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IMPROVED METHOD FOR DETERMINING MICROBIOLOGICAL CONTAMINATION OF FATTY ACID METHYL ESTERS AND BLENDED DIESEL FUELS

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Abstract. The process of degradation of biodiesel of vegetable origin (rapeseed and sunflower) under the influence of various factors is considered. Existing methods of determining microbiological contamination of substances are analyzed, their main advantages, disadvantages, and limits of use are determined. Based on the combination of existing methods, a method of qualitative and quantitative determination of the degree of microbiological (bacterial and mycological) damage to fuels has been developed. Quantitative and qualitative characteristics of microbiological damage to traditional and alternative diesel fuels have been established. The microorganisms that are the most active destructors of biofuels have been identified.

Keywords: blended fuels, diesel fuel, methyl esters of fatty acids, microbiological damage.

1. Introduction

Saving energy from petroleum products and increasing emissions from diesel engines are forcing most countries to look for ways of reducing the risk of heat engines being affected by the environment. Fatty acid methyl esters (FAME) are currently one of the most promising alternative energy sources both as a pure fuel and as an additive in blended diesel fuels.

FAME have a similar to diesel fuel calorific value and density and also improves the lubricating properties of mixed fuels. High cetane number, 51 and more, affects the efficiency of fuel ignition, especially in the conditions of cold start.[1] Increased almost 3 times the flash point of 393 K in a closed crucible, which is 3 times higher than that of alternative fuels provides high fire safety.

The main advantage of FAME is the reduction of harmful substances concentration in the fulfilled gases. Due to the higher cetane number, the use of FAME reduces hydrocarbon emissions by 40 %. The main advantage of FAME is the reduction of the concentration of harmful substances in exhaust gases. There is a 57 % reduction in CO emissions when using blended diesel fuels and a 15 % reduction in nitrogen oxide emissions.[2] Increasing the percentage of the alternative component in the blended fuel reduces the ignition delay and accelerates the reaction of the blend, resulting in reduced emissions of unburned hydrocarbons.

Diesel engines running on mixed fuel with the addition of FAME are considered the most environmentally friendly and promising. FAME undergoes almost complete biological decomposition: 99 % of FAME are processed by microorganisms in soil or water in 28 days, which minimizes pollution of rivers and lakes.[3]

However, the impact on most of the regulated performance indicators is ambiguous due to the fact that the characteristics of FAME differ depending on the raw material.

Because FAME consists of saturated and unsaturated fatty acids, over time they are more prone to oxidation than mineral diesel. In addition, corrosive ions contained in water promote the growth of microorganisms and can hydrolyze methyl esters to form more corrosive fatty acids at the fuel/water interface. Oxidation results in the formation of insoluble resins, as well as deposits and other secondary oxidation products, such as low molecular weight organic acids, aldehydes, and ketones, which increase the total acidity, viscosity, iodine value, and corrosion activity.[4]

Due to high hygroscopicity and biodegradability, FAME is more prone to microorganism contamination. FAME mineralization is 2-4 times higher than that of petroleum diesel fuel. The length of the fatty acid chain, the number and position of C=C double bonds, and the presence of antioxidant compounds contribute to the oxidative capacity and biological stability of FAME. The higher the concentration of saturated fatty acids, the greater the oxidative stability.[5]

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When FAME is added to petroleum diesel, the rate and completeness of petroleum diesel decomposition are increased several times, as microorganisms use fatty acids as a source of energy.[6]

Microbiological corrosion is the irreversible destruction of structural material by microorganisms. It is the result of the impact of microorganisms and their metabolic products on the metal surface. Groups of microorganisms are often attached to surfaces. The involvement of microorganisms can accelerate the rate of corrosion of structural materials up to ten times. For the development of microorganisms the necessary factors are elevated temperature (293-308 K), the presence of water (from 0.01 %) and nutrients in the fuel.[7]

