International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:7, No:7, 2013

Performance of Bio-Composite Carbonized Materials in Probiotic Applications

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***Abstract*—**A new composite sorbent based on carbonized rice husk (CRH) and immobilized on it living cells and inactivated cultural liquid containing antimicrobials metabolites of *Bacillus subtilis* CK-245 is developed. The sorption and antimicrobic activity of CRH concerning five species of *Enterobacteriaceae* is studied. Prospects of use of developed sorbent in medicine and veterinary science is shown.

***Keywords*—**CRH, probiotic, concentrated fugate, sorption and antimicrobial activity.

I. Introduction

THE efferent (detoxification) medicine is based on application of sorbents for binding and removal different toxins and microbic cells from blood or gastrointestinal tract.

One of the directions of chemistry and biotechnology is receiving composite medical preparations and enriched sorbents. These are composite materials consisting of carbon sorbent and biologically active components. Their basis is made by the carbon sorbent received of high-temperature carbonization of rice husk produced at the Combustion Problem Institute. The developed porous surface of CRH made it properties as highly effective adsorbent [1]. The surface of these sorbents can be modified by absorbing on it heteroatoms, various functional groups even the whole microbic cells or biologically active substances that allows to receive composites with a new combination of properties. Initially introduced as just an imitation of Mother Nature, artificial immobilization of cells and enzymes has now transformed into a valuable biotechnological instrument. Its growing practical application and development over years led to appearance of fascinating novel microbial and enzymatic technologies [2]. Research on the immobilized biocatalysts is currently conducted in many laboratories around the world. In some countries (Japan, USA and others) immobilized microbial cells have been successfully applied for adsorption of heavy metals from dilute solutions [3], for purification of sewage [4] as well as for intensification of microbiological processes (production of antibiotics, organic acids, sugar syrups, fermented drinks, etc.). It was shown that immobilized cells allow conducting biotechnological process over extended periods of time, under strict control of the process kinetics and microbial activity [5]. This methodical reception is called enrichment or functionalization and it allows receiving the heterogeneous biocomposites possessing antimicrobic, anti-inflammatory, antitoxic, immunomodulating, antiallergic and other types of biological activity [6].

Example is the immobilized probiotic "Riso-Lakt". On its surface immobilized three types of lactobacilli probiotics [7]. Probiotics represent preparations of living microorganisms and their metabolites which have positive physiological, biochemical and immunological effects on the host organism via optimization of the host's microbial ecosystem when introduced in a natural way.

The immobilized microcolonies of bacteria keep their viability and are attached to mucous intestines.

Due to the increase of resistance of bacteria to antibiotics, there was a necessity of development of alternative therapeutic agent, among which the important place occupies use of probiotics, including *Bacillus* possessing antimicrobial activity. Bacteria *B. subtilis* produces more than 70 various antibiotics [8]. One of the important problems of production of probiotics is development of technologies without waste, in particular, use fugates of microbic cultures which do not contain bacteria, but there are products of their metabolism and biosynthesis which can render antimicrobial effect.

The purpose of this research is to develop a new composition material on the basis of CRH sorbent and the cells and metabolites of sporogenous bacteria immobilized on it. It was expected that this material should possess sorption and antimicrobial activity against microorganisms-intestinal pathogens.

II. Materials and Methods

- *Carbonized rice husk.* Carbonized rice husk received at high temperature in anoxic condition at the Institute of Combustion Problems [6].
- *Microorganisms. Bacillus subtilis* CK-245 (antagonist). strains of microorganisms used for determination of antagonistic activity of fugate: *Salmonella typhi* RM-1; RM-2; Z-3; *Salmonella typhimurium* G-1; G-4; G-5; *Enterobacter aerogenes* PM-1; PM-2; *Escherichia coli* G-3; G-4; *Proteus vulgaris* PM-1 from the collection of biotechnology department Al-Farabi Kazakh National University.
- *Methods.* Cultivation of *Bacillus subtilis* CK-245 carried out in the liquid cellulose containing medium Omelyansky in bottles on a shaker-incubator ES-20 (Latvia) within 48 hours. Concentration of fugate carried out with use of separator ASG-3MB, "Alpha Laval".

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Efficiency of division of native culture of *Bacillus subtilis* CK-245 at separation determined by (1):

$$F= C_0\text{-}C_f/C_0x100 \quad (1)$$

where F - Efficiency of division of separator, %; C_0-C_f – concentration of cells, respectively, in cultural liquid and in concentrated fugate (CF). Antagonistic activity of CF determined by a diffusion method in agar and estimated on size of a zone of absence of microorganisms' growth round paper disks with CF.

