The influence of cultivation conditions on the immobilized acetic acid bacteria metabolic activity

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Annotation. Brewing is an innovative sector of the food industry. Non-alcoholic beer production increases every year. The largest volumes of non-alcoholic beer are obtained by regular beer dialysis. The beer dialysate formed in production contains ethyl alcohol, which entails the need for its rational use. One of the effective ways to use beer dialysate is food biochemical vinegar production. The purpose of the work is to conduct research for biotechnology development of food vinegar from beer dialysates generation using acetic acid bacteria (AAB) immobilization on bio-carriers of various nature. The objects of research were samples of beer dialysates (BD), concentrated to a volume fraction of ethyl alcohol of 7.5%, Acetobacter aceti AAB, vinegar from BD. To study the changes in metabolic activity of immobilized AAB on a bio-carrier, acetic acid fermentation was performed using Acetobacter aceti cells, immobilized on beech shavings, zeolite, and high-pressure polyethylene. Air was supplied to a reservoir by a microcompressor from below through a finely porous ceramic nozzle, providing uniform air dispersion in cultural fluid. Graphs of the cells number and Acetobacter aceti biomass dependence on cultivation duration, nature of the bio-carrier and the aeration method when obtaining vinegar from beer dialysate are presented. The experimental data of the volatile components composition, organic acids and beer dialysate amino acids and vinegar from it are presented. Electron microscopic research of Acetobacter aceti AAB during beech shavings and zeolite cultivation is presented. The results of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are presented.

1. Introduction

With the introduction of a new product category, which is non-alcoholic beer, it becomes necessary to solve the problem of the use of resulting secondary resource - beer dialysate. One effective way to use it is vinegar production.

To obtain special types of natural vinegar, special raw materials are used, in honor of which the final product is called [1–5].

The main methods for biochemical vinegar generation are depth and circulating (surface). The depth method is based on AAB cultivating directly in an aerated medium principle. The circulation cultivation method is characterized by the fact that AAB are fixed on a solid carrier, and the nutrient
fluid is constantly circulated from top to bottom through the carrier. There is a lot of information about various environmental factors influence on microorganism’s growth and reproduction [6, 7].

Currently, oak, beech, hornbeam, and birch shavings are used as wood shavings in biochemical vinegar production by the circulation method. There is a method in which zeolite is used as a carrier instead of traditional beech shavings [8]. Zeolites are microporous crystalline substances. Crystallinity provides mechanical and chemical zeolites’ stability, the same and controlled pores, channels, cavities size. This property determines zeolites’ use as molecular sieves, adsorbents and detergents [9, 10].

The accumulated scientific experience allows one to suggest the use effectiveness of beer dialysates for biochemical food vinegar production using AAB, immobilized on a bio-carrier.

The purpose of the work is to conduct research for biotechnology development of food vinegar from beer dialysates generation using AAB immobilization on bio-carriers of various nature.

2. Materials and research methods

The studies were conducted in the laboratory of All-Russian Scientific Research Institute of Brewing, Beverage and Wine Industry – Branch of V.M. Gorbatev Federal Research Center for Food Systems of RAS using analysis methods adopted in enochemistry, brewing, vinegar industry and set forth in the relevant GOST, and modern instrumental analysis methods. Electron microscopic analysis of acetic acid bacteria was carried out together with the Institute of Biochemistry and Physiology of Microorganisms named after G.K. Scryabin of RAS of the Federal Research Center "Pushchino Scientific Center for Biological Research of RAS".

The qualitative and quantitative composition of volatile compounds were determined according to GOST 33834-2016.

The qualitative and quantitative amino acids composition were determined on a liquid chromatograph with an Agilent Technologies 1200 diode array detector (Agilent, USA), equipped with an automatic system for collecting and processing information on the Method for measuring free amino acids mass concentration in alcoholic and non-alcoholic beverages using high performance liquid chromatography (certification No. 01.00225/205-48-12, the registration code of measurement procedure in the Federal Information Fund for Ensuring the Uniformity of Measurements F.R.1.31.2012.13428).

Specimens’ preparation for SEM was performed according to the scheme [11]. Samples were viewed in JSM-6510LV scanning electron microscope.

