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Microbiology

STUDIES ON THE CAUSES OF BIOLOGICAL ACTIVITY OF
WATER BACTERIA *AEROMONAS* NAD *PSEUDOMONAS* GENUS

BADANIA PRZYCZYN BIOLOGICZNEJ AKTYWNOŚCI BAKTERII WODNYCH
RODZAJU *AEROMONAS* I *PSEUDOMONAS*

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Investigated the factors responsible for antagonistic and stimulating activity of the selected bacteria strains of *Aeromonas* and *Pseudomonas* genus. By application of paper chromatography method and gel separation on Sephadex column, obtained several fractions of bacterial culture examined extracts. Determinations were made on the biological activity of fractions and on the presence in them of proteins, lipids and polysaccharides.

INTRODUCTION

Among many factors influencing the activity of bacteria strains, the basic importance is assigned to biologically active substances, which are responsible for inhibiting activity or growth stimulation of microbes.

On the presence of biologically active organisms within the sea and inland water environment, wrote Cviić (1953), Carlucci and Pramer (1960), Krasilnikov (1958), Chodyniecki (1972) and others. They reported that, the quantitative ratios and the specific composition of bacterial populations in water depends considerably from the antagonistic or stimulating activity of the microbes. Rosenfeld and Zo Bell (1946) also observed the antagonistic activity of natural sea water on the microbes.

Not much of data can be found in available literature, on any efforts made for determination of the nature of growthactive factors produced by bacteria *Aeromonas* and *Pseudomonas* sp. Landenberger (1952) separated the biologically active substances

produced by *Pseudomonas fluorescens* into two fractions. Both of them contained the fluorescent green pigment. The first one was acting antagonistically on *Bac. megatherium*, for what according to author, was responsible the green pigment. The second fraction stimulated the growth of *Bac. subtilis*, what was assigned to the properties of lactoflavine. Gräf (1958) investigated the factors produced by the specified strains *Pseudomonas fluorescens*, which inhibited the growth of ameba.

The present work is aimed for determination of the factors responsible for antagonistic and symbiotic activity of the selected bacteria strains of *Aeromonas* and *Pseudomonas* genus.

MATERIAL AND METHODS

For investigation used the bacteria-free extracts of 12 days broth cultures of water bacteria (Chodyniecki, 1972) of the recognized biological activity: *Aeromonas punctata* No 1, *Pseudomonas* sp. III group No. 11 possessing the stimulating properties and *Pseudomonas* sp. I group No. 9, 20, 21 having the antagonistic activity. As test strains, used the *Aeromonas punctata* No. 4 sensible to stimulating activity of extracts No. 1 and 11 and *Pseudomonas* sp. II group No. 12 sensitive to antagonistic properties of extracts No. 9, 20 and 21.

The presence of organic nitrogen in extracts was determined by biuret method according to Kingsley (Opieńska et al., 1957), while the counting was effected by Pulfrich photometer with filter S-53 of max. optical density 530 nm.

The separation of extracts by paper ascending chromatography method was effected on Schleicher and Schüll paper 2043 B. The chromatograms were unfolded according to Partridge arrangement: n-butanol, acetic acid and water (4:1:5) and developed by ninhydrin solution according to Fischer and Dörfer. The values R_f were determined for coloured strips and then the intensity of chromatogram bands colour was analysed by direct photometric method on apparatus MGB-151. The biological activity of the particular bands obtained in chromatographic separation were denoted on agar plates infected with test strain.

The separation of extracts by column chromatography on gel Sephadex G-50 was performed with application of phosphate buffer of pH 6.8 according to Linskens. Biological activity of the obtained fractions determined by method of dilutions and the antagonistic activity was related to test strain *Pseudomonas* sp. II group No. 12, while the symbiotic activity – to *Aeromonas punctata* No. 1.

The presence of proteins, lipids and polysaccharides determined on Schleicher and Schüll paper 2043 A. Discolouration of proteins was performed by application of the complex containing: bromine-phenol blue, ethanol and mercury chloride. To disclose the presence of lipids used the Sudan Black according to Swahn method. The polysaccharides determined according to Kōiw method by complex of anhydrous acid solution, sodium acetate and sulphite magenta solution. All the determinations were performed with three repetitions.

RESULTS

It was determined that, the extracts from cultures of broth strains *Aeromonas sp.* and *Pseudomonas sp.* and the control broth contained the nitrogen of protein or peptid origin. The highest content of nitrogen, viz.: 1.116 to 1.736 g/% shown the culture extracts of bacteria *Pseudomonas sp.* I group Nos. 9, 20 and 21. Within the culture extracts of *Aeromonas punctata* No. 1 and *Pseudomonas sp.* III group No. 11, the quantity of organic nitrogen amounted to 0.864–0.944 g/%, while in control broth – 0.704 g/% (Tabl. 1).

