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Chapter 10

Heterogeneous Composites on the Basis of Microbial Cells and Nanostructured Carbonized Sorbents

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Additional information is available at the end of the chapter

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1. Introduction

The fact that microorganisms prefer to grow on liquid/solid phase surfaces rather than in the surrounding aqueous phase was noticed long time ago [1]. Virtually any surface – animal, mineral, or vegetable – is a subject for microbial colonization and subsequent biofilm formation. It would be adequate to name just a few notorious examples on microbial colonization of contact lenses, ship hulls, petroleum pipelines, rocks in streams and all kinds of biomedical implants. The propensity of microorganisms to become surface-bound is so profound and ubiquitous that it vindicates the advantages for attached forms over their free-ranging counterparts [2]. Indeed, from ecological and evolutionary standpoints, for many microorganisms the surface-bound state means dwelling in nutritionally favorable, non-hostile environments [3]. Therefore, in most of natural and artificial ecosystems surface-associated microorganisms vastly outnumber organisms in suspension and often organize into complex communities with features that differ dramatically from those of free cells [4].

Initially introduced as just an imitation of Mother Nature, artificial immobilization of cells and enzymes has now transformed itself into a valuable biotechnological instrument. Its growing practical application and development over years led to appearance of fascinating novel microbial and enzymatic technologies [5-7]. Research on the immobilized biocatalysts is currently conducted in many laboratories around the world. In Japan, USA and other countries immobilized microbial cells have been successfully applied for adsorption of heavy metals from dilute solutions [8, 9], for purification of sewage [10] as well as for intensification of microbiological technologies (production of antibiotics, organic acids, sugar syrups, fermented drinks, etc.) [11]. It was shown that immobilized cells allow

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conducting biotechnological process over extended periods of time, under strict control of the process kinetics, product quality and microbial activity [12].

Immobilization of cells can be carried out mainly by two methods: by entrapment of the microorganisms into porous polymers or microcapsules or by binding to an organic or inorganic support matrix (adsorption methods). The latter is considered to be more suitable for retaining cell viability [13]. Adsorption is also one of the easiest methods of immobilization of microbial cells, especially those that adhere naturally to the surfaces of materials [14]. It should be noted here that rapid development of technology of receipt of the immobilized biocatalysts resulted in contradictory results. So, the first attempts of immobilization were related with adsorption of enzymes and cells on arboreal sawdust and coal. In these experiments, adsorption was accompanied by a considerable desorption. In this connection, regarding the simplicity and availability of adsorption immobilization it has been having a reputation like "easy come easy go". Though never forgotten, in the last decade adsorption methods of immobilization gained increasingly more interest caused by considerable expansion in assortment of carriers with outstanding absorption properties, by better understanding of mechanisms and approaches aimed on firm attachment of biocatalyst to a carrier and by development of new methods of surface conditioning [12].

The adhesion of microbial cells to surfaces is rendered mainly by Van der Waals forces, ionic and covalent interactions, with considerable contribution of various microbial exopolymers [13]. Traditionally, adsorption immobilization is regarded as consisting of several relatively distinct stages, including a) adsorption of dissolved macromolecules on the surface; b) diffusion and concentration of cells from the bulk phase to the surface; c) reversible attachment of cells; d) biosynthesis of anchoring polymers by the cells which leads to an irreversible attachment stabilized by covalent bonds and entropy-driven interactions.

Selection of an appropriate adsorbent, especially for industrial process is based on several criteria. Most important among them are: a) material's costs and availability in large amounts; b) simplicity and efficacy of the immobilization process; c) preservation of cell viability; d) adsorbent's specific surface (capacity). There is no an ideal material so far but many these requirements are met by inorganic (sand particles, ceramics, metallic hydroxides and porous glass) and organic (charcoal, wood shavings and cellulose, polyurethanes) carriers. For example, porous glass-based fixed-bed reactors are successfully used for of the aerobic [15] and anaerobic [16] biotechnological transformations.

The immobilization process can be characterized by several parameters: initial biomass loading, retainment of biomass, strength of the adhesion, retainment of the activity of the biocatalyst, effectiveness of mass transfer, engineering realization and general operational stability. When microorganisms are immobilized by adsorption the initial cell loading of the immobilization matrix is one of the limiting factors [17]. The cell loading on the adsorbent is influenced by the physical and chemical properties of the adsorption material, of the microorganism to be immobilized and by the composition and parameters of the surrounding medium. Another critical point for a system with the cells immobilized by adsorption is the retainment of the biomass on the surface. The retainment is generally ruled by the adhesion strength, which can be described in kinetic and in thermodynamic terms.

Concerning the biocatalyst viability/activity retaining, the immobilization by adsorption is probably the gentlest existing method [14]. Because the adsorptive fixation occurs under "standard" conditions, no changes of the cultivation parameters are necessary to produce the immobilized biocatalysts. Compared to cell entrapment in organic polymers it can generally be assumed that during adsorption also the enzymatic activity can be preserved at a high level. Very often the activity of only one enzyme is responsible of the catalytic process of interest. In such a process the stability is characterized by the half-life of the enzyme. Enzymatic "half-lives" up to two years have been reported [18].

Though adsorbed biocatalyst systems are easy to run and used for many years, there is still enough space for optimization [13]. Development and probation of new types of heterogeneous composite materials, possessing advanced properties for biological catalysts, as carrier systems, as filters etc. on the basis of attached enzymes or whole microbial cells is of great importance for biotechnological processes. These and other tasks are addressed by *engineering enzymology* – a scientific and technical discipline combining principles, theoretical approaches and practical methods of chemical and enzymatic catalysis, microbiology, chemical technology and biochemistry. Recent efforts in engineering enzymology are focused (among others) on the following directions:

- development and optimization of immobilization methods leading to novel biotechnological and biomedical applications;
- search of materials satisfying strict requirements of biotechnological processes (such as non-toxicity, mechanical stability, etc.);
- construction of bio-composite materials based on individual enzymes, multi-enzyme complexes and whole cells, targeted on realization of specific industrial processes;
- development of methods for modification of surface properties aimed on fine tuning and better control of the "biocatalyst-carrier" interface

In the light of these challenges, nanostructured carbonized materials appear as an attractive substrate for designing and production of cost-effective high-performance bio-composite materials.

2. Synthesis of nanostructured carbonized materials

Adsorption properties of carbonaceous adsorbents are used in purification and recovery of valuable substances for very long time. Active carbons are used in oil processing, petroleum chemistry, wine making, butter production, etc. [19-21]. They are increasingly applied in medicine, for example, to remove toxins from physiological liquids [22]. The last years are characterized by the intensive studies on carbon nanotubes and nanostructured carbon sorbents (NCS).

There are many methods suitable for synthesis of NCS, such as electric arc discharge, laser vaporization and chemical vapor deposition techniques [23-25]. In the Institute of Combustion Problems (Almaty, Kazakhstan) following methods are used for obtaining of NCS: flame carbonization, catalytic carbonization and synthesis of carbon nanotubes by

microwave plasma enhanced chemical vapor deposition (MPECVD). It was found that the transition metals like Fe, Ni, Co, their oxides and alloys are very effective catalysts for carbon nano-structuring. Another interesting approach used was the carbonization of walnut shells, grape seeds, apricot stones, wheat bran, rice husk, etc. in presence of activating agents. The samples were carbonized according to the procedure developed in the R.M. Mansurova Laboratory of Carbon Nanomaterials at the Institute of Combustion Problems, using a gas-flow setup **(Figure 1)** within temperature range of 250–900 °C in argon flow (50–90 cm^3/min).



Figure 1. Pilot setup for flame carbonization of diverse raw plant materials.

During carbonization the major mass loss occurred within temperature range 150-500°C, where large amount of volatile and liquid products (65-75 % of total mass) were released. In the case of rice husk, the reduction of mass was found to be around 50% which is related to high content of silicon in the samples.

