ISOLATION OF HALOPHYLIC MICROORGANISMS IN THE SALINE LAKES FROM OCNA SIBIULUI AND ANALYSIS OF RED PIGMENTS PRODUCTION

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Abstract: Ten salty lakes from the region of Ocna Sibiului in Romania were analysed for the occurrence of halophilic microorganisms. The lakes were classified depending on the total number of microorganisms as follows: Without Bottom> Mud> Red> Brâncoveanu> Flax> Ocnita> Black> Mine Entrance> Cats> Swallow. The ions, especially magnesium, show a high influence on the number and types of microorganisms present in these lakes.
From the isolated strains, only 17 are producing orange to red pigments. These microorganisms were characterised on solid and liquid substrates. The strain M1 was chosen as model microorganism for the analysis of pigments formation, because s producing a pink pigment with very intense colour and has the fastest grow compared with the other strain isolated. The procedure for the extraction and analysis of red pigments was developed. The cultivation in bioreactor of the strain M1 showed that the pigment is forming in parallel with biomass and at the end of the exponential phase the cells loss the red pigment very fast (hours).

Keywords: halophilic microorganism, salty lake, Ocna Sibiului, red pigment extraction

INTRODUCTION

The main attractions for the tourists visiting the Ocna Sibiului area in Romania are the salty lakes. 52 lakes are found in this region, with a total surface area of 35741 m^3 and with 265699 m^3 volume of accumulated water (Alexe et al., 2005). Excepting the lakes used for leisure purposes (the man-

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made lakes Horia, Closca and Crisan), they are formed on abandoned salt mines or on excavations.

The most important are:

- Lake without Bottom, which is declared protected area and has powerful heliothermic effect;
- Lake of the Abandoned Salt Mine (Avram Iancu), the deeper antroposalted lake in Romania;
- Brancoveanu Lake, the most salted lake in Ocna Sibiului, also protected area;
- Ocnita Lake, connected with the lake Abandoned Salt Mine;
- Flax Lake, Cats Lake, Mud Lake.

The lakes are visited for both curative and entertainment purposes. Their healing properties are due especially to the mud formed at the bottom of the lakes by some organisms, like *Amphora* and *Artemia* sp. (Ionescu et al., 1998). Even the salts concentration is high, some pathogenic microorganisms can grow here (Oprean et al., 2006a).

This research has as main goal the isolation of extreme halophylic microorganisms which inhabit these natural lakes. The cultural characteristics on solid and liquid substrates are also analysed.

For the microorganisms isolated, the influence of magnesium ions on their growth is analysed. This ion was chosen because it was found in high quantities in the studied lakes (Oprean et al., 2006b) and influences positively the growth of halophylic microorganisms, especially archea. (Rodriquez Valera et al., 1983) (Toreblanca et al., 1986). It is supposed that archaea are found in large numbers in the extreme saline lakes from Ocna Sibiului, they being present in all salty lakes (Oren, 1999) (Oren, 2001).

For the food industry, finding new sources of natural additives is very important. Actually, the most used sources of natural red pigments are the plants which produce carotenoidic pigments or flavonoids (like anthocians from grapes or other fruits (Goiffon et al., 1999)). Many microorganisms are producers of carotenoidic pigments (Nelis and De Leenherr, 1991). The extremely halophilic archaea are also known as producers of carotenoidic pigments (Kelly et al., 1970) (Orena and Rodriguez.Valera, 2001).

During isolation and analysis in the lab, it was observed that one of the microorganisms isolated from the lakes is producing pink pigments of carotenoidic type. So, the second aim of this research is the analysis of pigments production during cultivation of the microorganism in batch system in bioreactor.

Table1: Physical and chemical characteristics of the studied lakes from Ocna Sibiului

Lake	**pH**	**Dissolved Oxygen**	**Exceeding or deficit of oxygen**	**Dry mass**	**Ca^{2+}**	**Na^{+}**	**Mg^{2+}**	**Chloride**	**Sulphate**	**Durity**
	unit	**mg/l**	**mg/l**	**g/l**	**mg/l**	**g/l**	**mg/l**	**g/l**	**g/l**	**German degrees**
Brâncoveanu	5.3	5.44	-1.6	731	840	310	48	710	4.43	128.8
Black	5.8	0.64	- 6.49	1335	800	420	2359	1051	7	655.2
Red (with Island)	6.5	5.61	-1.52	3.90	84	420	243	397	3.2	89.6
Mud	6.5	3.97	-3.07	165	540		158	151	0.040	112
Ocnița	5.5	7.39	0.07	340	520	68	48	334	3	84
Swallow (Sf. Ioan)	7.3	8.66	1.34	119	160	27	6	14	0.3	12.6
Gura Minei	6	8.49	1.17	180	480	22	146	131	2.4	78.4
Flax (Pânzelor)	7	13.89	6.67	918	240	17	97	85	2.2	56
Cats (Mâțelor)	6.8	7.28	0.06	24.6	240	20	122	16	1.86	61.6
Without Bottom	6.5	7.2	-0.12	146	400	108	182	128	1.77	98

