THE VALIDATION OF MICROBIAL COUNTING METHOD FOR PIGMENTED YEASTS FOR THE TESTING OF EFFECTS OF NANOPARTICLES

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Abstract: At the moment, technologies based on the use of nanomaterials in various fields of medicine and industry are developing. The study of the influence of metal nanoparticles on microorganisms is an important area of modern biotechnology and microbiology. Of particular interest in this regard, is the use of microbiological methods for assessing the effects of metal nanoparticles on living organisms. Among other nanoparticles, zinc oxide nanoparticles are probably the most widely used nanomaterials. The cell viability is one of the important parameters for the determination of nanotoxicity. The present research paper contains information related to the validation of the method for microbial counting for pigmented yeasts cultivated in the presence of ZnO nanoparticles. The validation of this method was performed according to the USP 30-NF25 and method validation by U.S. Environmental Protection Agency. Pigmented yeasts were selected for this research. The microbial counting method was validated to ensure precision, accuracy, linearity and robustness and can be applicated safely for the study of the effect of nanoparticles on microorganisms cell viability.

Key words: validation; microbial count; yeasts; ZnO nanoparticles.

INTRODUCTION

At the present time the production of nanomaterials with wide industrial and scientific applications has lead to the increased interest in nanotoxicology research [7, 23, 26, 32]. Thereby, the need for microbiological and biochemical methods and assays to determine the effect of nanoparticles on living organisms has steadily increased. Zinc oxide nanoparticles (NPs) have been spreading for the manufacture of an increasing number of commercial products [15, 30]. ZnO NPs are a promising platform for use in biomedical research due to the anticancer properties and can be succesfully used for antidiabetic treatment, also [6, 8, 29].

Thus, it is necessary to evaluate risks for these nanoparticles. This paper contains information for the validation of microbiological method for testing the action of nanoparticles in yeasts. The cell viability is one of the important parameters for the determination of nanotoxicity. This one is the most commonly used method for cytotoxicity analysis induced by chemical or physical factors that exert toxic effects through different cellular changes that can affect cells' ability to divide [11]. The yeasts present an biotehnological object useful for many biotechnological applications [9].

To use the modern achievements of nano-technology in medicine, biotechnology, food industry, it is necessary to pay special attention to studies of the biological effect of nanoparticles on yeast cells [13, 16, 17, 18]. In this research, an microbial counting method was validated for the determination of yeasts cell viability in the presence of zinc oxide nanoparticles. Pigmented yeast strain *Rhodotorula gracilis* CNMN-Y-03 was selected as a biological model for evaluation of action of nanoparticles. Due to the cellular structure, the short generation time, functional organization and ability to response to stress conditions, yeasts can serve for elaboration and validation of microbiological tests for nanotoxicology studies.

In view of the above, the validation for microbial counting method would prove the effectiveness of method for estimation of effect of zinc oxide nanoparticles on yeasts. The validation of the microbiological test was performed to ensure the quality of the method used in the research. The validation report includes: the conditions under which validation is carried out (method specification, conditions, equipment, reagents) and verification of performance parameters **(**precision, accuracy, linearity and robustness).

MATERIALS AND METHODS

Culture media and nanoparticles

For validation of the microbial counting method YPD (yeast extract peptone dextrose) media specific to yeast strains was used. The submerged cultivation was carried out in depth capacity 1 liter Erlenmeyer flasks on shaker 200 rpm (revolutions per minute) at a temperature of 25…28^{0}C, the duration of cultivation 72 hours. Yeast cells in amount of 5%, $2 \cdot 10^6$ cells/ml were inoculated in liquid medium. The cultivation of yeasts was effectuated at the constant illumination of 2000 Lx.

Nanoparticles: ZnO nanoparticles with particle size <50 nm in form of nanopowder, purity >97%, surface area >10,8 m^2/g (ALDRICH) were used. The nanoparticle stock solution was prepared according to the method specified [20]. Deionized water was prepared in a filtered membrane (0.45 g/L). The solution was ultrasonically assisted using a 50 W ultrasonic processor at 50% amplitude for 10 minutes. The pH of the solution was adjusted to 6-7 using dilute NaOH or HCl, and the solution was stored at 4°C. Prior to use, stocks were equilibrated to room temperature 23 ± 2 °C and sonicated for 5 minutes at 50% amplitude. The nanoparticles were added as an emulsion at the inoculation stage of the strain. The concentrations of nanoparticles used in experiments constituted 1.0; 5.0;

10; 20 mg/L. The variant without application of nanoparticles was used as control sample.

