THE ASSESSMENT OF ORGANIC SUPPORT MATERIAL FOR *Rhodococcus rhodochrous* CNMN-Ac-05 CELLS IMMOBILIZATION

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Abstract. The aim of this study was to assess the utilization of agricultural waste as the support material for rhodococcus cells immobilization. Hazelnut (*Corylus avellana* L.) shell, common walnut (*Juglans regia* L.) shell, peanut (*Arachis hypogaea* L.) shell, pistachios (*Pistacia vera* L.) shell, pumpkin (*Cucurbita pepo* L.) and sunflower *(Helianthus annuus* L.) seeds husk were used to immobilize cells of *Rhodococcus rhodochrous* CNMN-Ac-05, destructor of persistent organic pollutants. The peanut shell as a support material demonstrated a good adsorption of bacterial cells – 7.95 mg dry cells per gram of carrier (34.3%), while the efficiency of cells immobilization on the sunflower's husk was very low – 6.2%. The reduction of carrier particle size to 100–500 µm was contributed to an increase in the number of cells adsorbed on supports made from peanut and hazelnut shells. The efficiency of bacterial immobilization was in 1.5 and 3 times higher than the initial one for supports made of peanut and hazelnut shell accordingly.

Keywords: *Rhodococcus rhodochrous*; organic supports; immobilized cells.

INTRODUCTION

Rhodococcus species are ubiquitous bacteria in pristine and contaminated environments, possess remarkable metabolic activities, can persist under harsh environmental conditions, compete successfully in complex bacterial populations, and therefore could be considered as having great potential in bioremediation applications [18]. Upon revealing new catabolic abilities of *Rhodococcus* species and isolation of environmental strains degrading a wide range of contaminants, these microorganisms have been increasingly explored for bioremediation of soils, waters, and air polluted with different recalcitrant and toxic organic chemicals [6, 16, 18].

Cell immobilization has become an important practice in biotechnology in the last years, resulting in increased performance and efficiency of the agricultural and economic process [15, 20, 31, 34]. The immobilized cells have commonly been used for various biotechnological applications, for examples: antibiotic production, soil bioremediation, biodegradation and biotransformation of xenobiotics in wastewater treatment plants [37]. The utilization of cells immobilized on organic supports ensures low cost, ease of handling of preparations and control of microbiological processes [9, 10, 20]. For cell immobilization are used media and methods well studied in the last two decades. The immobilization supports are classified as inorganic (montmorillonite, zeolite, diatomite, different clays, anthracite, porous glass, activated charcoal, etc.) and organic as cellulose (DEAE-cellulose), wood sawdust, delignified sawdust etc. Inorganic supports have been selected to immobilize microorganisms because they can survive microbial degradation and are thermostable [3, 32]. Selecting the right support for immobilization is an important factor that determines the activity of immobilized cells [17]. They could survive longer than free cells and it could be reused twice in the process of toxic pesticides degradation without the loss in activity [25]. For some species of bacteria and yeast, cell immobilization can induce changes in cell growth, physiology, and metabolic activity: improves oxygen diffusion and enhances dehydrogenase activity [3, 22]. Various scientific papers have revealed about the modified metabolic behavior of immobilized cells. Up to now, researchers concluded that cells have higher yield of biosynthesis or catabolism reactions [14, 23, 33].

Recently, various natural materials have been researched as supports for the immobilization of yeast cells, including grape peel, silkworm [27], sugarcane [29], plant sponge (*Luffa cylindrica* (L.) M.Roem.) and bacterial cellulose [28]. *Pseudomonas aeruginosa* and *Bacillus sphaericus* were immobilized on walnut shell, with the aim to use of immobilized individual and mixed cultures for decontamination of oil-polluted wastewater in Egypt [10]. Based on the above exposed, the aim of this study was identify a suitable agricultural waste as a support material for immobilization of actinobacteria *Rhodococcus rhodochrous* CNMN-Ac-05.

