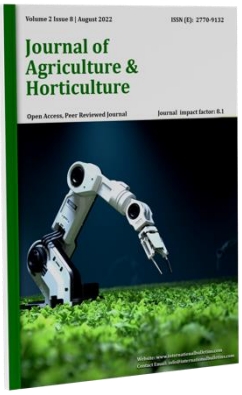

METHODS FOR PRODUCING BACTERIA OF THE TYPE AZOTOBACTER CHROOCOCCUM TYPE IN NUTRIENT MEDIUM BY BIOTECHNOLOGICAL METHOD IN THE PRODUCTION OF BIOORGANIC FERTILIZERS

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Abstract: This article describes biotechnological methods for the cultivation of free-living nitrogen-fixing bacteria of the Azotobacter chroococcum species in nutrient media. During the study, various nutrient media were tested for the cultivation of these microorganisms, among which Ashby mannitol agar was identified as the most effective medium. This medium is nitrogen-free, which enhances the ability of the bacterium to fix atmospheric nitrogen. Also, the ability to solubilize phosphates, the activity of antioxidant enzymes (catalase and peroxidase), and other useful biological properties of A. chroococcum were studied. The results obtained in the study expand the possibilities of using these microorganisms in the production of environmentally friendly, biologically active fertilizers.

Keywords: Azotobacter chroococcum, Ashby mannitol agar, nitrogen fixation, nutrient medium, biotechnology, phosphate solubilization, antioxidant enzymes, biofertilizer.

Introduction

In organic farming, natural and green manures from leguminous crops are the main source of nitrogen for crop plants. The aim of this study was to show that the total protein content and yield of spring barley grain grown in an organic farming system can be influenced by appropriate agrotechnological methods using innovative fertilization methods based on bacterial formulations. Azotobacter and Azospirillum bacteria are biologically characterized by the ability to convert nitrogen into ammonia, which is necessary for the synthesis of amino acids that make up plant proteins [1, 2, 3, 4 , 5 , 6] . Bacteria absorb N_2 only to meet the needs of cell metabolism, therefore they do not release solid nitrogen into the environment. Nitrogen reaches the soil environment only after the death of bacterial cells. Therefore, the plant rhizosphere, where the amount and availability of nutrients and energy-providing components are relatively high, is a favorable place for the development of Azotobacter spp. [7, 8, 9].

Biofertilizers promote plant growth by providing nutrients, including biologically bound nitrogen, or by increasing the availability of insoluble nutrients in the soil and by synthesizing substances that stimulate plant growth [10, 13]. Biofertilizers are an economically and environmentally attractive means of improving crop quality and quantity [12]. They are less expensive and improve crop growth and quality by directly or indirectly stimulating the release of plant hormones [11]. The plants complement each other because deep-rooted legumes can obtain water and minerals from the subsoil, while the root systems of grasses and cereals use the topsoil and nitrogen released into the soil by legumes.

The best understood effect of legumes on the soil is the enrichment of the soil with nitrogen fixed by Rhizobium nodule bacteria living in symbiosis with legumes [14, 15, 16, 17, 18 , 19 , 20, 21]. Research on this topic in Uzbekistan is significantly lacking. This work was an

attempt to at least partially fill this gap, as its goal was to determine the methods of studying the use of Azospirillum lipoferum and Azotobacter chroococcum bacteria, as well as methods for biotechnologically growing Azotobacter chroococcum bacteria in nutrient media for the production of bioorganic fertilizers in the agricultural system.

Materials and methods

The main object of the study was selected microorganisms of the Azotobacter chroococcum species. These bacteria were collected from agro-ecological zones rich in dry soil and peat, and were previously purified and identified in the laboratory.

Growing environment:

Ashby mannitol agar (AMA) was selected as the main nutrient medium for the cultivation and growth of microorganisms in the study (Table 1). This medium is a nitrogen-free medium for Azotobacter chroococcum , which clearly demonstrates its ability to fix nitrogen.

Table 1

Ashby's mannitol agar composition (per 1 liter):

No.	Substance	Quantity	Task
1	Mannitol	20 g	Carbon source (energy)
2	K2HPO4 (dipotassium phosphate)	0.2 g	Phosphorus source, pH buffer
3	$MgSO_4 \cdot 7H_2O$	0.2 g	Source of magnesium (for enzymes)
4	NaCl	0.2 g	Ionic balance
5	K2SO4	0.1 g	Source of potassium
6	$CaCO_3$	5.0 g	pH stabilization, low carbon source
7	Agar (only in agar medium)	15–20 g	For harsh environments
8	Distilled water	1 liter	Base liquid for solution
9	The pH should be around 7.0. There is no nitrogen (NH_4^+ or NO_3^-) — this allows Azotobacter to fix nitrogen.		