3. Surface structure and composition of the plant-derived carbonized sorbents

Carbon surface has a unique character. It has a porous structure which determines its high adsorption capacity; it has a chemical composition which enables numerous interactions with both polar and nonpolar molecules. Besides, it has active sites in the form of edges, dislocations and discontinuities which facilitate its chemical reactions with many compounds and functional groups.

Carbonized sorbents obtained by us on the basis of plant materials possess extended macro- and mesoporous structure, favorable for the adsorption of large molecules and cells [20, 22]. One can see in the **Table 1** that the specific surface (S_{sp}) and size of pores increased proportionally to the carbonization temperature up to 700 °C. However, further increase of temperature caused decrease of these parameters due to the increase of the density of the samples as reported also by Banerjee and coworkers [26].

Raw Material	T, °C	Size, μm			S_{sp}, m^2/g
		Macropores	Mesopores	Micropores	
Walnut Shells	300	25	12	1.8	250
	500	30	13	2.3	770
	600	30	16	2.4	780
	700	30	16	2.3	800
	800	28	14	1.7	830
	850	29	15	2.4	800
Grape Stones	300	18	12	3	200
	600	22	14	6	500
	700	27	15	7	530
	800	25	13	5	540
	850	26	14	6	500

Table 1. Specific surface and pore size of the samples carbonized at different temperatures

Electron microscopy images (**Figure 2**) show the meso- and macro-porous structure of the materials appeared as a result of flame carbonization. A drastic contrast is visible between the structures of the raw material and the material after temperature treatment. Interestingly, flame carbonization of the raw plant materials often led to formation of complex carbon nanostructures (**Figure 3**) of various size and morphology. Treatment at 500°C resulted in appearance of transparent thin membrane sheets of 20-40 μm size. Prolonged heating (>30 min) at 600°C cased the formed translucent films to roll into 1400 nm long tubular structures of a diameter 400-500 nm. Further increase in the carbonization temperature and duration initiated the appearance of variety of nanostructures of diverse morphologies.

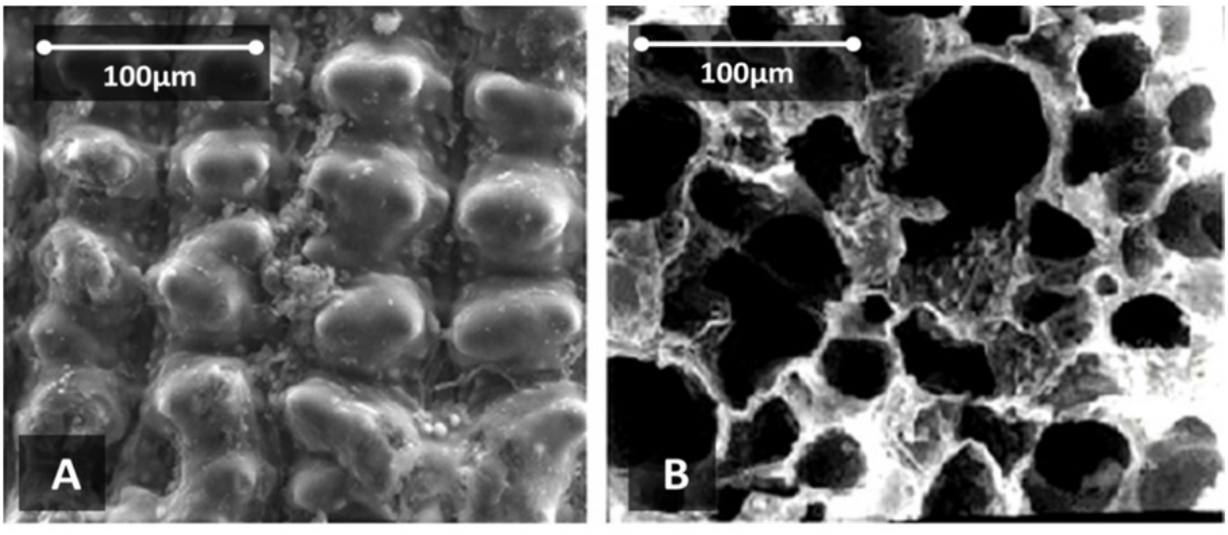


Figure 2. Electron microscopic images of rice shells in native state (A) and after carbonization at 650°C (B)

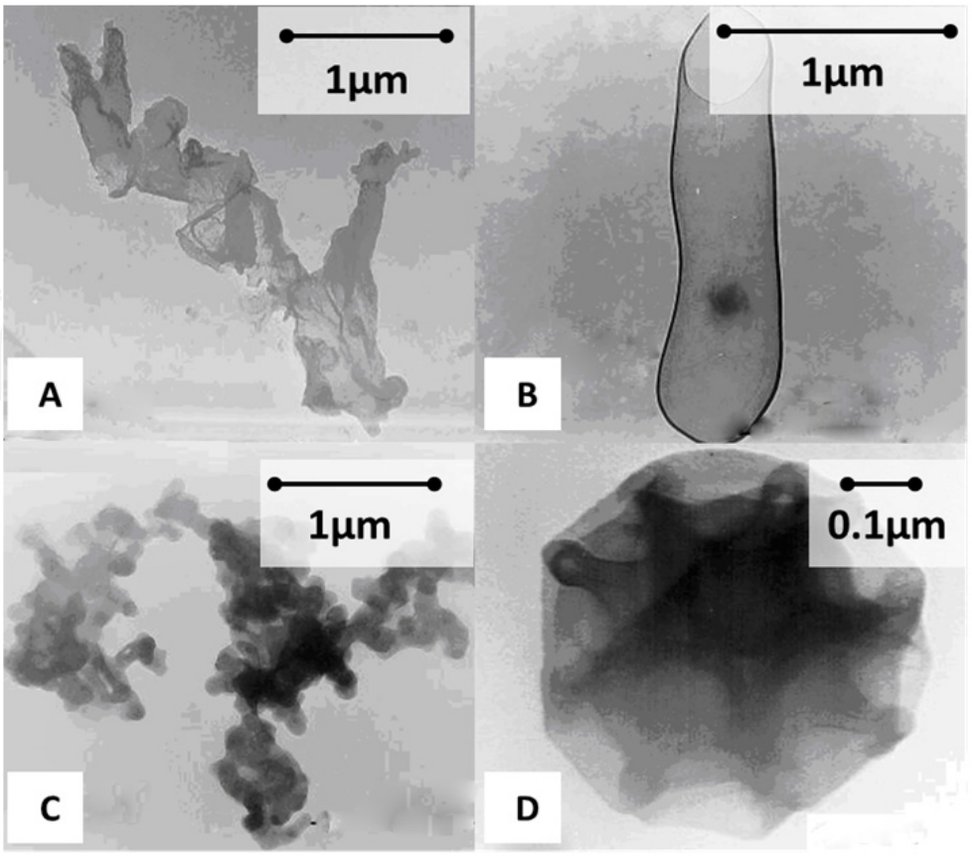


Figure 3. Diverse micro- and nanoscale structures observed with electron microscopy in apricot stones carbonized at different temperatures: (A) at 500°C; (B) at 600°C for 30 min; (C) at 700ºC; (D) at 750°C.

Together with flame carbonization, microwave plasma-enhanced chemical vapor deposition (MPECVD) is considered to be a very promising method for the carbon nanotubes synthesis due to the lower growth temperature, uniform heat distribution and the ability to control different growth parameters [27]. Carbon nanostructures having different shapes have been synthesized using this method: aligned and curly filaments, flat and coiled carbon nanosheets. We also investigated the impact of different growth parameters such as temperature, pressure and hydrogen/methane exchange rate on the morphology of the carbon nanotubes. The results showed that there is a strong dependence of the morphology of the carbon nanotubes on the experimental conditions. For example, the quality of the carbon nanotubes was greatly affected by nitrogen influx during the growth process. Moreover, the diameter of the carbon nanotubes became smaller as nitrogen concentration in the gas mixture dropped. This implies the potential way to control the diameter of the carbon nanotubes precisely. It was also found that the threshold field required for the field emission can be reduced if nitrogen gas was introduced.

