Saimaa University of Applied Sciences
Faculty of Technology, Imatra, Finland
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THE APPLICATION OF ENZYME AND YEAST

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Abstract
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This bachelor's thesis concerns the application of enzymes and yeasts for bio-industry. The purpose of this work is to understand the basic knowledge about enzyme and yeast, and meanwhile, to find out their different applications. Through comprehensive study, the knowledge was accumulated which brought a clear understanding for the enzyme structure and yeast microorganism, together with their working principles for the bioprocess.

For wood-based industry, the different enzymes used in bio-pulping process were studied. The lignin-oxidizing enzymes, e.g. laccase, lignin peroxidase and manganese peroxidase, are effectively applied in the biodegradation of lignin. On the other hand, the biodegradation of hemicelluloses needs the present of xylanase and mannanase. Furthermore, the production process for enzymes is introduced which contains raw materials preparation, filtration, concentration, purification, drying, etc.

Concerning the application of yeast, saccharomyces cerevisiae is commonly used in the bio-ethanol fermentation industry. In addition, the operating condition should be carefully selected in the fermentation process in order to improve the productivity. For the waste water treatment industry, the choice of yeast could bring the certain benefit comparing with the traditional process. The various yeasts, e.g. yarrowia lipolytica, candida utilis and candida parapsilosis are commonly applied concerning the waste water from the various industry processes.

Keywords: enzyme, yeast, bio-pulping, lignin-oxidizing enzyme, hemicellulase, bio-ethanol, fermentation

CONTENTS

1 INTRODUCATION 4
2 ENZYMES...5
3 CLASSIFICATIONS OF ENZYMES 6
3.1 Classification of enzymes by structure 6
3.2 Classification of enzymes by catalysed reaction types 9
4 PRODUCTION AND APPLICATIONS OF ENZYMES 12
4.1 Production of enzymes 13
4.2 Application of enzymes...15
4.2.1 Bio-pulping process 16
4.2.2 Biodegradation of lignin 17
4.2.3 Biodegradation of Hemicelluloses...22
5 YEASTS...25
6 APPLICATIONS OF YEASTS...29
6.1 Yeast application for wood-based materials 30
6.2 Yeast application in wastewater treatment 36
7 SUMMARY...41
FIGURES 43
TABLES 44
FORMULAE 44
REFERENCES 45

1 INTRODUCATION

Biotechnology has expanded tremendously in recent years. The significant advances of biotechnology opened up a new world for the paper and forest industry to reduce energy consumption and produce environment friendly, which is a major area of research and development in the paper and forest industry.

Enzymes, as industrial biocatalysts, are a class of proteins extracted from animals, plants, and microorganisms. It has a board range of applications in different industries such as food, textiles, pharmaceuticals, detergents, paper and pulp, waste management, etc. Modern enzyme industry is high-tech industry, which is characterized with high catalytic efficiency and specificity. The yeast is a single-celled micro-organism. It usually has a several thousands of proteins and has cells tissues. The application area is comprehensive, which refers to environmental technologies, biomedical research, fundamental biological research, health-care industries, food/chemical industries and fermentation industries.

In this thesis, the basic knowledge for enzyme is introduced, e.g. the different structures of enzymes and the various catalyzed reaction types. In follow, the application of enzyme for wood-based industry, e.g. bio-pulping process, is introduced to understand the principles of biodegradation of lignin and hemicellulose by a variety of enzymes. Three most important lignin-oxidizing enzymes for biodegradation of lignin and two enzymes for biodegradation of hemicellulose are described. On the other hand, the basic knowledge of yeasts and its application are studied. The example to apply the yeast in the bio-ethanol production and the wastewater treatment are discussed.

2 ENZYMES

Enzymes are giant macromolecules, biological catalysts and they mostly consisted of protein, which are polymers of amino acids and small amount of RNA. The molecular weight of enzyme is from 10,000 to 2000,000 Da. All enzymes contain four elements C, H, O, N. In the organisms, synthesis and degradation of protein, fat and carbohydrate, as well as many complex chemical changes in the life activities are closely related with the enzyme. [1.]

Enzymes are biological catalysts and therefore they have some basic properties as catalyst, e.g. they speed up the rate of chemical reactions without losing or changing by the reaction. Compared with non-biological catalysts, biocatalyst can react at normal temperature and pressure, and have the high catalytic efficiency. The temperature has effect on the activity of enzymes. Under lower temperatures, the speed of chemical reactions increases by temperature increasing for the enzymatic reaction. However, when the temperature exceeds a certain value, the enzymes denature and lead to decrease of catalytic activity. The optimum temperature for activity of enzymes is different due to different enzymes and the most enzymes have an optimum temperature around 37°C. When substrate, enzyme concentrations and temperature are constant, the range of pH on the reaction rate that enzyme-catalysed is from 5 to 8. [2.]

3 CLASSIFICATIONS OF ENZYMES

According to the structure of enzymes, enzymes can be classified as monomer enzyme, oligomeric enzyme, multienzyme system and multi-enzyme complex. On the other hand, all known enzymes are divided into six categories according to their catalysed reaction type. They are oxidoreductases, transferases, hydrolases, lyases, isomerase and ligases [3], [4], [5].

3.1 Classification of enzymes by structure

Enzymes can be classified by different structures. They are introduced as following texts,

- Monomer enzyme

The molecular weight of monomer enzyme is between 13,000 and 35,000 Da. Monomer enzyme normally belongs to the primary structure of enzyme, as shown in figure 3.1. Primary structure refers to AA order in the protein connection, including the location of the disulfide bond. It is the actual sequence of amino acids in the chain. The type of monomer enzyme is a few and generally they do catalyzed hydrolysis reaction. [6.], [7.]

Figure3.1. Primary Structure of Enzyme (circle is amino acid and line is peptide bond) [6]

- Oligomeric enzyme

Molecular weight of oligomeric enzyme is quite big, which is more than 35,000 Da. Oligomeric enzyme belongs to secondary structure. The secondary structure is the folding formed conformation by the main chain itself of the polypeptide chain along a certain steering wheel through hydrogen bonds. It involves only the main chain conformation of the peptide chain and hydrogen bonds formed in the chain or between chains. It mainly has α-helix and β-pleated sheet, β-turn, etc. Compared with the monomer enzyme, oligomeric enzyme together with regulatory factor will cause conformational change, which lead to the change in catalytic activity and hence make a regulating effect on the metabolism. [8.]

About the α-helix, hydrogen bonds formed in the peptide chain, hydrogen bonds are almost parallel with the axis. C=O oxygen of each amino acid residue forms hydrogen bond with subsequent N-H hydrogen of the fourth amino acid residues. The β-pleated sheet is also called β-structure or β-conformation. It is the second most common secondary structure in the protein. The β-pleated sheet is formed by two or more fully extended polypeptide chains arranging in parallel and by cross linking of hydrogen bonds between the chains, or is formed by hydrogen bonds between the different peptides in a peptide chain. The structure of polypeptide chain in secondary structure is shown in figure 3.2.

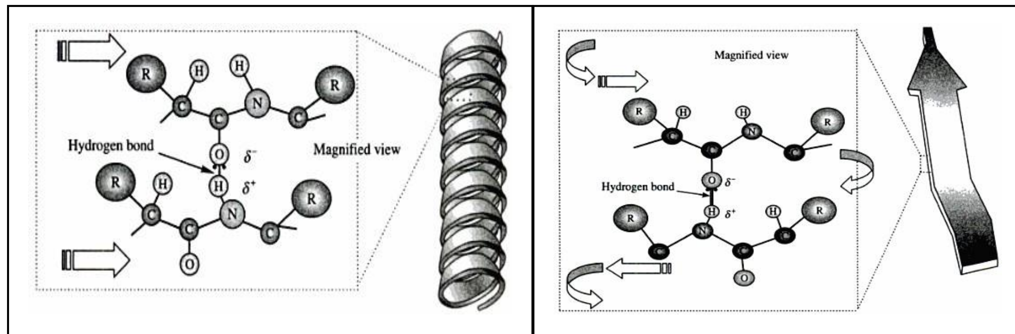


Figure3.2. Structure of polypeptide chain in secondary structure of enzyme (hydrogen bond in α-helix and β-pleated sheet) [1]

- Multienzyme system

Multienzyme system contains many enzymes, which are interconnected to form the reaction chain system. For example, ten kinds of enzymes in glycolysis constitute a multi-enzyme system. Generally, multienzyme system belongs to tertiary structure. The tertiary structure includes the main chain conformation and side chain. The chains interact and multi-directional curl along the three-dimensional space, and form a spherical molecular structure by further curling and folding. In three-dimensional structure of enzyme, proteins are well ordered and their interiors are well packed. The tertiary structure of enzyme is shown in figure 3.3.

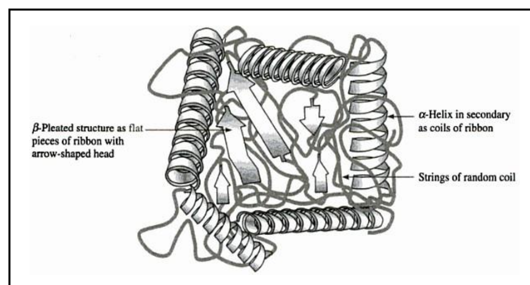


Figure3.3. Tertiary structure of enzyme [1]

- Multi-enzyme complex

Multi-enzyme complex is several enzymes embedded in each other and combined to form a fully functional complex with specific structure. Each multi-enzyme system has a molecular weight over two million. The quaternary structure is very important. The quaternary structure of enzyme is indicated in figure 3.4.

