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AN OBJECTIVE METHOD TO ASSESS BIOLUMINESCENT PROPERTIES OF SELECTED BACTERIAL STRAINS

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Abstract. Emission of light as a result of biochemical activities of some living bacteria *Vibrio fischeri* (in the past known as *Photobacterium phosphoreum*) makes it possible to monitor environmental changes in ecosystems. Toxicity testing as an international standard operating procedure based on the use of this method has already been accepted. The bioluminescent test offers a rapid, simple and sensitive method to test a wide spectrum of chemical substances and environmental samples including water, wastewater, sludge extracts, etc. In this study, aimed at characterising and comparing bioluminescent properties, four different bacterial strains were cultivated in four different liquid mediums and temperature conditions. The bioluminescent intensity of bacterial suspensions was measured using a laboratory BioOrbit 1253 luminometer during bacteria culture. Based on obtained results and mathematical calculations of RLU (relative luminescent units) values strain *Photobacterium phosphoreum* + NCBE medium were indicated as the variant demonstrating proper bioluminescence intensity and characteristics most suitable for further applications.

Key words: ecotoxicology, biotests, bioluminescence, *Photobacterium*

INTRODUCTION

Bioluminescence is a phenomenon consisting in the generation of light by some living organisms as a result of their biochemical and enzymatic activity. Luminescence of this type may be observed in insects, certain plants and fungi, but primarily in bacteria [ISO 113480, Kießling and Rayner-Brandes 1998].

Attempts have been made for many years to apply bioluminescent reactions in practice. It may be stated that the range of such possibilities connected with bioluminescence measurement is very wide, as it includes agriculture, environmental protection,

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food technology and food science, and in recent years also genetic engineering [Walker et al. 2002, Sousa 1998, Mercer et al. 1999].

The development of a method to isolate the luciferin-luciferase complex from tropical insects belonging to family *Lampyridae* offered a possibility for biochemical companies to commercially produce tests detecting high-energy cell components, such as e.g. ATP (adenosine triphosphate). Such tests are used to detect residues of organic pollutants on working surfaces of machines, equipment, walls, etc, and to promptly assess their hygienic condition. Such as assessment may also be used to evaluate the effectiveness of cleaning and disinfection procedures of process lines in industrial plants. Other examples of their applications include assessment of microbial counts in food samples based on measured luminescence, as well as the evaluation of effectiveness of thermal food processing or sterility of foodstuffs or cosmetics [Czajkowska 1997, Handbook... 1996, BioTox™... 1996].

At the same time it was observed that bioluminescence is a property of certain bacteria found in abundance in marine ecosystems and that the presence of chemical pollutants and toxins disturbs cell metabolism of bioluminescent bacteria, reducing the intensity of the emitted light [Kießling and Rayner-Brandes 1998, BioTox™... 1996].

Luminescent bacteria are facultative aerobes. The capacity for luminescence is closely related to the composition of the culture medium. In order for the bacteria to generate light the presence and access to oxygen are required. The end product of bacterial luminescence, which is frequently compared to respiration, is not adenosine triphosphate (ATP), but the excited chemical compound emitting light called luciferase. Bacteria glow very intensively when their cells are found in concentrated suspensions (photo 1) [Madanecki 2004].

a) b)