The preparation of acetic acid bacteria cells for TEM was carried out according to previously described methods [12, 13] and was additionally contrasted with lead citrate by Reynolds [14]. Ultrathin sections were examined in JEM-1200EX electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

To study changes in AAB metabolic activity, immobilized on the bio-carrier, acetic acid fermentation was performed using *Acetobacter aceti* cells, immobilized on beech shavings, zeolite, and high-pressure polyethylene. To select the optimal parameters of alcohol oxidation process in dialysate, the experiment was carried out in vertical glass tanks with a jacket with capacity of 1.5 dm³. Air was supplied to the reservoir by the microcompressor from below through a finely porous ceramic nozzle, providing uniform air dispersion in the cultural fluid. The oxidation process was carried out to residual alcohol volume fraction from 0.15% to 0.3%.

The amount of the introduced bio-carrier was calculated by specific surface in comparison with beech shavings. The optimal temperature (30±2) °C during cultivation was maintained by supplying water to the jacket. During vinegar obtaining, the parameters, controlled in production were determined: the biomass amount, the viable cells number, the acetic acid mass concentration, and the residual alcohol volume fraction.

To study the growth of *Acetobacter aceti* cells during immobilization on selected bio-carriers, the biomass amount and the cells number after plating on an agar medium were determined.
Acetobacter aceti VNIIPBT-66 (VKPM B-4578), provided by the National Bioresource Center All-Russian Collection of Industrial Microorganisms of State Research Institute of Genetics and Selection of Industrial Microorganisms («Genetika»), was used for acetic acid fermentation.

The strain cultivation was carried out on media, recommended in the strain passport by the gradual biomass accumulation method and increasing physiological bacteria activity by successive reseeding on nutrient media.

The liquid nutrient medium during cultivation for the lag phase: the acetic acid mass fraction, g/100 cm$^3$ - (3.5-4.0); ethyl alcohol volume fraction, % - (1.5-2.0); disubstituted ammonium phosphate, g/dm$^3$ - 2.0; monosubstituted potassium phosphate, g/dm$^3$ - 2.0; magnesium sulfate, g/dm$^3$ - 1.0.

Seeding of sterile nutrient medium by Acetobacter aceti was leading by adding a bio-carrier into it with followed leading of liquid AAB inoculum for their immobilization. Cultivation was carried out under aerobic conditions for 7-14 days.

The amount of the initial inoculum was taken from the biomass of 45 g/dm$^3$ with a cell content of 0.9x10$^6$ CFU/cm$^3$.

3. Discussion of research results

3.1 The research of changes in metabolic activity of immobilized on bio-carrier AAB upon vinegar from BD obtaining depending on the bio-carrier

Cells growth dynamics in first 14 days are shown in Figures 1 and 2. With further cultivation, starting from 15 day, a sharp decrease in indicators was observed.

Figure 1 shows that in first three days of cultivation, the biomass amount did not change, then gradually began to decrease.

The number of viable cells (Figure 2) initially increased during 4 days of cultivation (more actively on beech shavings and zeolite), then there was a decrease in CFU in all cases, which confirms the inhibitory effect of the resulting acetic acid.

3.2 The research of changes in AAB metabolic activity immobilized on a bio-carrier during vinegar from BD obtaining depending on aeration regimes

The intensification of cultivation process was carried out by changing the forced aeration regime. Single preliminary aeration was used for 24 hours, periodic aeration day after two days and continuous aeration for 7 days with regulated air flow rate of 9.0 m$^3$ per m$^3$ of medium per hour (Figure 3).
Figure 3. Dependence of the biomass amount (a) and cells (b) of *Acetobacter aceti* on the cultivation duration and aeration method for obtaining vinegar from BD by surface method on beech shavings and zeolite.

As can be seen from figure 3, different methods of aeration led to almost the same results. On the 4th day of cultivation, the number of viable cells increases, then this indicator gradually decreases, which is associated with inhibitory effect of the resulting acetic acid.

In addition to biomass amount and viable cells number in 1 ml, the controlled fermentation parameters were determined: acetic acid mass concentration and residual alcohol volume fraction (Figure 4).

Figure 4. Comparative diagram of the acetic acid mass concentration and the residual alcohol volume fraction depending on the bio-carrier in vinegar from BD obtaining by the surface method.
Figure 5. Acetic acid mass concentration in cultural fluid during immobilization of *Acetobacter aceti* on zeolite depending on method of aeration

Under optimal cultivation conditions, the greatest amount of acetic acid was formed in a medium with zeolite and amounted to 6.3 g/100 cm$^3$, while residual ethyl alcohol volume fraction was minimal and amounted to 0.5%.

As can be seen from Figure 5, on the seventh day of cultivation, the acetic acid mass concentration was almost the same. This makes it possible to significantly reduce air consumption during vinegar production using zeolite as a biocarrier (more than 2 times with periodic aeration and 7 times with preliminary medium aeration).

