Title: Norfloxacin degradation by Bacillus subtilis strains able to produce biosurfactants on a bioreactor scale

Author: Łukasz Jałowiecki, Joanna Żur, Grażyna Anna Płaza

Citation style: Jałowiecki Łukasz, Żur Joanna, Płaza Grażyna Anna. (2017). Norfloxacin degradation by Bacillus subtilis strains able to produce biosurfactants on a bioreactor scale. "E3S Web of Conferences" (Vol. 17 (2017), art. no. 00033), doi 10.1051/e3sconf/20171700033

Norfloxacin degradation by *Bacillus subtilis* strains able to produce biosurfactants on a bioreactor scale

Łukasz Jałowiecki[1*], *Joanna* Żur[2], and *Grażyna A.* Płaza[3]

[1]Department of Environmental Microbiology, Institute for Ecology of Industrial Areas,40-844 Kossutha 6 Katowice, Poland
[2]Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice,40-032 Jagielońska 28 Katowice, Poland
[3]Department of Environmental Microbiology, Institute for Ecology of Industrial Areas, Katowice, Poland

Abstract. The discharge of antibiotics into the environment has become a major concern since this group of pharmaceuticals influence on microbial communities not only by its mode of action, but also because of the risk of a worldwide dispersal of antibiotic resistance genes (ARG). Antibiotics residues have been found in various environments such as waters, sediments, and soils. Moreover, most WWTPs are not designed to treat such kind of pollutants, which remain incompletely removed. Currently, biodegradation processes which involved bacterial strains with increased degradation capabilities is one of the most promising technique. The aim of this study was to evaluate the norfloxacin biodegradation potential of the three *Bacillus subtilis* strains named T-1, T'-1 and I'-1a on a bioreactor scale. The aerobic degradation was conducted in a 5-liter bioreactor on minimal salts medium in co-metabolic culture supplemented with glucose. The degradation rate of norfloxacin was determined with the HPLC technique. The surface tension was determined using ring method in order to observe the changes in biosurfactants production. Also, the biofilm formation abilities of the bacteria with two quantitative methods, crystal violet (CV) method and TTC-based test and enzymes production were evaluated.

1 Introduction

Currently, the annual production of antibiotics ranging from 100,000 to 200,000 tons per year [1, 2]. The increasing intake of pharmaceuticals results in uncontrolled pollution of the environment with these substances and their metabolites. Simultaneously, the popular aggressive chemical methods used in wastewater treatment plants (WWTPs) e.g. ozonation, Fenton processes or photocatalysis with TiO_2 are not able to complete removal of that kind of pollutants. Recently, biodegradation processes, which involved microorganisms with increased degradation capabilities is one of the most promising method used in WWTPs.

* Corresponding author: jalowiecki@ietu.katowice.pl

In this study microbial consortium composed of three *Bacillus subtilis* strains (T-1, T'-1 and I'-1a) was used for norfloxacin, the next-generation fluoroquinolone antibiotic degradation on a bioreactor scale. Fluoroquinolones, the synthetic class of antibiotics derived from nalidixic acid are widely used against both Gram-negative and Gram-positive microorganisms. All of the examined strains are able to lipopeptide (LPs) active surface compounds produce namely surfactins, iturins and fengycins [3, 4]. Biosurfactants, despite their ability to reduce the surface and interfacial tension are also classified as one of the chemical class of quorum sensing (QS) molecules. QS is defined as the regulation of gene expression in response to changes in cell-population density. Microorganisms use quorum sensing communications to regulate different physiological activities i.e. virulence, metabolites synthesis, sporulation and biofilm production. It is also widely known that microorganisms, which are able to surface-active compounds produced are characterized by good abilities to rapid colonization of different surfaces and initiating of biofilm formation [5]. In this study, two quantitative methods used for evaluation of biofilm formation diverse in terms of detection sensitivity were applied. Microorganisms with increased degradation potential may be used for construction of bacterial consortia and its subsequent immobilization. Besides this, microorganisms represents an alternative source of useful enzymes e.g. inulinases, cellulases, proteases or lipases, which have widespread uses in biotechnology, industry and medicine.