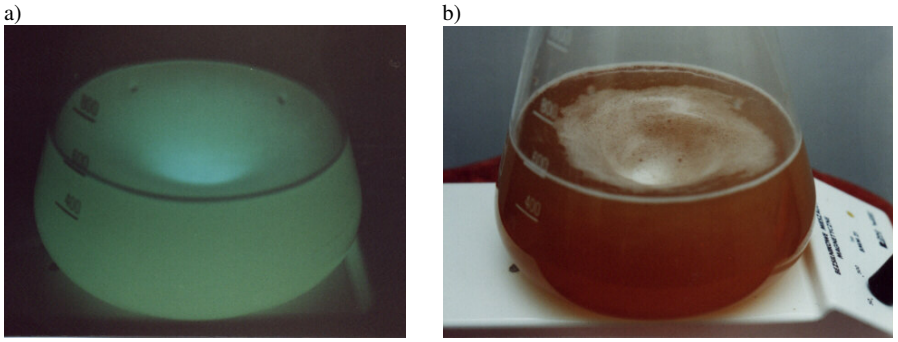


Photo 1. A photograph of *Photobacterium luciferum* culture on 'BOSS' liquid medium: a – a photo taken in daylight, b – a photo in the dark [Madanecki 2004]
Fot. 1. Zdjęcie hodowli *Photobacterium luciferum* na płynnym podłożu 'BOSS': a – zdjęcie wykonane przy świetle dziennym, b – zdjęcie w ciemności [Madanecki 2004]

From the scientific point of view advances in the isolation of bioluminescent bacteria as well as the determination of optimal culture conditions are of great importance. However, it seems that one of the less recognized methodological problems still remains the assessment of luminescence capacity of bioluminescent bacteria and its visualization

is usually limited to photography or special bacterial cell staining methods. The bioluminescent test using lyophilized *Vibrio fischeri* bacteria (formally: *Photobacterium phosphoreum*) was accepted as consistent with international IOS standards [ISO 11348 1994] and is at present used to assess toxicity of chemical compounds found in nature, including ecotoxicological analysis of water, sewage or sewage sludge and many other environmental samples [Kircher 1996]. Equipment and ready-to-use biotests using bacterial bioluminescence are commercially available, e.g. Microtox (Azur Environmental) or ToxAlert (Merck) [Konieczny et al. 1998, Mansel and Griffiths 1995, Nałęcz-Jawecki 2003], and their use is controlled by respective legal regulations [Ustawa... 2004].

In practice the precise measurement of the diverse effect of different substances on the development of bacteria with bioluminescent properties still determines the usability of bacterial bioluminescence. Assessment of such an effect may be suitable in the measurement of concentrations of these substances in different environmental samples. However, due to this diversity it is advisable to optimize the selection of specific strains and media for measurement purposes.

THE AIM AND SCOPE OF THE STUDY

The aim of this study was an objective, comparative assessment of the capacity to emit light exhibited by selected strains of luminescent bacteria under changing culture conditions on liquid media (temperature, medium composition, culture time). Using results of luminescence intensity measurements of selected bacteria taken with a laboratory luminometer it was attempted to identify strains and media providing intensive and stable luminescence of the system.

MATERIAL AND METHODS

Material for analyses consisted of four bacterial strains with bioluminescent properties, i.e. *Photobacterium phosphoreum* (classified to genus *Vibrio*, known as *Vibrio phosphoreum*), *Photobacterium luciferum*, *Vibrio fischeri* and *Vibrio harveyi*, obtained from the Department of Microbiology, the University of Gdańsk. Four liquid media prepared following the recommendations by Madanecki [2004] were used in the cultures [ISO 11348 1994].

LA medium	BOSS medium	LM medium	NCBE medium
NaCl 10 g Yeast extract 5 g Pepton (Bacto-peptone) 10 g Agar 15 g Made up with distilled water to 1000 ml	NaCl 30 g Glycerol 1 g Pepton (Bacxto-peptone) 10 g Meat extract 3 g Made up with distilled water to 1000 ml	Yeast extract 3 g Glycerol 3 g $CaCO_3$ 1 g Trypton 3 g Made up with sea water to* 1000 ml	Yeast extract 3 g Pepton (Bacto-peptone) 5 g distilled water 250 ml sea water* 750 ml

*Water prepared using sea salt used in aquariums.

Bacterial cultures were run at different temperature ranges (from 18 to 37°C) using magnetic agitators and maintaining identical mixing conditions for bacterial suspensions, while at the same time providing sufficient amounts of oxygen to cultures of the analysed bacteria.

