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Benzene Biodegradation by *Bacillus Subtilis* in Water and Validation System with Kefir Grains as Biological Indicator

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Abstract

Benzene is an aromatic molecule, which belongs to a particular class of compounds known as BTEX: benzene, toluene, ethylbenzene and xylene, such compounds harm to the environment and humans. An effective process for the degradation of these compounds is bioremediation. This technique aims the biological oxidation by microorganisms decomposing organic matter in simple substances such as CO2 and H2O. In this work, we studied the kinetics of well-Zeno degradation by Bacillus subtilis ATCC 6051. The experiment of biodegradation, early containing 100mg /L of benzene, developed in cell concentration of 1g /L. The saline decontamination level was evaluated with kefir grains, as a biological indicator. The biodegradation of 48% of benzene was confirmed (time less than 2 hours) for cell development. The decontamination of water promoted the development of kefir grains, about 2-3 times greater than the initial concentration, which used as a biological indicator of the biodegradation developed system.

1. Introduction

The alkyl benzenes are benzene and other aromatic molecules having only one ring. Among these monochrome hydrocarbons, the compounds named BTEX: benzene, toluene, ethylbenzene and xylene, are present in petroleum products, and widely used in many industrial and plastics, solvents, pharmaceuticals and fuels. Among the BTEX, benzene is high degree of dangerousness and quantities present in the environment. Chronic exposure to benzene can cause leukemia, lymphoma, depression, immunosuppression, peripheral neuropathy (JANBANDHU; FULEKAR, 2011). One way of treating wastewater and contaminated sites is the bioremediation of benzene, the monoaromatic hydrocarbon is degraded aerobically by microorganisms using an oxidative procedure wherein the first step of the pathway occurs through reactions catalyzed by enzymes oxygenase's, which introduce oxygen atoms on the aromatic ring which open the ring and make them accessible carbon atoms. (CAVALCA et al., 2000), transforming the compound in products with little or no toxicity (Tiburtius et al., 2004). In this work, we tested the benzene biodegradation process utilizing cells of *B. subtilis* ATCC 6051 as a degrading agent, this being a Gram-positive bacterium saprophyte, common in soil and water. Cells of *B. subtilis* are studied due to their ability to synthesize bio surfactants, using hydrocarbons as sole carbon source (MUKHERJEE et al., 2006). The biodegradation of benzene in water was validated taking as a bioindicator kefir grains. The size of the irregular gelatinous grains varies between 3,0 to 35,0 mm in diameter (IRIGOYEN et al., 2005). The grains form gelatinous and protein matrix in which more than 35 species of microorganisms live in symbiosis. (WITTHUHN et al., 2004) Among them *Saccharomyces cerevisiae* is one of the major contributor to the formation and growth of kefir grains. *S. cerevisiae* is widely used in the manufacture of biosensors to monitoring organic pollutants in the environment (Belkin S., 2003) and for the assessment of contamination by chromium (Jianlong. W. et al., 2003).

2. Objective

For the study of the kinetics of biodegradation of benzene, cells of *Bacillus subtilis* ATCC 6051 were developed, and kefir grains as bioindicator.

3. Materials and Methods

3.1 Study Microorganism

Cells of *B. subtilis* was kindly provided by Prof. Dr.. Thereza Cristina Vessoni Penna Department of Biotechnology, Faculty of Pharmaceutical Sciences of USP (FCFUSP).

3.2 Biological Indicator (bioindicator)

The collaborator Cristina Brasileira kindly provided the kefir grains in water, named Tibico.

3.3 Media and Stock Solutions

The compositions of the media and stock solutions, kept in the freezer, used in the experiments were:

- ✓ Stock solution of benzene: benzene (analytical grade) in ethanol (439.5 g / L).
- ✓ Growth medium Pre-inoculum: TSB (Tryptone Soya Broth): Pancreatic digestive Casein 17 g / L; Papain Enzyme 3.0 g / L; Hydrochloride sodium 5.0 g / L Glucose 2.5 g / L; Potassium dibasic 2.5 g / L.
- ✓ Detection method: (Bushnell-Haas (BH) - with yeast extract (2 g / l): Magnesium sulfate (MgSO4.7H2O) 0.2 g/L; Calcium Chloride (CaCl2) 0.02 g/L; Phosphate Potassium Monobasic (KH2PO4) 1.0 g / L, potassium phosphate Dibasic
- ✓ (K2HPO4) 1.0 g / L, ammonium nitrate (NH4NO3) 1.0g / L, Ferric chloride (FeCl 3) 0.03 g / L ; yeast extract 2g /L Source: (Adapted Bushnell Haas, 1941)..

