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Către

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Universitatea " Dunărea de Jos " din Galaţi vă face cunoscut că în data de_____, ora_____, în_____ , va avea loc susţinerea publică a tezei de doctorat intitulată: ***" STUDIUL ŞI MODELAREA BIOPROCESELOR CU ROL ÎN BIOREMEDIEREA APELOR REZIDUALE INDUSTRIALE "***, elaborată de domnul/doamna ***ing. PALELA MIHAELA,*** în vederea conferirii titlului ştiinţific de doctor în Domeniul de doctorat - ***Inginerie industrială.***

Comisia de doctorat are urmatoarea componenţă :

Președinte:	***Prof.univ.dr.ing. Petru ALEXE*** ***Decan – Facultatea de Ştiinţa şi Ingineria Alimentelor*** ***Universitatea "Dunărea de Jos" din Galaţi***
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Referent 3:	***Prof.univ.dr.ing. Sergiu CARAMAN*** ***Universitatea "Dunărea de Jos" din Galaţi***

Cu această ocazie vă transmitem rezumatul tezei de doctorat şi vă invităm să participaţi la susţinerea publică. În cazul în care doriţi să faceţi eventuale aprecieri sau observaţii asupra conţinutului lucrării, vă rugăm să le transmiteţi în scris pe adresa Universităţii, str. Domnească nr. 47, 800008 - Galaţi, Fax - 0236 / 461353.

R E C T O R,
Prof.dr.ing. Viorel MINZU

SECRETAR DOCTORAT,
Ing. Laiza AXINTE

„DUNAREA DE JOS" UNIVERSITY, GALAȚI
FACULTY OF
"FOOD SCIENCE AND ENGINEERING"

PhD thesis

STUDY AND MODELLING OF THE BIOPROCESSES INVOLVED IN THE INDUSTRIAL WASTEWATER BIOREMEDIATION
(Abstract)

Scientifical coordinator
prof. dr. eng. BAHRIM GABRIELA

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GALATI
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SCIENTIFIC OBJECTIVES

The humanity became conscious of the hazardous effect of the pollution which is a consequence of the demographic increase, intense urbanization and of the agricultural and industrial activities extinction, and tries to decrease the negative effects of the pollution by different ways and actions.

In Romania, the advanced wastewaters treatment problem has got proportions especially in the last 10 years. Galati city is one of the most pollutant harbour on the Danube river because of the economical life developed around the Naval Shipyard DAMEN SA, Port River, Arcelor-Mittal steel plant, and Mineral Port. All this units contribute to waters and soil pollution with heavy metals. The food industry is another industrial branch which has a high contribution to the environment pollution with organic compounds.

Thus, in the context of Romania's integration in European Union, it is imperative to take urgent and efficient measures in the environment protection field, meaning also the demands those regarding the treatment – through different methods - of the wastewater derived from economical agents and urban or rural communities. In this way it is necessary to mention that, in present, in Galati city two modern municipal wastewaters treatment plants are going to be implemented.

In the actual context of the modern tendencies of the research activities to find new efficient and eco-friendly solutions to reduce the environment pollution, the PhD thesis, entitled *The study and modelling of the bioprocesses responsible of the industrial wastewaters bioremediation,* reveal a number of original contributions for fundamental and applied research, with great importance for the identification of some efficient solutions to remove the biodegradable and persistent chemical compounds from wastewaters. This PhD facilitated an interdisciplinary research, techniques from classical and modern biotechnology, modelling and advanced control of the bioprocesses being applied. The solutions will contribute to the research recovery for biotechnology application in the environment protection and bioremediation fields in Romania.

The studies performed in this PhD project had the following **objectives**:

- The qualitative analysis of the dairy wastewater microbiota.
- Biochemical characterization and molecular identification of some wastewater isolated microorganisms with high biodegradation potential.
- Study on the bioprocesses responsible of the organic pollutants removal from the simulated dairy wastewaters, in pilot conditions.
- Modeling and simulation of the biological treatment processes of the

wastewater using submerse cultivation system and aerobic conditions.

- Kinetic and modelling of the heavy metals biosorption process.

To perform the research activities provided in the doctoral curriculum and which answer to the scientifical objectives of the PhD thesis, the infrastructure of the following modern laboratories was used:

- The wastewater pilot plant from the faculty of Food Science and Engineering from Dunarea de Jos University, Galati.
- Biotechnology Applied in Food Industry – Integrated Center for Research and Education - Bioaliment (www.bioaliment.ugal.ro), faculty of Food Science and Engineering from Dunarea de Jos University, Galati.
- Molecular Genetics and Bioprocess Engineering laboratories from the Institute of Biotechnology, and the Hydrogeology laboratory of the Institute of Geology Sciences from the Technical University from Berlin, Germany.

The financial and material support was offered by the following sources:

1. The project „Improving the Qualitative Indicators for Biological Treatment of Food Industry Wastewaters Using Advanced Systems, CEEX- MENER 717/ 2006, 2006 – 2008 founded by the National Research and Development Programme, MENER (project manager: Prof. dr. ing. Sergiu Caraman). The results of the modelling studies on the bioprocesses conducted in this project and showed in the PhD thesis have been analysed and interpreted under the carefully guidance of the research staff from the faculty of Automatic Control, Computers, Electrical and Electronics Engineering, prof.dr.eng. Sergiu Caraman, lecturer dr. eng. Barbu Marian and PhD student eng. George Ifrim.
2. The BD type fellowship got by competition from National Council of Scientific Research and Higher Educational System (ro. CNCSIS). The project topic was „Study and modelling of the bioprocesses responsible of biological treatment of the food industry wastewaters", project code 231, april 2008 – december 2010.
3. The DBU fellowship founded by Deutsche Bundesstiftung Umwelt: "The analysis of some efficient methods applied for xenobiotic compounds bioremediation: heavy metals biosorption." The stage was carried on in the laboratories of the Institute of Biotechnology, Technical University from Berlin, Germany, under the guidance of prof. dr. nat. Peter Neubauer and the research staff; may 2009 – june 2010. For the opportunity of this fellowship, the author acknowledges to professor dr. Carmen Socaciu, from the Agricultural Science and Veterinary Medicine University from Cluj-Napoca.

PhD THESIS STRUCTURE

The PhD manuscript is presented on 188 pages and it is divided into two parts as follows: **I. Review of literature**, which contains 4 chapters and **II. Experimental set-up** structured on 6 chapters. The manuscript contains 57 figures and graphs and 44 Tables.

I) REVIEW OF LITERATURE, divided into four chapters with the following topics:

- **Chapter 1,** entitled ***Applied biotechnologies for the wastewater biological treatment in aerobic conditions***, is based on the literature data which describe the wastewater features and the applied biotechnologies. It is divided into three subchapters which describe the legal conditions for the wastewater discharge into the natural receivers, physico-chemical characteristics of the industrial wastewater, as well as the treatment strategies.
- **Chapter 2,** entitled ***Microorganisms responsible of the wastewater biological treatment in aerobic conditions*** is divided into two subchapters and discusses about the biological methods frequently applied for the wastewater treatment, the biology of the activated sludge, and about the biochemical processes responsible for the biotransformation of the organic compounds.
- **Chapter 3,** named ***Heavy metal biosorption,*** covers, through six subchapters, informations about the implications of the heavy metals pollution on the environment. It presents the conventional and nonconventional techniques used to remove heavy metals, as well as the biochemical and molecular arguments to encourage the utilization of the microbial biomass as biosorbant for wastewaters bioremediation.
- **Chapter 4,** named ***Practical and theoretical basis applied on the modelling and simulation of epuration bioprocesses*** tells about the importance of the modeling field on the analysis and control of the bioremediation processes. All three subchapters describe the mathematical models recommended for utilization in the aerobic biological treatment of the wastewater, as well as for the heavy metals biosorption by microbial biomass.

II) ORIGINAL CONTRIBUTIONS AND PERSPECTIVES section covers the original results obtained in this research work and it is divided in six chapters, as follows:

- **Chapter 5**, ***Biochemical and genetical characterization of some microorganisms isolated from food industry wastewater***, shows the results obtained after the qualitative and quantitative evaluation of the wastewater isolated microbiota. The wastewater samples were collected from cheese production and general sewerage of a dairy plant in Galati city, before the wastewater being overflowed in the municipal collecting system. The materials, equipments and the

investigation methods are presented in six subchapters.

- **Chapter 6,** entitled ***Study and modelling of the aerobic biological treatment of the food industrial wastewater*** reveals the results of the research on the bioepuration process of the simulated wastewaters. The experiments were performed in wastewater pilot plant, under aerobic conditions. The methods and equipments used, as well as the experimental set-up are described in six subchapters. Thus, biodegradation potential of the organic pollutants from the synthetic dairy wastewater could be evaluated in both, batch and countinous systems, using as inoculum isolated microorganisms or activated sludge. The process conducted in the wastewater pilot plant was modelled using the Nejjari model.
- **Chapter 7,** ***Kinetics and modelling of the heavy metal biosorption process*** reveals the results of the study performed to obtain an efficient biosorbant to be used for the heavy metals removal from polluted wastewater. In this way, five subchapters describe the research performed to get the biomass used as biosorbant and the heavy metals biosorption process modelling. A new cultivation system called EnBase-Flo® is used for the first time in biosorption field to get a high yield of biomass. The influence of this new medium on the biosorption capacity of the activ or inactive biomass was studied. To describe the sorption process, Langmuir, Freundlich and Lagergren models were applied.
- **Chapter 8** covers the ***General conclusions*** which points out the scientifical characteristic and the application of the PhD thesis focused on the study and modelling of the bioprocesses responsible of the industrial wastewaters biological treatment.
- **Chapters 9 and 10** summarize the original contributions of the PhD research stage, the impact on the knowledge development, perspectives for the future researches, as well as the modalities of the results dissemination.

II. ORIGINAL CONTRIBUTIONS AND PERSPECTIVES

5. Biochemical and genetical characterization of some microorganisms isolated from food industry wastewater

5.2. Materials and equipments

- **Microorganisms**: 18 strains of microorganisms (8 bacteria, 5 yeasts, 5 moulds) have been isolated from dairy wastewater; 10 wastewater isolated strains were selected for genetical characterization (3 bacteria, 4 yeasts and 2 mould strains).

- **Growth media and buffers**
  - *Growth media for bacterial strains isolation and mentenance:* PCA (Plate Count Agar), LB (Luria Bertani)
  - *Growth media for moulds strains isolation and mentenance:* ME (Malt extract Agar), YEPD (Yeast Extract Peptone Dextrose)
  - *Mineral salt medium for bacteria growth (code BC*): supplimented with 1% lactose and 1% milk casein as unique carbon and nitrogen sources
  - *Mineral salt medium for yeasts growth (code Dj)*: supplimented with 1% lactose as unique carbon sources
  - *Mineral salt medium for moulds growth (Czapek salts, code Cza*): supplimented with 1% lactose, 1% lactic acid as unique carbon and 1% casein and nitrogen sources

Specifique primers used for PCR technique:

***Bacteria**:* 16 S ARN, Forw: 5'- AGAGTTTGATCCTGGCTCAG-3' , Rev: 5'GGTTACCTTGTTACGACTT-3,

***Fungi**:* 18 S ARN, C14/NL1Seq26Sforw/GCATATCAATAAGCGGAGGAAAAG C15/NL4Seq26Srev/GGTCCGTGTTTCAAGACGG

The DNA purification was performed with Hi-Yield PCR clean-up kit (QIAGEN).

In this research step, the equipments used for molecular characterization of the microorganisms, belonged to the infrastructure of the Molecular Genetics laboratories from the Institute of Biotechnology, from the Technical University, Berlin, Germany.

5.3. Methods of investigation

5.3.1. Sampling and microorganism's isolation methods

a) *Wastewater sampling sources*

The wastewater sampling was performed from a dairy plant, from two different sources: cheese production and general sewerage, before the wastewater being overflowed in the municipal colecting system. To respect the agent confidentiality, the unit will be called in this study, the dairy plant A.

b) *Wastewater sampling method*

Wastewater sampling was performed according to the instructions from the romanian standard SR ISO 5667-10.

The wastewater samples were collected at the beginning of January and at the end of February 2008, being kept at 0...4^{o}C and analysed in the Wastewater Treatment Lab of Food Science and Engineering Faculty, *Dunarea de Jos* University of Galati.

c) Microorganisms isolation

Wastewater microorganisms were isolated by Koch method based on cell dilution and spreading on the agar medium. From the individual colonies resulted on the medium, bacteria, yeasts and moulds pure cultures were obtained by inoculation in test tubes with sloping medium surface. The optimal conditions used were those recommended for the microorganism cultivation, namely the cultivation on MEA (malt extract agar) at temperatures of 25…28^{o}C, for 3–5 days (yeasts and moulds), and the cultivation on PCA (plate count agar) medium at temperature of 37^{o}C, for 48 hours (bacteria). The cultivation was repeated till pure cultures were obtained. The pure cultures were coded according to the Microorganisms Collection of the Bioaliment Platform Research Integrated Center of the University "Dunarea de Jos" (acronym MIUG) and kept as pure stock cultures for biochemical and molecular characterization and for use in the ulterior research steps. The isolated strains were preserved as lyophilisated cells and as 30 % glycerol stock in YEPD (yeasts), 50 % glycerol stock in ME (moulds) and 10 % glycerol stock in LB medium (bacteria). The glycerol stock cultures were freezed at -80°C.