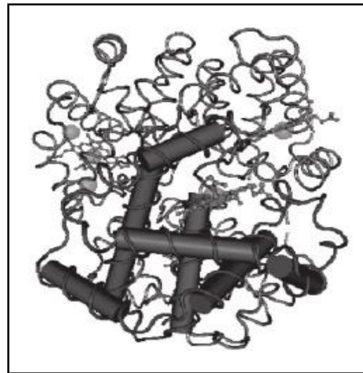


Figure3.4. Quaternary structure of enzyme [9]

Quaternary structure is protein molecules that by two or more polypeptides with tertiary structure according to a certain way polymerize a specific conformation. In the quaternary structure of protein, the forces between subunits mainly include hydrogen bonding, ionic bonding, Van der Waals force and hydrophobic bond. Hydrophobic bond is the main force. [5.]

3.2 Classification of enzymes by catalysed reaction types

Enzymes can be classified by catalyzed reaction types, which are oxidoreductases, transferases, hydrolases, lyases, isomerase and ligases.

- Oxidoreductases

Oxidoreductases, for example, oxygenases, oxidases, reductase, peroxidases, are an enzyme group which transfer of electrons or hydrogen atoms from one compound to another as shown in formula 1. The enzyme catalyses oxidoreductions of CH-OH, CH-CH, $CH\text{-}NH_2$, C-NH, etc. Oxidoreductases in the industry are mainly used in baking, brewing, textile, pulp and paper. [10.], [11.]

$$A_{reduced} + B_{oxidized} \rightarrow A_{oxidized} + B_{reduced} \quad (1)$$

- Transferases

Transferases are the enzymes that catalyse the transfer of same group or radical, R, from one compound to another, which is indicated in formula 2. Common transferases have aminotransferase, transmethylases, acyltransferase and protein kinase. The enzymes of this group catalyse the transfer of one carbon groups, aldehydic or ketonic residues, acyl, alkyl nitrogen and so on. The industrial application areas of transferases normally only are dairy and brewing. [10.], [11.]

$$AR + B \leftrightarrow A + BR \quad (2)$$

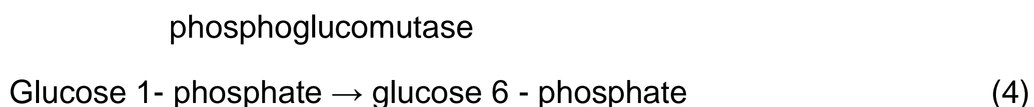
- Lyases

Lyases catalyse the addition of a group across a double bond. The chemical reaction is shown is formula 3. Lyases include decarboxylase, citrate lyase, dehydratase and deaminase. This group's enzymes act on carbon-carbon, carbon-oxygen, carbon-nitrogen, etc. The industrial application areas of lyases mainly include brewing, textile and fruit juice. [10.], [11.]

$$C - C \leftrightarrow C = C + X - Y \quad (3)$$

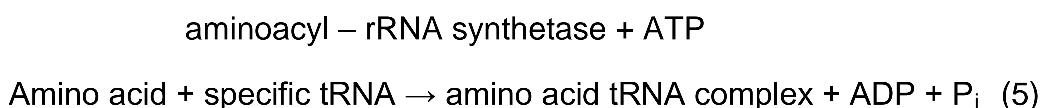
- Isomerase

Isomerases are the enzyme group which catalyses rearrangements in a molecule, converting one isomer to another, in which one compound is changed to another having the same number and kinds of atoms, but having different molecular structure, e.g. cis-trans isomerase, mutase, epimerase, racemase and phosphoglucomutase. The reaction is shown in formula 4. This group include enzymes catalysing interconversions of optical, geometrical and positional isomers. Isomerases in the industry can be used in grain wet milling, and some cosmetics. [10.], [11.]


phosphoglucomutase
Glucose 1- phosphate → glucose 6 - phosphate (4)

- Ligases

Ligases are the enzyme group which catalyses two molecular substrates into one molecular compound, e.g. pyruvate carboxylase, glutamine synthetase, glutathione synthetase and aminoacyl – rRNA synthetase. The reaction uses energy which from the hydrolysis of ATP to ADP and phosphate and the C-O, C-S, C-N and C-C bonds are formed in the reaction. A chemical example is shown in formula 5. The industrial application has food and drink, detergents and biosensors. [10.]


aminoacyl – rRNA synthetase + ATP
Amino acid + specific tRNA → amino acid tRNA complex + ADP + P_i (5)

- Hydrolases

Hydrolases are the enzyme group which breaks a large substrate molecular to two small products. The chemical reaction is shown in formula 6. The common hydrolytic enzymes have lactase, amylase, maltase, protease, peptidase and esterases. The enzymes of this group catalyse hydrolysis of ester, ether, peptide, etc. by addition of water. The application areas of hydrolases are quite wide, like baking, dairy, laundry, pulp and paper, textile, fruit juice, animal feed and so on. *[10.], [11.]*

Lactase

Lactose + water → glucose + galactose (6)

4 PRODUCTION AND APPLICATIONS OF ENZYMES

Commercial enzyme production is central to the modern biotechnology industry. Traditional enzyme production depended on the natural source as raw materials, like animal organs, plant materials. However, genetic engineering has now opened a choice for producing enzymes from micro-organisms and transgenic plants.

4.1 Production of enzymes

Currently, some most influential enzyme-making factories in the world are Novo Nordisk Company of Denmark, Gist-Brocades Company from Netherland and Genencor Company from America. The products from these companies together occupy 74.3% of global market. Three major enzymes, i.e. protease, amylase and lipase are produced in current industrial applications. Protease is used for detergents, dairy industry, leather industry. Amylase is used in baking, brewing, starch saccharification and textiles. Lipase is used in detergent, food and fine chemical industries. [10.], [12.], [13.] The products of enzymes of protease, amylase and lipase are indicated in the figure 4.1.

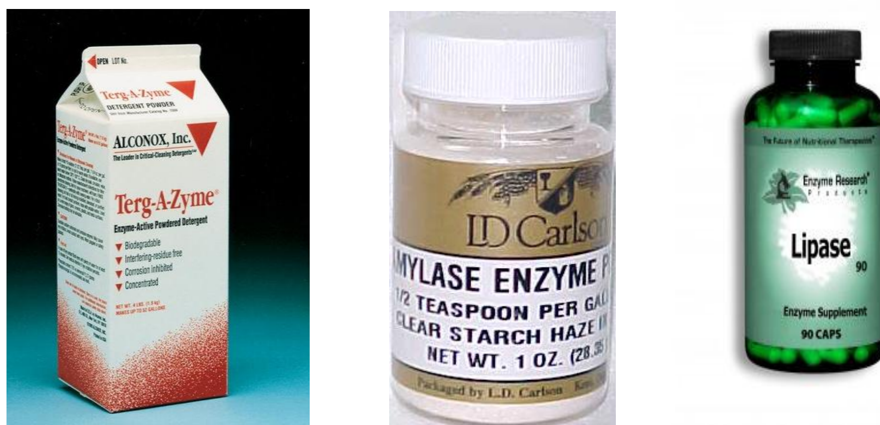


Figure4.1 Products of enzymes of protease, amylase and lipase

The main steps involved in the recovery of enzymes contain raw materials preparation, filtration, concentration, purification and drying [10]. Figure 4.2 shows the normal steps in the isolation of enzymes.

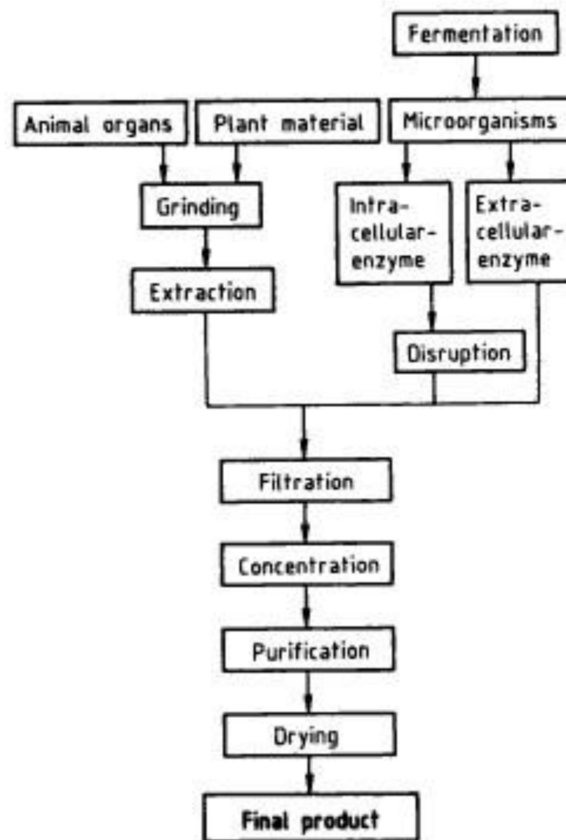


Figure4.2. Sequence of steps in the isolation of enzymes [10]

The primary consideration in the production of any enzyme is the choice of source. There are three kinds of raw materials that can be used, which are animal organs, plant materials and micro-organisms (bacteria, fungi and yeast). For the animal organs, the low temperature must be kept to protect activity of enzyme. Plant materials can be ground with different grinders and can be extracted with buffer solution as well. The most significant feature of the organisms for producing industrial enzymes is their safe status, and they must be non-toxic, non-pathogenic and not produce antibiotics. The micro-organisms can be cultured in the large quantities in a short period by fermentation and the enzymes often differ in composition and properties. When the fermentation is used to produce the enzyme, the organism is preferred which gives high yields of enzyme in shortest fermentation time. [10.]

