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Bidimensional gel electrophoresis analysis of *Enterobacter hormaechei* D15 proteins in response to Diclofenac

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Abstract

The continuous detection of pharmaceutical compounds in the aquatic environment due to their incomplete removal in WWTP has become a hot spot for researchers regarding their toxic effect to the environment and human health. The present study aims to attempt to analyze the protein profile of *E. hormaechei* D15 cells that were exposed to DCF at final concentration of 10mg/l. For this purpose, double dimensional gel electrophoresis analysis with isoelectric focusing from pH 3 through 10 and a 12.5% polyacrylamide SDS gel is performed using either cells grown in presence of DCF or cells grown in presence of glucose (control). The results revealed that the bacterium D15 is able to grow in presence of DCF as sole carbon and energy source. Bidimensional gel electrophoresis analysis showed that a total of 12 unique spots were observed in cytosolic fraction (soluble proteins), with 16 and 8 differentially spots in DCF and glucose treated samples, respectively. Also, a total of 21 unique spots were observed in insoluble fraction (Membrane proteins) with 11 and 7 differentially spots expressed in DCF and glucose treated samples, respectively. The observed proteins have a molecular weight ranging from 10 to 120 KDa and a pI ranging from 5 to 8.A difference in protein profile between DCF treated samples and control (glucose treated samples) indicated that specific proteins may be induced for DCF breakdown. Meanwhile, further studies to characterize enzymatic mechanisms involved in its biodegradation are needed.

1. Introduction

The emergence of pharmaceutical compound in different environmental matrices such as soil, water, and sediment has been an attractive issue in recent years, a great number of studies have been carried out to elucidate the harmful effect that pharmaceuticals may impose to the environment and health of living beings [1-3]. Diclofenac (DCF) is one of the persistent micropollutants in the environment; it is belonging to the group of non-steroidal anti-inflammatory drugs used in human and animal health [4-5].

In the environment it is detected in the range of ng to µg/l [6-7]. The removal of such micropolutants is essential to prevent environmental contamination and possible adverse effects [8-11]. In general biodegradation is considered to be the most important removal mechanisms. The capacity of various microorganisms to biodegrade persistent organic pollutants has been widely studied during the last decades to deal with the extensive contamination of the environment; the attention has been focused on bacteria and filamentous fungi [12- 13].

In general microorganisms used in the process of bioremediation have the important characteristics like the ability to transform the substrate, the specific rate of substrate removal and the resulting metabolites [14]. Only few studies have focused on the biochemistry of pharmaceuticals biodegradation. of studies carried out on DCF biodegradation in the environment have suggested the formation of

hydroxylated and carboxylated by products. These metabolites were used to propose degradative pathways of DCF by different microorganisms [15-16]. This method has a great important to understand DCF or pharmaceuticals microbial degradation; however, it remains to be elucidated if all different microorganisms degrade DCF with the same proposed pathways or by some other unknown mechanisms. Therefore, global analyses of protein expression profile maybe more informative tool for understanding the physiological mechanisms of biodegradation [17].

The present study deals with the analyses of the total protein profile of a DCF degrading bacterium *Enterobacter hormaechei* (D15) in presence and absence of DCF in order to elucidate the difference in protein profile expression using bidimensionnal electrophoresis.

2. Material and Methods

1.1.Bacterial strain and culture conditions

Diclofenac-degrading bacterium *Enterobacter hormaechei* D15 isolated from activated sludge was used in this experiment. An overnight culture of the selected strain was prepared in nutrient broth. The culture was centrifuged at 6000 rpm for 10 min; the pellet was washed twice with saline solution and resuspended in distilled water. 100 ml of the MMSM containing either 10 mg/l of DCF or 10 mg/l of glucose was inoculated with 5% (OD=1.2) of the bacterial suspension and incubated under shaking (150 rpm). The bacterium grown on MMSM supplemented with glucose was used as control. Optical density was measured at 600 nm for the monitoring of bacterial growth. When cultures reached the late exponential phase, they were harvested and centrifuged at 10000 rpm for 20 min at 4 °C. The pellet was washed tree time with saline solution (0.85% NaCl) and stored at -80 °C until use for proteomic analysis [18].

