EFFECTS OF AMARANTHS’ SEEDS ON DEHYDROGENASE ACTIVITY AND GASES EMISSION IN METHANGENIC BIOREACTORS

Víctor COVALIOV, Irina SENICOVSÇÃOA *, Vladimir NENNO, Valentin BOBICA, Olga COVALIOVA

Moldova State University

Institute of Pedology, Agrochemistry and Protection of Soil “Nikolae Dimo”

The influence of amaranths’ seeds as the source of squalene on the dehydrogenase activity and efficiency of methane production were investigated in methanogenic bench-scale (5000 ml) bioreactors used to treat the mixture of distillery wastes and farmyard manure. The adding of amaranth seeds to the methanogenic bioreactor has an inhibitory effect on the dehydrogenase activity and stimulates the process of methanogenesis. Dehydrogenase activity decreased with the increase of doses of squalene and its trend had a close connection with doses (R²=0.77-0.78). The methane content in the total amount of gases is 65.3-71.3% in a bioreactor with the additive of amaranth seeds in a dose of 50 mg l⁻¹, which is 22.1% higher than in the the control bioreactor without additives. The increase in squalene concentration higher than 0.0005% is not rational because its stimulating effect on the methanogenic process decreases. Anaerobic digestion of alcohol distillery industry wastes with manure is a complex nonlinear time-varying microbiological process. Dehydrogenase activity trends in the experiment are described by the power function for 5 hours observations and by the logarithmic function for 120 hours of observations. Trends of CH₄ are described by the polynomial function in all periods of testing. Correlation coefficients are 0.37 and 0.70 for CH₄ after 5 and 120 hours of the anaerobic digestion. Dehydrogenase activity is in the close negative connection with the amount of gases, including methane. Correlation analysis between dehydrogenase activity and the release of gases has revealed the moderate and strongly negative link during 24 hours after the start of the experiment.

Keywords: amaranths’ seeds, bioreactor, dehydrogenase activity, distillery grains, methane.

EFFECTUL SEMIȚELOR DE AMARANT ASUPRA ACTIVITĂȚII DEHIDROGENAZEI ŞI EMISSIONEI GAZELOR ÎN BIOREACTOARELE METANOGENE

In bioreactors of methanogens unite consecutively, with a volume of 5000 ml, utilized for the treatment of amestecul de borhot de la distilarea alcoolului cu gunoi de grajd, a fost studiată influența preparatelor din semințe de amaranț, ca sursă de scualen, asupra activității dehidrogenazei și eficienței producării metanului în procese anaeroobe. Adaugarea preparatelor de semințe de amarant în bioreactorul metanogen are un efect inhibitor asupra activității dehidrogenazei și stimulează procesul de metanogeneză. Activitatea dehidrogenazei a scăzut odată cu creșterea dozelor de preparat din semințe de amarant, iar tendența a fost în legată strânsă cu dozele (R² = 0,77-0,78).

În bioreactorul cu aditiv din semințe de amarant, în doză optimă de 50 mg l⁻¹, conținutul de metan în volumul total de gaze emise a fost de 65,3-71,3%, ceea ce este cu 22,1% mai mare față de bioreactorul de control, fără aditiv. Ridicarea concentrației aditivului, calculată în scualen, mai sus de 0,0005% nu este rațională, deoarece după această valoare efectul de stimulare a procesului metanogen scade. Digestia anaerobă a deșeurilor din industria de distilare a alcoolului cu gunoi de grajd este un proces microbiologic complex care variază neliniar în timp.

Trendul activității dehidrogenazei în experiment este descris de funcția de putere pentru 5 ore de observații și de funcția logaritmică pentru 120 ore de observații. Tendințele acumulării CH₄ în toate perioadele de testare sunt descrise de o funcție polinomială. Coeficienții de corelație pentru CH₄ sunt 0,37 și 0,70, respectiv pentru 5 și 120 ore de digestie anaerobă. Activitatea dehidrogenazei este în relație negativă cu cantitatea de gaze, inclusiv de metan. Analiza corelațiilor dintre activitatea dehidrogenazei și emisiile de gaze a indicat o legătura moderată și puternică negativă timp de 24 de ore de la inițierea experimentului.