3.4 Kinetics of Growth

The cultivation of pre-inoculum was developed in 250 mL Erlenmeyer flask type containing 50 mL of medium TSB (Tryptone Soya Broth). After 72 hours growth at 30 ° C in a shaker at 200 rpm, was measured optical density of the cell suspension of the strain 6051 UV-visible spectrophotometer (Shimadzu UV-1650 PC) at a wavelength of 660nm (DO660nm) (Mukherjee et al., 2006). To start monitoring the growth curve, calculate the volume (ml) required pre-inoculum to obtain a next initial DO660nm 1 g / L in the medium Bushnell-Haas (BH), contained in a bottle type Erlenmeyer 250mL. He retired, then the first to rate DO660nm reading at the initial time (0h). The culture was kept at 30°C in a shaker at 200 rpm, and cell growth was monitored by absorbance measured periodically until it reached a stable maximum value, representing the stationary phase.

3.5 Dry Weight

After cell growth as described in the above item 3.4, at steady state, the cells centrifuged for 25 minutes at 3500 rpm and the precipitate obtained dissolved in a minimum of distilled water, procedure twice repeated. The biomass was suspended in distilled water to give a final cell concentration, two ml of the suspension were placed in a dry pot and already weighed (in duplicate), drying it in an oven at 60 ° C and weighing the crucible with cells until constant weight. It calculated the concentration (g / L) suspension, dividing the cell mass (mg) obtained from the known volume (2 ml). This same slurry used for the calculation of the cell concentration, more dilutions were made for approximately 5 absorbance readings (DO660nm) between 0.1 and 0.5 corresponding to its respective reading each cell concentration.

3.6 Characterization of Benzene

3.6.1 Definition of Great Wavelength

To confirm the optimum wavelength for the measurement of benzene in spectrophotometer (Shimadzu UV-1650 PC) made up scan at a wavelength in the UV region (200-305nm). The results identified the peak of light absorption aromatic hydrocarbon to obtain a calibration curve.

3.6.2 Calibration curve for Benzene

The known diluted solutions of benzene made up of corresponding absorbance reading at the optimum wavelength determined. The values obtained were plotted absorbance versus the concentration of benzene to yield the aromatic hydrocarbon calibration curve.

3.7 Biodegradation experiment of Benzene

After cell growth have reached the stationary phase as described in section 3.4, the cells centrifuged for 25 minutes at 3500 rpm. Biomass dissolved in a small volume of distilled water and measured the resulting cell suspension DO660nm. With the aid of dry weight the calculations were carried out to know which volume (ml) of this cell concentrate must be added in flasks (250 ml) containing 100 ml of the detection means to obtain a cell concentration 1g / L in the experiments biodegradation. At the start of the biodegradability test obtained at the time it is added a certain amount of benzene stock solution so that the concentration thereof in the inoculated medium was already 1g / L. Was removed, then the first aliquot (about 5 ml) on the point 0h and the vials were placed at 30 ^{o} C under constant agitation 200 rpm. Aliquots were withdrawn periodically and analyzed immediately after filtration through a Millipore membrane 0, 45mm.Cada cell-free aliquot had its absorbance measured at the optimum wavelength for the predetermined benzene, to quantify the concentration of this compound over the calibration curve obtained in item 3.6.2. This procedure was performed until the end of test, in which degradation was observed, which was reached in 90 minutes, for a period of 150 minutes experiment. We used a vial free from cells *B. subtilis* ATCC 6051 as a control containing the same amounts of middle benzene, under the same conditions of time and temperature, to verify loss of the hydrocarbon by evaporation. The biodegradation experiments were performed in duplicate (Figure 1).

