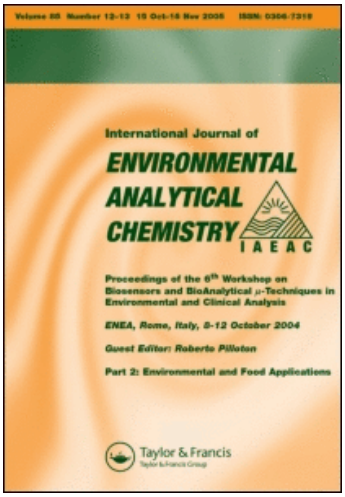

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Fluorescent bacteria detecting iron loading

Antoniea Poiata[a]; Alexandru Vlahovici[b]; Dorina-Emilia Creanga[b]; Petronela Tupu[b]
[a] University of Medicine and Pharmacy 'Gr. T. Popa', Faculty of Pharmacy, Iasi, Romania [b] University 'Al. I. Cuza', Faculty of Physics, Iasi, Romania

To cite this Article Poiata, Antoniea , Vlahovici, Alexandru , Creanga, Dorina-Emilia and Tupu, Petronela(2005) 'Fluorescent bacteria detecting iron loading', International Journal of Environmental Analytical Chemistry, 85: 12, 993 — 1000
To link to this Article: DOI: 10.1080/03067310500151235
URL: http://dx.doi.org/10.1080/03067310500151235

Intern. J. Environ. Anal. Chem.
Vol. 85, No. 12–13, 15 October–15 November 2005, 993–1000

Taylor & Francis
Taylor & Francis Group

Fluorescent bacteria detecting iron loading

ANTONIEA POIATA†, ALEXANDRU VLAHOVICI‡,
DORINA-EMILIA CREANGA*‡ and PETRONELA TUPU‡

†University of Medicine and Pharmacy 'Gr. T. Popa',
Faculty of Pharmacy, Iasi, Romania
‡University 'Al. I. Cuza', Faculty of Physics, Iasi, Romania

(*Received 4 October 2004; in final form 28 January 2005*)

Since iron pollution represents a real threat in the modern industrial era, while, in the meantime, the environment control using microorganisms has been developed, the present investigation was dedicated to iron sensing by means of bacterial cultures. *Pseudomonas aeruginosa* was chosen due to its ability to up-take the environmental iron in the form of complex iron compositions called siderophores, characterized by luminescent features. It was proved that wild strains of this human pathogen could be the biological components of an iron biosensor for both clinical and environmental applications.

Keywords: Fluorescent pyoverdine; Iron trace detection; Biotechnological tool; Microbial wild strain; Box-plot

1. Introduction

In environmental science microorganisms are known to have various applications, many research projects being focused on the removal of soil contaminants (herbicides, pesticides, chlorinated compounds), the degradation of oils in waste waters from the food industry, the degradation of petroleum in the sea, the minimization of wastes from the food industry, etc.

The main part of these applications was related to the presence of various bacteria species in waste waters (from swimming pool and re-circulatory cooling systems from petrochemicals, refineries and pharmaceutical units). We mention the anaerobic bacteria, the algae, the iron oxidizers, the sulphate reducers, the nitrifiers, the *Staphylococcus*, *Proteus* and *Pseudomonas* species, the phenol and hydrocarbon degraders and several species of fungi [1]. Regarding iron pollution, the iron bacteria [2] represent ubiquitous microorganisms found in ferruginous waters. In order to fulfill their energy requirements these microorganisms are provided with the ability to oxidize ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}). In contrast with the benign species,

*Corresponding author. Email: dorinacreanga@yahoo.com

International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis
DOI: 10.1080/03067310500151235

there are also iron consumers among the human pathogen germs, such as certain *Pseudomonas* bacteria.

The fungus *Rhizopus arrhizus* and various bacteria from the *Pseudomonas* genus (proteobacteria known for their capacity to colonize various ecological niches including mineral waters [3, 4] and the human body) are the most studied microorganisms that are able to consume iron when available in the environment.

Distinct from iron oxidizer bacteria, *Pseudomonas* species are able to up-take the iron by synthesizing complex iron combinations known as siderophores that can be further internalized by, for example, plant roots. Based on this, the avoidance of iron toxicity within the soil through regulation of bacterial iron transport was greatly considered a viable environmental solution [5, 6] in the frame of a more general issue – the bacterial control in environment [7–9].

