

II. МИКРООРГАНИЗМИ И ОКОЛНА СРЕДА

COMPARATIVE STUDY OF YEAST STRAINS' POTENTIAL TO ACCUMULATE CADMIUM IONS – A PERSPECTIVE PATHWAY FOR FUTURE BIOTECHNOLOGICAL NANOPARTICLE FORMATION

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Abstract. Nanoparticles' unique properties and applications define the practical interest towards them and the consequent elaboration of different synthesis strategies. Understanding the process of biomineralization and the identification of microorganisms capable of producing nanoparticles under defined conditions, unveils new perspectives for the nanotechnology and facilitate its fusion with biotechnology. The main objective of the present scientific work was to compare selected biosynthetic and metabolic activities of yeast strains isolated from soil. As the bionanoparticle-formation mechanisms in yeasts are strain- and cultivation conditions-specific, the foreseen experiments aimed eventually to recognize and introduce new promising yeast strains with high potential for the synthesis of CdS bionanoparticles. Soil-isolated yeast strain designated as *S. pombe* NK05/2 demonstrated highest levels of specific cadmium accumulation – 8.8 and 13.75 mg.g⁻¹ BDM (bio dry mass) for both media tested, and exhibit the best biosynthetic characteristics.

Keywords: nanoparticles, yeast, cadmium, biomineralization

1. INTRODUCTION

Ecological and biological balance in soils is preserved by bioremediation. This process is based on the ability of microorganisms to exploit the metabolic potential of their cells to transform, decompose and immobilize different contaminants, including heavy metals. Therefore metal-containing soils are a potential reservoir for nanoparticle - producing microorganisms with commercial value. In order to populate different ecological niches, microorganisms have adopted special cell functions. The three main reasons for them to produce nanoparticles are (1) chemolithotrophy for energy production, (2) use of these particles for special functions and (3) detoxification for survival in toxic environments. All those well-known mechanisms are the basis for a new, nature-inspired approach for inorganic nanoparticles production.

In order to survive in toxic environments microorganisms have developed several detoxification mechanisms. While some metal ions such as cadmium and mercury are nonessential and toxic, others like copper and zinc are essential for the normal physiological functions of living organisms [1]. Still, increased levels of essential metal ions can also become lethal. Therefore survival of the cells depends on the mechanisms and the capability to regulate the intracellular concentrations of these metal ions. Insoluble nontoxic nano-clusters of Ag⁰, Au⁰, ZnS, CdS and Ag₂S have been found in different microorganisms [2].

Strategies such as enzymatic oxidation or reduction, sorption on the cell wall and in some cases subsequent chelating with extracellular peptides or

polysaccharides have been developed and used by microorganisms. Some species can control the membrane transport of heavy metals towards, or their active efflux from the cell [3, 4]. Metal ion resistance via transport and passive mechanisms leading to extracellular precipitation is more characteristic for prokaryotes [5] whereas sequestration mechanisms are mainly adopted by eukaryotes [6]. Thus, cells can not only form complex molecules but also complex spatial structures, which are controlled by diffusion and self-organization of vesicles.

Of all the eukaryotes, yeast species are probably the most studied and applied in bioprocesses, which qualifies them as an attractive object for nanoparticles synthesis. Although recognized mainly for their ability to produce semiconductor nanoparticles, particularly cadmium sulfide (CdS), recent studies demonstrate the potential of yeasts to form other nanoparticle types [7, 8, 9].

Schizosaccharomyces pombe, referred also as fission yeast, and first described by P. Lindner in 1893 [10], are easy to cultivate and suitable for genetic and molecular manipulations, which qualifies them as a desired object for scientific experiments [11]. Profound studies explore the CdS nanoparticle-formation mechanism in *S. pombe*. The biomineralization of cadmium by this yeast specie is a metal-triggered biotransformation, in which metal ions are consequently chelated with small selective peptides (phytochelatins) and co-precipitated with inorganic sulfur, resulting in non-toxic CdS clusters [12].