In order to get the final enzyme product, separation methods including centrifugation, filtration, microfiltration or flocculation where solid-liquid are separated are used. In follows, the mild concentration procedures that do not inactivate enzymes can be employed because the enzyme concentration in raw material is very low. These contain thermal method, precipitation, and membrane filtration.

Then, the purification step is performed using column chromatography. There are several separation principles in the chromatography, such as gel filtration, size exclusion, ion exchange, hydrophobic interaction and affinity binding. Other methods for purification include precipitation with salts, crystallization and aqueous two-liquid phase extraction. Because of the expensive cost of some purification methods, enzyme purification often depends on cheaper separation processes, such as selective precipitation or crystallization. Another purification method used for small scale enzyme is the utilization of magnetic separation techniques. [10.] The principal separation methods used in purification of enzymes are shown in Appendix II.

4.2 Application of enzymes

Nowadays, enzymes have been widely used in the field of food processing as well as other industries, e.g. medicine, analysis and measurement, biological engineering, etc. In the industrial enzymes, 80% are hydrolytic enzymes, mainly used for the degradation of the polymer in nature, such as starch, protein, fat and other substances. For pulping and paper industry, enzymes used in the paper industry include: cellulase, hemicellulase, ligninase, amylase, lipase,

pectinase, laccase. The process includes bio-pulping, bio-bleaching of pulp and biological deinking of paper-making, etc. The application of enzymes in pulp and paper industry is described in the following sections. [14.], [15.]

4.2.1 Bio-pulping process

Traditional manufacturing industries generally use soda and sulphate in production of pulp. This process produces big wastewater emissions and contains large amounts of suspended solids, BOD, COD, toxic substances. The bio-mechanical pulping is a combination of biological method and traditional mechanical pulping process. Compared with the traditional pulping, the bio-pulping decreases energy consumption (33% energy saving) and environmental pollution. [16.] The overview of bio-pulping process is shown in figure 4.3.

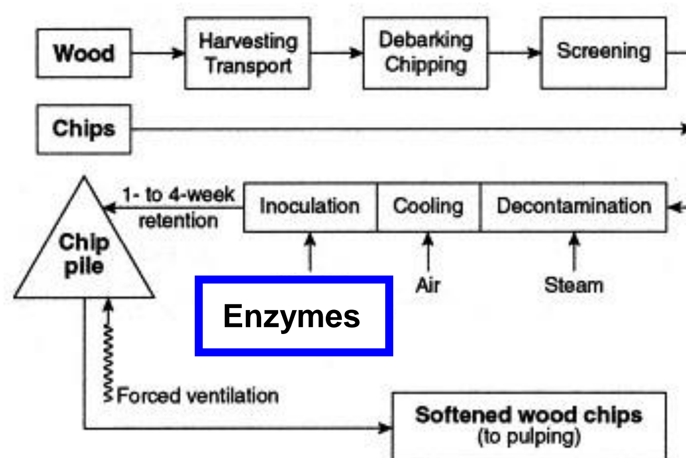


Figure4.3. Overview of Bio-pulping process [17]

As shown in the above figure, some enzymes are used to degrade lignin in bio-pulping process. Also, it is necessary to provide suitable process conditions to get a good bio-pulping effect. Different wood materials use different biological enzymes in bio-pulping process.

4.2.2 Biodegradation of lignin

Wood mainly composes of three main compounds, i.e. cellulose, hemicellulose and lignin. The basic mechanism of lignin degradation is that the lignin of the raw material changes to low molecular weight lignin using enzymes and hence the separation between lignin, cellulose and hemicellulose can be done. Common enzymes for lignin degradation are lignin-oxidizing enzymes. Lignin-oxidizing enzymes are mainly produced by white-rot-fungi. The Lignin-oxidizing enzyme can directly degrade residual lignin in the pulp and achieve the effect of delignification and bleaching pulp. Therefore, pulp's kappa number decreases and whiteness of pulp improves. [15.]

The most important lignin-oxidizing enzyme includes laccase, lignin peroxidase and manganese peroxidase. The lignin peroxidases can catalyse the oxidation of non-phenolic aromatic rings in lignin to cation radicals in the presence of hydrogen peroxide. Manganese peroxidises oxidize phenolic units in lignin. Laccase uses molecular oxygen as a cosubstrate and oxidize phenolic subunits in lignin and reduces oxygen to water. Besides, if adding readily oxidized substrates, the substrate range of laccases can be extended to non-phenolic subunits. The white rot fungi is the most important degrading bacterium to lignin

in the nature, it mainly includes lignin peroxidase, manganese peroxidase and laccase. [14.]

- Laccase

Appropriate substrate molecules of laccase are phenols, as well as aromatic and fatty amines. Laccase is an important lignin-degrading enzyme, which catalyses demethylation of lignin components. It is a monomeric glycoprotein containing 6.5% carbohydrate and having a molecular weight of 64,000Da. Laccase has an isoelectric point of 3.6 and pH range from 3 to 9, with optimum pH value being 4.5. Laccase contains copper and it is an extracellular oxidase. It has been used in many different processes such as sporulation, pigment production, rhizomorph formation and lignin degradation. Laccase as multi-copper-enzyme normally catalyzes phenol and polyphenols in the wood. Laccase substrates are major molecules of poor solubility, therefore, when choosing reaction conditions, it is often necessary to use organic solvents or immobilized laccase. The figure 4.4 shows the active sites of copper in laccase.

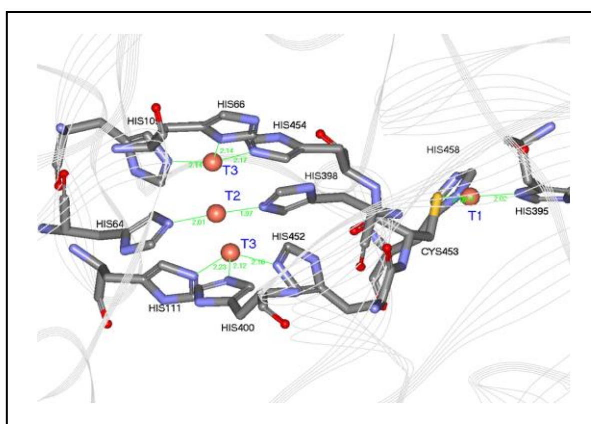


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Figure4.4. Environment of four coppers (The copper atoms are balls: T1, T2, T3) in the active site of laccases [19]

The active center of many laccases is composed of three various binding sides of the copper atoms. In type-1 copper (T1) (the binding side of the substrate), the Cu^{2+} ion is coordinated with the enzyme protein via cysteine and histidine. After the binding of the electrons (coming from the oxidized substrate) on type-1 copper, the electrons are transferred to the trinuclear cluster consisting of type-2 (T1) and type-3 copper (T1), where the O_2 reduces to water. [19.]

- Lignin peroxidases

Lignin peroxidase is the one of the most important enzymes related to degradation of lignin and it catalyses H_2O_2-dependent oxidation of lignin. It oxidizes both phenolic and non-phenolic substructures of lignin, and it is a more effective delignifying agent than lactase. Lignin peroxidase can oxidize those compounds with high redox potentials which cannot be oxidized by other enzymes. It is an oligomannose type of glycoprotein. There can be as many as 15 lignin peroxidase isozymes, ranging in molecular weight from 38,000 to 43,000Da. The spectrum of isozymes produced depends on culture conditions. [20.] The catalytic cycle of lignin peroxidase is shown in figure 4.5.

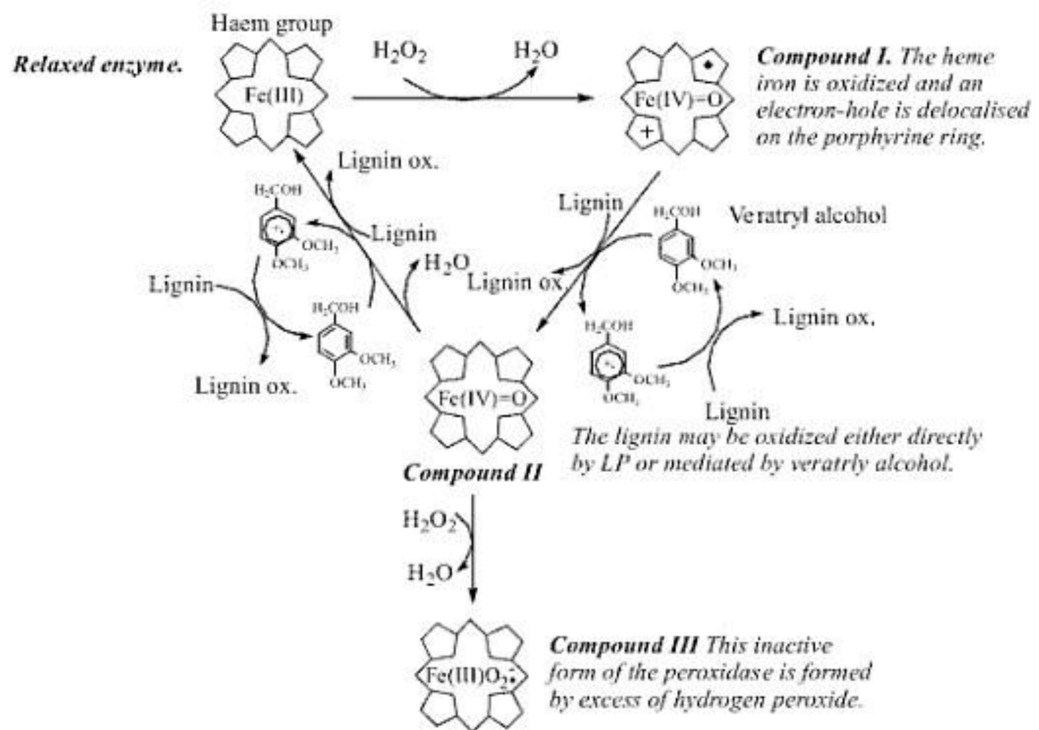

Figure4.5. Catalytic cycle of lignin peroxidase [15]