The *Pseudomonas* species that chelate the iron in the form of siderophores (greenish pigments known as pyoverdines [10]) are easy to recognize by visual inspection as well as by fluorescence emission under UV irradiation. Both *Pseudomonas fluorescens* and *P. aeruginosa* strains, although producing structurally different pyoverdines, have demonstrated highly efficient cross-reactions when tested for pyoverdine-mediated iron uptake. Antibiotic activity was evidenced in some strains of *Pseudomonas* pyoverdines [11–13], while the role of *Pseudomonas* in quorum-sensing processes was also emphasized [14, 15].

Metal biosensors based on *Pseudomonas putida* were reported by Perry *et al.* [16]: the sensor responded in a rapid time frame with altered light output when it was exposed to increasing activity of the toxic elements Cu(II), Zn, Pb, and Cd.

Iron biosensors based on *Pseudomonas* bacteria have been reported. The best-controlled sensitive devices based on the bacterial ability of iron detection have been obtained by using adequate mutants specialized in iron oxidization from different substrates.

Joyner and Lindow [17] reported an iron biosensor based on the ferric iron availability to cells of a *Pseudomonas syringae* mutant that exhibited iron-dependent fluorescence when iron was added to the growth media.

Geneticists such as Temple *et al.* [18] have designed an iron biosensor (namely an iron-regulated promoter) following engineering of tree *Pseudomonas* cells in order to estimate the relative abundance of iron on some tree blossoms. The rapid detection of trace levels of iron in biological samples represents the finality of a new procedure developed by scientists dedicated to agricultural science [19].

Iron loading describes not only an environmental issue but also a medical problem (the liver and the spleen being critical organs for colloidal iron so that colloidal ferrofluids can be used as contract agents in NMR), the rapid and easy observable iron loading representing a challenge for both physicians and physicists.

The investigation presented in the following was focused on colloidal iron sensing by means of the quantitative relationship between wild *P. aeruginosa* fluorescence and the concentration of colloidal iron oxide in the culture medium (designed to simulate either environmental or human organism pollution). The authors want to report the preliminary data of the experiments intended for the design of an iron biosensor. The first steps are aimed at testing wild strains of *P. aeruginosa* developed within the human body and to isolate the most sensitive ones that are able to detect low levels of iron in both biological specimens and environmental samples. In future experiments, various inorganic and organic components in the test culture medium

need to be studied in order to identify the optimal composition for fluorescence rapid and linear variation to the variation of low-level iron loading in the samples.

2. Experimental

2.1 *Biological material*

Biological specimens of wild strains of *P. aeruginosa* have been grown in sterile glass tubes with standard liquid culture medium (nutritive broth based on meat extract from OXOID, with 6.5 pH) supplemented with aqueous ferrofluid. The ferrofluid was prepared accordingly to ref. [20] (magnetite – i.e., iron oxide Fe_3O_4, stabilized with ammonia oleate), ferrophase volume fraction being 1.5%, saturation magnetization of 10.34 kA m^{-1}, and physical diameter ranging between 4 and 25 nm.

2.2 *Iron supply*

Ferrofluid concentration in the culture medium ranged between 0.003 and 1.0 mL L^{-1} (double serial dilutions) which is equivalent with the iron oxide loading ranging between 0.5 and 72.0 μg L^{-1}. So, the dilutions were: 1–0.500–0.250–0.120–0.060–0.030–0.015–0.075 mL L^{-1}, corresponding to iron oxide concentrations of 72.0–36.0–18.0–9.0–4.5–2.2–1.1–0.5 μg L^{-1}.

Initially 20 *P. aeruginosa* strains, isolated from different patients with gastro-intestinal diseases, were tested within the same colloidal iron oxide concentration range: three of them were eliminated later because of a high standard deviation noticed during repeated tests (hidden non-homogeneity within the apparently homogeneous biological specimens). Finally five representative behavioral strains were selected to describe the experimental results.

