Bacillus Pumilus as a Supplement for Waste Recycling by Insect

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Abstract. The possibility of enriching the intestinal microbiota of cockroaches of the species Pycnoscelus nigra with a cellulolytic strain - Bacillus pumilus IB-320 was reviewed, as well as an assessment of the effect of the used microorganism on process of bioconversion of various types of paper waste and soil humification. Based on the results of a model experiment with a period of 90 days, various degree of bioconversion of cellulose-containing wastes for the zoomicrobial comple were found (in descending order): grass hay, paper and soil substrate (mix of brick crumbs, softwood sawdust and coconut-fiber substrate). Treatment with strain IB-320 contributed to an increase in the total number of bacteria obtained from the substrates and fecal extracts of cockroaches by 1.5-1.7 and 1.5-3 times, respectively. The introduction of the selected strain also significantly increased the rate of destruction of such substrates as cardboard and egg trays by 40% and 25%, accordingly. In the variant with using the grass hay, the presence of strain IB-320 led to an increase in humic acids by 1.5. The prospects of application Bacillus pumilus IB-320 as part of an active zoomicrobial complex for more efficient disposal of cellulose-containing waste have been shown.

1. Introduction
Recycling solid organic waste through composting is a universal and economically profitable way of getting organic fertilizers, which are not only the source of nutrients, but also are antagonistic to phytopathogens [1]. Besides composting, a wide use is made of vermicomposting, these technologies can be applied both as independent ones, and in combination [2]. Applying insects in the bioconversion of waste enables us to enlarge the range of recycled waste, while producing compost, and besides, to get the biomass, used in cattle-breeding, pharmaceutics and in producing biofuels [3, 4]. It is a fact that the gut microbiota can directly influence the preferences of insects in feeding on this or that product, and it also determines the possibility of assimilation new substrates [5].

The cockroaches Pycnoscelus nigra [6] in the natural environment habitat in the moist soil of the evergreen tropic forests. They are preferably detritophagous, but also can be saprophages. They multiply by means of obligate parthenogenesis, which under favourable conditions leads to the population growth in geometrical progression. The cockroaches, hatched from eggs, get the gut microbiota from parents or by ingesting food, which creates the prospect of introducing new strains of bacteria into the intestines of cockroaches to expand the list of waste suitable for processing. The feces
of *P. nigra* look like biohumus of worms, and unlike the excrements of synanthropic cockroaches and do not have the smell [7]. The enumerated qualities make *P. nigra* the attractive organism for the use in composting in a community with microorganisms.

*Bacillus pumilus* are known by their high enzymatic activity in relation to cellulose- and hemicellulose-containing substrates and keratin-containing waste of poultry breeding [8]. Also *B. pumilus* is used in animal husbandry and poultry farming as probiotics, feed additives that improve physiological parameters, protect against various infections and have established themselves as nontoxic biopreparations [9]. Thus, taking into consideration all the positive characteristics of the strain, an increase in the process of bioconversion of the offered by the zoomicrobial complex was expected.

The aim of the present study is to clear out, whether the enrichment of the environment and the gut microbiota of cockroaches by bacterium-destroyer of cellulose will be successful and to assess the influence of the used microorganism on the process of bioconversion of the plant residues and different types of waste of the pulp and paper industry.

2. Materials and methods

The object of investigation were the cockroaches of *P. nigra* [6]. The test bacteria were represented by the strain *Bacillus* sp. IB-320 from the collection of cellulose- and xylanase-producing spore-forming bacteria of the UIB UFRC RAS. The strain IB-320 expressed hydrolyzing activity in relation to such substrates as carboxymethyl cellulose (CMC), xylan, wheat straw, maize cobs. According to its physiological and biochemical properties, the given strain was preliminarily related to the bacteria *B. pumilus*.

*P. nigra* were cultivated in the transparent plastic containers, volume 500 ml, with ventilation holes. As the soil, we used the mixture, containing 40 g of brick crumbs, 20 g of softwood sawdust, 30 g of coconut-fiber substrate. We added into some containers paper pulp: 1 g of cardboard + 4 g of egg trays + 1 g of office paper or 4 g of grass hay. All the components of the mixture had been sterilized beforehand by autoclaving. 10 cockroaches in the imago stage were put into the containers, having 3 variants of the substrate: 1) the main soil (G), 2) the main soil + the paper pulp (GP), 3) the main soil + grass hay (GH) in six replications each. The substrates of 3 containers from each replication were sprayed beforehand with 1 ml suspension of the culture *B. pumilus* IB-320 with titer 10⁸ CFU/ml (colony-forming units). At first, 70 ml of water were added into each container, and then the containers were sprayed, considering moisture vapourisation.

The spectroscopy analysis of humic acids was performed by extraction humic acids of samples in a 0.05 M NaOH. The optical density of the solution at 350 nm was measured at the spectrophotometer (UNICO 2800, USA) [10].

All bacterial inoculations associated with the determination of contamination of substrates and fecal extracts were performed according to standard tenfold dilutions, spread-plated in triplicate on LB agar media [11] and cultivated at temperature of 28 °C. CFU were counted and calculated per g of soil or feces. Morphologically different colonies were tested on the medium with Congo Red with the aim to identify cellulytic isolates, including a variant of the morphotype similar to the variant of *Bacillus pumilus* IB-320. This procedure included the following stages. In order to determine the endoglucanase activity, colony cells were transferred onto plates with LB medium containing 0.5% CMC. Cultivated 72 hours at 30 °C. Poured with 0.1% Congo Red dye solution for 30 minutes, washed 2 times with distilled water and poured with 1 M NaCl solution. After 15 min, the zones of CMCase activity were determined [12].

