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COD reduction ability of microorganisms isolated from highly loaded pharmaceutical wastewater pre-treatment process

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Abstract
Efficiency of a biological wastewater treatment process depends on microbial diversity and their ability to degrade specific pollutants. The aim of this study was isolation and identification of predominant microorganisms associated with pharmaceutical wastewater pre-treatment process and evaluation of a chemical oxygen demand (COD) removal efficiency of each individual isolate. There were 65 microorganisms isolated from activated sludge of the JSC "Grindeks" industrial wastewater biological pre-treatment process and subsequently tested for COD reduction in pharmaceutical wastewater containing 2300-3500 mg/l of COD. Out of all isolates 9 bacteria, 5 yeasts and 3 filamentous fungi showed COD reduction. The highest COD reduction levels among all isolates were observed from filamentous fungi *Fusarium udum* (88.7%) and *Fusarium solani* (89.4%). The most effective bacterial strains were *Aeromonas caviae* and *Sphingobacterium thalpophilum*, with 78.1% and 75.9% COD removal, respectively. *Rhodotorula mucilaginosa* was the most effective yeast strain and achieved 76.6% COD reduction.

Keywords: Pharmaceutical wastewater, biodegradation, COD removal, microorganisms

Introduction
Pharmaceutical wastewater is a complex mixture of different organic and inorganic compounds including residues of active pharmaceutical substances, solvents, toxic and biorecalcitrant chemicals that inhibit microbial activity of the activated sludge process and present great challenge for a proper treatment and downstream processing [1]. Furthermore, composition, flow and other characteristics (pH, salinity, etc.) of the wastewater may change considerably from day to day depending on the production stages and intensity, making the biological treatment and downstream processing even more difficult, and the pre-treatment plant of JSC "Grindeks" is no exception [2-3]. Microorganisms within the activated sludge have to face a toxic load and be able to degrade a wide range of chemical compounds in harsh, ever-changing conditions.
JSC "Grindeks" is the leading pharmaceutical company in the Baltic States and produces about 25 different kinds of active pharmaceutical ingredients in heart and cardiovascular, CNS and anticancer medication therapeutic groups. The wastewater from "Grindeks" pharmaceutical production facility is treated in a moving bed biofilm reactor (MBBR) type of pre-treatment plant, after which the wastewater is discharged into the municipal sewer system for the final treatment. Incoming COD load varies from 1 to 2.5 t and the total nitrogen load is in the range from 0.015 to 0.045 t per day, while the wastewater flow varies from 160 to 360 m^3/d. The hydraulic retention time (HRT) of the plant is in the range of 3-6 days and COD removal is 93-94% in average.
This study involved several steps of microbiological examination of the JSC "Grindeks" wastewater treatment process (WWTP) - isolation, identification and characterization of the microorganisms associated with the process. Also, batch experiments on the ability of individual cultures to biodegrade pharmaceutical wastewater containing 2300-3500 mg/l of COD were carried out.

2. Materials and methods
2.1. Isolation of microorganisms
Samples from MBBR process (suspended biomass and biofilm carriers) were collected from JSC "Grindeks" WWTP. The biomass was obtained from biofilm carriers by scraping off a thin layer of biofilm from their plastic surface with the sharp end of a stainless steel spatula, and suspended in sterile distilled water. All

samples were treated in an aseptic manner. Serial dilutions of the samples up to 10^{-8} were prepared for proper isolation of individual colony forming units. The isolation of bacteria involved the use of tryptic glucose yeast extract agar (Biolife) and R2A agar (Conda Laboratorios), and incubation at 28 ^{o}C for 72 hours, while the isolation of yeast and filamentous fungi was performed using the Sabouraud dextrose agar with chloramphenicol (Biolife) and incubation at 26 ^{o}C for 96 hours (yeast) and at 25 ^{o}C for 96 hours (filamentous fungi). Incubation times and temperatures were set to be in the range of optimal growth for mesophilic microorganisms with slightly lower temperatures and increased incubation times for yeasts and filamentous fungi.