A morphological evaluation through the examination of cultural characters of the colonies and by microscopic analysis of Gram stained cells, by using microscope Olympus 4BX, was performed for the isolated strains.

5.3.2. Methods of biochemical characterization of the wastewater isolated microorganisms

To distinguish the biochemical potential of the isolated cells, the capacity to grow and metabolize different unique carbon and nitrogen sources, the pure culture cells were inoculated by pricking the solid media surface. After cultivation in the same conditions mentioned before, the growth diameters were determined. In the case of the media supplimented with casein, the biotransformation potential was determined thrugh the hydrolysis index calculation. The following formula was applied:

$$I_h = D_{zh}/D_c$$

where I_h is the substrate hydrolysis index, D_{zh} represents the hydrolysis diameter and Dc, colony diameter.

All experiments were performed in triplicates and the data plotted represent the average values and standard deviations.

5.3.3. Molecular characterization of the microorganisms with high biodegradation potential of the organic compounds

For the identification of the isolates, colony polymerase chain reaction (PCR technique) was applied and the yeasts and bacteria genes were amplified using the specific primers 18S rRNA and 16S rRNA, respectively. The forward and reverse primers are presented in Growth media and buffers section.

The PCR products of 600 bp (yeasts) and 1500 bp (bacteria) were purified using Hi-Yield PCR clean-up kit. Sequencing was carried out by LGC Genomics Company (Berlin, Germany). The sequences were aligned with close matches using the Tuebingen multiple sequence alignment program (http://toolkit.tuebingen.mpg.de). Nucleotide sequence similarities were determined using BLAST (NCBI database; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amplified and sequenced 18S rRNA and 16S rRNA genes from the wastewater yeasts and bacterial isolates were uploaded to the National Center for Biotechnology Information (BLAST) website to search for similarity to known DNA sequences to confirm the species.

5.4. Results and disscusion

5.4.1. Morphological characterization of the wastewater isolated microorganisms

The aim of the experiments was the evaluation of the dairy wastewater microbiota and to identify some active strains adapted to the wastewater physical - chemical conditions, for using them as specialised inoculum in wastewater treatment on model systems.

În the Table 5.1, can be observed the diversity of the wastewater microbiota, the bacteria, yeasts and moulds strains building a complex community in correlation to the pollutants, carbon (lactose, lactic acid) and nitrogen (casein) compounds from the wastewater and the pollution degree.

Table 5.1. Wastewater isolated microorganism selected for characterization and use for biological treatment

Category of microorganisms	Strains code
Bacteria	LBc2, BBc2, BBc5
Yeasts	LDj3, BDj1, LDj1, LDj8
Fungi	LFg2, LFg3

5.4.2. Evaluation of the microorgnisms biodegradation potential on the wastewater organic compounds

The most frequent pollutant in the dairy wastewater is represented by lactose which is biodegraded to lactic acid, butiric acid, propionic acid, ethanol and gases such as carbon dioxide and hydrogen. Lactose and casein have been choosed because of their incidence in the dairy wastewater. Thus, the bacteria were tested for their capacity to metabolize lactose and casein through cultivation on BC medium supplemented with 1% lactose or 1% casein. After 48 hours of incubation at 37^{o}C, a good growth could be observed for 60% of the strains on media supplyed with 1% lactose, the colonies diameters being over 0.2 cm. The strains coded BBc2 and LBc5 showed the better potential to metabolise lactose (colony diameters of 0.7 and 0.5 cm, respectively) (Figure 5.1). Through cultivation on media supplimented with 1% casein as unique nitrogen source, the bacteria strains developed colonies of maximum 0.5 cm. The strain BBc5 is followed by other three strains LBc3, LBc5 and LBc6 with a maximum colony diameter of 0.4 cm. In comparison to proteins, hydrocarbons are more susceptible to biodegradation (Sarkar et al., 2006) and this explains why the tested strains presented a better growth on media supplimented with lactose than casein. The strains coded BBc2, BBc5 and LBc5 have been chosen for molecular charaterization.

The metabolization capacity of lactose was tested to the isolated yeast strains as well. After 4 days of cultivation on DJ medium supplemented with 1% lactose, and at the temperature of 25^{o}C, the colony diameters were measured and the hydrolysis index was calculated.

All yeast strains have the capacity to metabolise lactose, but the strain coded LDj3 distinguished with the best potential, the colony diameter reaching the value of 0.8 cm (Figure. 5.3). The strains coded LDj1, LDj3, LDj7 and LDj8 have been selected for genetical charaterization.

The pure moulds cultures isolated from the dairy wastewater showed a low potential of biotransformation of the tested organic compunds (casein, lactose and lactic acid). The performant strain *Geotrichum candidum* MIUG 1.15 from the MIUG Collection was used as positive control. The results are depicted in the Table 5.2. The moulds identified in the wastewater don't play a proeminent part in the organic compounds biotransformation.

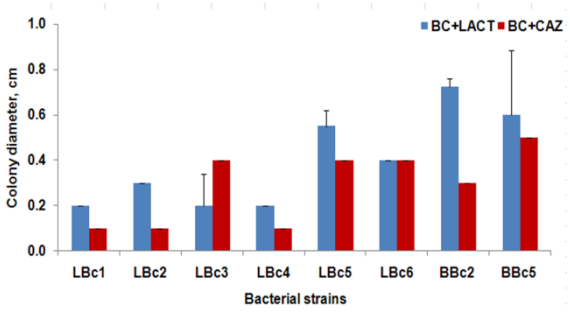

Fig. 5.1. Dairy wastewater bacteria capacity to metabolise lactose and casein

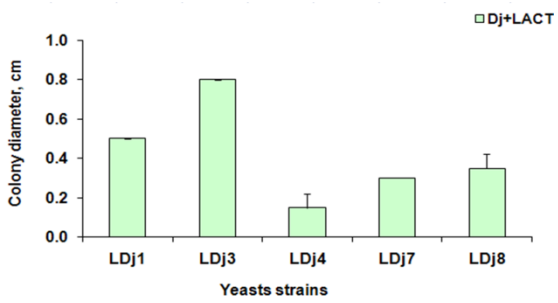

Fig. 5.2. Dairy wastewater yeasts to metabolise lactose

Table 5.2. Organic compounds biotransformation potential of fungi isolated from the dairy wastewater

Isolation source	**Strain code**	**Growth* on agar Czapek medium**	**Growth* on Czapek medium supplied with:**		
			lactose 1%	**lactic acid 1%**	**casein 1%**
Dairy wastewater	LFg1	+ + -	+ - -	- - -	+ - -
	LFg2	+ + -	- - -	- - -	+ - -
	LFg3	+ + -	- - -	+ + +	- - -
	LFg4	+ + -	- - -	+ - -	+ - -
	LFg5	+ + -	- - -	+ - -	- - -
MIUG collection	*Geotrichum candidum* MIUG 1.15	+ + +	+ + +	+ + +	+ - -

*Growth potential evaluation: - - - no growth; + - - low ; + + - medium; + + + high

5.4.3. Genetic characterization of the strains with high biodegradation potential

The isolated microorganisms with the better capacity to metabolise the carbon and nitrogen sources used in this study, have been characterized. Thus, 3 bacteria, 4 yeasts and 2 mould strains has been genetically identified. It was necessary to identify them for a comparison of the results to the literature data and for the evaluation of the data on a scientific basis. The PCR technique and genes sequencing revealed the genus and species of the studied strains. The wastewater isolated strains were identified as *Bacillus* sp., *Bacillus subtilis, Candida silvae*, *Pichia* sp., *Kluyveromyces marxianus* and *Aspergillus niger.*

The bacterial and yeast DNA extraction was performed by using the standard Colony PCR protocol. The cloned DNA fragments were submitted to the agarose gel electrophoresis technique and visualized by UV Gel Documentation system (Figure 5.3).

The sequencing of the purified DNA was carried out by LGC Genomics Company (Berlin, Germany). The sequences were aligned with close matches using the Tuebingen multiple sequence alignment program (http://toolkit.tuebingen.mpg.de). Nucleotide sequence similarities were determined using BLAST (NCBI database; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The Colony PCR method applied in this study, proved to be efficient for all bacterial strains selected for identification, since all lines showed a good DNA product with the length of 1500 base pares. The strain *Escherichia coli* W 3110 was used as positive control.

Only the strain coded BBc2 was 100% identified as *Bacillus subtilis*. The alignement of the sequences from the strains LBc2 and BBc5 showed 100% similarity between this two microorganisms proving that they pertain to the same taxon which belongs to *Bacillus* genus. The genetical methods were completed with the morphological characterisation. From microscopic analysis and Gram staining, the bacterial strains were found to be rod-shaped, Gram-positive bacteria.

The yeast strains were 100% identified as *Candida silvae* (strain LDj3) and *Kluyveromyces marxianus* (strains LDj1 and LDj8). In the case of the strain LDj7, only the genus was 100% identified as *Pichia*. The BLAST program showed two options with 100% coverage for the species: *Pichia fabiani* and *Pichia veronae*. From this consideration, the strain will be called *Pichia* spp.

In that's concern the mould strains, there were applied different mechanical and thermic disruption methods to break the cells and spors wall and to release the DNA. In the Figure 5.3 the DNA fragments of approximately 600 bp from the strains LFg2 and LFg3 can be seen. The two moulds were 100% identified as *Aspergillus niger*.

For all strains the DNA purification ratio values ($A_{260/280}$) belonged to the optimum interval (1,8-2) proving that the DNA product was pure and not contaminated. The morphological characteristics consolidate the genetical results.

The microorganisms identified in this study are classified as level 1 biohazard and they can be used without any risk for different studies related to the environment bioremediation.

Different microorganisms similar with those identified in this work were identified in milk products: *Candida silvae* (cheese), *Galactomyces geotrichum* (milk, yogurt), *Kluyveromyces marxianus* (cheese, kefyr, yoghurt) (Deak, 2008). The frequency of the identified strains in the dairy products confirm the possibility to find them in the corresponding wastewater. *Bacillus* spp. strain and the mould *Aspergillus niger* are worldwide spread.

The organic compounds biodegradation capacity of the identified strains was compared with data from scientifical literature. The microorganisms form this study showed a different behaviour. Unlike the strains studied by Uden (1963) and Walt (1971) cited by Deak, 2008, the yeast strains such as *Candida silavae* and *Kluyveromyces*

marxianus revealed a very good potential to assimilate lactose. *Pichia* spp. strain from this study developed a very good growth on the medium supplimented with lactose, in contrast to the srains presented by Deak (2008) which could not assimilate this carbohydrate. Should not be ignored that the strains from this study are coming from dairy wastewater which has a high concentration of casein, being considered an optimum environment for selective growth of the microorganisms adapted to biodegrade the pollutant organic compounds.

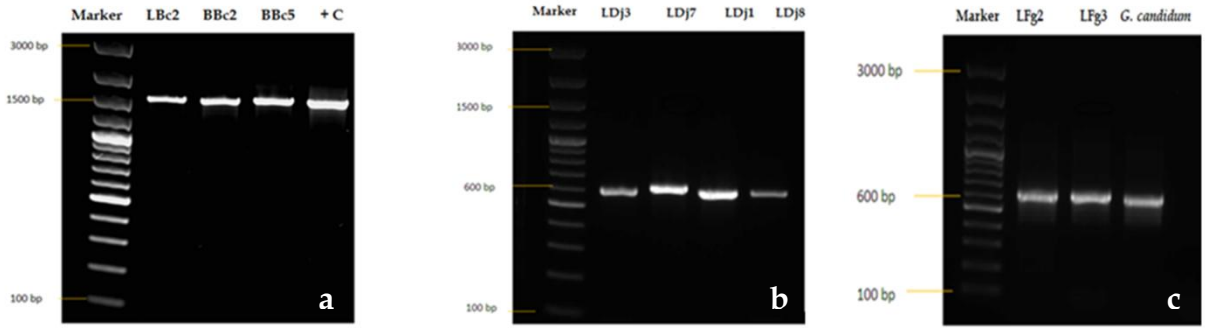

Fig. 5.3. The electrophoretic study of the DNA product from the isolated strains: bacteria (a), yeasts (b) and moulds (c). DNA Marker: 0.3 ng (0.1 ng/μL)

Table 5.3. Wastewater isolated strains taxonomy

Category	Strain code	Genus, species	MIUG code
Bacteria	LBc5	*Bacillus* sp.	MIUG 6.161
	BBc2	*Bacillus subtilis*	MIUG 6.160
	BBc5	*Bacillus* sp.	MIUG 6.162
Yeasts	LDj3	*Candida silvae*	MIUG 3.10
	LDj7	*Pichia fabiani/P. veronae*	MIUG 7.2
	LDj1	*Kluyveromyces marxianus*	MIUG 8.5
	LDj8	*Kluyveromyces marxianus*	MIUG 8.6
Fungi	LFg2	*Aspergillus niger*	MIUG 1.58
	LFg3	*Aspergillus niger*	MIUG 1.59

5.5. Conclusions

- The strains with the better biodegradive potential of some organic compounds associated with wastewater composition were selected in order to be used in the next steps of the research.
- Bacteria, yeast and mould strains with high capacity to metabolise the organic compounds pollutant in the dairy wastewater were isolated and identified as species belonging to the genus *Bacillus, Candida, Pichia, Kluyeromyces* and *Aspergillus.*
- The wastewater isolated strains are level 1 biohazard microorganisms.

