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SYNTHESIS OF HIGHER ALCOHOLS DURING ALCOHOLIC FERMENTATION OF RYE MASHES

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Formation of by-products during alcoholic fermentation is a complex process. Particular attention should be paid to generation of higher alcohols because of its complex mechanism and dynamics. In XIX century the higher alcohols were thought about as “bacterial metabolites of spoilage” that contaminate alcoholic beverages.

At the beginning of XX century Ehrlich proved that these compounds were produced by yeast from amino acids and they naturally occurred in all alcoholic beverages derived from spirits of agricultural origin. The quantity and profile of fusel alcohols in the wash depend on many factors such as raw materials used to prepare the sweet mash, yeast strain and the inoculum dose, supplements added to the mash. Investigations of many researchers prove that higher alcohols are formed through catabolic and anabolic pathways. They are either products of amino acid catabolism – as was found by Ehrlich or by-products of amino acid synthesis from pyruvate through the anabolic pathway. The occurrence of fusel alcohols in raw spirits from agricultural distilleries is a result of the presence of amino acids, sugars and products of their metabolism mainly aldehydes, in fermented mashes.

Introduction

One of Polish Standards regulates allowable concentrations of alcoholic fermentation by-products like methanol, aldehydes, esters and organic acids in raw spirits from agricultural distilleries but it does not refer to higher alcohols [1]. Concentration of the latter compounds is limited by another Polish Standard related to fuel alcohol (bioethanol) PN-A-79521 [2]. The majority of alcoholic fermentation by-products have a strong impact on taste and aroma of alcoholic beverages. Higher alcohols and esters make an essential contribution to the flavor of beer, wine and vodka [3].

Processes that yield higher alcohols were unknown till the beginning of XX century. In XIX century these compounds were believed to be bacterial metabolites of spoilage contaminating alcoholic beverages [4]. First theories assumed that they derived from sugars through bacterial fermentation taking place simultaneously with the alcoholic fermentation. For the first time they were identified by Scheele in 1785 in fermented starch mashes. It gave rise to the name of amyl alcohol, which was coined from the Latin word *amylum* – it means starch [5, 6].

The quantity and profile of fusel alcohols in the wash depend on many factors. Concentrations of higher alcohols vary between 0.1 and 0.7% in relation to ethanol produced. Isoamyl alcohol (60-80%), isobutanol (15-25%) and n-propanol (4-7%) are the most abundant of fusel alcohols [4, 5, 7].

Theories and mechanisms of higher alcohols formation during alcoholic fermentation

Investigations on higher alcohol formation gave rise to the development of theories of: Ehrlich, Neubauer and Fromherz, Guymon, Sentheshanmaganathan, and Genevois and Lafon. It was proved that higher alcohols derived through catabolic and anabolic pathways (Fig. 1) [8].

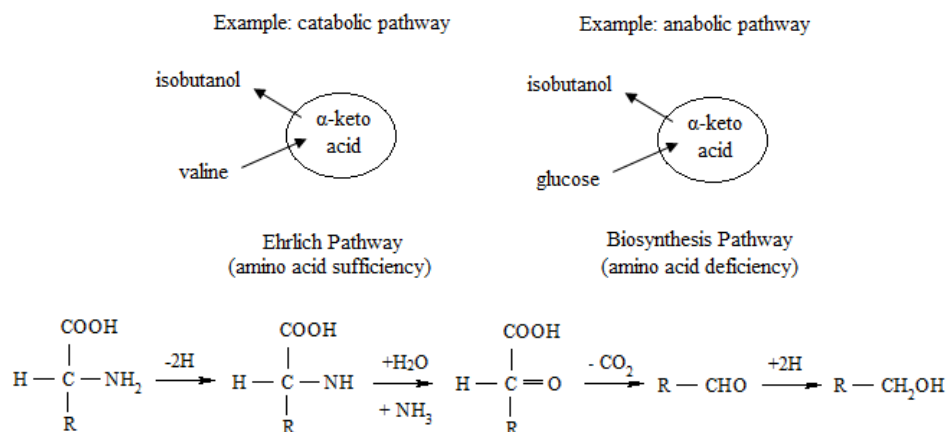


Fig. 1. Higher alcohols (fusel oil) are produced by yeast metabolism via two routes both of which utilize α -keto acid [8]

In the catabolic pathway proposed by Ehrlich higher alcohols derived from amino acids while in the anabolic process they derived from sugars as by-products of amino acid synthesis [9-11].