Bioluminescence was measured using a luminometer model 1253 by BIOORBIT. Intensity of emitted light was expressed in relative bioluminescence units (RLU) read directly from the meter, first at 12 h after inoculation. In all variants culture time did not exceed 120 h, when the last measurement of emitted light intensity was taken each time.

Results were subjected to statistical analysis using STATISTICA PL software, with the application of the analysis of variance or analysis of regression, depending on the requirements.

Table 1. Mean bioluminescence values – RLU of examined microorganisms cultivated in temperature 20-22°C
Tabela 1. Średnie wartości bioluminescencji RLU badanych gatunków bakterii hodowanych w temperaturze 20-22°C

Bacteria strain Gatunek bakterii	Culture time Czas hodowli h	Medium – Podłoże			
		LA	BOSS	LM	NCBE
Vibrio fisheri	12	1 280	17	9	817
	24	1 399	112	16	830
	36	1 488	117	11	901
	48	902	120	53	712
	120	880	184	189	453
Vibrio harveyi	12	316	10	10	13
	24	451	56	36	110
	36	850	73	48	215
	48	743	98	63	307
	120	283	118	43	289
Photobacterium phosphoreum	12	1 341	1 581	10	26
	24	1 386	1 611	51	204
	36	593	1 543	96	439
	48	310	1 281	198	706
	120	421	411	244	821
Photobacterium luciferum	12	31	11	251	110
	24	58	18	346	204
	36	381	43	307	520
	48	740	61	198	393
	120	861	160	364	756

DISCUSSION AND RESULTS

Obtained means of bioluminescence emitted by the tested bacteria under different culture conditions are presented in Tables 1, 2 and 3. Depending on the temperature at which a given bacterial strain was cultivated, measured RLU values varied considerably. All results were subjected to multifactorial analysis of variance. It turned out that the most important factor affecting changes in recorded values was culture temperature. The highest values, exceeding 1200 RLU, were obtained when cultures of individual strains on all media were run at 18-22°C. It was observed that under the other temperature conditions, i.e. 25-30°C and 35-37°C, some of the analysed bacterial cultures either did not exhibit any bioluminescent properties which could be recorded using a luminometer or it was luminescence with very low intensity.

Table 2. Mean bioluminescence values (RLU) of examined microorganisms cultivated in temperature 28-30°C
Tabela 2. Średnie wartości bioluminescencji (RLU) badanych gatunków bakterii, hodowanych w temperaturze 28-30°C

Bacteria strain Gatunek bakterii	Culture time Czas hodowli h	Medium – Podłoże			
		LA	BOSS	LM	NCBE
Vibrio fischeri	12	128	133	4	13
	24	133	107	7	16
	36	189	109	9	28
	48	196	111	8	15
	120	125	40	3	10
Vibrio harveyi	12	12	19	1	6
	24	16	21	1	8
	36	57	26	4	5
	48	24	13	3	3
	120	38	7	2	3
Photobacterium phosphoreum	12	66	47	11	10
	24	98	52	15	12
	36	93	48	26	25
	48	74	41	6	6
	120	86	8	2	3
Photobacterium luciferum	12	28	2	1	3
	24	33	3	2	2
	36	48	6	4	6
	48	52	9	2	2
	120	52	2	2	1

Table 3. Mean bioluminescence values (RLU) of examined microorganisms cultivated in temperature 35-37°C
Tabela 3. Średnie wartości bioluminescencji (RLU) badanych gatunków bakterii hodowanych w temperaturze 35-37°C

Bacteria strain Gatunek bakterii	Culture time Czas hodowli h	Medium – Podłoże			
		LA	BOSS	LM	NCBE
Vibrio fischeri	12	22	12	5	2
	24	45	9	7	1
	36	31	8	4	4
	48	16	7	1	2
	120	6	7	1	1
Vibrio harveyi	12	10	4	10	1
	24	14	8	10	8
	36	16	6	2	4
	48	15	1	1	2
	120	7	1	0	1
Photobacterium phosphoreum	12	45	3	0	1
	24	32	2	1	3
	36	36	1	5	2
	48	17	1	0	2
	120	2	1	0	1
Photobacterium luciferum	12	1	1	0	1
	24	2	1	0	2
	36	1	4	4	1
	48	1	3	1	0
	120	1	1	0	0

