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Promising Renewable Raw for Ethanol Biosynthesis

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Abstract

Ethanol is a valuable product and raw material for various industries, it is widely used as a solvent, fuel, and antiseptic substance. Biotechnology for the production of ethyl alcohol using the microbiological method is rapidly becoming an integral component of global production, as these processes become more efficient and cost-effective. In this regard, an urgent and practically significant direction of research is the development of new technologies and improvement of existing ones to product ethanol based on renewable raw materials. Therefore, it is necessary to systematize the current scientific information in this area. This review examines modern molecular biotechnologies for producing ethanol from various types of renewable raw materials of plant and animal origin. Particular attention we paid to technologies that use waste products from the processing industry, as milk whey, which is a good substrate for ethanol production by the microbiological method. Technologies with the use of various types of microorganisms are considered.

Keywords: ethanol, renewable raw materials, alcoholic fermentation, biosynthesis, milk whey, *Saccharomyces cerevisiae*.

1. Introduction

Ethanol is widely used in various chemical, fuel, food, perfumery and cosmetic industries. There are two main approaches to product ethanol, using chemical and microbiological methods. According to the first one, we have the so-called industrial alcohol, as a rule, by catalytic hydration of ethylene (Gao et al., 2019).

The reaction is carried out in the presence of high-pressure steam at a temperature of 300 °C, where the ratio of ethylene to steam is 5:3. Catalyst of this reaction is orthophosphoric acid supported on silica gel, activated carbon or asbestos. The reaction scheme is as follows:

$CH_2{=}CH_2 + H_2O \rightarrow CH_3CH_2OH$.

However, hydrocarbon reserves are being depleted and prices for them are steadily growing, therefore, modern technologies are increasingly switching to renewable sources of raw materials, distinguished by their availability and low cost.

Processing plant and animal raw materials led to generation of different organic wastes that require disposal, or secondary products with a lower nutritional value, and therefore having a low cost. Such biomass can be a suitable raw material for microbiological synthesis (biosynthesis) of ethanol for the needs of the medical and food industry. Ethyl alcohol obtained from raw materials of plant or animal origin is also called bioethanol (Busic et al., 2018). It is widely used as a component of fuel mixtures, as well as with a proper degree of purification in medicine and the

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food industry. Thus, the microbiological method for ethanol production is preferable. It allows to use the renewable raw materials, which are waste requiring disposal (Robak et al., 2018; Sarris, Papanikolaou, 2015; Rosales-Calderon, Arantes, 2019).

Ethanol biosynthesis is based on the process of alcoholic fermentation. It may be described as the biological anaerobic converting sugars such as glucose, fructose and sucrose. Microbial enzymes transform sugars into cellular energy without the participation of oxygen, ethanol and carbon dioxide are by-products. In the presence of oxygen, the ethanol biosynthesis is terminated, yeast switches to aerobic respiration, and this phenomenon is called the Pasteur effect (Busic et al., 2018).

Under anaerobic conditions, the rate of glucose metabolism become higher, but the ATP production decreases. When exposed to aerobic conditions, ATP production is increased and the rate of glycolysis slows down. The overall equation for the reaction of alcoholic fermentation is as follows:

$$C_6H_{12}O_6 + 2H_3PO_4 + 2\text{АДФ} \rightarrow 2CH_3CH_2OH + 2CO_2 + 2\text{АТФ}.$$

In addition to yeast, such bacteria as *Zymomonas mobilis, Sarcina ventriculi, Erwinia amylovora*, etc. can participate in alcohol fermentation, but bacterial fermentation differs in the amount and nature of by-products. Many studied strains of facultative anaerobic fungi have the same ability. The maximum rate of alcohol formation is characteristic for *Mucoraceae* as *Mucor*, *Rhizopus,* and *Zygorhynchus*. However, the ethanol yield in these cases is much lower and it is about 5-7 % (Bui et al., 2019; Thanh et al., 2016).