MATERIALS AND METHODS

Object of study was the strain *Rhodococcus rhodochrous* CNMN-Ac-05, deposited in the National Collection of Non-Pathogenic Microorganisms of the Republic of Moldova, which is able to grow and develop on a medium containing trifluralin as the sole source of carbon and energy [13]. This strain is an adapted variant of *R. rhodochrous OBT18*, offered by SEESIB Lab, Blaise Pascal University (Clermont-Ferrand, France).

Method of cultivation. The *R. rhodochrous* strain was preserved in the aliquot freezer on TS medium, with the following composition g/L: hydrolyzed casein – 17.0, soybean meal papaya extract – 3.0, NaCl – 5.0, $K_2HPO_4 \times 3H_2O$ – 2.5, glucose – 2.5, pH (at 25°C) – 7.3 ± 0.2.

For the production of bacterial biomass, the *R. rhodochrous* strain was grown under continuous aeration conditions on a stirrer 180-200 rpm at 28°C for 48 hours on TS medium. *R. rhodochrous* cell mass was separated by centrifugation for 30 minutes at 5000 rpm and washed twice with NaCl solution (0.8%).

The *R. rhodochrous* biomass was determined on the spectrophotometer by the optical density of *R. rhodochrous* cell suspensions, with subsequent recalculation of the dry mass of the cells according to the calibration curve. The dry biomass of *R. rhodochrous* was determined by gravimetric method, by drying at 105°C [31].

Supports. Six organic supports were selected for rhodococci cells immobilization: shell of hazelnuts (*Corylus avellana* L.), common walnut (*Juglans regia* L.) shell, peanut (*Arachis hypogaea* L.) shell, pistachios (*Pistacia vera* L.) shell, sunflower (*Helianthus annuus* L.) seeds husks, and pumpkin (*Cucurbita pepo* L.) seeds husk.

Supports preparation. Organic substrates were ground, sifted through sieve No. 0.5 and washed until clear water was obtained, then washed with distilled water in three repeats and passed through deionized water the same in three repeats. The supports were dried in an oven for 2-3 hours at 80-90°C to set up the constant weight. A 1.0 g sample was placed in a 250 ml Erlenmeyer flask, then the supports were sterilized at 1 atm for 15 minutes.

Obtaining of *R. rhodochrous* cells immobilized. To immobilize rhodococci cells, Knapp buffer was used with the following composition g/L: K_2HPO_4 – 1.0; KH_2PO_4 – 1.0; $MgSO_4 \times 7H_2O$ – 0.04; $FeCl_3 \times 6H_2O$ – 0.004, pH – 6.7.

R. rhodochrous whole cells, 150 mg, and 50 ml of Knapp buffer were added to a 250 ml Erlenmeyer flask with 1 g of sterile support. In other flask, only Knapp buffer was added, without the addition of cells. Flasks were placed under continuous aeration conditions on shaking 180-200 rpm for 20 minutes, t = 24°C, then placed in the refrigerator for 16-20 hours [12]. The content of the flasks were filtered through a capron filter. The support on the filter was washed three times with Knapp buffer, 50 ml for each wash. The filtrate was poured into a volumetric flask, adjusted to 250 ml with Knapp buffer.

The amount of immobilized biomass was estimated by two different methods: 1) indirect, or spectrophotometric, by measuring the D540 optical density of the cell suspension before and after immobilization; 2) by quantitating the number of viable bacterial cells (colony-forming units, CFU) inoculated on agar medium TS from the cell suspension before and after immobilization. The number of colony-forming units (CFU) was considered over a 4 day period of growth of rhodococci bacteria [36].

Statistical analysis was performed using MS Excel. All results were expressed as mean of three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$.

RESULTS

Before the immobilization, the initial suspension of *R. rhodochrous* CNMN-Ac-05 cells contained 23.17 mg dry cells (0.46 mg dry cells/ml), the colony forming units (CFU) concentration was 50.83×10^9 CFU/ml.

Following the methodology, it was observed that the filtration period during the washing step was varied for different supports; it was the fastest for the peanut shells, and the slowest – for the shells of the pumpkin seeds. The immobilization of *R. rhodochrous* cells on organic supports and the assessment of cell adsorption by two different methods, allowed to obtain data regarding the adsorbing properties of the carriers in study.

