CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649091

Biotechnological Strategies of *Pseudomonas fluorescens* Growth on Dibenzothiophene

Thayse. A. L. Silva*[a], Maria Luiza R. B. Silva[b], Ian Garrard[c], Elias. B. Tambourgi[d], Galba. M. Campos-Takaki[e]

[a]National Program of Postdoctoral CAPES (CAPES/UNICAP) – Post-Graduation in Development of Environmental Processes, UNICAP, Nunes Machado Street, n° 42, Boa Vista, Zip code: 50.050-590 Recife, Brazil.
[b]National Program of Postdoctoral CAPES / Agronomic Institute of Pernambuco (IPA), Genome laboratory, General San Martin Av., 1371 Bongi, Recife, Brazil.
[c]Brunel Institute for Bioengineering, Brunel University, Heinz Wolff 001, Brunel University,Uxbridge UB8 3PH , United Kingdom.
[d]School of Chemical Engineering, State University of Campinas, UNICAMP, Zip code: 13083-852, Campinas, SP, Brazil.
[e] Nucleus of Research on Environmental Sciences, Catholic University of Pernambuco, UNICAP, Nunes Machado Street, n° 42, Boa Vista, Zip code: 50.050-590 Recife, Brazil.
silva_tal@yahoo.com.br

The growth kinetics of a bacterium of the strain *Pseudomonas fluorescens* UCP 1514, which is indigenous in soil, has been investigated in batch cultures containing dibenzothiophene. In this paper the potential of the bacterium, when grown in medium with high concentration of dibenzothiophene and its potential for biodegradation were analyzed. The microorganism was grown in medium Luria Bertani – agar medium with 10mM of dibenzothiophene during 24 hours, to select acclimated colonies. After selection of the colonies, the microorganism was maintained in liquid medium with 2 mM of DBT dissolved in dimethylformamide. The kinetics of growth was evaluated through viability and pH. The results showed that the microorganism was able to metabolize DBT. The colonies physiologically adapted that grew on DBT, by DNA amplification using the PCR (polymerase chain reaction) technique and the specific primer BOX have also been identified.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise a large and heterogeneous group of organic contaminants that are formed and emitted as a result of the incomplete combustion of organic material. Sulfur-oxide gases (mostly SO_2) are released during the combustion of sulfur compounds, which results not only in serious air pollution, but also poison metal catalysts (Pawelec et al., 2011). Benzothiophene (BT), dibenzothiophene (DBT), and their alkylated derivatives, account for more than 50% of the total sulfur content of commercial diesel (Chi et al., 2011). These aromatic thiophenes are recalcitrant organic sulfur compounds and more resistant to hydrodesulfurization (HDS) treatment than other sulfur compounds such as mercaptans and sulfides (Hasan et al., 2012).
Hydrodesulfurization (HDS) is the most extensively employed method. However, some aromatic sulfur-compounds, such as 4- and 4,6-alkyldibenzothiophene DBT, and polyaromatic sulfur compounds, show resistance to be completely removed (Calzada et al., 2012).
Microbes have been in contact with crude oil since early stages of formation and maturation in different oil reservoirs. During this long history of contact, microorganisms have evolved enormous metabolic activities, which enabled them to utilize and transform nearly every chemical class in crude oil for their survival (Mohamed et al., 2015). Biodesulfurization (BDS), a microbial-mediated process for specific S removal from thiophenic compounds encountered in crude oils such as benzothiophene (BT), dibenzothiophene (DBT), and their alkylated homologs, has been reported in numerous microbial species (Xu et al., 2009).
Therefore, research regarding the degradation or desulphurization of such thiophenic compounds would be necessary for environmental aspects. Considerable development, in recent years, has occurred in the

Please cite this article as: Silva T., Silva M.L., Garrard I., Tambourgi E.B., Campos Takaki G., 2016, Biotechnological strategies of pseudomonas fluorescens growth on dibenzothiophene, Chemical Engineering Transactions, 49, 541-546 DOI: 10.3303/CET1649091

application of Molecular Biology Techniques, especially in PCR analysis (Polymerase Chain Reaction), which allows the amplification of given sequences of DNA molecule. In research on genetic diversity, PCR analysis has often been used with specifics primers, such as BOX, ERIC and REP, and the profiles obtained have resulted in products after reaction which allow the strains to be differentiated (Bustos-Jaimes et al., 2003). Genetic variation within rhizobia was assessed using BOX-PCR and was shown to be a useful method for differentiating closely related rhizobia. This method was used to assess whether the presence of heavy metals in soil led to noticeable changes in rhizobia diversity. BOX-PCR analysis was preferred to other genetic approaches such as (16S-DNA analysis) due to its ability to detect DNA mutations, insertions and deletions (Delorme et al., 2003).