2 Materials and methods

Bacterial strains. Three *Bacillus subtilis* strains were used in the study named: T-1, T'-1 and I'-1a. They were isolated from sludge of an oil-refinery in Czechowice-Dziedzice in Poland [6]. In the previous study, the strains were identified and characterized by Płaza et al. [3] and Bernat et al. [4].

Degradation of norfloxacin. Degradation experiment was performed in a bioreactor (BioFlo 415, New Brunswick Scientific, US) containing 5 liters of mineral salts medium (MSM) with the following composition, (g L^{-1}): 3.78 $Na_2HPO_4 \cdot 12H_2O$; 0.5 KH_2PO4; 5 NH_4Cl; 0.2 $MgSO_4 \cdot 7H_2O$; 0.01 yeast extract. The cometabolic culture was supplemented with glucose. The bacteria were cultivated on LB medium up to 48 h, under shaking conditions (130 rpm) at 30ºC. Then, the culture samples were centrifuged at 5 000 *g* for 10 min, and bacteria were resuspended in mineral salts medium. The suspensions of the each bacteria were added to the bioreactor. Norfloxacin was also added to the final concentration of 7.5 mg L^{-1}. The degradation rate of norfloxacin was determined with the HPLC technique using Merck Hitachi HPLC reversed-phase chromatograph equipped with a column Ascentis Express® C18, pre-column Opti-Solw® EXP, and UV/VIS DAD detector. The mobile phase was consisted of acetonitrile and 1% acetic acid (10:90 v/v; flow-rate 1 ml/min). The detection wavelength was set at 270 nm.

Determination of biosurfactants production. 50 ml of bacterial culture was centrifuged at 5 000 g for 15 min and the supernatant was used for the surface tension measurements. The surface tension was determined by DeNouya method using a ring with a tensiometer SIGMA 702 (Attension). Measurements were repeated at least three times, and an average value was used to express the surface activity of each sample.

Enzymes production.

Amylase activity. In order to detect the amylase activity, bacteria were plated on nutrient agar plates supplemented with 1% starch. The plates were then incubated at 28 ± 1ºC for 48 h. After the appearance of the colonies on the starch-agar medium, the culture plates were flooded with 1% Lugol's iodine solution to identify amylase activity.

Cellulase activity. For the detection of cellulose activities, isolates were grown on plates with Carboxymethyl Cellulose agar (CMC) and flooded with 1% Lugol's iodine

solution to identify the enzyme activity. The presence of cellulolytic activity was confirmed by the appearance of yellow colour around the colonies.

Inulinase activity. The bacteria were inoculated on MHI agar plates, which contained inulin as the sole carbon and energy source and incubated at 28 ± 1ºC for 3 to 5 days. Inulin was sterilized separately and added before pouring the medium into the plates, with the final concentration of 2%. After incubation, plates were flooded with Lugol's iodine solution for 5 min. A clear halo appeared around the colonies, where the inulin has been degraded.

Lipase activity. Lipolytic activity of the bacterial strains was determined on blood agar plates and incubated at 28°C for 48 h. After autoclaving, the blood agar was cooled, then 5% (v/v) defibrinated sheep blood was added. The positive samples were then plated again on the Tween 80 agar plates with phenol red indicator and incubated at 28°C for 4 days. The lipase activity will bring about change in coloration from pink to lemon yellow.

Protease activity. The extracellular protease production was evaluated with two methods. In the first method the isolates were plated on enriched nutrient agar (2% starch) plates and incubated at 28 ± 1°C for 48 h. The appearance of a clear zone around the colony after flooding the plate with 1% Lugol's iodine solution confirmed the presence of proteolytic activity. In the second method, Skimmed Milk Agar (SMA) was used as a screening medium for the protease production. The plates were incubated at 28°C for 48 h. Development of clear zones around the bacterial strains confirmed the proteolytic activity of the strains.

Urease activity. In order to evaluate the urease activity, the bacteria were streaked on the Urea Agar Base (UAB) and incubated at 28°C for 48 h. The urease activity was determined by the appearance of the purple colour around the colonies.