Cuvinte-cheie: semințe de amarant, bioreactor, activitatea dehidrogenazei, distilare, metan.

Introduction

Recycling of the wine and alcohol distillery industry wastes is the actual problem in the Republic of Moldova. Currently, about 30 alcohol distilleries that produce alcoholic beverages and bioethanol operate at the territory of country. The annual volumes of the production and accumulation of liquid wastes are considerable. It is known that 10-13 liters of liquid wastes (distillery grains) are formed during the production of one liter of rectified spirit. For example, discharge of distillery grains from the Bulboaca wine factory constitutes 1,500 m³ day⁻¹ or 30,000-35,000 m³ year⁻¹. The solution of the problem of utilization of liquid wastes of the
wine and alcohol distillery industry is possible through the elaboration of environmentally grounded technologies for their anaerobic digestion.

The development of technologies of the anaerobic fermentation of liquid and solid wastes from the agricultural production is one of the perspective directions in the biogas production from plant raw materials and organic wastes formed in agriculture. Liquid wastes of the wine, bear and alcohol industry can serve as energy materials for the biogas production. The creation of bioreactors allows not only to obtain biometan in industrial volumes, but also to process environmentally hazardous industrial organic wastes.

The mechanism of the methane’s energetic processes is not decoded yet; however the general principal statements have been established [1, 8, 13 and 14]. Obtaining of energy, at least during the oxidation of H2, associated with the reduction of CO2, is connected with the functioning of the electron-transport system, involving dehydrogenases, electron carriers and reductases. Hydrogenase and formiatdehydrogenase have been identified as dehydrogenases. The specific enzymes are typical for the methanogenesis, due to their presence the methane formation occurs from carbon dioxide and hydrogen with the methanogenic microorganisms under the anaerobic conditions. The detailed review regarding the structure and functions of the enzymes involved in the methane synthesis from carbon dioxide and molecular hydrogen was performed by Shima S. et al. in 2002 [15]. However, it remains unclear what is the connection of dehydrogenase with methane release and whether this index can be the criteria of the efficiency of methanogenesis processes?

Enhancing effectiveness of biogas production can be accomplished by the use of various biologically active substances of natural origin. As it is known, biomass and extracts of various plant species, such as lavender, goldenrod, yarrow and morning glory, amaranth [7, 11 and 12] render the stimulating effect on the methane-forming microorganisms. To ensure the effectiveness of the methanogenesis processes and to increase the biogas yield and to reduce CO2 contents in it, with the appropriate increase in methane contents, the new technologies anaerobic fermentation of winery industry wastes have been proposed according to MD patents [2, 4 and 6]. One of essential elements of the new anaerobic fermentation technology of wine wastes is the use of biologically active additives obtained from the amaranth and birch bark, into the treated wastes. Additives of squalene as biochemically active substance, which has a property to capture oxygen and saturate the microorganisms tissues with it during the simple biochemical interaction with water, ensure the more complete running of methanogenic process. In its turn, this promotes the higher biogas yield and higher contents of biomethane in it. The perspective additives for methanogenesis processes have been recognized to be squalene and betulinol.

It has been shown that squalene and betulinol stimulate the aerobic microorganisms’ multiplication, which can activate the aerobic stage of wastes treatment and improve their preparation for the methanogenesis process [5]. Testing of biologically active compounds in vitro has demonstrated the stimulating effect of squalene and betulinol in concentration of 0.033% with regard to dehydrogenases activity. Squalene activated dehydrogenases by 25%, and betulinol – 1.8 times [5]. In order to simplify and reduce the cost of technology further research was planned to conduct with natural sources of squalene and betulinol - with amaranth seeds and birch bark.

*The purpose of the research* was to determine the effect of amaranth seeds as the source of squalene on the dehydrogenase activity and methane yield in the anaerobic fermentation of the mixture of distillery grains and farmyard manure and to evaluate the relationship between dehydrogenase and the release of gases.

