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Original Research

Intraspecific or Intraspecies Differentiation of *Saccharomyces cerevisiae* Strains Isolated from Fish, Sewage and Odra Waters Based on Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) Technique

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Abstract

Saccharomyces cerevisiae strains isolated from fish and subsurface layers of the Odra Estuary waters were examined. Statistical comparison showed that the increase in numbers of *S. cerevisiae* strains was related to an increase in the total number of yeasts and yeast-like fungi in tested samples. In case of analysis of fish mycoflora, correlation wasn't observed between fish species, places of catch and total viable count of yeasts and yeast-like fungi. RAPD-PCR analysis enabled us to distinguish three main groups; ten genotypes each were determined.

Keywords: *S. cerevisiae*, RAPD-PCR, water, fish

Introduction

Qualitatively and quantitatively varied microflora is present in the aquatic environments and creates characteristic structures typical of particular water reservoirs. Atypical groups of microorganisms occur in the environment affected by human activity. Based on their adaptation abilities, such microorganisms may either survive and become an inseparable part of the aquatic biocoenosis or undergo slow extinction. Survival of microorganisms in the environment depends on the ability of cells to adjust to so-called minimal ecosystems created by biofilms and microbiological nets. In such populations a function of species rather than the species itself is usually regarded. The presence of microorganisms in new ecological structures may be determined either by genetic differentiation of strains or by their phenotypic flexibility. Serious problems may be posed by microorganisms which replace natural microflora. No competition or antagonistic relationships within the aquatic environment promotes the occurrence of dominant strains which may be agents of waterborne infections for people and animals.

The most recent studies show that each year a significant increase in the number of mycosis caused by 'emerging pathogens' is observed [1, 2]. Surprisingly, *Saccharomyces cerevisiae*, considered as non-pathogenic, commonly known and widely used in industry and household, has been included in the group of pathogenic yeasts since the 1990s [1, 3-6], however, it has not been frequently isolated from natural water bodies [7, 8]. According to Sage et al. [9] the increased number of *S. cerevisiae* isolated from water samples is linked to industrial

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and household activities. Presently, studies are conducted on the possible application of *S. cerevisiae* as a water contamination index [10, 11].

The aim of the work was to determine the intraspecies differentiation of *S. cerevisiae* strains isolated from fish and the Odra estuary (the second largest Polish river) and to evaluate whether the strains were a stable element of the aquatic microflora.

Experimental Procedures

Collection of Water Samples and Their Mycological Analysis

The area of studies covered the Odra estuary:

(i) Odra Szczecińska,

(ii) Roztoka Odrzańska with the Szczecin Lagoon, and

(iii) the Pomeranian Bay (Fig. 1).

Stations were chosen based on the indices of hydrochemical pollution (WIOŚ, 2000) and the characteristics of catchment area (Table 1).

Collecting the 56 water samples was performed according to protocols recommended by an accredited laboratory of the Regional Sanitary Inspectorate in Szczecin. All samples were collected from the subsurface water layer once a month from April to November, 2001. The evaluation of the total viable count (TVC) of yeasts and yeast-like organisms was performed as previously described [8].

Collection of Fish Samples and their Mycological Analysis

A total number of 90 fish – bream (*Abramis brama*), roach (*Rutilus rutilis*) and perch (*Perca fluviatilis*) – were caught seasonally (spring, summer, autumn). A characteristic of fish is presented in Table 2. Fish were killed immediately and placed in sterile boxes separately. They were delivered to the laboratory in a collective cooling box within an hour and then microbiological analyses were carried out. Mycocoenoses were evaluated based on samples of mucus, gills and intestines. Mucus samples (1g) were collected from fish surfaces and vortexed for 5 min. Gill and intestinal samples were prepared using sterile surgical instruments and ground in mortars with glass beads.

Water and fish samples were suspended in sterile saline solutions, cultured on Sabouraud Dextrose Agar supplemented with chloramphenicol (Oxoid) and incubated at 20°C for 3-5 days. All the samples were duplicated three times.