The nutrient medium does not contain nitrogen compounds , which activates the ability of Azotobacter chroococcum to fix free nitrogen.

Additional media: Formulations prepared from various organic and inorganic components were tested as alternative media in the experiment. The effectiveness of these media was evaluated based on the growth rate of microorganisms, colony count, pigment production, and enzyme activity (catalase, peroxidase).

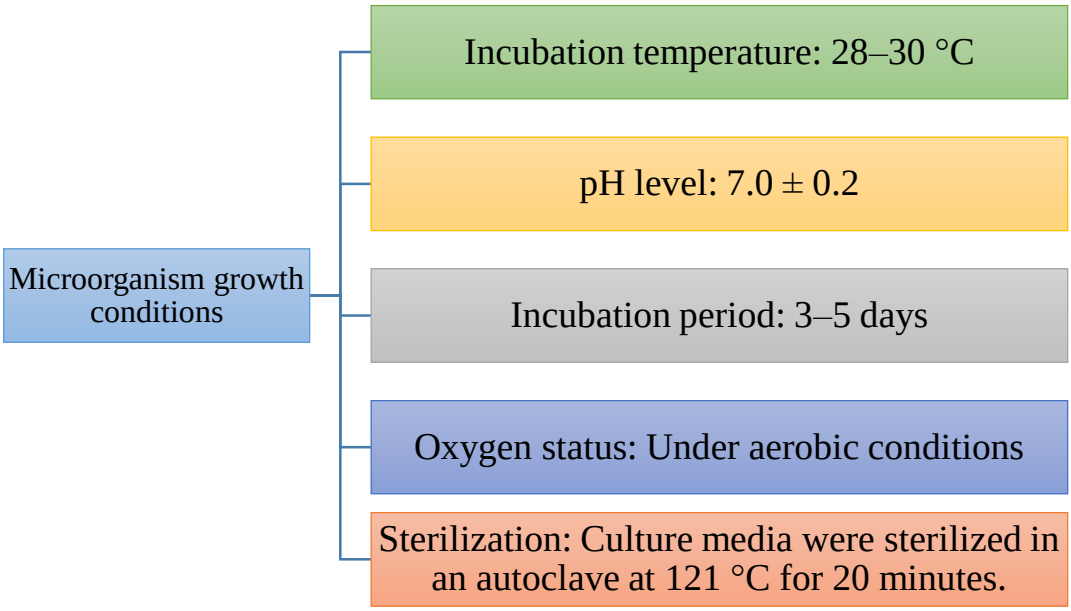

Figure 1 Microorganism growth conditions

The diagram shows the main parameters required for the cultivation of the microorganism Azotobacter chroococcum in the laboratory. For effective growth of microorganisms, it is important to maintain the incubation temperature in the range of 28–30 °C and the pH of the medium around 7.0 ± 0.2. The incubation period is usually 3–5 days. The bacteria are grown under aerobic conditions — that is, in the presence of oxygen. The culture media are sterilized in an autoclave at 121 °C for 20 minutes before experimental use. These conditions are optimal for nitrogen fixation, biomass growth, and the production of biologically active metabolites by A. chroococcum.

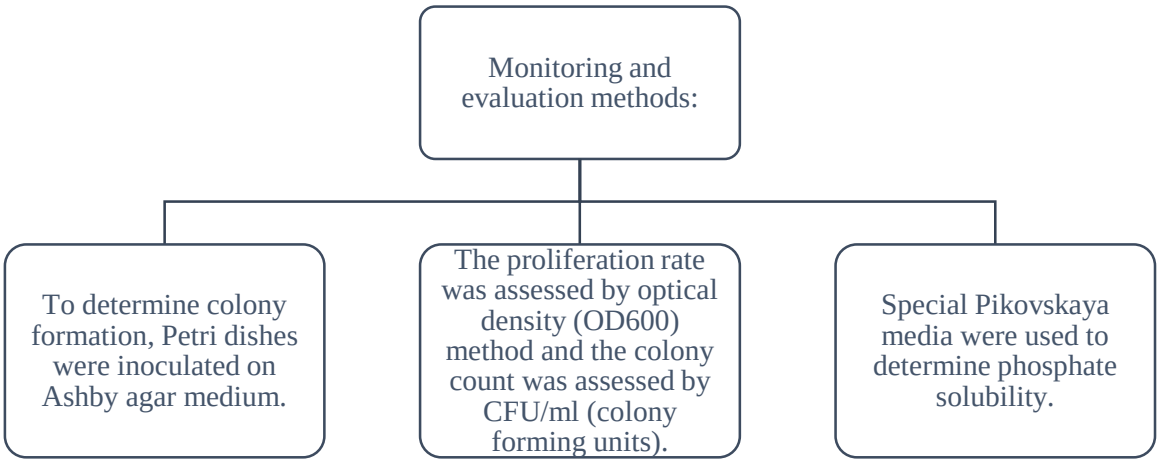

Figure 2. Monitoring and evaluation methods

This diagram shows the monitoring methods used to assess the growth activity and biological properties of the microorganism Azotobacter chroococcum. In the first stage of the microbiological analysis, colony formation was monitored by inoculation on Ashby agar medium in Petri dishes. The growth rate of bacteria was assessed by counting optical density (OD600) and CFU/ml (colony forming units), which allowed to determine the growth rate and cell density. In addition, in order to determine the agrobiological usefulness of microorganisms, phosphate solubility was tested using special Pikovskaya media. This

