# Influence of nanoparticles on the solventogenesis of bacteria *Clostridium beijerinckii* IMV B-7806, *Clostridium acetobutylicum* IMV B-7807

**Serhii Skrotskyi, Serhiy Voychuk, Liudmyla Khomenko, Olga Vasyliuk, Valentyn Pidgorskyi**

*D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Science of Ukraine, Kyiv, Ukraine*

**Keywords:**

Bacteria
Clostridium
Biobutanol
Nanoparticles

**Article history:**

Received 10.10.2018
Received in revised form 19.12.2018
Accepted 28.03.2019

**Corresponding author:**

Serhii Skrotskyi
E-mail:
skro.science@gmail.com

**DOI:** 10.24263/2304-974X-2019-8-1-12

## Abstract

**Introduction.** Given the fact that nanoparticles (NPs) have long been used by the nature, the problem of use of alternative human-designed NPs for achievement of desired biological or medical effects remains relevant for today. In the field of biotechnology NPs can appear as catalysts of biochemical processes, as well as protectors (cryo-, osmo-, etc.), sorbents of toxic metabolites, conductors and mediators, signaling molecules, etc.

**Materials and methods**. The influence of nanoparticles of metals (iron oxides, cerium, silver, gold and gadolinium) on the biosynthesis of butanol by the strains of acetone-butyl bacteria *Clostridium beijerinckii* IMV B-7806 and *C. acetobutylicum* IMV B-7807 was studied.

**Results and discussion.** Synthesis of main products of ABE-fermentation (acetone, butanol and ethanol) was affected in presence of NPs in the culture medium. It was shown that for the strain *Clostridium beijerinckii* IMV B-7806, all studied NPs suppressed the synthesis of butanol compared to control. In case of presence of silver oxide NPs during butanol synthesis by strain *C. acetobutylicum* IMV B-7807 there was observed a tendency ($p \leq 0.16$) for increase of butanol yield from 9.0 ± 0.6 g/L in control up to 11.1 ± 1.8 g/L and 11.1 ± 1.1 g/L in the presence of 0.1 mkM and 10 mkM NPs in medium respectively. The nature of changes depended on type of NPs and their concentration. The optimal concentrations of the studied NPs were estimated. Also assumptions on possible mechanisms of the NPs` effect on the ABE fermentation process were formulated. The regulatory potential of the NPs for the coordination of the ABE-fermentation processes and synthesis of fatty acids has been studied in order to increase the yield of the target product.

**Conclusion.** The effect of the NPs on the synthesis of organic solvents by acetone-butyl bacteria is strain-specific and determined by the growth properties of bacteria and by the functioning of specific enzyme systems as well.

## Introduction

More and more attention has been paid to the biological synthesis of chemicals, which expands the capabilities of industrial microbiology by reducing production costs, and this respectively allows to get high profits. Therefore there exist constantly increasing interest in obtaining bioobutanol, bioethanol, and other biofuels through microbial conversion processes of sugars and various starch-containing substances [1–4].

The main problem in obtaining biofuels is the toxicity of end products for microorganisms themselves This significantly limits the potential of microbial synthesis [3]. Today, different approaches are used to solve this problem and to intensify the process of ABE fermentation by using more stable and overactive strains of bacterial producers, obtained by methods of genetic engineering, as well as by optimizing production through using more advanced systems of accumulation and discharge of end products, etc [3, 4]. One of the most promising areas of modern science is the study of properties of nanoparticles (NPs) and nanomaterials (NMs). NPs and NMs can not only influence certain individual biological processes, but simultaneously change both the biological properties of the organism (microorganism) and also properties of the external environment, and thereby create new conditions of existence [5–8]. As a result of this living organisms acquire new features - resistance to pressure of stress factors, intensification of physiological and biochemical functions, and so on. The unique properties of NPs are increasingly used in various spheres of life - medicine, technology, biology, agro-industrial complex and other areas [6-11]. Nanoparticles (NPs) are part of the nature itself, since many of them are natural compounds and perform certain biological functions in cells. This indicates the promise of the use of alternate human-designed NPs to obtain the desired effect [7]. There are more and more applications of nanoparticles in biology and medicine: direct use of them in “as is” form, for example as antimicrobial agents [8]; as selective indicator molecules for some pathogenic microorganisms [9]; sorbents for immobilization of enzymes [10], as delivering agents for diagnostic and therapeutic substances [11, 12]. For medical use nowadays there already exist biodegradable nanoparticles synthesized from albumin [13], polyalkylcyanoacrylate [14], polylactategluclate [15], and solid lipids [16]. However, in recent years nanoparticles based on metal oxides are gaining more and more popularity [13,17,18]. In the field of biotechnology, NPs can act as catalysts of biochemical processes, as well as various protectors (cryo-, osmo-, etc.), sorbents of toxic metabolites, conductors and mediators, signaling molecules, etc.