TVC of yeast and yeast-like fungi was evaluated. Ten colonies per plate were isolated to confirm species iden-

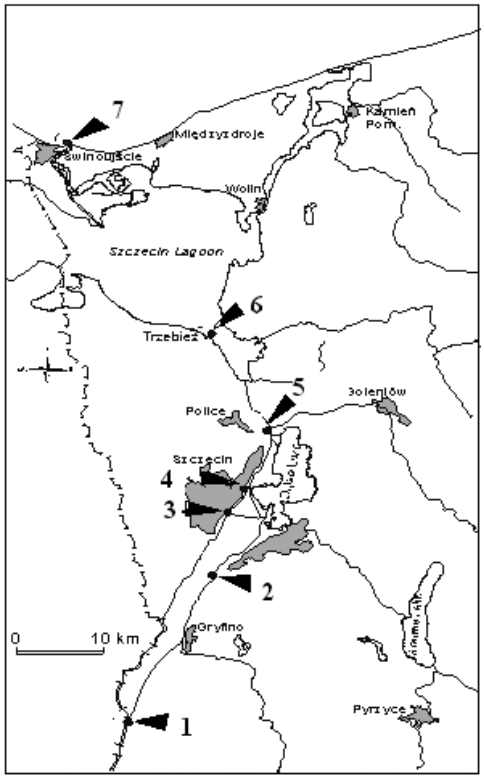

Fig. 1. Map of sampling station: **1**-Widuchowa, **2**-Highway, **3**-Ling Bridge, **4**-Maritime office, **5**-Police, **6**-Trzebież, **7**-Świnoujście

Table 1. A characteristic of sample-collection sites within the Odra Estuary

Area	No.	Sites	Potential source of contamination	Microflora*		Water quality**
				S. cerevisiae	yeasts	
Odra Szczecińska	1	Widuchowa	Birds, wetlands	0.3×10^1	4.0×10^2	II
	2	Highway	Floods, non-arable fields	1.0×10^1		III
	3	Ling Bridge	Industrial and municipal wastes	3.5×10^3	5.1×10^3	>**
	4	Maritime office		8.6×10^2	1.8×10^3	>
Roztoka Odrzańska with the Szczecin Lagoon	5	Police	Chemical plant	1.3×10^2	5.2×10^2	III
	6	Trzebież	Port	0.6×10^1	7.7×10^3	I
The Pomeranian Bay	7	Świnoujście	Port	none	2.5×10^1	I

*according to WIOŚ [20], **> out of standards

tity based on colony and cell morphology. Preliminary identification was carried out according to Vaughan-Martini and Martini [12]. Species identification of *S. cerevisiae* was confirmed using biochemical tests API ID 32 C (bioMèrieux) according to the manufacturer's protocol.

DNA Extraction

DNA was extracted from 24 h cultures in 2 ml of YPD medium at 30°C using QIAamp DNA Mini kits (Qiagen) plus sorbitol buffer and lyticase according to the manufacturer's instructions.

RAPD-PCR Assay

The set of RP1-4 (5' TAG GAT CGA A 3') and SOY (5' AGG TCA CTG A 3') was chosen [13]. Primers were synthesized by TIB MolBiol (Poland). Optimization of the assay was performed according to an orthogonal array designed by Taguchi and Wu [14] and modified by Cobb and Clarkson [15].

PCR mixture contained 500 mM KCl, 100 mM Tris-HCl pH 8.3 (at 25°C), 3.5 mM of $MgCl_2$, 0.625 mM of each dNTP, 12.5 pmol/mL of each primer and 1U of *Taq* DNA polymerase (Eppendorf) and 20 ng/µL of template DNA.

The thermal profile (Mastercycler Gradient, Eppendorf) consisted of one initial cycle: denaturation at 95°C, annealing at 36°C, elongation at 72°C performed for 5 min each and followed by 30 cycles of a 94°C denaturation for 1 min, a 72°C annealing for 1 min and a 72°C elongation for 2 min. At the end of amplification the mixture was subjected to the final extension at 72°C for 10 min.

Products were analyzed by electrophoresis in 2% agarose gel (Prona Agarose Plus) stained with ethidium bromide (0.5 µL/mL) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and examined under UV light (GelDoc, BioRad). Quantity One (Bio-Rad) and BioGene (Vilber Lourmat) software were used to estimate the similarity (Dice 2%) within a group of isolates.

Statistical Analysis

Statistical analysis included the mean, standard deviation, significance and correlation tests performed using (Statistica PL).