The following problems arise as a result of the development of microorganisms: increase in acidity, the change in physicochemical properties of fuel (the increase in kinematic viscosity, refractive index, pH, actual resin content), the appearance of sediment, turbidity, and pungent odor[6] sludge deposits on the inner walls of fuel systems; damage of paints and varnishes (swelling, destruction, deterioration of adhesion) and elastomeric products; colonies of microscopic fungi on rubber and sealants intensify the condensation of water vapor, impair the mechanical and dielectric properties of these materials.[8]

Species composition and properties of strains (aggressiveness to the material) differ depending on the material, design, and technological features of the fuel system and the climatic area of operation of the product. Fungi of the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Alternaria* and bacteria of the genus *Bacillus* can destroy a variety of materials. At the same time, many microorganisms are destructive only to some materials.[9]

More than half of all cases of microbiological destruction of oils and lubricants occur as a result of fungi of the genus *Aspergillus*, *Penicillium*, *Fusarium*, and *Scopulariopsi*. Microbiological damage of polymer and paint materials is a consequence of the activity of fungi of the genus *Penicillium*, *Stemphylium*, *Chaetomium*, *Trichoderma*.[6]

The main microorganisms that cause biodegradation of fuels are bacteria of the genera *Pseudomonas*, *Micrososis*, *Mycobacterium*, as well as fungi *Cladosporium*, *Aspergillus*, *Repicillum*, *A1ternaria* and others. At the same time, the bacterium *Psaerugenosa* and the fungi *Cladosporium Resinae* ("kerosene fungus") are found more often than others in petroleum products.[10]

Cladosporium Resinae ("Kerosene fungus") is the most common in diesel fuels. Unlike other genera of fungi, "kerosene fungus" produces much more biomass, which causes technical malfunctions in the fuel system. Since this fungus grows on the border of the "fuel-water" phases, it needs only a drop of moisture to grow. Then the mycelium begins to cover the drop, holding it in place, and continues to grow, producing even more water through its metabolism.[11]

It has been proved that the process of decomposition of aliphatic compounds begins with the help of monooxygenase and dioxygenase enzymes. The initial decomposition process occurs under aerobic conditions by oxidation of hydrocarbons. This process converts aliphatic compounds to primary and secondary alcohols.[12]

The speed and depth of microbial oxidation of fuel depend on its hydrocarbon composition. According to study,[13] the trend of diesel fuel decomposition under the action of microorganisms is in descending order in the following series: *n*-alkanes> branched alkanes > monoaromatic substances > cycloalkanes > polyaromatic.

n-Alkanes are more sensitive and more vulnerable to the action of microorganisms than other forms of hydrocarbons. Therefore, fuels that contain mostly paraffinic hydrocarbons are destroyed by microorganisms faster than fuels, which consist of more aromatic compounds.[7]

It follows from the above that almost all materials used in engines are exposed to microorganisms, so an important task is to study the microbiological stability of materials and develop ways to protect them.

One of the main ways to prevent microbiological contamination is biocidal additives, which are chemical compounds, which are able to kill microorganisms or inhibit their reproduction, preventing fuel degradation. Currently, active work is underway to develop biocidal fuel additives.[14] The selection of biocidal additives is a complex, multi-purpose task. The additive must be heat-resistant, non-toxic, as universal as possible in relation to various microorganisms, and must not affect the physicochemical properties of the fuel.

Regular sludge drainage is used as the main prevention measure for all fuel tanks. If microbiological contamination is suspected, it should be tested.

The methods of determining microbiological contamination include:

– A method of direct counting the number of cells under a microscope. This method is rarely used due to the presence of emulsion and uneven cell distribution in the fuel. It is impossible to identify viable cells among the dead ones.

– In the gravimetric method, the mycelium of fungi is separated on filters under a vacuum. The resulting biomass is washed, dried, and adjusted to the constant weight. The method gives an accurate result only in the presence of microbiological contamination, which can be seen with the naked eye.