3.8 System validation with kefir grains

After the degradation, the samples were filtered through 0.45 µ membrane and then added 1 g of brown sugar and 1 g of kefir grains to 100 ml of medium contained in a bottle type Erlenmeyer. The controls were divided into positive and negative, the positive control being free of benzene residue being in ideal conditions for the development of kefir grains, and the negative control was added 100 mg of benzene. Subsequently the jars were placed in a temperature-controlled oven at 25 ° C, are realized growth readings of kefir grains after 24 hours and 48 hours (Figure 1).

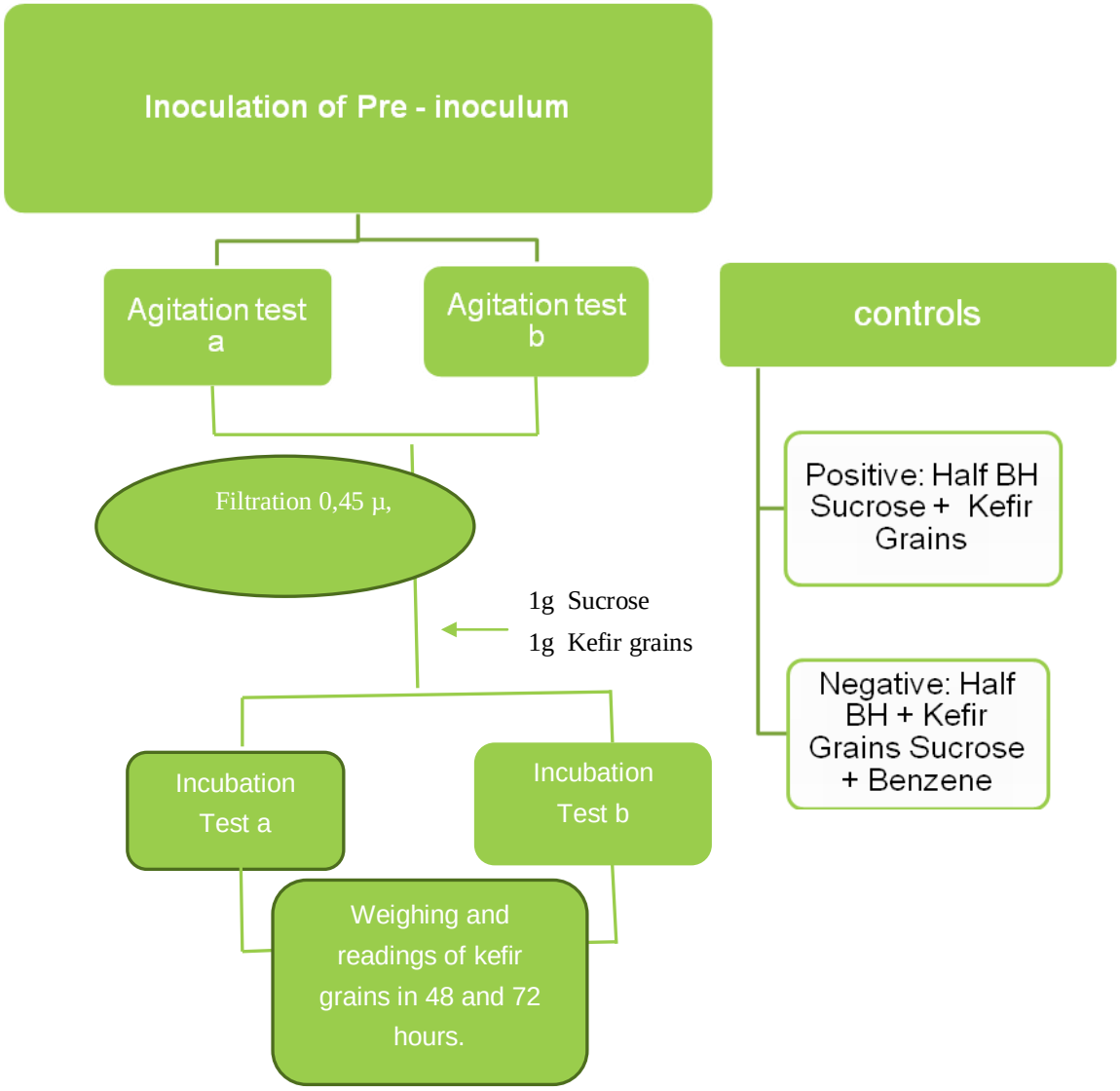

Figure 1: Flow chart of the system validation test with kefir grains.