Thus further discussion of results was limited to the values presented in Table 1, assuming culture temperature of 20-22°C to be the most suitable for the selected bioluminescent bacteria and tested media. The statistically significant effect (for $\alpha \leq 0.05$) of both the type of the tested strain and applied medium on bioluminescence intensity was evident. As it results from microbiological practice [Trojanowska 2004], for most cases of bacterial cultures of this type there is a parabolic dependence of the recorded RLU value on culture time. This means that there is a certain optimum time at which the highest RLU value is obtained and that there are different culture times at which RLU values are going to be identical. Also in this study such a dependence was observed (Fig. 1 a, b, c, d).

A factor frequently observed in the course of microbiological analyses and considerably hindering immediate interpretation of results is their large scatter. Thus it was assumed that the character of changes in RLU values in a given time range should be close to linear, with uniform and possibly large increments in a time unit. An additional condition was to maintain a unidirectional increase in the analysed values over the entire time interval.

These conditions were most fully met in the following cases:
- for strain *Photobacterium phosphoreum* and LM medium as well as NCBE medium,
- for strain *Photobacterium luciferum* and LA and Boss media.

In turn, the most inform increase and a high gradient value were recorded for the variant of *Photobacterium phosphoreum* and NCBE medium.

In relation with the above, the values obtained for cultures run for 48 h were adopted in further considerations. The starting point consisted of culture results recorded after 12 h. In order to facilitate further computations, the obtained results were transformed into relative values (assuming the RLU value after 12 h as 1,00). For the analysed time intervals they are presented in Table 4. Such modified results were used in the calculations of interrelationships and regressions. On the basis of the presentation of results of the performed calculations (Table 5) it may be concluded that in each case a high value of the coefficient of determination was obtained (R^2). At the same time, the lack of very high significance for certain correlations may be explained by a small number of testing points, and thus also a low number of degrees of freedom. The analysis of values recorded for parameter "b" (the slope of a straight line) in the obtained regression equations indicates a varied suitability of analyzed systems e.g. in testing the presence of pollutants in the environment.

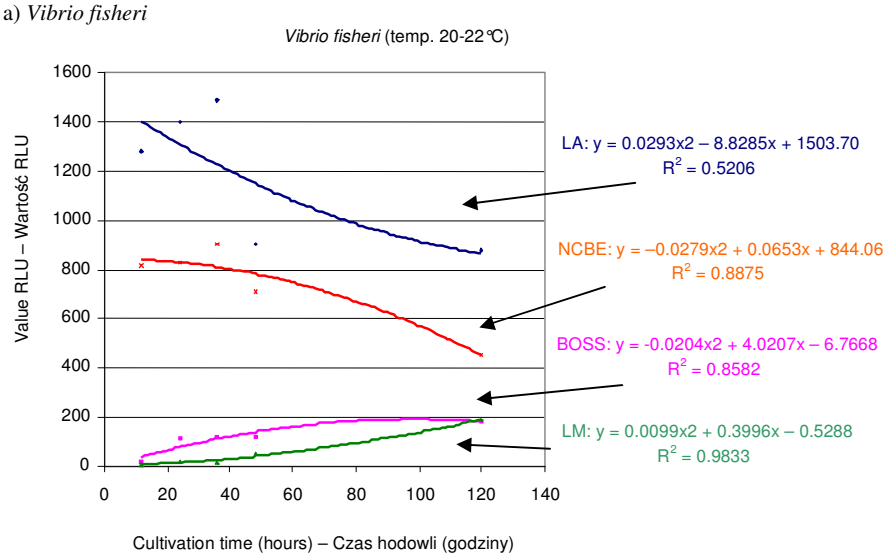
Table 4. Relative values of bioluminescence (RLU) of selected microorganisms
Tabela 4. Względne wartości bioluminescencji (RLU) wybranych gatunków bakterii