– The centrifugation method is based on the centrifugation of contaminated fuel samples that have been

previously homogenized. The length of certain layers in a test tube is read from the calibration graph.

– A method for counting microorganisms on fixed-colored Gram smears. The disadvantages of this method are the uneven distribution of the emulsion on a glass slide and the difficulty of preparing a drug of a strictly known area.[15]

– Method for determining the presence of microorganisms by estimating changes in certain fuel parameters (aqueous phase pH, acidity, sulfide content, component composition, metal content, changes in flash point, viscosity, and acidity). This method gives inaccurate information due to the indirect determination of the presence of microorganisms.

– Indicator method – direct fuel analysis using indicators. Interacting with ninhydrin microorganisms acquire color from blue to pink. Chloride triphenyl tetrazolium (TTC) in the presence of bacteria is converted into insoluble red pigment formazan. In this case, the concentration of bacteria is determined by comparing the intensity of staining of experimental and control samples. Dye Coomassie Brilliant Blue R-250 acquires a blue color. The Lowry method is distinguished: the solution turns blue as a result of the interaction of proteins produced by microorganisms with the Folin's reagent. Indicator-based methods provide only qualitative information on biological contamination.[16]

– The radiometric method measures the intensity of β radioactive isotope (indicator) that accumulates in microorganisms. The intensity of radiation is directly proportional to the size of biomass.

– Method for determining the presence of a microbiological microorganism by the presence of metabolic products: protein compounds (determined by IR), carboxylic acids (chromatography), vitamin B6 (fluorescence spectroscopy method), enzymes (biochemical method).

– Luminescent method. When irradiating prestained with fluorochrome microorganisms with UV light with a wavelength of 360-365 nm, a green glow can be observed.

Express methods are currently being developed that significantly reduce the time for this test. Existing methods for assessing microbiological damage are, in most cases, complex and time-consuming. Currently, the question of the degree of microbiological damage to traditional and alternative fuels is beginning to develop, but there is no method for quantifying microorganisms. At present, indicator methods for determining microbiological contamination are used, but these methods provide only a qualitative assessment, not a quantitative one, which does not fully determine the degree of microbiological contamination of fuel.[7]

2. Experimental

2.1. Materials and Methods

In this study, an improved microbiological method for the quantitative counting of colonies of microorganisms in fuel has been developed. The improved methodology is based on a combination of microbiological and gravimetric methods.

Using the method of cultivation on dense nutrient media in a Petri dish allows you to detect colonies of microorganisms that are present on the filter, in concentrations that cannot be determined gravimetrically. In turn, the use of additional paper filters can increase the accuracy of microscopic examination, avoiding errors due to uneven distribution of microorganism cells in the fuel. The advantages of this method are simplicity and reproducibility. This method provides important information about the diversity of microorganisms and allows to detect the viable microorganisms.

The following reagents were used for microbiological studies: alcohol, enzymatic peptone, 5 % glucose, microbiological agar, sodium citrate, yeast, hydrochloric acid 1 mol/L, sodium hydroxide 10 %.

This study used the following equipment: glass measuring cylinders with nominal capacities of 100, 400, and 1000 mL and glass ground lids of appropriate size; glass bottles with a capacity of 1000 mL; glass pipettes with a nominal capacity of 1, 2, 10 mL with 0.1 mL graduation; disposable sterile plastic tips for test tubes; sterile paper filters “Blue Tape” with a pore size of 3–5 microns; metal tongs with blunt tips; thermostat capable of maintaining a temperature of 298 ± 2 K; alcohol burner; drying cabinet capable of maintaining a temperature of 453 ± 5 K; laboratory microscope “Biolam” C-11; glass trowels; aluminum foil; sterile cotton wool; paper stickers and markers for marking Petri dishes; parchment paper; sterile glass Petri dishes with a diameter of 90 mm.