Ehrlich was a pioneer in the studies on the mechanism of higher alcohols synthesis. He was the first researcher who noticed that higher alcohols derived from essential amino acids in beverages fermented by yeasts of the genus

Saccharomyces. Ehrlich proved that these yeasts released ammonia from molecules of amino acids and assumed that it was incorporated into yeast proteins while the higher alcohols resulting from this metabolic process were secreted by cells to the environment [3, 12, 13]. Ehrlich named his theory “the alcoholic fermentation of amino acids”. He found that 2-methylbutanol, 3-methylbutanol and isobutyl alcohols were obtained through decarboxylation and deamination of leucine (Fig. 2), isoleucine and valine, respectively [5].

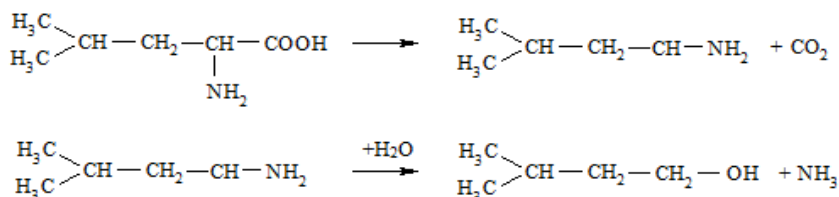


Fig. 2. The scheme of isoamyl alcohol synthesis according to Ehrlich pathway [5]

Studies of Buchner and Meisenheimer showed that Ehrlich theory was reasonable but the reaction of deamination preceded the decarboxylation of amino acids (Fig. 3) [5].

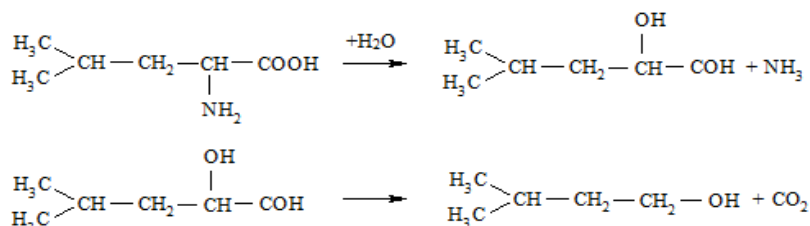


Fig. 3. The conversion of leucine to isoamyl alcohol according to Buchner and Meisenheimer [5]

Neubauer and Fromherz also tried to verify Ehrlich pathway and postulated that the synthesis of higher alcohols from amino acids was more complex. Their experiments revealed that amino acids added to the fermentation medium were at first converted to corresponding keto acids which were subsequently metabolized to alcohols. Their theory was based on the formation of hypothetic imino acid (through the reaction of oxidation) which was further deaminated to the corresponding keto acid. The latter was subsequently decarboxylated and reduced as shown in Fig. 4 [3, 5].

The catabolic route of higher alcohols synthesis was further elucidated by Sentheshanmuganthan who revealed that keto acids were formed through transamination in the first phase of amino acid metabolism [13, 14]. This process is catalyzed by yeast transaminases. In the second step the keto acids are decarboxylated to aldehydes and the latter are reduced to corresponding higher

alcohols. His investigations were limited to synthesis of tyrosol from tyrosine and 3-methyl-1-butanol from leucine.