After the immobilization, the 7.95 and 5.45 mg of dry cells were adsorbed on the peanuts and hazelnut shells, the degree of immobilization was 34.29 and 25.50%, respectively. The other options of the experiment demonstrated a low degree of immobilization – from 6.20 on the husk of sunflower seeds to 18.11% on the husk of pumpkin seeds (Fig. 1).

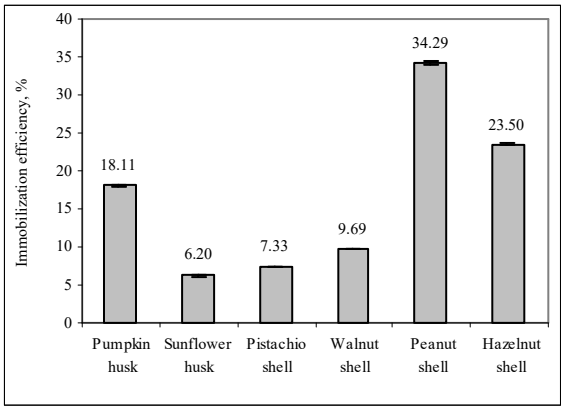

Figure 1. The immobilization efficiency of the organic carriers after the adsorption of *R. rhodochrous* cells

The results of microbiological sieving of a suspension of *R. rhodochrous* cells, remaining in the Knapp buffer after the immobilization on organic substrates were recorded in Table 1.

It is necessary to note the high level of compliance of the immobilization degree values calculated by this method with the values obtained by spectroscopy (Table 1; Fig. 1). The only exception was the option "hazelnut shell"; the difference between the values calculated in two different ways was 4%. The quantity of CFU adsorbed on such supports as hazelnut shell and peanut shell was 14.97 and 17.90 CFU $\times 10^9$/ml suspension accordingly, that was considered the sufficient concentration for use in pesticides degradation.

In order to increase the degree of immobilization of *R. rhodochrous* cells on supports, the best options for

Table 1. The number of *R. rhodochrous* cells before and after immobilization on organic supports and the degree of immobilization (%)

Number of cells *R. rhodochrous*	**Supports, experimental options**						
	Control	Pumpkin husk	Sunflower husk	Pistachio shell	Walnut shell	Peanut shell	Hazelnut shell
CFU not immobilized, CFU × 10^9/ml	50.83 ± 3.74	41.40 ± 2.64	47.40 ± 3.95	46.40 ± 2.64	45.87 ± 4.02	32.93 ± 6.16	35.87 ± 4.02
CFU immobilized, CFU × 10^9/ml	-	9.43 ± 0.50	3.43 ± 0.29	4.43 ± 0.25	4.97 ± 0.44	17.90 ± 3.35	14.50 ± 1.44
Immobilization, %	-	18.56 ± 0.99	6.75 ± 0.56	8.72 ± 0.50	9.77 ± 0.86	35.21 ± 6.58	29.44 ± 2.83

Table 2. Immobilization of *R.rhodochrous* cells on the peanut and hazelnut shells, in dependence of support dimension (%)

Immobilization of *R.rhodochrous*	**Supports, experimental options**			
	Peanut shell		Hazelnut shell	
	500-1000 μm	100-500 μm	500-1000 μm	100-500 μm
Biomass immobilized, mg dry cells/g	7.95 ± 0.06	18.31 ± 0.45	5.45 ± 0.03	25.14 ± 0.64
Immobilization, %	34.29 ± 0.26	51.35 ± 1.25	23.50 ± 0.15	70.49 ± 1.78

the experiment – the peanut and hazelnut shells are crushed to obtain a fraction of 100–500 μm (Table 2). We found that the supports particle size reduction to 100–500 μm contributed to an increase in the area surface suitable for bacterial adhesion, and an increase in the number of cells adsorbed on the supports. A high level of bacterial immobilization was demonstrated, which was in 1.5 and 3 times higher than the initial one for supports made of peanut and hazelnut shell accordingly.