Immobilization of CF on CRH in the ratio 1:1 was performed during 2 hours. Then it was flushed with isotonic solution. CRH mix with CF immobilized on it displayed on shelves, then dried up in a drying cabinet.

Sorption efficiency of CRH and composition material (CRH+CF) of was derived from the difference between the cell counts in the culture medium before and after sorption. Antagonistic activity of the received composite material concerning microorganisms determined by a way of joint cultivation in nutritious broth within 18 hours at 37°C on a shaker-incubator ES-20 (Latvia).

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 *Bacillus subtilis CK-245* in nutrient broth 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 5mg/ kg body mass; the first group, in addition, was fed with liquid suspension of *Bacillus subtilis CK-245*; the second and the third groups received, after the induced dysbacteriosis, the same amounts of *Bacillus subtilis CK-245* 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*: Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris Proteus mirabilis, Enterobacter aerogenes, Salmonella typhimurium, Shigella zonnei* and *Shigella fleexneri* were considered as a measure of the antagonist 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.

III. Results and Discussion

Our previous experiments have proved that the hydrophobicity level of both cells and nanostructured carbonized sorbents (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 (Fig. 1). 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 very diverse applications.

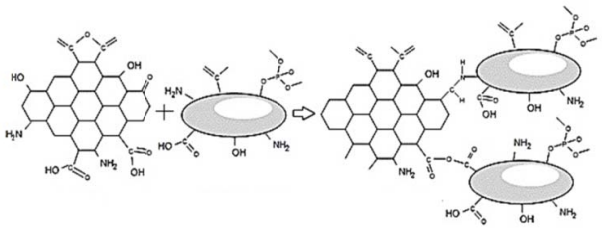

Fig. 1 Formation of covalent bonds forming between the surfaces of microbial cells and the carbonized materials considerably contributes to stability of the bio-composite materials [6]

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 10^8 colony-forming units (~viable cells) per gram of CA. The microbial cells are distributed on the surfaces not homogenously but rather form 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 a critical factor for bacterial survival and adaptability.

Fig. 2 shows subsequent stages of rice husk colonization by *Bacillus subtilis CK-245*. 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).

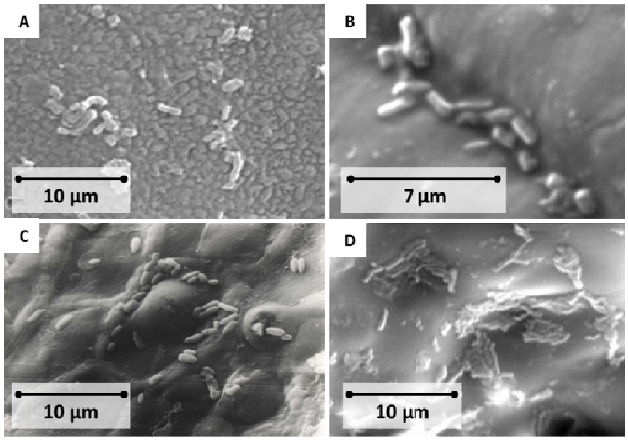

Fig. 2 Subsequent stages of colonization of CRH by *Bacillus subtilis CK-245*. A: carbonized rice husk, initial adsorption; B: carbonized grape stones, initial adsorption, C and D – carbonized grape stones, micro-colony formation

In our model experiments in vitro, CRH 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.

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 undesired effect, several approaches have been suggested so far.

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 a protective media for probiotic bacteria immobilized in their pores.

Twenty four hours after immobilization *Bacillus subtilis CK-245* displayed very good growth rate and began forming micro-colonies on the CRH. The data on gastric juice resistance of suspended and immobilized preparations of *Bacillus subtilis CK-245* are shown in the Table I.

TABLE I
INFLUENCE OF IN-VITRO GASTRIC JUICE TREATMENT ON VIABILITY OF SUSPENDED AND IMMOBILIZED CELLS OF *BACILLUS SUBTILIS* CK-245

Experimental group	Concentration of viable cells, ml^{-1}	
	Before treatment	After treatment
Suspended culture	$3{,}7 \times 10^9$	$5{,}2 \times 10^5$
CRH	$1{,}1 \times 10^9$	$8{,}2 \times 10^7$

In the suspended culture *Bacillus subtilis CK-245* after the gastric juice treatment the concentration of living cells decreased more than 7000 times. In contrast to that, the cells inside of the bio-composite materials were showed significantly (~500 times) better survival rate. The obtained data strongly suggested the protective action of CRH on the immobilized bacterial cells. These results look very encouraging in respect of construction of highly efficient bio-composite materials having extended probiotic activities.