In this investigation the ability of the bacterium *Pseudomonas fluorescens* to grow in the presence of dibenzothiophene and its influence on microbial metabolism were studied together with the DNA amplification using the PCR (Polymerase Chain Reaction) technique and the specific primer, BOX. Since DBT derivatives are the most frequently encountered in organic sulfur compounds, DBT is generally employed as a model molecule in microbiological studies of heavy oil desulfurization.

2. Material and Methods

2.1. Microorganism and culture medium

The experiments were carried out using pure cultures of *Pseudomonas fluorescens* (UCP 1514) deposited in the Nucleus of Research in Environmental Sciences – NPCIAMB, of the Catholic University of Pernambuco. The bacterial culture was kept in solid medium agar nutrient consisting of meat extract (5.0g), peptone (10.0g), NaCl (5.0g), agar (15.0) per litre of distilled water, at 4°C as a stock of bacterial innoculum.

2.2. Selection of DBT- acclimated colonies

The strain of *Pseudomonas fluorescens* was submitted with a gradient of DBT, using the technique described by Szybalski and Bryson (1952). The culture medium used for performing the technique was Luria Bertani (LB), containing tryptone (10.0g), yeast extract (5.0g), NaCl (10.0g) and glucose (5.0g), agar (17.0g) per litre, with pH 7.0, as per Konish et al.(1997). The solution of DBT (Sigma-Aldrich®) was prepared in dimethylformamide (DMF) at a concentration of 1 M, filtered and sterilized with a Millipore® filter. To the culture medium was added 1ml of DBT in the concentration of 10mM in Petri plates kept in a sloping position (so that the half reached 1 cm away from the part of most discharge on the border of the plate), until the DBT gradient solidified. Then, after solidification of gradient DBT, a suspension of 106 UFC/ml of *Pseudomonas fluorescens* was added in 9.9 ml of medium, with the plates maintained in a horizontal position. The plates were kept at 37°C for 24 hours. After incubation time, the colonies grown in higher concentration of DBT were observed. All experiments were performed in triplicate and with control. The physiologically adapted colonies were transferred for tubes containing culture medium LB with DBT at 10mM.

2.3. Batch experiments

The culture was grown in LB liquid medium as described earlier. The following DBT concentration and solvent was employed as stock solution: 1M DBT in dimethylfomamide (DMF) (Setti et al., 1995). The solution was sterilized in a Millipore® filter. The Erlenmeyer flasks were autoclaved at 120°C for 30 minutes. Batch kinetic experiments were conducted in 250 ml Erlenmeyer flasks by adding 2 mM DBT solution to 100 ml liquid culture medium. Another culture was prepared as innoculum just before the batch experiment and, while the bacteria were in the exponential phase, a volume of 5ml consisting of 108 cells/ml was transferred to 100 ml of liquid medium. All experiments were performed in triplicate by control trials without innoculum, performed on a rotary shaker operating at 150 rpm at a constant temperature of 37°C for 144 hours. Samples were taken every 24 hours. Bacterial growth was monitored through cell viability using the pour plate technique and the pH by using a pH meter (Orion 310). The specific growth speed (μesp) and generation time (TG) were determined as per Pirt (1975) For the specific growth rate, the following Eq. (1) was used:

$$\mu esp = (\ln x - \ln x0/T - T0) \quad (1)$$

Where:
X= Final biomass
X0= Initial biomass
T= Final time
T0= Initial time
The generation time was determined by:
TG=ln2/ μesp

After culture time, the fermentations were centrifuged at 5,000 g for 15 minutes at 5°C to separate the biomass and metabolic liquid. After separation, the metabolic liquid was utilized to determinate the consumption of glucose and the quantification of protein.

2.4. Analytical methods

The glucose consumption was determined by the colorimetric method (LABTEST® Diagnotisc-Brazil) at 505 nm and the total protein was determined as per the Bradford (1976) method, using Bovine Serum Albumin (BSA) as standard.