6. Study and modelling of the aerobic biological treatment of the food industrial wastewater

6.2. Materials and methods

- **Cultivation media and reactives**
  - ***simulated wastewater*** obtained by diluting acid whey (from cheese production) with water.
- **Inoculum:**
  - mixed culture obtained by combination (1:3:3 ratio) of the wet biomass from the following selected cultures (g): *Bacillus* spp. MIUG 6.161, *Candida silavae* MIUG 3.10 and *Geotrichum candidum* MIUG 1.15;
  - activated sludge from the wastewater treatment plant from S.C. Rompak, Pascani city.
- **Hanna kits** used for the offline parameters determination**: HI 93754C -** Chemical Oxygen Demand (COD); **HI 93767A -** Total nitrogen (N_{tot}); **HI 93758B -** Phosphates (PO_4^{3-})
- **Equipments**
  - 1L Applikon bioreactor from the Cultures and Fermentation laboratory of the Integrated Research Center - BioAliment of University "Dunarea de Jos", Galati (Figure 6.1);
  - Wastewater treatment pilot plant from the Wastewater treatment laboratory of the Faculty of Food Science and Engineering from the University "Dunarea de Jos", Galati (Figure 6.2).

Wastewater treatment pilot plant characteristics: feeding tank capacity - 100 L provided with refrigeration equipment; aeration tank working volume – 35 L; maximum feeding flow 12.5 L h^{-1}; maximum aeration flow 30 L min^{-1}; electrods for online determination of pH, temperature, dissolved oxygen (DO), oxidation-reduction potential (ORP), total suspended solids (TSS); air-lift system for sludge recirculation.

A HMI (Human-Machine Interface) facilitates the process control and monitoring.

Fig. 6.1. 1L Aplikon Bioreactor

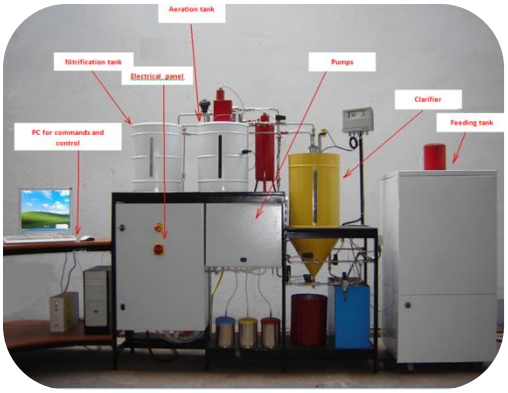

Fig. 6.2. Wastewater treatment pilot plant

6.3. The experimental protocol

Experiment A (submerse cultivation on orbital shaker, batch system)

The aim of the experiment was the simulated wastewater biological treatment in batch conditions, in submerged cultivation system, on orbital shaker by using the activated sludge. The experiment was unfolded on a period of 8 days, in 0.5 mL Erlenmeyer flasks, at 200 rpm and 25°C. The medium was obtained by diluting acid whey in a ratio of 1:5 and 1:10 with drinking water to obtain the synthetic dairy wastewater with an initial concentration of COD of 14 628 mg L^{-1} and 8671 mg L^{-1}, respectively. A volume of 1 mL of activated sludge was inoculated on 0.2 mL sterile medium. The experiment was performed in triplicates and the data plotted represent the average values and standard deviations.

Experiment B (submerse cultivation, batch system)

In this experiment a study on the biological treatment of simulated wastewater was performed by exploating the wastewater isolated strains capacity to remove organic compounds.

The synthetic dairy wastewater was obtained by diluting acid whey in a ratio of 1:4 with drinking water to obtain an initial concentration of COD of 13.500 mg L^{-1}. The treatment was made in batch system on a periode of 12 days in 1L Applikon bioreactor, in a cultivation volume of 70%, at constant temperature of 25°C. The aeration rate was constantly maintained at 2 L min^{-1} and the mixing speed was constantly maintained at 300 rpm. The medium was inoculated with a mixed culture obtained from precultures by cultivation of each strain in 0.2 mL diluted acid whey, in 0.5 mL Erlenmeyer flasks, at 200 rpm and temperature of 25°C, for 18 hours, on the orhizontal shaker (Medline SI 300R). The pH value was continously adjusted to 5.5 by 1N NaOH.

Controlling the physico - chemical parameters of cultivation, the development degree of microorganisms was evaluated through the multiplication growth curve based on the turbidity measurement. The treatment degree was determined by evaluating the capacity to remove polluting organic matters from the culture media, the removal time and the technical limit of the treatment. Since the inoculum used (formed by bacteria, yeasts and moulds) has not the capacity to form flocks, able to sediment easily, samples were centrifuged at 9000 rpm for 10 minutes. The chemical analysis for COD and N_{tot} were made on the obtained supernatant.

Experiment C (submerse cultivation, continous system)

In this study the improving of the wastewater treatment process through biological method was the main interest. In this way, 0.5 L of activated sludge was inoculated on synthetic dairy wastewater obtained by diluting whey in a ratio of 1:40 to get an initial concentration of COD of 1924 mg L^{-1}. The treatment process has been run in continous system, in the wastewater treatment pilot plant for 91 hours. The activated sludge recirculation rate was 2.4 L min^{-1}. The treatment degree was determined offline and online by evaluating the following parameters in the aeration tank: chemical oxygen demand, turbidity (as well as in the treated effluent), total nitrogen, phosphates concentration, pH, dissolved oxygen, oxidation - reduction potential.

The influence of the feeding flow rate and aeration flow rate in dynamic system on the epuration process has been studied. Thus, two feeding rates have been applied: 2.2 L h^{-1} in the first 50 hours and 4.5 L h^{-1} till the end of the experiment. The aeration flow rates applied are the following: 5 L min^{-1} in the first 9 hours, 10 L min^{-1} between 9 and 21 hours, 8 L min^{-1} between 21 and 67 hours, 10 L min^{-1} after 67 hours.

The analysis and interpretation of the online data was based on the average of hours. The treatment process modelling has been done to obtain an adequate mathematical model for the conditions applied in the wastewater treatment pilot plant and for advanced control of the process.

6.4. Investigation methods

- Determination of chemical oxygen demand (COD)
- Evaluation of turbidity (NTU)
- Determination of total nitrogen (N_{tot})
- Determination of phosphates
- Evaluation of pH
- Measuring of the dissolved oxygen concentration (DO)
- Evaluation of oxidation - reduction potential (ORP)
- Temperature measuring

– Mathematical modelling of bioprocesses: Model used - Nejjari; Software - Matlab-Simulink

6.5. Results and disscusion

6.5.1. Study on the biodegradation of the dairy wastewater organic compunds in aerobic conditions

Evaluation of the activated sludge potential to biodegradate the organic compounds from dairy wastewater (Experiment A)

The analysed parameters were the biomass and the organic compounds concentration through turbidity and the chemical oxygen demand measurement. The biodegradation potential and the substrate bioavailability of the chemical oxygen demand on two different initial concentrations was established. The total COD efficiency was calculated. The values are depicted in the Table 6.1.

In the Figure 6.3, the simulated wastewater treatment process evolution is showed. The biomass presents a growth dynamic more advanced in the case of the dilution 1:5 (initial chemical oxygen demand, CCO_i, of 14 628 mg L^{-1}), than the dilution 1:10 (CCO_i = 8 671 mg L^{-1}). When a higher initial concentration of the organic compounds (dilution 1:5) is applied, the growth curve of the microorganisms, which represents the dynamic growth of different categories of cells (bacteria, yeasts, moulds etc.) from the activated sludge, presents an ascending profile in the first 4 days of treatment. In the same time, an indirect correlation between growth and organic compounds concentration could be observed till the decline phase of the growth appears. The organic compounds biotransformation reaches the maximum efficiency of 89% after 6 days of the aerobic treatment. The increase of the turbidity indicates the multiplication of the microorganisms which are using the available organic compounds. The activated sludge efficiency was lower in the case of the initial organic compounds concentration of 8 671 mg L^{-1}, the percentage of 75% being reached after 6 days of treatment (Figure 6.4). This fact can be explained by the microbial diversity of the activated sludge and by the high food to microorganism ratio.

Low substrate concentration can also affect biodegradation capacity of microorganisms. Organisms growing on very low substrate concentration have a high affinity for substrates (i.e., very low half-saturation constant Ks). Some microorganisms in the environment may not be able to assimilate and grow on limited organic substrates. Similar situation could be observed on the medium obtained by diluting whey in a ratio of 1:10 with water. Most biodegradation studies have been carried out using relatively high substrate concentrations. This is not a realistic approach as environmental concentrations are much lower (ppm or ppb level) than those that are sometimes used under laboratory conditions (Bitton, 2000).

Microorganisms respond to low nutrient concentrations by adapting morphologically, leading to cells with high surface-to-volume ratios (Bitton, 2000).

Table 6.1. The parameters values obtained during the simulated dairy wastewater aerobic biological treatment with activated sludge

whey:water ratio	Time, days	Turbidity, NTU	COD, mg L^{-1}	COD removal efficiency, %
1:5	0	28.15	14 628.6	
	1	40.53	10 322.6	29
	2	49.64	11 332.1	23
	3	141.70	10 044.1	31
	4	150.55	5 930.3	59
	5	140.73	2 877.1	80
	6	105.14	1 644.0	89
	7	109.62	1 820.5	88
1:10	0	23.47	8 671.0	
	1	34.61	7 609.2	16
	2	43.93	4 403.7	49
	3	88.23	3 429.7	60
	4	74.14	2 673.8	69
	5	123.81	2 289.9	74
	6	112.69	2 172.5	75
	7	147.22	2 673.8	69

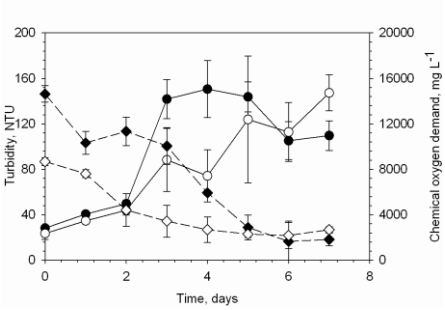

Fig. 6.3. The biomass growth and chemical oxygen demand evolution (full symbol- dilution 1:5, empty symbol- dilution 1:10; circle- NTU, rhombus-COD)

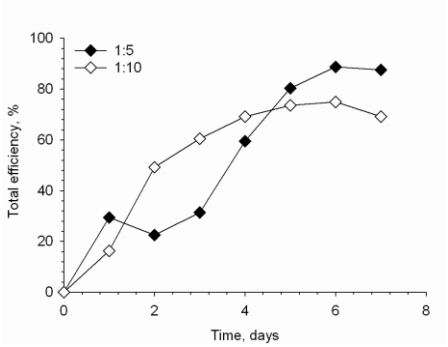

Fig. 6.4. The epuration process efficiency

Evaluation of the organic compounds removal capacity of the wastewater isolated microorganisms (Experiment B)

The ability to mineralize the organic compounds from the simulated wastewater, using wastewater strains selected for their capacity to metabolize lactose (*Bacillus* spp., *Candida silvae)*, casein (*Bacillus* spp., *Geotricum candidum*) and lactic acid (*Geotricum candidum*), has been studied. The main analysed parameters, as well as the results obtained during the wastewater treatment in batch system, are presented in the Table 6.2.

The initial turbidity value of 175 NTU was obtained by inoculating 6.8 g of wet biomass in a medium with the working volum of 700 mL. The Figure 6.5 reveals the rapid microbial growth; the turbidity reached the maximum value of 2810 NTU in the first 3 days. In the next days, a decrease of the biomass profile could be observed. The

organic compounds concentration continues to decrease inspite the descending profile of the turbidity starting with the 4th day of the experiment. The cellular constituents released in the aqueous environment as the result of the cell lysis phenomenon became nutritients and energy sources for the viable cells involved in the organic compounds oxidation reactions, leading to the indirect correlation between the biomass accumulation and organic compounds removal process. The maximum epuration efficiency of 94% could be registered after 6 days of treatment.