2.2. Identification of the microorganisms

2.2.1. Biolog™ Microbial Identification System

As the primary and basic tool for identification of the isolates the GEN III MicroPlate™ and FF MicroPlate™ test panels of the Biolog™ Microbial Identification System (Biolog Inc., USA) were used. The identification was carried out according to the manufacturer's instructions, and the results were processed using the Biolog's identification system software OmniLog® Data Collection.

2.2.2. PCR method

Genomic DNA was extracted from approximately 0.25 g of mycelia or bacterial, or yeast biomass using the method developed by Cenis [4]. Extracted DNA was amplified by PCR with primers ITS4 [5] and ITS1F [6] for fungi or FORB and REVB for bacteria [7-8].

The PCR reactions in Mastercycler Personal (Eppendorf, Germany) were carried out in 50 µl volume. The mixture contained 0.4 µl Hot Start *Taq* DNA Polymerase, 5 µl 10X Hot Start PCR Buffer, 5 µl dNTP Mix, 2 mM each, 4 µl 25 mM $MgCl_2$, 0.75 µl Bovine Serum Albumin 20 mg ml^{-1} (all reagents from Thermo Scientific Fermentas Molecular Biology Solutions, Lithuania), 1 µl of each 25 µM primer (OPERON Biotechnologies, Germany), 30.85 µl sterile distilled water and 1 µl of DNA template. The PCR conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C (for fungi) and 56 °C (for bacteria), 1 min of primer extension at 72 °C (30 cycles) and final extension for 10 min at 72 °C.

Amplified DNA fragments were treated with FastAP™ Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Scientific Fermentas Molecular Biology Solutions, Lithuania) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). The double stranded sequences of PCR amplicons were assembled using Staden Package 1.6.0. A homology search was performed against the National Centre for Biotechnology Information GenBank nucleotide database using the Basic Local Alignment Search Tool.

2.3. Preparation of the pharmaceutical wastewater

The wastewater samples were collected from JSC "Grindeks" WWTP buffer tank containing an agitated wastewater from the production department, and filtered through 1.6 µm Whatman GF/A glass microfiber filters and afterwards through 0.45 µm Millipore Durapore® membrane filters in order to remove the suspended and particulate matter, followed by filtration through 0.2 µm Sartorius Minisart® high flow polyethersulfone membrane syringe filters for a cold sterilization of the wastewater just before the use in batch experiments. The samples with COD values ranging from 2300 to 3500 mg/l were used. When necessary, dilutions with purified water from Millipore RiOs-DI 3 UV water purification system were made. The pH was adjusted using the 20% H_2SO_4 and 30% NaOH. pH values of the wastewater for bacteria and yeast tests were set to pH 7.0 ± 0.2 and for filamentous fungi to pH 5.6 ± 0.2.

2.4. Batch experiments

COD degradation in batch experiments was performed in 250 ml Erlenmeyer flasks, incubated and shaken in a shaker-incubator Biosan ES-20 with a rotation speed set to 250 rpm and temperature set to 28 ^{o}C for bacteria isolates, to 26 ^{o}C for yeast isolates and to 25 ^{o}C for filamentous fungi isolates. To maintain desired pH value, a Sørensen's phosphate buffer solution (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4) for bacteria and yeasts, and a phosphate-citrate acid buffer solution (0.2 M Na_2HPO_4, 0.1 M citric acid) for filamentous fungi of 1/5 of total volume was added. Turbidity measurements were done on 0 and 5th day with Lovibond turbidimeter TurbiDirect. The initial turbidity measurement was done to determine relative starting concentration of the pure cultures suspended into the wastewater and the turbidity was desired to be not lower than 10 and higher than 70 nephelometric turbidity units (NTU). In case of an exceedingly low initial concentration of the microorganism, more of them were added and suspended from the solid media, and if the concentration was too high a filtered wastewater was added. All experiments were carried out in sterile conditions with sterilized flasks and by

observing aseptic work techniques at all time, and prior to test cultures the pH buffer solutions were added. Flasks were sealed with sterile cotton corks.
COD analyses were done by using HACH LANGE cuvette tests LCK014 and LCK514 and the spectrophotometer Hach DR 5000. During 0, 1st, 3rd and 5th experiment days COD was measured. Samples for COD measurements were filtered through 1.6 µm Whatman GF/A glass microfiber filters.