Phytochelatin coated nanoparticles derived from *S. pombe* are analogous to the semiconductor quantum dots studied in solid state physics. The semiconductor

properties of some nanoparticles are the basis for the fabrication of better batteries [13], catalysts [14, 15], solar [14, 16] and fuel cells [14, 17]. Biocompatible peptide-covered nanoparticles are part of specific sensor systems used in natural sciences [18, 19]; applied in molecular recognition based immunoassays [18] and used as biomarkers for in vivo investigations of intracellular processes [16]. Yeast synthesis mechanism overcomes one of the biggest disadvantages of physical and chemical synthetic pathways - the agglomeration into larger particles. Nanocrystals derived from yeast are naturally stabilized by the phytochelatin layer, which also effectively controls particle size – 1.8 nm for *S. pombe* [20].

The main objective of the present scientific work was to compare selected biosynthetic and metabolic activities of yeast strains isolated from soil. The investigations were focused on identifying the most relevant and effective yeast characteristics regarding the future biotechnological nanoparticle formation. As the bionanoparticle-formation mechanisms in yeasts are strain- and cultivation conditions-specific, the foreseen experiments aimed eventually to recognize and introduce new promising yeast strains with high potential for the synthesis of CdS bionanoparticles.

2. MATERIALS AND METHODS

Yeast strains

The following yeast strains were used: *Schizosaccharomyces pombe* var. *pombe* Lindner L972h-(DSMZ No. 70576), as well as *S. pombe* NK05/1, *S. pombe* NK05/2 and *S. pombe* NK05/3, isolated from soil samples. All strains were kept under 4°C in test tubes containing MYGP-agar (malt extract yeast extract glucose peptone) medium.

Cultivation media and conditions

“Rich” medium with composition g.l⁻¹: malt extract 6.0, yeast extract 6.0, D-Glucose 20.0, peptone 10.0, ammonium sulfate 1.4, pH 5.8±0.2 at 25°C.

“Poor” medium with composition g.l⁻¹: yeast extract 5.0, D-Glucose 10.0, ammonium sulfate 1.4, pH 5.8±0.2 at 25°C.

Cultivations were conducted in 500 ml flasks, containing 100 ml medium described above. Each flask was inoculated with 5 % 24 hours inoculum culture and incubated at 30°C on a rotary shaker Unimax 2010 (Heidolph, Germany) at 120 min⁻¹. Cadmium ions in concentration of 1 mM as a Cd(NO₃)₂ solution (Merck, Germany) were

introduced under sterile conditions twelve hours after the inoculation. Cultivations' duration was 28 hours, as the end of the process was determined based on the biomass concentrations and for the cultivations with cadmium addition – based on the alteration of cadmium concentration. All samples were taken under sterile conditions in laminar flow bench.

Analysis

Biomass concentration (c_x) was determined either gravimetrically by drying the centrifuged (7400×g for 10 min) and three times washed cells with MilliQ water (>18MΩ) from two 20-ml samples dried at 80°C until constant weight – bio dry mass (BDM), or via optical density at 550 nm (OD550), measured in a Varian-Cary 50 Conc. UV-Vis Spectrometer with the calibration factor $c_x/OD550$ being 0.155 g l⁻¹.

Glucose concentration was determined photometrically using a glucose test-kit (Glucose liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH).

Cadmium concentrations were analyzed via inductively coupled plasma optical emission spectroscopy (ICP OES) (Vista-Pro, Varian). Samples were diluted in 1% HNO₃ prior to analysis.

3. RESULTS AND DISCUSSION

The successful isolation and correct identification of yeast species is essential for the selection of new strains – producers of nanoparticles. In the present work microbial species were primary isolated from soil samples, obtained from regions, contaminated with heavy metals [21]. Environmental pollutions are complex and affect the whole ecosystem. The selection of natural sample source was based on the reports regarding the influence of heavy metals as a stress factor upon the soil microbiota [22, 23]. Processing of the collected soil samples resulted in 144 isolates containing fungi, actinomycetes and yeasts. After identification procedures, including microscopy analysis and investigation of morphological and physiological characteristics (results not presented), from the yeast quota out of 26 isolates successfully identified were three, classified as *Schizosaccharomyces pombe* (Fig. 1). Specifics and activities of the identified three strains were further tested in comparison to a collection strain *S. pombe* DSMZ No. 70576 with known characteristics.

