Microbial communities of activated sludge: a potential contribution to the production of vitamin B12

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Abstract. The chemical synthesis of vitamin B12, an important nutrient for living organisms, is complex due to the asymmetric structure of the vitamin molecule. This opens up significant prospects for the development of biotechnological approaches to cyanocobalamin synthesis, which forms the basis of this work. The study examines the potential of activated sludge generated during wastewater treatment as a source of vitamin B12 products for the production of feed concentrates. As a nutrient medium, the possibility of using waste from alcohol processing production – stillage, is being considered. Together, this can make it possible to ensure the economic viability, sustainability and feasibility of biosynthesis of feed vitamin B12 on an industrial scale. The cultivation process was monitored by monitoring a set of indicators, including the pH of the medium, the amount of dry matter, dihydrogenase activity, weight gain and B12 content. The analysis of the data highlights the importance of understanding the complexity of the relationship between individual cultivation parameters to optimize vitamin B12 production processes. In general, the achieved level of vitamin B12 synthesis was 430 mcg/l, which allows to consider active sludge and distillery stillage as potentially promising components of the vitamin B12 biosynthesis process.

1 Introduction

The use of vitamin B12 is directly related to maintaining the health and optimal functioning of a living organism. Vitamin B12 (cyanocobalamin) plays an important role in a variety of biological processes such as DNA synthesis, red blood cell formation and the normal functioning of the nervous system. Its deficiency can lead to serious health consequences, including anemia, neurological disorders and other diseases [1, 2]. Therefore, providing adequate levels of vitamin B12 through nutrition or feed supplements is essential to maintain overall health and prevent various diseases.

In the context of animal husbandry, the relevance of obtaining vitamin B12 is related to the need for its use in animal feed. The optimal level of vitamin B12 in the animal feeding diet is one of the factors that ensure the required level of animal health and productivity.
The complexity of obtaining vitamin B12 preparations is due to the peculiarities of the asymmetric structure of its molecule and the complexities of chemical synthesis. Existing methods of obtaining vitamin B12 are not always highly effective or sustainable, so modern research in this area is aimed at finding or developing new production technologies that would be more effective, cost-effective and sustainable. Research in the field of microbiological synthesis, fermentation, genetic engineering and biotechnology opens up new prospects for optimizing vitamin B12 production [2-6].

Active sludge formed as a result of the wastewater treatment process, which is a complex microbial community including a variety of heterotrophic microorganisms, is considered as one of the promising areas. The active sludge is dominated by various groups of bacteria, including Betaproteobacteria, Enterobacteria, vibrios, pseudomonas, Zoogloea spp., Sphaerotilus natans and others. These microorganisms play a key role in the biological removal of biogenic elements such as nitrogen and phosphorus from wastewater [3, 7-10].

The process of activated sludge formation is accompanied by flocculation of microbial cells, organic and inorganic particles into extracellular polymeric substances. As a result, a structural and functional community is formed, where microorganisms closely interact with each other. This community is effective in the biological treatment of wastewater from organic pollutants.

Given that propionic acid bacteria are producers of vitamin B12, and active sludge contains a variety of heterotrophic microorganisms, including ammonium and nitrite oxidizing bacteria, phosphate accumulating organisms and foaming bacteria, it can be assumed that active sludge can be used to produce vitamin B12 [1, 4, 11-13].

In addition, when using activated sludge for the production of feed concentrates based on fermentation waste containing vitamin B12, methane-forming bacteria can also play an important role. These bacteria can provide the necessary conditions for the formation of vitamin B12 during waste treatment and the formation of feed concentrates. Currently, in a number of countries around the world there are industrial productions of vitamin B12 based on activated sludge [3, 5, 14, 15].

The choice of distillery stillage as a substrate for obtaining vitamin B12 using activated sludge is justified by its biological availability. Distillery stillage contains organic substances that can be metabolized by microorganisms of activated sludge. It contains carbohydrates, nitrogenous compounds and other important nutrients necessary for the growth and reproduction of microorganisms capable of synthesizing vitamin B12.