Currently, an attention is only beginning to be focused on the study of the influence of nanoparticles on microorganisms of various genera and species. Progress in research related to this problem is complicated by the extremely small number of publications, the lack of a broad methodological base, the need for developing new control methods, etc [1, 2, 6, 9, 17, 18]. The structure of NPs is largely determined by the method of their production. By the spatial structure 3 main classes of NPs [5] are known: three-dimensional particles obtained by explosion of conductors, plasma synthesis, the restoration of thin films, etc .; two-dimensional objects - films obtained by methods of molecular and ionic stratification, etc .; one-dimensional objects - nanowiskers, nanotubes, nanofibers, which are obtained by the method of molecular stratification, the introduction of substances into cylindrical micropores, etc [8, 12, 13]. Also, there are nanocomposites - materials obtained by introducing NPs to any matrix. Nanoparticles of metals have different shapes and in most cases are crystallic, although some of them can be amorphous.

In this paper, we have focused on the study of the effect of NPs of metal oxides on the synthesis of butanol, which is the final product of acetone-butyl fermentation. This topic was

almost not studied, despite heightened interest of scientists around the world in the synthesis of biofuels. There is only small number of publications on this subject [18].

The aim of research was to analyze the potential use of metal nanoparticles for intensification of ABE fermentation processes of the acetone-butyl bacteria to increase the synthesis of butanol.

## Materials and methods

Bacteria *Clostridium beijerinckii* strain IMV B-7806 and *C. acetobutylicum* strain IMV B-7807 that are deposited in the Depository of the D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine were used in the study.

**Nanoparticles.** Magnetic nanoparticles were prepared by the method [10] and were kindly given for the study by Dr. Pud O. A. from the Institute of Bioorganic Chemistry of NAS of Ukraine. Other nanoparticles were kindly presented by Dr. Zholobak N.M. from the D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine.

**Electronic microscopy.** Determination of the size of nanoparticles and their general morphology was carried out by electron microscopy. For this purpose, 5 mkl suspensions of nanoparticles were dropped on a surface of a carbon coated copper grids and dried at room temperature. After that, the nanoparticles were analyzed using a transmission electron microscope JEM-1400 (Jeol, Japan) at an accelerating voltage of 80 kV and an instrumental magnification of x50,000 - x100,000.

**Analysis of nanoparticles sizes.** The size of nanoparticles was determined from the digital images obtained by electron microscopy. Images analysis was done with a help of the image analysis software ImageJ version 1.50 (National Institutes of Health, USA).

**Study of the influence of nanoparticles on the synthesis of butanol.** Suspension of bacterial cells in the active phase of growth was obtained by cultivation on liquid thioglycolic nutrient media (Himedia) of the following composition (g/L): tryptone -15.0; yeast extract - 5,0; glucose -50.0; sodium chloride -2,5; L-cysteine - 0.5; sodium thioglycolate - 0,5; sodium rezazurin - 0,5; agar-agar - 0.75. After sterilization (1.1 atm for 15 minutes), the medium was cooled to 25 ° C and poured into test tubes. After that in order to provide anaerobic conditions of cultivation 1.5 ml of sterile vaseline oil was added in each tube. For investigation prepared nutrient media were sowed with 5% of the inoculum using a 18–24 h bacterial culture of the corresponding *Clostridium* strain.