2.3 *Spectral devices*

Pseudomonas cell density in the test samples was nephelometrically controlled using a C. Zeiss-Jena spectrophotometer. Fluorescence measurement was carried out in quartz cells of 5 mm width, using a laboratory assembled installation (figure 1);

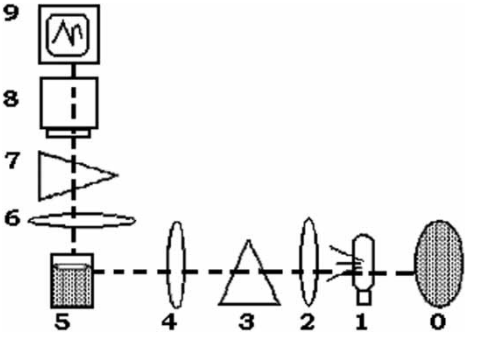

Figure 1. Fluorescence installation. 0, mirror; 1, xenon lamp; 2, 4, 6, quart focalization lens; 3, excitation monochromator (prism); 5, sample cell; 7, analysis monochromator (prism); 8, photomultiplier with supply source; 9, direct current amplifier and recorder.

fluorescence excitation was done using UV light having a wavelength of 300 nm; fluorescence quenching was avoided by 1:10 dilution in distilled water.

2.4 *Statistic analysis*

All measurements have been carried out on five repetitions of the bacterial culture samples. Average values and standard deviations have been used for the graphical plots. Statistical significance of the differences between the control and the iron polluted sample was assessed by applying a *t*-test (pair type, two-tailed). Statistical comparison was carried out by using the box-plot representation technique [21].

3. Results and discussions

The fluorescence spectrum of the *P. aeruginosa* sample is represented in figure 2. It is evident that only one large fluorescence band appears, in the blue-greenish range of visible radiation (centered on the wavelength of 470 nm), no shift being observed in iron polluted samples in comparison to the control sample.

In figure 3 the fluorescence of five strains of *P. aeruginosa* specimens cultivated in conditions of iron loaded medium is presented in linear-logarithmic scale (average values and standard deviations corresponding to five repetitions for each iron oxide concentration).

We can see that the tendency for fluorescence to increase exists for the relatively low iron level revealed in the case of three strains (P.s.1, P.s.2, P.s.3), while for the highest level of iron (36–72 $\mu g\,L^{-1}$) a diminution of fluorescence intensity is revealed in all five strains. The amplitude of the fluorescence variation is about 45% in P.s.2.

The five strains that the authors consider to be representative for the 17 strains maintained in the frame of results analysis did not exhibit the same sensitivity, nor the same fluorescence level in control samples or in the iron loaded samples.

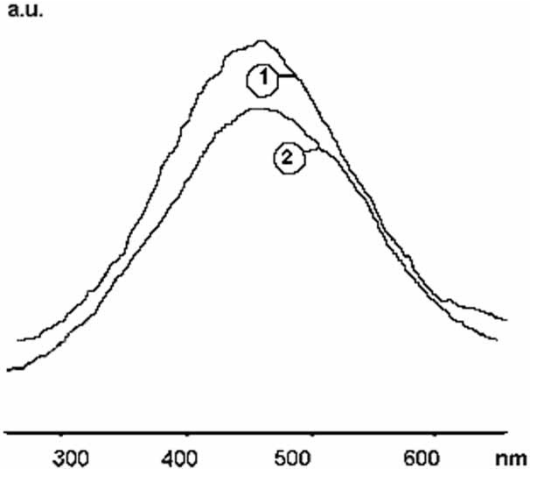

Figure 2. Fluorescence of *Pseudomonas* siderophore in control sample (1) and in one iron polluted sample (2).