Table 6.2. Dynamic of the mineralization process of the organic compounds by the selected wastewater selected microorganisms

Time, days	Turbidity, NTU	Specific growth rate, h^{-1}	COD, mg L^{-1}	COD removal efficiency, %	N_{tot} mg L^{-1}	N_{tot} removal effciency, %	Dissolved oxygen, %
0	175.0	0.000	13 625.0		287.5		38.9
1	700.0	0.602	13 418.0	2	140.0	51	9.3
2	1456.5	0.318	8626.0	37	15.5	95	1.5
3	2810.0	0.285	6126.0	55	99.0	66	0.8
4	1953.0	-0.158	2862.0	79	108.0	62	0.2
5	1676.5	-0.066	1144.0	92	14.8	95	6.7
6	1442.5	-0.065	788.0	94	17.0	94	17.7
7	1234.5	-0.068	1100.0	92	18.4	94	16.6
8	586.5	-0.323	1214.0	91	60.4	79	9.1
9	550.0	-0.028	1150.0	92	50.0	83	10.1
10	406.5	-0.131	1136.0	92	10.2	96	14.7
11	394.0	-0.014	1234.0	91	80.4	72	19.2
12	339.5	-0.065	1180.0	91	86.4	70	25.3

The chemical oxygen demand (COD) reached the stabilization level (maximum limit of organic compounds transformation) after 5 days of treatment. This level is characteristic for each applied system. In practice, the stabilization procedure is named aerobic treatment with extended aeration and it is applied after the main treatment to reduce the amount of volatile organic compounds (VOC). The stabilization process reduces the bad smell, destroys the pathogen bacteria and produces biofertilisers.

Another factor essential for the epuration process evolution, is the dissolved oxygen concentration. There is an indirect correlation between the oxygen concentration and turbidity (Figure 6.5) because of the high oxygen consumption during microbial cells respiration and organic compounds oxidation. After 8 days of treatment, the oxygen concentration increases as a result of the low consumption rate on the biomass entered in the decline phase.

Because the medium was obtained from diluted whey, which contains high amount of proteins, high initial total nitrogen concentration of 287 mg L^{-1} was identified. The isolated microorganisms showed a maximum nitrogen removal efficiency of 96%

after two and ten days of experiment. Thus, a reduction of approximately 19 times of the nitrogen occurred because of the proteins mineralization and denitrification processes (Table 6.2, Figure 6.6). The organic compounds loading rate in dairy wastewater is determined by the amount of lactose, fats and proteins (Perle et al., 1995). The relation between these substances can be extremely varied, affecting their susceptibility to biological treatment (Janczukowicz, 2007).

A dirrect correlation could be observed between the COD and DO as long as the involved microorganisms in the epuration process were in the exponential growth.

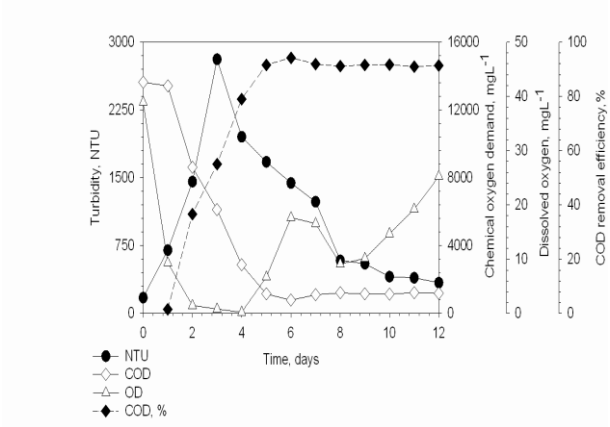

Fig. 6.5. Turbidity, chemical oxygen demand, dissolved oxygen concentrations and organic compounds removal efficency during the aerobic treatment with selected microorgansms

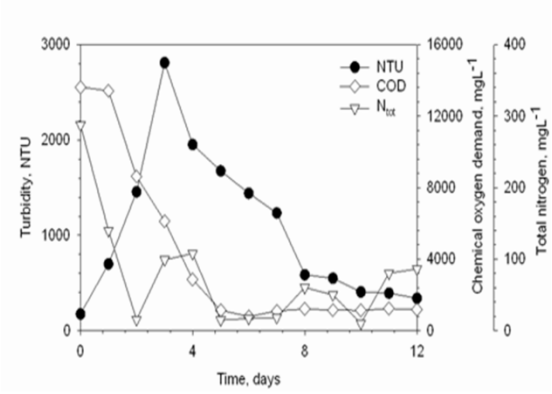

Fig. 6.6. Correlation between turbidity, COD and total nitrogen during the aerobic treatment with selected microorganisms

Study of the biodegradability in continous system of the organic compounds from the simulated dairy wastewater (Experiment C)

The aim of this study was the optimization of the parameters with influence on the organic compounds biodegradation at pilot scale. In this experiment, the following previews results were taken into account: the activated sludge and the inoculum formed by selected microorgasims showed a maximum mineralization efficiency of 89% and 94% respectively, after 6 days of treatment in batch system. Because the activated sludge has the ability to form flocks with high settling velocity, it is recommended for the wastewater treatment in continous system. Thus, the activated sludge was choosed to evaluate the organic compounds biodegradation in the wastewater treatment pilot plant.

For a better correlation between the real physico-chemical values of the dairy wastewater parameters and those obtained from the experiment performed in model system, the real dairy effluents coming from the outlet system have been characterized. The results are shown in the Table 6.3.

The influence of the feeding and aeration flow rates in the dynamic system on the epuration process has been studied. Three different aeration flow rates have been applied (5, 8 and 10 L min^{-1}). The short increasing of the pH values in the first 3 hours results from the protons consumption by cells during the intense growth phase, but the protons release after the intense oxidation of the organic compounds decreased very fast the pH. A direct correlation between pH and the aeration flow rates could be observed (Figure 6.7). The point when the pH becomes constant coincids with the increase of the feeding flow rate from 2.2 L h^{-1} to 4.5 L h^{-1} after 50 hours of treatment.

Table 6.3. Physico-chemical characteristics of the dairy wastewater and the maximum accepted values for the effluents discharged in the natural receivers

Analysed parameters	This study	Maximum accepted values according to NTPA 001/2002
TDS*, mg L^{-1}	179-2140	35 ÷ 60
COD, mg L^{-1}	16 000-16 490	70 ÷ 125
pH	4.7-5.0	6.5 ÷ 8.5

*Total dissolved solids

Table 6.4. Physico-chemical and biological parameters variation during the aerobic biological treatment in continous system and with activated sludge recirculation

Time, h	pH	Oxidation-reduction potential, mV	Air input, L min^{-1}	Turbidity in the aerated tank, NTU	COD, mg L^{-1}	N_{tot}, mg L^{-1}	PO_4^{3-}, mg L^{-1}	Turbidity in the treated water, NTU
0	7.56	-131.80	5.07	31.20	1924.00	21.20	32.96	11.52
21	8.28	-246.45	9.45	390.78	1274.00	6.40	44.00	157.40
46	8.30	-243.08	7.99	729.05	666.00	13.20	35.65	83.30
54	8.18	-289.29	7.99	805.98	870.00	-	-	77.26
69	8.18	-293.67	9.98	657.07	966.00	12.00	34.50	103.00
76	8.15	-321.60	10.03	591.38	670.00	-	-	163.00
91	8.15	-182.26	10.02	487.49	416.00	6.40	36.72	199.60

The intense oxygenation increased the organic compounds oxidation through the microbial cell respiration in aerobic conditions, resulting acids, carbon dioxide and water (tricarboxilic acid cycle). In the aerobic conditions, the electron acceptor is represented by oxygen and the donor, by the organic compounds. Thus, a significant decrease of the oxygen concentration takes place in the first 9 hours and, a gradual one, after 21 hours till the end of the epuration process. A direct correlation could be seen between pH and the DO concentration till the increase of the feeding rate. Thus, the aeration rate directly influenced the pH and DO evolution in the medium.

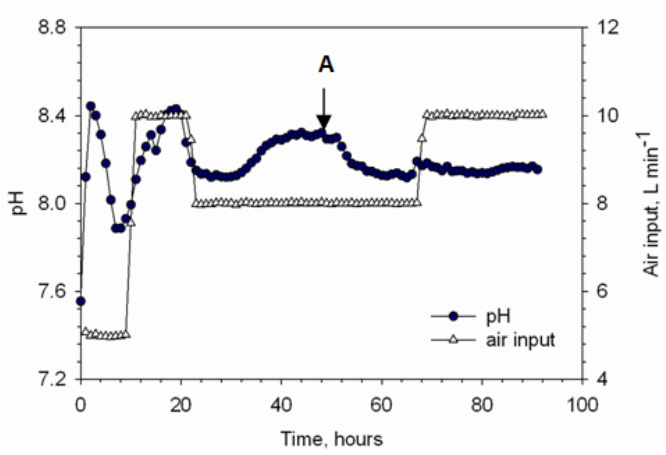

Fig. 6.7. Aeration and wastewater feeding rate (A) influence on pH evolution in continous system

A direct correlation between the turbidity and pH can be observed in the Figure 6.8. The modulations produced by the aeration flow rate variations on the pH profile after 9, 21 and 67 hours, can be found also in the turbidity profile.

The efficient wastewater epuration treatment is characterized by flocks formation through including the anorganic solid particles, bacteria, yeasts, moulds, algea and protozoa, which are associated by the extracellular polyglucides mainly sinthesized by Gram negative bacteria. In the conditions of a corresponding sludge recirculation and aeration rate, the higher sludge age, the higher flocks dimension and the settling spead in the clarifier. The doubling feeding rate after 50 hours, reduced the retention time in the aeration tank, the biomass being washed out and increasing the turbidity in the pilot plant clarifier (data not showed). The dirrect correlation between turbidity and COD has been influenced by the feeding rate increase after 50 hours. In the Figure 6.9 the variation of the organic compounds can be catched in the frame of three intervals: **I.** the COD concentration decrease as the biomass consumption rate increases (situation observed also by Gray, 2005**); II.** the feeding rate increasing leads to the organic compounds accumulation, the biomass enters in the decline phase, **III**. the turbidity continues to decrease together with the organic compounds concentration which tends to the threshold of stabilization (data not showed). According to Ndegwa (2007), the stabilization process in the wastewater treatment plant is indicated by the increase of the oxydation - reduction potential from negative to positive values, or by the easy modulation on the pH profile. In the aerobic conditions, the chemical reactions induce a positive ORP values (> +200 mV), and lower values than + 50 mV under anaerobic conditions (Bitton, 2000). Although the aerobic conditions have been mentained, in this study, the ORP showed negative values during all experiment (Figure 6.9). This fact can be explained by the high oxygen concentration which can decrease the oxygen difussion rate in the aqueous environment; the oxygen limitation forces the microorganisms to perfom their metabolic activities under anoxic conditions (Gray, 2005). The carbon atoms from the carbon dioxide resulted from the reduction processes, have the maximum oxidation

state. Higher the energetic state of the electrons, lower the ORP values are (Bitton, 2000). The increase of the ORP values after 67 hours is correlated with the increase of the aeration rate from 8 to 10 L min^{-1} and the cells lysis which release in the medium cellular constituents influencing the oxidation – reduction potential evolution.

A dirrect correlation between the dissolved oxygen and pH has been obtained. A similar phenomenon has been observed by Ndgewa et al. (2007).

The activated sludge reaches the maximum organic compounds removal efficiency of 78% after 91 hours of treatment.

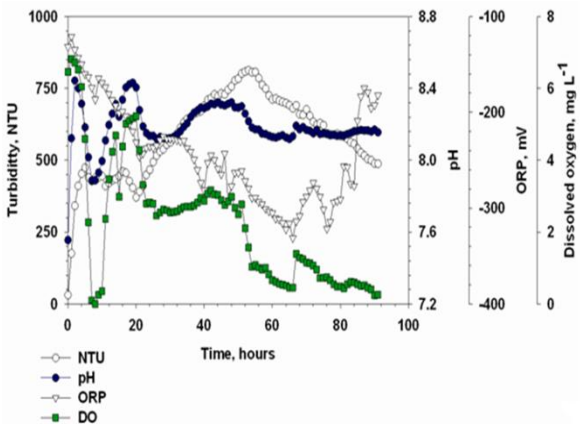

Fig. 6.8. Correlation between turbidity, pH and oxidation reduction potential

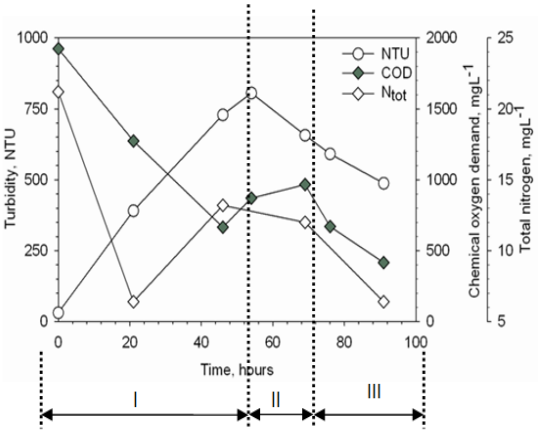

Fig. 6.9. Correlation between biomass concentration, chemical oxygen demand and total nitrogen in the pilot plant aeration tank

The total nitrogen and chemical oxygen demand follow almost the same profile (Figure 6.9). The main component in the simulated wastewater is casein, a complex protein with a high nitrogen concentration. As a result of the intense proteins mineralization and denitrification processes, a nitrogen removal efficiency of 70% has been achieved in the first 20 hours. An accumulation of the total nitrogen was registered in the moment of decreasing the aeration rate from 10 to 8 L min^{-1} because of the better oxygen diffusion and utilisation by cells in nitrification processes. The total nitrogen values as well as the ORP results confirm the oxygen limitation conditions induced by the high aeration rates applied to the dairy synthetic wastewater.