1.2.Protein extraction

The cells cultivated in presence of DCF or glucose was used for protein extraction. The extraction was carried out using the Ready Prep^{TM} protein extraction kit (Soluble/ Insoluble) according to the manufacturer's instructions. The resulted soluble and insoluble proteins were divided in aliquots and stored at -80°C until the 2D electrophoresis analysis.

1.3. Protein quantification

Total protein concentration present in the samples was measured using Bradford method. 10 µl of protein samples (soluble/Insoluble) was mixed with 90 µl of Bradford reagent (Coomassie blue) in 96-well plates. The samples were incubated in dark for 1h, after that the OD was measured at 595nm. The protein concentration was estimated using BSA standard calibration curve (In the appendix) and expressed as mg/l. the presence of proteins in the samples was confirmed by SDS-PAGE Electrophoresis.

1.4. Double dimension electrophoresis

1.4.1. Rehydration of the samples

Double dimension electrophoresis analysis was carried out according to Ahmed et al., (2015). 20 µl of the extracted protein was mixed with 125 µl of rehydration buffer containing (7 M urea, 2 M thiorea, 2% (w/v) CHAPS, 15 mM DTT and 0.5% (V/V) Immobilized pH gradient (IPG) buffer (pH 3-10) (Biorad). The strips gel (7 cm, pH 3-10) was placed gently on the prepared mixture and covered with 2 ml of mineral oil to prevent evaporation during rehydration process. Samples were covered and incubated at room temperature overnight (11-16 h) to rehydrate the IPG strips and load the protein samples.

1.4.2. Isoelectric focusing step (IEF)

The IPG strips were removed from rehydration tray and transferred to the corresponding channel in the focusing tray and covered with 2 ml of mineral oil. The samples were placed into the protein IEF cell and programmed using 3 steps protocol (250 V for 20 min, 4000 V for 2 h and 4000 V for 3 h). When the electrophoresis run has been completed, the IPG strips were removed from the focusing tray and transferred into a new and clean rehydration/equilibration tray and conserved at -70°C until further analysis.

1.4.3. IPG strips equilibration

The stored IPG strips were removed from the freezer and placed onto the lab bench to thaw. After that the strips were covered with 2.5 ml equilibration buffer I containing (1.5 mMTris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace bromophenol blue, and 10 mg/l of DTT) and placed on orbital shaker for 15 min. After the incubation, the strips were removed from the equilibration buffer I and placed in the equilibration buffer II (1.5 mMTris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace bromophenol blue, and 250 mg/l of Iodoacetamide). The samples were returned to the orbital shaker for 15 min.

1.4.4. SDS- PAGE electrophoresis

After the end of the equilibration step, the IPG strips were washed with 1X Tris/ glycine/SDS running buffer (25 mMTris, 192 mM glycine, 0.1% SDS, pH 8.3) and placed on the top of a 12.5% continuous polyacrylamide vertical gel (16 mm×16 mm), and sealed with preheated agarose solution (25 mMTris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue). Electrophoresis was performed at a constant current of 120 V/gel for 3–5 h until the advancing bromophenol blue dye reached the bottom of the gels. The gels were stained overnight using Coomassie Brilliant Blue R-250 stain method.

1.4.5. Coomassie Brilliant Blue R-250 stain method

At the end of SDS-PAGE, the gels were placed into a tray containing the stain (Coomassie blue). The gels were placed on an orbital shaker for at least 60 min. the stained gels were destained with a destain solution (10% acetic acid and 40% methanol in water) until the background staining is acceptable. Destain solution was changed several times to obtained good results. Gels were stored in a solution of 10% acetic acid until the image analysis.

1.4.6. Image analysis

After the Coomassie Brilliant Bleu R-250 stain, three gels of each sample were imaged using a densitometer (Bio-rad's GS-800 calibrated imaging densitometer) and the gel images were proceeded with Adobe photoshope5.0 software and saved in the TIFF image file. The images were then matched and analyzed using PD Quest 2D gel analysis software (version 8, Bio-rad). Protein expression was considered different between the samples if the mean normalized spot volume varied at least two-fold. This was confirmed by analysis of variance at a significant level of $p<0.05$.