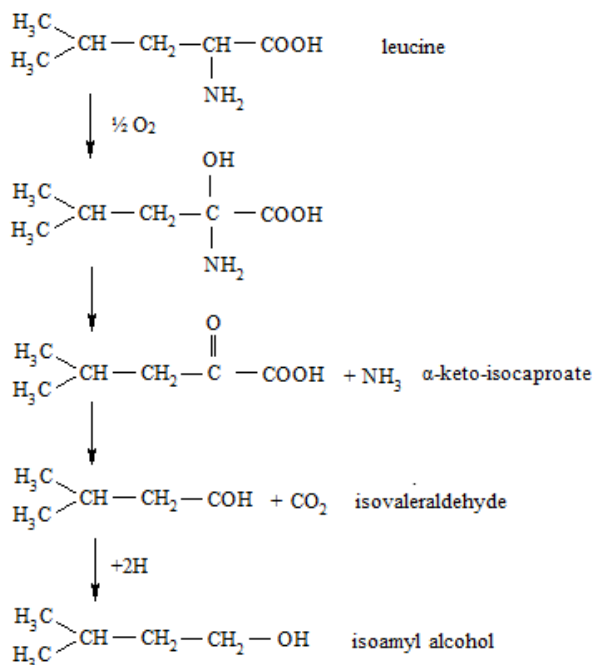
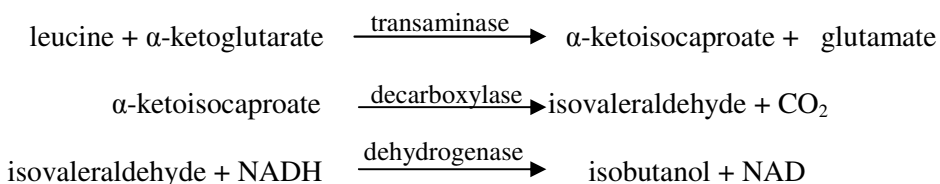


Fig. 4. The conversion of leucine to isoamyl alcohol according to Neubauer and Fromherz [3, 5]

The conversion of leucine is shown reaction [11]:



Studies of Yoskizawa, Suomalainen, Nordstrom and Guymon showed that higher alcohols could also derive from keto acids through their decarboxylation to corresponding aldehydes which are subsequently reduced by specific alcohol dehydrogenases to alcohols. Guymon determined the concentration of organic acids produced during alcoholic fermentation and found that yeasts converted α -ketobutyric acid to n-propanol while condensation of this acid with pyruvate yielded isoleucine.