The phosphate concentration is another parameter analysed in this experiment. The results are presented in the Table 6.4. No significant changes in the phosphate concentration could be observed during 91 hours of experiment. The final concentration (C_f = 36 mg L^{-1}) was even higher in comparison with the initial phosphate concentration (C_f = 36 mg L^{-1}). In the wastewater composition, phosphorus can be found as soluble orthophosphates, poliphosphates, and in the structure of the organic compounds. In this work, the alkalin pH impedes the reactions of complexation with metal ions such as Fe, Ca, which determine the compounds precipitation and phosphorus removal from the aqueous environment. The low pH

can facilitate the H_2S formation, able to interact with the iron phosphate and to release the orthophosphates from the environment (Bitton, 2000). The final concentration of 36 mg L^{-1} detected in this study exceeds the limit of 20 mg L^{-1} accepted for the total phosphorus in wastewater. Thus, there are necessary more investigations to improve the phosphorus removal method.

6.5.2. Mathematical modelling of the aerobic bioepuration process, set-up in the wastewater treatment pilot plant, in batch system and with sludge recirculation

The practical identification of the mathematical modelling, as well as the variants of the model, was performed using the experimental data obtained in the experiment C. A method based on the identification of the model parameters proposed by Nejjari et al. (1999) was applied.

It was considered the θ vector in the case of the parameters to be identified using the experimental data; the following equation was used:

$$\theta = [\mu_{\max} k_o k_{do} k_s Y \alpha \beta]^T \quad [6.1]$$

For the θ vector identification, the following criterion was defined:

$$E = \sum_{k=1}^{N} \left[\xi_1[k] - \hat{\xi}_1[k] \right]^2 \quad [6.2]$$

Where: N represents the experimental data number from one experiment; $\xi_1[k]$ represents the vector of the measurable variables in the biological wastewater treatment; $\hat{\xi}_1[k]$ is the vector of the state variables corresponding to the measurable variables. It can be written in the following way:

$$\xi_1 = L \cdot \xi \quad [6.3]$$

L is a matrix which divides the vector ξ in two parts: measurable and nonmeasurable variables. In the case of the Nejjari model, the vector ξ contains 4 state variables, such as:

$$\xi = [X\ S\ DO\ X_r]^T \quad [6.4]$$

The first three measurable parameters are: biomass (activated sludge, X) measured through the turbidity electrode, dissolved oxygen concentration (DO) measured directly with the DO electrode and the substrate (organic compounds, S) measured through the correlation between the oxidation-reduction potential (ORP) and the chemical oxygen demand (COD) and dissolved oxygen (DO).

For the parameters identification, the exerimental data between 1500 and 2000 minutes have been used, periode of time when the three online measurable variables are very good correlated. In this way, the external perturbative phenomenon could be avoided.

Two cases have been considered: **I.** the β parameter was considered zero because no activated sludge was removed during the process and **II.** where the dissolved oxygen variation equation has been separated identified, the model being composed by three equations (biomass, organic substrate, recirculated biomass).

The following starting points have been applied in the first case, to search the parameters: μ_{max} = 0.15, k_o = 0.5, k_{do} = 2, k_s = 100, Y = 1.5 şi α = 0.018. The following values have been identified for the Nejjari model: μ_{max} = 0.7275, k_o = 0.125, k_{do} = 13.975, k_s = 68.75, Y = 1.4375 şi α = 0.0055.

Because the dissolved oxygen dynamic was more rapid than the others, the second case was choosed to identify the model parameters, μ_{max}, k_{do}, k_s and Y. The following values has been obtained: μ_{max} = 0.0462, k_{do} = 1.8625, k_s = 103.75 and Y = 1.1125.

The Nejjari model can not describe the dissolved oxygen dynamic and that's why it can be divided in two parts: a subsystem expressed by the biomass concentration dynamic and the one of the substrate, and the second subsystem, expressed by the dissolved oxygen dynamic (Figure 6.10 a and b).

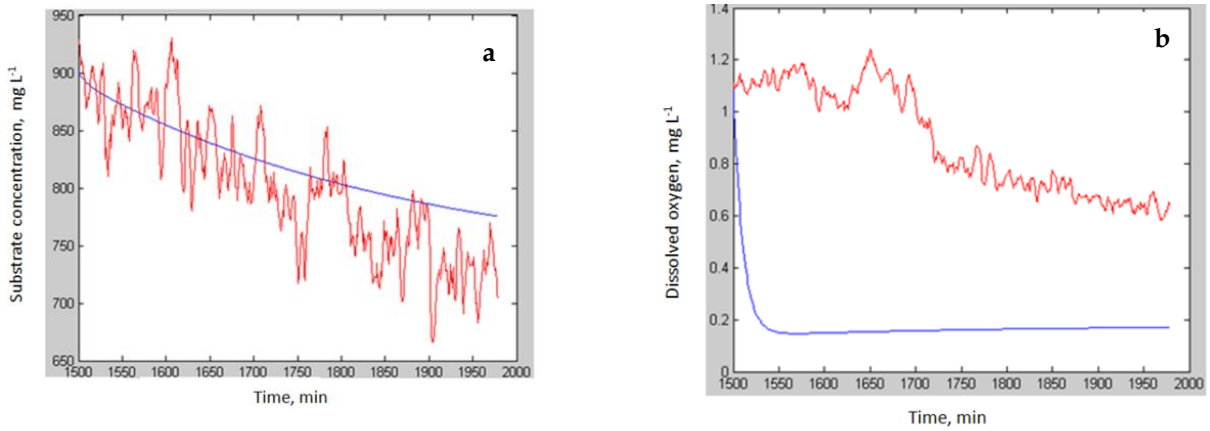

Fig. 6.10. Evolution of the substrate (a) and dissolved oxygen (b) concentration: red – experimental data, blue - model

The identification and optimisation of the correlations between the independent variables which influence the treatment process, play a particularly important role to the optimum function in continous system of the wastewater treatment plant. The variables taken into consideration during the treatment process are: wastewater and activated sludge recirculation flow rates, organic loading rate of the activated sludge in the aeration tank. For a correct evaluation of the treatment process, the correlation

between turbidity, pH, redox potential, dissolved oxygen concentration and chemical oxygen demand has to be considered.

6.6. Conclusions

- The activated sludge inoculated in a dairy simulated wastewater and in batch system showed a maximum organic compounds removal efficiency of 89% on initial organic load of 14 628 mg L^{-1}, and 75% in the wastewater with two times lower organic compounds concentration.
- The mixted specialized inoculum composed by bacteria, yeasts and moulds wastewater isolated strains prooved a very good efficiency of 94% for the organic compounds mineralization from the simulated wastewater, but it can not be used in the industrial processes because of its incapacity to form flocks with high settling velocity.
- A dirrect correlation could be established between the following parameters: pH - aeration flow rate, pH-turbidity, pH- dissolved oxygen, redox potential – dissolved oxygen concentration.
- An indirect correlation could be established between the following parameters: turbidity - dissolved oxygen, turbidity - redox potential, turbidity – chemical oxygen demand (correlated with the exponential growth phase of the biomass).
- The maximum substrate removal efficiency of 78% has been reached after 91 hours by the activated sludge inoculated on a simulated wastewater and in continous system.
- The wastewater treatment effciency can be improved by the control and optimisation of the aeration and feeding rate which influences the evolution of the bioconversion processes of the organic compounds from the wastewater composition.
- The modelling studies revealed the complexity of the biological treatment processes.

7. Kinetic and modelling of the heavy metal biosorption process

7.2. Materials and equipment

Cultivation media used for growth optimisation of the selected strains and for heavy metal biosorption study: Luria Bertani (LB), Yeast Extract Peptone Dextrose (YEPD), Yeast Nitrogen Base 10× (YNB), Malt Extract (ME), EnBase - Flo® (Enzyme-based-substrate delivery).

Heavy metal solutions (mono - component system)

The following metal salts were used to get metal ions stock solutions of 1 g L^{-1}: $Zn(NO_3)_2$, $Cd(NO_3)_2 \times 4H_2O$, $Pb(NO_3)_2$. Ultrapure water was used for heavy metal salts dilution.

Microorganisms:

- growth optimisation studies: *Bacillus* spp. (strains MIUG 6.161 and MIUG 6.162), *Bacillus subtilis* MIUG 6.160, *Candida silvae* MIUG 3.10, *Pichia* sp. MIUG 7.2, *Kluyveromyces marxianus* MIUG 8.5, *Aspergillus niger* MIUG 1.59, *Escherichia coli* W3110 (positive control).

- biosorption studies: *Bacillus* spp. MIUG 6.161, *Bacillus subtilis* MIUG 6.160, *Candida silvae MIUG 3.10, Pichia* spp. MIUG 7.2, *Kluyveromyces marxianus* MIUG 8.5.

In this research stage, the infrastructure used belonged to the Molecular Genetics and Bioprocess Engineering laboratories from the Institute of Biotechnology, and to the Hydrogeology Department, Institute of Geology Sciences from the Technical University, Berlin, Germany**.**

7.3. Methods of investigation

7.3.1. Monitoring of the microbial growth

Identification of the optimum cultivation medium for biomass preparation

A novel fed-batch type cultivation approach called EnBase®-Flo (BioSilta, Oulu, Finland) was used to obtain a high amount of biomass for the biosorption studies. It was used for the first time for wastewater isolated microorganisms cultivation. The growth of the bacteria, yeast and mould strains on EnBase (mineral salt complex media) was tested by supplying the medium with different concentrations of amyloglucosidase enzyme (3, 6, and 12 UL^{-1}).

Bacteria and yeast cells cultivation was performed in 24 deepwell plates, using 2 mL sterile medium, and 200 rpm. A shake flask incubator with 5 cm shaking amplitude and a shaking frequency of 200 rpm was used.

The mould strain was cultivated in 500 ml Erlenmeyer shake flasks with 100 ml culture medium at 25°C. A shaking frequency of 180 rpm was applied.

Cultivations were performed at 37°C (bacteria), 30°C (yeasts) and 25°C (moulds) temperature. All cultivations has been performed in triplicates.

In order to monitor the microbial growth, the optical density was measured at an wave length of 600 nm with an Ultraspec 3300 spectrophotometer and 1 mm cuvettes against a water blank. The mould growth was monitored through mycelia dry weight recorded as g L^{-1}.

Evaluation of the viable cells tolerance to different heavy metals concentration

Viable cells of two bacterial strains (*Bacillus* spp. MIUG 6.161, *Bacillus subtilis* MIUG 6.160) and three yeast strains (*Candida silvae*, *Pichia* spp., *Kluyveromyces marxianus*) were inoculated in sterile standard growth media (LB and YEPD respectively) supplied with different concentrations of metal solutions. Thus, sterilized stocks of $Zn(NO_3)_2$, $Cd(NO_3)_2$ and $Pb(NO_3)_2$ were aseptically added to reach the final metal ions concentration from 25 to 100 mg L^{-1} for bacteria and to 200 mg L^{-1} for yeasts strains. The cultivation conditions are mentioned in the previews section. Free heavy metal media were used as control.

7.3.2. Preparation of the inactive biomass used as biosorbant

Appropriate volumes of the overnight culture were inoculated into 100 mL EnBase®-Flo medium in 500 mL shake flasks with an initial cell density OD_{600} of about 0,15. The same culture conditions as for overnight culture were applied for the EnBase cultures. The cultures were harvested during glucose limited growth after 30 and 48 hours and centrifuged at 5 000 and 4 000 ×g, respectively. The biomass was washed several times with bidistilled water until the conductivity of the supernatant was below 20 μS. The biosorbent for the experiments was obtained by drying the bacterial and the yeast cells at 60°C and 80°C, respectively, to constant weight for 24 hours. After pulverization to a uniform maximum particle size of 0,18 mm, using an electric grinding apparatus (A10, IKA, Staufen), mortar, pestle and standardized sieves, the biomass was stored at 4°C.

7.3.3. Evaluation of heavy metals biosorption capacity of the inactive and active biomass

All sorption experiments were performed in 10 mL propylene tubes treated before overnight in 5% nitric acid and for 24 hours in pure water. The batch equilibrium technique was carried out using an orbital rotary shaker (Heidolph Instruments) with an agitation rate of 15 rpm for 24 hours. All biosorption studies were started at pH values of approximately 5.0 and the pH was not adjusted during the experiments.

A total of 8 mg inactive biomass was mixed with 8 mL of metal ion - containing solution to get a final dosage of 1 g L^{-1}. The metal concentration ranged from 5 to 200 mg L^{-1} for bacteria and from 3 to 350 mg L^{-1} for yeasts.

The bioaccumulation properties of the viable cells from *Bacillus subtilis* and *Candida silvae* strains were studied. Thus, the strains were grown on EnBase medium until the maximum cell density was reached. The viable biomass was centrifuged and 80 mg of bacterial wet biomass or 40 mg of yeast wet biomass (equivalent to 8 mg of dry weight biomass) were combined with 8 mL of metal solution to get a final biomass dosage of 1g L^{-1}. After 24 hours of contact time with the single metal solution, the biomass was centrifuged at 10 000 rpm for 5 minutes and the metal concentration from the supernatant analyzed using Absorption Atomic Spectrometer novAA300/400 from Jena Analitik Company.