3. Results and discussion

The present study aims to attempt to analyze the protein profile of *E. hormaechei* D15 cells that were exposed to DCF at final concentration of 10mg/l. For this purpose, double dimensional gel electrophoresis analysis with isoelectric focusing from pH 3 through 10 and a 12.5% polyacrylamide

SDS gel was performed using either, cells grown in presence of DCF and cells grown in presence of glucose (control). Bacterial growth of the selected strain was monitored over the period of the experiment in the two samples (control and DCF samples). The results revealed that the bacterium D15 was able to grow in presence of DCF as sole carbon and energy (Figure 1). The same results were obtained in the previous experiment of biodegradation of DCF as sole carbon source by the strain D15[19], were the elimination rate of DCF was 52.8%. This transformation of DCF led to the appearance of1-(2, 6 dichlophenyl) -1.3-dihydro-2H-indol.2.one as DCF transformation product. The highest growth rate was obtained in the samples with glucose (OD = 0,535). The expressed proteins in the samples were first revealed by SDS PAGE. Bidimentional gel electrophoresis analysis showed that, a total of 12 unique spots were observed in cytosolic fraction (soluble protein) with 16 and 8 differentially spots in DCF and glucose treated samples, respectively. And a total of 21 unique spots were observed in insoluble fraction (Membrane protein) with 11 and 7 differentially spots expressed in DCF and glucose treated samples, respectively. The observed proteins have a molecular weight ranging from 10 to 120 KDa and a pI ranging from 5 to 8 (Figure 2, Table 1).

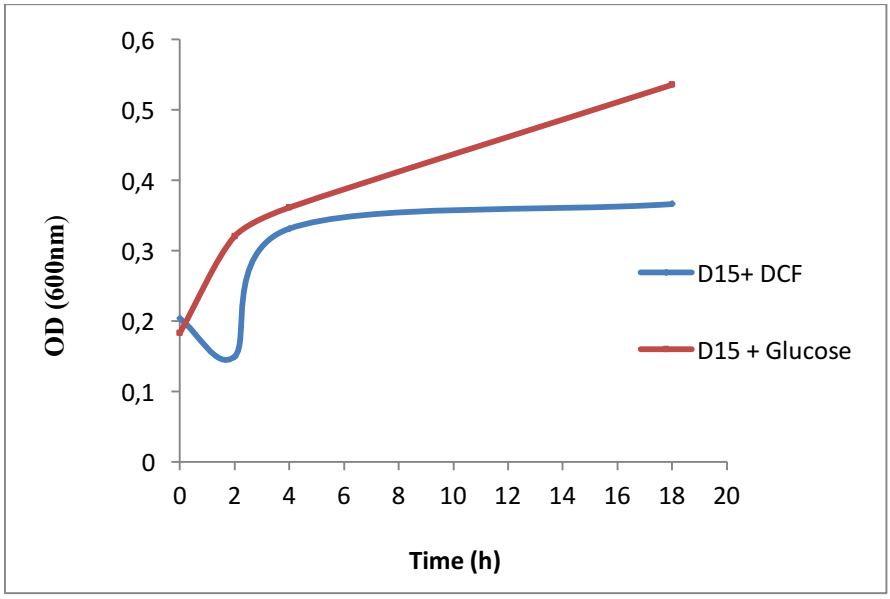


Figure 1: *E. hormaechei* D15 growth in presence of glucose (10 mg/l) and in presence of DCF (10 mg/l) as sole carbon source in MMSM

Table 1: *E. hormaechei* D15 proteins in presence of glucose or DCF

DCF exposed samples		**Glucose exposed samples**	
Soluble proteins	**Insoluble proteins**	**Soluble proteins**	**Insoluble proteins**
28±1 spots	32±3 spots	20±2 spots	28±1 spots

Bardot et al [6] reported that, when the bacterium *B. megaterium* was exposed to mesotrione as sole source of carbon, a stress situation was observed. In this case several proteins were up regulated compared to the control. Kulkarni et al [12] also reported that the exposition of microbial cells to different physical and chemical stresses induce synthesize of several proteins for the bacterial protection. Shimizu et al [15] stated that microbial organism's synthesis different enzymes for the breakdown of organic compounds to generate energy for cell growth. These enzymes change dynamically in response to the change in growth conditions and cell state.