The use of distillery stillages as a substrate makes it possible to make the process of obtaining vitamin B12 with the help of activated sludge stable. This is due to the uniformity of the substrate composition and the optimal composition, which promotes the activity of microorganisms.

Distillery stillage is often a byproduct in the production of alcoholic beverages or biofuels. Its use as a substrate for the production of vitamin B12 makes it possible to effectively use production waste and reduce raw material costs. The possibility of using it on an industrial scale is due to the fact that distillery stillage is produced in large volumes at industrial enterprises, which makes it affordable and suitable for use on the scale of industrial production of vitamin B12.

The above determines the relevance of this study, the purpose of which is to assess the potential for obtaining vitamin B12 based on the use of activated sludge and distillery stillage as a nutrient medium as producers of the microbial community.
2 Materials and Methods

2.1 Metrics of the cultivation process

Cultivation is carried out under anaerobic conditions at a temperature of +54°C in a semi-continuous manner with no more than 5% of fresh nutrient medium added to the culture liquid daily. The addition of the nutrient medium was continued until the volume of the culture liquid reached 1 liter. Cultivation was carried out for 70 days.

The composition of the nutrient medium per 1 liter of the test substrate (stillage): CoCl₂ – 10 mg; molasses – 2 g; 5,6 – dimethylbenzimidazole – 10 mg; methanol -20 ml.

2.2 Control of the cultivation process

The cultivation process was monitored for pH and dry matter content, assessment of dihydrogenase activity, ammonia content and the amount of accumulated vitamin B12. The frequency and methods of control are presented in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Indicators</th>
<th>Meas. unit</th>
<th>Measurement technique</th>
<th>Frequency of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth of microbial biomass (bottle weight)</td>
<td>d</td>
<td>gravimetrically</td>
<td>daily</td>
</tr>
<tr>
<td>2</td>
<td>pH</td>
<td></td>
<td>potentiometrically</td>
<td>daily</td>
</tr>
<tr>
<td>3</td>
<td>Dry substances</td>
<td>%</td>
<td>refractometrically</td>
<td>daily</td>
</tr>
<tr>
<td>4</td>
<td>Dehydrogenase activity (DHA)</td>
<td>Sec</td>
<td>qualitative reaction with methylene blue</td>
<td>daily</td>
</tr>
<tr>
<td>5</td>
<td>Ammonia (NH₃)</td>
<td>g/l</td>
<td>spectrophotometrically</td>
<td>1 time per week</td>
</tr>
<tr>
<td>6</td>
<td>Vitamin B12</td>
<td>Mg/l</td>
<td>TLC, HPLC, microbiologically according to GOST R 57201-2016</td>
<td>1 time in 20 days</td>
</tr>
</tbody>
</table>

3 Results and Discussion

It is known that the synthesis of vitamin B12 can proceed in two ways: aerobic and anaerobic. Both of these synthesis pathways can be divided into four sections: synthesis of ALA, synthesis of the corrin ring component, construction of the lower axial ligand and synthesis of cobalamin. In nature, only some representatives of archaea and eubacteria have the ability to produce vitamin B12 in two ways, including about 30 enzyme-mediated stages. One of them is the oxygen-dependent pathway that exists in R. Sphaeroides and Pseudomonas denitrificans. The other one is the oxygen-independent pathway, which has been found in Bacillus megaterium, Salmonella enterica and Propionibacterium freudenreichii. The main difference between these two pathways lies in the process of corrine ring compression and cobalt chelation, in which they diverge in precorrin 2 and merge to form Cob(II)irinate a, c-diamide. Nevertheless, the regulated mechanisms of both aerobic and anaerobic pathways of vitamin B12 biosynthesis are still unclear. The main strategies for increasing the yield of vitamin B12 are based on the selection of the composition of the nutrient medium, cultivation conditions and random mutagenesis to create strains producing vitamin B12 with high yields [3, 8, 12].