2.2. Basic Preparations and Sterilization

The fuel was left at rest for 1 hour, after which it was visually inspected for visible mechanical impurities or free water. If there is foreign contamination in the sample, the fuel was cleaned and dehydrated, and then carefully moved to evenly distribute the cells of microorganisms.

Glassware was wrapped in parchment paper, the pipette holes were closed with cotton plugs, paper filters were wrapped in parchment paper envelopes one by one, and then kept for 2 hours in an oven at a temperature of 453 ± 5 K. Metal tools were placed in a glass with alcohol so that the working ends were completely immersed.

The finished nutrient medium was brought to a temperature of 338-343 K and kept at this temperature for 1 hour. During this time, all vegetative cells died, and only spores remained viable. The medium was then cooled to a temperature optimal for spore germination (298 ± 2 K) and reheated to 343 K every other day. This cycle was repeated three times.[17]

2.3. Determination of Microbiological Pollution

2.3.1. Preparation of Medium

To prepare the medium, 60 g of glucose-peptone agar was suspended in 1 liter of distilled water and heated until completely dissolved. The molten medium was poured into a sterile Petri dish and, after solidification, was checked with a pH meter. If pH is outside the range of 5.4 ± 0.2, which is optimal for good growth of most bacteria and fungal spores, it was adjusted using hydrochloric acid or sodium hydroxide. After preparation, the medium was poured into sterile glass bottles and sterilized according to (2.2).

20 mL of nutrient agar medium molted in a boiling water bath was poured into sterile Petri dishes. To compensate the lyophobicity of the agar surface in relation to the fuel, pre-calcined filter "blue tape" was set with sterile forceps on the medium surface. To prevent condensation, the cups were left on a horizontal surface until completely solidified and then kept for 3 days at 298 ± 2 K with the lids down to dry the surface of the medium and check its sterility. Paper labels with the date and number of sowing were glued to the cups.

2.3.2. Incubation of Microorganisms

A known volume of pre-mixed fuel was placed on a surface of agar medium in a Petri dish with a sterile pipette and distributed over its surface with a sterile spatula. The mixed fuel was distributed on the surface in circular motions, simultaneously with the rotation of the cup. Glass and metal tools were immersed in alcohol and held over an alcohol burner before and after use.

Petri dishes were aged for 7 days at a temperature of 298 ± 2 K without direct sunlight. To prevent the surface from re-sowing with fungal spores, the samples were stored with lids upwards.

2.3.3. Counting the Number of Colonies of the Forming Elements

During and after incubation, the number of colonies of each class of microorganisms was counted and the number of viable bacteria and fungi present in this volume of the initial fuel sample was counted. Measurement of bacteriological contamination was carried out after an incubation period of 48 hours, mycological – after 7 days.

Counting the number of colonies of the forming elements (CFE) can be done manually or using an automatic microorganism colony counter, for example, SCM-1, SCM-2. The counter counts the colonies of microorganisms in Petri dishes by applying dots with an electropen to the bottom of the dish in the places where the colonies are found. The device registers the fact of touch and displays the result on a digital display.

In this work, the number of colonies of microorganisms was counted manually. The Petri dish was placed on a light background and the total number of all visible colonies was counted with the naked eye.

To count the number of CFE, the Petri dish was placed on a light background and the total number of all visible colonies was counted, marking them at the bottom of the cup with a marker. Once the colony has been counted, it is not re-counted, even if it has increased in size.

To calculate the number of colonies of cells of microorganisms in 1 liter of the initial suspension summed the results of parallel seeding and determined the average number of colonies by the formula:[18]

$$M = \frac{a \cdot 1000}{V} \quad (1)$$

where a is the average number of colonies during seeding, V is the volume of the suspension taken for inoculation, mL.

The accuracy of the technique depends on the number of colonies formed on the plate with a dense nutrient medium. Accuracy can be specified with a 95 % confidence interval. These limits determine the range with the number of colonies with a probability of 95 %. The distribution of organisms in the fuel sample is random and corresponds to the Poisson series.