At the end of the experiments, the solutions were centrifuged at 5 000 ×g for 10 min. The supernatant was filtered through Rotilabo® Nylon membranes with a diameter of 0.20 μm (Carl-Roth) and the metal concentration was determined by Absorption Atomic Spectroscopy (AAS) with flame atomization in a NOVA 300/400 spectrometer (Analytik Jena AG, Jena, Germany). The results were recorded as mg of metal per g dry weight biomass. The uptake capacity at equilibrium was calculated using mass balance equation [7.1] for the biosorbent (Vieira and Volesky, 2000):

$$q = V(C_i - C_e)/S \quad [7.1]$$

The removal efficiency was calculated with the equation [7.2]:

$$R_e = \frac{C_i - C_e}{C_i} \cdot 100 \quad [7.2]$$

7.3.4. Models applied for biosorption study

The empirical Freundlich and Langmuir isotherm models were used for interpreting the lead, cadmium and zinc biosorption equillibrium.

The linearized Langmuir isotherm model is

$$\frac{C_e}{q_e} = \frac{1}{q_m b} + \frac{C_e}{q_m} \quad [7.3]$$

The linearized Freundlich equation is given by

$$\log q_e = \log k_F + {}^{1}/_{n_F} \log C_e \quad [7.4]$$

7.3.5. Models applied for biosorption kinetics

The bacterial and yeast sorption kinetic was investigated at room temperature using a constant adsorbent concentration of 1 g L^{-1} and an initial metal concentration of 100 mg L^{-1}. Samples were taken at different time intervals up to 240 min. The adsorption kinetics of cadmium, lead and zinc were analyzed using Lagergren pseudo-first order and Ho pseudo-second order kinetic models.

The liniarized equation for the pseudofirst-order kinetic model is expressed by

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t \qquad [7.5]$$

The liniarized equation for the pseudosecond-order kinetic model is expressed by

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t \qquad [7.6]$$

7.4. Results and discussion

7.4.1. Identification of the optimum growth medium for biomass production

It was necessary to identify an optimum basal growth media to obtain a **high amount of biomass** to be applied on heavy metal biosorption. Thus, a comparison between the novel EnBase-Flo® cultivation method and the standard growth media was performed.

Bacillus spp. strains produced a significant amount of biomass on EnBase medium supplied with 3 and 6 U L^{-1} amylase, reaching the maximum OD_{600} of arround 30 after only 24 hours. For *Bacillus* spp. MIUG 6.161, unlike the LB medium, the EnBase medium improved the growth with approximately 13 times (Figure 7.1). All bacteria strains showed a high prefferance for EnBase medium supplied with enzyme. All *Bacillus* strains showed a maximum growth rate of approximately 0.4 h^{-1} on LB medium and 0.3 h^{-1} on EnBase medium in the case of all tested amylase concentrations.

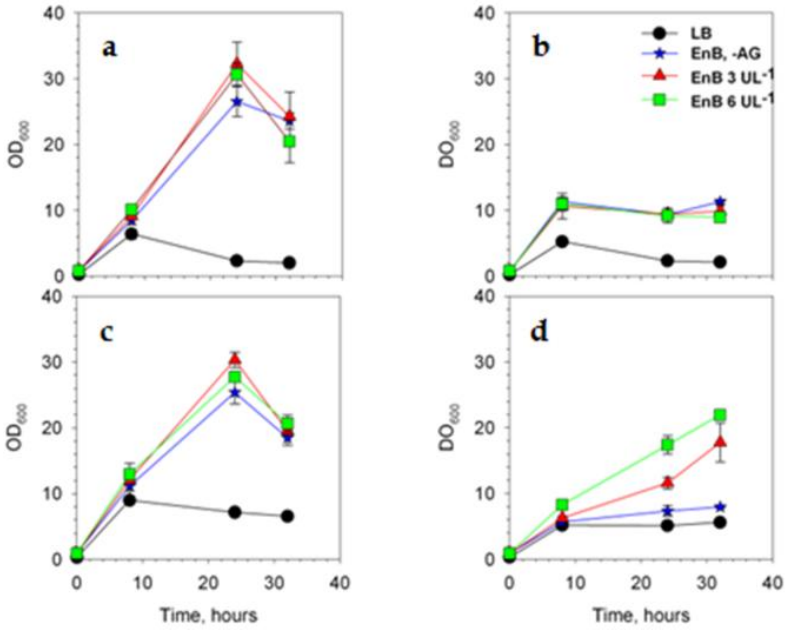

Fig. 7.1. Growth dynamic of the bacteria cultivated on EnBase (EnB) supplimented with different concentrations of amyloglucosidase (AG): a) *Bacillus* spp.MIUG 6.161; b) *Bacillus subtilis* MIUG 6.160; c) *Bacillus* spp. MIUG 6.162; d) *Escherichia coli* W3110

The LB cultivation is a typical fed-batch process characterised by a high initial glucose concentration which steadily decreases over 32 hours of cultivation. Unlike the LB medium, the EnBase-Flo® is a Fed-batch technology where the amyloglucosidase enzyme controls the amount of glucose released through the dextrine degradation; the growth phase is extended as long as the cells find the carbon and energy sources necessary for their metabolism.

The yeast strains *Candida silvae*, *Pichia sp.* and *Kluyveromyces marxianus* strains presented a maximum OD_{600} of 60, 53 and 54, respectively, after 72 hours of cultivation on EnBase medium supplied with 3 U L^{-1} amylase, the growth being improved of almost 10 times compared with the standard YEPD cultivation medium (Figure 7.2). Higher the enzyme activity, higher the cellular growth rate could be calculated.

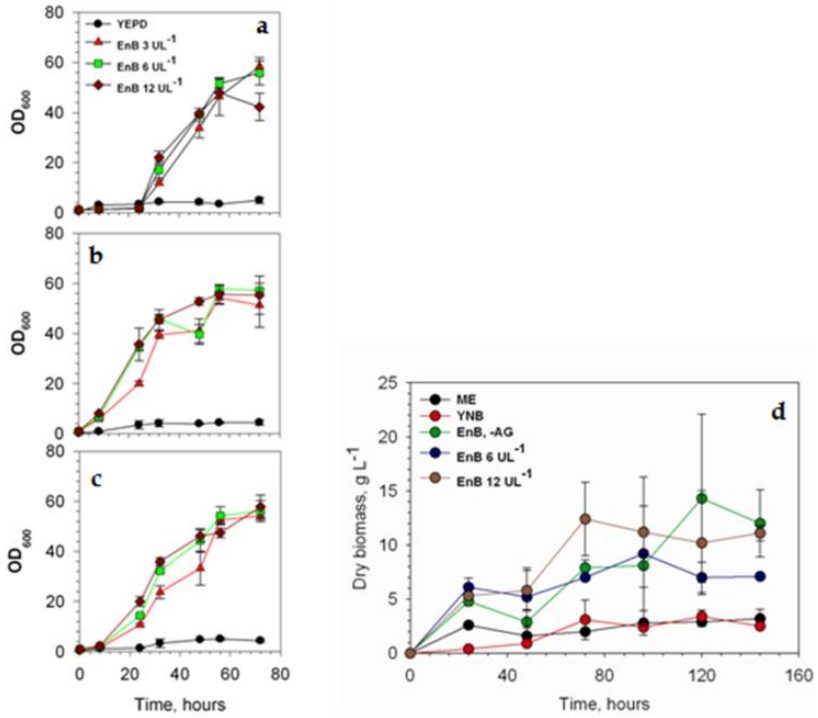

Fig. 7.2. Growth dynamic of the yeast and mould strains cultivated on EnBase (EnB) supplimented with different concentrations of amyloglucosidase (AG): a) *Candida silvae*; b) *Pichia* spp.; c) *Kluyveromyces marxianus.*; d) *Aspergillus niger*

Aspergillus niger mycelia growth was montored by drying the biomass at 60°C for 24 hours. After 120 h of cultivation, a maximum biomass concentration of 14.3 g L^{-1} could be registered on EnBase medium without amylase. The mould strain produced a maximum biomass concentration of 12 g L^{-1} on EnBase medium supplied with 12 U L^{-1} amylase only after 72 hours. The amylase produced by *A niger* increases the amylase concentration in the growth medium, the bioavalible glucose amount, and

consequently the growth rate of the mould cells which leads to the rapid carbon and energy sources exhausting. This explains why the decline phase occurs after 72 hours of cultivation on EnBase supplied with enzyme, faster than on the EnBase medium without enzyme. The novel EnBase medium improved the mould growth with 4 times comparing with the standard growth media (Figure 7.2 d).

Here we cultivated successfully wastewater isolated bacteria, yeasts and mould strains. For all tested strains the final cell densities on EnBase medium were between 4 and 13 times higher compared to the standard cultivation media. The biomass obtained on EnBase was submitted to biosorption studies.

7.4.2. Heavy metal tolerance of the selected microorganisms

Before to evaluate the heavy metal uptake by the wastewater isolated strains, it was necessary to monitor the behaviour of this strains in the presence of different concentrations of metal ions. Thus, two bacteria and three yeast strains tolerance to Zn^{2+}, Cd^{2+} and Pb^{2+} ions was studied. The initial metal concentrations ranged from 25 to 200 mg L^{-1}. The bacterial and yeast cells were inoculated on standard culture media such as LB and YEPD respectively and the optical density (OD_{600}) was determined.

It was interesting to see that the wild strains isolated from wastewater with an organic composition, could grow and resist to high concentrations of heavy metals: 75 mg L^{-1} Zn^{2+} and 100 mg L^{-1} Pb^{2+} (bacteria); 200 mg L^{-1} Zn^{2+} and Pb^{2+} (yeasts) (figure 7.3). Cadmium concentrations higher than 25 mg L^{-1} inghibited the bacteria cells growth. The maximum Cd^{2+} concentration tolerated by the yeast strains was 100 mg L^{-1} (*Candida silvae* and *Pichia* spp.*)* and 50 mg L^{-1} (*K. marxianus)*.

In this study, the viable yeast strains showed a better tolerance to heavy metals than bacteria. Unlike the bacteria cells inoculated on the media supplimented with Zn^{2+} and Cd^{2+} ions, those inoculated on the medium supplimented with Pb^{2+} ions, developed a higher amount of biomass.

It was reported in the literature that a municipal wastewater strain of *Pichia guillermondii* could tolerate maximum 168 mg L^{-1} Cd^{2+} (Balsalobre et. al., 2002). Yazgan (1993) found that the strain *K. marxianus* ATCC 8608 could tolerate a minimum Zn^{2+} and Cd^{2+} concentrations of 653 mg L^{-1} and 281 mg L^{-1} respectively. However it may be remarked, that the specific numbers from different studies must be compared with caution, due to the different experimental conditions employed (pH control, temperature, equilibrium time and biomass dosage) in the different research works.

The isolated strains can represent very good candidates for heavy metal biosorption, taking into account that this microorganisms have a great ability to adapt themselves to different emvironmental conditions.

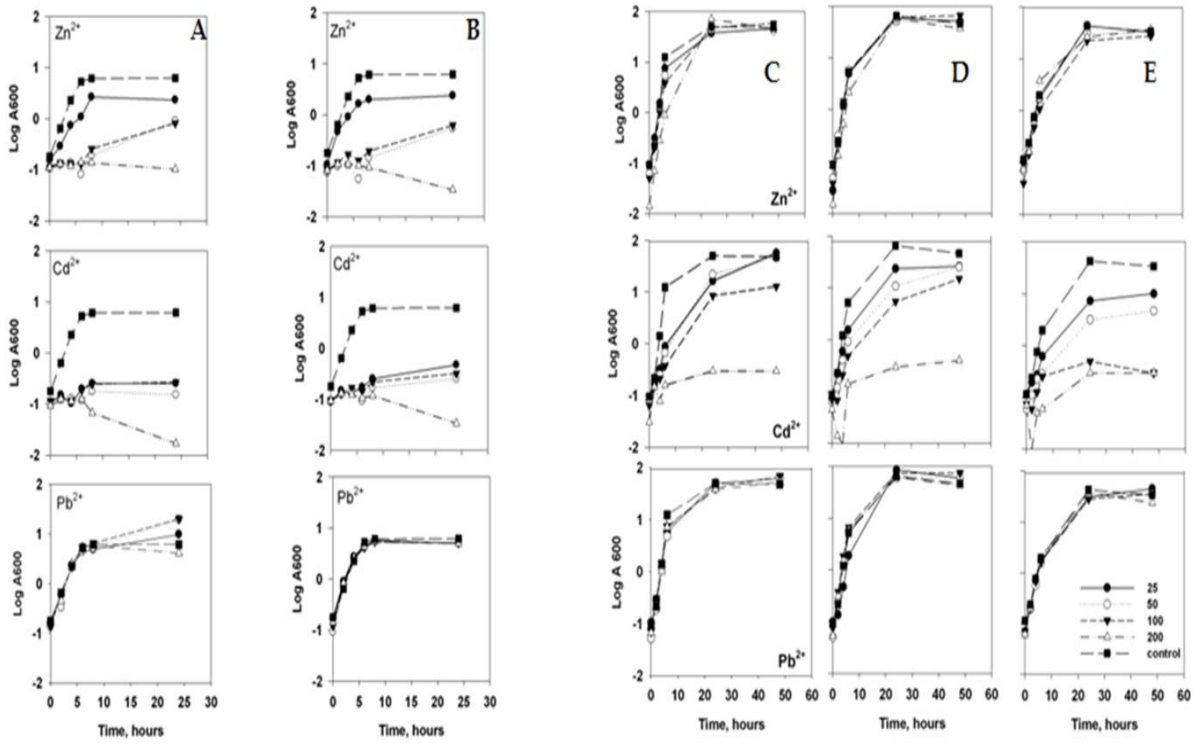

Fig. 7.3. Heavy metal tolerance of the bacteria and yeast strains isolated from dairy wastewater: A. *Bacillus* spp; B. *Bacillus subtilis;* C. *Candida silvae;* D. *Pichia* spp.; E. *Kluyveromyces marxianus*

7.4.3. Influence of growth medium on sorption capacity

There are not so many studies concerning the influence of the growth medium on heavy metal uptake capacity. The majority of scientific data present results with biomass obtained from fermentation processes or strains isolated from different environments and maintained on standard growth media or grown on mineral salt media. To accurately characterize metal speciation, the chemical composition of the medium must be known. This requires use of a chemically defined media to ensure that all components capable of interacting with metals are taken into consideration (Twiss et al. 2001).