Biofilm production. The abilities to biofilm production of the tested strains was performed according to procedures described by Stepanović et al. [7] for crystal violet (CV) and Brown et al. [8] for 2,3,5-triphenyltetrazolium chloride (TTC) reduction, with certain modifications. Each well of 96-well tissue culture plate were filled with 200 µl sterile LB medium and 20 µl of individual overnight culture strain dilution of 1:100 in fresh LB medium. The plates were incubated for 48 h at 30°C. Then, the wells were washed three times with 200 µl per well of sterile phosphate buffer saline (PBS, pH 7.2), emptied and left to dry. Next, the plates were fixed with 200 µl per well of methanol for 30 min, dried and then stained with 200 µl per well of 0.1% crystal violet dissolved in acetone:ethanol mixture (1:4, v/v) solution for 20 min. After staining followed by brief drying, 200 µl per well of 30% acetic acid was added into each well in order to resolubilized the dye bound to the adherent cells and incubated for 30 min. For TTC analysis, each well of the plate were filled with 200 µl sterile LB medium and 20 µl of overnight culture diluted to OD_{600} = 0.05. After incubation, the wells were washed with 200 µl per well of sterile phosphate buffer saline (PBS, pH 7.2), emptied and left to dry. Afterwards, 0.5% TTC solution were added to each well and incubated for 24 h. After the incubation period, the wells were emptied and stain with 200 µl of acetone/ethanol (1:4, v/v). Negative controls were obtained by incubating the wells only with 200 µl per well of LB medium, without bacteria. The optical density (OD) of the obtained solutions was measured at 600 nm and 490 nm for crystal violet and TTC assay, respectively, using a microtiter plate reader (Eppendorf).

3 Results and discussion

The concentration of antibiotic residues detected in the environment usually ranging from ng/L to µg/L level in natural water and wastewater and from µg/kg to mg/kg level in soil and sludge. Currently, antibiotics are considered to be emerging pollutants due to the risk of development, maintenance, transfer or spread of antibiotics resistant bacteria and

antibiotics resistance genes (ARG) [2]. Norfloxacin is a synthetic fluoroquinolone antibiotic used for the treatment of conjunctivitis, gastrointestinal, urinary tract and skin infections with broad-spectrum antibacterial activity against most Gram-negative and Gram-positive bacteria, widely used in both human and veterinary medicine. Norfloxacin and other quinolones may enter into the environment mainly from hospital and municipal wastewaters. Simultaneously, the most intensively used techniques for wastewater treatment e.g. ozonation or UV/H_2O_2 and Fenton processes are not sufficient to complete removal of different class of pharmaceuticals e.g. antibiotics, non-steroidal anti-inflammatory drugs, hormones or lipid regulators [2, 9]. Moreover, it is widely known that antibiotics from fluoroquinolone class, i.e. ciprofloxacin and norfloxacin, are poorly biodegradable. Up to now, literature data on microbial degradation of norfloxacin remain scarce. Parshikov et al. [10] reported that species from *Escherichia*, *Pseudomonas*, *Microbacterium*, *Mycobacterium* and *Streptomyces* genera are able to transform norfloxacin by site-specific hydroxylation. Metabolism of norfloxacin *via* *N*-acetylation, oxidation, and breakdown of the piperazine ring has been reported for humans and fungi. *Microbacterium* sp. isolated from wastewater have been shown to transform the norfloxacin to four main metabolites, namely: *N*-acetylnorfloxacin, desethylene *N*-acetylnorfloxacin, 8-hydroxynorfloxacin and 6-hydroxynorfloxacin. Kim et al. [11] observed that during growth of the strain in medium supplemented with norfloxacin four main metabolites were produced. All of them were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) analyses. As results showed the metabolites were identified as: 8-hydroxynorfloxacin, 6-defluoro-6-hydroxynorfloxacin, desethylene norfloxacin, and *N*-acetylnorfloxacin [11]. As Lindberg et al. [12] and Santos et al. [2] reported more than 70% of fluoroquinolones present in the wastewater remained adsorbed to sludge, which may results in their releasing into the environment i.e. as biosolids, manures or wastewaters for agriculture. As many authors highlighted these antibiotic residues may affect microbial activity and structure in both soil and water environments. It is widely known that antibiotic residues in wastewater may results in occurrence of selective pressure on bacteria for development of resistance. However it is worth to note, that resistance to fluoroquinolones is multifactorial and include chromosome-mediated spontaneous mutation, production of multidrug-resistance (MDR) efflux pumps, modifying enzymes or target proteins [11, 13].