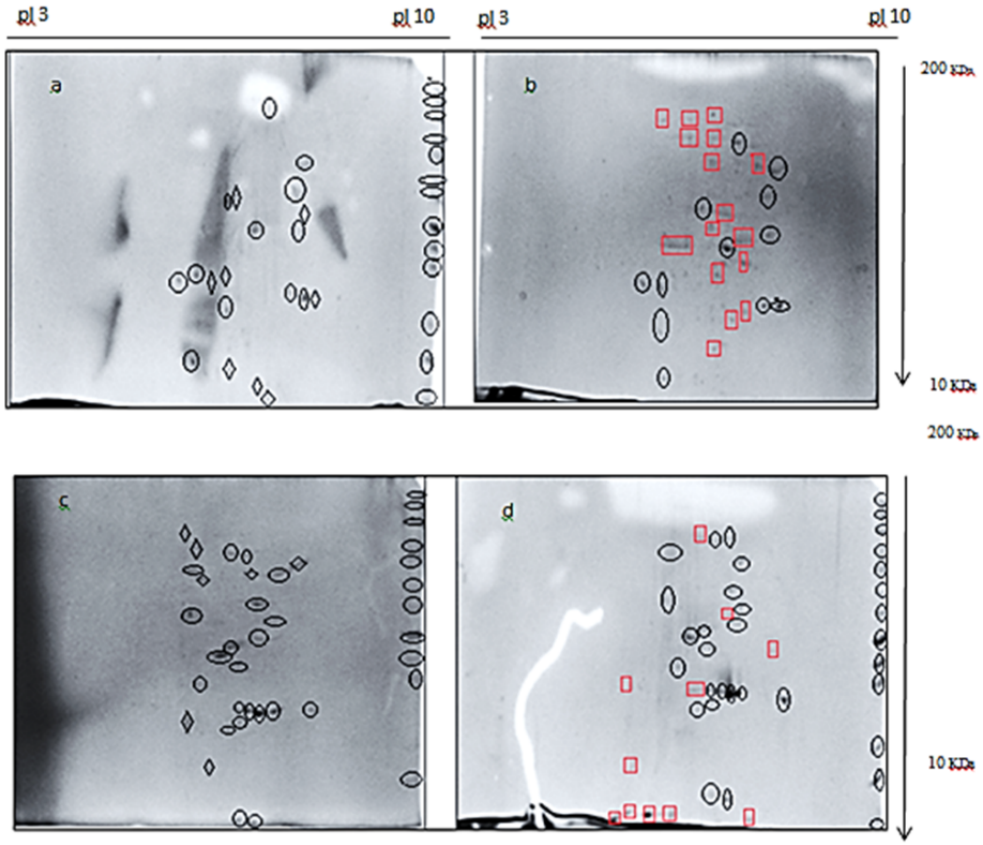
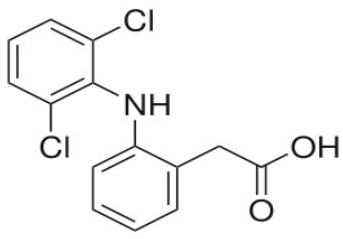


Figure 2: Comparative two-dimentional electrophoresis gel images showing the proteome of *E. hormaechei* D15: (a) soluble fraction in presence of glucose as carbon source, b) soluble fraction in presence of DCF as carbon and energy source, c) insoluble fraction in presence of glucose as carbon source, d) insoluble fraction in presence of DCF as carbon source ◇: spots present in control and DCF samples, ▭ : spots present only in the control, ⬭ : spots present only in DCF samples.

Generally, under stress conditions bacterial cells synthesize only the necessary elements to preserve for growth. This may explain the disappearance of some spots in DCF supplemented samples (present in glucose treated samples). DCF is a drug with a complex structure; specific protein may be induced for its breakdown. The expression of new proteins in DCF supplemented samples was noticed. In another study conducted by Schmidt et al [10] the sublethal effects of DCF on the protein profiles of marine mussels (Mytilus spp.) was evaluated. Twelve protein spots were significantly increased or decreased by diclofenac, seven of which were successfully identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These proteins were involved in energy metabolism, oxidative stress response, protein folding, and immune responses.



Diclofenac molecule

Kraigher et al., [20] investigated the influence of pharmaceuticals on bacterial community in activated sludge exposed to increasing concentrations of diclofenac in fed-batch reactors over 41 days. They

reported that nitrification activity was not detectably influenced by the addition of diclofenac, while the main change of the bacterial community structure was detected only at the end of incubation (after 41 days) when diclofenac was added to artificial wastewater as the only carbon source.