Modern technologies for bioethanol production are represented by two main types: periodic (1) and continuous (2) processes (Robak et al., 2018).

The technologies for producing ethanol using microorganisms differ depending on the feedstock and the specific type of producing organisms. These factors are the main affecting the complexity of the technological scheme, the yield of the target product, and other important indicators to determine the economic efficiency of ethanol production. Next, we will consider the main types of raw materials for ethanol production and the modern technologies corresponding to them.

2. Results and discussion

Starchy raw materials

Root crops as many types of potatoes and grains of corn, barley, and wheat contain a large amount of starch. Starch from various sources can be used for further microbiological conversion to ethanol. Figure 1 shows the structure of starch-containing raw materials used in Russia for ethanol production. As it follows from the diagram, maize accounts for 40 % of all starch-containing raw materials, wheat accounts for 35 %, quarter of all raw materials are rye and other crops (Turshatov et al., 2019).

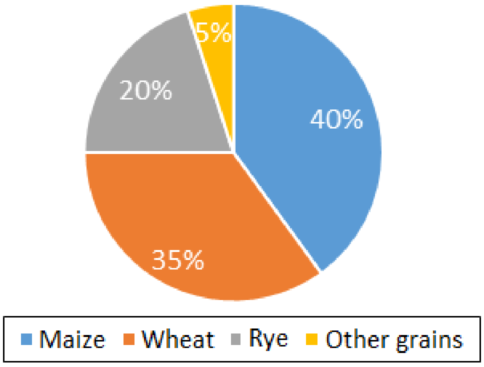

Fig. 1. Structure of use of starch-containing raw materials for ethanol production in Russia (data for 2019)

Due to processing raw materials from corn dry and wet methods are useful. Starch-containing raw materials require preliminary hydrolysis with enzyme preparations so that the yeast can convert the substrate into ethanol. Enzymes hydrolyze 1,4- or 1,6- glycoside bonds in amylose and amylopectin, while the first to break the glycoside bonds, using a water molecule as an acceptor. Hydrolysis of starch is carried out under the action of amylolytic enzymes, mainly α-amylase, β-amylase, and glucoamylase- with the formation of dextrins, oligosaccharides, maltose, or glucose (Mohanty, Swain, 2019; Shahsavarani et al., 2013).

In addition to yeast, there are known methods that use *Bacillus licheniformis* bacteria and genetically modified strains of *Escherichia coli* and *Bacillus subtilis* bacteria that produce α-amylase, as well as mold fungi *Aspergillus niger* and *Rhizopus sp*. producing glucoamylases.

In addition to yeast, methods are known where bacteria *Bacillus licheniformis* and genetically modified strains of bacteria *Escherichia coli* and *Bacillus* subtilis, which produce α-amylase, as well as molds *Aspergillus niger* and *Rhizopus sp*. producing glucoamylases (Busic et al., 2018).

Technologies based on the production of ethyl alcohol from starch are mainly divided into two groups, which differ in the method of boiling down: extrusion processing using pressure (1) and mechanical-enzymatic processing (2).

The first group involves the pretreatment of wheat grains, where coarse grains are mixed with water. The amount of water depends on the starchiness of the grain. After feeding the water emulsion into the the brewer, the batch is subjected to overpressure at an elevated temperature of over 100 °C. Such harsh conditions, allow to torn the grain shell and destroy the cell structure. Next, the mass is fed into the boil-over. The next stage includes saccharification with the addition of enzyme preparations and subsequent fermentation (Ryabova et al., 2014).

Some of technologies for producing ethanol from wheat use mechanical-enzymatic treatment. For example, the crushed grain is added to water heated to 50 ° C for 30 minutes with preparations containing α-amylase and xylase. The ratio of raw mass and water is 1: 3. The boiling down stage consists of raising temperature to 70 °C for 1.5 h, with a subsequent increase to 90 °C for 1 hour. After this stage, the process of saccharification of the wort follows with a glucoamylase-containing preparation. The alcohol yield is 65 ml per 100 g of starch. The adding a proteolytic enzyme preparation into saccharified wort increases the alcohol yield to an average of 70 dal/t of starch (Mussatto et al., 2010).