In this study, the minimal growth mediu with known composition, EnBase was choosed to get biomass. The EnBase®-Flo medium and technology has been described extensively in recent publications (Glazyrina et al. 2010; Krause et al. 2010).

A comparison between the metal uptake capacity of the bacterial biomass grown on standard medium LB and the biomass grown on the EnBase medium was done for two bacterial strains: *Bacillus* spp. MIUG 6.161 and *Bacillus subtilis* MIUG 6.160. The same cultivation conditions as those described in Section 7.3.3 have been applied. The initial meatal ion concentrations ranged from 5 to 200 mg L^{-1}. The Langmuir model was used to estimate the maximum uptake capacity of the inactive biomass.

Bacillus sp. and *B. subtilis* biomasses both showed higher Cd^{2+} maximum uptake capacities for the EnBase®-Flo grown cells, than for the cells grown in LB. The maximum uptake values obtained for the cells grown in EnBase®-Flo medium indicate an improvement of 18 % and 44 %, respectively. *Bacillus* sp. and *B. subtilis* showed a better maximum uptake capacity for Pb^{2+} if grown in EnBase®-Flo medium compared to LB with an improvement of 7.5 % and 55 % respectively. For Zn^{2+} no enhancement of the uptake capacity for EnBase®-Flo grown cells was detected (Figure 7.4).

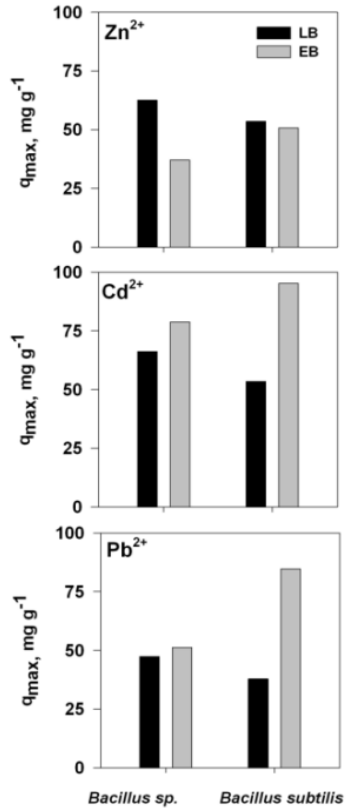

Fig. 7.4. The growth culture influence on heavy metal uptake by *Bacillus* strains (LB-Luria Bertani; EB-EnBase

Taking into account that the medium EnBase contains a small amount of Zn^{2+} ions (80 µg L^{-1}), it is possible that a high number of active sits to be already occupated during the cells growth on this medium. Thus, the biomass composition and the growth medium are very important for the metal ions uptake mechanism (Sing and Tripathi, 2007). In recent years, interests has been focused on increasing the sorption capacity of the biomass. As sorption takes place on the biomass surface, increasing/activating the binding sites on the surface would be an effective approach for enhancing the biosorption capacity. In this study we showed that a new method can be applied to

improve the sorption ability. There are necessary more studies to be performed on this topic.

In this study, the minimal growth mediu with known composition, EnBase, was choosed to get biomass used in the biosorption experiments.

7.4.4. Study and modelling of the bisorption capacity of the inactive and active biomass

Biosorption process to the inactive biomass

The metal uptake capacity of two bacteria strains (*Bacillus* spp. and *Bacillus subtilis)* and three yeast strains (*Candida silvae, Pichia* spp. and *K. marxianus)* was determined.

The Langmuir and Freundlich adsorption models were used for the mathematical description of the biosorption equilibrium and isotherm constants were also evaluated. A pseudo-first and second order models were applied to describe the uptake rate for all tested isolates.

The wastewater isolated strains showed the following order regarding the maximum Zn^{2+} uptake capacity: *B. subtilis* > *Bacillus* spp.> *Pichia* spp. > *C. silvae* > *K. marxianus* (Table 7.1 A). Nevertheless, the maximum sorption rate k_2 of $3.58 \cdot 10^{-2}$ g mg^{-1} min^{-1} was determined for the yeast strain *Pichia* spp., followed by *Candida silvae,K. marxianus, B. subtilis* şi *Bacillus* spp. (Table 7.2). For the maximum Cd^{+2} uptake capacity the order of the strains was *B. subtilis* > *Bacillus* sp. > *K. marxianus* > *C. silvae* > *Pichia* spp. (Table 7.1 B), and the highest rate (k_2 = 6.53×10^{-3} g mg^{-1} min^{-1}) was calculated for *C. silvae*. The maximum Pb^{2+} adsorption capacity was estimated for *K. marxianus* followed by the *Bacillus* isolates, *C. silvae* and *Pichia* spp. (table 7.1 C).; *Pichia* sp. had the highest uptake rate (5.30×10^{-1} g mg^{-1} min^{-1}).

The experimental data reveal that the Langmuir and pseudo-second order models describe succesfully the sorption and kinetic processes for the inactive biomass used as biosorbent. The experimental data validation for the Langmuir model reveal a monolayer sorption mechanism and an homogenous distribution of the active sites on the sorbents surface. Only the Pb^{2+} ions uptake by *B. subtilis* biomass was better described by the Freundlich model which reveals that the ions sorption takes place on a heterogenous surface of the biosorbent.

Tabelul 7.1. Adsorption isotherm parameters for biosorption of Zn^{2+} (A), Cd^{2+} (B) and Pb^{2+} (C) ions with nonviable wastewater isolated microorganisms

A

Strain	Langmuir isotherm				Freundlich isotherm			
	q_{max}, mg g^{-1}	q_{max}, mM g^{-1}	b, L mg^{-1}	R^2	k	1/n	n	R^2
Bacillus spp.	37.04	0.56	0.07	0.98	5.89	0.37	2.72	0.98
B.subtilis	50.76	0.77	0.16	0.99	6.34	0.47	2.14	0.92
C. silvae	8.67	0.13	0.30	1.00	1.31	0.36	2.80	0.95
Pichia spp.	4.58	0.07	0.51	1.00	1.29	0.31	3.21	0.88
K.marxianus	8.16	0.12	0.04	0.73	0.86	0.46	2.19	0.92

B

Strain	Langmuir isotherm				Freundlich isotherm			
	q_{max}, mg g^{-1}	q_{max}, mM g^{-1}	b, L mg^{-1}	R^2	k	1/n	n	R^2
Bacillus spp.	78.74	0.70	0.05	0.91	7.33	0.48	2.07	0.91
B.subtilis	95.24	0.85	0.13	0.99	9.98	0.55	1.83	0.83
C.silvae	53.76	0.47	0.06	0.97	2.10	0.70	1.42	0.82
Pichia spp.	53.76	0.47	0.16	0.99	4.37	0.52	1.91	0.81
K.marxianus	57.14	0.50	0.21	0.99	5.20	0.52	1.90	0.85

C

Strain	**Langmuir isotherm**				**Freundlich isotherm**			
	q_{max}, mg g^{-1}	q_{max}, mM g^{-1}	b, L mg^{-1}	R^2	k	1/n	n	R^2
Bacillus spp.	51.28	0.24	0.07	0.99	4.85	0.49	2.03	0.99
B.subtilis	84.75	0.40	0.01	**0.36**	2.43	0.63	1.59	**0.91**
C. silvae	48.31	0.23	0.02	0.94	1.19	0.72	1.39	0.74
Pichia spp.	22.62	0.11	0.20	1.00	2.33	0.47	2.13	0.80
K.marxianus	59.88	0.29	0.05	1.00	3.61	0.56	1.79	0.94

Tabelul 7.2. Pseudosecond-order model rate constants for adsorption of Zn^{2+}, Cd^{2+} and Pb^{2+} on wastewater isolates biomass powder

Strain	**Zn^{2+}**		**Cd^{2+}**		**Pb^{2+}**	
	k_2, g mg^{-1} min^{-1}	**R^2**	**k_2, g mg^{-1} min^{-1}**	**R^2**	**k_2, g mg^{-1} min^{-1}**	**R^2**
Bacillus spp.	3.14×10^{-3}	0.96	8.62×10^{-4}	0.94	9.76×10^{-4}	0.97
B.subtilis	5.58×10^{-3}	1.00	3.28×10^{-3}	1.00	4.28×10^{-3}	1.00
C. silvae	2.23×10^{-2}	1.00	6.53×10^{-3}	1.00	9.64×10^{-2}	1.00
Pichia spp.	3.58×10^{-2}	1.00	4.51×10^{-3}	1.00	5.30×10^{-1}	1.00
K.marxianus	7.03×10^{-3}	0.99	5.82×10^{-3}	0.99	5.82×10^{-3}	1.00

Other studies on the uptake capacity of *B. subtilis* showed maximum values of 63 mg g^{-1} for Zn^{2+} (Doyle et al. 1980), 22 mg g^{-1} for Cd^{2+} and 124 mg g^{-1} for Pb^{2+} (Volesky et al. 1994). In our study, the maximum uptake capacities for our strain of *B. subtilis*

were in the same order of magnitude, and even higher for Cd^{2+} (95 mg g^{-1}). This encourages us to use this strain in future studies. Due to their good performance for Cd^{2+} and Pb^{2+} removal, the yeast strains will be used in further studies too.

The pseudo-second order model fitted very well the experimental data for the biosorption rate of all three metals, the correlation coefficients (R^2) being higher than 0.97. The uptake rates of yeast strains were higher than for the tested bacteria and metal ions. Eventually this may be the explanation for the faster saturation of the available binding sites for yeast biomass compared to the bacteria.

The biosorbant classification according to Chojnacka (2010) is the basis of the Langmuir isotherms evaluation in this study. Thus, the bacterial strains can be classified as sorbent type 1 for the adsorption of all metal ions, while the yeast strains are classified as sorbent type 4 for Zn^{2+} and as sorbent type 3 for Cd^{2+}. For Pb^{2+} ions, *K. marxianus* showed the characteristics of the sorbent type 1, while *C. silvae* and *Pichia* sp. could be characterized as biosorbents of type 2 and 3, respectively. The sorbents possessing characteristics such as material 1 and 3 are recommended to reduce the concentration of the metals from the polluted environment.

The contact time between the metal and adsorbent has been identified. For all three metal ions the system equilibrium was achieved almost within 15 min for the yeast biomass and within 30 min for the bacterial biomass, respectively (data not sowed). According to the literature, metal ion adsorption reaches the equilibrium within 5 to 30 min (Nuhoglu et al. 2003), this informations supporting very well the experimental data from this study.

Heavy metals bioaccumulation

The maximum metal uptake capacity of the active cells of *Bacillus subtilis* and *Candida silave* strains was tested. The Langmuir and Freundlich models were applied to predict the maximum uptake capacities of Zn^{2+} and Cd^{2+} ions. The maximum uptake capacity of Pb^{2+} ions could be evaluated with the freundlich model which validated the experimental data with an efficiency higher than 96% (Table 7.3).

A comparison between the inactive and active biomass metal uptake capacity could be realized. Thus, the monolayer adsorption capacity of the active cells of *B. subtilis* was 87 mg g^{-1} wet biomass for Zn^{2+} ions and 108.7 mg g^{-1} wet biomass for Cd^{2+} ions. *Candida silvae* viable cells maximum uptake capacity was determined to be 8.67 mg g^{-1} and 6.37 mg g^{-1} wet biomass for Zn^{2+} and Cd^{2+}, respectively. Analysing the parameters of the Freundlich isotherm from the Table 7.3, it can be concluded that the strain *Bacillus subtilis* has a superior lead uptake capacity than *Candida silvae* strain. Even the viable cells of bacterial and yeast strains showed a better uptake capacity for Zn^{2+}, Cd^{2+} and Pb^{2+} ions than the dried nonviable cells, the letter one are preffered because of the superior advantages: the biosorption is a passive one, not controlled by metabolism, nutrients are nor required, the process is reverisble, high metal

concentrations without toxic effect, the absorption rate is faster, high metal affinity, high capacity of biosorbent regeneration with the possibility of the metal recovery, high sorption capacity etc. (Vijayaraghavan et. al., 2008).

