Improvement of Culture Medium for Cultivation of Citrobacteria

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Abstract. The article presents the results of research on the cultivation of citrobacteria on culture media. In the experiment, culture media were used, both selective ones for citrobacteria – Simmons and Endo media, and universal ones for facultative anaerobes – meat-peptone agar (MPA). The composition of the studied culture media was injected with various concentrations of a TS-1 growth stimulant of microorganisms, which is a culture liquid of kombucha, with the production and manufacturing technology of which the authors worked. As a result of the studies, the authors found that the introduction of the 2% TS-1 growth stimulator into Simmons agar medium during the cultivation of citrobacteria gives an increase in the bacterial mass by 1.5 times more on the MPA medium and by 2 times more on the Endo medium in comparison to other concentrations of TS-1.

1. Introduction

The principal possibility of large-scale cultivation of pathogenic microbes on standard media with the known composition was first proved by the Russian microbiologist N. Ushinsky in 1893. Subsequently, the use of synthetic media became the most important problem in the biological industry, but such media, proposed at the level of science at the end of the 19th and beginning of the 20th century, were of little use for industrial purposes.

Meat media have partially retained their importance and the best of them are used today. Now industrial enterprises, in addition to meat media, use media from non-food raw materials. They are usually named after the main protein raw material, or in accordance with the enzyme used for hydrolysis.

Culture media include the following additives:
- substances that help to reduce surface tension and eliminate foam;
- substances that provide proper oxidation-reduction potential.

Combinations of hydrolysates, extracts and growth stimulators of microorganisms in nutrient media, rather than an increase in the amount of the same ingredient, allows obtaining a greater yield of biomass of microorganisms. If possible, production culture media should be cleaned of protein ballast substances by precipitation with triacetic acid. In some cases, production culture media must be clarified by means of supercentrifugation and ultrafiltration using high-performance separators and powerful supercentrifuges.

In the production of bacterial preparations for culture media, the following hydrolysates are most often used: hydrolysates with a high degree of protein breakdown; hydrolysates with shallow breakdown of the protein molecule; hydrolysates that contain a large amount of amino acids and peptones.
Hydrolysis carried out by exoenzymes is called digestion. Hydrolysis carried out by proper enzymes of raw material is called autolysis. There is also chemical hydrolysis carried out by acids or alkalis (less often).

In recent years, yeast autolysates have been widely used for the preparation of culture media in the biological industry. In industrial production, the following types of hydrolysates are used: acid casein hydrolysates; fish-corn hydrolysates; acid hydrolysates of fish and bone meal; meat acid hydrolysates; tryptic hydrolysates of casein; tryptic casein-yeast hydrolysates; casein-mushroom hydrolysates; fish and mushroom hydrolysates; meat and mushroom hydrolysates; papain digestion meat hydrolysates; soybean seed hydrolysates; gelatin hydrolysates; fresh fish hydrolysates; whale meal hydrolysates; tryptic meat hydrolysates; open-hearth peptone; digests or hydrolysates according to Hottinger. Culture media are also produced on the basis of hydralizates, which act as stimulators of the growth of microorganisms. In industrial production, culture media are made on the basis of extracts, infusions or dialysates.

Against the background of the available culture media, the list of our studies has a goal to develop a culture medium that would be optimal for the cultivation of citrobacteria and the accumulation of the maximum concentration of bacterial mass on it. As a bioadditive to the culture media used in the field of biotechnological research, we used the previously studied microorganism growth stimulator (TS-1 SRM TS-1), which is based on the culture liquid of kombucha.