To optimize the enzymatic stage Romaniuk et al. (2015) use especial glucoamylase-containing preparation 'Glucogam' for hydrolysis of wheat at a temperature of 55 ° C. The subsequent fermentation of saccharified mass by the yeast *S. cerevisiae* IMB Y-5007 with feeding diammonium phosphate provided the alcohol yield of 60.7 dal/t of conventional starch. In case of *S. cerevisiae* XII application, the alcohol yield was of 60.6 dal/t of conventional starch.

There are data on the production of ethanol from potato waste using *S. cerevisiae*. A homogeneous suspension consisted of potato flour and water in a ratio of 1:10. The suspension was liquefied with α-amylase at 80 °C for 40 min, followed by saccharification with glucoamylase at 65 °C for two hours. Fermentation of the hydrolyzate with S. cerevisiae at 35 °C for two days resulted in an ethanol yield of 33 g/L (Noufal et al., 2017).

Researchers are also improving methods for producing ethanol using barley. As an example, Bharti B., Chauhan (2016) describe saccharification technology of boiled barley mass with an enzyme preparation of glucoamylase at a temperature of 50–65 °C and pH 4.0–5.5 for 120 min. It was found that pH has a multifaceted effect on the saccharification process. On the one hand, hydrogen ions change the ionization of the active center and the conformational state of glucoamylase. On the other hand, they affect the stability of the tertiary structure of glucoamylase. The maximum accumulation of glucose is observed at pH 4.5. The use of multi-enzyme composition results in increasing the glucose amount by 34.7 % compared to the control. The degree of starch hydrolysis increases, since it becomes more accessible for the action of saccharifying enzymes due to the hydrolysis of protein substances and barley grain shells containing hemicelluloses. The use of a multienzyme composition can reduce the consumption of glucoamylase.

The bioconversion of wort prepared on the basis of corn flour into ethanol demonstrates the advantage of extrusion processing in comparison with the traditional mechano-enzymatic method (Peralta-Contreras et al., 2014).

Figure 2 summarizes the process of alcohol production from grain crops.