2.4. Identification of Microbiological Contamination

The genus and representative of the bacterial isolates were determined by evaluating the morphological and cultural characteristics (Gram stain, shape, and size of cells, relief). To determine morphological features and prepare preparations, samples were cultured on dense nutrient media.

Special nutrient media were used for different classes of microorganisms. Saburo agar with glucose was used to identify unicellular and mycelial fungi. Chapek-Dox agar with glucose was used to cultivate fungi and yeast. The peculiarity of this nutrient medium is the slow growth of microorganisms, making it convenient to observe the process of culture development and isolate individual colonies.

Petri dishes with colonies were first examined with the naked eye or through a magnifying glass and then placed upside down and examined with a microscope at low magnification and narrowed diaphragm.

The colonies were described using a color scale indicating the pigmentation of the colonies and the surrounding agar, as well as morphological features (shape, size, features of the colony).

Prepared smear preparations. A small part of the bacterial material was removed from the largest colony with a sterile spatula, evenly distributed on a glass slide, fixed in an alcohol burner flame, and examined with an immersion Biolam C-11 microscope using conventional methods. The Gram staining method was used to distinguish Gram-negative and Gram-positive bacteria. Two dyes are used in the method – the main and additional dyes. Gram-positive microorganisms acquired a purple color, and Gram-negative – red (pink) color.[19]

Fungi were characterized and identified based on the morphology of their colonies and microscopic features. To characterize and identify fungi and yeast, the methods of cultivation on dense agar medium and microscopic examination describing the size, shape, structure of colonies, and their color were used. The relief of the colonies and their contours were determined with a magnifying glass. Colonies that differed in at least one feature were treated as several types. A mycological atlas was used to confirm the identity of each fungus.[20]

After 48 hours of incubation at a temperature of 298 K, there was the growth of fungi with the formation of a colony with a diameter of 1-5 mm. Each type of microorganism has a certain type of colony, so each type of colony was judged on the diversity of microbiological studies of the studied samples of fuel.

3. Results and Discussion

The susceptibility of fuel to microbial degradation depends on several factors, such as its chemical composition and the presence of water and nutrients. FAME is more prone to microbiological damage than conventional diesel fuel because its molecules are more easily broken down by microorganisms. In addition, increased use of additives, higher hygroscopicity, and lack of strict maintenance of storage tanks create the necessary conditions for the rapid development of microbial populations.[21] The microbiological degradation of FAME is mainly oxidative and hydrolytic in nature and depends on the raw material for FAME production. The main factors are the nature of fatty acids present in the raw material and the degree of unsaturation of esters that are part of it. FAME obtained from vegetable oils such as sunflower seeds used in this study has a higher content of unsaturated fatty acids, mainly oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3). These products not only affect the properties of alternative fuels, but are also aggressive towards structural materials and create problems during engine operation.[7]

More saturated fatty acids, as well as the presence of methyl ester of oleic acid in high concentrations, increase the resistance to oxidation. These characteristics reduce FAME's susceptibility to chemical and biological degradation, which is crucial for the long-term storage of FAME.[22]

3.1. Results of Determining the Degree of Microbiological Pollution

The degree of microbiological contamination was established for samples of FAME from sunflower and rapeseed and diesel fuel. For each type of fuel, three crops of 0.15, 0.20 and 0.2 mL were carried out. These volumes were selected by a series of inoculations to find the optimal number of colonies in the cup. The calculations considered only those Petri dishes that contained at least 20 and at most 300 colonies of microorganisms. The upper limit is due to the ability to distinguish with the naked eye the individual colonies.

The results of the study of the degree of bacteriological and mycological contamination of different fuels are presented in Figs. 1 and 2.