The isolated strains *S. pombe* NK05/1, *S. pombe* NK05/2 and *S. pombe* NK05/3 were investigated regarding their dynamics, growth on rich and poor

media and the capacity to accumulate cadmium. Parallel, under the same conditions, experiments with the strain *S. pombe* var. *pombe* Lindner L972h- (DSMZ No. 70576) intended to reveal a possible impact of the high soil cadmium content upon the

metabolism and the detoxification capacity of the isolated strains. Figure 2 represents the alteration of biomass and glucose concentrations of the four yeast strains tested as time functions, under the shaking flask conditions described

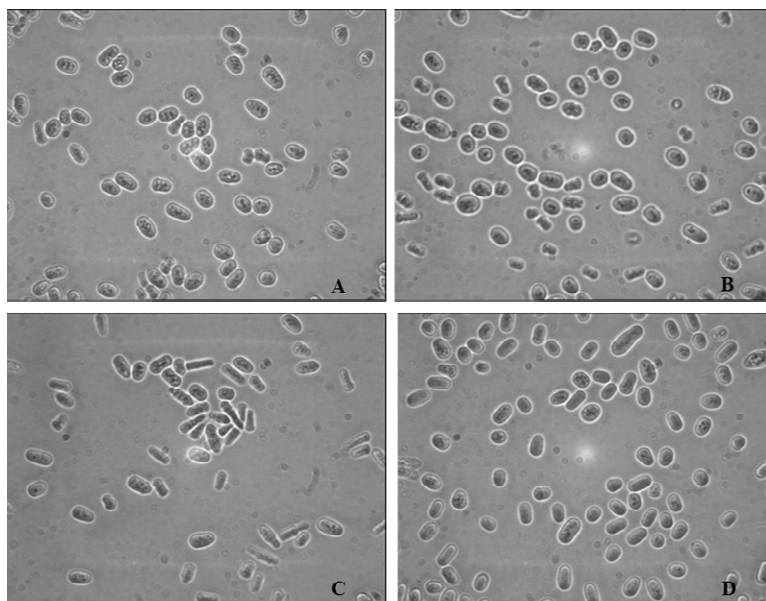


Fig. 1. Microscopic images of the four tested yeast strains: A - *S. pombe* NK05/1; B - *S. pombe* NK05/2; C - *S. pombe* NK05/3; D - *S. pombe* var. *pombe* Lindner L972h- (DSMZ No. 70576)

Growth kinetics revealed that the strains enter exponential phase after four hours of cultivation except for the strain *S. pombe* NK05/3 entering exponential phase after six hours. Different pattern is demonstrated also regarding the utilization of the main carbon source – glucose. During the experiments strain *S. pombe* NK05/3 metabolized after four hours 70 % of the glucose, introduced in the medium, without sufficient influence upon biomass' concentration. The exponential growth and the biomass accumulation started only after the sinking of the glucose concentration in the medium below 1 g.l^{-1} . A possible explanation for these results is the initial ethanol formation and accumulation and the following diauxie – a process described for several *S. pombe* strains [24]. The glucose metabolization was slowest for strain *S. pombe* NK05/2 where twelve hours after process' start the glucose concentration in the medium was 45 % from the initial one, with 30 % and 8 % for strains *S. pombe* NK05/1 and *S. pombe* DSMZ No. 70576 respectively. Significant from practical point of view is the biomass yield regarding the substrate, designated with the coefficient Y ($\text{g biomass.g substrate}^{-1}$). This coefficient was highest for strain *S. pombe* NK05/3 - $Y=0.27$ and for *S. pombe* NK05/1 - $Y=0.22 \text{ g biomass.g substrate}^{-1}$. The yields for the rest two strains were $Y=0.18$ for *S. pombe* NK05/2 and $Y=0.19$ for *S.*

pombe DSMZ No. 70576 respectively. These values are slightly higher or close to results obtained by different authors experimenting with other *S. pombe* strains. Barford [25] reports yield of $Y=0.174 \text{ g biomass.g substrate}^{-1}$ after cultivating *S. pombe* in shaking flask conditions. This result is comparable with yield values from *Saccharomyces cerevisiae* and characteristic for microorganisms with predominantly fermentative metabolism – low biomass yield and ethanol formation [25]. Comparative study of basic culture characteristics for the four strains tested is presented on Table 1.