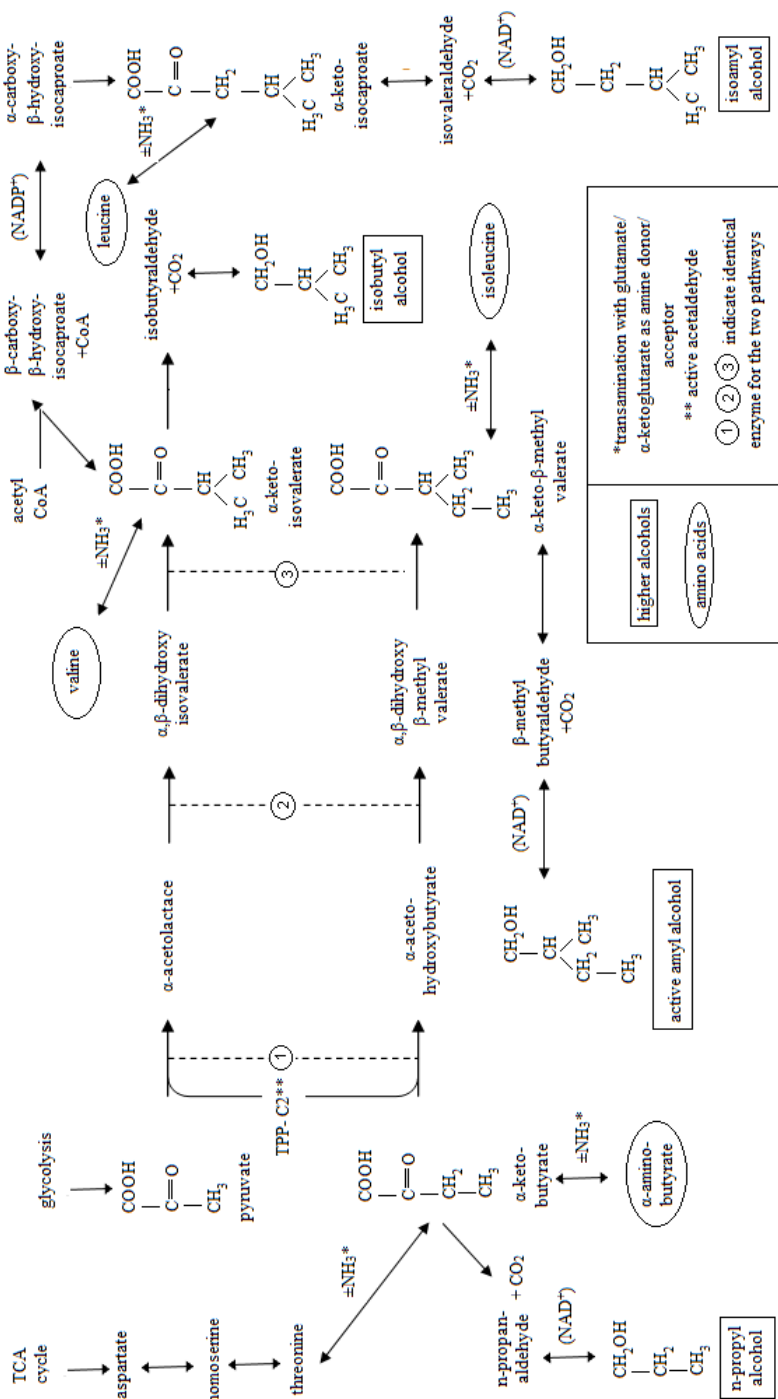


Fig. 5. Mechanism of higher alcohols formation from amino acids and sugars [12]

Drews, Szpecht and Schafer postulated the connected routes of synthesis of amino acids such as leucine, isoleucine, valine and threonine and higher alcohols such as n-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol. The central position in the scheme proposed by these researchers is occupied by pyruvate and activated acetic acid (acetyl-CoA). Pyruvate, ketobutyrate and ketoisovalerate are keto acids that are further decarboxylated to higher alcohols. They are products of either transamination of corresponding amino acids or intermediates in their synthesis. The sequence of these reactions is shown in Fig. 5 [12].

Pyruvate which is a product of glycolysis is further converted to corresponding higher alcohols and amino acids through reactions catalyzed by enzymes participating in catabolic pathways. The last two steps of this pathway such as decarboxylation of keto acids and reduction of aldehydes are catalyzed by the same enzymes which catalyze the conversion of pyruvate to ethanol [9]. It was proposed by Thonkis, Castor, Genevois and coworkers who demonstrated that not all fusel alcohols accumulated during alcoholic fermentation derived from amino acids available in fermentation medium. Genevois and Lafon proved that higher alcohols could be the products of carbohydrate metabolism and were formed from acetic acid and acetaldehyde [11]. They postulated that 2 molecules of acetic acid condense to form acetoacetate, which undergoes decarboxylation to acetone. The latter can be either reduced to isopropanol or condensed with acetaldehyde to β -methylcrotonaldehyde which is further converted to 3-methyl-1-butanol (Fig. 6) [11]:

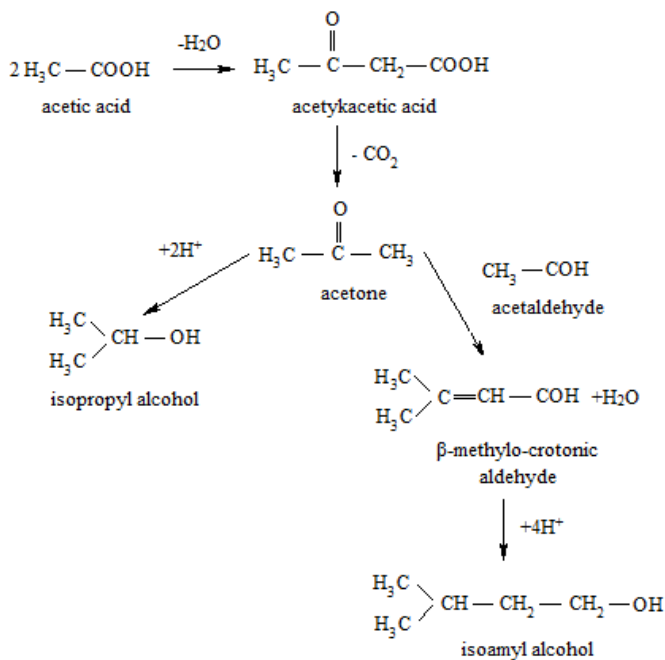


Fig. 6. Formation of isoamyl alcohol according to Genevois and Lafon

Factors affecting higher alcohols quantity in rye mashes

The quality of raw spirits and in particular quantities of alcoholic fermentation by-products such as aldehydes, organic acids and higher alcohols have always attracted attention of researchers involved in improvement of alcoholic fermentation processes. Many factors affect the quality of raw spirits in terms of by-products level [15, 16]. These factors comprise: the sort and quality of raw materials; conditions of mashing process, of starch liquefaction and saccharification, of alcoholic fermentation of mashes (temperature, strain and quantity of yeasts, sort and quantity of supplements added to mashes, type and activity of enzymes) [17-21].

Raw materials

Significant changes related to raw materials have been observed in Polish distilleries during the last several decades. About 20-30 years ago more than 80% of spirit was produced from potatoes but in 1997 their share dropped to only 12.4%. Recently, spirit manufacturing by agricultural distilleries has been based on cereals – mainly on rye, but also on maize [22]. Traditional rye breeding in Poland is justified by poor quality of soils. Light and very light soils having low or even very low pH account for more than 30% arable land in Poland. In consequence, yields of crops are relatively small. Rye is an exception to this rule. In Poland, an average yield of rye reaches 2.74 t/ha. The volume of ethanol produced in distilleries approaches 390 l per 1 tone of rye (for a mean content of starch in rye grains of 62%) [23]. However, breeding of rye has been systematically decreased, like that of oats, because of limitation of arable land.

In 1996 researchers from the Department of Food Biotechnology at SGGW in Warsaw compared the quality of raw spirits derived from rye, wheat-rye and amarantus. All these commodities contain approximately 50-60% starch on dry mass basis. Mashes prepared from these raw materials were fermented for 4 days by yeasts of strain B₄. Based on measurements of a decrease in mass caused by evolving of carbon dioxide it was found that the initial phase of fermentation of amarantus mashes was considerably shorter compared to the other two. Fermentation rate was the lowest for rye mash and therefore duration of this process was the longest. Also the degree of fermentation of mashes obtained from wheat-rye and amarantus was higher compared to rye mashes. The content of reducing substances remained after fermentation was the lowest for amarantus mashes, slightly higher for wheat-rye mashes while in rye washes it was relatively high.

Also profiles of by-products generated during alcoholic fermentation of these mashes were different. Spirits obtained from wheat-rye contained slightly less acids, esters, methanol and higher alcohols than rye spirits. Spirits obtained from amarantus contained 5-fold more aldehydes compared to rye spirits. Quantities of 3-methylbutanol and 2-methylbutanol in the first spirits were relatively low while the content of esters was high [24].

Yeasts

Yeasts require appropriate proportions of fundamental nutrients such as water, carbon and nitrogen sources, other macroelements necessary for synthesis of cell components such as phosphorus and magnesium as well as microelements, vitamins and other factors that are crucial for growth and metabolic activity. Microorganisms can utilize various nitrogen sources to synthesize proteins and other nitrogenous intracellular molecules. Yeasts can assimilate low molecular weight nitrogenous compounds, either organic like peptides, amino acids, amides or mineral like ammonium salts, ammonia, or urea. Each live cell dies when deprived of assimilable nitrogen sources for too long time. Determination of nitrogen balance is very difficult but regulation of microbial metabolism can be achieved by proper selection of culture medium components.