4. Results and Discussion

4.1 Determining the wavelength Great for benzene

It is known that compounds having aromatic ring absorb light in the ultraviolet region. To set the value of the optimum wavelength of benzene, ie, where the light absorption is maximal made whether a scan spectrum in the UV region (200-305 nm). The values obtained allowed to build the following graph (Figure 2).

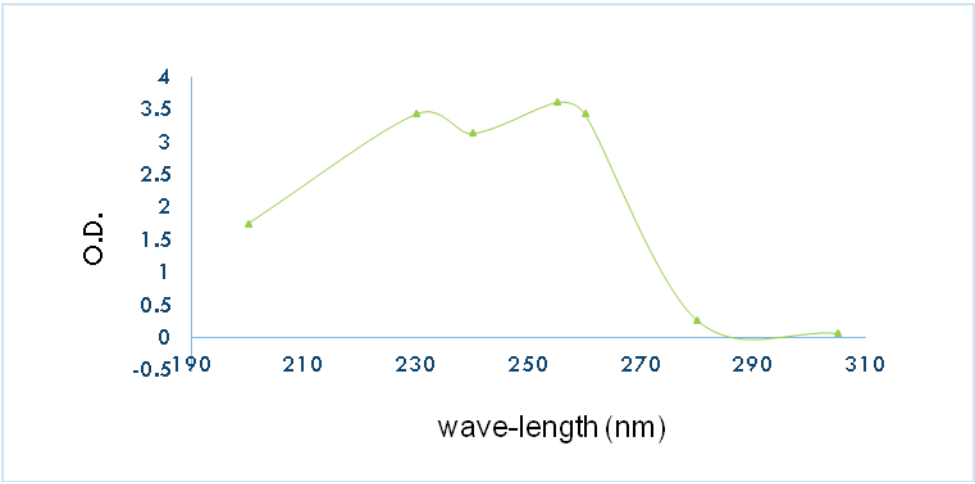

Figure 2. Benzene scan spectrum for the UV region.

For the chart it can be seen that at 255nm has the largest absorbance, thereby defining the optimum wavelength for benzene.

4.2 Calibration curve for benzene

Some dilutions and their respective absorbance at 255nm were performed to construct the Figure 3, in which a good linear correlation was observed (R2 = 0.9986) and a direct relationship between the concentration of benzene (g / L) and the optical density read.

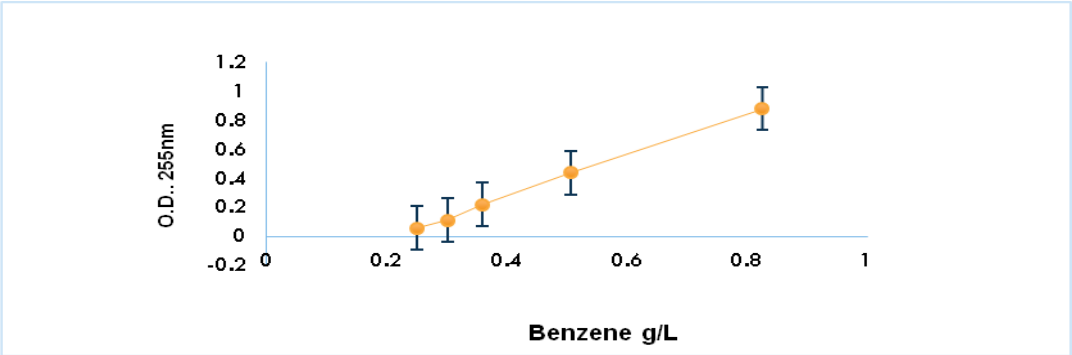

Figure 3. Calibration curves for benzene in the detecting means.

With the equation obtained straight:

$$\textbf{O.D. } 255_{nm} \textbf{ = (1,4468x [Benzene] - 0,308)}$$

It was possible to determine the concentration of Benzene (g / L) in the detecting means, measuring the absorbance of the filtered sample of optimum wavelength.

4.3 Correlation between absorbance and concentration Mobile

Some dilutions were performed in order to correlate the cell concentration to absorbance at 660nm as shown in Figure 4.