Adsorption behavior of the NCSs cannot be interpreted on the basis of surface area, pore size and nanostructural features alone. Specificity, affinity and capacity of such materials are strongly determined by chemical groups on their surface. These groups mostly appear due to controlled oxidation of carbon material's surface and can be roughly classified as phenolic (hydroxyl), carbonyl, carboxyl, ester, lactone and other groups (**Figure 4**).

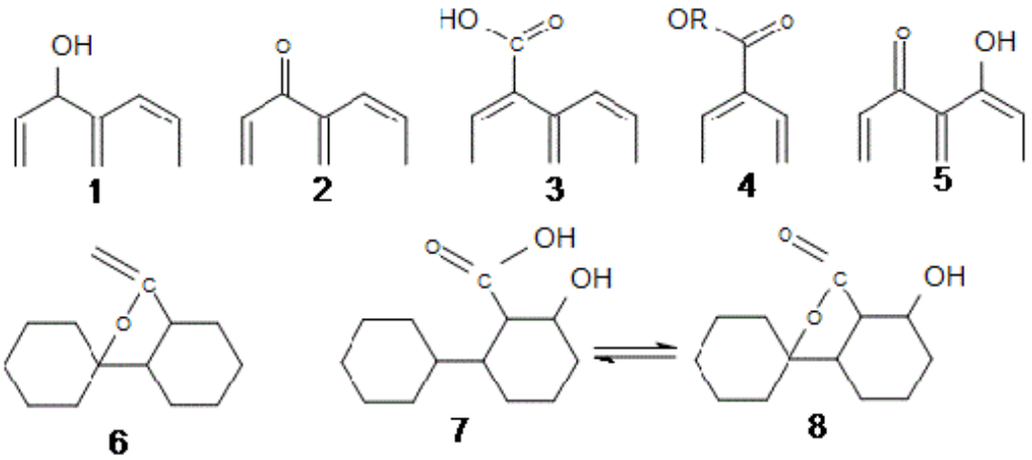


Figure 4. Major functional groups on the surface of carbonized adsorbents: 1) phenol (hydroxyl); 2) quinone (carbonyl); 3) carboxyl; 4) ester; 5) enol; 6)-8) different kinds of lactone groups.

Functional chemical groups on the NCS were analyzed by infrared spectroscopy using IR-spectrophotometer UR-20 (Zeiss Co., Germany). The IR-spectra of the native (raw) plant materials were mainly composed of characteristic absorption bands of NH_2 (3431.92 cm^{-1}), OH (3009.97 cm^{-1}), C=O (1643.25 cm^{-1}), C–O (1241.55 cm^{-1}), C–OH (1055.64-1157.28 cm^{-1}), C=C, C=N (1662.55 cm^{-1}) groups (**Figure 5a**). After carbonization at temperatures of 600–850 °C, a sharp (10-fold) drop of the intensity of characteristic bands of OH and NH groups was observed. In turn, the intensity of characteristic C–O–C bands increased. Also, bands related to CO_3^{2-} groups appeared and their intensity increased substantially with temperature rise. We observed also the bands related to CO_2 group in the region 2486.19 cm^{-1} (**Figure 5b**). Carbonization of apricot stones and rice husks proceeded similarly, but the latter displayed lower intensity of the corresponding bands.

In general, the higher was the temperature of the carbonization process the more intense were the characteristic absorption bands of the groups of NH_2, COH, C=O, OH as well as valence vibrations of CH-group in the aromatic rings. Furthermore, the IR-spectra obtained after carbonization exhibited characteristic absorption bands at 883 and 1050 cm^{-1} corresponding to C=C deformation vibrations of the aromatic ring, also those related to valence vibrations of aromatic ring 1600, 1578 and 1510 cm^{-1} as well as –C-H-vibrations at 3053 and 3030 cm^{-1}. In the sorbents carbonized at 300-850°C, the following polyaromatic hydrocarbons were identified: pyrene, (due to the presence of its characteristic absorption bands at 720 and 850 cm^{-1}); coronene: 547 and 1320 cm^{-1}; fluoranthene: 600, 740 and 820 cm^{-1}. Thus, we established the appearance of multiple polyaromatic hydrocarbons structures after carbonization. Further increase of carbonization temperature led to increase in intensity of absorption bands related to aromatic condensed systems.

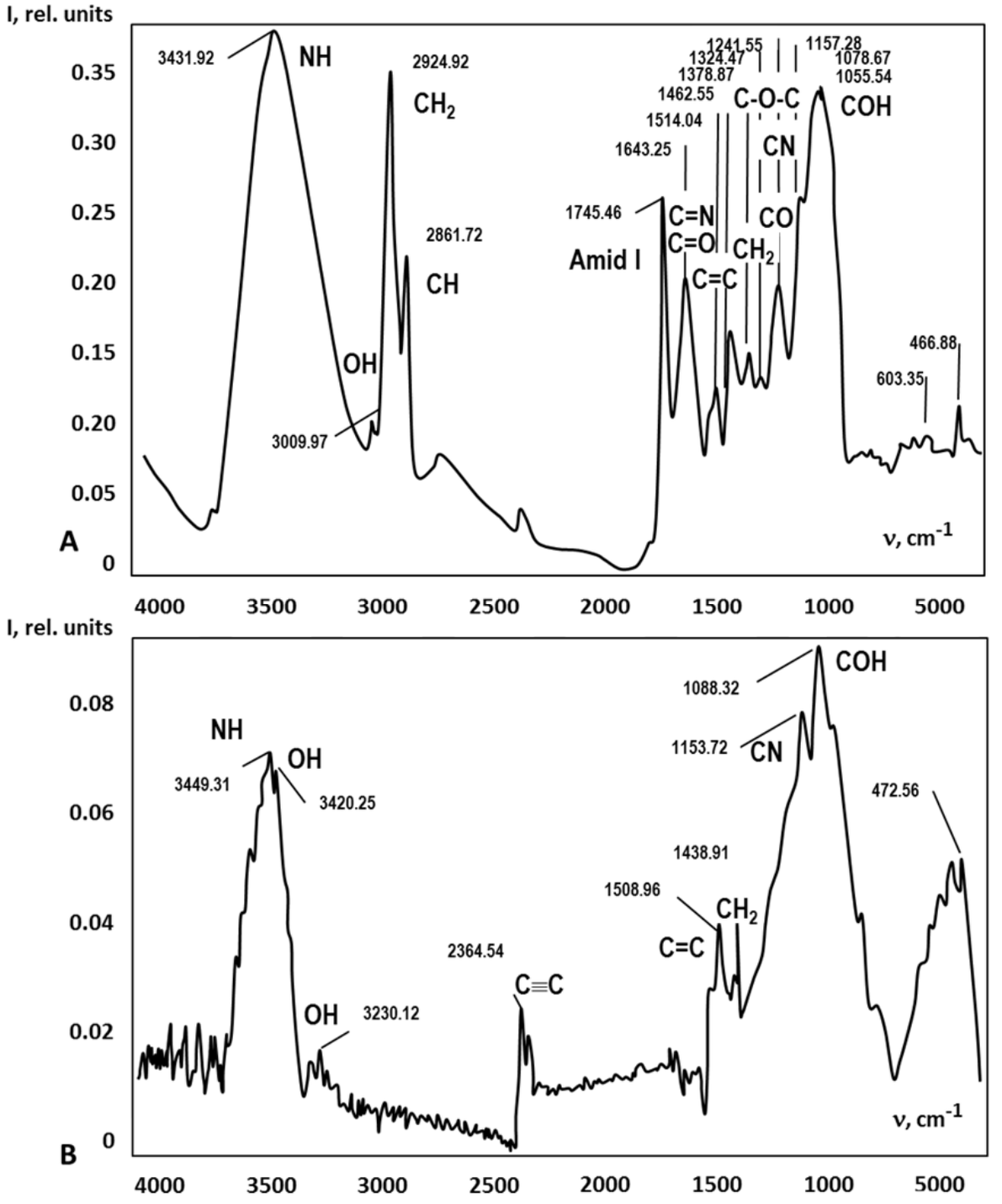


Figure 5. IR-spectra of native (non-carbonized) (A) and carbonized at 800°C (B) apricot stone surfaces.