Disperse nanoparticle systems were added to the sterile medium at a concentrations of 10.0; 1.0; 0.1 ηM. Subsequently, the contents of the test tube were thoroughly mixed with vortex (Mancor) for 30 seconds and inoculum (5%) was added. As a control we used non-inoculated tubes containing same medium with or without addition of nanoparticles. Cultivation of cells was carried out under anaerobic conditions at a temperature of 37 ° C. The content of butanol and other ABE-fermentation products was determined at the end point after 72 hours of cultivation.

**The determination of butanol content was carried out on a gas-liquid chromatography.** The quantitative determination of short-chain fatty acids was studied by gas chromatography - mass spectrometry (GC-MS) with use of GC-MS instrument Agilent

6890N / 5973inert (Agilent Technologies, USA), HP-INNOWax capillary column (30m × 0.25mm × 0.25μm) (J & W Scientific, USA). The separation was carried out with a temperature gradient of 20 ° C / min from 40 to 250 °C, using helium as gas carrier, a flow rate through a column of 1 ml / min and volume of injection - 0.2 μl. As an internal standard, isovalerylate acid was used. The identification of short-chain fatty fatty acids was performed using libraries of NIST02 mass spectra and standard short chain fatty acid solutions (Sigma-Aldrich, USA).

**Statistical analysis** was performed with the help of Microsoft Excel and STATISTICA version 10 (StatSoft, Inc. 2011) software packages. The significance of difference between the mean values was determined with *t*-test and was considered reliable at $p \leq 0.05$. A two-way ANOVA was used to evaluate the influence of different factors on the studied parameters.

## Results and discussion

Much attention is paid to the study of the interaction of microorganisms with metal ions, due to their key role in various biotechnological and natural processes. Analysis of the nanoparticles that we used in the study with electron microscopy showed that the smallest size had gold nanoparticles that ranged from 4 nm to 13 nm (Table 1, Figure 1).