Test object. Pigmented yeast strain *Rhodotorula gracilis* CNMN-Y-30, producer of proteins and carotenoids, was selected for microbiological test. The strain is stored in the Collection of Nonpathogenic Microorganisms of Institute of Microbiology and Biotechnology of Republic of Moldova.

Methods

Culture Media. For inoculation and submerged cultivation of yeasts was used specific YPD fermentation media [2]. From the stock cultures in bacteriological agar, yeasts were transferred on a liquid medium beer wort. Yeasts cultures were incubated for 24 hours at 28...30°C. Serial dilutions in 0.9% sodium chloride solution were effectuated and the last three were plated and incubated for 24 hours at 30...32°C to obtain a standardized suspension to 10^2 CFU/mL (Colony-Forming Unit). For the validation of the microbiological test the method for determining the total number of colonies was selected. From the experimental groups, 1.0 mL volume were taken and deposited in Petri dishes with 0.3 or 1.0 mL of standardized suspension of yeasts cells. Thus, plates were incubated at 30 ± 2 °C for 48 hours. After this period were effectuated counting of the colonies of yeasts. The results were presented in colony forming units/plate. Validation of the microbiological method was performed using repeatability parameters, intermediate precision, linearity, accuracy and robustness by using the USP30-NF25 and method validation of U.S. Environmental Protection Agency (EPA) [3-5, 21, 36].

The precision was determined at two levels – repeatability and intermediate precision. The repeatability was evaluated with two levels of microbial incrimination, the first with a 10-30 CFU/plate and the second with a 30-300 CFU/plate. The criterion used to prove the repeatability was as recommended by USP 30-NF25 according to which, relative standard deviation must be 25% or 15% for groups with 10-30 CFU/plate or 30-300 CFU/plate, respectively. The eligibility criterion for intermediate precision was relative standard deviation, which should not exceed 10%. The obtained results were statistically analyzed.

The accuracy was defined as the measure of the closeness of the experimental data in the analysis. Fixed volumes of 0.3 mL and 1.0 mL of yeasts suspension standardized in 10^2 CFU/mL were used to frame the analysis groups, with incrimination levels of 10-30 CFU/plate and 30 - 300 CFU/plate. The eligibility criterion to prove the accuracy of the method proposed by USP 30-NF25 presented the yeasts cells recovery of less than 70%.

The linearity was the ability (within a given range of application) to produce test results that are directly proportional to the concentration (quantity) of the studied parameter in the sample. The linearity of the method was evaluated through the correlation among different volumes (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mL) and their corresponding Colony Forming Units. The results were analyzed by determination of the linear regression using linear correlation (R^2) that was at least 0.95.

The robustness of the method presents an ability to remain unaffected by intentional variations of the parameters of method. Robustness was evaluated through the application of changes to the microbiological method conditions. This was performed by different medium and incubating at different temperatures.

Statistical analyses. Statistical processing of results was done using program Statistica 7.0. that is a data analysis and visualization program. Relative standard deviation (RSD) is a special form of the standard deviation and was used to determine the precision of obtained data. The average result, $\bar{x}$ is calculated by summing the individual results and dividing this sum by the number (n) of individual values:

RESULTS

For the validation of the microbiological test the method for determining the total number of colonies was selected. Precision is usually expressed by relative standard deviation (RSD). The eligibility criterion should not exceed 10%. In the repeatability parameter, the experimental group inoculated with two different yeasts concentrations (10 30 CFU/plate and 30-300 CFU/plate) had lower coefficients of variation than those recommended by USP-30. *Repeatability.* Five groups of experimental samples with concentrations of 0 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L were analyzed on the same day and under the same conditions (Table 1). RSD ranges from 3.11-4.89% for the 10-30 CFU/Petri dish batch and between 0.67-1.49% for to 30-300 CFU/Petri dish respectivley. The repeatability results indicate that the test method is accurate, with the relative standard deviation being less than 10%.

Determination of the intermediate precision was done by 2 analytical procedure in the research. This is an experiment, data are obtained from day 1 and from day 2 by the two analysts. Were analyzed nanoparticles of ZnO of concentrations of 0 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L (Table 2). Research was carried out in three repetitions. The eligibility criterion was expressed by the relative standard deviation, which should not exceed 10%. RSD values vary within the limits of 2.32-4.80%, indicating that the test method is accurate under the conditions of the intermediate precision.

Accuracy. The accuracy of the method was demonstrated by microbiological recovery using 5 experimental samples. Determination of recovery percentage has shown that values prevail 70% (Table 3). The data goes within the limits of microbiological recovery established under the USP 30-NF25 protocol.

The test method is accurate and confirmed by the Student test.