4. Adsorption characteristics of the nanostructured carbonized materials in respect of microbial cells

Activated carbons are known as excellent adsorbents. Their applications include the adsorptive removal of color, odor, taste, undesirable organic and inorganic pollutants from drinking and waste water; air purification in inhabited spaces; purification of many chemical, and pharmaceutical products, etc. [19-21, 28]. Their use in medicine and health applications to combat certain types of bacterial ailments and for the adsorptive removal of certain toxins and poisons, and for purification of blood, becomes increasingly popular [29, 30]. Studies in last years have brought data on high adsorption ability of carbonized materials in respect of mammalian cells [31], microbial cells [32] and enzymes [33, 34].

The samples we used for microbial adsorption had been carbonized according to the procedure developed at the Laboratory of Hybrid Technologies in the Institute of Combustion Problems, Almaty, Kazakhstan. A flow set-up was used with following parameters: temperature range 650-800°C in argon flow (50-90 cm^3/min). Different temperatures and flow regimes caused alterations in the pore structure and therefore resulted in different properties of the activated carbon [35, 36]. The adsorbents obtained from plant material showed themselves as very versatile and efficient because of their extremely high surface area, multiple functional groups and the macro-pore structure which is highly suitable for bacterial adhesion.

The interaction between the cell and the adsorbing surface is dictated by multiple physicochemical variables, reviewed in many brilliant works [37-39]. Obviously, an effective attachment depends on chemical and physical properties of both adsorbent and cells. The chemical groups on the surface of the carbonized materials were mentioned in the previous section. In this respect, microbial cells demonstrate even larger versatility. Their surfaces can be hydrophilic or hydrophobic, carry positively or negatively charges; expose various specialized chemical groups and even release polymers (adhesive glycoproteins, polysaccharides, proteins, teichoic acids, etc. (**Figure 6**.).

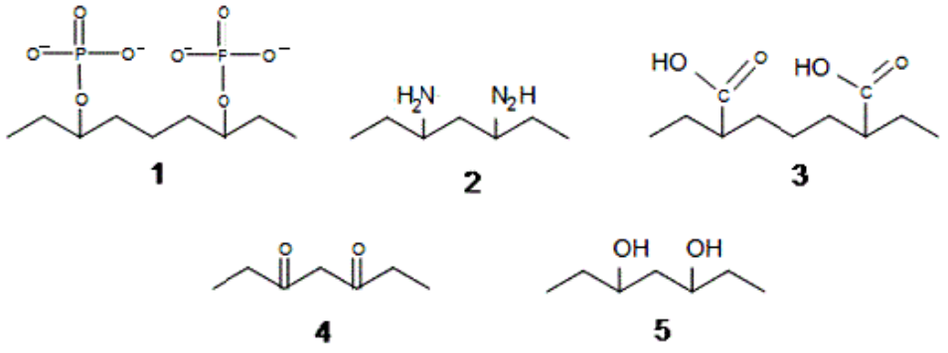


Figure 6. Principal functional groups on the surface of microbial cells: 1) phosphate; 2) amine; 3) carboxyl; 4) carbonyl; 5) hydroxyl.

Molecular biological and biochemical studies on cell adhesion focus predominantly on identification, isolation and structural analysis of attachment-responsible biological molecules and their genetic determinants. Physiological aspects of cellular adsorption

concern mainly the influence of cultivation parameters (temperature, nutrition compounds, oxygen concentration, presence of antibiotics and vitamins) on bacterial adherence-related phenotype, adhesion molecules metabolism and surface structural organization [40]. Once in initial contact with a surface, microbes develop different types of attachment behaviors. Motile attachment behavior of *P. fluorescens* allows the flagellated cells to move along surfaces in a semi-attached condition within the hydrodynamic boundary layer, independent of the flow direction [41]. Reversible adhesion of *E. coli* cells with residence times of over several minutes on a surface has been described as "near-surface swimming"[42]. In the case that microbes can no longer move perpendicularly away from the surface the term "irreversible attachment" is used [14].

A net electrostatic charge on the NCS and the cell surfaces affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the surface that results in the formation of an electric double layer. This layer consists of two parts: an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated.

Thermodynamically, spontaneous cell adsorption onto a surface results in decrease of Gibbs free energy but sometimes there is a significant energy barrier due to electrostatic repulsion. Existing theoretical models predict that there are two regions where the strongest attraction forces between two surfaces occur (the "primary" and "secondary" minima, at distances of ~0.5 nm and ~5 nm, correspondingly). Generally it is assumed that microbes adhere reversibly to the "secondary minimum" and irreversibly to the "primary minimum" with the aid of cell surface appendages that can pierce the repulsive energy barrier [12, 14].

Our previous experiments have shown that, together with electrostatic properties, the hydrophobicity of both cells and NCSs plays a crucial role in both adsorption capacity and biomass retainment on the surface. Hydrophobicity of the carbonized materials can be easily controlled by the activation of the surfaces by water steam. The nature of the exposed chemical groups enables formation of multiple covalent bonds between the surfaces (**Figure 7**). Large number of different interactions involved in the cellular attachment to the carbonized surfaces makes possible fine tuning of the immobilization process in order to achieve versatility and adaptability of the bio-composite materials for different applications.

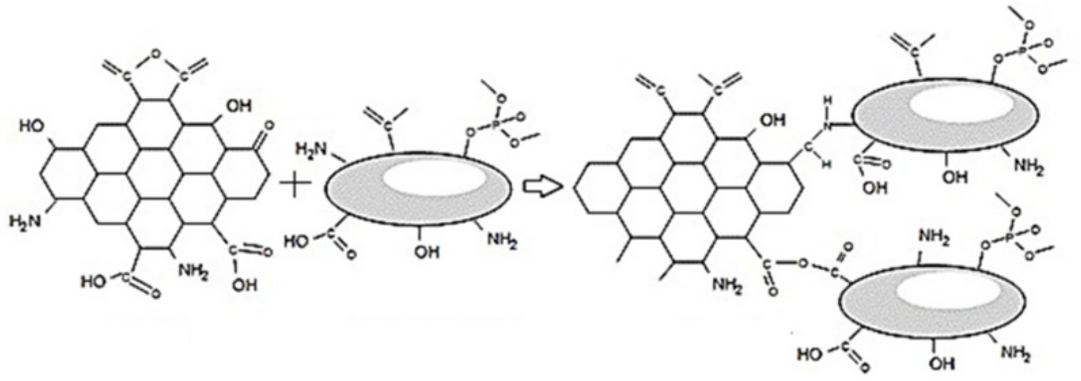


Figure 7. Formation of covalent bonds between the surfaces of microbial cells and the carbonized materials considerably contributes to stability of the bio-composite materials.

Electron microscopy examinations suggested that there is strong bonding interaction between microbial cells and the NCSs. In case of optimal incubation parameters, the cell load reaches ~62 %, corresponding to ~10^8 colony-forming units (~viable cells) per gram of NCS. The microbial cells were distributed on the surfaces not homogenously but rather formed clusters (micro-colonies). Taking into consideration potential intestinal and biomedical applications of the bio-composites, this fact is of particular importance because inter-cellular interactions and aggregation processes in the micro-colonies point out initial stages of biofilm formation, which in turn is an essential factor for bacterial survival and adaptability.

