influences the costs of the process, making it more cost effective [Takahashi et al., 1998]. There are other examples of enzymes displayed on baker's yeast surface such as the lipase CaLIP4 from *Candida albicans* [Breinig et al., 2002], lipase A from *Bacillus subtilis* [Mormeneo et al., 2008; Roustan et al., 2005] and lipase L1 from *Bacillus stearothermophilus* [Breinig et al., 2002].

The other tendency in *S. cerevisiae* modifications reflects the broadening of kinds of carbon sources that can be utilized by yeast cells for biodiesel production purposes

Though biodiesel synthesis is the main area of cell wall engineered yeast application nevertheless it was employed in some other esterifications. To improve cost-efficiency a whole-cell biocatalysts CALB-displaying was constructed using the Flo1p short (FS) anchor system. Lyophilized yeast cells were applied to an ester synthesis reaction at 60°C using adipic acid and butanol as substrates [Tanino et al., 2007]. Similar whole cell catalyst have been applied in the esterification of hexanoic acid with ethanol, yielding 98.2% of the ester under optimum conditions [Han et al., 2009].

Recombinant *R. oryzae* lipase (ROL) displayed on the *S. cerevisiae* cell surface was used in the resolution of enantiomers of (*R,S*)-1-phenylethanol, which serves as one of the important chiral building blocks. During enantioselective transesterification of this alcohol with vinyl acetate (Fig. 17) the yield of (*R*)-1-phenylethyl acetate reached 97 % with 93 % enantiomeric excess [Matsumoto et al., 2004].

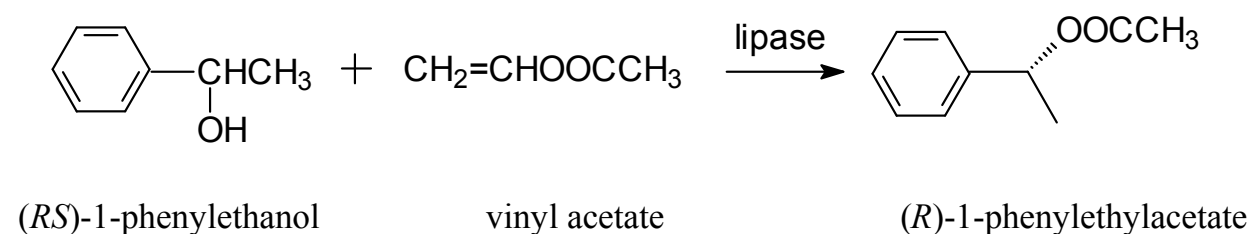


Fig. 17. Synthesis of (*R*)-1-phenylethylacetate

The ROL-displaying yeast whole-cell biocatalyst catalyzed the stereospecific hydrolysis of the pharmaceutical precursor (*R,S*)-1-benzyloxy-3-chloro-2-propyl monosuccinate. The isomer (*R*) was isolated with the ee up to 95,5% (Fig. 18). *Rhizopus oryzae* lipase was displayed on the cell surface of *Saccharomyces cerevisiae* via the Flo1 N-terminal region (1100 amino acids), which corresponds to a flocculation functional domain [Nakamura Y., 2006].

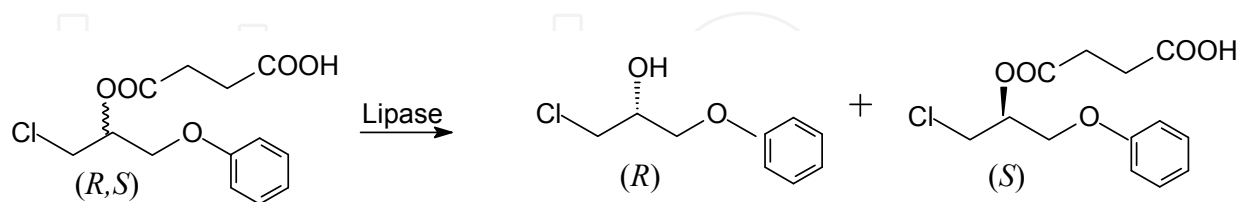


Fig. 18. Stereospecific hydrolysis of of (*R,S*)-1-benzyloxy-3-chloro-2-propyl monosuccinate

4.4 L-malic acid production

Biotransformation, using whole cells of modified *S. cerevisiae* can be applied in the synthesis of L-malic acid (Fig. 19). Besides the food industry (acidulant E 296), malic acid has extensive application in the pharmaceutical and cosmetic industries. L-malic acid is enzymatically produced via the hydration of maleic or fumaric acid (FA) catalysed by fumarase (EC 4.2.1.2) from bacteria such as *Brevibacterium ammoniagenses* and *B. flavum* [Yazawa et al., 2010].