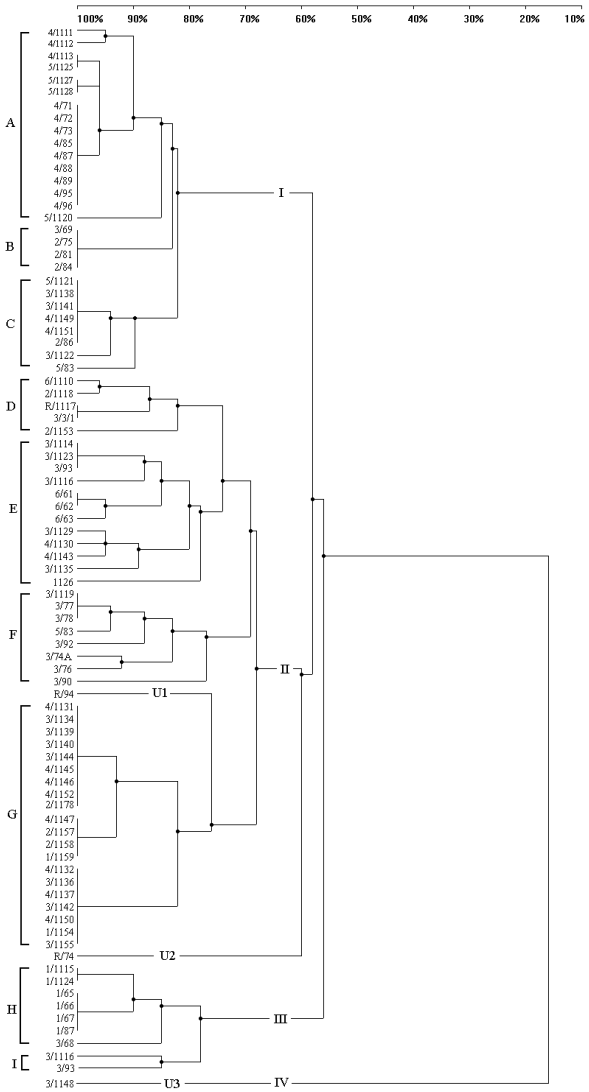

Fig. 2. Dendrogram demonstrating the genetic relationship isolated of *S.cerevisiae.*

Table 2. A characteristic of analyzed fish.

Species	Sites	Number	Fish characteristic		
			Weight (g)	Length (cm)	Injuries
Bream (*Abramis brama*)	1.3 6	21 9	1250-1400 1300-1640	48.0-50.0 47.5-51.0	none
Roach (*Rutilis rutilis*)	1.3 6	21 9	37.1-92.3 45.0-55.0	14.5-20.5 18.0-21.0	none
Perch (*Perca fluviatilis*)	1.3 6	21 9	30.5-75.5 30.0-70.0	16.5-17.5 30.0-80.0	none

Results

Analysis of Aquatic Mycoflora

Analysis of spatial distribution of mycoflora revealed a relatively low number of *S. cerevisiae* in waters ingoing towards Szczecin. Significantly higher numbers of yeasts and yeast-like fungi were observed within the inhabited area of Odra Szczecińska with the highest level of mycocoenoses (5.1×10^3 cfu/mL) found at station 3, which included the main sewage dumping site for the community of Szczecin (Table 1). TVC of yeasts (1.8×10^3 cfu/mL) and *S. cerevisiae* (8.6×10^2 cfu/mL) increased up to site 4. The slight decrease in the number of analyzed forms was observed not earlier than at site 5. The number of *S. cerevisiae* strains was statistically significantly higher at sites 4-6 than that observed at the other sites (Table 1). A significant decrease in TVC of yeasts was noticed only within Roztoka Odrzańska at sites 6 and 7 where the numbers were comparable (Table 1). The number of *S. cerevisiae* in samples from site 6 was similar to results obtained for sites 1 and 2 (Table 1). *Saccharomyces* sp. were not detected within the area of the Pomeranian Bay.

Analysis of Fish Mycoflora

Results presented in Table 3 revealed statistically insignificant quantitative differentiation of fungi in particular fish parts. The lowest quantitative differentiation was established among gills, mucus, and intestines in summer. The level of microorganisms isolated from fish in all analyzed environments did not exceed 10^2 cfu/g. However, according to Table 3, only in this season of studies was the frequency of fungi isolated from fish intestines (mainly bream and perch) higher. It was also reported that TVC of yeasts and yeast-like fungi was significantly lower ($p<0.05$) in summer than in spring and autumn. Mucus was the most contaminated medium, whereas the amounts detected in gills and intestines were comparable. *S. cerevisiae* was found sporadically only in roach and perch. No correlation was observed between fish species, places of catch and TVC of yeasts and yeast-like fungi.