Table 1

Content of organic nitrogen within the examined extracts

Extract No.	Obtained culture from strain	g/%
1	<i>Aeromonas punctata</i> No. 1	0.944
11	<i>Pseudomonas sp.</i> II gr. No. 11	0.868
9	<i>Pseudomonas sp.</i> I gr. No. 9	1.488
20	<i>Pseudomonas sp.</i> I gr. No. 20	1.736
21	<i>Pseudomonas sp.</i> I gr. No. 21	1.116
K		0.704

K – control

On the chromatograms of examined extracts and of control, obtained seven coloured distribution bands and the fifth fraction was clearly visible within the extracts Nos 9, 20, 21 only (Fig. 1). The chromatograms examined under Wood light demonstrated the fluorescence of various intensity. It appeared most clearly within distribution bands of the same extracts, viz. Nos 9, 20 and 21. Probably, the presence and the quantity of fluorescent substance is in connection with the activity of the examined extracts.

The values R_f for particular coloured badns are presented in Tab. 2. It appears from the table that, the differences of values for the particular fractions of the examined and control extracts were within the limits of experimental error. Therefore, more detailed interpretation of the results was not possible.

The quantity of determined coloured substances resulting from intensity colouring of chromatographic distribution bands within the particular fractions are illustrated by the absorbtion curves shown on Fig. 2. The closest quantitative ratios occured within the bands marked with digits V, VI and VII and are characteristic for extracts Nos 9, 20 and 21. The remaining bands show clear quantitative oscillation. The analyses of biological activity of particular fractions from chromatographic separation on agar plates gave the negative results.

The separation of extracts on gel column Sephadex G-50 proved the presence of four fractions, out of which, only some possessed the antagonistic properties. This related solely to fraction „2” of culture extracts from strains *Pseudomonas sp.* I group No. 21 in

Fig. 1. Distribution of bacteria-free extracts by ascending paper chromatography

Table 2

Value of coefficient R_f of bands from chromatographic separation of extracts

Extract No.	Separation bands						
	I	II	III	IV	V	VI	VII
1	0.14	0.18	0.24	0.39	—	0.52	0.63
11	0.14	0.17	0.22	0.40	—	0.53	0.62
9	0.12	0.19	0.25	0.37	0.41	0.54	0.64
20	0.13	0.20	0.24	0.38	0.42	0.53	0.63
21	0.12	0.19	0.25	0.37	0.41	0.53	0.64
K	0.12	0.19	0.24	0.40	—	0.54	0.63

K — control

undiluted form and to fraction „3” in dilution of ratio 1:2. At the same time, the sum of all four fractions of this extract inhibited the growth of analysed strains in dilution of ratio 1:4. The separation fractions of extracts No. 9 and 20 did not show any antagonistic activity. However, the sum of fractions from extract No. 9 inhibited the growth of test strain in dilution 1:4 and from extract 20 in dilution 1:2. Additionally, ascertained that, in undiluted condition, the sum of fractions from extracts Nos 9 and 20 caused the increased growth of strains *Aeromonas punctata* No. 4.

Fig. 2. Absorption curves for filtrate fractions obtained by direct photometering method of chromatograms

The stimulating activity of obtained fractions presented in Tab. 4. The properties for growth stimulation of test strain *Aeromonas punctata* No. 4 demonstrated the fraction „3” of extract No. 1 only.

The activity of examined fractions was relatively low and was occurring in dilutions of 1:2–1:4. It is characteristic that, the sum of fractions from particular extracts demonstrated similar low activity. Thus, it is possible that, the active factor was lost during the preparation of particular extracts; it perhaps was absorbed on Seitz filters or was present in particular fractions, but in under-threshold quantities.

The determinations of proteins, lipids and polysaccharides in examined extracts gave the characteristic results. In biuret test, obtained the positive results, which are

Table 3

Antagonistic activity of fractions from extracts *Pseudomonas* sp. I group Nos 9, 20, 21
obtained on gel column Sephadex G-50

Test strain	Extract	Dilution	Fraction				Sum of fractions
			"1"	"2"	"3"	"4"	
Pseudo- monas sp. II group No. 12	9	0	+++	+++	+++	+++	—
		1:2	+++	+++	+++	+++	—
		1:4	+++	+++	+++	+++	±
		1:8	+++	+++	+++	+++	+++
	20	0	+++	+++	+++	+++	—
		1:2	+++	+++	+++	+++	+
		1:4	+++	+++	+++	+++	+++
		1:8	+++	+++	+++	+++	+++
	21	0	+++	++	++	+++	++
		1:2	+++	+++	++	+++	++
		1:4	+++	+++	+++	+++	++
		1:8	+++	+++	+++	+++	+++
	K		+++	+++	+++	+++	+++

— = no growth; ± = doubtful growth; + = weak growth; ++ = average growth; +++ = strong growth;
K = control.