The next series of experiment was devoted to comparative analysis of the probiotic 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 II.

TABLE II
INFLUENCE OF THE PROBIOTIC BIO-COMPOSITES CONTAINING *BACILLUS SUBTILIS* CK-245 ON THE QUANTITY OF *ENTEROBACTERIA* IN THE INTESTINE OF RATS AFTER CIPROFLOXACIN-INDUCED DYSBACTERIOSIS

Experimental group	Number of bacteria in 1g			
	Large intestine		Small intestine	
	wall	Contents	wall	contents
Before the treatment with ciprofloxacin				
The control	$(3.0\pm0.3)\times10^4$	$(6.9\pm0.7)\times10^6$	$(1.1\pm0.2)\times10^2$	$(1.5\pm0.4)\times10^3$
1 day after treatment with ciprofloxacin				
Without probiotics	$(2.9\pm0.5)\times10^5$	$(7.9\pm0.6)\times10^7$	$(2.9\pm0.3)\times10^4$	$(1.2\pm0.4)\times10^5$
Suspended probiotic	$(9.7\pm0.6)\times10^4$	$(1.2\pm0.3)\times10^7$	$(7.8\pm0.8)\times10^2$	$(8.9\pm0.2)\times10^3$
Probiotics on RH**	$(6.9\pm0.4)\times10^4$	$(9.5\pm0.2)\times10^6$	$(0.9\pm0.3)\times10^2$	$(5.3\pm0.8)\times10^3$
15 days after treatment with ciprofloxacin				
Without probiotics	$(2.4\pm0.4)\times10^5$	$(2.1\pm0.3)\times10^8$	$(8.4\pm0.2)\times10^4$	$(9.5\pm0.8)\times10^5$
Suspended probiotic	$(1.2\pm0.7)\times10^5$	$(9.6\pm0.6)\times10^7$	$(2.9\pm0.4)\times10^3$	$(2.8\pm0.4)\times10^4$
Probiotics on RH**	$(3.4\pm0.4)\times10^4$	$(8.5\pm0.7)\times10^6$	$(1.2\pm0.3)\times10^3$	$(1.2\pm0.7)\times10^3$

*CRH: carbonized rice husk

The data in the table show that after ciprofloxacin-induced dysbacteriosis, significant increase (~2 orders) of the *Escherichia*-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 led to significant suppression in Enterobacteria proliferation and spread. Being immobilized on CRH, probiotic bacteria effectively inhibited growth of undesirable 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.

We speculate that all the above-mentioned mechanisms could contribute to the increased activity of immobilized probiotic strains. Our previous date suggest that the immobilization of *Lactobacillus* on carbonized vegetable raw material (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 physic-chemical conditions on the interface "sorbent/microbe" [9]. 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 beneficiary effects.

The great binding strength and capacity of the material in respect of cells and dissolved compounds is mainly created by extended network of nanotubes but also appears due to high hydrophobicity of the surface. Although many more studies and tests are necessary, and a lot of work needs to be done, we can now envision creation in the nearest future of new generation of CRH-based probiotic preparations, effectively normalizing the intestinal microflora, bringing relief to millions of patients around the world.

Base biological activity which should possess probiotics is antagonistic activity in relation to pathogens. The main reason for use of enterosorbents is food poisonings and the diseases caused by pathogen and opportunistic Enterobacteria.

The studied samples of CF were active against all test strains of *Salmonella* (Fig. 4). Antagonistic activity of CF was considered zero at width of a zone of absence of test strains growth to 1.0mm, low – at 1.1-4.9mm, average - at 5.0-8.9 mm, high – at 9.0mm and more.

At the following stage of antimicrobic activity of CF against other species of bacteria of *Enterobacteriaceae* family (Table II) was defined. As target microorganisms used: *Echerichia coli* G-3; G-4 (enteropatogenic intestinal bacteria - the causative agent of colibacteriosis); *Proteus vulgaris* PM-1 (food spoil); *Enterobacter aerogenes* PM-1; PM-2 (opportunistic enteric infections).

It possesses antagonistic activity on the relation to all used test microorganisms with growth inhibition zones to *Enterobacter aerogenes* PM-1-19±0,6; *Enterobacter aerogenes* PM-2 - 10,8±0,6; *Echerichia coli* G-3 - 16±0,7; *Echerichia coli* G-4 - 19,04±0,5; *Proteus vulgaris* RM-1 – 20,03 ± 0,4.

In Table III, it is shown the sorption activity of CRH and composite material concerning microbic cells.