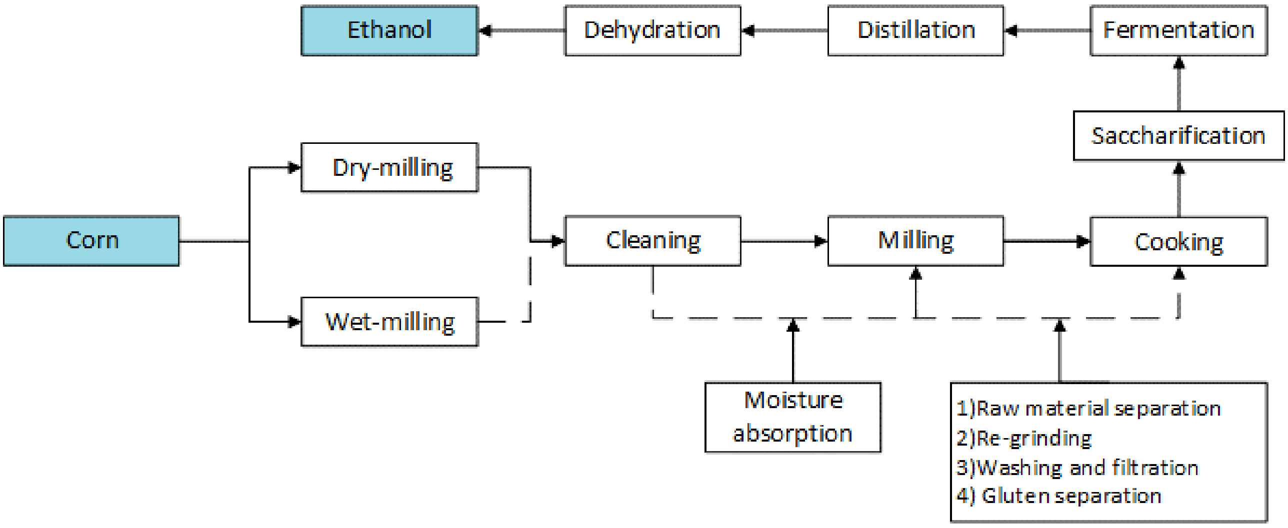

Fig. 2. Generalized process of ethanol synthesis of from cereals containing starch

Most of the research is aimed to increase the efficiency of the fermentation through the use of new purified enzyme, microorganisms, and their combinations, as well as by varying the technological parameters of the preliminary feedstock preparation.

Sugar-containing raw materials

Sugar cane and beets are the main sources of sugar in the world. Two-thirds of the world's sugar production comes from sugarcane and one third comes from sugar beets. Sugarcane as a raw material for ethanol production has many advantages, as it is a crop that does not require costly agricultural technologies. Sugar cane has a relatively low cost and high reproducibility (Busic et al., 2018). They can be easily hydrolyzed by the β-fructofuranosidase, which is typical enzyme for most *Saccharomyces* species (Reis et al., 2013). Consequently, pretreatment is not required, and this fact makes this process more preferable in comparison to use the starch-contain raw materials. Sugar crops only require a grinding process to extract the sugars into the fermentation medium. Ethanol can also be produced directly from juice or molasses (Eggleston et al., 2010).

In Russia and Europe, sugar production is mainly based on the use of sugar beets as raw materials. The juice formed during the sugar beet processing as an intermediate product, as well as crystalline sugar, can be a raw material for ethanol production. Molasses, being the main by-product of the sugar industry, is widely used in biotechnological industries, including ethanol production. The total residual sugar in molasses can account for 50–60 % of the total mass by volume, the sucrose is about 60 % of this mass, which makes this substrate suitable for large-scale ethanol production (Palmonari et al., 2020). Also, molasses is obtained as a by-product in the drying out citrus pulp with a total sugar content of at least 45 %. The glucose production from starch also produces molasses. Starch molasses contains about 43 % sugars and 73 % solids (Busic et al., 2018).

S. cerevisiae are microorganisms that are most often used for the bioethanol production from sugar-containing raw materials. They easily break down sucrose into glucose and fructose. *S. cerevisiae* cells require a small amount of oxygen for the synthesis of fatty acids and sterols during the production of bioethanol; therefore, aeration is an important parameter of this process (Bharti, Chauhan, 2016; Lip et al., 2020). *S. cerevisiae* does not tolerate high concentrations of sugar and salt in the medium and they are also temperature sensitive. Cane molasses medium has the highest osmolarity due to moderate sugar and salt concentrations, which negatively affects ethanol synthesis (Sarris, Papanikolaou, 2015).

Many studies have looked for *S. cerevisiae* strains with higher resistance to salt and temperature (Tekarslan-Sahin et al., 2018; Subodinee et al., 2019; Lip et al., 2020). *Schizosaccharomyces pombe* yeast is also used for bioethanol production as it withstands high salt concentrations and high solids content. In the production of bioethanol, the possibility of using other microorganisms, such as *Zymomonas mobilis, Klebsiella oxytoca, E. coli, Thermoanaerobacter ethanolicus, Pichia stipitis, Candida shehatae, Mucor indicus,* was investigated (Subodinee et al., 2019). However, no adequate alternative to *S. cerevisiae* has yet been found.

Figure 3 shows schematically a generalized process for the synthesis of ethanol from molasses.