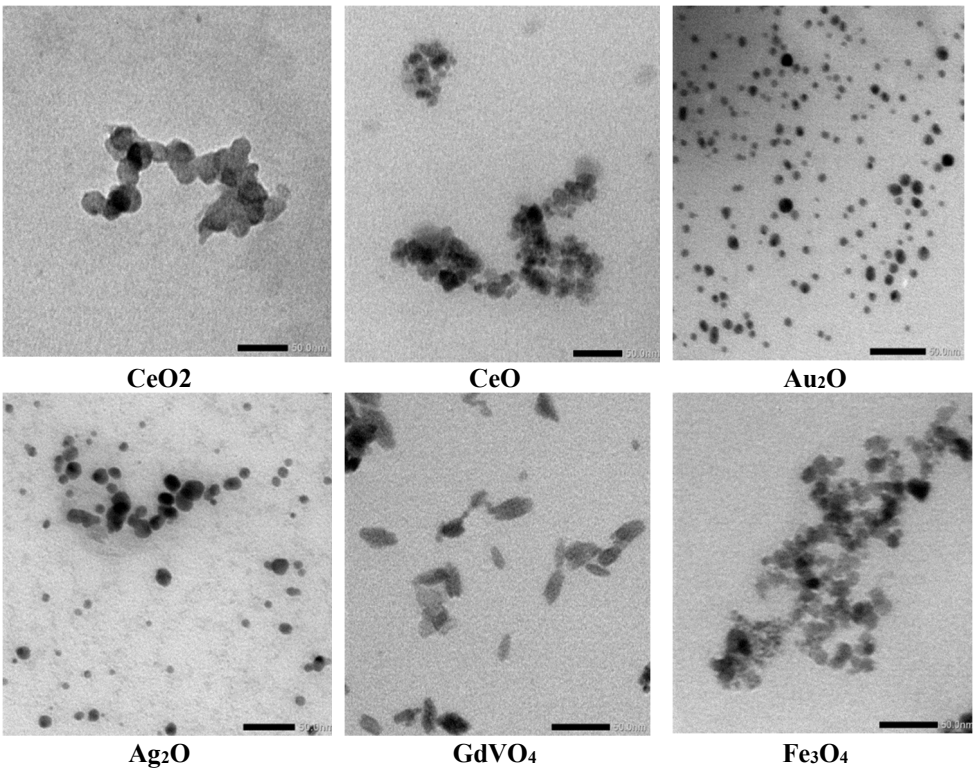


**Figure 1. Electron microscopy of the nanoparticles that were used in the study. Bar is 50 nm. Magnification ×80 000**

**Table 1**

**Nanoparticles sizes (in *nm*)**

| Nanoparticle | Mean ± Sd | Maximum | Minimum |
|---|---|---|---|
| $CeO_2$ | 22.6±1.5 | 24.6 | 20.4 |
| CeO | 13.7±5.6 | 22.3 | 7.4 |
| $Ag_2O$ | 13.2±5.3 | 22.1 | 5.6 |
| $Au_2O$ | 7.6±3.1 | 13.9 | 4.0 |
| $GdVO_4$ | 26.6±6.2 | 35.3 | 18.5 |
| $Fe_3O_4$ | 12.3±5.2 | 20.8 | 5.1 |

The addition of nanoparticles (NPs) to the nutritional medium affected the synthesis of butanol which respectively altered the growth rates of bacteria and largely depended from NPs toxicity level. In particular for the strain *Clostridium beijerinckii* IMV B-7806 all investigated NPs inhibited the synthesis of butanol compared to control (tab.2). The effect of NPs had strain-specific and/or species-specific character. Also for the strain *C. beijerinckii* IMV B-7806 there was observed a general decrease in the formation of butanol in the presence of all the studied NPs. Significant inhibition of the final product synthesis was detected in the presence of iron and cerium oxides. Other NPs also have shown a tendency to suppress the butanol synthesis in this strain. The intensity of effect depended on the concentration of NPs in the culture medium. With the increase in NPs concentration, the effect of butanol synthesis inhibition was more expressed and approached to statistically significant. The opposite trend was observed for the butanol yield obtained during cultivation in presence of different NPs for the other strain - *C. acetobutylicum* IMV B-7807 (tab. 2). The presence of iron and cerium nanoparticles in the medium in general produced stimulating effect on the synthesis of butanol in this strain.

**Table 2**

**Synthesis of butanol in *Clostridium* strains under the influence of nanoparticles**

| **Nanoparticle** | **Butanol, g/L** | | ***p*** |
|---|---|---|---|
| | **Mean** | **Sd** | |
| ***C. beijerinckii* B-7806** | | | |
| Control | 10.4 | 1.8 | - |
| $Fe_3O_4$ | 8.3 | 0.8 | **0.020** |
| $GdVO_4$ | 9.3 | 0.9 | 0.193 |
| CeO | 8.1 | 0.8 | **0.011** |
| $CeO_2$ | 8.9 | 1.1 | 0.094 |
| $Ag_2O$ | 8.7 | 1.3 | 0.052 |
| $Au_2O$ | 9.2 | 0.5 | 0.158 |
| ***C. acetobutylicum* B-7807** | | | |
| Control | 9.0 | 0.6 | - |
| $Fe_3O_4$ | 9.4 | 0.8 | 0.644 |
| $GdVO_4$ | 8.8 | 1.2 | 0.803 |
| CeO | 8.5 | 0.9 | 0.582 |
| $CeO_2$ | 9.4 | 1.2 | 0.670 |
| $Ag_2O$ | 10.4 | 1.6 | 0.155 |
| $Au_2O$ | 9.2 | 1.4 | 0.817 |
| ***C. acetobutylicum* B-7807** | | | |
| Control | 9.0 | 0.6 | - |

However, the effective use of cerium oxide (CeO2) and iron oxide resulted in a slight stimulative effect compared to control. An increase in butanol synthesis was observed up to a maximum level of 9.4 g / l.