Figure 8 shows subsequent stages of rice husk colonization by Lactobacilli. It is clearly visible that the number of cells in a micro-colony varies between around 20 and 200 corresponding to the natural micro-colony structure in the epithelial layer of the intestine. The appeared bacterial colonies demonstrated almost irreversible adhesion in the absence of a competitive substrate (intestinal surface).

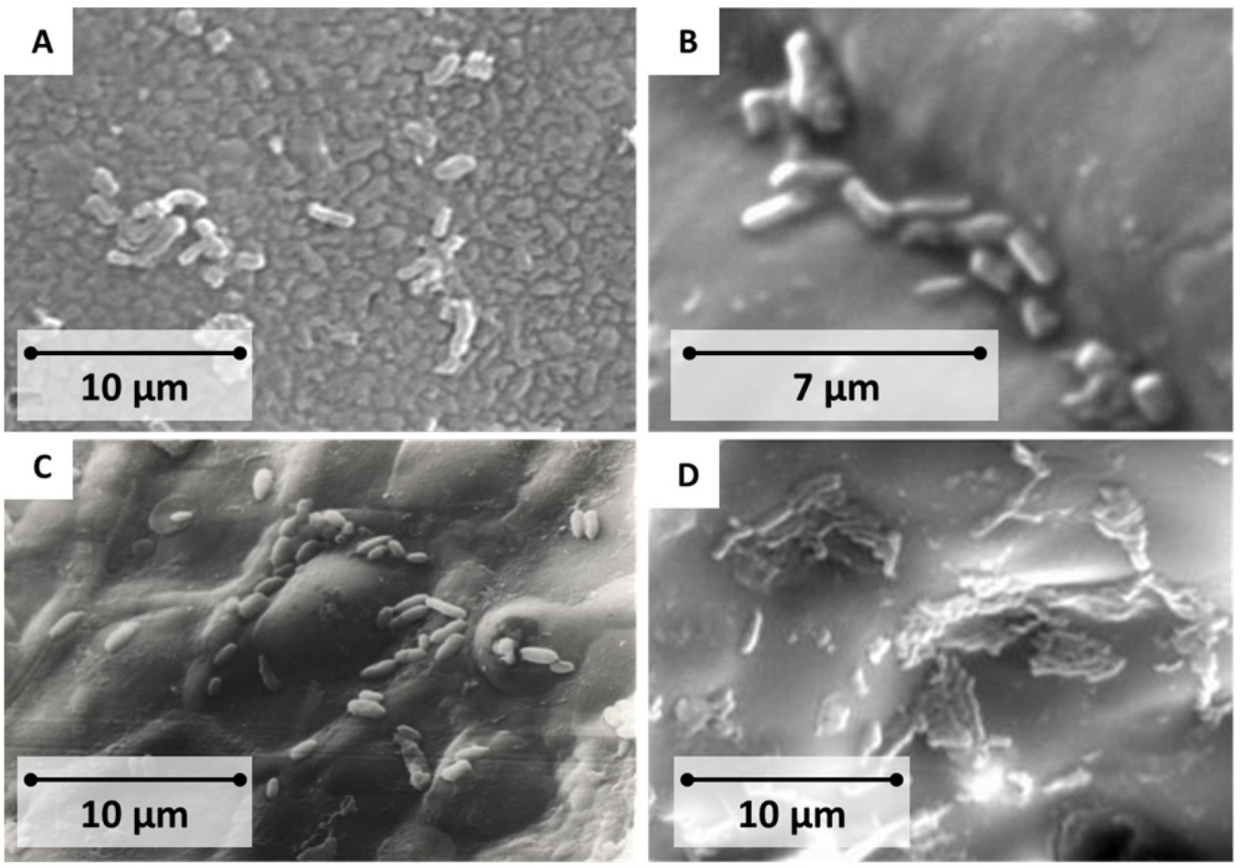


Figure 8. Subsequent stages of colonization of NCSs by Lactobacilli. A: carbonized rice husk, initial adsorption; B: carbonized grape stones, initial adsorption, C and D – carbonized grape stones, micro-colony formation

5. Performance of bio-composite carbonized materials in probiotic applications

In our model experiments in vitro, NCSs showed outstanding compatibility with many bacterial strains, indicating their high potential in miscellaneous branches of biotechnology and medicine. One of such applications of great interest is design and approbation of new generation of probiotic preparations for preventions and correction of micro-ecological disorders in gastrointestinal tract of the humans and animals. Environmentally, nutritionally and infection-induced pathologic shifts of gastrointestinal tracts' micro-ecology often lead to the increase in amount of gram negative bacteria, particularly of *Enterobacteria*. It leads to the translocation of bacterial toxic products from bowels to other organs causing development of endotoxinemia and other pathologies.

Probiotic is a viable mono- or mixed culture of beneficial microorganisms applied to animals or humans that sustainably improves properties of the indigenous microflora. The term "probiotics" was first coined by Lilley and Stillwell in 1965 [43]. R. Fuller later defined probiotics as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." [44]. Over years, the term "probiotics" has undergone several more definitions arriving at the final one, officially adopted by the International Scientific Association for Probiotics and Prebiotics, outlining the breadth and scope of probiotics as they are known today: "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" [45]. Mechanisms of probiotic action are numerous and include:

- Prevention of adhesion of pathogen to host tissues;
- Stimulation and modulation of the mucosal immune system by reducing the production of pro-inflammatory cytokines through action on NF-kB pathways;
- Improvement of intestinal barrier integrity and up-regulation of mucin production;
- Killing or inhibiting the growth of pathogens through the production of bactriocins or other products such as acids or peroxides, which are antagonistic toward pathogenic bacteria.

Over past decades, probiotics have been extensively studied for their health-promoting effects and have been successfully used to control gastro-intestinal diseases. As shown above, the mechanisms of probiotic action appear to link with colonization resistance and immune modulation. Since *Bifidobacterium* and *Lactobacillus* species belong to normal intestinal microflora of humans, majority of probiotics was created on the basis of these bacteria. Lactic acid bacteria can produce numerous antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, bacteriocins, as well as adhesion inhibitors, which strongly affect microflora.

Many probiotic preparations serve for an improvement of micro-ecological situations in bowels. Therefore, to reach the destination place, probiotic preparations have to pass through the stomach and the small intestine, which is unavoidably connected with significant reduction probiotic bacteria viability. To reduce this undesirable effect, several approaches have been suggested so far. Montalto et al. administered probiotic mix both in

capsules and in liquid form without observing statistically significant difference in bacterial survival [46]. A specially designed tube with a reservoir containing probiotics has been suggested by Çaglar et al [47] with some encouraging results. However, the search for most suitable means of delivery and dosages of probiotics continues. One of our aims in this respect was to investigate the capacity of the carbonized materials as protective media for probiotic bacteria immobilized in their pores.

6. Biological objects

The type strain of lactic acid bacteria, *Lactobacillus fermentum AK-2* was used in our probiotic studies. It possesses excellent probiotic potencies due to its high antagonistic and adhesive activities. Rice husk and grapes stones carbonized were produced in the Institute of Combustion Problems at the al-Farabi Kazakh National University in Almaty as described above. *Lactobacillus* cells were adsorbed onto the carrier for 24 hours. Unattached cells were rinsed away by the isotonic NaCl solution and the firmly attached bacteria were incubated for several more days for micro-colony formation. After that the prepared bio-composite material was examined microscopically to ensure successful settlement of bacteria.

In bacteria survival experiments, gastric conditions were modeled in vitro by using gastric juice received from clinical gastroscopy. Different preparations of *L. fermentum* in MRS-1 medium were incubated in the gastric juice for 1 hour. After that the number of viable cells was quantified.

In vivo experiments were conducted on 6-8 week old wild rats, previously subjected to an experimental dysbacteriosis induced by the antibiotic ciprofloxacin. The animals were divided into several experimental groups. The control group received only the antibiotic in therapeutic dose of 5 mg/ kg body mass; the first group, in addition, was fed with liquid suspension of *L. fermentum* AK-2; the second and the third groups received, after the induced dysbacteriosis, the same amounts of *L. fermentum* but the bacteria were immobilized on grape stones and rice husk, correspondingly.