The activity of lignin peroxidase has been recommended to rely on veratryl alcohol. Lignin peroxidase can oxidize veratryl alcohol to a radical. veratryl alcohol as a redox mediator can work as a coenzyme working close to enzyme active site of enzyme and it can even protect the enzyme. However, in the absence of veratry alcohol, lignin peroxidase can also directly oxidize lignin polymer into radicals using a second active site. [15.]

- Manganese peroxidase

Manganese peroxidase has been considered to be the most important lignin-oxidizing enzyme that can be used for kraft pulp biobleaching. Manganese peroxidase treatment can reduce energy consumption in mechanical pulping, which results in 25% energy saving in the post-refining stage. The brightness of pulp can be increased and manganese peroxidase does not affect strength

properties, viscosity and yield of pulp. [21.] The reaction catalyzed by manganese peroxidase is shown in figure 4.6. The figure 4.7 shows the structure of manganese peroxidase and the active site of manganese peroxidase.

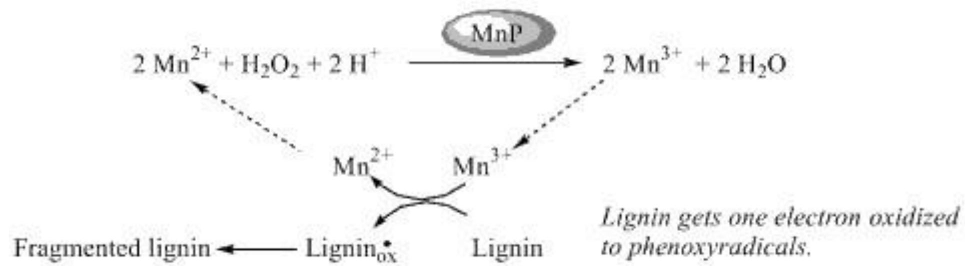

Figure4.6. Reaction catalyzed by manganese peroxidase [15]

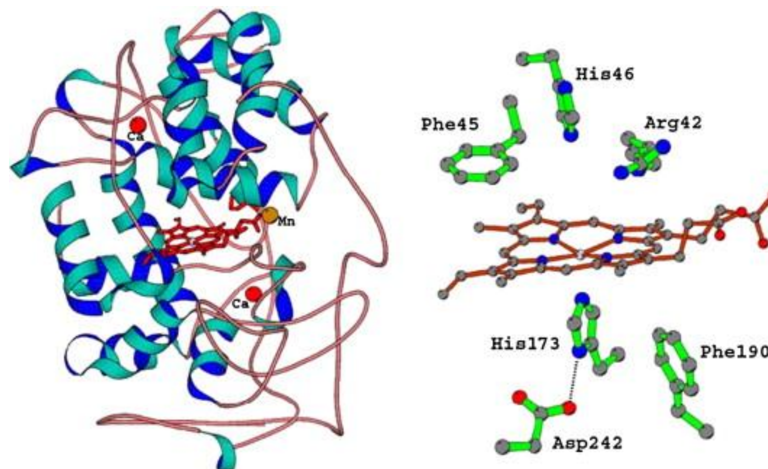

Figure4.7 Structure of manganese peroxidase and the active site of manganese peroxidise [18]

Manganese peroxidase is a relatively small enzyme that consisting of around 350 amino acid residues, two structural calcium ions, 478 solvent molecules, etc. Peroxidase hydrogen (H_2O_2) is essential for the reactions catalysed by manganese peroxidase. Manganese peroxidase uses Mn^{2+} as the reducing substrate for H_2O_2 by oxidizing it to Mn^{3+}**.** Molecular weight of Manganese

peroxidase is from 43 000 to 49 000 Da and the optimum pH value is around 4. The manganese peroxidase creates low molecular weight oxidising agents that diffuse into the lignin substrate and are able to oxidise phenolics residues in the lignin. [22.]

4.2.3 Biodegradation of Hemicelluloses

Hemicelluloses are biologically degraded by cleavage of the chemical bonds between the sugar residues with heimicellulases. Hemicellulases mainly include xylanase and mannanases. Hemicellulases have a positive role in the pulp and paper industry to remove the hemicelluloses from the dissolving pulps.

- Xylanase

The complex of polymeric carbohydrates include xylan, xyloglucan, glucomannan, galactoglucomannan, arabinogalactan. Xylan is a major structural polysaccharide in plant cells. In addition to cellulose, xylan is the most abundant polysaccharide in nature and it is the major component of hemicellulose. Xylanase is an enzyme in the degradation of hemicellulose xylan. It is hydrolytic enzyme, which are produced by plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods. Xylanase is a widespread group of enzymes and can be classified as different families. The structure of xylan and action of enzymes of the xylanase complex are shown in the following figure 4.8.

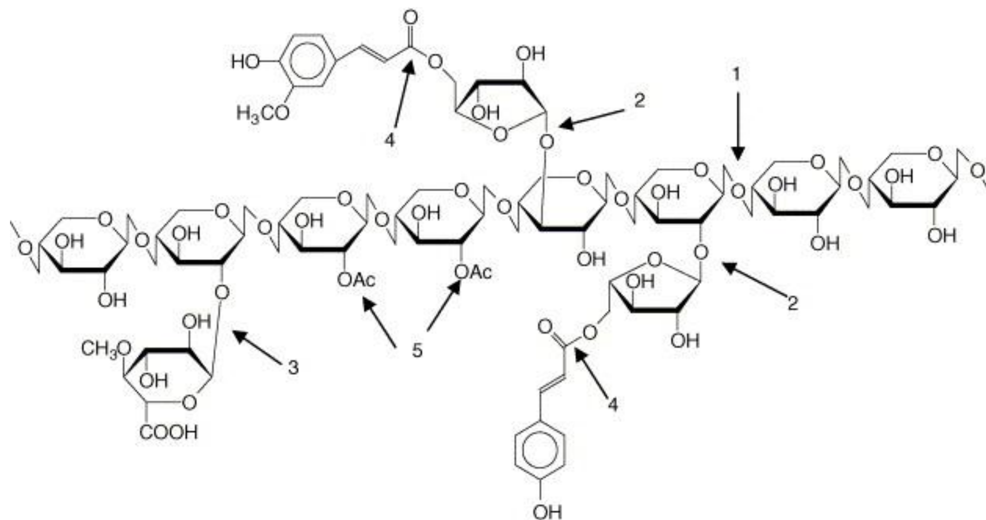

Figure4.8. Structure of xylan and site of action of the enzymes of the xylanase complex. (1: endoxylanases; 2: α-L-arabinofuranosidases; 3: glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases) [23]

There are various substances in xylan and one kind of xylanase is not enough to finish the process of biodegradation. Therefore, the biodegradation of xylan needs a set of xylanase complex. Different xylanase has different functions during the process. The endoxylanases hydrolyze the main chain of xylan and produce a mixture of xylooligosaccharides. The α-L-arabinofuranosidases can remove L- arabinofuranose side chains. The glucuronidases normally hydrolyze the methyl glucuronate residues. The acetyl xylan esterases could hydrolyze acetate groups from the main chain. [23.]

- Mannanases

Mannanase has been used in paper industry as well. There is a wide variety of mannanases involved in the degradation of hemicelluloses. Mannans have two

forms, glucomannan and linear mannan, in nature. For example, mannases are enzymes that degrade 1.4-β and 1.6-α glycosidlic bonds of mannans. Endo-β-1.4-mannanases (β-mannanases) are mainly functional to enzymatic depolymerization of mannan backbone. [24.] The structures of different forms of mannans and the enzymes required for their hydrolysis are shown in figure 4.9.