3. Results and discussion

3.1. Identity of the isolates

Table 1 shows summary of identified species which showed COD reduction. Most of the isolates belong to bacteria but yeasts and filamentous fungi were also present.

Table 1: List of species with COD reduction

Taxonomic groups		
Bacteria	Yeasts	Filamentous fungi
Acidovorax delafieldii	*Candida inconspicua**	*Fusarium solani**
Aeromonas caviae	*Galactomyces pseudocandidum**	*Fusarium udum*
Flavobacterium johnsoniae	*Rhodotorula mucilaginosa**	*Pseudallescheria boydii**
Moraxella osloensis	*Trichosporon asahii**	
*Paracoccus versutus**	*Trichosporon domesticum**	
Pseudomonas aeruginosa		
Pseudomonas pseudoalcaligenes		
*Sphingobacterium thalpophilum**		
Tsukamurella inchonensis		

* Identified by PCR method

There were 65 microorganisms isolated from WWTP in total, 33 of which were bacteria, 22 were yeasts and 10 were filamentous fungi. Activity of bacteria was the highest comparing to yeasts and filamentous fungi in the wastewater samples, however only for nine bacterial isolates that showed COD reduction greater than a negative control, the identification was carried out. Other isolates were considered as irrelevant for the further batch tests. Three out of nine identified isolates *Aeromonas caviae*, *Moraxella osloensis* and *Sphingobacterium thalpophilum* achieved a total COD degradation higher than 70% during 5 days of contact time (Fig. 1). Other bacteria showed relatively low degradation activity on pharmaceutical wastewater. It could mean that most of bacteria are involved only in the final stage of biodegradation when the complex chemical compounds already are converted to readily biodegradable and non-toxic molecules. Or it could also indicate on their poor utilization of majority of the substances present in the wastewater. Although it was evident that COD degradation curves continue to increase, experiments were finished on the 5th day, due to hydraulic retention time of JSC "Grindeks" WWTP being no longer than 6 days.
Activity of the yeast isolates was lower than that of the bacteria and only five isolates showed higher COD reduction than it was for negative control. Two isolates out of five (*Galactomyces pseudocandidum* and *Rhodotorula mucilaginosa)* were able to reduce COD above 70%. Reason for that is considered to be the same as for the bacterial isolates.
Activity of the filamentous fungi was the weakest among all isolates, while COD reduction for the identified isolates was the highest, exceeding 75% for each isolate, and some like *Fusarium solani* reached its maximum reduction already after 72 hours after coming into contact with the tested water. The comparatively strong reduction of COD for the filamentous fungi isolates is believed to be caused by the specific enzymatic activity of the isolates.

3.2. COD degradation ability of bacterial isolates

As it can be seen in Figure 1, most of the nine bacteria isolates from the batch tests showed similar COD reduction patterns. The specific contact time of 5 days for all batch experiments was chosen to match the HRT of WWTP, therefore, reduction limits could not be determined and even though there is no practical

significance, the reduction could potentially be higher if the batch tests were carried out for longer periods of time.

As mentioned before, each batch test was provided with a negative control with no microorganisms added to the wastewater. COD reduction of negative control varied from 38.6% to 51.2% and it can be explained by the high concentration of volatile organic compounds (mainly solvents) present in the tested wastewater. Figure 2 shows individual COD reduction ability of each bacterial isolate, obtained by subtracting COD reduction of negative control from the total COD reduction result. The net COD reduction performance of individual isolates varied from 2.1% to 31.6%. The worst result was provided by *Flavobacterium johnsoniae*.