As an indicator of the probiotic activity, the number of viable *Enterobacteria* in different parts of the rat intestine was measured. Changes in detected amounts of gram-negative *Enterobacteria* such as *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis, Enterobacter aerogenes, Salmonella typhimurium, Shigella zonnei* and *Shigella fleexneri* were considered as a measure of the antagonistic action strength of the preparations. For *Enterobacteria* quantification, suspended gut content was incubated on Petri dishes with Endo agar. The analyses were conducted for 15 days, every day starting from a day of antibiotic treatment. Finally, the amount of *Lactobacillus* cells attached to the rat intestinal epithelium was directly counted as described elsewhere [48].

7. Results

Twenty four hours after immobilization *Lactobacillus* displayed very good growth rate and began forming micro-colonies on the NCS. The data on gastric juice resistance of suspended and immobilized preparations of *Lactobacillus* are shown in **Table 2.**

Experimental group	Concentration of viable cells, ml^{-1}	
	Before treatment	After treatment
Suspended culture	3.7×10^9	5.2×10^5
Grape stone-based bio-composite	1.6×10^9	3.1×10^6
Rice husk-based bio-composite	1.1×10^9	8.2×10^7

Table 2. Influence of in-vitro gastric juice treatment on viability of suspended and immobilized cells of Lactobacillus fermentum AK-2

In the suspended *Lactobacillus* culture after the gastric juice treatment the concentration of living cells decreased more than 7000 times. In contrast to that, the cells being a part of the bio-composite materials showed significantly (~500 times) better survival rate. The obtained data strongly suggested the protective action of NCSs on the immobilized *Lactobacillus* cells. These results look very encouraging in respect of construction of highly efficient bio-composite materials having extended probiotic activities.

The next series of experiments was devoted to comparative analysis of the antagonistic activity of suspended and immobilized probiotic preparations. After induced dysbacteriosis, intestinal microflora of rats was observed for the period of 15 days. The data are presented in the **Table 3**.

Experimental group	Number of bacteria in 1g			
	Large intestine		Small intestine	
	wall	lumen	wall	lumen
Before treatment with ciprofloxacin				
The control	$(3.0\pm0.3) \times 10^4$	$(6.9\pm0.7) \times 10^6$	$(1.1\pm0.2)\times 10^2$	$(1.5\pm0.4)\times 10^3$
1 day after treatment with ciprofloxacin				
Without probiotics	$(2.9\pm0.5) \times 10^5$	$(7.9\pm0.6) \times 10^7$	$(2.9\pm0.3) \times 10^4$	$(1.2\pm0.4) \times 10^5$
Probiotics on GS*	$(9.7\pm0.6) \times 10^4$	$(1.2\pm0.3) \times 10^7$	$(7.8\pm0.8) \times 10^2$	$(8.9\pm0.2) \times 10^3$
Probiotics on RH**	$(6.9\pm0.4) \times 10^4$	$(9.5\pm0.2) \times 10^6$	$(0.9\pm0.3) \times 10^2$	$(5.3\pm0.8) \times 10^3$
15 days after treatment with ciprofloxacin				
Without probiotics	$(2.4\pm0.4) \times 10^5$	$(2.1\pm0.3) \times 10^8$	$(8.4\pm0.2) \times 10^4$	$(9.5\pm0.8) \times 10^5$
Probiotics on GS*	$(1.2\pm0.7) \times 10^5$	$(9.6\pm0.6) \times 10^7$	$(2.9\pm0.4) \times 10^3$	$(2.8\pm0.4) \times 10^4$
Probiotics on RH**	$(3.4\pm0.4) \times 10^4$	$(8.5\pm0.7) \times 10^6$	$(1.2\pm0.3) \times 10^3$	$(1.2\pm0.7) \times 10^3$

*GS: grape stone based carbonized adsorbent, **RH: rice husk-based carbonized adsorbent.

Table 3. Influence of the probiotic bio-composites containing L. fermentum AK-2 on the quantity of Enterobacteria in the intestine of rats after ciprofloxacin-induced dysbacteriosis.

The data in the table show that after ciprofloxacin-induced dysbacteriosis, significant increase (~2 orders) of the undesirable *Enterobacteria*-group microflora was observed, manifesting even more during the following 15 days after the antibiotic administration. This occurred both in the gut lumen and in its walls. Application of probiotics in the bio-composite forms, using carbonized rice husk and carbonized grape stones led to significant

suppression in *Enterobacteria* proliferation and spread. Being immobilized on NCS, probiotic bacteria effectively inhibited growth of unhealthy bacterial forms, this counteracting development of dysbacteriosis. The measured inhibitory effects were much higher than those shown by suspended probiotic preparation. This effect can have been brought by different mechanisms, including better survival of probiotic bacteria (as was demonstrated above), by their increased antagonistic metabolic activity, and possibly also by exchange of the bacteria adsorbed on NCS and the bacteria attached to the intestinal cell walls.

The possibility that some exchange between the cells adsorbed in different locations indeed could take place has been demonstrated by counting of lactic acid bacteria cells attached on the surface of intestinal epithelium after NCS-adsorbed *Lactobacillus* cells were brought in contact with cultured intestinal cells (**Figure 9**).

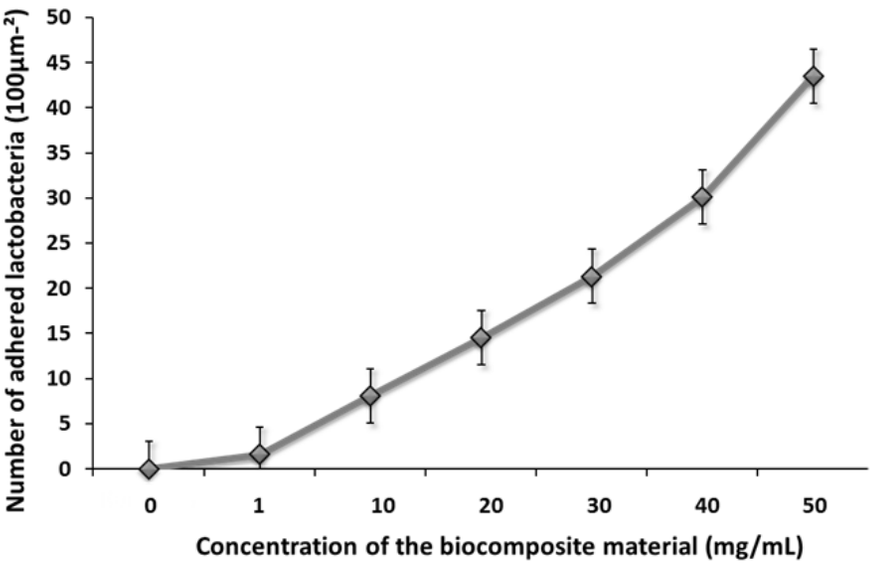


Figure 9. Colonization of cultured intestinal epithelial cells by Lactobacillus initially immobilized on the carbonized rice husk as a function of the applied bio-composite material.

As shown in the Figure 9, increase in concentration of the applied bio-composite material resulted in corresponding rise in the number of *Lactobacillus* cells firmly attached to the intestinal epithelium. Most active adhesion of probiotic cells occurs within the first two hours of incubation. Our calculations also showed that around 50% of the cells detached from the carbonized adsorbent later strongly attach to the surface of the epithelial cells. Since attachment to the recipient's cells is one of the most important indicators of a probiotic preparation activity, the obtained data suggest that the created bio-composite probiotic preparations successfully performed their functions.