2.5. HPLC analysis

DBT quantification was carried out by HPLC analysis - Varian UV-VIS detector, model 320. Separation was carried out with a C18 RP column (4.6 x 250 mm), SOLVENT LIBERATION SYSTEM, model 210 Varian Star®, with the following separation conditions: mobile phase acetonitrile 75% and phosphate buffer 10 mM (pH 6.0). The eluate was detected at 232 nm.

2.6. Genomic DNA extraction

For DNA extraction, the microorganism was grown in Luria Bertani-liquid medium at 37°C with 150 rpm at an early exponential state. After fermentation, the cells were put on ice for 15 minutes, then centrifuged at 13,000 g, at 4°C for 10 minutes. The supernatant was removed and the cells, washed by adding 50µl of isotonic solution and centrifuged again (13,000 g , 4°C for 10 minutes), were resuspended in 1/50 the volume of isotonic solution. The experiment was performed by adding cell lysis solution, equal to the volume of the resuspended solution, mixed by inversion and put on ice for 5 minutes. The sample was extracted with chloroform isoamilic alcohol 24:1(v/v), mixed by inversion, centrifuged as described earlier and the organic phase was removed. The DNA was extracted in the aqueous phase with 1:3 volume of ammonium acetate (7.5M), mixed by inversion, adding 2.5 times the volume of ethanol, mixed again by invertion and kept for 15 minutes at room temperature; it was centrifuged for 15 minutes at 12,000 rpm and the supernatant removed by adding 1ml of alcohol 70%, centrifuged at 13,000g , 4°C for 5 minutes, and thereafter dried at room temperature. The DNA was dissolved in TBE buffer and quantified in agarose gel to 1%, stained with SyBr Gold 1X (Invitrogen®). Electrophoresis was performed in a horizontal system using TBE buffer 0.5 X for 50 minutes at 80 Volts. The gel was visualized in a UV chamber.

2.7. DNA amplification

The DNA was amplified by PCR (Polymerase Chain Reaction) with primer BOX(5'-ACGGCAAGGCGACGTCGACG-3'), with repetitive regions of cromossomic DNA (Versalovic et al., 1994). The amplification reaction was performed with the following volumes: milli-Q sterile water, 16.36 µl; dNTPs, 0.4 µl (stock solution with 20mM each base); buffer 10X, 2.5 µl; MgCl2, 1.25 µl (50mM); oligonucleotide, 1.0 µl (50pmol µl-1); DNA, 1.0 µl (50mg); Taq, 50 µl (5U/ µl). The amplification was performed using the following cycles: (i) one cycle of initial denaturation 7 min at 95°C; (ii)35x [denaturation 1min at 94°C, hybridization 1min at 53°C, extension 8min at 65°C]; (iii) one cycle of final extension 16 min at 65°C, maintained at 4°C. The samples were then submitted to gel eletrophoresis in agarose gel, at 1.5% (20x25cm), at 100V in TBE buffer 1X (10.8 g Tris-base 5.5g boric acid , 4ml of EDTA 0.5M pH 8.0, per liter of solution) which, after six hours, was stained with Ethyl bromide and visualized in a UV chamber (Fernandes et al., 2003). The bands were analyzed by Biometra Program 65.

2.8. Electrophoresis of proteins SDS-PAGE

The analysis was performed in line with Laemmli (1970). The samples were diluted in buffer (Tris-HCl 12mmol/l pH6.8, glycerol 5%, SDS (Sodium dodecyl sulphate) 0.4%, 2-mercaptoethanol 2mmol/l and bromophenol blue 0.02%) and denatured at 90°C for 5 minutes. Aliquots were applied in polyacrylamide gel containing SDS. The concentration of polyacrylamide on separator and ordered gel were 12% and 14%, respectively. The ratio of acrylamide to bisacrylamide was 30:8 (w/w). The separator gel contained 2.5ml of Tris-HCl 1.5mol/l pH 8.8, 100ml of SDS 10% (m/v), 50µl of ammonium persulphate 10% (m/v) and 5µl of TEMED. The electrophoresis was performed at 150V for 2 hours in Tris-HCl buffer, 25mmol/l pH8.3, glycine 192mmol/l and SDS 0.1%, using Höefer Gel Systems. The gels were stained with Coomassie Brilliant Blue solution, 250 R (Coomassie Blue 0.15%, methanol 53% and acetic acid 7% in bi-distilled water. The gels were immersed in a dryer solution (methanol 50% and glycerol 1% in bi-distilled water) for 2 hours.