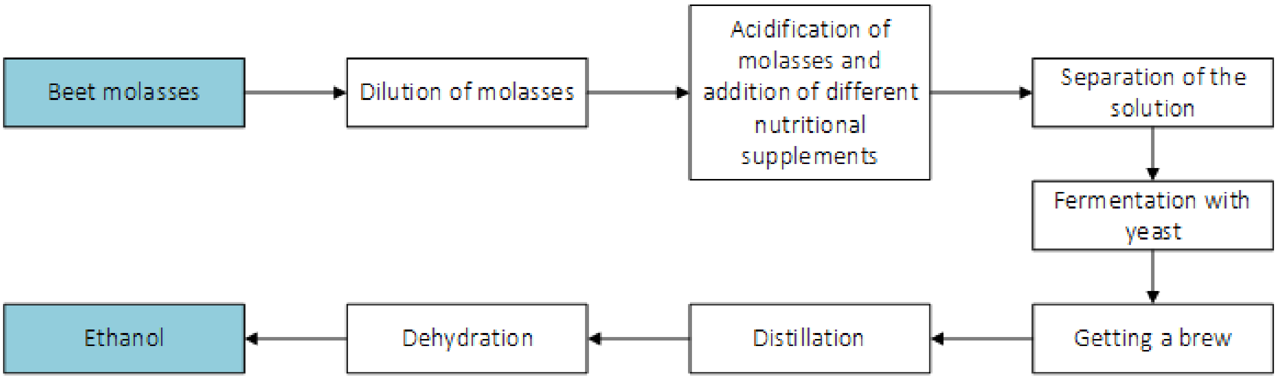

Fig. 3. Generalized process for the synthesis of ethanol from molasses

Since the technology for ethanol producing from molasses and other sugar-containing raw materials is simpler in comparison with starch-containing ones, the main research here is aimed at finding exactly new types and strains of microorganisms.

Lignocellulose-containing raw materials

The cellulose is surrounded by lignin. In terms of chemical structure, it is a linear β-glucan D-glucose polymer linked by β-1,4-glycosidic bonds. The structure of cellulose is difficult to destroy without enzymatic hydrolysis due to its crystalline nature. The linear cellulose chain consists of 500-14,000 D-glucose units. To transform this rigid crystalline structure from microfibrils in the cell wall into an amorphous structure in water, high temperature and pressure conditions are required at 320 °C and 25 MPa, respectively. These requirements are obviously higher than for starchy raw materials (Subodinee et al., 2019).

Lignocellulosic renewable raw material is promising for ethanol production, since it is also renewable. This raw material can be divided into four groups: plant residues as various cakes, different types of straw and rice husks (1), wood (2), cellulose waste (3), and grass biomass (4). The average lignocellulose mass contains 43 % cellulose, 27 % lignin, 20 % hemicellulose, and 10 % other components (Robak et al., 2018). The diversity of lignocellulosic biomass composition is a disadvantage, since there is a need for more complex and expensive technologies.

The complex structure of lignocellulose requires an additional preliminary stage of raw material preparation. The main problem is that it is necessary to hydrolyze lignocellulose to glucose in order to make it available to microorganisms. Methods of exposure to raw materials include acid and alkaline hydrolysis, enzymatic hydrolysis, transformation under the action of microorganisms, and physico-chemical applications using pressure, steam, radiation, ultrasound, etc. (Amores et al., 2013; Benazzi et al., 2013).

Figure 4 presents a general scheme of this process.