The effect of nanoparticles depended on their concentration, and increase of NPs level in medium led to both stimulating and depressing effects on the synthesis of organic solvents by studied acetone-butyl bacterial strains.

The stimulation of the butanol synthesis with iron nanoparticles produced insignificant influence on increasing of nanoparticles concentration in the culture medium, and presence of cerium oxide nanoparticles resulted in rapid increase in the butanol yield at NPs concentrations of 1 ηM and 10 ηM.Effect of gold nanoparticles in the *C. acetobutylicum* strain IMV B-7807 occurred only at a concentration of 0.1 ηM in which the yield of the synthesized butanol was 10.3 g/L, compared with 9 g/L in the control (Table 2). The maximum yield of butanol (almost 11.1 g/L) was fixed in presence of 0.1 ηM and 10.0 ηM of silver oxide NPs in the medium. In case of use of these NPs in concentration of 1.0 ηM the amount of synthesized butanol reduced to 8.9 g/L, indicating an inhibitory effect.

For the strain *C. acetobutylicum* IMV B–7807 the yield of butanol during cultivation in presence of various NPs in concentrations 0.1-10 ηM remained unchanged. In case of silver oxide NPs presence there was observed a tendency ($p \leq 0.16$) for increase of butanol yield in compare with control level 9.0 ± 0.6 g/L up to 11.1 ± 1.8 g/L and 11.1 ± 1.1 g/L in presence of NPs in concentrations 0.1 and 10 ηM respectively.

Observed effect of iron oxide NPs indicates that synthesis of organic solvents in acetone-butyl bacteria is determined not only by growth parameters but also by functioning of enzyme systems themselves independently from bacterial growth process.

The nanoparticles of iron oxide and cerium used in the study were able to increase the synthesis of butanol by strain *C. acetobutylicum* IMV B-7807 on average in 1.5 times. And for strain *C. beijerinckii* IMV B-7806, the butanol synthesis rates remained unchanged in presence of nanoparticles and in control experiments. Obtained result indicates the existence of certain strain characteristics, which are probably related to absence of some special enzyme systems. Exactly this would allow to metabolize existing organic matter with this strain and synthesize ABE-fermentation products at the sufficient level. The primary and probably the most important factor is the presence of a set of certain enzymes or enzyme systems, that is the basis of specific features of one or another strain. The presence of a complete set of enzyme systems is a key factor that allows the full use of the metabolic potential of bacterial cells, while the presence of NPs is a secondary factor that can be either a mediator, or a catalyst for these processes, or perform other functions whose characteristics require separate scientific researches.

The fermentation time in strain *C. beijerinckii* IMV B-7806 decreases almost twice in the presence of iron oxide, and does not change in the presence of other NPs. In the strain *C. acetobutylicum* IMV B-7807 the total fermentation time on the contrary increases almost twice in the presence of iron, cerium and gold NPs. No significant correlation dependencies have been detected between formation of the final product andstart time of gas formation or the fermentation duration time.

Therefore, the effect of NPs was shown to be strain-specific. Efficacy of NPs action is concentration-dependent. Observed decrease of the butanol yield in the strain *C. beijerinckii* B-7806 and its insignificant induction in the presence of silver oxide in strain *C.acetobutylicum* IMV B-7807 have no dependence on the basic parameters of fermentation (start time of gas formation, duration of fermentation), and therefore is probably determined by other factors.

**Results analysis**

The possibility of widespread introduction of nanoparticles in various aspects of human life points to the high potential of these structures, in particular in the field of biotechnological production of liquid fuels, mainly through stimulation of fermentation processes. The study of the influence of these nanoparticles on the biosynthesis of butanol with clostridia was carried out for the first time. Despite the lack of literature data on the interaction of these nanoparticles with clostridia, our data suggest the prospective and feasibility of use of some nanoparticles to enhance the release of target products of ABE-fermentation. However, it is obvious that for other biotechnological processes, the use of these nanoparticles can also be effective.