DCF biodegradation was reported in several studies by both bacterial and fungal cells, but much less information is available about the enzymatic systems involved in this biodegradation. According to the literature, most of the available studies are conducted on the fungal enzymes involved in DCF biodegradation but the information about bacterial transformation/ biodegradation of polycyclic NSAID is scarce. In this context, Domaradzka et al [8, reported that, biodegradation of DCF by *Phanerochaetesordida*YK-624 resulted in the formation of hydroxylated DCF (4' OH DCF, 5 OH DCF and 4'-5 OH DCF). The authors suggested that DCF hydroxylation is due to the cooperation of monooxygenase with cytochrome P-450 but the role of extracellular ligninolytic enzymes was not excluding. Groning et al [9] revealed that DCF was degraded in river sediment and the 5-hydroxyDCF-*p*- benzoquinone imine was considered as the major metabolites. The authors suggest that *p*-benzoquinone imine was formed by dehydration but no enzymes were proposed.

Kjeldal et al [12] studied the proteomic of gemfibrozil- degrading Bacillus sp Ge 10; they observed that the abundance of several proteins increased when the bacterial cells are exposed to gemfibrozil and they identified the cytochrome P450 along with an alcohol dehydrogenase and a potential catechol-2, 3-dioxygenase as candidate enzymes involved in the transformation of the drug. Almeida et al [1], studied the proteomic of IBU-degrading *Patulibacter* sp. strain I11, they found that, a number of proteins are up-regulated in response to active degradation of IBU and some of these proteins are known to be involved in aromatic compounds degradation. Similarly, Maravic et al [15] reported that after the exposition of the strain *E. hormaechei* sub sp. *Steigerwatii*51 to cefotaxime, 1072 spots from the whole-cell proteome were detected and 35 proteins were differentially expressed. The authors revealed that 50% of these proteins were involved in cell metabolism, then cell wall organization/ virulence, stress response and transport.

Conclusion

Proteomic is a powerful tool for acquiring information on gene/protein function and has an important role in the development of environmental biotechnologies. Further studies for the identification of the proteins expressed in the presence of DCF are needed to understand the metabolic pathway involved in biotransformation/biodegradation of DCF by *E.hormaechei* D15 and to provide a better understanding of DCF biodegradation in the environment.

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References

1. B. Almeida, Oehmen, R. Marques, D. Brito, G. Carvalho, C. Barreto, Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture, *Bioresource Technology*,133 (2013): 31-37. https://doi.org/10.1016/j.biortech.2013.01.035
2. I.A.M. Ahmed, M.E. Eltayeb, N. Mori, J. Arima, H. Tanaka, T. Taniguchi, N. Yamanaka, Proteomic analysis of homocholine catabolic pathway in Pseudomonas sp. strain A9, *Process Biochemistry*, 50 (2015) 1735-1747. https://doi.org/10.1016/j.procbio.2015.07.001