approach allowed to determine not only the growth of microorganisms, but also their metabolic activity related to phosphorus.

Research results

The most important factor affecting the growth of microorganisms and their biosynthesis of various biologically active substances is the composition of the nutrient medium, as well as the conditions for its preparation and sterilization. The production nutrient medium should be complete, that is, it should contain a rational and balanced set of components necessary for the construction of cell mass and the synthesis of the target product. The results of the study allow for the effective use of local agrobiological raw materials, thereby creating the basis for the development of microbiological preparations that serve to increase soil fertility in an environmentally safe and sustainable way.

Ashby's mannitol agar or liquid medium allows this bacterium to grow in a nitrogen-free environment, demonstrating that it is a free-living nitrogen fixer. Table 1

Table 2 compares the growth efficiency of Azotobacter chroococcum on various media. The highest growth rate was observed on Ashby mannitol agar, which was considered optimal for the bacteria. On this medium, the maximum growth phase was recorded within 3 days and the colony count reached 180×10^6 CFU/ml.

Although growth was relatively good in glucose-based medium, it was 120×10^6 CFU/ml, and maximum growth occurred on day 4. In peptone medium, growth was low, and bacterial activity was significantly reduced — 95×10^6 CFU/ml.

Table 2.

Growth rate of A. chroococcum **in different nutrient media**

No.	Type of food environment	Maximum growth time (days)	Colony count (CFU/ml, $\times10^6$)	Analysis
1	Ashby mannitol agar	3	180	Highest growth, productive environment
2	Glucose-based medium	4	120	Average growth
3	Peptone medium	4	95	Low growth, as a supportive environment
4	Starchy environment	5	80	Minimal growth, not recommended

The lowest growth was recorded on starch medium, where the adaptation of microorganisms was poor and the colony count did not exceed 80×10^6 CFU/ml. Therefore, this medium is not recommended for the growth of microorganisms.

These results show that the growth and metabolic activity of microorganisms are directly dependent on the components of the nutrient medium surrounding them. Ashby mannitol agar not only creates favorable conditions for growth, but also enhances nitrogen fixation, which is an important factor for biotechnological purposes. During the study , the growth rate, nitrogen fixation activity, phosphate solubility, and antioxidant enzyme activity of Azotobacter chroococcum were evaluated by growing them on different nutrient media.

The experiments showed that Azotobacter chroococcum showed the most effective growth on **Ashby mannitol agar**

Discussion

Nitrogen fixation activity:

A. chroococcum cells grown in Ashby medium under nitrogen-free conditions showed a strong ability to fix atmospheric nitrogen. This bacterium's **ability to independently meet its nitrogen needs** was confirmed in practice. Compared with the control (nitrogen-rich medium), a 30–40% increase in biomass was observed in the mannitol-based nitrogen-free medium.

Growth dynamics of microorganisms:

The growth rate and density of microorganisms in different media were monitored. In Ashby medium, bacteria reached the maximum growth phase on day 3, which allowed us to consider this medium as effective even in industrial conditions. In alternative media (glucose-based, peptone, and starch-based), bacterial growth was slower.

Phosphate solubility:

Azotobacter chroococcum was tested on Pikovskaya medium with a special indicator. The formation of a bright ring around the bacterium proved its **ability to solubilize inorganic phosphates** . This condition facilitates the absorption of phosphorus element by plants.

Antioxidant enzyme activity:

Azotobacter chroococcum was found to have high levels of catalase and peroxidase enzyme activity, which allowed the bacterium to be evaluated as an organism that **is resistant to oxidative stress and capable of degrading toxic substances in the soil.**

Conclusion:

As a result of the conducted research, **Ashby mannitol agar was confirmed** as the most optimal nutrient medium for the bacterium Azotobacter chroococcum . This medium maximally demonstrated the nitrogen fixation, growth rate, phosphate solubility and enzymatic activity of the bacterium. Such microorganisms can be widely used in the preparation of environmentally safe, biologically active fertilizers. The results of the research further expand the possibilities of biotechnological cultivation of Azotobacter species on an industrial scale.

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