According to Williams et al. [26] the CdS nanoparticles yield could be enhanced by increasing the biomass yield. From all four strains studied, the highest final biomass concentration demonstrated strain *S. pombe* NK05/1, which in addition exhibit yield coefficient of $Y=0.22$, that is higher than the average for that yeast species. The best strain, regarding this coefficient, is *S. pombe* NK05/3, characterized by the second highest final biomass concentration. In biotechnological processes, where the biomass concentration is directly proportional to the product yield, strains *S. pombe* NK05/1 and *S. pombe* NK05/3, based on their characteristics, could be recognized as most perspective ones.

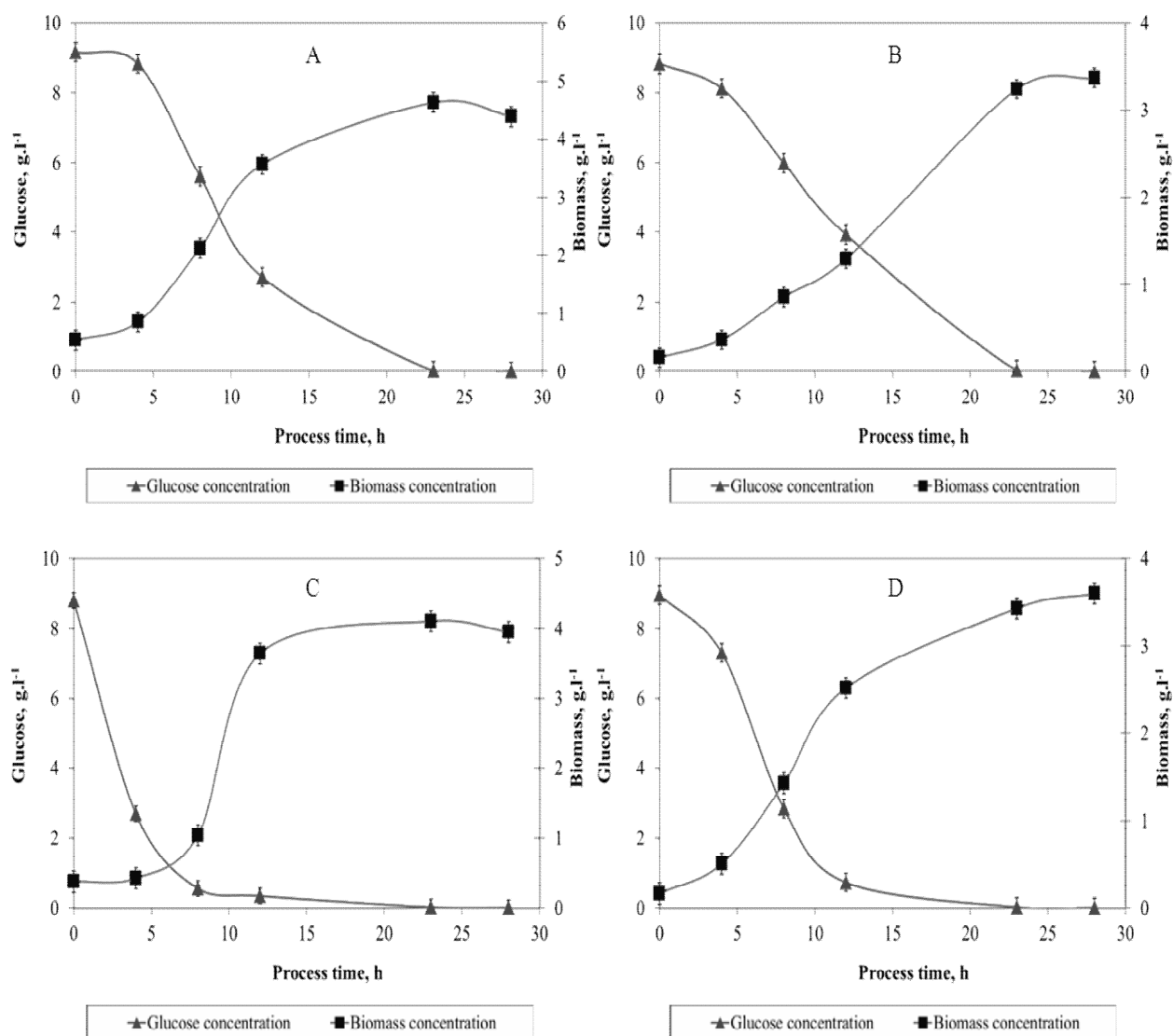


Fig. 2. Growth kinetics of the investigated yeast strains: A - *S. pombe* NK05/1; B - *S. pombe* NK05/2; C - *S. pombe* NK05/3; D - *S. pombe* var. *pombe* Lindner L972h-(DSMZ No. 70576)