Studying of efficiency of *Enterobacteria* sorption has shown that, first CRH possesses high sorption characteristics concerning microbic cells. Secondly, that the capacity of sorption of *Enterobacteria* of composite material is at the same level. Therefore, the immobilization of CF does not influence sorption of CRH properties concerning microbic cells.

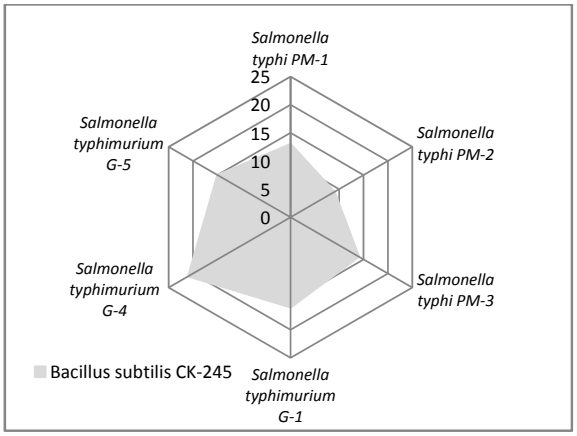

Fig. 3 Antagonistic activity of concentrated fugate of *Bacillus subtilis* CK-24 concerning *Salmonella*

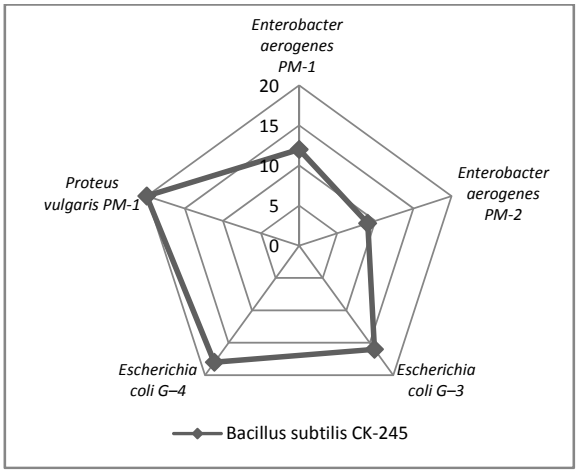

Fig. 4 Antagonistic activity concentrated fugate of *Bacillus subtilis* CK-24 against *Enterobacteriaceae*

TABLE III
EFFICIENCY OF SORPTION OF BACTERIA ON CRH AND CM

Test organism stains	Initial concentration of cells x10^9	Capacity of cell sorption (10^8 to 1 g sorbent)	
		CRH	CM
S. typhi PM-1	2,5±0,4	62,1±4,9	57,1±2,9
S. typhimurium G-1	2,0±0,3	73,4±5,5	69,4±5,6
E. coli G-3	2,3±0,08	84,1±3,8	79,9±4,9
E.aerogenes PM-2	2,6±0,5	56,1±1,9	48,2±3,8
P. vulgaris PM-1	2,8±0,6	64,1±4,8	59, 3±2,4

Antimicrobic properties of the received composite material estimated at joint cultivation with target-microorganisms in nutrient broth within 18 hours (Table IV). In system brought 100ml of a suspension of bacteria in concentration of 10^8 CFU/ml, 10g of sorbent or 10g of composite material.

Abscopal effect of materials is determined by percent of the survived cells of test strains.

TABLE IV
ANTIMICROBIC ACTIVITY OF THE COMBINED SORBENT AT JOINT CULTIVATION WITH TEST ORGANISMS

Variants	Number of viable cells of test strains, % to control				
	S. typhi PM-1	*S. typhimurium* G-1	*E. coli* G-3	*E. aerogenes* PM-2	*P. vulgaris* PM-1
CF+CRH	0,8±0,01	0,6±0,03	2,3±0,02	1,6±0,03	1,8±0,03
CRH	67±2,6	70±4,6	72±5,3	77±5,6	57±4,7
Control			100		

IV. CONCLUSION

The composition material has the expressed negative effect on all 5 test cultures from *Enterobacteria* family. At the same time sorbent are capable to attach about 28-43% of these microorganisms. Therefore using the received preparation there is an effective suppression of test cultures. It means that the composite material possesses both sorption and antimicrobic activity against *Enterobacteria* – activators of food poisoning. The new probiotic on the basis of CRH and antimicrobic metabolites of sporogenous bacteria can be alternative to antibiotics. The high antimicrobial activity of a biological component will interfere with a microbic contamination and to promote safety of medicinal forms.

The researches directed on receiving carbonized sorbent on the basis of nonconventional secondary vegetative raw materials-rice husk and development of ways of its functionalization for the purpose of receiving the new biological products possessing high sorption and antibacterial, wound healing activity for their application in efferent therapy are represented perspective.

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