Linearity. The studied method showed a linearity between the number of CFUs determined in 5 experimental samples and the volume of the cell suspension (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mL) (Table 4). Correlation coefficient (R^2) values range from 0.922-0.970. These values coincide with the values set by USP 30-NF25, $R^2 \geq 0.95$. The data are confirmed by the Fisher criterion. **Robustness**. The robustness of the method has been investigated through variation in temperature and cultivation medium. The determinations were repeated 3 times for each sample tested (Table 5). Considering changes in temperature, cultivation medium, the results of determining the number of colonies are not statistically different from those obtained under standard conditions.

Table 1. Repeatability of the method of determining the total number of colonies

Number of sample	CFU/Petri dish	RSD %
Sample 1 - Control	10-30	**3.77**
	30-300	**0.84**
Sample 2 – 1.0	10-30	**4.89**
	30-300	**1.02**
Sample 3 – 5.0	10-30	**4.15**
	30-300	**1.49**
Sample 4 – 10.0	10-30	**3.65**
	30-300	**0.67**
Sample 5 – 20.0	10-30	**3.11**
	30-300	**0.75**

CFU=Colony Forming Units, RSD= relative standard deviation

Tabel 2. The intermediate precision of the method for determining the total number of colonies for the strain *Rhodotorula gracilis* CNMN-Y-03 with F tab and F cal values, 95% confidence interval

Analytical	Sample 1 - Control CFU	Sample 2 -1.0 mg/L CFU	Sample 3 -5.0 mg/L CFU	Sample 4 -10.0 mg/L CFU	Sample 5 -20.0 mg/L CFU
1	155	166	170	244	208
1	170	180	184	228	228
1	162	173	177	236	218
$\bar{x}$	162	173	177	236	218
RSD, %	4.62	4.04	3.95	3.38	4.58
$\mathbf{F_{tab}}$	9.11	9.11	9.11	9.11	9.11
$\mathbf{F_{cal}}$	1.62	1.62	1.62	1.62	1.62
2	160	160	165	240	210
2	168	178	180	220	215
2	167	174	179	229	220
$\bar{x}$	165	170	175	229	215
RSD, %	2.64	5.53	4.80	4.36	2.32
$\mathbf{F_{tab}}$	9.11	9.11	9.11	9.11	9.11
$\mathbf{F_{cal}}$	1.45	1.45	1.45	1.45	1.45

CFU=Colony Forming Units, $\bar{x}$= average, F tab= F distribution tabulated and F cal= F distribution calculated

Table 3. The accuracy of the method for determining the total number of colonies for the *Rhodotorula gracilis* CNMN-Y-03 strain with t tab and t cal values, the confidence interval of 95%

ZnO NPs (50 nm)	CFU ±SD	R^2	F_{tab}	F_{cal}
Sample 1 - Control	174±8.62	0.945	**9.117182**	**1.83**
Sample 2 -1.0	184±5.97	0.970	**9.117182**	**1.09**
Sample 3-5.0	204±7.79	0.943	**9.117182**	**2.04**
Sample 4-10.0	247±6.88	0.942	**9.117182**	**2.32**
Sample 5-20.0	262±3.20	0.922	**9.117182**	**0.56**

CFU=Colony Forming Units, SD= standard deviation, R^2= correlation coefficient, F tab= F distribution tabulated and F cal= F distribution calculated

Table 4. Linearity of the total colony counting method, 95% confidence interval, SD-relative deviation, R^2-correlation coefficient

Concentrations of ZnO NPs (<50 nm), mg/L	10-30 CFU/Petri dish			30-300 CFU/Petri dish		
	%R	t_{tab}	t_{cal}	%R	t_{tab}	t_{cal}
Control	100		2.30	100		1.41
1.0	113		1.63	132		1.72
5.0	127	2.77	2.52	128	2.77	1.75
10.0	153		1.36	118		1.76
20	167		1.51	115		1.63

CFU=Colony Forming Units, R= recovery percentage, t_{tab} =T distribution tabulated, t_{cal}= T distribution calculated

Table 5. The robustness of the method for determining the total number of colonies with the respective values F tab and F cal, the 95% confidence interval.