Table 1. Basic culture characteristics of the yeast strains tested

Strain	Biomass BDM, g.l ⁻¹	μ_{max} , h ⁻¹	Y, g bio70mass.g substrate ⁻¹
<i>S. pombe</i> NK05/1	4.5	0.178	0.22
<i>S. pombe</i> NK05/2	3.3	0.158	0.18
<i>S. pombe</i> NK05/3	4.0	0.269	0.27
<i>S. pombe</i> DSMZ No. 70576	3.6	0.200	0.19

As the biotechnological production of CdS nanoparticles is a complex process directed by series of factors and conditions, the choice of adequate strain is determined not only by the biosynthetic potential of the cells but also by their performance in cadmium containing media.

Shaking flask cultivation using poor medium aimed a mimic reproduction of nature's soil conditions – natural habitat for the isolated strains, to observe their behavior in these conditions and by the use of less-components media to introduce economic aspect into the approach. Of interest was the performance of the strains in media with and without cadmium (Fig. 3).

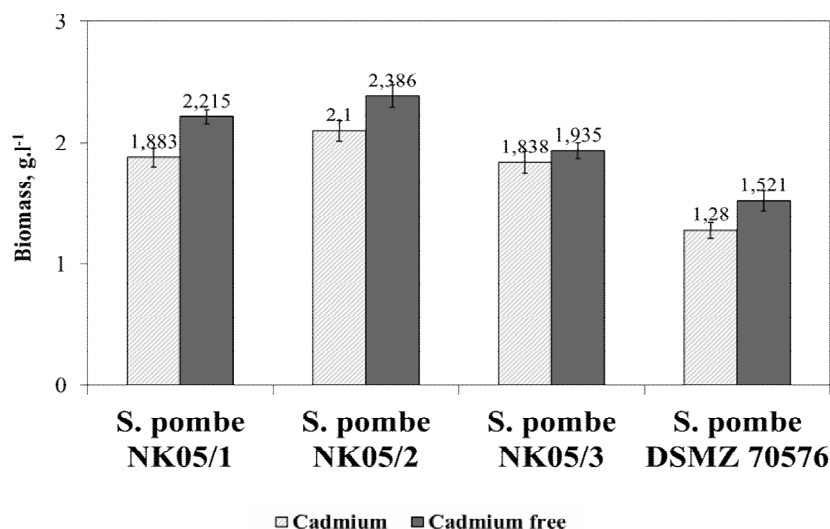


Fig. 3. Final biomass concentration BDM after poor medium shaking flask cultivation with and without cadmium

The objective of this experiment was to demonstrate the level of impact that cadmium ions have upon yeast cells' metabolism. Cadmium ions in the form of $\text{Cd}(\text{NO}_3)_2$ solution were introduced into the culture media twelve hours after the start of the cultivation. The time period for cadmium addition was selected in order to avoid inhibition of yeast growth in a cultivation phase with lower cell concentration and more importantly the introduction of cadmium in mid- to late exponential phase ensures maximum formation of CdS nanoparticles [27, 28].

The final biomass concentrations from strains *S. pombe* NK05/1, *S. pombe* NK05/3 and *S. pombe* DSMZ No. 70576, cultivated using poor medium, were respectively with 50 %, 52 % and 58 % lower than the concentrations, obtained by cultivations using rich medium. Of interest was the final biomass concentration of strain *S. pombe* NK05/2 cultivated with poor medium, which was only 28 % lower than the one derived from cultivation with rich medium. The combination of this yeast strain and a poor medium led to a better cell growth, respectively higher biomass concentration, compared with the other three strains, presumably as a result from the better adaptation of the metabolism of *S. pombe* NK05/2 towards habitat's conditions and the

ongoing natural selection of beneficial properties and activities. This assumption is supported by the comparison between the three soil-isolated strains and the one originating from culture collection. The strain *S. pombe* DSMZ No. 70576, cultivated with poor medium, demonstrated the lowest biomass formation from all four strains tested, with final biomass concentration 30 % lower than the one from the other strains. The cultivation of all four compared *S. pombe* strains, with addition of cadmium ions in the medium, had no significant effect on the final biomass concentrations. The results demonstrate that all strains possess well-functioning mechanisms to neutralize the stress factor being the cadmium ions.