This drink has been known at all times, but recently interest in it has grown significantly not only in everyday life, but also in industrial technologies for the production of functional food products, including drinks. According to many authors, the culture liquid produced by kombucha is a good tool for the prevention of many diseases resulting from violations of environmental protection and a general weakening of people's immunity under stress [1]. However, there is little evidence that this drink is very well used in biological plants to stimulate the growth of microorganisms in the production of biological preparations [2]. In addition, the culture liquid of kombucha and the body of kombucha itself are used in cosmetology and the food industry. According to the results of some studies, it has an antibacterial and antimicrobial effect, has an antitoxic effect on the body, normalizes pressure, and good results appear in the treatment of various diseases of the gastrointestinal tract, since after taking antibiotics and other chemotherapeutic drugs, the kombucha actively restores the gastric microflora [1, 3]. Antibiotic properties are found in the culture liquid of kombucha. A detailed study of the biochemical composition of this drink revealed the products of glycolysis: acetic acid, glycerin, gluconic acid, succinic acid, lactic acid, malic acid, P, C, B1, B2, B6 vitamins, enzymes, ethanol. It was suggested that these qualities of the culture liquid affected the active growth of some microorganisms. In addition to the above, Luneva N.M. et al (2016) conducted a number of studies in which it was found that in the culture liquid of “kombucha” there is an acidic protease, which acts at pH 3.4-3.6. After 1.5 hours, associations are formed in solutions at different pH values. In addition, changes in total protein concentration were studied, which varied depending on the pH of the solution [4, 5].

The culture liquid of kombucha under the name of TS-1 growth stimulant of microorganisms was tested in the biological industry in order to cultivate enterobacteria. During these studies, in the tested microorganisms there were no changes. This was confirmed by the results of studies on the response of representatives of the intestinal microflora to antibiotic drugs. Industrial technologies are being developed for the production of culture media and the cultivation of enterobacteria on them, including media with the addition of the SRM TS-1. This microorganism growth stimulator was introduced at various concentrations into the composition of production culture media [6].

Formed by several types of yeast (Zygosaccharomyces sp., Saccharomyces sp.) And bacteria (Acetobacter sp., Gluconobacter oxydans, Bacterium gluconicum, Torula, Dekkera, Pichia sp.) kombucha is a symbiont. To study the effects of environmental conditions, symbiotic relationships during the cultivation of kombucha and their influence on the adaptive abilities of the body are interesting.

Kombucha is proposed to be used in courses to treat or alleviate the course of gastrointestinal diseases, lung diseases, dermatitis of various etiologies, weight loss, as an adjuvant that helps to
normalize blood pressure and has an immunostimulating effect, and also reduces alcohol withdrawal syndrome [7].

Many authors who studied this symbiont used black tea or, less often, green tea as a base for cultivating the body of kombucha. To feed the microorganisms, sugar or glucose was added to the infusion and the recommended cultivation temperature was 20–23 °C. To find growth stimulants alternative to kombucha, they tried to add caffeine instead of tea, which can also stimulate bacteria to produce cellulose. But this experiment was not distinguished by a high accumulation of body mass of the kombucha and the body itself did not differ in cartilaginous formation [8].

Studies have been conducted in which the mushroom Medusomyces gisevi was used in the preparation of bread. It has been experimentally established that the ratio of kombucha to obtain 1 ton of bread is 1: 4. But how the process of the kinetics of the yeast process goes on has not yet been studied. However, a higher amylase activity was noted in comparison with the control in the medium with the addition of kombucha and cyclodextrins. Subsequently, in combination with a significant amount of acidic proteases, it retains this activity. Thus, it was confirmed that cyclodextrins could stabilize enzymes and that fact could be applied in practice [9].

Most enterobacteria are undemanding to culture media. Bacteria of the Citrobacter genus are isolated from environmental objects and food products. They are representatives of the normal flora of the gastrointestinal tract of animals (including rabbits) and humans. An increase in their concentration as a result of feeding or other environmental factors can lead to gastrointestinal upset [10, 11]. Energy is obtained by membrane and respiratory phosphorylation. They are catalase-positive (with rare exceptions, for example, S. dysenteriae) and, in the overwhelming majority of cases, oxidase-negative. Citrobacteria can be isolated from the gastrointestinal tract of many animals. Therefore, it is relevant to improve the nutrient media for their cultivation [12, 13].

In malonate broth, the growth of bacteria of the genera Enterobacter, Klebsiella and Citrobacter leads to a blue discoloration of the medium, and the growth of bacteria of the genera Escherichia, Salmonella, Shigella, Edwardsiella, Yersinia, Serratia, Morganella, Proteus and Providencia is not accompanied by a change in the color of the medium or it turns yellow.

On a medium with urea, enterobacteria capable of utilizing urea form ammonia, which stains the medium pinkish-red. Proteidae cause reddening of the environment within 2–6 hours, and enterobacters, citrobacters and Klebsiella – for a longer time (up to 3–5 days). On a urease medium (according to Christensen), many enterobacteria give such a color reaction faster.