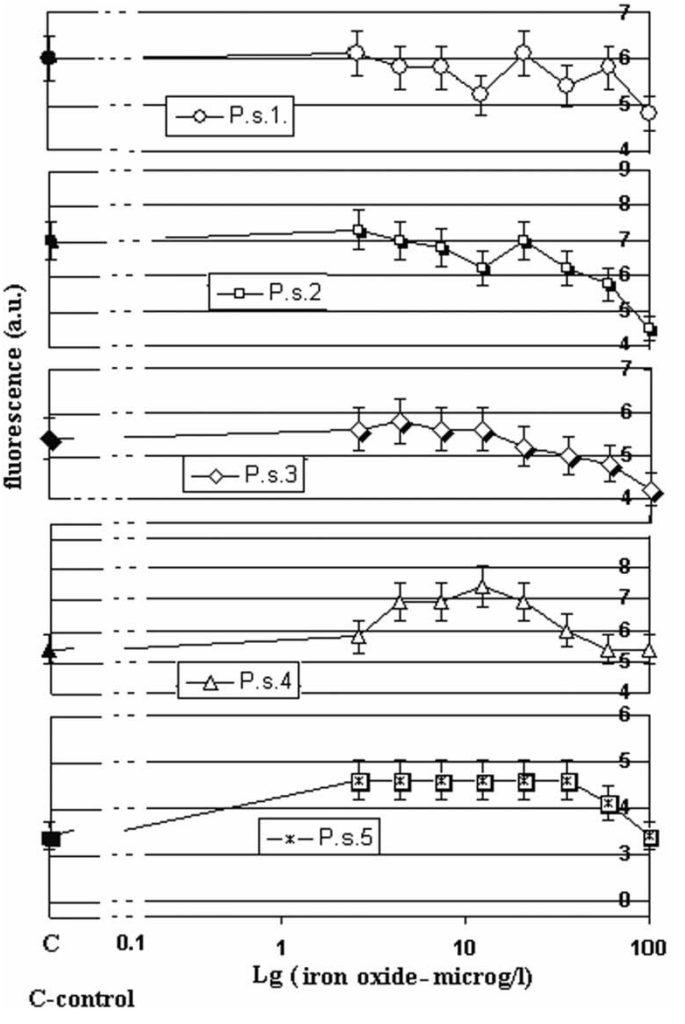

Figure 3. *Pseudomonas* fluorescence in the presence of iron oxides pollution (P.s.1, . . . , P.s.5 are the notations for the presented strains).

The bacteria of P.s.1, P.s.2 and P.s.3 types are less sensitive (P.s.3 responding the most coherently to the chemical stimulus) in the range of low iron level, but present more than 40% diminished fluorescent emission for relatively high iron level, while P.s.4 and P.s.5 present significant coherent fluorescence increase in the range of low iron loading (about 35%). The fluorescence curve of P.s.4 is characterized by a plateau while for P.s.5 one-maximum curve is shaped.

The fluorescence being generated by the *Pseudomonas* siderophores (pyoverdines) shows that the presence of colloidal iron oxide in the culture medium is able to influence iron uptake by the *P. aeruginosa* bacterial cells.

As we know, ferrofluids are based on ferrophase particles, coated by long chain molecules and suspended in a carrier liquid (water in the present case). Since magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are the main iron compounds forming the

ferrophase in most ferrofluids based on iron, it is plausible that the data obtained by us represent the result of siderophore production stimulation by colloidal iron loaded media.

The modality used by *P. aeruginosa* to metabolize iron is still not explained. There is no distinct mention about the possibility that these bacteria are able to oxidize the internalized iron. In their review, Vasil and Ochsner [22] stress underline that many data reveal the implication of a ferric uptake regulator (Fur) and a Fur-regulated alternative sigma factor which are essential to the ability of the bacteria to perform iron acquisition, intake and trafficking, but no clarification upon the distinct iron acquisition mechanisms of *P. aeruginosa* may yet be done (neither for bacteria hosted in soil, in a pipeline, on plants or in the lungs of cystic fibrosis patients).

Considering the presence of *P. aeruginosa* bacteria not only in waste industrial and domestic waters, but also in the human body, as mentioned above, it seems that the quantitative iron sensing by means of the pyoverdine fluorescence intensity represents a viable project for medical applications.

The *P. aeruginosa*-based biosensor is intended to carry out fluorescence measurements on fixed wavelength of 470 nm (in relation to a fluorescence calibration curve) in biological specimens as well as in environmental samples, the computer system comparing the raw data with a calibration curve in order to deliver the iron concentration value.