3. Results and Discussion

3.1. Kinetic study and effect of DBT on the growth of *Pseudomonas fluorescens*

The colonies visualized after the growth period with DBT on the Petri plates did not show morphological alteration, maintaining the structures and sizes characteristic of the species. During the fermentation, Pseudomonas fluorescens UCP 1514 grew as well in the control as in DBT medium (Figure 1). The beginning of the exponential phase was observed at 6 hours of culture. The specific velocity of growth on control was 0.25 h-1 and in experiments with DBT was 0.22 h-1, with a generation time of 2.77 and 3.15 hours for the control and DBT culture, respectively (Table 1). The pH was not modified significantly during the cultivation. With the control, growth was observed until 48 hours of culture. After this period, the decline phase of microorganisms began. In experiments with DBT, The isolated DBT desulfurizing bacterial isolates showed maximum growth in the mesophilic temperature range and at neutral pH value. (Mohamed et al., 2015). The protein concentration was analyzed in the samples of metabolic liquid extracted each 2 hours until 12 hours of culture and thereafter at periods of 24 hours until 144 hours, at the end of fermentation.

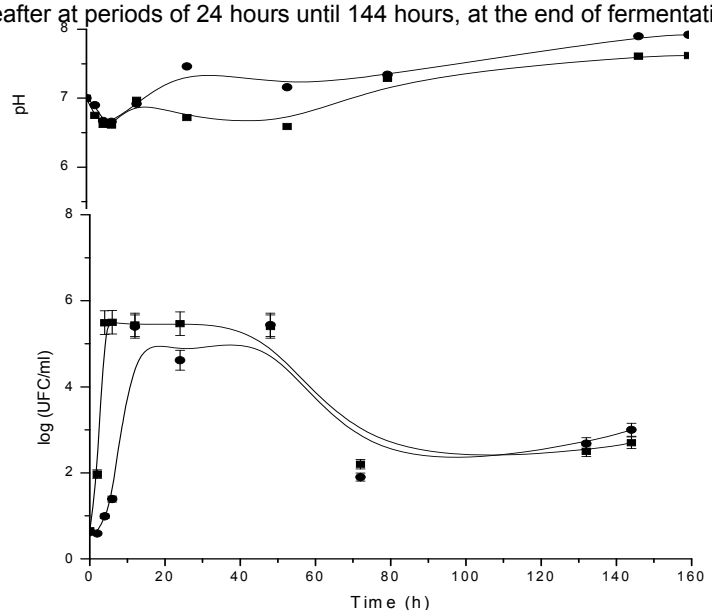

Figure 1. Kinetic growth and pH of Pseudomonas fluorescens in Luria Bertani medium. (▪) Control; (•) 2mM de DBT.

Table1. Values of specific velocity of growth µesp and generation time TG of kinetic growth of Pseudomonas fluorescens in Luria Bertani Medium during 144 of culture.

Experiments	μ_{esp}	T_G
Control	0,25 h^{-1}	2,77 h
Medium with 2mM DBT	0,22 h^{-1}	3,15 h

Figure 2 shows that the extracellular proteins produced increased after 24 hours of culture. Then the microorganisms produced an expressive proportion of enzymes to adapt to the compound, as well as to degrade it with the cleavage of carbon-carbon bonds, and reducing the compounds into less complex molecules. The same behavior was observed in *Pseudomonas fluorescens*; at first there was an adaptation period to the compound that resulted in the production of extracellular proteins. In the presence of recalcitrant compounds, in the beginning of the growth period, the microorganisms seek to adapt and utilize the compounds as a carbon source. The glucose consumption was expressive at the beginning of the fermentation as shown in Figure 3, due to the intensive activity of the microorganisms to remain viable in the presence of dibenzothiophene and then to degrade it. The glucose was consumed during the first hours of fermentation, as glucose is an easy source of energy and carbon, probably in this period, the dibenzothiophene was used by *Pseudomonas fluorescens* as an energy source and even sulfur, for the biosynthesis of vitamins and aminoacids.

3.3 Molecular biology studies

Molecular biology studies were realized to identify the modifications on physiology and metabolism of the microorganism, with DNA amplification by PCR and electrophoresis of proteins with SDS-PAGE. The comparison with the standard amplification of genomic DNA fragments generated by repetitive elements amplified by primer BOX is shown in Figure 4. The reaction of amplification of the DNA genomic region of *Pseudomonas fluorescens* using PCR with primer BOX demonstrated a significant number of bands. Figure 4 shows that the bands varied from 400 to 2000kb.