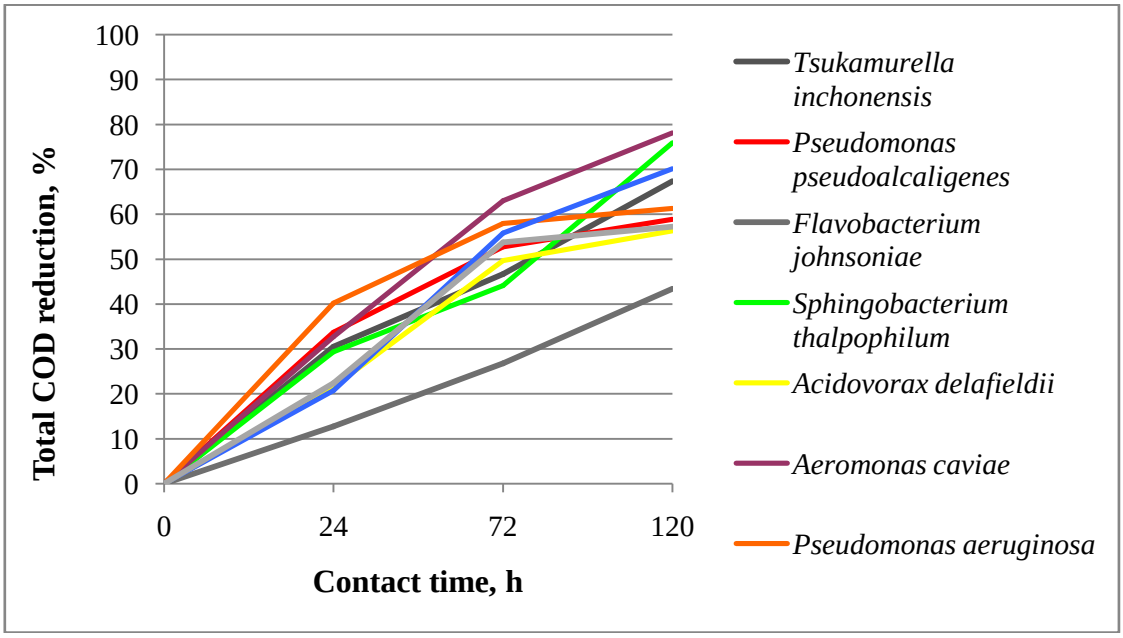

Figure 1: COD reduction of bacterial species

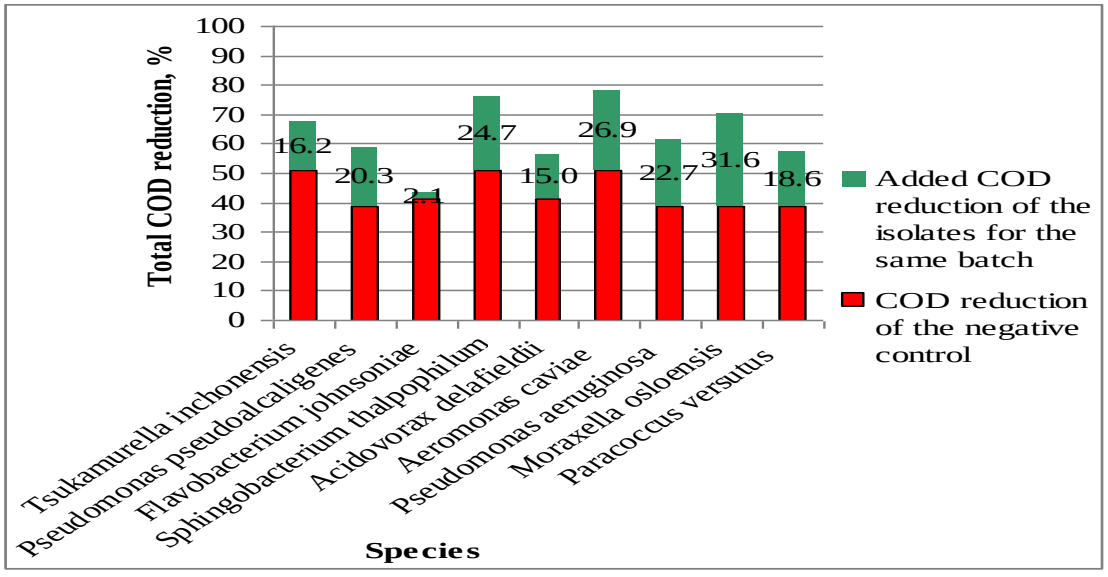

Figure 2: COD reduction performance of the bacterial isolates

Bacteria which showed the highest efficiency in "Grindeks" WWTP are also present in biological treatment of other types of industrial wastewater. *Aeromonas caviae* is known as an efficient biosorbent of toxic heavy metal Cr(VI) from industrial wastewater [9]. *Moraxella osloensis* also has the ability to degrade the acrylamide [10] and synthetic azo dyes in textile industry wastewater [11]. *Sphingobacterium thalpophilum* is known to possess deemulsification properties, and therefore is useful in microbial deemulsification process of oil emulsions in order to recover oil from them [12].

Calculation showed a weak correlation between COD degradation and the increase of turbidity of the batch samples. For example, *Paracoccus versutus* showed only 18.6% of COD reduction, while the turbidity increase for this isolate was the highest, 623.5%. *Pseudomonas pseudoalcaligenes* achieved 20.3% COD degradation, but the turbidity increase was only 165.1%. Increase of turbidity indicates the increase of cell concentration, and from the economical point of view it is not desirable, because of the increased costs for excess sludge removal and recycling.

3.3. COD degradation ability of yeasts and filamentous fungi

Yeasts and filamentous fungi isolates composed almost half of all identified microorganisms with the ability to reduce COD (Table 1). As shown in Figure 3, the potential for the identified yeasts to biodegrade chemical pollution is considerable, and Figure 4 indicates that an average net COD reduction was higher than from bacterial isolates. COD removal efficiency of the five yeast isolates varied from 68.3% to 76.6% with an average net COD reduction of 25.6% compared to bacterial 19.8%. The highest net biodegradation potential of yeasts belonged to *Rhodotorula mucilaginosa*, which is known by its significant ability to absorb heavy metals and to degrade refractory organic pollutants, including petroleum hydrocarbons and polychlorinated biphenyls [15-18].