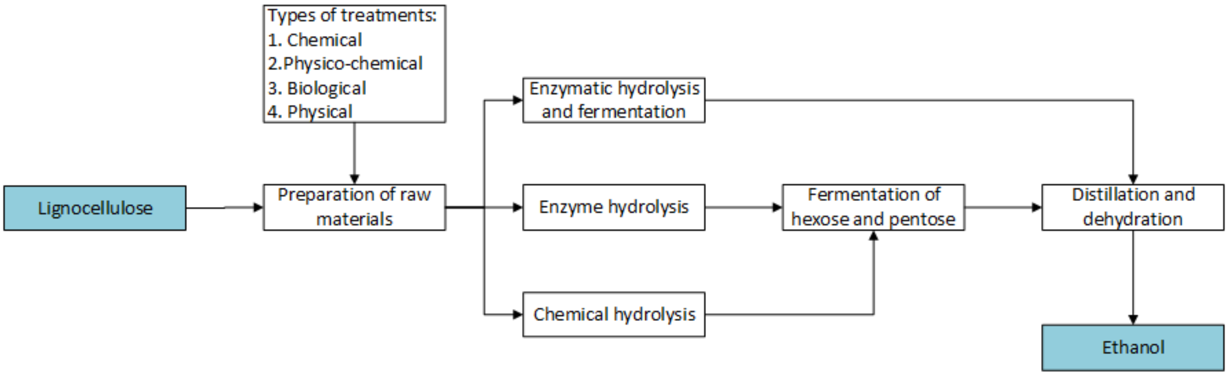

Fig. 4. Generalized process for the synthesis of ethanol from lignocellulose

The choice of pretreatment depends on the nature of the feedstock and by-products. This has a great impact on all subsequent stages of ethanol production. Therefore, for the effective use of lignocellulose as a raw material for ethanol production, a careful selection of the preparation stage is required. Further studies of this issue will make it possible to effectively use a large amount of plant waste to obtain a valuable product.

To product the ethyl alcohol, not only vegetable raw materials and also raw materials of animal origin including whey which can be used.

Milk serum

Whey being liquid residue after sedimentation and removal of casein from milk during cheese making, contains a large amount of nutrients, about 55 % milk substances. It should be noted that about 9 liters of whey is produced for every 1 kg of cheese. Whey contains lactose of 40 g/L, soluble proteins of 6 g/L, lipids of 4 g/L, vitamins and mineral salts, lactic acid and citric acid, urea and uric acid, β-lactoglobulin, α-lactoglobulin, immunoglobulins, serum albumin and lactoferrin, amino acids, creatine, creatinine, ammonia, etc. Water-soluble vitamins and part of the fat-soluble vitamins of milk as A, B1, B6, C, E, and choline are almost completely transferred to whey. This is a stimulating factor for the development of microorganisms (Dasa et al., 2016).

Whey is the main by-product of the dairy and cheese industry, which is not always or not fully used, therefore it become a liquid waste with a high organic load. The annual production of whey in the world is more than 115 million tons, and according to some estimates, more than 160 million tons, with 47 % ending up in the sewers (Papademas, Kotsaki, 2019). In Russia, according to theoretical calculations, more than 5 million tons per year are produced. At the same time, no more than 30 % of the whey is processed, a small part is used for feeding animals, the rest goes to wastewater (Isina, 2020). In Europe and the USA, more than 80 % of milk whey is used for processing (Pasotti et al., 2017).

Lactose is the main component of whey, which contributes to a high biological oxygen demand (up to 60 g/L) and chemical oxygen demand (up to 80 g/L). If whey enters wastewater, oxygen depletion of water occurs. According to Kieselmann Ru specialists, one ton of whey discharged into a reservoir is, in terms of the damage caused to nature, equal to 100 tons of ordinary household wastewater (Isina, 2020).

In 2019, in the Russian Federation, amendments were made to Federal Law No. 416-FZ of December 7, 2011 ‘On Water Supply and Wastewater Disposal’, which toughen the requirements for the wastewater pollution transferred by factories to water utilities.

Thus, the production of ethanol from whey reduces the need for complex and expensive wastewater treatment processes required for whey disposal. Despite the fact that the conversion of whey to ethanol has proven to be effective in combating liquid waste, the commercialization of ethanol production from whey on an industrial scale is still slow. However, some large-scale whey ethanol production facilities are already operational (Papademas, Kotsaki, 2019; Zabed et al., 2017).