The high resistance of bacteria to fluoroquinolones results also from the multifunctionality of mechanisms responsible for this resistance, since MDR and RND (resistance-nodulation-division) pumps which are also involved in heavy metals or different agents i.e. ethidium bromide or hydrocarbons removal. Yang et al. [9] suggested that microorganisms play a crucial role in norfloxacin degradation in soil. Slightly changed nitrogen transformation and negligible effect of antibiotic on soil respiration suggested that the environmental concentration of norfloxacin may have a inconsiderable effect on microbial activity. Pan and Chu [14] suggested that the microbial transformation and degradation of norfloxain can be attributed to microbial resistance against fluoroquinolones. In this study, the bacterial consortium consist of the three examined strains from the *Bacillus* genus was able to degrade of 5.5 mg L^{-1} of norfloxacin during 26 days in cometabolic systems with glucose. (Fig. 1). The estimated reduction of norfloxacin was 75% during the experiment time. Species from the *Bacillus* genus are characterized by different industrial and biotechnological applications due to their good degradation abilities and production of various molecules, i.e. lipopeptide (LP) biological surface active compounds include surfactins, iturins, bacillomycins, fengycins and lichenysins. LP biosurfactants greatly reduce surface and interfacial tension and they are known as good emulsifiers, dispersing and foaming agents used in chemistry, food production, agriculture, cosmetics and in environmental biotechnology for soil and water treatment. One of the

tested strain, *B. subtilis* I'1a synthetized iturin A C14 and C15, which antimicrobial effects on planktonic and sessile form were confirmed for *Escherichia coli*, *Serratia marcescens*, *Enterobacter cloacae*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterococcus faecalis* [4]. During the time of the experiment the changes in surface tension were also measured. As results showed during norfloxacin degradation the surface tension of the bacterial culture was significantly decreased (Fig. 2).

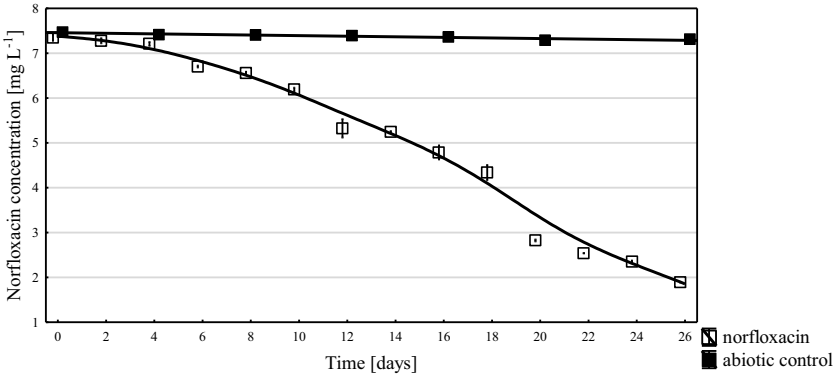

Fig. 1. Degradation of norfloxacin in the cometabolic culture by the bacterial consortium.

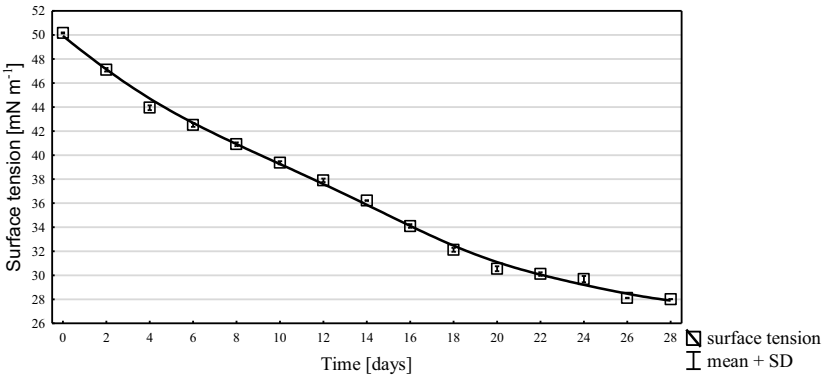

Fig. 2. Changes of the surface tension during the experiment period.