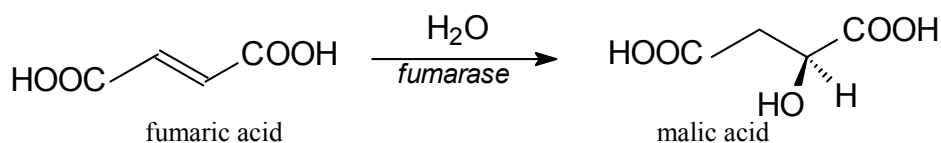


Fig. 19. Biotransformation of fumaric acid to L-malic acid

In the 1990s, production of L-malic acid from fumaric acid using *S. cerevisiae* cells was studied extensively with the amount of by-products minimized using modified *S. cerevisiae* cells with many copies of the fumarase gene as a biocatalysts [Presečki et al., 2007]. Conversion of fumaric acid to L-malic acid was carried out also in a bioreactor divided into three compartments by two supported liquid membranes. The yeast was immobilized in small glasslike beads of an alginate-silicate solgel matrix, allowing the reaction to reach almost 100% the conversion with only small amount of succinic acid produced. This was much higher than the 70% conversion rate found in industrial processes [Peleg et al., 1990].

5. Conclusions

S. cerevisiae has an enormous potential for the production of low and high molecular weight compounds by acting as an heterologous host and expressing biosynthetic enzymes or pathways. By coupling multiple enzymes, metabolic pathways in a single cell are created. This eliminates the need for purification of the chemical intermediates and the desired products can be prepared from simple, inexpensive and renewable materials. In the future the metabolic engineering of yeast may lead to an alternative production systems, helping overcome the limited availability of biologically active, commercially valuable and nutritionally important

plant secondary metabolites compounds. It should be possible to significantly reduce development and costs of these microbial cell factories.

S. cerevisiae may be used as a whole-cell biocatalysts in the biotransformations, mostly in reduction or hydrolysis reactions. Modern research has lead to improvements in the catalytic ability of baker's yeast, including the surface display of enzymes, optimization or increase of the cofactor availability in bioreduction reactions and gene knock-out, which eliminates the activity of enzymes with conflicting, unwanted stereoselectivity.

It was shown that genetically engineered baker's yeast has a great potential as a biocatalysts in many branches of chemistry. The fact is that it is impossible to enumerate all of the published application of modified *S. cerevisiae* cells as to improve valuable chemicals production. It is worth mentioning that there are still problems not solved. Many studies regarding *S. cerevisiae* metabolic engineering for lignocellulosic biomass utilization have been provided, but this process involving baker's yeast is still insufficient for industrial bioprocesses mainly due to low rate of reaction. Nevertheless it can be predicted that engineered baker's yeast would be an efficient tool in chemical processes as to improve people lives.

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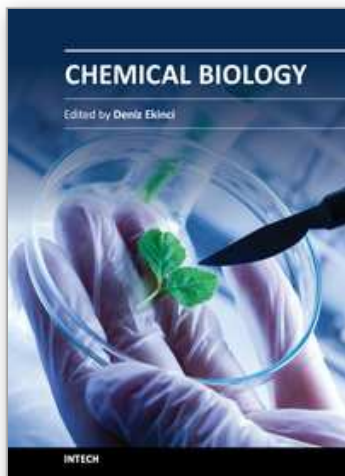
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Chemical biology utilizes chemical principles to modulate systems to either investigate the underlying biology or create new function. Over recent years, chemical biology has received particular attention of many scientists in the life sciences from botany to medicine. This book contains an overview focusing on the research area of protein purification, enzymology, vitamins, antioxidants, biotransformation, gene delivery, signaling, regulation and organization. Particular emphasis is devoted to both theoretical and experimental aspects. The textbook is written by international scientists with expertise in synthetic chemistry, protein biochemistry, enzymology, molecular biology, drug discovery and genetics many of which are active chemical, biochemical and biomedical research. The textbook is expected to enhance the knowledge of scientists in the complexities of chemical and biological approaches and stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of chemical biology.

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