The final CdS nanoparticle yield of a single cultivation is determined by two major factors: the initial glucose concentration in the feeding medium, which determines the final biomass concentration, and the CdS saturation level, that limits the amount of intracellular CdS nanoparticles in every cell [26]. The biosynthetic potential of all four strains to produce CdS nanoparticles was investigated by comparing the levels of specific cadmium accumulation – an indicator revealing the relation between the amount of the accumulated cadmium

and the yeast biomass (BDM) at the end of the cultivation process. Figure 4 presents the levels of specific cadmium accumulation from all four strains tested on both rich and poor medium.

The results obtained from cultivations, using rich medium (Fig. 4 A), revealed that the isolated from the soil strains demonstrate lower levels of specific cadmium accumulation in comparison with the collection strain. From the three nature isolated strains *S. pombe* NK05/2 demonstrated highest level

of specific cadmium accumulation of 8.8 mg.g^{-1} BDM, although regarding other indicators such as final biomass concentration, specific growth rate and yield it remained last from all four strains tested. *S. pombe* NK05/3 characterized by the highest μ and Y , demonstrated the lowest level of specific cadmium accumulation of 4.58 mg.g^{-1} BDM, suggesting that for the production of CdS nanoparticles, biomass concentration is important but not decisive criterion for the strain effectiveness.

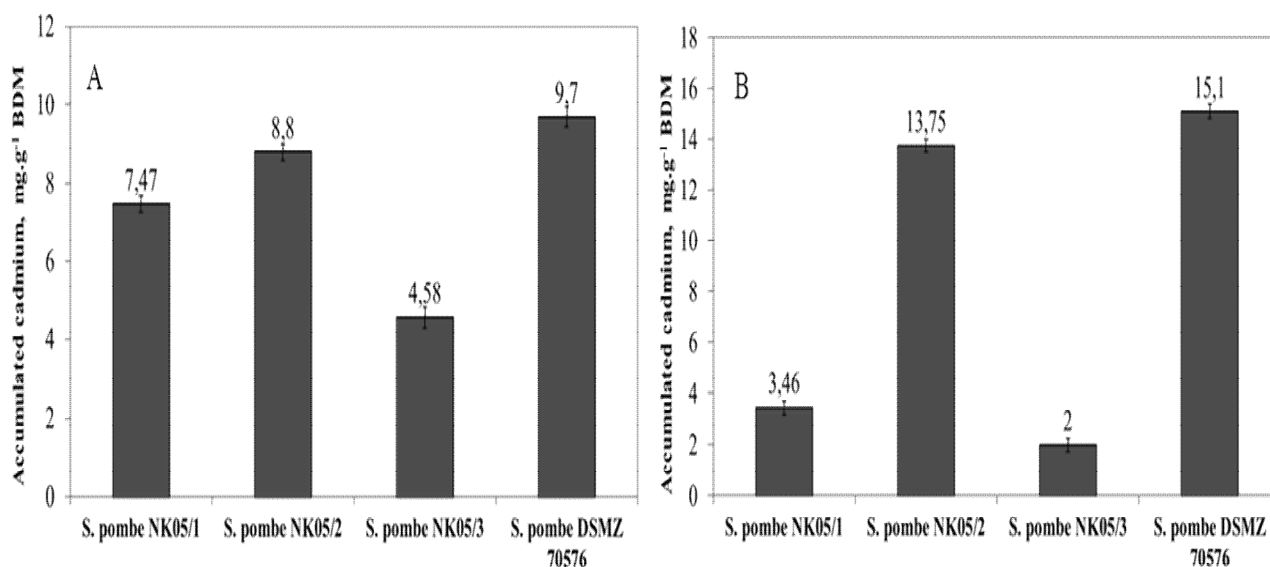


Fig. 4. Levels of specific cadmium accumulation of investigated strains and different media:
A – rich medium; B – poor medium