Despite the wide use of various yeast types, attention should be paid to *S. cerevisiae*, since they are actively applied in the alcohol industry, are safe, affordable and the most studied (model) type of microorganisms in biotechnology. One of the most prominent and unique features of these yeasts is their ability to rapidly convert sugars to ethanol both under anaerobic and aerobic conditions. In addition, they are able to withstand a high percentage of medium ethanol compared to other ones (Dong et al., 2015).

The metabolism of *S. cerevisiae* is characterized by the presence of the Crabtree effect known also as catabolic repression, concluding the glucose can partially convert to alcohol despite intense aeration even in an environment with a high sugar concentration. The Crabtree effect is characterized by the simultaneous action of aerobic and anaerobic energy pathways. In this case, the biomass yield varies widely depending on the mass fraction and the nature of the carbohydrate in the medium. It was found for *S. cerevisiae* to suppress synthesis of tricarboxylic acid cycle enzymes, cytochromes and enzymes of the respiratory chain after increasing medium concentration of glucose or fructose more than 0.1 %, even the absence of an oxygen limit. The Crabtree effect is more expressive, when glucose and fructose are used as carbohydrate food, compared with maltose, maltotriose, or galactose application (Robak et al., 2018).

The analysis of modern methods of alcohol obtaining from whey based on lactose fermentation allows to choose four main approaches, which vary depending on the concentration of lactose: the using native unconcentrated whey with a low lactose content (1); the using concentrated whey with a lactose content of 15–20 % (2); production of alcohol from whey after enzymatic hydrolysis of lactose (3); and the using immobilized microorganisms (4) (Panesar, Kennedy, 2012).

The simplest technologies for the production of ethanol are technologies with application of natural unconcentrated whey. However, it has been found that increasing the lactose concentration in the substrate up to 200 g/L inclusive improves the efficiency of the fermentation (Dasa et al., 2016).

Zohri et al. (2014) carried out a comparative study of natural cheese whey containing 50 g/L of lactose and whey with preliminary deproteinization and concentration of lactose up to 140 g/L. Two strains of *Kluyveromyces marxianus* and four strains of *S. cerevisiae* were studied. Also, authors studied the effect of different initial pH values and addition of four different nitrogen sources to whey. All yeast strains studied were found to be capable of growing and producing ethanol from both original and treated whey. Ethanol production levels range from 3.4 to 18.5 g/L in case of original whey use and from 24.1 to 57.7 g/L when treated whey was applied. Optimum starting pH was 5.5 and yeast extract was the best added nitrogen source. The maximum ethanol levels produced by *K. marxianus* ZMS3GU133329 and *S. cerevisiae* EC1118 from treated whey adjusted to pH 5.5 and supplemented with 0.3 % yeast extract reached 69.9 and 65.4 g/L, which corresponds to 97.8 and 91.4 % of theoretical values, respectively.

The combined use of yeast and lactose-fermenting bacteria is the most effective way of alcohol production, since these microorganisms alone cannot ferment whey without prior hydrolysis. This method does not imply additional power supplies. The presence of lactic acid bacteria in fermentation primarily promotes the hydrolysis of lactose to glucose and galactose. The same time organic acids are produced that can promote the formation of aromatic compounds and lower pH, inhibiting the growth of harmful microorganisms. The adding lactic acid bacteria in the amount of 1 % does not inhibit the growth of yeast. When whey proteins are precipitated using high temperatures, the yield of yeast biomass increases. Heat treatment of whey changes the biological value of the medium. Partial hydrolysis of lactose and whey proteins occurs with the release of more easily digestible half-life products. Therefore, the environment becomes more favorable for yeast growth (Vikhareva et al., 2019).

Figure 5 shows producing ethanol from cheese whey as a generalized diagram.