Ions of other elements such as potassium, magnesium, iron, sulphur and phosphorus are assimilated by yeasts directly from culture media in the form of organic and inorganic compounds. Sulphur is essential for synthesis of proteins contained in the protoplasm. Its sources are water-soluble sulphates or organic compounds such as sulphur amino acids. Phosphorus is assimilated mainly as ammonium, potassium or sodium phosphates. It plays important roles as a component of lecithin and nucleoproteins and a participant of numerous enzymatic processes. Also magnesium is very important because it is essential for proper development of protoplasm and functioning of many fermentation processes [25, 26].

Alcoholic fermentation consisting in the conversion of sugars to ethanol and carbon dioxide is a result of metabolic processes conducted by yeast *Saccharomyces cerevisiae*. Agriculture distilleries need yeast strains capable of fast sugar fermenting and resistant to ethanol concentrations up to 10% and elevated temperature, even above 35°C. Therefore yeast strains have been continually improved [17, 27]. Higher alcohols are significantly affected by yeast inoculum size. Concentration of fusel oil increased with increasing yeast inoculum levels.

Supplements

Mineral salts, vitamins and unsaturated fatty acids present in fermentation media affect the yield of ethanol synthesis positively and can reduce the quantity of by-products in raw spirits. It was found that these activators of fermentation enhanced utilization of acetaldehyde by yeasts thereby reducing its concentration in fermentation medium [28-31]. Experiments of Nordstrom and Carlsson who added 2,4-dinitrophenol to fermentation media and investigations of Gutierrez who supplemented these media with sulphates revealed that the presence of yeast growth inhibitors decreased the quantity of higher alcohols. The course of alcoholic fermentation and the content of higher alcohols are also affected by vitamins and mineral salts [32].

The most often used in distillery technology supplements that activate fermentation process are: superphosphate, diammonium sulphate, diammonium phosphate, urea, ammonium water, potassium phosphate, and magnesium sulphate. Doses of mineral salts depend on the chemical composition of raw materials which can be deprived of some nutrients that are crucial for growth and metabolism of yeasts. Mineral salts are essential for yeast metabolism as activators of enzymes or structural components of molecules building yeast cells [29].

Supplementation of mashes with inorganic nitrogen causes a decrease in formation of higher alcohols. It can be observed when urea was used as a source of this element. However, urea combines with ethanol to form ethyl carbamate and therefore its addition is not recommended [30, 31]. Addition of ammonium salts (diammonium phosphate, diammonium sulphate) improves the yield of ethanol and speeds up fermentation process but decisive factor is dose and chemical composition of the salt. Nitrogen compounds present in fermentation media are metabolized by yeasts and used for growth, cell development and reproduction. The enrichment of fermentation media with ammonium salts at the beginning of the stationary phase stimulates yeast metabolism [33].

It was found that the addition of a mixture of a few mineral salts such as: sulphates of ammonium and magnesium, and magnesium hydrophosphate improved all fermentation parameters and increased the yield of ethanol synthesis from starch with the concomitant decrease of quantities of higher alcohols and esters in raw spirits. Fortification of rye mashes considerably at 19.5°Blg with a mineral mix raised the ethanol synthesis yield and the quality of raw spirit due to the joint effect of ammonium sulphate, potassium hydrophosphate and magnesium sulphate. The content of higher alcohols in this raw spirit was decreased by 48% as compared to the reference raw spirit obtained without the mineral mix. When the mixture of salts was replaced by a mixture of calcium pantothenate and thiamine the level of higher alcohols was reduced by 18% as compared to the reference [34].