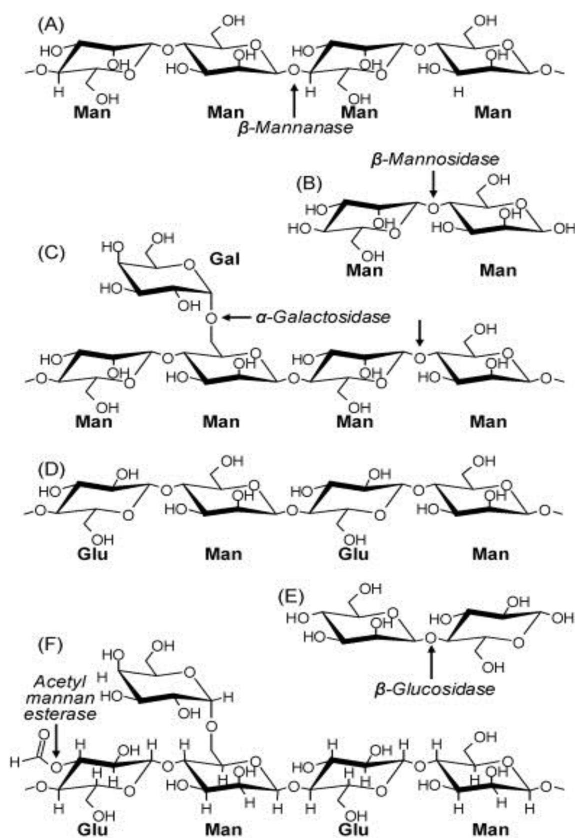

Figure4.9. Structures of different forms of mannans and the enzymes required for their hydrolysis [24]

From the figure shown above, it can clearly be found out that different mannanases act on different hemicelluloses. Major enzymes, involved in the hydrolysis of linear mannans and glucomannans, are 1.4-β-D-mannan mannohydrolases (β-mannanases), 1.4-β-D-mannopyranoside hydrolases (β-mannosidases) and 1.4-β-D-glucoside glucohydrolases (β-glucosidases). The β-mannanases are endo-acting hydrolases, attacking the internal glycosidic

bonds of the mannan backbone chain, releasing short β-1,4-manno-oligosaccharides. The β-mannosidases are exo-acting hydrolases, releasing mannose from the oligosaccharides by attacking the terminal linkage at the non-reducing end as well as cleaving mannobiose into mannose units. The β-glucosidases remove the 1,4-glucopyranose units at the non-reducing end of the oligomers derived from the degradation of glucomannan and galactoglucomannan. [24.]

The broad substrate specificities of β-mannanases and their specialised functionality cause a board application. The β-mannanases have been widely applied into nutraceutical production, pharmaceutical applications, food and feed applications, animal feeds, commodity production, paper and pulp production and detergent formulations, etc. The β-Mannanases are commonly found as a part of the hemicellulase of hydrolases produced by ascomycetes fungi. In paper and pulp industy, the β-mannanases are applied in the bleaching of pulp. The presence of β-Mannanases can facilitate bleaching, eliminating residual lignin, increasing paper brightness and reducing loss of fiber yield. [24.]

5 YEASTS

Yeasts are eukaryotic micro-organisms classified in the Fungi. Yeast is a single-cell protein and its protein-content is around 50%. It has plenty of amino acid content and rich B vitamins, enzymes and a variety of physiologically active substances with high economic value. Generally, yeasts are 3–4 μm in diameter, but some can reach over 40 μm. The size of yeasts depends on diverse species as well as the cell age and environmental conditions. Yeast is widely distributed

in nature, mainly grown in acidic sugary environment. It is present in fruits, vegetables, nectar, plant leaf surface, orchard soil, milk, animal excrement and air, etc. The figure 5.1 shows the yeast cells and yeast products. [25.]

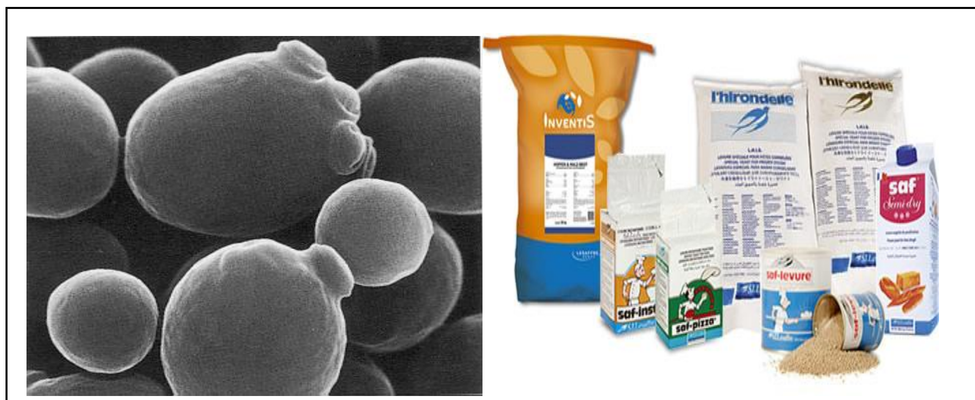

Figure5.1. Yeast cells and yeast products

The organelles and compartments in yeast cell mainly consist of cell wall, plasma membrane, periplasmic space, bud scars, nucleus, mitochondria and so on. The cell wall is composed of 6-8% protein, 8-14% liquid and a small amount of chitin (1-2%). It constitutes 15-25% of the dry weight of the cell. The cell wall has two polysaccharides that are 30-35% glucan, 30% mannan and minor percentage of chitin. Other components of cell wall are proteins, lipids and inorganic phosphate. The plasma membrane is about 7 nm thick. The plasma membrane controls what enters and what leaves the cytosole, as well as the extrusion of hazardous molecules to the cell. The main function of the cell membrane is selectively transported nutrients for excreted metabolites. Meanwhile, it is also biosynthesis and assembly base of macromolecular component and it is the fraction of enzyme synthesis and effect place.

The cell nucleus is eukaryotes, and has completely nuclear in the cell. It is a round-lobate organelle, some 1.5 μm in diameter. The main component of the

nucleus is deoxyribonucleic acid (DNA), which is the control center of the metabolic processes and plays an important role in breeding and genetics. The mitochondria are granular or rod-like organelles and located in the cytoplasm. Each cell has 1 to 20 mitochondria. The important factors affected contain partial oxygen pressure, glucose concentration, presence of unfermentable substrates, availability of sterols and fatty acids, and of particular metal ions (Mg++). [26.] The organelles and compartments in yeast cell are shown in the figure 5.2.

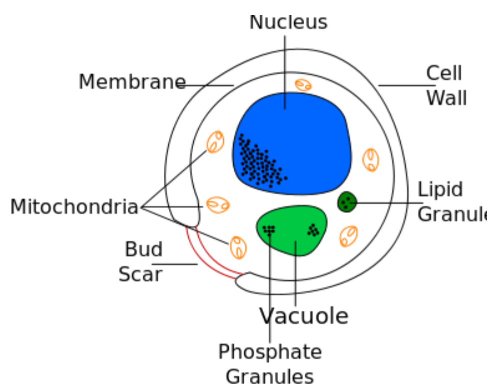

Figure5.2. Organelles and compartments in yeast cell

There are various types of commercial yeast, like fresh, compressed-fresh and dehydrated, they have different moisture content and are shown in figure 5.3. The chemical composition of fresh yeast is shown in the figure 5.4. Most of yeasts favour a pH around 4.5-5.0. The range of temperature for growing is various. For example, Leucosporidium frigidum grows at -2 to 20 °C, Saccharomyces telluris grows at 5 to 35 °C, and Candida slooffi grows at 28 to 45 °C. The cells can survive freezing under certain conditions, with viability decreasing over time. [27.], [28.]

Fingure5.3. Fresh yeast, compressed fresh yeast and dehydrated yeast

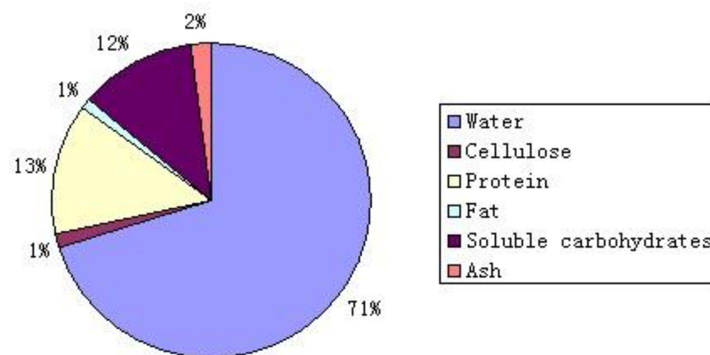

Figure5.4. Chemical composition of fresh yeast [28]

Yeast, like other living organisms, requires growth factor of oxygen, carbon, nitrogen, phosphorus, trace elements. All wild-type yeasts use glucose, mannose and fructose. Some yeasts also use sugars, organic acids and nitrates, and some yeasts only use ammonium salts. Some species can synthesize the growth factors, but many species must be supplied the growth factors. In an environment with oxygen, yeast changes glucose into water and carbon dioxide. Under anaerobic conditions, glucose is broken down into carbon dioxide and alcohol.

6 APPLICATIONS OF YEASTS

Yeasts are a useful application of valuable micro-organisms. They are micro-organisms that are diverse, reactive, robust, genetically manipulable. Yeast has a very early application and closed relationship with human beings. It plays an important role in the brewing, food, medicine and other kinds of industries. Different yeasts have different characteristics and biotechnological applications. Some species play beneficial roles and some are detrimental organisms of human disease. According to the application areas, the yeast application can be divided into six parts, i.e. for environmental technologies, for biomedical research, for fundamental biological research, for health-care industries, for food/chemical industries and for fermentation industries. [29.]