Analysis of Intraspecies Differentiation of *S. cerevisiae* Strains

S. cerevisiae strains isolated from the lower part of the Odra (Widuchowa – the Szczecin Lagoon) and fish

Table 3. Mycological analysis of fish.

Species	Catch site	Yeasts (CFU/g)			Occurrence of *S. cerevisiae*		
		Mucus	Gills	Intestines	Mucus	Gills	Intestines
Bream (Abramis brama)	spring	-	-	-	-	-	-
	summer	1.2×10^2	2.3×10^2	8.9×10^2	-	-	-
	autumn	4.2×10^3	4.8×10^3	4.2×10^2	-	-	-
	spring	2.8×10^4	8.2×10^3	3.8×10^3	-	-	-
	summer	$2.2\times10^{2*}$	1.2×10^2	7.8×10^2	-	-	-
	autumn	5.6×10^4	4.4×10^3	1.3×10^3	-	-	-
Roach (Rutilis rutilis)	spring	5.6×10^4	9.4×10^3	9.8×10^1	-	-	-
	summer	$1.1\times10^{2*}$	$1.4\times10^{2*}$	5.9×10^2	-	-	-
	autumn	3.2×10^4	1.2×10^4	4.8×10^2	-	-	+
	spring	3.6×10^4	7.2×10^3	6.2×10^2	-	-	-
	summer	$2.8\times10^{2*}$	$2.2\times10^{2*}$	8.2×10^2	-	-	-
	autumn	6.0×10^4	8.6×10^3	4.2×10^2	-	-	-
Perch (Perca fluviatilis)	spring	3.1×10^4	7.2×10^3	1.3×10^2	-	-	-
	summer	$2.8\times10^{2*}$	$2.2\times10^{2*}$	8.9×10^2	-	-	-
	autumn	4.5×10^5	2.8×10^4	9.3×10^1	-	-	+
	spring	2.1×10^4	8.4×10^3	2.3×10^2	-	-	-
	summer	$5.8\times10^{2*}$	$6.2\times10^{2*}$	8.9×10^2	-	-	-
	autumn	4.0×10^4	1.8×10^4	5.3×10^2	-	-	-

* – significant differences

were analyzed using RAPD-PCR technique. Fig. 2 presents results of cluster analysis. Three main groups (I-III) including nine genotypes (A-I) each were determined. *S. cerevisiae* strain (R/1117) isolated from the perch intestines belonged to group I-D whereas roach strains (R/74 and R/94) were determined as unique.

Discussion of Results

Degradation of natural water bodies promotes distribution of pathogens. In Poland it is estimated that 20% of sewage is delivered directly to surface waters, 52% of which is a source of drinking water for humans. Theoretically, sewage delivered to natural waters should not pose a microbiological hazard or destabilize natural biocoenoses, and its fecal index should not exceed the indices observed in waters into which it is introduced. Increasing numbers of sewage sources, especially in large cities, calls for novel and rapid water-quality tests.