Due to many channels and pores, the zeolite is able to regulate the composition of the gaseous medium in the liquid (the amount of dissolved oxygen and carbon dioxide) and provide the necessary amount of air for the cultivation of bacteria and oxidation of the nutrient medium during natural aeration, allowing only the preliminary aeration of the nutrient medium.

3.3 Study of BD biochemical composition and vinegar obtained from it

The research results of qualitative and quantitative composition of volatile components and amino acids are shown in Tables 1 and 2.

| Table 1. Mass concentration of volatile compounds, mg/dm$^3$ |
|---------------------------------------------------------------|
| Names of volatile components | Beer dialysate | Vinegar |
|                              | beech shavings | zeolite | polyethylene |
| Acetaldehyde                | 1.3            | 3.1     | 4.8          | 40          |
| Ethyl acetate               | 18.2           | 42.6    | 38.4         | 29.5        |
| Methanol                    | 1.9            | 1.1     | 0.6          | 0.9         |
| 2-propanol                  | 0.3            | not found | not found | not found |
| 2-butanol                   | 0.1            | not found | not found | not found |
| 1-propanol                  | 21.5           | 3.0     | 2.4          | 2.8         |
| Isobutanol                  | 33.3           | 2.0     | 1.1          | 1.7         |
| Isoamyl acetate             | 1.2            | not found | not found | not found |
| 1-butanol                   | 0.9            | 0.1     | 0.2          | 0.4         |
| Isoamylol                   | 99.3           | 6.4     | 4.7          | 5.8         |
| Ethylcaproate               | 0.3            | not found | not found | not found |
| Hexanol                     | 0.1            | not found | not found | not found |
| Ethyl lactate               | 0.4            | 0.8     | 0.5          | 0.6         |
| Ethylcaprylate              | not found      | 0.6     | 0.4          | 0.3         |
| Phenylethyl alcohol         | 28.7           | 35.4    | 37.3         | 32.1        |
| Total                       | 207.5          | 95.1    | 90.4         | 78.1        |
The content of volatile components in vinegar decreased by 2-2.5 times compared to BD depending on the bio-carrier, mainly as a result of decrease in mass concentration of higher alcohols of isoamylol, 1-propanol and isobutanol, which has positive effect on vinegar aroma and reduces product toxicity. At the same time, the content of β-phenylethyl alcohol, ethyl lactate esters and ethyl caprylate, which determine the specificity of the product, increased.

### Table 2. Mass concentration of amino acids, mg/dm³

| Name of amino acid | Beer dialysate | beech shavings | Vinegar | zeolite | polyethylene |
|--------------------|----------------|----------------|---------|---------|--------------|
| Aspartic acid      | 3.0            | not found      | 1.9     | 1.9     | 1.5          |
| Glutamic acid      | 2.7            | 1.9            | not found | 1.9     | 1.8          |
| Asparagine         | 3.4            | 2.5            | 2.6     | 1.2     | 0.9          |
| Histidine          | 4.0            | 3.3            | 3.3     | 3.7     | 2.5          |
| Serine             | 9.7            | 3.3            | 3.3     | 3.7     | 2.5          |
| Glutamine          | 4.7            | 3.3            | 3.3     | 3.7     | 1.8          |
| Arginine           | 1.2            | 0.3            | 0.3     | not found | 0.9          |
| Glycine            | 4.1            | 2.2            | 2.2     | 3.8     | 1.7          |
| Threonine          | 6.0            | 3.3            | 3.3     | 3.8     | 3.5          |
| Alanine            | not found      | 2.0            | 2.0     | 2.7     | 2.8          |
| Tyrosine           | not found      | 3.7            | 3.7     | 3.8     | 3.1          |
| Valine             | 2.8            | not found      | 1.6     | not found | 1.6          |
| Methionine         | 4.5            | 1.2            | 1.4     | not found | 1.4          |
| Tryptophan         | 4.4            | 2.6            | 2.5     | not found | 2.5          |
| Isoleucine         | 5.3            | 90.4           | 102.7   | 95.2    | 95.2         |
| Phenylalanine      | not found      | 20.1           | 22.8    | 21.6    | 21.6         |
| Leucine            | 5.2            | 1.6            | 1.7     | 0.8     | 0.8          |
| Lysine             | not found      | 0.5            | 0.5     | 3.0     | 3.0          |
| **Total**          | **61.0**       | **140.4**      | **158.5** | **138.4** | **138.4** |

As can be seen from Table 2, amino acids mass concentration in BD is low and amounts to 61.0 g/dm³, which confirms the results of previous research [15].