Our studies have shown that during the entire cultivation period there was a stable increase in the biomass of producers. The weight increase of the bottle increased from 282.27
g to 1091.72 g, which may indicate active reproduction of microorganisms or accumulation of metabolites.

The pH value of the cultivated mixture ranged from 7.52 to 7.95, probably reflecting changes in the biochemical processes in the culture. In general, maintaining pH values in a range close to neutral is critically necessary, since a number of studies show that cyanocobalamin production stops at pH 4.5 and below [1, 8]. An increase in the pH value from 7.52 to 7.95 may also be associated with an increase in the concentration of ammonium in the culture fluid. A high concentration of ammonium can stimulate microorganisms to more intensive growth and metabolic activity, which can lead to an increase in the mass of the culture medium [13-15].

The concentration of solids ranged from 1.0% to 4.0%, possibly reflecting the accumulation of metabolites or changes in the composition of the medium. The dehydrogenase activity varied for 70 days starting from 20 seconds and reaching 12 seconds by the end of the period, which may indicate an increase in the metabolic activity of the culture. The concentration of ammonium increased from 1.75 g/l to 4.00 g/l, which may be due to metabolic activity or the use of ammonium in growth processes. These changes indicate a dynamic process of growth and development of culture, which requires the regulation of cultivation conditions to achieve the desired results. The cumulative dynamics of the cultivation process indicators is shown in Fig. 1.

Collectively, the established fluctuations in these indicators showed the need to add a nutrient substrate (distillery stillage) to ensure further growth of microorganisms or maintain optimal conditions for their growth and development conditions. The nutrient medium was updated by adding a fresh portion of stillage every 14 days in a volume of 2.67 – 17.38 ml, depending on the recorded values of the indicators.

![Fig. 1. Dynamics of changes in the parameters of the cultivation process control.](image)

In general, the results obtained indicate the complexity and interconnectedness of various aspects of crop cultivation and emphasize the importance of an integrated approach to the analysis and regulation of cultivation conditions.

The key objective of the study was to quantify the biosynthesis of vitamin B12 in the formed cultivation conditions. The results of the chromatographic analysis are shown in Fig. 2.
The determination of vitamin B12 content in the culture fluid was carried out using potassium cyanide (KCN). The volume of the sample for analysis was 1.1 ml.

The concentration of vitamin B12 in the culture fluid after 70 days of cultivation was 0.43 mcg/ml, which is comparable with the results of other studies presented in the scientific literature. At the same time, further research is required to improve the efficiency of the vitamin B12 biosynthesis process, including the search for vitamin synthesis stimulants.

4 Conclusion

The study confirms that the activated sludge obtained as a result of wastewater treatment contains a variety of microorganisms capable of acting as producers of biologically active substances, namely vitamin B12. It is a complex microbial community that includes various groups of bacteria, such as ammonium and nitrite oxidizing, phosphate accumulating, and others. The key factor is the presence of propionic acid bacteria in the active sludge, which differ in their ability to synthesize vitamin B12 in commercially significant amounts. This opens up prospects for the use of activated sludge in the production of animal feed concentrates.

The choice of distillery stillage as a substrate for the production of vitamin B12 using activated sludge is due to its biological availability, the availability of necessary nutrients, economic feasibility, process stability and the possibility of industrial use.

From the analysis of the data, it can be seen that parameters such as an increase in the mass of the culture medium and the addition of fresh medium can affect the concentration of ammonium and dehydrogenase activity in the culture. This indicates the relationship between different parameters, which may be important for optimizing vitamin B12 production processes using activated sludge and distillery stillage.

The synthesized amount of vitamin B12 was 430 mcg/l, which generally indicates the need to find approaches to optimize biosynthesis processes. The key areas here may be the use of biosynthesis process stimulants such as cobalt, as well as the search for new producers.
References