We suppose that all the above-mentioned mechanisms (better survival, shifts in physiological activity, etc.) could contribute to the increased activity of immobilized probiotic strains. Our previous data suggest that the immobilization of *Lactobacillus* on carbonized materials (rice husk, grape stones) increased their physiological activity and the quantity of the antibacterial metabolites by 25-60%, which consequently would lead

to increase of the antagonistic activity of *Lactobacillus*. High in-vitro and in-vivo efficiency of the immobilized probiotics can be also ascribed by the specific micro-environmental physicochemical conditions on the interface "sorbent/microbe" [14]. Moreover, besides delivery of bacteria in intestine the NCSs can possibly contribute to detoxification by absorption of intestinal toxins by the active sites on the surface not occupied by microbial cells. All these considerations suggest synergistic summation of multiple beneficial effects.

The collected evidences clearly demonstrate that the use of the nano-structured carbonized sorbents as delivery vehicles for the oral administration of probiotic microorganisms has a very big potential for improving functionality, safety and stability of probiotic preparations. A novel probiotic preparation named "Riso-Lact" has been recently developed at the al-Farabi Kazakh National University. The preparation consists of the *Lactobacillus fermentum AK-2* cells immobilized on rice husk under well-defined optimized laboratory conditions and will possibly find its application in treatment of dysbacteriosis in humans and animals. The great binding strength and capacity of the material in respect of cells and dissolved compounds are mainly conditioned by the extended network of nanotubes but appears also due to high hydrophobicity of the surface. Although many more studies and tests are necessary, and a lot of work needs yet to be done, we can now envision the creation of a new generation of NCS-based probiotic preparations, effectively normalizing the intestinal microflora, bringing relief to millions of patients around the world.

8. Future prospects for biomedical and environmental engineering applications

In the finalizing part of this chapter we would like to give a short overview of possible further applications of the bio-composite materials based of nanostructured carbonized adsorbents. Without any doubt, the field of future applications of such materials is vast and hardly foreseeable. The topics presented bellow will underline mostly biomedical and environmental applications where certain preliminary experimental material has been collected by our and other working groups.

8.1. Bio-composite material on the basis of carbonized rice husk and micro-algae *Spirulina*

As we have shown above, NCSs with immobilized *Lactobacillus* cells have a good potential as future probiotic preparations. One of key elements of their probiotic action is the involvement of the immobilized cells into the physiological processes on the surface of the intestinal epithelium. The NCS themselves also are able to adsorb significant amounts of gram-negative cells and their toxins, thus contributing to the beneficial effects of the preparations.

Together with known probiotic strains, we have recently attempted to apply the approaches previously had been probed on *Lactobacillus*, on micro-algae *Spirulina* in order to check its

behavior as a component of bio-composite materials. *Spirulina platensis* is a blue-green alga (photosynthesizing cyanobacterium) having diverse biological activities. Due to high content of highly valuable proteins, indispensable amino acids, vitamins, beta-carotene and other pigments, mineral substances, indispensable fatty acids and polysaccharides, *Spirulina* has been found suitable for use as bioactive additive [49]. *Spirulina* produces an immune-stimulating effect by enhancing the resistance of humans, mammals, chickens and fish to infections, has capacity of influencing hemopoiesis, stimulating the production of antibodies and cytokines [50]. Moreover, *Spirulina* preparations are regarded as functional products contributing to the preservation of the resident intestinal microflora, especially lactic acid bacilli and bifidobacteria, and to a decrease in the level of undesirable microorganisms like *Candida albicans* [51].

In our experiments, both NCSs and *Spirulina* cells (the strain CALU-532m) applied alone to cultured rat epithelial cells, (IEC-6) at concentrations 5-50 μg/mL stimulated their proliferation and regeneration (**Figure 10**). Remarkably, being combined together in a bio-composite material, they showed a distinct synergy in their action, seemingly enhancing each other's activities.

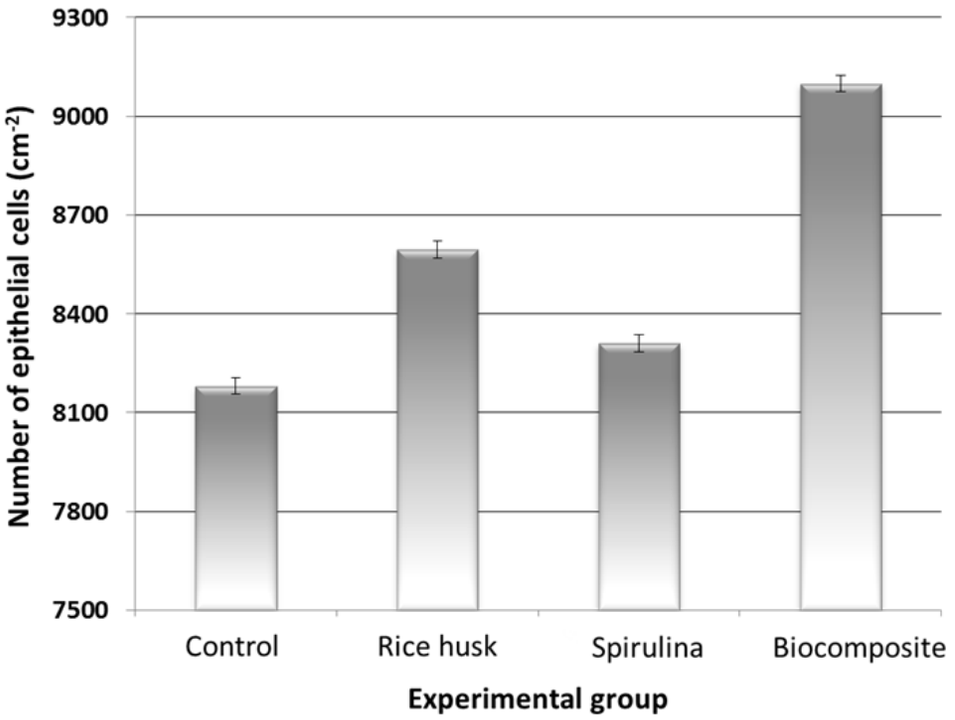


Figure 10. Stimulation of growth of cultured intestinal epithelial rat cells by carbonized materials (rice husk) and Spirulina platensis cells applied alone and in combination.

The epithelial cells treated by the NCS-Spirulina bio-composite reached confluence after 30 hours of incubation, whereas the control group required 48 h. The number of viable cells per square centimeter was found to be 9100±4.6 (SD) and 8180±3.5(SD), respectively.

8.2. Nanostructured carbonized materials for treatment of chronic wounds and sores

Problem of intractable wounds is one of persisting challenges in current clinical practice. In spite of modern antibiotics, hormonal and anti-inflammatory drugs, some chronic wounds and sores resist any treatment for weeks and months. The problem is especially serious in case of diabetic patients. The contributing factors are well-known and include high acidic wound environment, high concentration of bacteria (typically over ~10^5 cells per gram tissue) and their toxins, continuous production of inflammatory cytokines, products of tissue necrosis and high osmotic pressure.

All chronic wounds are colonized by bacteria. The delayed closure for many chronic and acute wounds is associated with high levels of bacteria in the wounded tissues. Even at lower levels bacteria hinder wound healing due to toxin secretion either directly from viable cells (exotoxins) or as a result of cell lysis (endotoxins). These toxins tend to cause local necrosis and disrupt the delicate balance of critical mediators such as cytokines and proteases necessary for healing progression. The swelling of the surrounding tissues (edema) often causes disturbances in oxygen delivery, which in turn leads to gangrenous and secondary necrotic processes. Therefore, control and removal of toxins and bacteria should be considered as key elements of a successful wound-healing therapy.

Taking these circumstances into account, it becomes obvious that chemical (pharmacological) treatment alone is rather inefficient in treatment of such wounds. A common topical agent used to combat bacterial burden in chronic wounds is disperse silver [52]. Recent comparative evaluation of various therapeutic methods has shown that the application of carbonized adsorbing materials to the necrosis zones is even more efficient: it lowers intoxication, stabilizes the blood level of glucose, improves indices of immunological reactivity, promotes a more dynamic course of a wound process, and reduces the time of treatment [53]. Some possible factors contributing to the efficiency of the NCS in wound healing are presented in **Figure 11**.