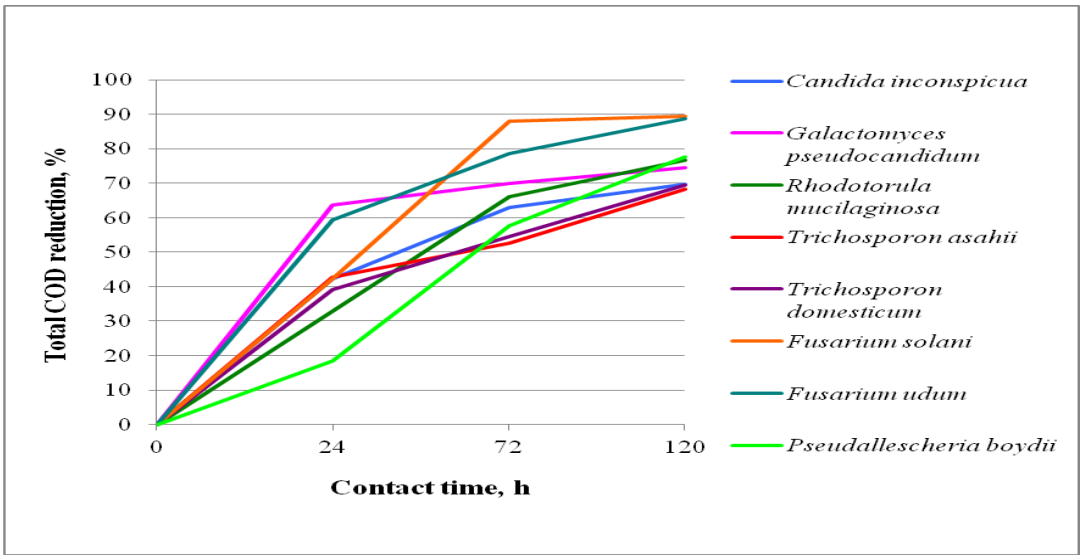

Figure 3: COD reduction of yeasts and filamentous fungi species

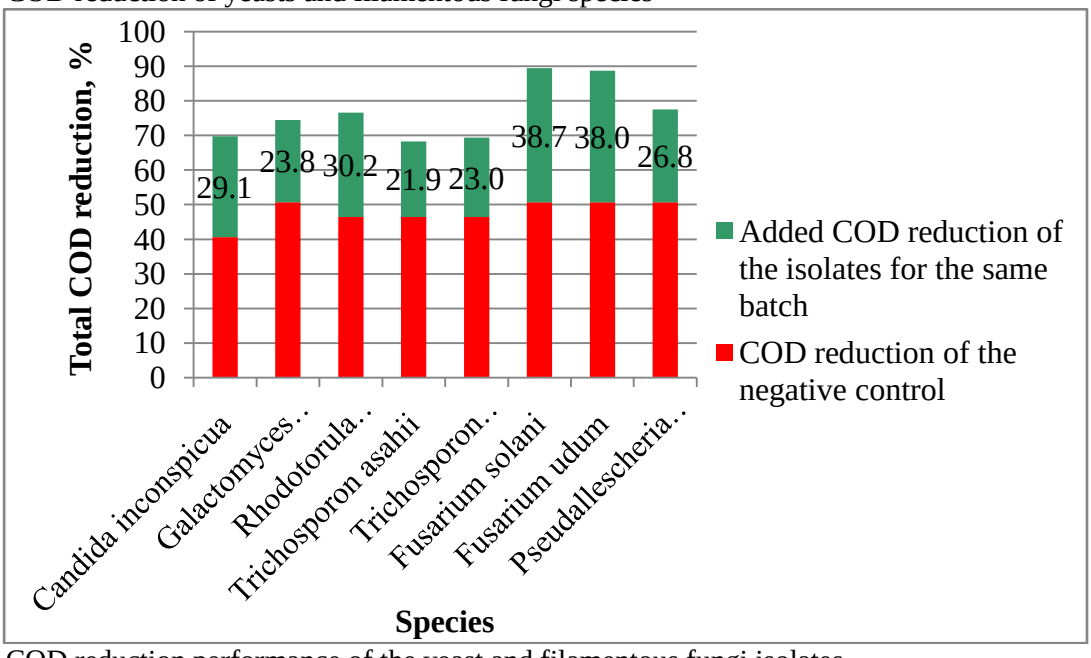

Figure 4: COD reduction performance of the yeast and filamentous fungi isolates

Filamentous fungi showed the highest COD removal efficiency compared to bacteria and yeasts (Fig. 3). The maximum COD reduction of 88.7% and 89.4% was observed from *Fusarium udum* and *Fusarium solani*. Also, the average net COD degradation was 34.5% and the reduction speed was the fastest as well. These filamentous fungi produce laccase enzyme, which can be used for removal/degradation of a number of environmental pollutants, including dyes, phenolic compounds, endocrine disrupting chemicals, polycyclic aromatic hydrocarbons and others xenobiotics [13]. *Fusarium solani* is also useful as a biosorbent for Cr(VI) removal from industrial effluents [14].
As mentioned before the average COD removal efficiency of JSC "Grindeks" wastewater treatment process is 93-94% which is higher than the COD degradation efficiency of individual isolates described in this article, because in real wastewater treatment process activated sludge consist of consortia of microorganisms and in a right combinations they are capable to reach better treatment results.

Conclusions

The highest COD degradation in "Grindeks" pharmaceutical wastewater pre-treatment process was showed by the filamentous fungi species *Fusarium udum* and *Fusarium solani*, which achieved 88.7% and 89.4% COD reduction during the 5-day-long contact time. *Aeromonas caviae* and *Sphingobacterium thalpophilum* showed higher COD degradation efficiency in comparison with other bacterial isolates. The COD degradation achieved by these cultures was 78.1% and 75.9%, respectively. *Rhodotorula mucilaginosa* reduced COD by 76.6% and was determined as the most effective yeast isolate.

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