Analyzing the obtained results, it is seen that mineral diesel fuel and FAME based on sunflower oil had medium contamination with fungal spores and strong bacteria. FAME based on rapeseed oil was heavily contaminated with bacteria and fungal spores.

There are no generally accepted limits on the amount of microbiological contamination of motor fuels. The amount of CFE depends on a number of factors, but there are approximate limits of moderate and severe microbiological contamination. Moderate pollution is from 4,000 to 20,000 CFE/L, severe pollution is more than 20,000 CFE/L. These limits are intended to indicate in advance that the growth of microorganisms is occurring in the fuel.

As a rule, higher levels of contamination are required for operational problems to occur or fuel suitability to be adversely affected, but when using FAME, these problems are acute and can cause serious problems.

3.2. Results of isolation of microorganism isolates

Description of morphological features and identification of bacterial isolates are given in Table 1, and the identification of mycological isolates is presented in Table 2.

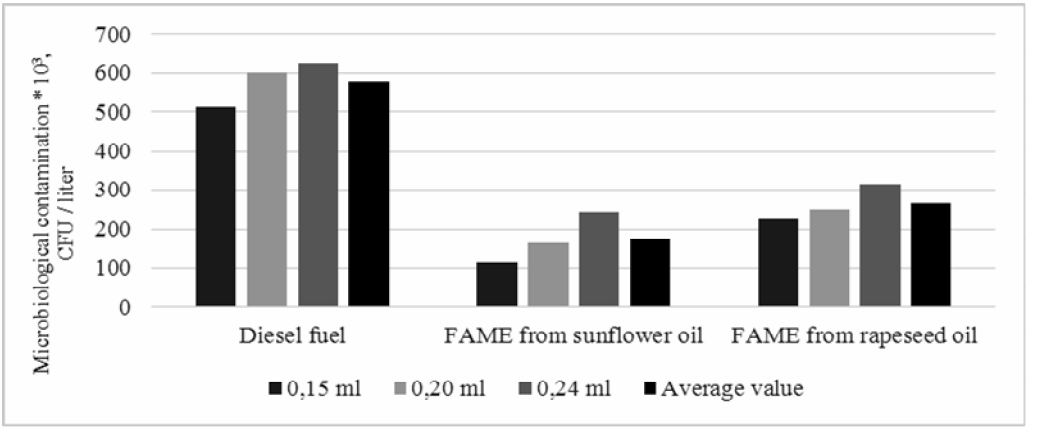

Fig. 1. Degree of bacteriological contamination of different fuel samples

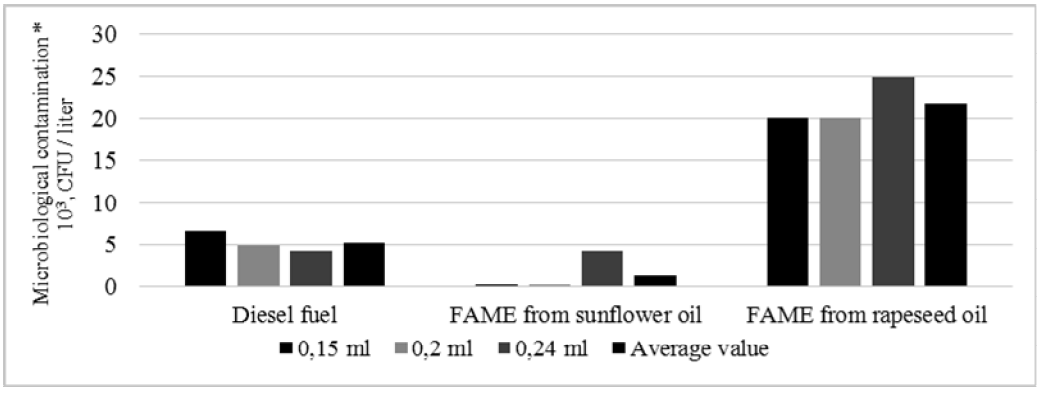

Fig. 2. Degree of mycological contamination of different fuel samples

Table 1. Description of the bacterial isolates morphology

No.	Form of colony	The size of colony	Relief of colony	Color	Genus and representative
1	Rounded	Small (1-2mm)	Flat	Colorless, muddy	*Alcaligenaceae*
2	Complex form	Large (4-6 mm)	Buggy with convex center	Lemon	*Pseudomonas Putida*
3	Rounded	Small	With a convex center	Lemon	*Micrococcus luteus*
4	Rounded	Small	Buggy	Light pink	*Pseudomonas chlororaphis*
5	Rounded	Large	Domed	Cream	*Klebsiella aerogenes*
6	Rounded	Medium (2-4 mm)	Bulging	Red and pink	*Serratia marcescens*

Table 2. Description of the fungal isolates morphology

No.	Relief of the colony	Color	Path	Genus and representative
1	Difficult to determine	Translucent, dark gray	Corrugated	*Rhizopus sp*
2	With a convex center, wavy	Light yellow with brown spots and white spraying, white outline	Corrugated	*Aspergillus baeticus*
3	With a pronounced center with waves from the center	Black with translucent white outline	Equal	*Aspergillus niger*
4	Mesh, buggy	White	Uneven	*Aspergíllus candidus*
5	With a pronounced center	Dark green with light outline	Corrugated	*Cladosporium C.*
6	Rough	Gray-blue with white outline	Equal	*Penicillium commune*