On Drigalski agar, E. coli colonies are yellow, and most other enterobacteria are blue or blue-green. Enterobacteria (Klebsiella, Enterobacters) capable of utilizing ammonium dihydrogen phosphate and sodium citrate grow on Simmons citrate agar and are one of the most popular sources of nitrogen and carbon. This process can be observed by changing the color of the environment from green to blue.

On MIO medium, enterobacteria are differentiated according to 3 criteria: mobility (turbidity of the medium and / or propagation of growth beyond the seeding line), decarboxylation of ornithine (with a negative reaction, the medium at the bottom of the test tube turns yellow, while with a positive reaction, it turns purple) and the formation of indole (a positive reaction manifests itself as redness of the medium after adding 3–4 drops of Kovacs reagent and shaking) [14, 15].

2. Materials and methods
Based on the studies of the above authors, we conducted research on the selection of culture media in order to obtain the largest amount of bacterial culture of citrobacteria. For this purpose, various concentrations of the TS-1 growth stimulator were added to standard culture media for the cultivation of citrobacteria. The growth stimulator was added to each portion of the culture media in the amount of 1%, 2%, 3%; 4% and 5%. After thorough mixing, they were sterilized by autoclaving at 134°C for 30 minutes. Agar culture media (Simmons, Endo and meat-peptone agar) were poured into sterile Petri dishes. After solidification under sterile conditions, in a laminar flow cabinet, 1 ml of a culture of the VKPM B-13235 Citrobacter freundii collection culture was applied on each medium at a concentration of 10 bln. microbial cells in 1 ml (3 pcs. of each medium). Meat-peptone agar (MPA) does not quite
correspond to the nutritional properties for the cultivation of citrobacteria, therefore it was used as a control in the experiment. The cultivation was carried out in a thermostat at 37 °C for 20–24 hours. A day later, a weak growth of Citrobacter freundii colonies appeared on the nutrient media, so all Petri dishes were placed again in a thermostat for further cultivation. In general, the exposure of the cultures in the thermostat was 40 hours, after which the final count of the colonies was carried out.

3. Research results
Colonies obtained in Petri dishes were counted by the visual method and counted in the BIOSTAT software according to Student's t test. The results of these studies are presented in table 1.

| Accumulation of bacterial mass in the culture medium, mg / ml, with different concentrations of SRM TS-1, % | Nutrient media | Endo medium | Simmons medium | MPA |
|---------------------------------------------------------------|---------------|------------|---------------|------|
| 1,0 test, M±m | 12.24±1.5 | 8.9±2.1 | 10.5±2.1 |
| % of accumulation to control | 45.5 | 42.6 | 54.8 |
| 2,0 test M±m | 13.05±2.4 | 14.25±0.9 | 7.98±0.9 |
| % of accumulation to control | 55.2 | 128.3 | 17.7 |
| 3,0 test M±m | 11.18±2.4 | 11.57±1.0 | 9.24±1.4 |
| % of accumulation to control | 32.9 | 85.4 | 36.28 |
| 4,0 test M±m | 8.9±0.7 | 9.9±2.5 | 12.5±1.0 |
| % of accumulation to control | 5.8 | 58.6 | 84.3 |
| 5,0 test M±m | 9.9±0.7 | 10.4±1.1 | 11.4±1.5 |
| % of accumulation to control | 17.7 | 66.6 | 68.1 |
| control | test M±m | 8.41*±0.46 | 6.24±0.5 | 6.78±0.56 |

Note: * P >0.05

From the data presented in table 1, it can be seen that, despite the slight difference in the accumulation of microbial cells of Citrobacter freundii, on the surface of the improved culture media there are differences from culture media with different concentrations of the TS-1 growth stimulator. So, on the Endo medium, the best concentration was the medium with the addition of the 2% kombucha culture liquid, which is 50% higher in relation to the content with 4% concentration. With the addition of the 1% growth stimulator, the culture concentration was only 10% less than in the medium supplemented with the 2% TS-1.

On the Simmons medium, the bacterial cell count with the addition of 2% exceeds the results of all other media and is 128.3%, which is 42.9% more than the highest result on the Simmons medium with the addition of the 3% TS-1 microorganism growth stimulator.