To find other factors affecting concentration of higher alcohols in rye spirits the pH of mashes was regulated by using hydrated calcium and ammonium water. Raw spirits obtained from mashes with pH regulated by hydrated calcium contained more fusel alcohols (3.45g/dm^3) than those obtained from mashes which pH was not regulated (3.14g/dm^3). In raw spirits derived from mashes which pH was regulated with ammonium water concentration of higher alcohols was the highest and decreased (2.73g/dm^3). Results of this experiment indicate that calcium ions added to the mash to adjust its pH probably stimulate enzymatic decarboxylation and deamination of amino acids thereby contributing to the increased quantity of higher alcohols. By contrast, ammonium ions contained in fermentation medium inhibit formation of these by-products [35].

Investigations of Gutierrez revealed that the deficiency of such vitamins as biotin, thiamine, pantothenic acid and pyridoxine, and microelements such as boron, manganese, zinc, and iron, affects production of ethanol, pyruvate and higher alcohols such as n-propanol, isobutanol and isoamyl alcohol. Thiamine is a coenzyme of pyruvate decarboxylase which is responsible for decarboxylation of

pyruvate to acetaldehyde. The deficiency of thiamine in fermentation medium results in accumulation of pyruvate and a decrease in the content of isoamyl and other alcohols. Pantothenic acid is essential for synthesis of coenzyme A (CoA) and acetyl-CoA so it is also essential for production of isoamyl alcohol. The deficiency of this vitamin causes a considerable decrease in isoamyl alcohol quantity and a concomitant rise in the content of isobutanol. It was also found that a deficiency in pyridoxine stimulated synthesis of isobutyl and isoamyl alcohols because this vitamin is a prosthetic group of transaminase. Yeast growth was not retarded by a deficit of boron, manganese and iron ions because these elements are thought to be accumulated in cells during successive steps of yeast propagation [35]. In turn, the deficit of zinc reduced the growth of yeasts by 18.7% and thereby decreased the yield of ethanol synthesis. This phenomenon resulted from the dependence of activity of a few key enzymes such as alcohol dehydrogenase, phosphatase, and aldolase on zinc ions. Moreover the quantity of higher alcohols was substantially decreased by a deficiency of zinc [36].

The enrichment of fermentation media with salts of magnesium also has a strong impact on metabolic activities and growth of yeasts. Magnesium ions affect the integrity and permeability of cytoplasmic membrane because they form complexes with phospholipids building the bilayer. Magnesium deficit brings about a decrease in the permeability of these membranes which in turn results in elevated intracellular concentration of calcium and sodium ions and decreased contents of potassium and phosphorus ions [37]. Magnesium ions are components of ribosomes and participate in stabilization of DNA and RNA molecules [38].

These ions are essential for aggregation of ribosomes into polysomes and thereby decide on processes of translation and activation of more than 300 enzymes, including: synthetases, phosphofructokinase and membrane ATP-ase which are involved in active transport of saccharides and amino acids into cells. Mg^{2+} ions also protect aforementioned cell components from the harmful effect of other metal ions [38]. Supplementation with magnesium ions increases the rate of yeast growth and the degree of utilization of saccharides contained in fermentation medium. Besides, these ions play the very important role in protection of cells from harmful environmental factors like the elevated concentration of ethyl alcohol in fermentation medium [33].

Addition of amino acids to fermentation medium can enhance the synthesis of higher alcohols although 50% or more of them derived from sugars. Graceva who studied the effect of supplementation with certain amino acids on the quantity of fusel oils formed during alcoholic fermentation found that it depended on the structure of their side groups. For instance, addition of α -alanine and α -aminobutyric acid caused a 21-fold increase in n-propanol content while supplementation with glycine increased isobutanol synthesis yield only by 1.85-fold [34].

Liquefaction and saccharification of starch

The pressureless starch liberation method (PLS) is an energy-saving alternative to mashing of raw materials. The PLS method consists in the replacement of high-pressure mashing of raw materials by their mechanical disintegration. The thorough disintegration is a prerequisite of the effectiveness of this method. The ground raw material is mixed with water and heated up to 80-90°C (in high pressure-thermal method it is heated up to 151.4°C) that eliminates some disadvantageous chemical reactions and saves thermal energy, even by 50% [40].