The wild strain presented products of amplification about of 650 to 2000kb, while the strain acclimated with DBT showed products of amplification between 400 to 2000kb. The band of molecular mass of 500kb is very expressive on samples 1 and 2, treated with DBT, but did not appear on sample 1, wild strain. The band of 100pb of the marker presented three different forms on the bands. Mohamed et al. (2015) studied the 16S-rRNA gene amplification and sequencing for identification and phylogenetic alignment of the isolated bacteria SA11, SA21, and SA31 biocatalysts. Blast search sequence analysis of around 1000 bp of the amplified 16S rRNA from both the 5 and 3 termini in at least 10 clones for each biocatalyst was performed to align the biocatalysts to the most closely relatives in GenBank database.

The activity of total proteins in denaturing gel (SDS-PAGE) of *Pseudomonas fluorescens* grown in Luria Bertani medium at 30°C identified the proteins. The profile obtained from samples showed different bands with proteins with of different molecular mass. The molecular weight of samples 1, 2 and 3 in Figure 5 were determined to be 116; 97.4; 97.4 kDa, respectively. The results showed that *Pseudomonas fluorescens* presented a physiological modification in its genomic profile, occurring a reorganization of the genes.

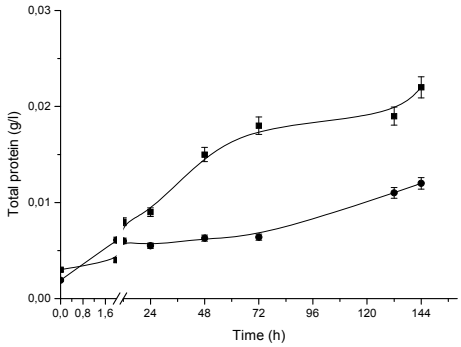

Figure 2. Total protein during metabolism of Pseudomonas fluorescens in Luria Bertani medium. (▪) Control; (•) 2mM de DBT.

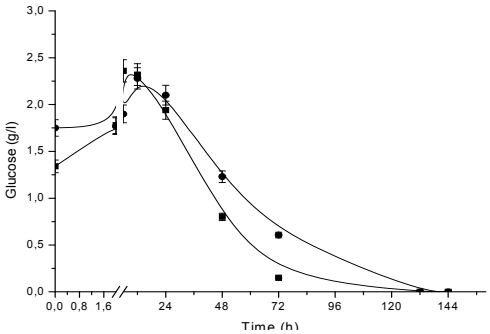

Figure 3. Glucose consumption during metabolism of Pseudomonas fluorescens in Luria Bertani médium. (▪) Control; (•) 2mM de DBT.

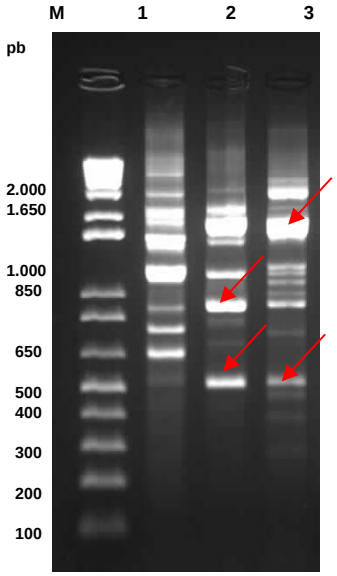

Figure 4. DNA profile 1Kb Plus DNA Ladder PCR (primer BOX). M. Marker; 1.Strain Pseudomonas fluorescens without DBT; 2. Strain with 10mM DBT; 3. Strain with 10mM DBT with liquid medium.

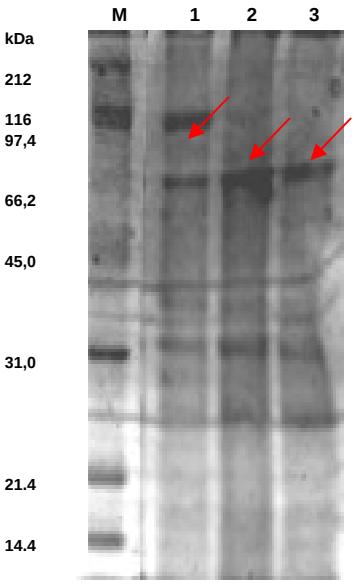

Figure 5. Protein profile. SDS-PAGE. M. Marker; 1.Strain Pseudomonas fluorescens without DBT; 2. Strain with 10mM DBT; 3. Strain with 10mM DBT with liquid medium.