Biological surface active compounds and biosurfactants-producing microorganisms are widely used in hydrocarbon and metal-contaminated environments. Singh and Cameotra [15] used the lipopeptide biosurfactants, surfactin and fungycin, obtained from *Bacillus subtilis* A21 strain for heavy metals i.e. cadmium, cobalt, zinc, copper, lead, nickel and petroleum hydrocarbon removal from the soil samples collected from industrial landfill. As results showed soil washing technique with the use of biosurfactant solution significantly removed the petroleum hydrocarbon (64.5% of initial amount) and heavy metals (from 44.2% for cadmium to 32.07% for zinc) content. Barin et al. [16] investigated the impact of bacterial consortium consist of microorganisms able to biosurfactants produce and with increased biodegradation abilities on the improvement of biodecontamination processes. The results revealed that the consortium of *Bacillus subtilis* tb1 and *Pseudomonas aeruginosa* was able to kerosene hydrocarbons decontamination and efficiency of the removal was at least 25% higher than single species. Moreover, the biosurfactants showed potent antimicrobial activity for both Gram-positive, Gram-negative and fungi. The negative influence of surface-active compounds on *Bacillus megaterium* and

Saccharomyces cerevisiae [17], *Legionella pneumophila* [18], *Listeria monocytogenes* or *Bacillus cereus* [19], is well documented. It is worth to note, that up to now there is no scientific literature on the role of biosurfactants in the antibiotic degradation. One of the aim of this study was also to determine the biofilm formation abilities of the tested strains. Biofilm is composed of highly structured multispecies bacterial communities embedded in a self-produced polymeric matrix called extracellular polymeric substances (EPS) [20, 21]. Biofilms as a mode of the bacterial growth predominate in a different ecosystems. Microorganisms in biofilms are characterized by increased tolerance to xenobiotics and various environmental condition (e.g. chemical and shear stresses, acidification). Spatial organization, varied genes expression and different metabolic pathways exhibited by bacteria in biofilms allow to biodegradation, biotransformation or detoxification of various toxic compounds. In recent years biofilm-mediated bioremediation processes are widely used for environmental purposes. As Edwards and Kjellerup [22] noticed that indigenous bacterial communities are able to metabolize and/or degrade organic contaminants and oxidizing heavy metals, however their inadequate abundance and activity and limited access to nutrients results in insufficient rate of degradation. Thus, the introduction of bacteria with increased degradation abilities and biofilm formation capabilities seems to be reasonable. Biofilm formation was determined by two different quantitative methods, with the use of crystal violet (CV) and reduction of 2,3,5-triphenyltetrazolium chloride (TTC) (Tab. 1).

Table 1. Biofilm production by the *Bacillus* strains.

Strain	CV		TTC (mg/ml)
	Absorbance 600 nm	Stepanović classification	
I'-1a	2.761±0.16	strong biofilm producer	1.026±0.009
T'-1	0	no biofilm producer	0.0015±0,0008
T-1	0.972±0.09	weak biofilm producer	0,245719±0,001

Stepanović classification: nonbiofilm producer (OD less than or equal to ODc); weak biofilm producer (OD greater than ODc and less than or equal to 2xODc); moderate biofilm producer (OD greater than 2xODc and less than or equal to 4xODc) and strong biofilm producer (OD greater than 4xODc). This classification is based upon the cut-off value called ODc (ODc means average OD of negative control + (3 x standard deviations of negative control).