Kim and co-authors used nanoparticles to increase the ethanol yield by *Clostridium ljungdahlii*. The fermentation of *C. ljungdahlii* with nanosized silicon dioxide more effectively increased the mass transfer. The concentrations of dissolved CO, CO2 and H2 increased by 272.9%, 200.2% and 156.1% respectively. Production of ethanol and acetic acid increased by 166.1% and 29.1%, correspondingly, as well [1].

Biomass, ethanol and acetic acid yield increased by 227.6%, 213.5% and 59.6%, respectively, in the presence of CoFe2O4 SiO2-CH3 nanoparticles [2].

While there are studies on the antimicrobial effects of nanoparticles on bacteria of the genus *Clostridium* [6].

The results obtained by us indicate the perspectivity and necessity of use of certain nanoparticles to increase the yield of the target product in ABE-fermentation, but it is obvious that use of NPs can be also effective for other biotechnological processes. Taking into account our previous studies and the results obtained at this stage, we noted that in order to establish the potential for the influence of the NPs on the synthesis of organic solvents in the ABE-fermentation, the effect of these NPs on the metabolic or physiological parameters of bacterial growth can be taken as the basis.In most cases it was established that the most effective in increasing the synthesis of butanol were NPs that effectively influenced (stimulated) growth processes and showed a positive influence on the economic and metabolic coefficients [18]. An example of such NP was the oxides of iron and cerium. However, the ability of silver oxide (at low concentrations) to stimulate the synthesis of butanol by cells of acetonobutyl bacteria of the strain *Clostridium acetobutylicum* IMV B-7807 indicates that, in addition to physiological and biochemical parameters, the nature of the stimulating and suppressing effects of the NPs should also be taken into account. Those NPs which are able to exhibit bacteriostatic effects (inhibiting growth processes) but at the same time do not inhibit the biochemical pathways for synthesis of target products may be more suitable for use in the biotechnological production of butanol. Such assumption requires additional experimental verification [1, 2, 18].

## Conclusion

The NPs can directly or indirectly participate in certain enzymatic reactions and act as catalysts, mediators, etc. Role of NPs which they play in ABE fermentation process requires further investigations, which would allow to derive a theory explaining the biological effect of the NPs in ABE fermentation process. At current stage due to the lack of special studies, we can only confirm the presence of biological effect itself. However, the obtained result, namely – an increase of biobutanol yield in the presence of NPs of iron oxide and cerium, as well as its relative repeatability for different strains of acetone-butyl bacteria, allows the

recommendation of these NPs for further study at pilot plants with purpose of introduction into the biotechnological process. This would allow not only to receive organic solvents (the main products of ABE-fermentation - acetone, butanol and ethanol), but also to use the waste biomass for extraction of lipid fraction with further transformation into biodiesel.

**Acknowledgements**. Research was carried out within the project (DR No 0115U004278) of the targeted complex multidisciplinary program of scientific researches of Ukraine "Development of scientific principles of rational use of natural resources and sustainable development. Scientific basis for the rational use of natural resources and waste management problems".