For environmental technologies, yeasts can be used in bioremediation, waste utilization, crop protection, biosorption of metals. For example, yeasts are widely used in production of single-cell proteins by many liquid wastes; vegetable and fruit processing wastes contain starch, cellulose, organic acids and are suitable substrates for yeasts utilizing organic acids; waste brine generated from kimchi production was proposed for cultivation of osmotolerant yeast; acid hydrolysate of shrimp-shell wastes also can be used for yeast biomass production. On the other hand, the wastewater from lemonade-processing can be assimilated by yeasts. The yeasts can remove approximately 80%, 70%, and 90% of waste BOD, nitrogen and phosphorus respectively in 16 hours; Yeasts also can reduce the wastewater strength of potato-processing wastewater along with the recovery of biomass. [30.], [31.]

Yeasts are used plenty in biomedical research, e.g. cancer, AIDS, drug metabolism, genotoxicity screens, human genetic disorders. In the study of cancer, the best treatment is to try to control proteins that lead to unlimited reproduction of cancer cells and using yeasts to find this type of proteins. Yeasts contain the genes and these genes are also present in human cells. Gene is made of DNA and it contains the instructions of the manufacture of proteins, these proteins determine the structure of the cell and control over their activities. More than 70% of the genes of the yeasts are similar with genes of human cells. For health-care industries, yeasts are used in pharmaceuticals, vaccines, probiotics, hormones, blood factors. The yeast-derived vaccine was first introduced in the United States in 1986. It has proven to be safe and effective. *[28.], [33.]*

Yeast is also used for fermentation industries, e.g. brewing, bioethanol, novel processes and fermentation product. For example, in the paper mill, wood chips are treated by the sulphite or sulphate method. In the sulphite or sulphate process, chips are cooked in liquor which containing sodium, magnesium, ammonium. The obtained waste liquor is called sulphite spent liquor (SSL). After removing sulphur dioxide, sulphurous acid and furfural from the liquor, the huge amounts of SSL can be used in alcohol fermentation, in the production of protein biomass for animal feed. [34.]

6.1 Yeast application for wood-based materials

Yeasts have an application in wood-based materials. They can be used for the conversion from wood to ethanol or from sulphate pulping waste to ethanol.

During these processes, the yeast, saccharomyces cerevisiae, is the most commonly used.

<u>Conversion from wood to ethanol</u>

When the wood is used as the raw materials, the hemicellulose and cellulose polymers are hydrolyzed with enzymes. The purpose is to release sugars. The sugars after the pre-treatment process and enzymatic hydrolysis step can be fermented by saccharomyces cerevisiae. After final distillation and evaporation, the ethanol is produced. The rest part of the lignin and other solid parts of biomass remaining can be burnt to provide heat and electricity for the process. [35.] The process of conversion from biomass to ethanol is shown in figure 6.1.

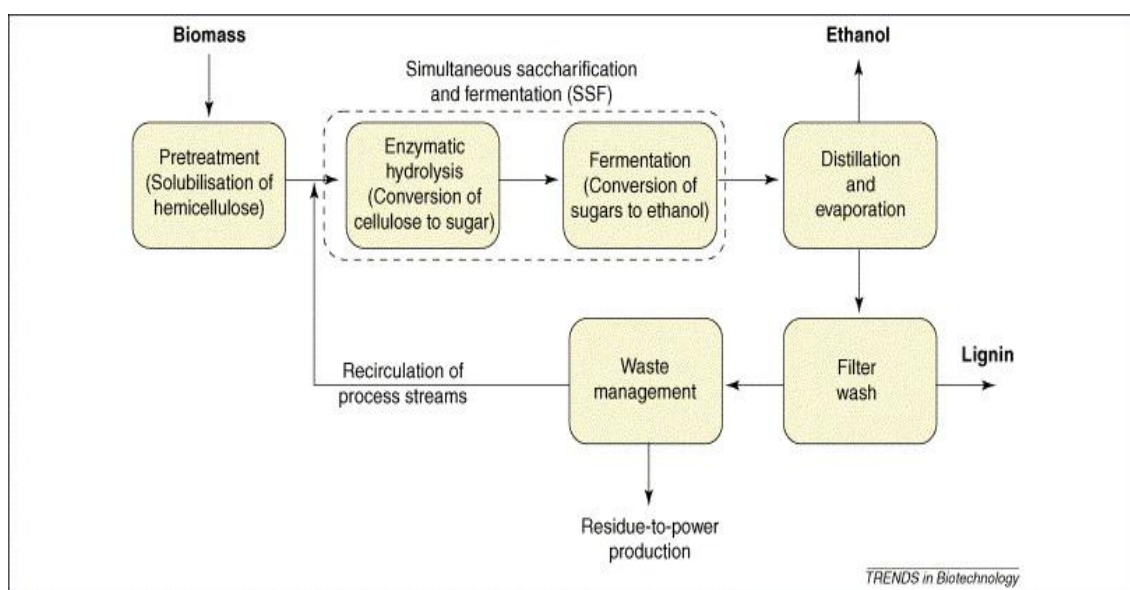

Figure6.1. Flow sheet for the conversion of biomass to ethanol [35]

Conversion from sulphate pulping waste to ethanol

The waste liquor (black liquor) is produced during sulphate pulping process. In the process, wood chips are cooked with white liquor, which contains sodium hydroxide (NaOH) and sodium sulphide (Na_2S). The composition of black liquor is shown in table 6.1.

Table6.1. Composition of sulphate waste liquor [36]

Component	Concentration (g/l)
Total solid	110-140
Ignition residue	15-17
Volatile organic acid	5-7
Sulphonic acid	0.6-1.2
Acetic acid	4.2-5.2
Calcium oxide	6.5-9.5
Sulphur (total)	10-12
Sulphite	1.3-2.5
Free sulphurous acid	1.4-3.4
Organic solvent compounds	9.5-14.2
Permanganate value ($KMnO_4$)	400-4000
Methyl alcohol	0.7-1.3
Ethyl alcohol	0.15-0.23
Acetone	0.1-0.13
Furfural	0.24-0.29
Lignin	56-69
Carbohydrates	20-21
Pentoses	2.5-3.4
Hexoses	17-18

After cooking, the pulp is separated from residual liquor, which contains wood lignin, organic materials and inorganic compounds. The residual liquor experiences a set of evaporation processes to increase the concentration of solid. Therefore, many chemicals are contained in the black liquor, e.g. Vanillin, lignosulfonates, alcohol, sugars, etc. [32.]

In order to use black liquor for alcoholic fermentation, waste liquor must firstly experience pre-treatment process that is shown in figure 6.2.

Figure6.2. Pre-treatment process of waste liquor

Firstly, the hot waste liquor goes through air stripping to exclude volatile harmful substances SO_2. Secondly, lime water or other alkaline solution is used to neutralize acid in waste liquor until the pH value is 5.4-5.5. Thirdly, using precipitation and decantation methods are used to remove calcium sulphate and sulphate calcium, which are produced in the neutralization process. Lastly, nitrogen and phosphorus source are added in the clear liquid. Commonly used nitrogen sources are ammonium sulphate, ammonia, urea, etc. and commonly used phosphorus source is calcium superphosphate. [37.], [38.]

Yeasts used in ethanol fermentation

The most common yeast used for ethanol-producing from sugar is saccharomyces cerevisiae. The principles of the glucose fermentation can be indicated as the chemical reaction below,

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad (7)$$

Meanwhile, E. coli and zymomonas mobilis as two basic bacteria are always mentioned for ethanol fermentation, as shown in the following table 6.2.

Table6.2. Comparison of ethanol fermentation by saccharomyces cerevisiae, E. coli and zymomonas mobilis [39]

Species	Saccharomyces cerevisiae	E. coli	Zymomonas mobilis
Substrates	Glucose, sucrose, xylose	Glucose, xylose	Glucose, fructose, sucrose, xylose
Medium	Simple medium	Simple medium	Simple medium
Culture pH	pH 5	pH 6-8	pH 7
Product yield	0.47g/g glucose 0.43g/g xylose	0.46g/g glucose 0.46g/g xylose	0.49g/g glucose nil from xylose
Product concentration	120g/L from glucose	~50g/L	120g/L from glucose
Productivity	>1.4g/L·h	0.83g/L·h	>2g/L·h with glucose

The saccharomyces cerevisiae mainly consists of proteins, glycoproteins, polysaccharides, polyphosphates, lipids, and nucleic acids. It is the cheapest

yeast for the conversion of biomass substrate. It is generally ellipsoidal in shape ranging from 5 to 10 μm at the large diameter and 1 to 7 μm at the small diameter. Mean cell volumes are 29 μm^3 for a haploid or 55 μm^3 for a diploid cell. The cell size increases with age. [40.]

The saccharomyces cerevisiae grows both under aerobic and anaerobic conditions and performs well in industrial fermentation conditions. The influencing parameters that affect the production of bio-ethanol from sugar are optimized. The pH range of saccharomyces cerevisiae is wide, with an optimum at acidic pH environment. It tolerates temperatures up to around 40°C, with an optimum temperature around 30-35°C. The optimum substrate concentration, enzyme concentration and fermentation period are 300 gm/l, 2 gm/l and 72 h respectively. Under this optimum operating condition the maximum of 53% bio-ethanol yield is achieved. [40.], [41.]