The experiments, using poor medium (Fig. 4 B) revealed double decrease in the specific cadmium accumulation for strains *S. pombe* NK05/1 and *S. pombe* NK05/3 compared to the results from the rich medium cultivations. Of interest are the levels of specific cadmium accumulation from strains *S. pombe* NK05/2 - 13.75 mg.g^{-1} BDM and *S. pombe* DSMZ No. 70576 - 15.1 mg.g^{-1} BDM, respectively with 56 % and 55 % higher than the levels demonstrated from the same strains but grown using rich medium. These results suggest that the amount of accumulated cadmium is related to greater extent to the medium composition. The significance of the medium composition for the formation of CdS nanoparticles with peptide layer is outlined by a number of authors. For the first time Dameron et al. report that the type of the peptide layer, stabilizing the nanoparticles, is predetermined by the rich or poor medium for the cultivation of the yeast *Candida glabrata* [29]. Our results confirm this correlation for strains from the *S. pombe* species.

For both experiments with rich and poor media, the results obtained for accumulated cadmium are several times higher than previously reported. Kowshik et al. describes, under similar conditions, specific cadmium accumulation of 1.4 mg.g^{-1} BDM [30]. This comparison demonstrates the feasibility and the perspective of the soil-isolated strains.

Conclusion and analysis of the results reveals that from the three isolated strains best characteristics, regarding the biosynthesis of CdS nanoparticles, possess strain *S. pombe* NK05/2. For the two media tested this strain demonstrated highest levels of specific cadmium accumulation – 8.8 and 13.75 mg.g^{-1} BDM for rich and poor medium respectively. From all four strains tested it expressed the best poor medium growth with highest final biomass concentration. These characteristics define strain *S. pombe* NK05/2 as most perspective, from the soil-isolated strains, for further investigations together with *S. pombe* DSMZ No. 70576 that exhibit the best biosynthetic characteristics.

4. CONCLUSIONS

The level of specific cadmium accumulation is a good but indirect indicator for the biosynthetic potential of yeast cells to produce CdS nanoparticles. The amount of accumulated cadmium is not directly equal to the amount of intracellular nanoparticles formed. Part of the accumulated cadmium could be later discharged from the cell by means of controlled cell-membrane efflux mechanisms or detoxified by other chelating molecules. The results obtained clearly demonstrate the difference in the metabolism and the detoxification potential of all four yeast strains tested and their ability to survive under metal-stress conditions. The present work reveals Nature's potential, after well-directed selection of natural sample-sources, to function as a pool for adapted strain-producers with naturally improved characteristics and properties, interesting for further applications. The full potential and applicability of the isolated and compared yeast strains can be revealed only after further profound and specialized studies regarding properties and characteristics of CdS bionanoparticles, isolated from the cells, which is a prospect for our further investigations.

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СРАВНИТЕЛНО ИЗСЛЕДВАНЕ НА ВЪЗМОЖНОСТИТЕ НА ДРОЖДЕВИ ЩАМОВЕ ДА АКУМУЛИРАТ КАДМИЕВИ ЙОНИ – ПЕРСПЕКТИВЕН ПОДХОД ЗА БЪДЕЩО БИОТЕХНОЛОГИЧНО ПОЛУЧАВАНЕ НА НАНОЧАСТИЦИ

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Резюме. Уникалните свойства и приложения на наночастиците определят практическия интерес към тях, а от там и разработването на различни синтезни технологии. Познаването на процеса на биоминерализация и откриването на микроорганизми способни да формират наночастици при определени условия, разкрива нови перспективи пред нанотехнологията и осъществява връзката и с биотехнологията. Основната цел на настоящата научна работа бе сравняването на избрани биосинтетични и метаболитни активности на изолирани от почвата дрождеви щамове. Характерният механизъм на детоксификация при дрождите предполага, че щамовата специфичност и условията на култивиране ще оказват силно влияние върху формирането на наночастиците. Експерименталната работа имаше за цел да селектира и предложи нови, обещаващи щамове с потенциал за синтез на кадмиево-сулфидни наночастици. Изолиран от почвени образци дрождеви щам обозначен като *S. pombe* NK05/2 демонстрира най-високи нива на специфично акумулиран кадмий - 8.8 и 13.75 mg.g⁻¹ абсолютно сухи дрожди, при двете тествани хранителни среди и прояви най-подходящите биосинтетични характеристики.

Ключови думи: наночастици, дрожди, кадмий, биоминерализация

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