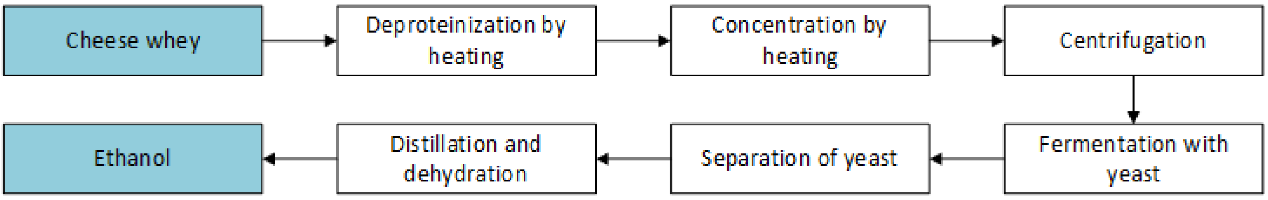

Fig. 5. Generalized process of ethanol synthesis from cheese whey

Recent developments in this area are aimed at finding new microorganisms capable of efficiently fermenting sugars with the formation of ethanol.

Japanese scientists discovered the ability of the brown rot fungi *Neolentinus lepideus* to ferment lactose into ethanol. The ability of the fungus to ferment lactose is not affected by the addition of glucose or calcium, as is the case with yeast. The fermentation is effective at pH = 2–3 and temperature 28 °C; the fermentation time is 48 hours. The process occurs regardless of the whey concentration, but it needs to be deproteinized. Therefore, *N. lepideus* may be useful in the ethanol production from materials consisting mainly of lactose, such as cheese whey or expired cow's milk (Okamoto et al., 2019).

Whey permeate is the lactose-rich liquid remaining after protein extraction from cheese whey. Artificially created microorganisms specially designed for fermentation of milk waste have been proposed for this technology. Eight *E. coli* strains were metabolically constructed using a new expression plasmid with genes of enzymes converting pyruvate to ethanol. The best strain among the candidates was selected according high ethanol yield. It has been shown for this constructed microorganism to be capable of efficiently fermenting whey permeate without food additives. The selected strain of *E. coli* lends itself to further optimization of metabolism and, according to the authors opinion, represents a step forward in the direction of efficient production of bioethanol from industrial waste (Pasotti et al., 2017).

Since *S. cerevisiae* yeast does not have a lactose metabolism system, modern research is aimed at finding ways to improve their strains. To construct lactose-consuming *S. cerevisiae* strains, approaches involving the expression of lactose genes of the phylogenetically related yeast *Kluyveromyces lactis*, as well as lactose genes from *E. coli* and *A. niger*, were used (Domingues et al., 2010).

Various bioengineering strategies have been applied including the use of genes for lactose metabolism from bacteria *E. coli*, yeast *K. lactis*, and *A. niger*. In study the metabolic engineering of *S. cerevisiae* cells for converting lactose to ethanol, the best results were obtained with recombinants engineered with *K. lactis* genes. Nevertheless, direct cloning of LAC4 and LAC12 from *K. lactis* with its own promoter did not allow direct selection of transformants in samples with lactose and led to recombinants with a slow growth phenotype (Malakar et al., 2020). Thus, we can conclude that this method is laborious and unstable.

3. Conclusion

The microbiological method for ethanol producing is promising both from the point of view of its increasing efficiency, and from the point of view of utilizing waste of plant and animal origin. Technologies differ depending on the feedstock and the specific type of producing organisms. The most promising raw material is milk whey, since its processing into alcohol is of particular environmental importance allowing to reduce the amount of wastewater and pollution of natural water reservoirs.

The search for new types and strains of microorganisms with low pathogenicity, resistant to harsh environmental conditions, allowing the process to be carried out with a maximal alcohol yield is the main scientific direction in this field. Here the most promising are further studies using bioinformatics methods and various bioengineering approaches.

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