3. B. Almeida, H. Kjeldal, I. Lolas, A.D. Knudsen, G. Carvalho, K.L. Nielsen, M.B. Crespo, A. Stensballe, J.L.Nielsen, Quantitative proteomic analysis of ibuprofen-degrading Patulibacter sp. strain I11, *Biodegradation* 24(2013) 615-630. doi: 10.1007/s10532-012-9610-5
4. P Paíga, Correia M, Fernandes MJ, Silva A, Carvalho M, Vieira J, Jorge S, Silva JG, Freire C, Delerue-Matos C (2019) Assessment of 83 pharmaceuticals in WWTP influent and effluent samples by UHPLC-MS/MS: Hourly variation. Sci Total Environ 648:582–600. https://doi.org/10.1016/j.scitotenv.2018.08.129
5. Kumar R, Sarmah AK, Padhye LP (2019) Fate of pharmaceuticals and personal care products in a wastewater treatment plant with parallel secondary wastewater treatment train. J Environ Manage 233:649–659. https://doi.org/10.1016/j.jenvman.2018.12.062
6. C. Bardot, P. Besse-Hoggan, L. Carles, M. Le Gall, G. Clary, P. Chafey, C. Federici, C. Broussard, I. Batisson, How the edaphic Bacillus megaterium strain Mes11 adapts its metabolism to the herbicide mesotrione pressure, *Environmental Pollution* 199 (2015) 198-208. doi: 10.1016/j.envpol
7. S. Aissaoui, E. Fagnani, S. Pérez, H. Ouled-Haddar, M. Sifour, Removal of diclofenac by a local bacterial consortium: UHPLC-ESI-MS/MS analysis of metabolites and ecotoxicity assessment, Brazilian Journal of Microbiology (2021):03-25 .DOI: 10.1007/s42770-021-00464-9
8. D. Domaradzka, U. Guzik, K. Hupert-Kocurek, D. Wojcieszyńska, Cometabolic Degradation of Naproxen by Planococcus sp. Strain S5, *Water, Air, & Soil Pollution* 226(2015) 297. DOI:10.1007/s11270-015-2564-6
9. J. Gröning, C. Held, C. Garten, U. Claußnitzer, S.R. Kaschabek, M. Schlömann, Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate, *Chemosphere* 69 (2007) 509-516. DOI:10.1016/j.chemosphere.2007.03.037
10. Courtier A, Cadiere A, Roig B (2019) Human pharmaceuticals: Why and how to reduce their presence in the environment. Curr Opin Green Sustain Chem 15:77–82. https://doi.org/10.1016/j.cogsc.2018.11.001H
11. Schmidt, W, Rainville, L.- C, McEneff G, Sheehan Dand Quinn B. A proteomic evaluation of the effects of the pharmaceutical's diclofenac and gemfibrozil on marine mussels (*Mytilus* spp.): evidence for chronic sublethal effects on stress- response proteins. Drug Test. Analysis, 6 (2014): 210-219. Doi:10.1002/dta.1463
12. Kjeldal, N.A. Zhou, D.K. Wissenbach, M. vonBergen, H.L. Gough, J.L. Nielsen, Genomic, Proteomic, and Metabolite Characterization of Gemfibrozil-Degrading Organism Bacillus sp. GeD10, *Environmental Science and Technology* 50 (2015) 744-755. DOI:10.1021/acs.est.5b05003
13. A.G. Kulkarni, B. Kaliwal, Proteomic Profiling of *Escherichia coli* in Response to Carbamate Pesticide-Methomyl, , *In* Soloneski S ,Marcelo(eds), *INSECTICIDES–BASIC AND OTHER APPLICATIONS,* (2012) Intech.DOI: 10.5772/2446
14. Singh VK, Singh R, Madhav S, Ahamad A, Pal DB, Tiwary D, Mishra PK (2020) Bioremediation: a sustainable approach for management of environmental contaminants. Elsevier Inc
15. A. Maravić, S. Cvjetan, M. Konta, R. Ladouce, F. A. Martín, Proteomic response of β-lactamases-producing Enterobacter cloacae complex strain to cefotaxime-induced stress, *Pathogens and Disease* 74 (2016). DOI:10.1093/femspd/ftw045

16. K. Shimizu, Metabolic regulation of a bacterial cell system with emphasis on Escherichia coli metabolism, *ISRN Biochemistry*, 18(2013):645-983. DOI:10.1155/2013/645983
17. A.B. Caracciolo, E. Topp, P. Grenni, Pharmaceuticals in the environment: biodegradation and effects on natural microbial communities. A review, *Journal of Pharmaceutical and Biomedical Analysis*, 106 (2015) 25-36. https://doi.org/10.1016/j.jpba.2014.11.040
18. S. Gupta, B. Pathak, M.H. Fulekar, Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review. *Reviews in Environmental Science and Bio/Technology*, 14 (2015) 241-269. https://doi.org/10.1007/s11157-014-9353-3
19. S. Aissaoui, H. Ouled-Haddar, M. Sifour, K. Harrouche, H. Sghaier, Metabolic and co-metabolic transformation of diclofenac by Enterobacter hormaechei D15 isolated from activated sludge, *Current Microbiology* 74(2017) 381–388. doi: 10.1007/s00284-016-1190-x
20. B. Kraigher and I.Mandic-Mulec, Influence of Diclofenac on Activated Sludge Bacterial Communities in Fed-Batch Reactors, Food Technol. Biotechnol. 58 (4) 402–410 (2020). https://doi.org/10.17113/ftb.58.04.20.6424

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