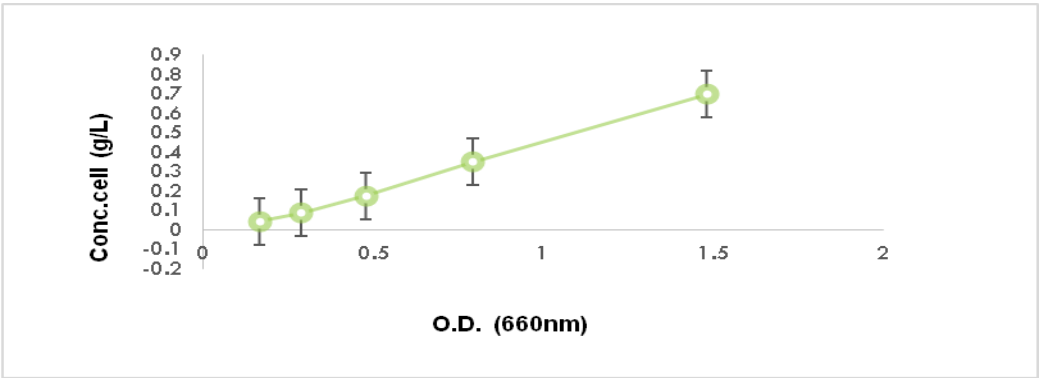

Figure 4. Correlation between absorbance measured at 660nm and cell concentration *Bacillus subtilis* ATCC 6051.

With the data obtained gave the following line equation with a good linear correlation (R2 = 0.9986):

$$\textbf{[Cell] = (0,5058x O.D. }_{660nm} \textbf{ – 0,0535)}$$

This allowed calculation of a correlation cell concentration (g / L) used in all subsequent experiments

4.4 Determination of Growth Curve

Having the correlation between cell concentration and optical density (item 4.3), went up to characterize the growth profile of the strain ATCC 6051. In Figure 5, there is the beginning of the exponential phase in about 2 hours, whereas, to achieve characterized the phase stabilization of growth (stationary phase), it took over 20h.

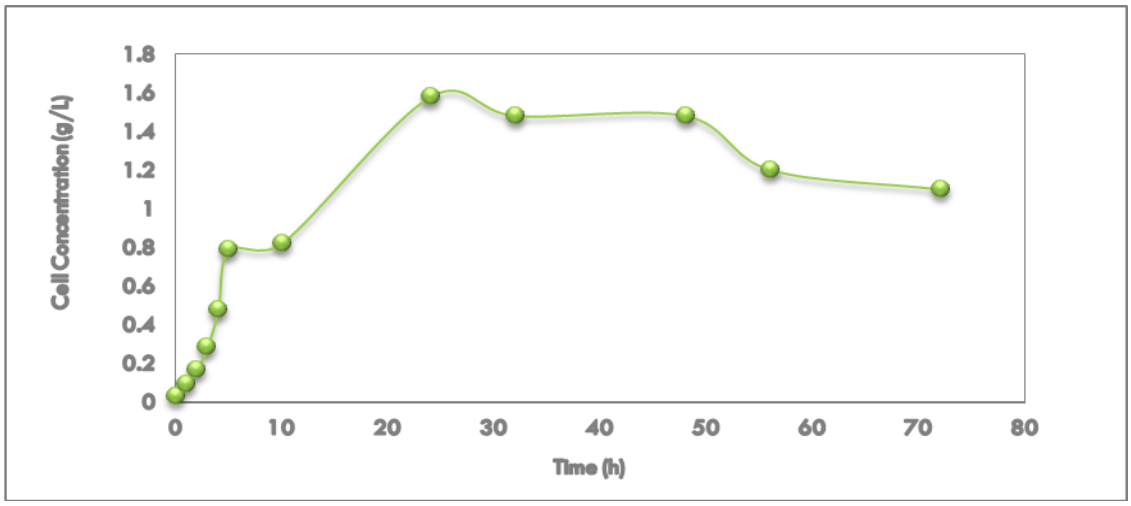

Figure 5. Bacillus growth curve subtilis ATCC 6051 amid TSB 30OC and 200rpm.

Through this curve, it is known how long the cells need to enter the exponential and stationary phase, this information is extremely important to carry out further tests. The benzene biodegradation experiment, for example, the cells used were in the stationary phase of growth.