3.1 *The limitations of the* P. aeruginosa *iron sensing*

The ability of *P. aeruginosa* strains to scavenge iron from the environment is limited. In the case of this experiment, the ferrofluid concentration ranged between 0.075 and 0.5 mL L^{-1} (the correspondent iron oxide range: 0.05–36 μg L^{-1}), suggesting that for higher iron concentration growth of the iron-consuming bacteria is negatively influenced. The statistical significance of the iron effect on the biosynthesis of fluorescent pyoverdine is given in table 1 (related to the significance thresholds of 0.05 and 0.01): only for the highest iron concentration is the difference control-sample not significant, when the iron seems unable to influence the pyoverdine level. Further experimental improvement is planned in order to increase the sensitivity of this detection method (i.e., the slope of the curve giving the fluorescence intensity *versus* iron oxide concentration) as well as the amplitude of the iron concentration range. In this respect new sets of wild bacterial *P. aeruginosa* strains isolated from the human body are to be tested while the composition of the culture medium is also intended to be adjusted by adding new protein and/or mineral components able to stimulate the bacterial metabolism in yielding pyoverdine in the presence of very low concentrations of iron.

Table 1. The statistical significance of the differences between the average values of fluorescence intensity in iron polluted media in comparison to the control.

Iron oxide concentration	0.5 μg L^{-1}	1.1 μg L^{-1}	2.2 μg L^{-1}	4.5 μg L^{-1}
Statistic significance	0.05	0.01	0.01	0.01
Iron oxide concentration	9.0 μg L^{-1}	18.0 μg L^{-1}	36.0 μg L^{-1}	72.0 μg L^{-1}
Statistic significance	0.01	0.01	0.01	> 0.05[a]

[a]Non-significant.

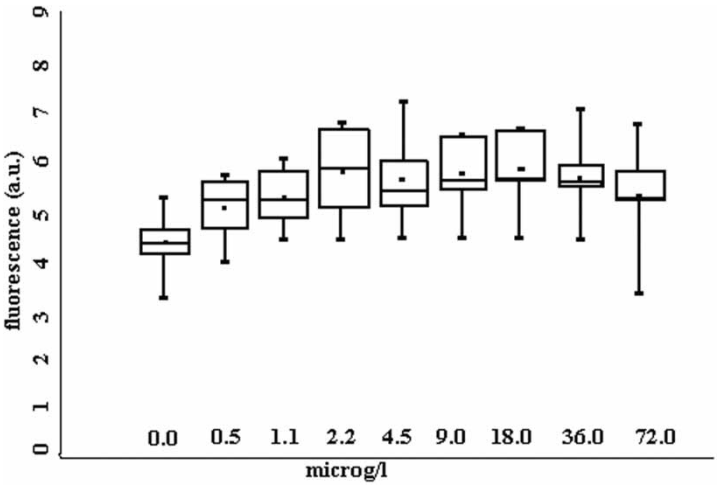

Figure 4. Comparison of the fluorescence intensity distribution curves by using the box-plot representation.

Further discussion regarding the differences between different *P. aeruginosa* strains has been done by means of the box-plot graphical technique.

The analysis of the distribution curves obtained for the nine series of data provided by all the 17 bacterial strains, as represented in figure 4, revealed the following:

- no exceptional large or low values exist within the nine data series taken into account (rather uniform behavior of the tested strains for all the nine iron oxide concentrations);
- for the highest iron oxide concentration the dispersion is the largest (the box length is the highest) and the median overlapped onto the box lowest edge (due to highest weight of higher data values in comparison to the small ones), which is concordant with the lack of the statistical significance of the fluorescence modification by the highest iron oxide level.

Therefore, the low level of colloidal iron oxide loading can be detected by means of fluorescent measurement in certain wild *P. aeruginosa* bacteria. This research intended to emphasize that, instead of artificial mutants some human wild pathogens may be utilized suggesting that clinical applications (the fluorescence of the biological specimens presenting *P. aeruginosa* contamination could indicate the iron loading of the patient) could be of much interest as the environmental ones. This would be the main specific characteristic of the projected biosensor that would be destined to detect the colloidal iron loading in the human body as well as in the environment.

A complementary investigation was carried out applying the agar diffusion microbiological test (on agar nutritive broth). The putative antimicrobial activity of the pyoverdine biosynthesized by the fluorescent *P. aeruginosa* cultivated on iron-polluted media was tested against three pathogen germs in order to get as indirect measurement method for the iron oxide pollution level. For relatively high ferrophase concentrations two of the three germs (*Sarcina lutea* and *Staphyloccocus aureus*) have responded coherently but for relatively low concentrations the experimental data are rather fuzzy. Moreover, in the range of high ferrophase concentrations, a rapid saturation

tendency of the pyoverdine antimicrobial activity *versus* colloidal iron oxide concentration was revealed – which is not convenient for a sensitive measurement method.