Due to the large size, thick cell wall and resistance to bacterial, the saccharomyces cerevisiae can produce a higher ethanol yield than E. coli and zymomonas mobilis. But zymomonas mobilis produces ethanol at a much faster rate than yeast. The saccharomyces cerevisiae and E.coli only ferment limited sugars, but the zymomonas mobilis can ferment all sugars. Except usage in the ethanol producing industry, the saccharomyces cerevisiae also has a board application in the aspect of baking, dairy, brewing, wine fermentation, fruit juices, alcoholic beverages and so on. [42.]

6.2 Yeast application in wastewater treatment

The wastewater from different industries contains various chemicals and substances. The use of yeast in biological treatment of industrial wastewater has been used in many decades. It has special advantages of high efficiency, high sludge load, excess sludge can be recycled as feed protein, etc.

The figure 6.3 shows that the carbon and nitrogen removal system can be altered with anaerobic and yeast treatment system. The comparison between yeast and anaerobic treatment process is presented in table 6.3.

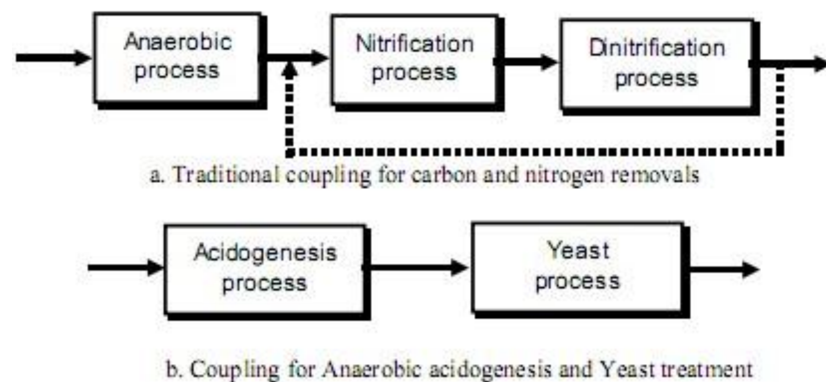

Figure6.3. Carbon and nitrogen removal system can be altered with anaerobic and yeast treatment system [43]

Table6.3. Comparison between yeast and anaerobic treatment process [43]

Yeast process	Anaerobic process
Cannot degrade complex organic compounds	Degrades cellulose
High nutrient requirement (BOD5:N:P)	Low nutrient requirement
Need for oxygen and agitation	Slight agitation
Exothermal reaction → need for cooling	Sensitive to variation of temperature
Can consume VFAs produced from acidogenesis	Dependent on two phases: liquefaction and gasification
High organic loadings	Low organic loadings
short HRT	High HRT (minimum 10 days)
Valuable biomass	Poor sludge production

In some high organic strength wastewater, there are carbon and nitrogen that have to be removed. The traditional processes of removal are anaerobic and aerobic processes, nitrification and denitrification. Nowadays, more and more yeasts are introduced to the processes. The fermentative bacteria transform the carbonaceous substrates and organic nitrogen to ammonia and VFA which are degradable substrates for yeast growth.

Yeast used in wastewater treatment in olive oil manufacture

The wastewater in olive mill mainly includes high concentration of fats, sugars, phenols, volatile fatty acids (VFA) that contribute to a high COD concentration (100-200 g/L). Using yeast, like yarrowia lipolytica, can reduce the COD level of

olive oil processing wastewater by 80% in 24 hours. Yarrowia lipolytica is aerobic yeast with the ability to degrade efficiently hydrophobic substrates such as n-alkanes, fatty acids, fats and oils. Yarrowia lipolytica's growth and metabolite secretion are effected by different environmental factors, which contain the amount of oxygen and pressure in the culture medium. It grows at pH values of 4.0-6.0 and at temperature of 10°C. [44.]

Yarrowia lipolytica has been considered a suitable model for dimorphism studies in yeasts, because it has an efficient system for genetic engineering transformation. In contrast to saccharomyces cerevisiae, yarrowia lipolytica does not produce true filaments and exhibits pseudo-hyphae growth under nitrogen limited conditions. Yarrowia lipolytica is a reliable, versatile and popular system for the expression of heterologous proteins with different features.

Yarrowia lipolytica is routinely isolated from different food media, such as cheeses, sausages. The yarrowia lipolytica has no ability to ferment sugars and it has very strong lipase and protease activity. One of the most important products secreted from yarrowia lipolytica is lipase. When Yarrowia lipolytica is grown under nutrient-limited conditions, it can be used to produce citric acid from a variety of carbon sources, including alkanes, plant oils, starch hydrolysates and raw glycerol. [44.]

Yeast used in wastewater treatment in silage manufacture

The silage is produced from fermentation of a kind of crop with high moisture content, such as grass or maize. This silage is used to animal feeding. The

wastewater during processes is extremely polluting and it has high BOD (30-80 g/L) and low pH, that is from 3.0 to 4.5. The yeast, i.e.Candida. utilis, could effectively remove 74-95% COD, 85-99% VFA, 82-99% phosphate in 24 hours. In the treatment process, the pH initial values of 3.7-5.8 normally increase to 8.5-9.0. [43.]

The candida utilis is a yeast-like fungus of the genus Candida. Its yeast cells are round or oval. The protein and vitamin B content in candida utilis are higher than saccharomyces cerevisiae, the remaining being represented by lipids, polysaccharides, etc. It uses urea and nitric acid as a nitrogen source and can grow in the medium without any growth factor. The candida utilis can ferment glucose, sucrose, raffinose, but it cannot ferment maltose, galactose, lactose and melibiose. It does not break down fat and is able to assimilate nitrate.

<u>Yeast used in wastewater treatment in dairy industry</u>

Wastewater from dairy industry contains plenty of milk constituents such as casein, lactose, fat and high inorganic salt. After treatment by yeast, i.e. Candida parapsilosis, the maximum BOD (90%) and COD (82%) could be removed. [43.]

The candida parapsilosis is a yeast-like fungus of the genus candida. The candida parapsilosis is a nonfermenting yeast and is a strictly aerobic organism that has attracted attention due to its mitochondria. The candida parapsilosis has been isolated from nonhuman sources such as domestic animals, insects or soil. Also, it is one of the fungi most frequently isolated from the skin, hands and

mucous membranes of healthy people. Nowadays, the candida parapsilosis is one of the most commonly isolated Candida species from blood cultures in Europe and is comprehensively applied in hospitals.

7 SUMMARY

Biotechnology has played a vital role in the development of mankind. This theoretical study has offered the knowledge to understand enzymes and yeasts and their application in our lives. Enzymes are biological catalysts, which have been used for a long time by human being. According to the structure of enzymes, enzymes can be classified as monomer enzyme, oligomeric enzyme, multienzyme system and multi-enzyme complex. On the other hand, the enzymes are divided into six categories according to their catalyzed reaction type, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerase and ligases. Yeasts are eukaryotic microorganisms. The Organelles and compartments in yeast cell mainly consist of cell wall, plasma membrane, periplasmic space, bud scars, nucleus, mitochondria etc.

Nowadays, the enzymes have been applied plenty in the field of food, dairy, detergents, leathers, textiles, chemical and pulp and paper industry, etc. For wood-based industry, application of enzymes decreases the energy consumption and environmental pollution. Wood is mainly composed of three main compounds, i.e. cellulose, hemicellulose and lignin. For treatment of lignin, the enzymes of laccase, lignin peroxidase and manganese peroxidase can degrade lignin by different catalyzed circles. For biodegradation of hemicelluloses, the xylanases and mannanases have played the most important roles.

Yeasts have been involved in many application areas, such as environmental technologies, fundamental biological research, food/chemical industries, fermentation industries etc. When using the wood as the raw material, the bio-ethanol is usually produced from the fermentation by the yeast. The most

common yeast used for ethanol producing from sugar is saccharomyces cerevisiae. The important parameters for the fermentation process are pH, temperature, substrate concentration, concentration of enzyme as well as fermentation period. For biological treatment of wastewater, yarrowia lipolytica, candida utilis and candida parapsilosis are commonly used for the various industry processes to efficiently remove BOD, COD or other pollutants.

FIGURES

Figure 3.1. Primary Structure of Enzyme

Figure 3.2. Structure of polypeptide chain in secondary structure of enzyme

Figure 3.3. Tertiary structure of enzyme

Figure 3.4. Quaternary structure of enzyme

Figure 4.1. Products of enzymes of protease, amylase and lipase

Figure 4.2. Sequence of steps in the isolation of enzymes

Figure 4.3. Overview of Bio-pulping process

Figure 4.4. Environment of four coppers in the active site of laccases

Figure 4.5. Catalytic cycle of lignin peroxidase

Figure 4.6. Reaction catalyzed by manganese peroxidase

Figure 4.7 Structure of manganese peroxidase and the active site of manganese peroxidase

Figure 4.8. Structure of xylan and site of action of the enzymes of the xylanase complex

Figure 4.9. Structures of different forms of mannans and the enzymes required for their hydrolysis

Figure 5.1. Yeast cells and yeast products

Figure 5.2. Organelles and compartments in yeast cel

Figure 5.3. Fresh yeast, compressed fresh yeast and dehydrated yeast

Figure 5.4. Chemical composition of fresh yeast

Figure 6.1. Flow sheet for the conversion of biomass to ethanol

Figure 6.2. Pre-treatment process of waste liquor

Figure 6.3. Carbon and nitrogen removal system can be altered with anaerobic and yeast treatment system

TABLES

Table 6.1. Composition of sulphate waste liquor

Table 6.2. Comparison of ethanol fermentation by saccharomyces cerevisiae, E. coli and zymomonas mobilis