Culture time Czas hodowli h	Relative value of RLU – Względne wartości RLU				
	Photobacterium phosphoreum		*Photobacterium luciferum*		
	LM	NCBE	LA	BOSS	NCBE
12	1.0000	1.0000	1.0000	1.0000	1.0000
24	5.1000	7.8462	1.8710	1.6364	1.8545
36	9.6000	16.8846	12.2903	3.9091	4.7273
48	19.800	27.1538	23.8710	5.5455	–

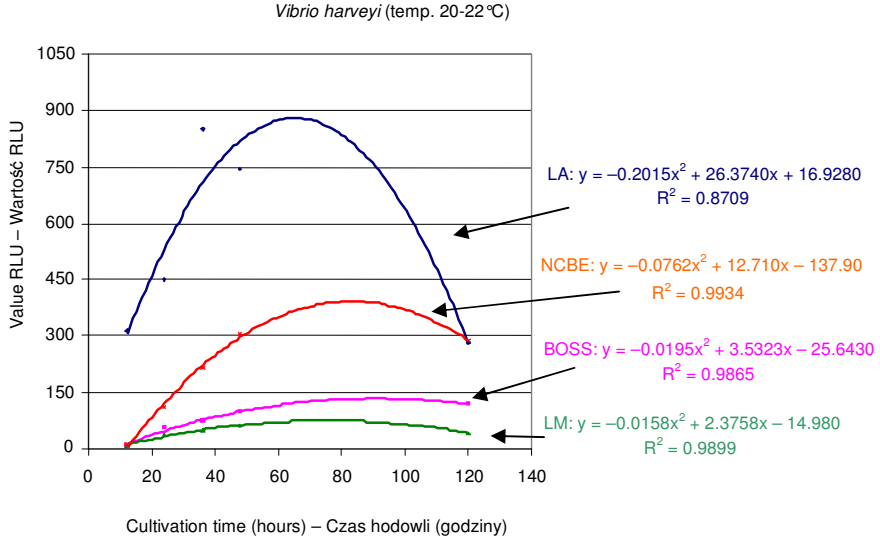
The usability of a given system for this purpose is determined by two parameters:
- a possibly high significance of the calculated dependence between the investigated factors,
- a possibly high value of factor "b" in the calculated regression equations.

In case of variants analysed in this study it may be ordered as follows: *Photobacterium phosphoreum* + NCBE > *Photobacterium luciferum* + LA > *Photobacterium phosphoreum* + LM. The other variants may be rejected in further investigations. Figure 2 gives an example of a graphic presentation of linear regression for the *Photobacterium phosphoreum* + NCBE system.