These two methods are different in terms of discrimination strength, crystal violet allow to detection the whole biofilm biomass include live and dead cells or acidic polysaccharides as well, while TTC staining allows to detect only metabolically active cells. On the basis of obtained results, for CV method, the examined strains were divided into the following categories using the classification of Stepanović et al. [7]. According to obtain results from CV method, the strain I'-1a was classified as strong biofilm producer, T'-1 as weak biofilm producer, while T-1 strain was unable to form the biofilm. The results from TTC staining were similar as obtained from CV methods, e.g. I'-1a and T'-1 produced 1.02 mg/ml and 0.24 mg/ml of formazan, respectively. The concentration of formazan produced by T-1 strain was estimated about 0.0015 mg/ml. Abilities of LPs produced by bacilli to affecting the bacterial adhesion properties, flagella development or to disturb the integrity of membranes and inhibition of biofilm formation is well documented [4]. The negative influence of mixture consist of fengycin and surfactin produced by *B. subtilis* and *B. licheniformis* on *E. coli* and *S. aureus* adhesion was observed by Rivardo et al. [23]. Wang et al. [24] suggested that bacteria coordinate i.e. biosurfactants and exopolysaccharide

production which mediated biofilm formation by mechanism in which competition for common sugar precursors mannose-1-phosphate (Man-1-P) and glucose-1-phosphate (Glc-1-P) used for both expopolysaccharides and rhamnolipids synthesis serves as control of the bacterial motility, biofilm formation and surfactants production. Surface active agents secreted by Gram-positive bacteria often serves also as signaling molecules in quorum sensing process. Quorum sensing is a cell–cell communication mechanism that synchronizes gene expression in response to population cell density. Only when the population density is high, the accumulation of the signal in the extracellular environment is sufficient to activate the response. Bacteria which can self-produce biosurfactants possess some biological advantages related to colonization of hydrophobic surfaces and initiating of biofilm formation. Production of biosurfactants enable also rapid surface colonization which called swarming. This type of surface translocation is defined as coordinated movement across solid or semi-solid surface considered as the fastest type of bacterial motility [5]. Our results also confirmed that strain I'1a which produced the highest concentration of surfactants was classified as strong biofilm producer. Rhamnolipids produced by *Pseudomonas aeruginosa* have been shown to factor which influence of maintaining the channels between mushroom-shaped biofilm which allow the distribution of oxygen and nutrients and removal of metabolites from the biofilm structure. Boles et al. [25] demonstrated also that secretion of the right amount of rhamnolipids was critical for proper biofilm development. Solano et al. [5] highlighted that quorum sensing up-regulates the biosurfactants production. Further, the abilities of the particular strains to different enzymes production were also evaluated Obtained results showed that all of the examined strains produced inulinases, ureases, proteases, lipases and amylases. Celullases were produced by two strains, T-1 and T'-1. All of the tested enzymes are widely used for biotechnological and industrial purposes e.g. inulinases are used for ultra-high fructose syrup obtaining from inulin, bioethanol production and some chemicals production, like citric acid or lactic acid. Ureases are used for urea content analysis in urine, blood, wastewaters or heavy metals content in natural environments. Proteases, lipases and amylases have great commercial potential for detergents, pharmaceuticals, leather, dairy, food, chiral alcohols, carboxylic acids, amines or epoxides and dairy production. Microbial cellulases are of great interests in various industrial applications including pulp and paper, textile, biofuel and food industry, brewing, and agriculture[26- 28].

In conclusion, three *Bacillus subtilis* species have the specific properties, e.g. production of lipopeptide biosurfactants and various enzymes, biofilm formation, and their consortium was able to norfloxacin degrade in the cometabolic culture. The results obtained from the biodegradation experiment are promising. To our best knowledge, there are no available reports on the norfloxacin biodegradation by the strains which produced biosurfactants. Moreover, the role of biosurfactants in antibiotic degradation is still unknown.

This work resulted from the BONUS no 2112932-1 project was supported by BONUS (Art 185), funded jointly by the EU and The National Center for Research and Development (Poland).