Conclusions

The results showed that in implementing this genetic screen, unknown genes and enzymes responsible for desulfurization have to be identified. Structural analyses of enzymes for DBT desulfurization are in progress, and such genetic engineering techniques will enhance and improve the desulfurizing enzymes, making them suitable for use in novel biodesulfurization processes.

References

Bradford M.M., 1976, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye binding, Analytical Biochemistry, 72, 248-254.

Bustos-Jaimes I., Amador G., Castorena G., Le Borgne S., 2003, Genotypic Characterization of Sulfur-Oxidative Desulfurizing Bacterial Strains Isolated from Mexican Refineries, Oil & Gas Science and Technology - Rev. IFP, 58, 4, 521-526.

Calzada J., Alcon A., Santos V.E., Garcia-Ochoa, F., 2012, Extended kinetic model for DBT desulfurization using Pseudomonas Putida CECT5279 in resting cells, Biochemical Engineering Journal, 66, 52– 60.

Chi Y, Li C, Jiao Q, Liu Q, Yan P et al. , 2011, Desulfurization by oxidation combined with extraction using acidic room-temperature ionic liquids. Green Chem 13: 1224-1229. doi:10.1039/c0gc00745e.

Delorme T.A., Gagliardi J.V., Angle J.S., Berkum P.V., Chaney R.L, 2003, Phenotypic and Genetic Diversity of Rhizobia Isolated from Nodules of Clover Grown in a Zinc and Cadmium Contaminated Soil, Soil Sci. Soc. Am., 67,1746–1754.

Fernandes M.F.F., Fernandes R.P.M., Hungria M., 2003, Caracterização genética de rizóbios nativos dos tabuleiros costeiros eficientes em culturas do guandu e caupi, Pesquisa. Agropecuária Brasileira, 8, 911-920.

Hasan Z, Jeon J, Jhung SH, 2012, Oxidative desulfurization of benzothiophene and thiophene with WOx/ZrO2 catalysts: effect of calcination temperature of catalysts. J Hazard Mater 205-206: 216-221.

Konishi J., Ishii Y., Onaka T., Okumura K., Suzuki M., 1997, Thermophilic carbon-sulfur-bond targeted biodesulfurization, Applied Environmental Microbiology, 63, 3164-3169.

Laemli U.K.,1970, Clevage of structural proteins during the assembly of the head of bactiophage t4, Nature, 227, 680-685.

Mohamed M E, Zakariya , Al-Yacoub Z H, Vedakumar J V, 2015, Biocatalytic desulfurization of thiophenic compounds and crude oil by newly isolated bacteria, Frontiers in Microbiology, v. 6 p. 1-12.

Pawelec B, Navarro RM, Campos-Martin JM, Fierro JLG, 2011, Towards near zero-sulfur liquid fuels: a perspective review. Catal Sci Technol 1: 23-42. doi:10.1039/c0cy00049c. doi:10.1016/j.jhazmat.2011.12.059. PubMed: 22245512.

Pirt S.J., 1975, Principles of Microbe and Cell Cultivation, Scientific Publications, Blackwell, London.

Setti L., Lanzarini G., Pifferi P.G,. 1995, Dibenzothiophene biodegradation by a Pseudomonas sp. In model solutions, Process Biochemistry, 30,721-728.

Szybalski W., Bryson V., 1952, Genetic studies on microbial cross restitance to toxic agents. I. Cross restitance of Escherichia coli to fifteen antibiotics, Journal Bacteriology, 64, 489-499.

Versalovic J., Schneider M., De Bruijn F.J., Lupski J.R., 1994, The effects of heavy metals on Genomic fingerprinting of bacteria using repetitive sequence-based dinitrogen fixation by Rhizobium- white clover in a range of longpolymerase chain reaction, Methods Cell Biology, 5, 25–40.

Xu, P., Feng, J., Yu, B., Li, F., and Ma, C. 2009, Recent developments in biodesulfurization of fossil fuels. Adv. Biochem. Eng. Biotechnol. 113, 255–274. doi: 10.1007/10_2008_16.