Taxonomic identification *Bacillus* sp. IB-320 and of the isolates with CMC activity from the guts of cockroaches was held, basing on the analysis of gene 16S pPHK. The gene fragment of 16S rRNA amplification was carried out using universal bacterial primers 27F (5’ - AGAGTTTGATC(A/C)TGGCTCAG 3’) and 1492R (5’ - ACCG(C/T)TACCTTGTGACGACTT - 3’) following standard protocols. Sequences were manually edited in MEGA7 [13] and identified by a basic local alignment search tool (BLAST) search of the GenBank database (http://www.ncbi.nlm.nih.gov/blast).
3. Results and discussion
Analysis of the 16S rRNA gene sequence revealed 100% gene homology of the *Bacillus* sp. IB-320 with gene of *Bacillus pumilus* NBRC 12092 T (NR_112637). The nucleotide sequence of the 16S rRNA gene of strain IB-320 with a length of 1450 bp was included in the Genbank database under the number MT914510. Thus, considering all the positive characteristics of the strain, the process of bioconversion of the offered substrates with the help of cockroaches was expected.

The analysis of the bioconversion rate of the paper pulp with cockroaches of *P. nigra* explicited that primarily, the cockroaches ate office paper. Introducing *B. pumilus* IB-320 led to the speeding of paper destruction by 1.5 times. In the following experiments office paper was excluded from the list of nutrient substrate, because with its presence, the cockroaches practically ignored the cardboard and egg trays. The speed of consuming cardboard was lower, but with treating the substrate with *B. pumilus* IB-320 also rose by almost 40% (figure 1). The egg trays proved to be the most difficult substrate for conversion. Their speed of destruction with strain IB-320 rose by 25%.

![Figure 1. Increase in substrate destruction rate by cockroaches in the presence *B. pumilus* IB-320 for a month period.](image)

When plating the extracts from the soil and fecal extracts on the test-plates with Congo Red, the bacterial colonies with cellulolytic activity of two phenotypes were identified. The belonging of one of them, having the phenotype of colonies like *Bacillus pumilus* IB-320, was later proved by the sequence-analysis of 16S RNA in the gene with 100% homology to *Bacillus pumilus*, and the second colonial morphotype was identified as *Bacillus subtilis*.

The comparison of bioavailability of substrates for cockroaches (without introducing bacteria) was held 3 months after the cultivation of insects. According to the results of the model experiment, in figure 2 you can see that the highest microbial contamination is present in the substrate GH. In the substrate GP there were less bacteria, and from G - the microorganisms were obtained almost by 2 times less. It is noteworthy that the percent of isolates with CMC-activity, present in the common pool of microorganisms, sown from the substrates GH and GP, were alike and made 2-5% from the common bacterial titer, while such isolates were not identified from the G-soil. It is supposed that contamination took place due to the cockroaches own commensal microflora through excrements. According to the experiments, cockroaches do not survive in the sterile soil G, and therefore sterile soil, cannot serve as a nutrient substrate. Processing the soil with *B. pumilus* IB-320 enabled the cockroaches to use this hard accessible substrate.
After inoculation of the substrates by spraying with the test culture, after 90 days, *Bacillus pumilus* was not only present on all substrate variants, but also remained in the guts of insects, as evidenced by the CFU data in Figures 2 and 3. Thus, the principle of adequate coexisting in the zoomicrobial complex is confirmed: when the composition of the gut microflora reflects the composition of bacteria in the environment. Spraying with the bacterium *B. pumilus* IB-320 enhanced the general number of bacteria, sown from the used substrates and fecal extracts by 1.5÷1.7 and 1.5÷3 times accordingly. The proportion of the test-culture IB-320 in the microbiome of cockroaches, feeding on the grass hay, exceeded the percent of other cellulolytics, developing under the similar conditions, by several times and made up 50% from the general number of microorganisms. The percentage of the test-culture in the substrate with the grass hay was not so high, but tangible, and provided not only the constant influx of bacteria with the food into the guts of cockroaches, but also contributed to the increase of other cellulolytic bacteria. In this variant of the experiment (GH) in the presence of *B. pumilus* IB-320 the increase in quantity of humic acids in the substrate was fixed as 1.5 times higher.

Interesting facts were obtained from a variant of the experiment of *B. pumilus* IB-320 inoculation of paper (GP). At this treatment a significant growth of the general bacterial population in the GP substrate and fecas extracts was achieved due to the dominant presence of strain IB-320, whose percent made up 80% and 90% accordingly. This fact can point to the high competitiveness of the introduced strain *B. pumilus* IB-320 among the other cellulolytics, minor proportions of which were present in the GP substrate and in the cockroaches guts in the beginning of the experiment. It is in feeding the cockroaches with paper, processed with the strain IB-320 that the maximal increase of 3÷15 times of the microflora in the guts of cockroaches was fixed as compared to the data get on other substrates.
Figure 3. The number of CFU in the fecal extracts, after 3 months of development of the cockroach colony. 1 - without the introduction of bacteria, 2 - with Bacillus pumilus IB-320.

The achieved results confirm the prospects of using the selected strain in the processes of bioconversion of cellulose-containing waste with the zoomicrobial complex, consisting of two and more biodestructors. The strain Bacillus pumilus IB-320 can be used both as a probiotic for the inoculation of cockroaches, and as a biofertilizer, stimulating the destruction of the substrate of the cellulose-and-pulp nature, contributing to the humification of the soil.

4. References
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