References

1. W. Baran, E. Adamek, J. Ziemiańska, A. Sobczak. J. Hazard. Mater, **196**, 1 (2011)
2. L.V. Santos, A.M. Meireles, L.C. Lange, J. Environ. Manage.**154**, 8 (2015)
3. G.A. Płaza, M. Pacwa-Płucieniczak, Z. Piotrowska-Seget, R. Brigmon, E. Król *Environmental Sustainability*, 173 (2015)
4. P. Bernat, K. Paraszkiewicz, P. Siewiera, M. Moryl, G. Płaza, J. Chojniak, World J. Microbiol. Biotechnol, **32**, 157 (2016)

5. C. Solano, M. Echeverz, I. Lasa, Curr. Opin. Microbiol,**18,** 96 (2014)
6. C.J. Berry, S. Story, D.J. Altman, R. Upchurch, W. Whitman, D. Singleton, G. Płaza, R.L. Brigmon. Oxford University Press, Oxford, UK, pp 87 (2006)
7. S. Stepanović, D. Vuković, V. Hola, G. Di Bonaventura, S. Djukić, I. Ćiraković, F. Ruzicka. APMIS, **115**, 891 (2007)
8. H.L. Brown, A.H.Vliet, R.P. Betts, M. Reuter, J. Appl. Microbiol, **115,** 1212 (2013)
9. J. Yang, G. Ying, S. Liu, L. Zhou, J. Zhao, R. Tao, P. Peng, J. Environ. Sci. Health. Part B, **47**, 288 (2012)
10. I.A. Parshikov, J.B. Sutherland J Ind Microbiol Biotechnol, **39**,1731 (2012)
11. D.W. Kim, T.M. Heinze, B.S. Kim, L.K. Schnackenberg, K.A. Woodling, J.B. Sutherland. Appl. Environ. Microbiol, **77**, 6100 (2011)
12. R.H. Lindberg, U. Olofsson, P. Rendahl, M.I. Johansson, M. Tysklind, B.A.V. Andersson. Environ. Sci. Technol, **40**, 1042 (2006)
13. L.S. Redgrave, S.B. Sutton, M.A. Webber, L.J.V. Piddock, Trends Microbiol, **22,** 438 (2014)
14. M. Pan, L.M. Chu, Sci. Total Environ, **545**, 48 (2016)
15. A.K. Singh, S.S. Cameotra, Environ. Sci. Pollut. Res, **20**, 7367 (2013)
16. R. Barin, M. Talebi, D. Biria, M. Beheshti, Int. J. Environ. Sci. Technol, **11,** 1701 (2014)
17. M.I. Sriram, K. Kalishwaralal, V. Deepak, R. Gracerosepat, K. Srisakthi, S. Gurunathan, Colloids and Surfaces B: Biointerfaces, **85,** 174 (2011)
18. C. Loiseau, M. Schlusselhuber, R. Bigot, J. Bertaux, J.M. Berjeaud, J. Verdon. Appl. Microbiol. Biotechnol, **99**, 5083 (2015)
19. K.J. Perez, J.S. Viana, F.C. Lopes, J.Q. Pereira, D.M. Santos, J.S. Oliveira, R.V. Velho, S.M. Crispim, J.R. Nicoli, A. Brandelli, R.M Drummond Nardi, Front. Microbiol. **8**, 61 (2017)
20. T. More, J.S.S. Yadav, R.D. Tyagi, R.Y. Surampalli. J. Environ. Manage, **144**, 1 (2014)
21. A. Mitra, S. Mukhopadhyay. AIMS Bioengineering, **3**, 44-59 (2016)
22. S.K Edwards, B.V.Kjellerup, Appl.Microbiol.Biotechnol, **97**, 9909 (2013)
23. F. Rivardo, R.J. Turner, G. Allegrone, H. Ceri, M.G. Martinotti. App. Microbiol. Biotechnol, **83**, 541-553 (2009)
24. S. Wang, S. Yu, Z. Zhang, Q. Wei, L. Yan, G. Ai, H. Liu, L.Z. Ma, Appl. Environ. Microbiol, **80**, 6724 (2014)
25. B.R. Boles, M. Thoendel, P.K. Mol. Microbiol, **57**, 1210 (2005)
26. C. Neagu Bonciu, G. Bahrim, Innov. Rom. Food Biotechnol**, 9**, 1 (2011)
27. R.C. Kuhad, R. Gupta, A. Singh, Enzyme Research, **2011,** 1 (2011)
28. J.L. Adrio, A.L. Demain. Biomolecules, **4**, 117-139 (2014)