However, application of the PLS method can lead to microbial contamination of mashes. The most harmful for manufacturing of spirits are contaminations with acid-producing bacteria. All species of lactic acid bacteria such as members of genera: *Streptococcus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Lactobacillus* are characterized by the capability of lactic acid synthesizing thereby leading to the acidification of the medium. These bacteria are sensitive to the deficit of nutrients such as vitamins and amino acids. Mashes obtained by the PLS method contain more of these nutrients than those obtained by traditional mashing. The lower temperature of this process is less detrimental for microbial contamination and the availability of nutrients additionally stimulates their development. Lactic acid bacteria that contaminate mashes metabolize saccharides destined for synthesis of alcohol.

Products of their metabolism acidify the environment that in turn reduces the activity of amylases responsible for dextrin hydrolysis. The remained dextrans cannot be assimilated by yeasts. Besides, acetic acid produced by heterofermentative lactic acid bacteria strongly inhibits yeasts growth and stops alcoholic fermentation at the concentration of 0.02%. Genevois and Lafon proved that higher alcohols could derive from acetic acid and acetaldehyde which are by-products of sugar conversions. This route begins from condensation of 2 molecules of acetic acid to acetoacetate [41]. Concentration of higher alcohols in raw spirits obtained from mashes produced by pressure cooking method is lower than in spirits derived from mashes produced by PLS method.

Conclusions

Higher alcohols and their esters, have a significant impact on the flavor of alcohol. In a mixture of these compounds, isoamyl alcohol has the largest share, and the smallest n-propanol. Higher alcohols can be formed by the metabolism of yeast and bacteria by decarboxylation of ketoacids, which are intermediates of biochemical changes of leucine, isoleucine, valine and threonine. Addition of these amino acids to fermenting medium may intensify the synthesis of higher alcohols, although 50% or even more arise in the mash of sugar. However, presence of inhibitors of yeast growth in the medium probably leads to a reduction of their

synthesis. Several studies have shown the impact of excess and deficiency of various vitamins and minerals on the production of higher alcohols during fermentation. Addition of mineral nutrients to mash stimulates the activity of yeast increase the efficiency of fermentation, as well as improves quality of raw spirit.

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SYNTEZA WYŻSZYCH ALKOHOLI W ZACIERACH ŻYTNICH

Streszczenie

Powstawanie poszczególnych produktów ubocznych podczas fermentacji alkoholowej, to proces bardzo złożony. Na szczególną uwagę, ze względu na mechanizm i dynamikę powstawania, zasługują wyższe alkohole. W XIX wieku, dominująca była opinia, że alkohole wyższe to „bakteryjne metabolity zepsucia”, którymi skażone były napoje alkoholowe. Na początku XX wieku znane już były prace Ehrlicha, który wykazał, że wyższe alkohole są syntetyzowane przez drożdże z aminokwasów, a zatem są składnikami występującymi naturalnie we wszystkich napojach alkoholowych wyprodukowanych z alkoholu etylowego pochodzenia rolniczego.

Na ilość fuzli w zacierze odfermentowanym, jak również na ich skład jakościowy, ma wpływ wiele czynników m.in.: rodzaj surowca użytego do przygotowania zacieru, rodzaj i wielkość inokulum drożdży oraz rodzaj pożywek, a także sposób prowadzenia fermentacji. Prace analityczne różnych autorów dowiodły, że wyższe alkohole mogą powstawać na drodze szlaku katabolicznego i anabolicznego. Szlak kataboliczny dotyczy przemian aminokwasów- w/w teoria Ehrlicha. Prekursorem w szlaku anabolicznym jest pirogronian, a fuzle są produktami pobocznymi, powstającymi podczas syntezy aminokwasów. Można jednoznacznie wnioskować, że występowanie fuzli w destylatach rolniczych jest efektem obecności w środowisku fermentacyjnym aminokwasów, cukrów oraz produktów ich przemiany, głównie aldehydów.