The possibility of employing yeasts and yeast-like fungi as indices for monitoring the quality of waters, suggested previously by El-Tawel and Shaban [19], was confirmed in our studies. Results revealed significant spatial differentiation of TVC of yeasts and yeast-like fungi depending on the site of sample collection (Table 1). A link between the municipal sewage affecting the quality of hydrochemical system [20] and the increase in number of isolated yeasts was noticed (Table 1). El-Tawel and Shaban (2001) reported that increasing water contamination was accompanied by the growing TVC of yeasts, mainly *Candida albicans*. Our results proved that such a correlation existed for TVC of yeasts and *S. cerevisiae.* It leads to the conclusion, supported by the dominance of particular genotypes in particular sites, that the level of TVC of yeasts in the most municipally contaminated areas is formed by *S. cerevisiae* (Table 1). Genotypes were divided into *municipal* (A, B, C, D, E, F and I) and environmental (G and H) types (Table 4, Fig. 1). Only the presence of G and H strains was detected at site 1 not affected by municipal influence. At site 2 a slight increase in the number of *S. cerevisiae* was observed as well as the occurrence of new genotypes – B, C and D – due to the inflow of waters from the fish cage cultures along the Lower Odra. Faid et al. [21] reported that the activity of some microorganisms, including *S. cerevisiae*, depended on the presence of sewage from fish cultures. Genotypes B, C, D, G and H were detected at sites 3 and 4 (Table 4), which highlighted ecological flexibility of analyzed *S. cerevisiae* strains. Changes in hydrochemical conditions [20] within a studied area lower water quality but at the same time provide nutrients for the outgrowth of yeasts including *S. cerevisiae* (Table 1). Similar correlation of sewage amounts with the increasing number of yeasts was observed by Sage et al. [9]. However, it cannot be excluded that sewage is not only a medium for the outgrowth of yeasts in natural environment but serves as a vector for yeasts introduced directly to the aquatic environment from inhabited/industrial areas. Four new genotypes (A, E, F, I), probably of sewage-origin, were detected at sites 3 and 4. Such strains had to be highly tolerant to environmental stress caused by toxicity of sewage medium. Probably, due to their susceptibility, environmental genotypes (G and H) were eliminated at subsequent sites 5 and 6. Genotypes A, C, D, E, and F were still detected but their presence was rather temporary (Bogusławska-Wąs, *oral communication*). According to Bogusławska-Wąs and Dąbrowski [8] *S. cerevisiae* is rarely isolated from the Szczecin Lagoon (site 6) where the dominant genus has been *Rhodotorula*.

The highest TVCs of yeasts were detected in mucus and gills of all analyzed fish, probably due to microbiological

Table 4. The occurrence of *S. cerevisiae* genotypes.

	Sites					
	1	2	3 and 4	5	6	7
Genotypes	A	A	**A**	**A**	A	A
	B	**B**	**B**	B	B	B
	C	**C**	**C**	**C**	C	C
	D	**D**	**D**	D	**D**	D
	E	E	**E**	E	**E**	E
	F	F	**F**	**F**	F	F
	G	**G**	**G**	G	G	G
	H	H	**H**	H	H	H
	I	I	**I**	I	I	I

Bold – genotypes present. 1 – Widuchowa, 2 – Highway, 3 – Ling Bridge, 4 – Maritime office, 5 – Police, 6 – Trzebież, 7 – Świnoujście

contamination of the aquatic environment. Yet our results did not confirm correlation between mycocoenoses of the aquatic environment and the occurrence of yeasts on fish. Mucus prevents microbiological colonization via a competition mechanism [16]. However, microbiological balance requires proper health condition of fish, which is affected by physicochemical properties of the environment, waterborne pathogens, and food accessibility. Andlid [17] reported that hydrophobic properties of bacterial cell wall determined its colonization e.g. to the surface of epithelium of fish intestines. A period of starvation played an important role in adherence of yeasts within a population of wild fish [17]. Our results did not reveal such a precise correlation. During the summer season gastrointestinal tracts of analyzed fish were filled and the presence of nutrients might promote the outgrowth of hydrophobic yeasts. TVC of yeasts and yeast-like fungi evaluated in fish gastrointestinal tracts during summer was higher than in mucus and gills. However, the results were not statistically significant (Table 4). Similar levels of mycocoenoses were observed in fish caught in autumn, probably due to the utilization of mucus as a source of nutrients by microflora [17]. Our results did not report significant presence of *S. cerevisiae* in fish material. According to Andlid et al. [18] results of 2-D PAGE analysis for fish isolates of *S. cerevisiae* revealed the presence of unique strains. In case of our studies, fish strains were too rare to confirm whether they comprised a separate fish genogroup(s).