**Material and Methods**

*Experiment.* Researches of the influence of biologically active substances on the process of methanogenesis were carried out on a specially designed set of four bioreactors [5]. The equipment consists of a bench-scale bioreactor, placed in the thermostatically controlled volume, devices to remove and control the volume of obtained biogas, formed sludge and purified liquid. This equipment makes it possible to study the biogas purification process from admixtures of CO2 and H2S simultaneously. The total volume of each bioreactor is 5000 cm³, the useful volume - 3500 cm³. To ensure the additional surface for the development of immobilized methanogenic bacteria, the grapevine rods have been introduced in bioreactor, with the total package surface of 8000 cm² [3].

The nutrition mixture with milk and sugar was gradually substituted by the mixture of alcohol distillery industry grains (60%) and farmyard manure (40%). The distillery grains indices were as it follows: dry substances – 8.4-8.8 %; Corg in the dry matter – 4.25%; Corg in the raw matter – 49.5%; BOD5 = 17800 mg O2 l⁻¹; COD = 28250 mg O2 l⁻¹; pH = 5.4.
Bioreactors were maintained in the regime of stationary growth phase of methanogenic culture. Three concentrations of squalene were studied at the same time, and one of bioreactors was determined to be the control. Observations were performed in the dynamics, after 5, 24, 96 and 120 hours of the incubation.

**Biologically active substances.** As the source of squalene were used pounded seeds of amaranth. Seeds contained 10% of squalene. The following concentrations of biologically active additives have been selected for the research: 0.0005%, 0.001% and 0.002% which were equivalent to the introduction in bioreactor of each 50, 100 and 200 mg of seeds or 5.0, 10 and 20 mg of active substance per each liter of the mixture introduced.

**Methods.** Dehydrogenase activity was determined with colorimetric method, according to the triphenylformasane (TPF) contents, formed from TTC (2,3,5-triphenyltetrasolium chloride), which was added to the sample of waste [9]. The substrate was 0.1M glucose solution. The calculations were made per 10 ml of waste for 24 hours of incubation. The volume of gas was measured by the volumetric method, the methane content – by the gas chromatographic method [10].

**Statistical analysis.** Standard statistical analysis (variation and correlation analysis) was conducted using MS Excel.

**Results and discussions**

**Effect of amaranths’ seeds on gases and methane emissions.** The additive of squalene in the composition of amaranth seeds in dose of 50 mg l⁻¹ has increased 1.6-2.4 times the methanogenesis efficiency after 5, 24 and 96 hours of the anaerobic fermentation of the mixture of distillery grains and farmyard manure; has rendered the significant effect on the biochemical process rate (Table 1).

<table>
<thead>
<tr>
<th>Variant</th>
<th>5 h</th>
<th>24 h</th>
<th>96 h</th>
<th>120 h</th>
<th>Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total CH₄</td>
<td>total CH₄</td>
<td>total CH₄</td>
<td>total CH₄</td>
<td>total CH₄</td>
</tr>
<tr>
<td>Control (mixture without additives)</td>
<td>87.5</td>
<td>45.9</td>
<td>125.0</td>
<td>72.3</td>
<td>140.0</td>
</tr>
<tr>
<td>Amaranth seeds 50 mg l⁻¹</td>
<td>150.0</td>
<td>98.0</td>
<td>250.0</td>
<td>173.0</td>
<td>180.0</td>
</tr>
<tr>
<td>Amaranth seeds 100 mg l⁻¹</td>
<td>120.0</td>
<td>64.8</td>
<td>175.0</td>
<td>108.9</td>
<td>175.0</td>
</tr>
<tr>
<td>Amaranth seeds 200 mg l⁻¹</td>
<td>120.0</td>
<td>64.6</td>
<td>150.0</td>
<td>95.3</td>
<td>150.0</td>
</tr>
</tbody>
</table>

The highest values of gas and methane emissions in the variants with the addition of seeds of amaranth have been recorded in 24 hours after start of the experiment. The control variant has demonstrated a gradual increase in the amount of gases.

The methane content in the total amount of gas increases by an average of 22.1% at the dose of amaranth seeds of 50 mg l⁻¹, accounting for 65.3-71.3%, while on the control remains approximately the same – 52.5-58.4%. The methane content in the total volume of the gas increases towards the end of the experiment from 65.3% to 71.3% on the variant with the dose of 50 mg l⁻¹ of amaranths’ seeds, from 54.0% to 70.3% with 100 mg l⁻¹ and 53.8% to 68.2% on the variant with the dose of 200 mg l⁻¹.