Table 4

Stimulating activity of fractions from culture extracts *Aeromonas* sp. No. 1
and *Pseudomonas* sp. II group No. 11 obtained on gel Sephadex G-50

Test strain	Extract	Dilution	Fraction				Sum of fractions
			"1"	"2"	"3"	"4"	
Aeromas punctata No. 4	1	0	+	+	++	+	++
		1:2	+	+	+	+	+
		1:4	+	+	+	+	+
	11	0	+	+	+	+	++
		1:2	+	+	+	+	+
		1:4	+	+	+	+	+
	K		+	+	+	+	+

+ = normal growth; ++ = stimulation; K = control.

non-specific for proteins, exclude the presence of amino acids, but show the possibility of peptides occurrence. The positive result of reaction by ninhydrin excludes the proteins and

shows the presence of free amino acids and certain peptides **Opieńska-Blaut et al.**, 1957). The absence of coloured spots in application of bromine-phenol blue permits the assumption that, the peptides and amino acids occur in the analysed material. The separation of filtrates in dextran gel Sephadex tends to confirm the presence of proteins and peptides (**Flodin**, 1962).

Table 5

The results of determination of substance contained in extracts

Item	Particulars	Result obtained	Specific method for:		
			proteins	peptides	amino acids
1	Biuret test	+	—	+	—
2	Reaction with nihydrin	+	—	+	+
3	Colouring by bromine-phenol blue	—	—	+	+
4	Separation on gel column Sephadex	+	+	+	—

Basing on the performed analyses, in tab. 5 are presented the results of determinations of the particular components contained in extracts. Such summary permits to assume that, in composition of the examined filtrates, controls and their fractions, dominate primarily the peptides and than the free amino acids. Already **Gräf** (1958) called an attention to polypeptid compounds as the factors being responsible for the antagonistic properties of the examined by him strains *Pseudomonas fluorescens*, while **Cvić** (1953) and **Krasilnikov** (1958) indicated towards the presence of gramicidin in sea water. It is known that this antibiotic is the cyclic polypeptid.

CONCLUSIONS

1. In all examined extracts dominated the low molecular weight peptides and next, the free amino acids.

2. The varying quantities of peptides and amino acids could influence various biological activity of the examined extracts. It is likely that, only some of them show the antagonistic or stimulating activity.

3. It seems that, the presence of fluorescent substance was connected with biological activity of the examined extracts.

4. More exact determination of the factors responsible for biological activity of microbes *Aeromonas* and *Pseudomonas* genus require further investigations.

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RODZAJU *AEROMONAS* I *PSEUDOMONAS*

Streszczenie

Posługując się metodą chromatografii bibułowej i rozdziatu na kolumnach w żelu Sephadex, badano przyczyny biologicznej aktywności szczepów rodzaju *Aeromonas* i *Pseudomonas*. W ekstraktach hodowli bakteryjnych otrzymano szereg frakcji. Określano następnie aktywność biologiczną tychże frakcji oraz obecność białek, lipidów i polisacharydów. Wykazano, że we wszystkich badanych ekstraktach dominowały niskocząsteczkowe peptydy a następnie wolne aminokwasy. Na zmienną aktywność biologiczną badanych ekstraktów mogą mieć wpływ różne ilości peptydów i aminokwasów, ponadto tylko niektóre z nich mogą wykazywać działanie antagonistyczne lub stymulacyjne. Wydaje się, że z aktywnością badanych szczepów bakteryjnych posiada związek obecność substancji fluoryzujących.

ИССЛЕДОВАНИЯ ПРИЧИН БИОЛОГИЧЕСКОЙ АКТИВНОСТИ
ВОДНЫХ БАКТЕРИЙ РОДА *AEROMONAS* И *PSEUDOMONAS*

Р е з ю м е

Пользуясь методом хроматографии на бумаге и разделения в колонках в геле *Sephadex* исследовали причины биологической активности штаммов бактерий рода *Aeromonas* и *Pseudomonas*. В экстрактах бактериальных культур получен ряд фракций. Затем была определена биологическая активность этих фракций и наличие белков, липидов и полисахаридов. Установлено, что во

всех исследуемых экстрактах преобладали низкомолекулярные пептиды и свободные аминокислоты. На изменчивость биологической активности исследуемых экстрактов может иметь влияние разное количество пептидов и аминокислот, а кроме того, только некоторые из них могут проявлять антагонистическое или стимулирующее действие. Кажется, что с активностью исследуемых бактериальных штаммов имеет связь наличие флуоресцирующих субстанций.

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