The third germ (*Bacillus cereus*) showed no sensitivity to the pyoverdine action.

Within the above-presented preliminary results the iron level in the test liquids was measured only by means of the fluorescence phenomenon. Further developed biosensor alternate methods for controlling iron during *P. aeruginosa* growth are to be considered. The fluorescence measurements are to be carried out at a well-established wavelength of 470 nm while the raw data are processed in a computer-assisted system. The most sensitive isolated *P. aeruginosa* strain will be carefully preserved on the most adequate culture medium variant – the composition able to give specific fluorescence control being identified. The actual problem in the present stage of our work is still the test sample manipulation in order to avoid fluorescence quenching (which is not yet an automated operation) but not to expand the assay duration.

4. Conclusion

Considering the fluorescence increase in wild bacterial cultures following the enhancement of colloidal iron oxide level, we can consider that the accuracy of this measurement method is acceptable as a preliminary biosensing tool able to control colloidal iron loading either in the environment or within the human body. Further improvements of culture medium composition and pH as well as careful selection of *P. aeruginosa* strains are designed in order to validate bacterial biocontrol possibilities. Combined measurement methods are to be used for the control of iron microbiological processing in the studied samples while deeper investigation needs to be designed with the goal of highlighting the iron oxidization processes.

References

[1] P.A. Chablain, G. Philippe, A. Groboillot, N. Truffaut, J.F. Guespin-Michel. *Res. Microbiol.*, **148**, 153 (1997).
[2] Edstrom Industries http://www.edstrom.com/Resources.cfm
[3] M. Elomari, L. Coroler, B. Hoste, M. Gillis, D. Izard, H. Leclerc. *Int. J. Syst. Bacteriol.*, **46**, 1138 (1996).
[4] S. Verhille, N. Baida, F. Dabboussi, D. Izard, H. Leclerc. *Syst. Appl. Microbiol.*, **22**, 45 (1999).
[5] V. Braun. *Biol. Chem.*, **378**, 779 (1997).
[6] V. Braun, H. Killmann. *Trends Biochem. Sci.*, **24**, 104 (1999).
[7] S.T. Chancey, D.W. Wood, E.A. Pierson, L.S. Pierson. *Appl. Environ. Microbiol.*, **68**, 3308 (2002).
[8] B.K. Duffy, G. Défago. *Appl. Environ. Microbiol.*, **66**, 3142 (2000).
[9] J.E. Loper. *Phytopathology*, **78**, 166 (1988).
[10] J.M. Meyer. *Arch. Microbiol.*, **174**, 135 (2000).
[11] J. Sebat, A. Paszczynski, M. Cortese, R.L. Crawford. *Appl. Environ. Microbiol.*, **67**, 3934 (2001).
[12] J.C. Stolworthy, A. Paszczynsk, R. Korus, R.L. Crawford. *Biodegradation*, **12**, 411 (2001).
[13] K. Audenaert, T. Pattery, P. Cornelis, M. Höfte. *Mol. Plant-Microbe. Inter.*, **15**, 1147 (2002).
[14] A. Stintzi, K. Evans, J.M. Meyer, K. Poole. *FEMS Microbiol. Lett.*, **166**, 341 (1998).
[15] M. Whiteley, K.M. Lee, E.P. Greenberg. *Proc. Natl. Acad. Sci. USA*, **96**, 13904 (1999).
[16] M. Perry, J. McLean, A. Anderson, C.D. Miller. In *Proceedings of Subsurface Science Symposium*, Boise, Idaho (2002); in *Inra News*, **2**, 10 (2002); http://www.inra.org
[17] D.C. Joyner, S.E. Lindow. *Microbiology*, **146**, 2435 (2000).
[18] T.N. Temple, V.O. Stockwell, K.B. Johnson, J. Loper. *Phytopathology*, **94**, 1286 (2004).
[19] B. Hardin. Agricultural Research Service, USDA, 309,681 (1999); http://www.ars.usda.gov/is/pr/thelatest.htm
[20] C. Cotae. Romanian Patent 77199/1981.
[21] W. Koopmans. *The Contemporary Statistics*, Academic Press, New York (1987).
[22] M.L. Vasil, U.A. Ochsner. *Mol. Microbiol.*, **34**, 399 (1999).