DISCUSSION

The use of microbial cells, immobilized for the purpose of biosynthesis or biodegradation reactions remains one of the most researched technologies of recent years [22]. Numerous biotechnological processes are advantaged by immobilization techniques and therefore several such techniques and support materials have been proposed [15, 30]. Immobilized cells are frequently used for the biotransformation due to advantages such as the prevention of the elution of impurities from the cells, easy separation of the cells from a reaction mixture, repeated use of the immobilized cells, and enhanced stability of the cells [2, 10, 25].

Microorganisms adsorb spontaneously on a wide variety of organic and inorganic supports. Adsorption is the earliest and easiest known method of preparing immobilized bacterial cells, and includes physical adsorption of a cell onto an inert support such as charcoal, bentonite or polysaccharides. The method generally requires contacting an aqueous suspension of the cells with the carrier. Cell adhesion to support material is dependent on physicochemical properties of using carriers: the pore size and structure, as well as on the hydrophobicity/hydrophilicity and surface charge [7, 31]. Binding of cells occurs through interactions such as Van der Waals forces, ionic bonds, hydrogen brides or covalent interactions. Microbial cell exhibit a dipolar character and behave as cations or anions, depending on the cell type and environmental conditions such as pH of the solution. Furthermore, cell physiology has a significant influence on the strength of the adhesion [31]. For *Rhodococcus* cells, having a hydrophobic cell-surface, was required a special treatment, physicochemical modification (hydrophobization) of carriers, for better adhesion to the matrix [17].

The support selection is one of the crucial decisions to be made in the course of preparation of the immobilization process [35]. In 1996, Leenen et al., suggested, that for treatment of wastewater, support materials need to meet the following criteria: insoluble, not easy degradable, non-toxic, nonpolluting, light weight; flexibility in overall shape, high mechanical and chemical stability, high diffusivity, simple immobilization procedure, high biomass retention, minimal attachment of other organisms and preferably a low cost price [19]. It is now believed that for successful immobilization, the support should be cell-friendly, user-friendly, resistant and biodegradable [4, 5, 21].

Agricultural waste had become alternative support materials for cell immobilization because they are environmental friendly, locally available and cheaper than synthetic polymer. Researchers indicated successful use of various agricultural residues to immobilize microbial cells for bioremediation purpose [1, 8, 9]. Immobilized cells could survive longer than free cells and they could be reused twice without the loss in toxic pesticides degradation ability [1].

When selecting supports for immobilization of *R. rhodochrous* cells, we considered the following criteria: existence the researches of successful use of support in rhodococci immobilization, the availability

of material in the country, and the prime cost reduced. Researches has shown that walnut shell have absorbent properties, being considered as industrial waste present in enormous quantities in the Republic of Moldova, as well as fruit and grape seeds [12]. The peanut and hazelnut shells are made up of lignin, cellulose, proteins, and carbohydrates. The shells consist of a fibrous skeleton, which supports a cellulosic layer. The cellulose and hemicelluloses contain in shell were used as an organic carrier for microorganisms, and, as well as protein, could serve as a source of nutrition for bacteria [24]. The organic solid supports selected to immobilize *R. rhodochrous* cells, such as walnut, hazelnut, pistachio, peanut shells, husks from pumpkin and sunflower seeds, in general, assume the criteria given as important to achieve the aim of the study, as they are readily available organic waste products, low-cost carriers suitable for bacterial immobilization. For example, walnut shell has absorbing properties and is considered as agricultural waste, present in large quantities in the Republic of Moldova, as well as fruit stones and grape seeds [12]. The peanut shells are often landfilled, but, due to their biodegradable and absorbent structure, they could be utilized as animal food filler, absorbents, or carriers for pesticides or fertilizers [11, 20].

The reduction of particle size could contribute to enlarging the particle surface, available for cells adhesion. The reduction of peanut and hazelnut shell carriers' particle size to 100–500 μm could improve the efficiency of rhodococci cells immobilization, without special treatment.

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