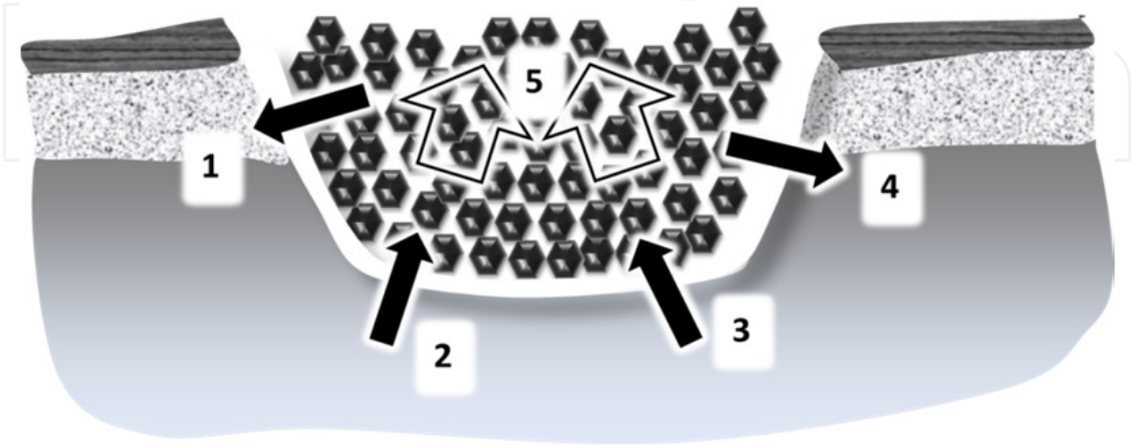


Figure 11. Hypothetical mechanisms of beneficial effects of NCS in wound healing processes: 1) stimulation of tissue regeneration; 2) binding of microorganisms and their toxins; 3) adsorption of inflammation factors and products of necrosis; 4) direct antimicrobial action; 5) capillary drainage.

Our studies have demonstrated that the use of NCS indeed can develop into an outstanding method for stimulation of wound healing. We produced in rats infected injuries, which healing typically occurs in 10-12 days. In the case the NCS were applied directly after the injury, an improvement and acceleration in wound healing was systematically observed (**Figure 12**).



Figure 12. Dynamics of infected wound healing in rats. A: Nanostructured carbonized sorbents were applied after injury. B: Wound healing in the control group.

Multiple data obtained on rats with different levels of bacterial contamination suggest that NCSs may offer multiple specific advantages in topical wound management through their high adsorption ability in respect of both gram-positive and gram-negative bacteria as well as bacterial toxins. High adsorbing activity in respect of bacterial lipopolysaccharides has been measured by our group in a model studies. The other possible mechanism of beneficial action of the NCS, such as stimulation of tissue regeneration and binding of inflammation mediators, are yet need to be studied.

8.3. NCS in bioremediation

Bioremediation is the use of microorganisms, their structures and their metabolic pathways to remove pollutants. Bioremediation is the most promising and cost effective technology widely used nowadays to clean up both soils and wastewaters containing organic or inorganic contaminants [9]. Discharge of pollutant-containing wastes has led to destruction of many agricultural lands and water bodies. Utilization of various microbes and their products to adsorb, transform and inactivate pollutants enhances the efficiency of the environment decontamination significantly. For bioremediation purposes, microbial cells (bacteria, fungi, algae, etc.) can be applied alone or in combination with some adsorbent, which can greatly enhance the viability and activity of the biological component. Such bio-composite sorbent, unlike mono-functional ion exchange resins, contains variety of functional sites including carboxyl, imidazole, sulphydryl, amino, phosphate, sulfate, thioether, phenol, carbonyl, amide and hydroxyl moieties.

Compared to "classical" sorbents, the bio-sorbents are cheaper, more effective alternatives for the removal of metallic elements, especially heavy metals from aqueous solution. Therefore, the bio-composite sorbents are increasingly widely used for heavy pollutants removal. This is now a field of intensive investigations focusing on microbial cellular structure, biosorption performance, material pretreatment, modification, regeneration/reuse, modeling of biosorption (isotherm and kinetic models), the development of novel bio-sorbents, their evaluation, potential application and future. A potent supportive discipline in bioremediation studies is molecular biotechnology, capable to elucidate the mechanisms at molecular level and to construct engineered organisms with higher biosorption capacity and selectivity.

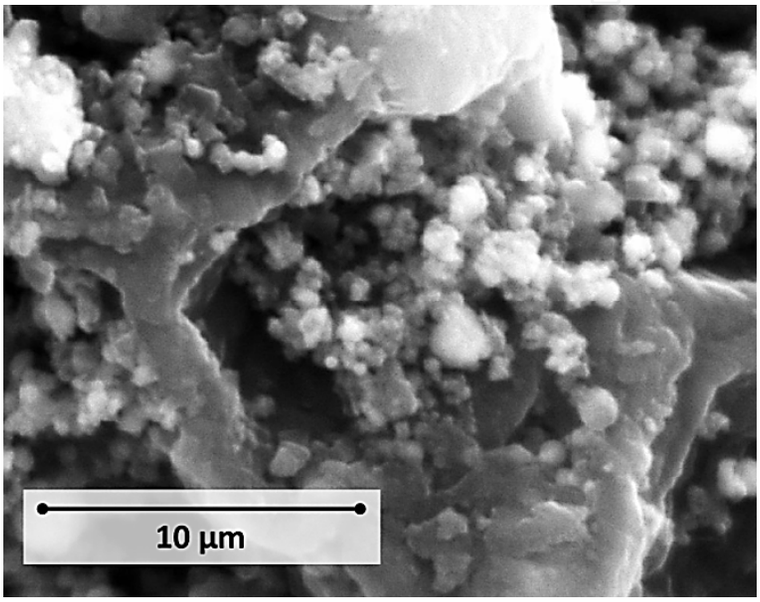


Figure 13. Electron microphotograph of the heterogeneous bio-composite bioremediation material created on the basis of NCS and bacterial cells Pseudomonas aeruginosa.

Due to their remarkable properties, nanostructured carbon materials such as carbonized grape stones and rice husk can be used as sorbents for extraction of toxic and radioactive elements. Current joint research conducted at the al-Farabi Kazakh National University (Microbiology Dept. of the Biology Faculty together with the Institute of Combustion problems) is aimed to create cost-effective and sustainable bio-composite materials on the basis of microbial cells adsorbed NCS of plant origin. Electron microscopy observations confirmed that multiple bioremediation-valuable cells can successfully attach, survive and proliferate inside the porous network of the NCS (**Figure 13**). The resulting heterogeneous biological composite materials possess outstanding pollutant-binding and transforming properties accompanied by high specificity, depending on the particular microbial strain used. In our model experiments, the obtained materials specifically adsorbed up to 95% metals from solutions. For cleaning of oil-polluted soils, we are currently developing a heterogeneous composite on the basis of carbonized sorbent and an immobilized microbial consortium consisting of bacterial strains with high oil-oxidizing activity. First encouraging results were obtained in the field experiments on oil-polluted soils.

Summarizing, we would like to emphasize that further studies and better understanding of the interactions between CNS and microbial cells are necessary. The future use of living cells as biocatalysts, especially in the environmental field, needs more systematic investigations of the microbial adsorption phenomenon. For this purpose it is necessary to develop and expand interdisciplinary collaboration networks connecting biologists, chemists, physicists and biochemical engineers.

This newly gained interdisciplinary knowledge could significantly stimulate development of novel immobilized bio-catalysts possessing high activity, selectivity and stability. Taking into account the wide spectrum of abilities of microorganisms and carbonized surfaces carriers, this ambitious mission does not look like an impossible one. Undoubtedly, in the coming years we will see the expanding of the application spheres of CNS-based heterogeneous composite biomaterials.

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