The increase in bio-additive concentration higher than 50 mg l⁻¹ is not rational, as it is not accompanied any more with the gas release rate acceleration and CH₄ emissions.

**Effect of amaranths’ seeds on the dehydrogenase activity.** Testing the amaranth seeds like bio-stimulator for the process of anaerobic decomposition of the organic matter in bioreactors has demonstrated its significant impact on the dehydrogenase activity at all doses (Table 2). Dehydrogenase activity in bioreactors with amaranth was not only statistically significant lower compared with the control, but different on the order at the beginning of the experiment. Already after 5 hours after the start of the experiment there was a decrease in the activity of 10.8-21 times. The differences were smoothed out through the 96-120 hours, but they continued to be statistically significant. Differences between the dose of amaranth of 50 mg l⁻¹ and doses of 100 and 200 mg l⁻¹ were statistically significant during the whole experiment. Differences between doses of amaranth 100 and 200 mg l⁻¹ were not significant. These results suggest that the dehydrogenase index reliably reflects the decline
of the content of organic substances in fermenting wastes as the result of their anaerobic fermentation with the gases release and is in the close negative connection with the amount of resulting gases, including methane.

### Table 2

<table>
<thead>
<tr>
<th>Variant</th>
<th>5 h</th>
<th>24 h</th>
<th>96 h</th>
<th>120 h</th>
<th>Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (mixture without additives)</td>
<td>4.84</td>
<td>5.26</td>
<td>0.27</td>
<td>0.20</td>
<td>2.64</td>
</tr>
<tr>
<td>Amaranth seeds 50 mg l(^{-1})</td>
<td>0.45</td>
<td>0.39</td>
<td>0.22</td>
<td>0.13</td>
<td>0.30</td>
</tr>
<tr>
<td>Amaranth seeds 100 mg l(^{-1})</td>
<td>0.23</td>
<td>0.21</td>
<td>0.17</td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td>Amaranth seeds 200 mg l(^{-1})</td>
<td>0.30</td>
<td>0.22</td>
<td>0.13</td>
<td>0.10</td>
<td>0.19</td>
</tr>
<tr>
<td>LSD (_{0.5}) (all variants)</td>
<td>1.85</td>
<td>2.03</td>
<td>0.06</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>LSD (_{0.5}) (variants with additives)</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

**Connection between the gasses’ release and dehydrogenase activity.** The database of dehydrogenase activity, the total amount of gas and methane were processed separately by the correlation and regression analysis during periods of observations (Figure 1 and 2). Regression equations with the highest correlation coefficients were chosen from all of the regression equations. Dehydrogenase activity trends in the experiment are described by the power function for 5 hours observations and by the logarithmic function for 120 hours of observations. Trends have the high correlation coefficients: 0.78 and 0.77 accordingly.

![Fig. 1. Trends of dehydrogenase activity and gas emissions in the anaerobic decomposition of the mixture of distillery grains and farmyard manure as a result of the amaranth seeds application in different concentrations during 5 h incubation: 1-3 – control, 4-6 – amaranth seeds 50 mg l\(^{-1}\), 7-9 – amaranth seeds 100 mg l\(^{-1}\), 10-12 – amaranth seeds 200 mg l\(^{-1}\).](image1)

**Fig. 1.** Trends of dehydrogenase activity and gas emissions in the anaerobic decomposition of the mixture of distillery grains and farmyard manure as a result of the amaranth seeds application in different concentrations during 5 h incubation: 1-3 – control, 4-6 – amaranth seeds 50 mg l\(^{-1}\), 7-9 – amaranth seeds 100 mg l\(^{-1}\), 10-12 – amaranth seeds 200 mg l\(^{-1}\).

![Fig. 2. Trends of dehydrogenase activity and gas emissions in the anaerobic decomposition of the mixture of distillery grains and farmyard manure as a result of the amaranth seeds application in different concentrations during 120 h incubation: 1-3 – control, 4-6 – amaranth seeds 50 mg l\(^{-1}\), 7-9 – amaranth seeds 100 mg l\(^{-1}\), 10-12 – amaranth seeds 200 mg l\(^{-1}\).](image2)

**Fig. 2.** Trends of dehydrogenase activity and gas emissions in the anaerobic decomposition of the mixture of distillery grains and farmyard manure as a result of the amaranth seeds application in different concentrations during 120 h incubation: 1-3 – control, 4-6 – amaranth seeds 50 mg l\(^{-1}\), 7-9 – amaranth seeds 100 mg l\(^{-1}\), 10-12 – amaranth seeds 200 mg l\(^{-1}\).