Various coloration of colonies was noted – a number of colors were clearly species-specific (Table 1). Colonies began to acquire color on the 3–4 day of the experiment, and the final intense color was acquired after 7 days. All samples of the studied fuels were infected with bacteria of the genus *Pseudomonas*, which do not have a significant impact on the performance of the fuel. The fungus *Cladosporium Resinae* was present in diesel fuel, *Cladosporium Resinae* and *Aspergillus* in FAME from rapeseed oil. Both genera of fungi have a devastating effect on fuel and structural materials of the engine and fuel system, in addition, fungi of the genus *Aspergillus* are pathogenic to humans.

4. Conclusions

1. Thus, based on the analysis of the literature on the subject of the study, currently the question of the degree of microbiological damage to traditional and alternative fuels is beginning to develop, but there is no method for quantifying microorganisms. At present, indicator methods for determining microbiological contamination are used, but these methods give only a qualitative assessment, not a quantitative one, which does not fully determine the degree of microbiological contamination of fuel.

2. We have developed an improved method for detecting the presence of microbiological damage to fuel and quantifying its degree.

3. The degree of microbiological damage of different samples of motor fuels was determined. Isolated, characterized, and identified microorganisms were in the fuel, including active destructors of fuels and structural materials. It was found that the tested fuel samples were contaminated with fungi and a large number of bacteria that can cause problems with fuel use.

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УДОСКОНАЛЕНА МЕТОДИКА ВИЗНАЧЕННЯ МІКРОБІОЛОГІЧНОГО ЗАБРУДНЕННЯ МЕТИЛОВИХ ЕСТЕРІВ ЖИРНИХ КИСЛОТ І СУМІШЕВИХ ДИЗЕЛЬНИХ ПАЛИВ

Анотація. *Розглянуто процес деградації біодизельного палива рослинного походження (ріпакового і соняшникового) під впливом різних чинників. Проаналізовано методики визначення мікробіологічного забруднення речовин, визначено їхні основні переваги, недоліки і межі використання. На основі комбінації наявних методів розроблено методику якісного і кількісного визначення ступеня мікробіологічного (бактеріального і мікологічного) ураження палив. Встановлені кількісні та якісні характеристики мікробіологічного ураження традиційних і альтернативних дизельних палив. Ідентифіковані мікроорганізми, які є найактивнішими деструкторами біопалив.*

Ключові слова: *дизельне паливо, метилові естери жирних кислот, мікробіологічне ураження, сумішеві палива.*