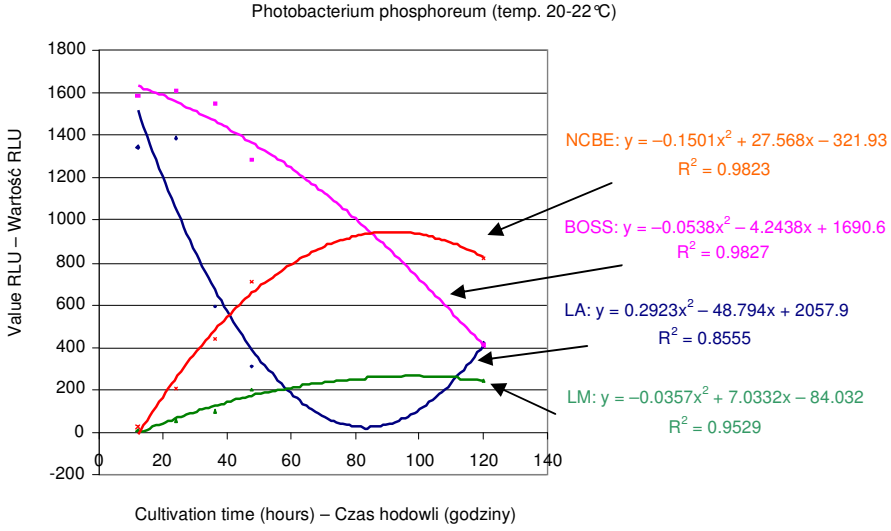
a) *Vibrio fisheri*



b) *Vibrio harvei*



c) *Photobacterium phosphoreum*



d) *Photobacterium luciferum*

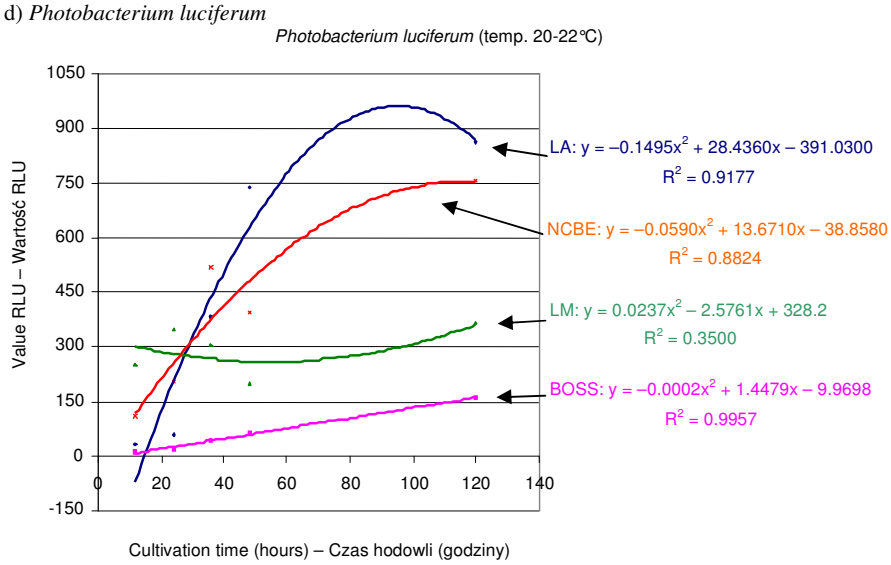


Fig. 1. Changes in bioluminescence (RLU) values of tested microorganisms as dependent on cultivation time and medium type (culture temperature: 20-22°C)
Rys. 1. Zmiany bioluminescencji (RLU) badanych mikroorganizmów w zależności od czasu hodowli i rodzaju podłoża (temperatura hodowli: 20-22°C)

Table 5. Correlation between bioluminescence values (RLU) and incubation time (x) for selected bacteria and medium type
Tabela 5. Zależności pomiędzy wartościami bioluminescencji (RLU) i czasem hodowli (x) dla wybranych bakterii i rodzajów podłoża

Bacteria strain Gatunek bakterii	Medium Pożywka	Linear regression Regresja liniowa $y = a + bx$	Straight line regression Regresja prostoliniowa $y = bx$	Power regression Regresja potęgowa $\log y = A + b \cdot \log x$ $y = ax^b$	Exponential regression Regresja wykładnicza $\ln y = A + bx$ $y = ae^{bx}$
Photobacterium phosphoreum	LM	$r^2 = 0.9523$**	$r^2 = 0.8856$	$r^2 = 0.9728$**	$r^2 = 0.9998$***
		$y = -42.00 + 6.125x$	$y = 4.625x$	$\log y = 0.1307 + 1.352\log x$	$\ln y = 2.945 + 0.0635x$
	NCBE	$r^2 = 0.9986$**	$r^2 = 0.9778$**	$r^2 = 0.9999$***	$r^2 = 0.9786$**
		$y = -78.333 + 20.9167x$	$y = 18.119x$	$\log y = 0.9341 + 1.2194\log x$	$\ln y = 4.5688 + 0.0558x$
Photobacterium luciferum	LA	$r^2 = 0.9991$***	$r^2 = 0.8105$	$r^2 = 0.9724$**	$r^2 = 0.9029$*
		$y = -320.00 + 28.4167x$	$y = 16.988x$	$\log y = -1.8105 + 3.0524\log x$	$\ln y = 1.9712 + 0.1362x$
	BOSS	$r^2 = 0.9912$**	$r^2 = 0.9096$*	$r^2 = 0.9761$**	$r^2 = 0.9096$*
		$y = -13.333 + 1.7917x$	$y = 1.3155x$	$\log y = -1.1039 + 1.8330\log x$	$\ln y = 1.1447 + 0.0892x$