Trends of total emissions of gases are described by the polynomial function for 5 h observations and by the logarithmic function for 120 hours of observations. Correlation coefficients are 0.47 and 0.73 respectively.
Trends of CH$_4$ and other gases are described by the polynomial function in all periods of testing. Correlation coefficients are 0.37 and 0.70 for CH$_4$ and 0.89 and 0.77 for other gases.

These results indicate that the dehydrogenase activity in the experiment was closely linked to bio-additives. The average values of correlation coefficients at the beginning of the experiment (0.47 for total gas and 0.37 for methane) shows that amaranth seeds are used by microorganisms for the metabolism almost immediately, thereby adapting the microbial community to the new substrate and reducing the lag phase.

The correlation analysis between the dehydrogenase activity and the release of gases has revealed the moderate and strong link (Table 3).

### Table 3

The correlation coefficients between the dehydrogenase activity and gas emissions in the experiment with laboratory bioreactors (n=12 for each period of observation)

<table>
<thead>
<tr>
<th>Time of observation</th>
<th>Gas emissions</th>
<th>CH$_4$</th>
<th>Other gases</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 h</td>
<td>-0.81</td>
<td>-0.66</td>
<td>-0.97</td>
</tr>
<tr>
<td>24 h</td>
<td>-0.59</td>
<td>-0.59</td>
<td>-0.56</td>
</tr>
<tr>
<td>96 h</td>
<td>-0.21</td>
<td>-0.36</td>
<td>0.83</td>
</tr>
<tr>
<td>120 h</td>
<td>0.79</td>
<td>-0.62</td>
<td>0.76</td>
</tr>
</tbody>
</table>

The strong negative correlation of the dehydrogenase with the release of gases has noted in 5 hours after the start of the experiment ($R^2 = -0.81$), the average negative correlation with the amount of CH$_4$ ($R^2 = -0.66$) and the strong negative correlation with other gases ($R^2 = -0.97$). Probably the latter fact is caused by the amount of CO$_2$ produced in the initial stage of fermentation. After 24 and 96 hours the connection between the dehydrogenase activity index and gases release weakened, but after 120 hours became strong again, but positive, except for the connection with the CH$_4$.

### Conclusions

The use of amaranth seeds as the source of squalene has an inhibitory effect on the dehydrogenase activity and stimulates the process of methanogenesis in bioreactors used to treat the mixture of distillery grains and farmyard manure. The methane content in the total amount of gases is 65.3-71.3% in a bioreactor with the additive of amaranth seeds in a dose of 50 mg l$^{-1}$, which is 22.1% higher than in the the control bioreactor without additives. Dehydrogenase activity’s trends have a high positive correlation ($R^2=0.77-0.78$) with doses of amaranth seeds under laboratory conditions in bioreactors. The increase in squalene concentration higher than 0.0005% (or amaranth seeds of 50 mg l$^{-1}$) is not rational because its stimulating effect on the methanogenic process decreases.

Anaerobic digestion of alcohol distillery industry wastes with manure is a complex nonlinear time-varying microbiological process. Dehydrogenase activity reflects the decline in the content of the organic matter of the mixture of distillery grains and farmyard manure as a result of anaerobic fermentation with the release of gases and is in the close negative connection with the amount of gases, including the methane. Correlation analysis between dehydrogenase activity and the release of gases has revealed the moderate and strongly negative link during 24 hours after the start of the experiment.

The database of the dehydrogenase activity has a practical importance for an operative evaluation of the processes of the organic wastes’ decomposition in methanogenic bioreactors. Future research in the domain of biochemical processes in bioreactors will be directed to a more fully understanding of the interaction between enzymes, the organic matter of wastes and biologically active substances. The important aspect of researches will be the elaboration of the methods to control the process of anaerobic digestion of organic materials.

### Bibliography:


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