4.5 Biodegradation tests

The benzene biodegradation experiment (C = 100mg / L), the cell concentration 1g / L, was done in duplicate and compared to a control cell absent. Aromatic was periodically quantitated spectrophotometrically at 255nm and the concentration was calculated from the calibration curve previously performed (section 4.2).

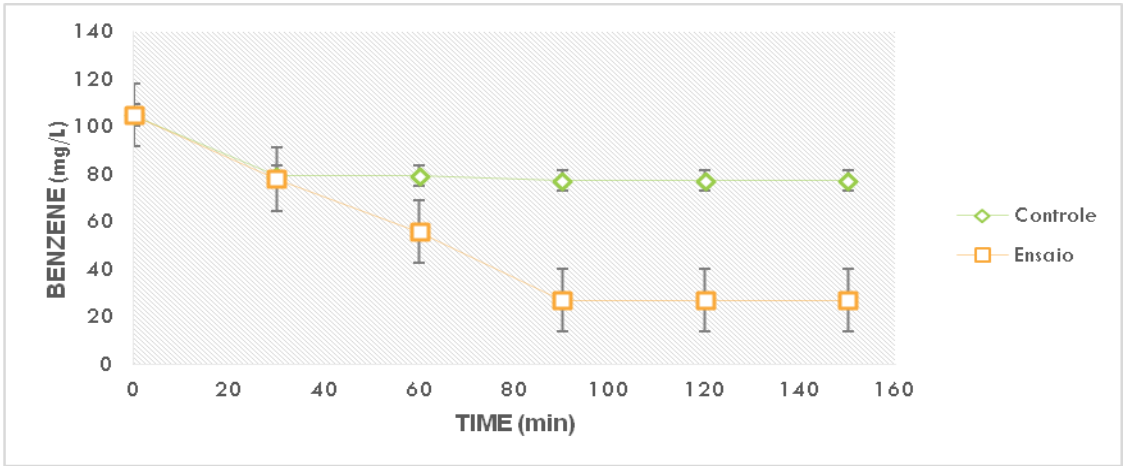

Figure 6. Benzene biodegradation kinetics by *Bacillus subtilis* to 280C and 200 RPM.

Figure 5 shows 30 minutes were required to start the process of biodegradation under the conditions tested. This range explains the time required for the production of enzymes responsible for the degradation and / or diffusion of the benzene through the cell membrane. After this time, it is remarkable a decrease in the concentration of benzene and a stabilization of this 27 mg / L after 90 minutes of test, resulting in effective removal of 48%. Such a biodegradation value was close to that obtained by *Bacillus vietnamiensis* UFRGS62 (50%) in 72 hours of testing. (Morales et al., 2008).

The results of the experiment and hydrocarbon removal percentage are shown in Table 1.

Table 1 - Biodegradation results after 150 minutes test

	Benzene (mg/L)	% Degradation
Test	27	74,2
Control	74,4	26,2

The initial decrease of benzene concentration, observed in the control, was 26.2%. The decrease of benzene was due to partial volatilization of the molecule from the vials remained in constant agitation during the whole test period, discounting portion of such percentage removal of the assay (74.2%), obtains an actual removal of 48% benzene in less than 2 hours, showing thus a potential of *B. subtilis* ATCC 6051 strain to degrade benzene.

4.6 System Validation with kefir grains

After the incubation period of kefir grains, so its size increased by 55% and 105% in 72 hours at 25 ^{o}C.

5. Conclusions

✓ The maximum absorption of benzene confirmed at 255nm. At this wavelength, there was obtained Benzene calibration curve DO255nm = (1,4468x [Benzene] - 0.308) (R2 = 0.9986) in the concentration range of 0 to 0.879 g / l.

✓ The growth profile of *Bacillus subtilis* ATCC 6051 strain was characterized by the start of the exponential phase of approximately 2h and stabilization of it (stationary phase) for more than 20h.

✓ Benzene biodegradation experiment, the cell concentration 1g / L, demonstrated the potential for degradation of Bacillus subtilis, since 48% Benzene was removed in 90 minutes.

✓ The decontamination of water confirmed with the development of kefir grains, about 2-3 times greater than the initial concentration, and used as a biological indicator of the developed system.

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