It has been shown that AAB in addition to actively consuming almost all determined amino acids, intensively synthesizes isoleucine, as well as cyclic aromatic amino acid phenylalanine, which is involved in addition of specific aroma. As can be seen from the table, the total of amino acids in vinegar is higher than in initial dialysate by 2.3-2.6 times depending on the bio-carrier, which increases the nutritional value of the product.

Vinegar, obtained from BD has characteristic specific organoleptic properties, with light beer aroma in combination with bread tones.

### 3.4 Electron-microscopic research of Acetobacter aceti AAB.

Figure 6 shows electron-microscopic images of zeolite samples with immobilized AAB by SEM. AAB cells under these conditions are characterized by the following sizes: length from 1.1 to 2 μm, sometimes 3.5 and 4.0 μm; cells thickness 0.7-0.8 μm. On the zeolite granules surface, fissile cells are also detected.

In the upper left and lower parts of the figure, a loose fibrillar-granular matrix (probably of polysaccharide nature) is visible, in which AAB cells are partially immersed.

The SEM results of AAB cells on the surface of beech shavings are presented in Figure 7.
Figure 6. *Acetobacter aceti* AAB cells on the surface of zeolite granule under conditions of prolonged cultivation on medium with BD

Figure 7. *Acetobacter aceti* AAB cells on the surface of beech shavings under prolonged cultivation on medium with BD

The density of *Acetobacter aceti* cells on the surface of beech shavings is significantly higher than on zeolite granules. Under these conditions, AAB cells are more uniform in size, with many dividing ones. The cell length is from 1 to 1.3 μm, the thickness is 0.7-0.8 μm. Spherical cells up to 1 μm in diameter are found.

On the surface of the beech shavings, along with AAB cells, complexly organized large crystals (from 5 to 9 μm) are appeared, collected from flat oval petals and, according to the stacking type, resembling a rose flower. The most probable assumption is that these are crystals of Mg pyrophosphate [16]. It can be assumed that they are formed from mineral salts (ammonium and potassium phosphates and magnesium sulfate) added to the medium for feeding acetic acid bacteria. However, the fact of their absence on the surface of zeolite granules under the same conditions remains unclear.

An analysis of the obtained data indicates that conditions for growth on beech shavings for AAB are more favorable.

Research of ultrathin sections of AAB inoculum cells immobilized on zeolite and beech shavings by TEM.

Figure 8. Ultrathin sections of *Acetobacter aceti* AAB cells. (a) – inoculum, (b) – zeolite, (c) – beech shavings

Analysis of inoculum ultrathin sections cells (Figure 8) showed that cytoplasm of AAB cells is highly heterogeneous, granular. Small and large electron-dense granules are located both inside the cell and outside, and are probably sorbed on the surface of the cells. Sometimes granules are released into the intercellular space. It can be assumed that the high electron density of these granules is due to polyphosphates presence.

On ultrathin sections of free cells from the sample under cultivation in zeolite granules presence (Figure 8b), it is seen that mainly multiple electron-dense granules are located in the intercellular space and partially connected with the cell surface. In general, the number of free extracellular and electron-dense granules bound to the cell surface is significantly higher than that in the inoculum. The
cell cytoplasm under these conditions is more enlightened, electronically transparent, in comparison with inoculum cells.

On the sample of cells sections from beech chips, the structure of the cytoplasm of cells is dense, heterogeneous. The cell population under these conditions is characterized by a large number of electron-dense granules on the cell surface. Sometimes small dense granules are evenly distributed over the entire surface of the cell, forming a microcapsule around it. This is a characteristic feature of free bacterial cells under cultivation on beech shavings.

4. Conclusion
Research of the BD biochemical composition and vinegar obtained from it showed that the content of volatile components in vinegar is 2-2.5 times lower than that of dialysate, depending on the used bio-carrier. In addition to the fact that they consume amino acids, AAB intensively synthesizes isoleucine, as well as the cyclic aromatic amino acid phenylalanine, which is involved in the addition of final product specific aroma.

The use of zeolite as a biocarrier makes it possible to significantly reduce air consumption during vinegar production (more than 2 times with periodic aeration and 7 times with preliminary aeration of the medium). An analysis of the obtained data as a result of electron microscopic research of Acetobacter aceti AAB cells immobilized on various bio-carriers indicates that the growth conditions for AAB on beech shavings are more favorable.

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