## References

1. Kim Y.K., Park S.E., Lee H., Yun J.Y. (2014), Enhancement of bioethanol production in syngas fermentation with *Clostridium ljungdahlii* using nanoparticles, *Bioresour. Technol*., 159, pp. 446-450, DOI:10.1016/j.biortech.2014.03.046
2. Kim Y.K., Lee H. (2016), Use of magnetic nanoparticles to enhance bioethanol production in syngas fermentation, *Bioresource Technology*, 204, pp. 139–144, DOI: 10.1016/j.biortech.2016.01.001
3. Jones D.T., Woods D. (1986), Acetone-butanol fermentation revisted, *Microbiology Reviews,* 50, pp. 484–524.
4. Papoutsakis E.T. (2008), Engineering solventogenic clostridia, *J. Current opinion in biotechnology,* 19, pp. 420–429
5. Andrusishina I. (2011), Nanochastitsy metallov: sposoby polucheniia, fiziko-khimicheskie svoistva, metody issledovaniia i otsenka toksichnosti, Suchasni problemi toksikologiï, 3, pp. 5–14.
6. Lee W.T., Wu Y.N., Chen Y.H., Wu S.R., Shih T.M., Li T.J., Yang L.X., Yeh C.S., Tsai P.J., Shieh D.B. (2017), Octahedron iron oxide nanocrystals prohibited *Clostridium difficile* spore germination and attenuated local and systemic inflammation, *Scientific Reports,* 7(1), 8124, DOI: 10.1038/s41598-017-08387-y.4.
7. Zinchenko A. (2014), Nanochastitsy kak transportery diagnosticheskikh i terapevticheskikh substants. Mikrobnye biotekhnologii: fundamentalnye i prikladnye aspekty: sbornik nauchnykh trudov, Belaruskaia navuka, Minsk, 6. pp. 36–50
8. Antonovskaia L.I., Skorb E.V., Sviridov D.V., Beliasova N.A. (2008), Antimikrobnaia aktivnost plenochnykh dioksid-titanovykh fotokatalizatorov po otnosheniiu k grampolozhitelnym i gramotritsatelnym bakteriiam, *Doklady NAN Belarusi*., 52(2), pp. 83–87
9. Poleshchuk N.N, Astashonok A.N, Rubanik L.V., Zhavnerko G.K., (2012), Nanotekhnologicheskie podkhody k izucheniiu strukturnoi organizatsii i detektsii Chlamydia trachomatis, *Zdravookhranenie*, 11. pp. 33–38.
10. Beresnev A. I.,. Kvach S. V, Zinchenko A. I. (2014), Immobilizatsiia bakterialnykh nukleozidfosforilaz na magnitnye nanochastitsy, *Mikrobnye biotekhnologii: fundamentalnye i prikladnye aspekty. Sb. Nauchnykh trudov,* 6. pp. 26–36.
11. Parveen S., Misra R., Sahoo S. K. (2012), Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging, *Nanomedicine,* 8(2), pp. 147–166, DOI:10.1016/j.nano.2011.05.016

12. Marty J.J., Oppenheim R.C., Speiser P. (1978), Nanoparticles – a new colloidal drug delivery system, *Pharm. Acta Helv.,* 53, pp.17–23.
13. Couvreur P., Kante B., Roland M., Guiot P., Bauduin P., Speiser P. (1979), Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties, *J. Pharm. Pharmacol,* 31(5), pp. 331–332.
14. Gurny R., Peppas N.A., Harrington D.D., Banker G.S. (1981), Development of Biodegradable and Injectable Latices for Controlled Release of Potent *Drugs, Drug Development and Industrial Pharmacy*, 7(1), pp. 1–25. DOI: 10.3109/03639048109055684.
15. Siekmann B, Westesen K., (1992), Submicron-sized parenteral carrier systems based on solid lipids, *Pharm Pharmacol Lett*., 1, pp. 123–126.
16. Rao C. N. R., Ramakrishna Matte H. S. S., Voggu Rakesh and Govindaraj A. (2012), Recent progress in the synthesis of inorganic nanoparticles, *Dalton Trans.,* 41, pp. 5089–5120.
17. Mongkolkajit J., Pullsirisombat J., Prichanont S., Tantayakom V., Prasitchoke P., Phisalaphong M. (2010), 2 γ-Alumina Doped Alginate Gel for Cell Immobilization in Fermentation Processes, *AJChE*, 10(1). pp. 1–7.
18. Durán-Padilla V.R., Davila-Vazquez G., Chávez-Vela N.A., Tinoco-Valencia J.R., Jáuregui-Rincón J. (2014), Iron effect on the fermentative metabolism of Clostridium acetobutylicum ATCC 824 using cheese whey as substrate, Biofuel Research Journal, 4, pp. 129–133, DOI: 10.18331/BRJ2015.1.4.5