Tabelul 7.3. Metal ions bioaccumulation by the active biomass

Strain	Metal	Langmuir isotherm			Freundlich isotherm			
		q_{max}, mg g^{-1}	b, L mg^{-1}	R^2	k	$1/n_F$	n_F	R^2
B. subtilis	Zn	87	0,123	0,9982	9,169	0,6098	1,640	0,9799
	Cd	108,7	0,029	0,9912	3,340	0,7745	1,291	0,9833
	Pb	151,52	0,107	0,4632	13,750	0,7479	1,337	0,9677
C. silvae	Zn	9	0,09	0,9988	2,169	0,3045	3,284	0,9889
	Cd	6,37	0,12	0,9956	2,966	0,1536	6,510	0,9995
	Pb	1,45	-	0,0504	3,421	0,0892	11,211	0,1563

7.4.5. Influence of the initial solut concentration and of the solution pH on the biosorption process

The metal uptake capacity of the inactive biomass of the isolated strains was investigated at equilibrium conditions as a function of the initial metal ion concentration with solutions which contained each one of the metal ions.

Both, bacteria and yeast strains showed a similar behavior regarding the effect of the initial metal concentration on the uptake capacity. An indirect correlation between the removal efficiency and the initial metal concentration was observed with all types of biomass and metal ions used in this work. On the opposite, the uptake capacity at equilibrium for all metals and strains tested was increased with an increasing initial metal ion concentration (Figure 7.5).

The results showed that there is a strong dependence of the initial metal concentration on the removal efficiency. At high metal concentrations the number of sites available for sorption become fewer compared to the number of moles of solute (Baysal et al. 2009). From the low uptake capacity of yeasts at higher initial metal concentration compared to the bacteria we conclude that the saturation of the available binding sites occurred faster with yeast biomass than with bacteria.

Generally the pH is decreasing with an increasing initial metal ion concentration. The difference between initial and final pH values varies from 1.0 unit for Zn^{2+} and Cd^{2+} to 2.0 units for Pb^{2+} ions (data not showed). This fact proves that ion-exchange mechanisms take place during metal uptake. Thus, metal biosorption depends on the protonation or deprotonation of the functional groups on the cell wall: at low pH, i.e. a high concentration of protons, metal binding sites become positively charged and metal cations and protons compete for binding sites, which results in lower uptake rate of the metal ion (Fourest et al. 1997).

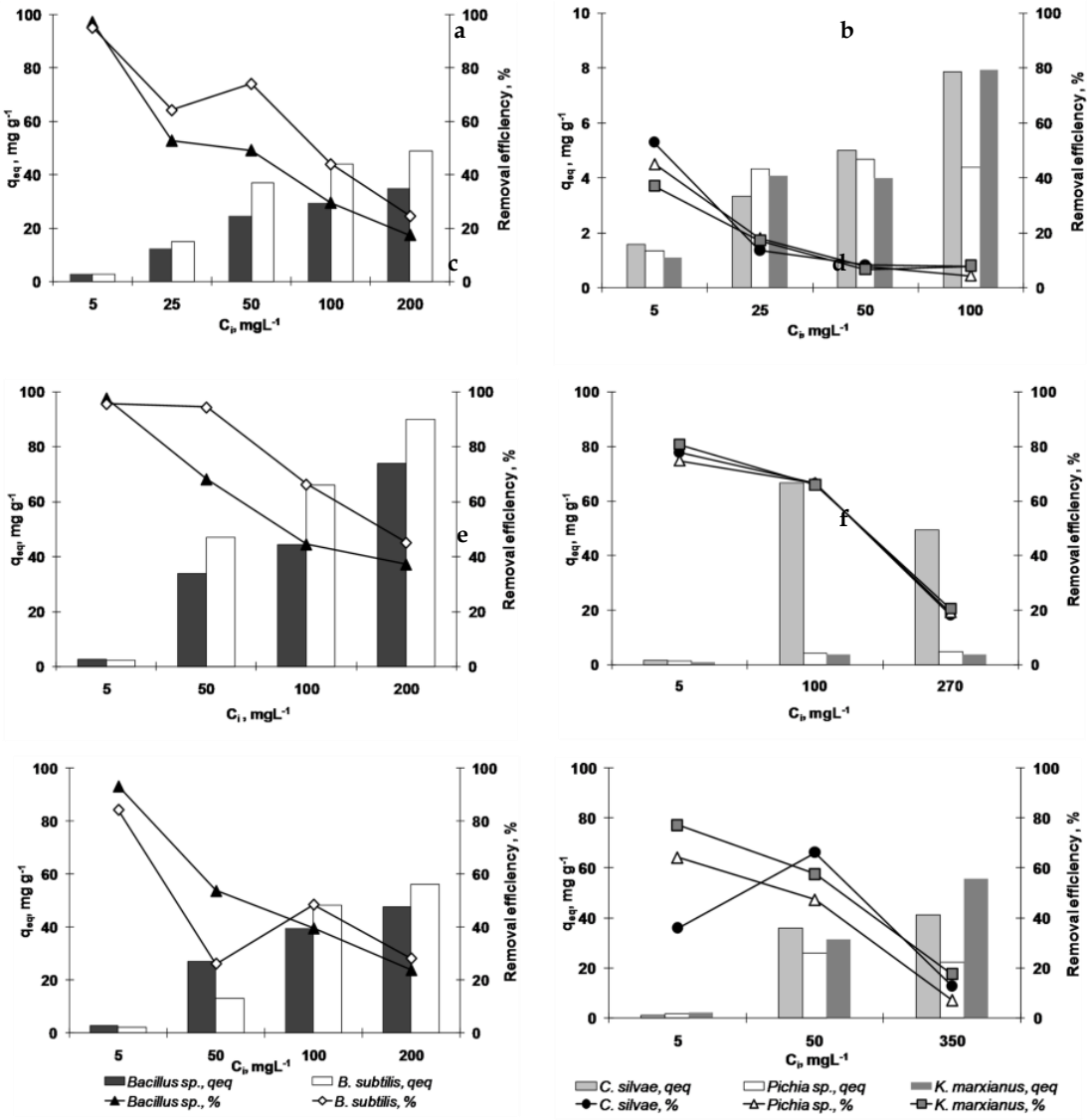

Fig. 7.5. Effect of increasing concentration of Zn^{+2} (a, b), Cd^{+2} (c, d) and Pb^{+2} (e, f) on uptake capacity at equilibrium of inactive dry biomass of wastewater isolated bacteria (a, c, e) and yeast (b, d, f)

7.5. Conclusions

- This study revealed that the new cultivation technology EnBae-Flo® can be successfuly applied to get high amout of biomass of wastewater isolated microorganisms. For all tested strains the final cell densities on EnBase medium were between 4 and 13 times higher compared to the standard cultivation media.

- The bacteria and yeast strains isolated from the dairy wastewater could tolerate high concentrations of Zn^{2+} and Pb^{2+} metal ions.
- The sorption studied on the inactive biomass revealed the following:
    - The EnBase-Flo® medium improved the sorption capacity of Cd^{2+} and Pb^{2+} ions with a percentage value of 44 % and 55 %, respectively.
    - The empirical models Langmuir and Freundlich described 91% and 74%, respectively, from the experimental data.
    - The bacteria strains showed a better heavy metals uptake capacity in monocomponent system, than the yeast strains.
    - The bacterial biomass was classified as sorbent type 1, and the yeast biomass as sorbent types 1 , 2 and 3.
    - The Pseudo-second order Kinetic model describes better the experimental data for all tested strains and metals.
    - The time necessary for bacterial and yeast strains to reach the equilibrium phase was identified to be 30 and 15 minutes, respectively.
    - A dirrect correleation between the uptake capacity and the initial solut concentration has been established.
    - The pH decreasing proves that ion-exchange mechanisms take place during the metal uptake.
- The active (viable) bacterial biomass showed a superior metal bioaccumulation capacity than the yeast biomass.

8. Final conclusions

The researches realised according to the scientific objectives of the PhD thesis, which aimed the identification of some efficient solutions for the biodegradable organic compounds and heavy metals removal from the industrial wastewater, conducted to the following final conclusions:

1. Different cathegories of microorganisms (bacteria, yeasts and moulds) were isolated from the dairy wastewater and the strains with the highest capacity to bioconvert organic compounds such as lactose, lactic acid and casein were selected for the wastewater biological treatment.
2. The taxonomy of the isolated and selected strains, identified by molecular biology tools, revealed the following species: *Bacillus* spp., *Bacillus subtlis, Candida silvae, Pichia* spp., *Kluyeromyces marxianus, Aspergillus niger*.
3. The aerobic treatment of the dairy sinthetic wastewater, performed in batch systems, established that the maximum organic compounds removal efficiency of the activated sludge and wastewater microoganisms was 89% and 94%,

respectively; a maximum effciency of 78% was registered for the activated sludge in contous system.

4. The wastewater treatment effciency can be improved by the control and optimisation of the independent variables which influence the evolution of the bioconversion processes of the organic compounds from the wastewater composition.
5. The correlations established between the treatment parameters lead to a better understanding of the wastewater biological treatment process.
6. The automated control of the treatment process offers the possibility to monitore and guide the process variables such as dissolved oxygen, organic substrate, biomass growth as well as the possibilty to remove the perturbations which occur during the continous system.
7. The new fed-batch cultivation technology, EnBase-Flo®, can be successfully applied for all cathegories of microorganisms, to obtained high amount of biomass with improved metal uptake qualities.
8. The wastewater isolated and identified strains represent very good candidates for Zn^{2+}, Cd^{2+} and Pb^{2+} ions removal from aqueous solutions, in monocomponent systems.
9. The modelling studies revealed the complexity of the biological treatment processes.

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9. Original contributions and perspectives

The PhD thesis researches bring the following original contributions:

- The biochemical, microbial and genetical characterization of some dairy wastewater isolated microorganisms, as well as the selection of some performant strains for the bioremediation field was performed.
- The aerobic biological treatment process conducted both in batch and continous systems in a wastewater treatment plant, has been studied and modeled using synthetic dairy wastewater. The most important parameters for the biotreatment efficiency improving were identified using as starter cultures wastewater isolated and selected microorgansims or activated sludge.
- The optimum biotechnological conditions for the production of biomass used as biosorbent has been established by applying the novel fed – batch cultivation system, EnBase-Flo®.
- The heavy metals Zn^{2+}, Cd^{2+} and Pb^{2+} biosorption process has been studied and modeled using as biosorbent inactive and active biomass coming from the microorganisms (bacteria, yeasts) isolated from the dairy wastewater.

In the context of the geographical position of the Galati city (situated on the left bank of the Danube, at the junction of the Siret River and Prut, near Lake Brates) and of the intense economic life developed arround the metalurgical and food industries, the studies regarding the wastewater bioremediation through cheap nonconventional methods are necessary for the equillibrium maintenance between the socio-economic life and the surrounding aquatic ecosystems. The results obtained encourage us to continue the researches for the industrial wastewater treatment process optimisation aimed to help the local companies for a better management of the water resources, to the environment protection and to improve the life quality.

10. Results dissemination

The results obtained in the frame of the doctoral stage have been disseminated in 2 scientific articles published in ISI journals (one of them being under review at World Journal of Microbiology and Biotechnology) and 3 papers presented in representative national and international conferences.

A. Articles published in ISI journals

Mihaela Palela, George Ifrim, Marian Barbu, Gabriela Bahrim, Sergiu Caraman, **2010.** Strategies for the aerobic biological treatment of the dairy wastewaters in controlled conditions, *Environemental Engineering and Management Journal,* 9(3), 399-405**.**

Mihaela Palela, Gabriela Bahrim, Eva Brand, Julia Glazyrina, Peter Neubauer, 2011. Removal of heavy metals using microbial isolates from industrial wastewaters: a valuable alternative for Romanian polluted waters. Under review to the *World Journal of Microbiology and Biotechnology*.

B. **Articles published in Indexing Data Basis journals**

Mihaela Palela, George Ifrim, Gabriela Bahrim, 2008. Microbiological and biochemical characterization of dairy and brewery wastewater microbiota**,** *The Annals of the University Dunărea de Jos of Galați, Fascicle VI–Food Technology*, New Series, II (XXXI), ISSN 1843 – 5157, 23-30;

http://www.ann.ugal.ro/tpa/FT%202008.htm

C. Scientific contributions presented in representative national and international conferences

Mihaela Palela, George Ifrim, Marian Barbu, Gabriela Bahrim, Sergiu Caraman, 2009. The metabolic activity evaluation of a specialised inoculum used for aerobic biological treatment of a simulated dairy-processing wastewater, INTERNATIONAL WORKSHOP, BENA, GALAȚI.

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