*$\alpha = 0.10$, **$\alpha = 0.05$, ***$\alpha = 0.01$.

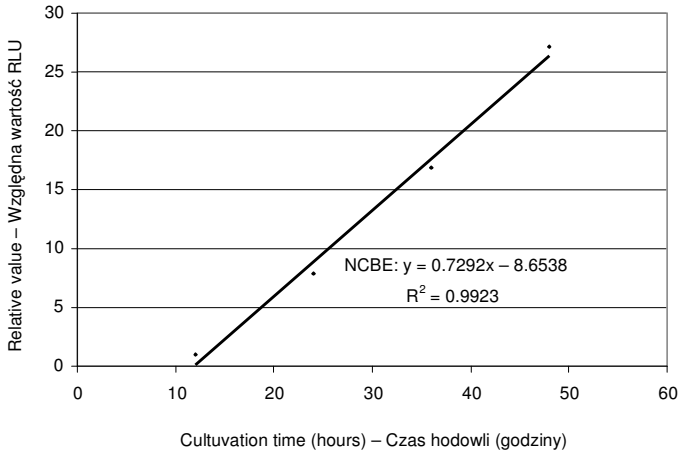


Fig. 2. Changes in bioluminescence (RLU) values of *Photobacterium phosphoreum* cultivated on NCBE medium type in temperature 20-22°C for incubation time until 48 hours (linear regression)
Rys. 2. Zmiany bioluminescencji (RLU) bakterii *Photobacterium phosphoreum* hodowanej na podłożu NCBE w temperaturze 20-22°C w czasie inkubacji do 48 godzin (regresja liniowa)

CONCLUSIONS

1. The described method of bioluminescence measurement using the tested bacterial strains and media may be applied for diagnostic purposes.

2. The best suitability was found for the experimental system of *Photobacterium phosphoreum* and NCBE.

3. It is advisable to test the sensitivity of selected systems to specific chemicals being major sources of pollution found in the environment, including those reported in practice connected with food processing.

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OBIEKTYWNA METODA OCENY WŁAŚCIWOŚCI BIOLUMINESCENCYJNYCH WYBRANYCH SZCZEPÓW BAKTERII

Streszczenie. Świecenie jako rezultat biochemicznej aktywności żywych bakterii *Vibrio fischeri* (dawniej znanych jako *Photobacterium phosphoreum*) stwarza możliwość monitorowania zmian środowiskowych w ekosystemach. Testy toksyczności wykorzystujące bioluminescencję zostały uznane za międzynarodowy standard postępowania. Tworzą szybką, łatwą i czułą metodę badania wielu substancji chemicznych i próbek środowiskowych, np. wody, ścieków, ekstraktów z osadów. W pracy podjęto próbę scharakteryzowania i porównania właściwości bioluminescencyjnych czterech różnych szczepów bakterii hodowanych na czterech podłożach płynnych w zróżnicowanych warunkach temperaturowych. Natężenie bioluminescencji emitowanej podczas hodowli mierzono za pomocą luminometru BioOrbit 1253. Na podstawie wykonanych pomiarów natężenia światła, wyrażonych w jednostkach RLU (*Relative Light Units*), i obliczeń matematycznych do dalszych aplikacji wytypowano szczep *Photobacterium phosphoreum* hodowany na podłożu NCBE.

Słowa kluczowe: ekotoksykologia, biotesty, bioluminescencja, *Photobacterium*

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