The results of the growth of the bacterial mass of citrobacteria on the ordinary meat-peptone agar were significantly lower compared to the other test media. The highest concentration was noted in Petri dishes with the MPA and the 4% TS-1 growth stimulator and amounted to 84.3%, which is 44% lower than the culture of citrobacteria grown on the Simmons medium with the addition of the 2% TS-1 microorganism growth stimulator.
4. Conclusion
Based on the foregoing, it can be concluded that the well-known culture liquid called *kombucha* (Medusomyces gisevii) can be recommended as a growth stimulator of microorganisms for active use in biotechnological production in order to obtain biomass of citrobacteria.

To accumulate the maximum amount of bacterial mass, we recommend using a 2% concentration of a microorganism growth stimulator in Simmons nutrient medium, since it was found in the experiment that the concentration promoted the accumulation of the bacterial mass of citrobacteria 1.5 times (65%) more than the growth marked on the control medium (MPA). In comparison to the Endo medium, on the tested culture medium the growth of the bacterial mass of citrobacteria was almost 2 times more (by 73.1%).

References
[1] Byazrova K Z 2017 Microflora of kombucha and its practical uses *Bulletin of scientific works of young scientists, graduate students and undergraduates* (Vladikavkaz: FSBEI HE (Federal State Budgetary Educational Institution of Higher Education) Gorsky SAU (State Agrarian University) 83–85
[2] Yurkevich D I and Kutyshenko V P 2002 Medusomycete (kombucha): scientific history, composition, physiology and metabolism features *Biophys.* 47 1116–29
[3] Dash H R, Mangwani N, Chakraborty J, Kumari S and Das S 2013 Marine bacteria *Appi Microbiol. and Biotechnol* 97–2 561–571
[4] Luneva N M 2016 Protein composition of native solution of Kombucha (Medusomyces gisevii lindau) *Modern trends in the development of science and technology* 5–1 21–24
[5] Wassels S, Axelsson L and Hansen E B 2004 The lactic acid bacteria, the food chain, and their regulation *Trends in Food Science and Technology* 15 498–505
[6] Oliferova E V 2001 Influence of the TS-1 microorganism growth stimulator on the main properties of enterobacteria In the collection Problems of the development of biology and chemistry in the North Caucasus. (Stavropol - Stavropol SAU (State Agrarian University) 113–115
[7] Elango V, Yuvakkumar R, Jegan S and Kannan N A 2008 Simple strategy to purify cyanobacterial cultures *Biotechnol* 7 4 23–24
[8] Hanson K E 2016 The First Fully Automated Molecular Diagnostic Panel for Meningitis and Encephalitis: How Well Does It Perform, and When Should It Be Used? *Jour. of Clinical Microbiology* 54-9 2222–24
[9] Bulanov M D and Glazova N V 2019 Optimization of cultivation conditions for kombucha Medusomyces gisevii lindau Innovations in the health of the nation *VII All-Russian scientific and practical conference with international participation* (St. Petersburg: St. Petersburg State University of Chemical Pharmacy) 119–123
[10] Pulcharovskaya L P, Vasiliev D A and Zolotukhin S N 2017 Isolation of bacteria of the genus Citrobacter *Bulletin of the Ulyanovsk SAA (State Agricultural Academy)* 3 (39) 83
[11] Kudrevatykh I A and Shumilina N N 2018 Assessment of the microbial landscape of the intestines of rabbits *Perm Agrarian Bulletin* 1 (21) 21–24
[12] Lighthart B and Mohr A J 2012 *Atmospheric microbial aerosols: theory and applications* (New York : Springer Science & Business Media) 397 p
[13] Zhumabekova K A 2005 Management of the composition of the mixed culture Kombucha 2005 *Biotech. theory and practice* 1 88–90
[14] Xu Q, Dziejmian M and Mekalanos J J 2003 Determination of the transcriptome of Vibrio cholerae during intraintestinal growth and midexponential phase in vitro 2003 *Proc. Natl. Acad. Sci. USA* 100 (3) 1285–91
[15] Guthke R, Linde J, Meeh F and Fisse T 2012 Systems biology of microbial infectious *Front Microbiol* 3 328