Temperature (˚C)	Sample 1-Control (X±SD)	Sample 2- 1.0 (X±SD)	Sample 3-5.0 (X±SD)	Sample 4-10.0 (X±SD)	Sample 5 -20.0 (X±SD)
20±2	97.5±1.53	52±2.92	100±5.00	116.5±1.28	139±6.72
25±2	105±0.95	73.5±0.68	110±1.82	134.5±3.34	123±3.4
30±2	115±3.47	95±5.26	132±5.8	131±4.58	152±2.6
F_{tab}	9.01	9.01	9.01	9.01	9.01
F_{cal}	1.13	2.84	3.6	1.72	2.29
Cultivation medium	**Sample 1-Control (X±SD)**	**Sample 2- 1.0 (X±SD)**	**Sample 3-5.0 (X±SD)**	**Sample 4-10.0 (X±SD)**	**Sample 5 -20.0 (X±SD)**
YPD (1)	162.5±4.3	173±4.04	177±3.95	218±4.58	236±3.38
YPD (2)	158±6.0	172±6.97	171±3.50	207±3.38	218±1.60
Malt wort	120±3.33	122±2.45	128±0.87	152±2.63	154.3±1.62
F_{tab}	9.01	9.01	9.01	9.01	9.01
F_{cal}	1.6	2.1	1.74	0.88	0.90

X=average, SD=standard deviation, F_{tab} =F distribution tabulated, F_{cal}= F distribution calculated

DISCUSSION

A series of previous studies has indicated that data referring to the behaviour and biological and toxic effect of nanoparticles is an important part of scientific literature [8, 12, 25, 27]. The development of nanotechnology creates preconditions for interpretation, validation of results referring to the interaction of cell and nanoparticles. Living organisms can serve as objects for testing of nanoparticle action. Our data are consistent with the scientific data of researchers which have demonstrated that the effect of various nanoparticles can be studied using microorganisms and bacteria[1, 19, 35]. There are few researches referring to the potential impact of nanoparticles on the microbial growth aspects [22, 33]. Thus recent research have studied the effect of metal nanoparticles on microbial growth dynamics and their toxic and biological effects [24, 32, 34]. Therefore, development of a rapid, sensitive and accurate method for counting of bacteria in the presence of nanoparticles is necessary for cosmetic, food industry and medicine.

This paper validates a microbial counting method for the testing of effects of nanoparticles with the utilization of yeasts. The test demonstrates that the methodology is adequate for the purposes to determine the effects of metal oxide nanoparticles on microorganisms. The study can be applied to evaluate the effects of nanomaterials at the cellular level. Microbial counting method for pigmented yeasts differ fundamentally from known physicochemical and biochemical methods. Therefore, it is not enough to use well-known validation procedures with approved parameters.

According to Zheng (2010), a new method with the utilization of luminous bacteria can be used for rapid determination of nanotoxicity [35]. The similar method of evaluation of the toxicity of nanoparticles using the test of bacterial bioluminescence was proposed by Sizova [28]. The bioluminescent method is more expensive and selective. It is not valid for all types of nanoparticles. Other techniques used, such as optical density tests have some disadvantages. Thus, only viable bacteria are counted with this method. These tests may provide questionable results referring the nanoparticle toxicity. The proposed in this paper test for determination of total colony number has the capacity for counting of any type of microorganisms. This optimized method could bring additional info to the existing conventional methods such as light scattering spectrometry and flow cytometry [14, 31].

The obtained results in this research are consistent with the results of Oliveira (2019) [19]. Thus, the validation of a microbial enumeration method - the membrane filtration method was studied. Other method for the validation colony counting system was described by Frost et al. (2016) [10]. The method is expensive, time-consuming and unselective. Whilst automated counting procedures have been validated for a number of microorganisms, the process has not been successful for all bacteria due to the requirement for a relatively high contrast between bacterial colonies and growth medium.

The microbial counting method using agar plates could be used as a method to estimate the influence of metal nanoparticles on yeast cells. The possibility of using the microbiological method to study the effect of minimum concentrations of nanoparticles on yeast cells is evidentiated. So, proposed in this research microbial counting method is used to determine the effects of nanoparticles on living organisms. This method with the utilization of yeasts has some advantages because there is no need to use animals for testing, simplicity and possibility to be performed under controlled conditions. The validation was performed in order to ensure the safety of microbial counting method. The high performance of the technique is associated with its ability to be easily implemented in routine lab applications. Thus, there is a necessity to adapt various measurement techniques for testing of nanomaterials.

The elaborated new valid tests that establish the effect of nanoparticles on yeasts cells might contribute to the enhancing of the utilization of various kinds of nanomaterials offering a potential platform for innovative application. Thus, method has proved precise, accurate, linear and robust. We have to mention that the proposed method is the promising one for the testing the effects of ZnO nanoparticles on microorganisms. The obtained results could have an

impact on the utilization of different types of nanoparticles in such fields as medicine, pharmaceutical and cosmetic industry.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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Received: August 19, 2021
Accepted: March 31, 2022
Published Online: Aprilie 4, 2022
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House