References

1. HAZEN K.C. New and emerging yeast pathogens. Clin. Microbiol. Rev. **8**, 462, **1995**.
2. SWOBODA-KOPEĆ E., ROKOSZ A., SAWICKA-GRZELAK A., WRÓBLEWSKA M., KRAWCZYK E., STELMACH E., ŁUCZAK M. Etiological agents of fungemias in hospitalized pathiens. Med. Dośw. Mikrobiol. **53**, 291, **2001.**
3. SOBEL J.D., VAZQUEZ J., LYNCH M., MERIWETHER C., ZERVOS M.J. Vaginitis due *Saccharomyces cerevisiae*, epidemiology, clinical aspects, and therapy. Clin. Infect. Dis. **16**, 93, **1993**.
4. XU J., BOYD C.M., LIVINGSTON E., MEYER W., MADDEN J.F., MITCHELL T.G. Species and genotypic diversities and similarities of pathogenic yeasts colonizing women. J. Clin. Microbiol. **37**, 3835, **1999.**
5. PONTON J., RUCHEL R., CLEMONS K.V., COLEMAN D.C., GRILLOT R., GUARRO J., ALDEBERT D., AMBROISE-THOMAS P., CANO J., CARRILLO-MUNOZ A.J., GENE J., PINEL C., STEVENS D.A., SULLIVAN D.J. Emerging pathogens. Med. Mycol. **38**, 225, **2000.**
6. GARCIA-MARTOS P., DOMINGUEZ I., MARIN P., GARCIA-AGUDO R., AOUFI S., MIRA J. [Antifungal susceptibility of emerging yeast pathogens.] Enferm. Infecc. Microbiol. Clin. **19**, 249, **2001.**
7. COMI G., RUFFONI B., CANTOMI C., PSINO L. Indigine ecologica sui lieviti delle acque di alcune specie di perci del Lago Maggiore. Arch. Vet. Ital. **34**,1., **1983.**
8. BOGUSŁAWSKA-WĄS E., DĄBROWSKI W. The seasonal variability of yeasts and yeast-like organisms in water and bottom sediment of the Szczecin Lagoon. Int. J. Hyg. Environ Health. **203**, 451, **2001**.
9. SAGE L., BENNASSER L., STEIMAN R., SEIGLE-MURANDI F. Fungal microflora biodiversity as function of pollution in Oued Sebou (Morrocco). Chemosphere **35**, 751, **1997**.
10. IKEBUKURO K., MIYATA A., CHO S.J., NOMURA S.M., CHANG Y., YAMAUCHI Y., HASEBE Y., UCHIYAMA S., KURUBE I. Sensor for monitoring river water. Int. J. Biotechnol. **48**, 73, **1996.**
11. TERZIYSKA A., WALTSCHEWA L., VENKOV P. A new sensitive test based on yeast cells for studying environmental pollution. Environ. Pollut. **109**, 43, **2000**.
12. VAUGHAN-MARTINI A., MARTINI A., CARDINALI G. A taxonomic key for the genus *Saccharomyces.* System. Appl. Microbiol. **16**, 113, **1993.**
13. LEHMAN P. F., LIN D., LASKER B. A. Genotyping identification and characterization of species and strains within the genus *Candida* by using Random Amplified Polymorphic DNA. J. Clin. Microbiol. **30**, 3249, **1992.**
14. TAGUCHI G., WU Y. Introduction to off-line quality control. Japan Quality Control Organisation. Nagoya Japa, **1980**.
15. COBB B.D., CLARKSON J.M. A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res. **22,** 3801, **1994**.
16. BORDAS M.A., BALEBONA M.C., ZORRILLA I., BORREGO J.J., MORINIGO M.A. Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). Appl. Environ. Microbiol. **62**, 3650, **1996.**
17. ANDLID T. Yeast isolated from the intestine of rainbow trout adhere to and grow in intestinal mucus. Mol. Marine Biol. Biotechnol. **7**, 115, **1998**.
18. ANDLID T., BLOMBERG L., GUSTAFSSON L., BLOMBERG A. Characterization of *Saccharomyces cerevisiae* CBS 7764 isolated from rainbow trout intestine. Syst. Appl. Microbiol. **22**, 145, **1999**.
19. EL-TAWEL G.E., SHABAN A.M. Microbiological quality of drinking water at eight water treatment plants. Int. J. Environ. Health Res. **11**, 285, **2001.**
20. WIOŚ The report on the condition of the environment in Western Pomeranian region in 2000. The Nature Preservation Inspection. The Regional Nature Preservation Inspectorate in Szczecin. The Library of the Environmental Monitoring. Szczecin, **2000.**
21. FAID M., ZOUITEN A., ELMARRAKCHI A., ACHKARI-BEGDOURI A. Biotransformation of fish waste into a stable feed ingredient. Food Chem. **60**, 13, **1997**.