Table 6.3. Comparison between yeast and anaerobic treatment process

FORMULAE

Formula 1. $A_{reduced} + B_{oxidized} \rightarrow A_{oxidized} + B_{reduced}$

Formula 2. $AR + B \leftrightarrow A + BR$

Formula 3. $\overset{X}{C} - \overset{Y}{C} \leftrightarrow C = C + X - Y$

Formula 4. Glucose 1 - phosphate $\xrightarrow{\text{phosphoglucomutase}}$ glucose 6 - phosphate

Formula 5. Amino acid + specific tRNA $\xrightarrow{\text{aminoacyl – rRNA synthetase + ATP}}$ amino acid tRNA complex + ADP + P_i

Formula 6. Lactose + water $\xrightarrow{\text{Lactase}}$ glucose + galactose

Formula 7. $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

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APPENDICES

APPENDIX I

The enzyme classification system developed by the Enzyme Commission (EC), a commission of IUPAC (International Union of Pure and Applied Chemistry) [3]

Enzyme classes and sub-classes	functions	Remarks
1 Oxidoreductases 1.1 at –CH-OH 1.2 at-C=O 1.3 at-C=C-	Oxidation-reduction reactions	Cosubstrate required, Two-substrate reactions
2 Transferases 2.1 C1-groups 2.2 Aldehyde- or ketogroups 2.3 Acyl-roups 2.4 Glycosyl-groups	to promote the exchange or transfer of certain chemical groups between the molecules of different substances	Two-substrate reaction, one substrate must be activated
3 hydrolases 3.1 Ester bonds 3.2 Glycoside bonds 3.3 Ether bonds 3.4 Peptide bonds 3.5 Amide bonds	to promote the hydrolysis reaction	Two-substrate reaction, one of these is H_2O
4 lyases 4.1 C-C	to catalyst reaction of baseplates group	One-substrate reaction→

4.2 C-O	or off groups on the double bond of the substance molecule, promote a compound is split into two compounds, or two compounds synthesis to a compound	bond breaking, Two-substrate reaction→ Bond formation
5 isomerases 5.1 Racemizations 5.2 Cis-trans-isomerizations 5.3 Intramolecular oxidoreductases	to promote of mutual transformation of isomers, catalyst the rearrangement reaction of substrate molecule inside	One-substrate reaction
6 ligase 6.1 C-O 6.2 C-S 6.3 C-N 6.4 C-C	to promote two molecules compounds get together, breaking the high-energy phosphate bond in the ATP molecule	Require ATP as cosubstrate, Two-substrate reaction

Appendix II

Principal separation methods used in purification of enzymes

Property	Method	Scale
Size or mass	Centrifugation	Large or small
	Gel filtration	Generally small
	Ultrafiltration	Generally small
Polarity		
(a) Charge	Ion-exchange chromatography	Large or small
	Chromatofocusing	Generally small
	Electrophoresis	Generally small
	Isoelectric focusing	Generally small
(b)Hydrophobic character	Hydrophobic chromatography	Generally small
Solubility	Change in pH	Generally small
	Change in ionic strength	Large or small
	Decrease in dielectric constant	Generally small
Specific binding sites or structural features	Affinity chromatography	Generally small
	Immobilized metal ion chromatography	Generally small
	Affinity elution	Large or small
	Dye-ligand chromatography	Large or small
	Immunoadsorption	
	Covalent chromatography	

APPENDIX III

Overview of industrial enzyme applications [10]

E.C. number Name	Application areas	Functions
Class 1: Oxidoreductases		
	Baking	Increase gluten strength
1.1.3.4 Glucose oxidase	Brewing	Shelf life improvement
	Dairy	Milk preservation
	Textile	Indirect enzymatic bleaching
	New industrial use	Tooth paste
1.1.3.5 Hexose oxidase	Baking	Increase gluten strength
1.10.3.2 Laccase	Textile	Prevention of backstaining in enzymatic stone washing
	Pulp and paper	Pulp bleaching
	New industrial use	Cork treatment
	New industrial use	Polymerization of lignin for production of wood fiberboards
1.11.1.6 Catalase	Brewing	Shelf life improvement
	Dairy	Milk preservation
	Textile	Hydrogen peroxide removal
	New industrial	Wastewater treatment,

	use	Hydrogen peroxide removal
1.11.1.7 Peroxidase, lactoperoxidase	Baking	Dough improvement
	Dairy	Milk preservation
1.13.11.12 Lipoxygenase	Baking	Whitening of breadcrumb
Class 2: Transferases		
2.3.2.13 Transglutaminase	Dairy	Texture improvement in yoghurt, whipped cream
2.4.1.5 Dextransucrase	Brewing	Production of isomaltooligosaccharide beer
Class 3: Hydrolases		
3.1.1.3 Triacylglycerol lipase	Baking	Bread improvement
	Dairy	Cheese clotting
	Laundry detergent	Removal of greasy stains
	Pulp and paper	Pitch removal
3.1.1.11 Pectin methylesterase	Fruit juice	Apple and red berry juice, citrus fruit peeling
3.1.1.26 Galactolipase	Baking	In situ formation of surfactants in dough for better gas retention
3.1.3.8 3-phytase	Brewing	Mashing
	Animal feed	Phosphate release from phytic acid in animal feed
3.1.3.26 6-phytase	Brewing	Mashing

	Animal feed	Phosphate release from phytic acid in animal feed
3.2.1.1	Baking	Antistaling in dough
	Fruit juice	Apple juice production
	Brewing	Mashing, fermentation
	Laundry detergent	Removal of starch containing stains
	Automatic dishwashing	Removal of starch containing stains
	Grain wet milling	Starch hydrolysis
	Animal feed	Improved digestion of starch in maize feed
	New industrial use	Viscosity control in oil drilling
	textile	Textile desizing
3.2.1.2 ß-amylase	Brewing	Mashing
3.2.1.3 Glucoamylase, amyloglucosidase	Fruit juice	Apple juice production
	Brewing	Mashing
	Grain wet milling	Hydrolysis of maltooligosaccharides
	New industrial use	Toothpaste
3.2.1.4Endo-1,4-ß-glucanase, cellulase	Brewing	Fermentation
	Laundry detergent	Removal of particular soils, softening, improved color brightness

	textile	Cotton finishing, denim ageing
3.2.1.6 4Endo-1,4(3)-ß-glucanase, cellulase	Animal feed	Improved weight gain and feed efficiency in poultry and swine
3.2.1.8 4Endo-1,4-ß-xylanase	Baking	Improved dough handling, dough stability
	Animal feed	Increase digestibility of cereals
	Textile	Flax retting
	Pulp and paper	Pulp bleaching
3.2.1.55 Arabinosidase	Fruit juice	Apple juice production
3.2.1.60 Glucan 1,4-α-maltotetraohydrolase	Baking	Antistaling in dough
3.2.1.67 Exopolygalacturonase	textile	Cotton scouring
3.2.1.78 Endo-1,4-ß-mannanase	Industry detergent	Removal of guar gum containing stains
	Textile	Flax retting
	Pulp and paper	Pulp bleaching
	New industry use	Viscosity control in oil drilling
3.2.1.91 Exo-cellobiohydrolase	Laundry detergent	Removal of particular soils, softening, improved color brightness
	Textile	Cotton finishing
	Pulp and paper	Mechanical pulping
3.4.X.X	Textile	Silk degumming, wool

		antishrinking
	New industry use	Artificial-denture cleaning
3.4.21.62 Subtilisin	Laundry detergent	Removal of proteinaceous stains
	Automatic dishwashing	Removal of proteinaceous stains
	Animal feed	Improved digestibility of proteins in animals feed
	New industry use	Membrane cleaning
3.4.21.63 Oryzin	Animal feed	Improved digestibility of proteins in animals feed
3.4.22.X Cysteine endopeptidases	Brewing	Filtration acid
	New industry use	Skin care
3.4.23.4 Chymosin	dairy	Cheese clotting
3.4.23.18 Aspergillopepsin I	Animal feed	Improved digestibility of proteins in animals feed
3.4.23.22 Endothiapepsin	Dairy	Cheese clotting
3.4.23.23 Mucorpepsin	Dairy	Cheese clotting
3.4.24.28 Bacillolysin	Animal feed	Improved digestibility of proteins in animals feed
Class 4: Lyases		
4.1.1.5 α-acetolacetate decarboxylase	Brewing	Diacetyl removal for flavour enhancement
4.2.2.2 Pectate lyase	Textile	Cotton scouring
4.2.2.10 Pectin lyase	Fruit juice	Apple and red berry juice, citrus fruit peeling
Class 5: Isomerases		

5.3.1.5 Xylose isomerise	Grain wet milling	Fructose production
5.3.4.1 Protein disulfide isomerase	New industrial use	Cosmetics, hair waving

APPENDIX Ⅳ

Organic acids obtained using the yeast yarrowia lipolytica [45]

Acid	Substrate	Product	
		$g\ L^{-1}$	yield (% substrate)
KGA	Petrolatum	109	120
	Ethanol	50	50
PA	Glucose	50	50
	Glycerol	61	71
CA+ICA	Petrolatum	102	142
ICA	Petrolatum	60	60
	Ethanol	66	66
CA	Petrolatum	217	145
	Ethanol	120	88
	Head fraction of ethanol	116	88