MATERIALS AND METHODS

Materials

The lakes analysed were: Lake without Bottom, Brancoveanu Lake, Black Lake, Red Lake, Ocnita Lake, Flax Lake, Cats Lake, Mud (Panzelor) Lake, Swallow Lake and Mine Entrance (Gura Minei) Lake. The physical and chemical characteristics of the lakes are mentioned in Table 1.
Samples of the lakes waters (10 cm deep) were taken in sterile bottles.
A solid growth medium was used to isolate the extreme halophylic microorganisms (Rodriguez-Valera, 1995). Due to the high salinity of the cultivation medium, only this small category of microorganisms could grow in these conditions.The composition of M_I is presented in Table 2. For the analysis of the influence of magnesium ions on the microorganisms growth, medium M_I was poured in Mg^{2+} and the medium M_{II} was obtained (Table 2).
For the analysis of the growth characteristics on liquid media, M_{III} and M_{IV} were used (Table 2).
After preparation, all cultivation media were sterilised (121°C, 20 minutes).

Table 2. Composition of the cultivation media used for the isolation and analysis of halophylic microorganisms

No.	Component	Quantity, g/l			
		M_I	M_{II}	M_{III}	M_{IV}
1.	NaCl	125			
2.	$MgCl_2$*$6H_2O$	50	25	50	25
3.	K_2SO_4	5			
4.	$CaCl_2$*$2H_2O$	0.134			
5.	Yeast extract	5			
6.	Peptone	5			
7.	Glucose monohydrate	2.2			
8.	Agar	18		0	

Methods

The isolation and characterisation of microorganisms was made in three steps:

a) Isolation of the microorganisms by plating on solid media of a quantity of 0.5 ml sample from each lake. The plates were maintained at 30°C.

b) Counting of the formed colonies using a Funke-Gerber colony counter.
c) Analysis of the cultural characteristics (Oprean and Mironescu, 2000) of the microorganisms which presented pigmentation (red, pink or orange):
   - Gram staining, pigmentation, shape (dimension), elevation and edge shape on solid media;
   - Pigmentation, motility, oxygen requirements (aerobe, facultative anaerobe or anaerobe) and type of growth at surface (pellicle or ring).

The selected pigment-producer microorganism was cultivated in a small bioreactor (two liter volume) in the conditions: pH 7, temperature 45°C, aeration rate 1liter air/minute and continuous agitation (500 rpm). The M_I medium was used for the cultivation in bioreactor.

For the biomass determination (measured as cell dry weight), sample broth was centrifuged at 11000 rpm, 4°C for 30 minutes. In order to remove the salts without cells lysis, the residue was washed twice with NaCl 10% and 5% solutions (Mironescu et al., 2003). After each washing, the samples were centrifuged (11000 rpm, 4^oC for 20 minutes). The washed cells were then dried at 80^oC for 24 h, the remaining salt crystals at the surface were removed with distilled water and dried again to constant weight.

The pigment is localised in the cellular membrane (Oren and Rodriguez-Valera, 2001). The procedure for its extraction from the resulted biomass was established in this research and has the following steps:

- Separation of cells from the medium by centrifugation at 10000 rpm, 30 minutes. The supernatant obtained contained all salts from M_I and the sediment contained the microbial biomass.
- Cells lysis. As previous researches show (Mironescu et al., 2003), in liquid media with reduced salt concentration (less than 3%) the cells are lysed. In order to produce cells lysis, the sediment resulted from centrifugation was diluted in ratio 1:50 = sediment: distilled water and maintained at 4°C for 24 hours.
- Separation of distilled water by centrifugation at 10000 rpm, 30 minutes.
- Extraction of pigments from the lysed biomass using 90% acetone solution (Kelly et al., 1970) in ratio 1:5 = biomass : acetone (until the pigments pass in the acetone solution).
- Separation of the biomass by centrifugation at 10000 rpm, 30 minutes. The supernatant consists on acetone solution containing pigments.
- Determination of the pigments spectrum intensity through measuring the absorption of the acetone solution with pigments at wavelengths 465 nm,

490 nm and 530 nm (that correspond to the absorption maxims of the one type of carotenoidic pigments called bacterioruberin (Kelly et al., 1970)). Pigments spectrum intensity was calculated with the formula:

$$I = A_{465} + A_{490} + A_{530}$$

Absorption was measured in a UV-VIS Perkin-Elmer Lambda 1 spectrofotometer.

RESULTS AND DISCUSSIONS

After two weeks of maintaining at 20°C, small isolated colonies appeared in the Petri dishes. The number of colonies varied between the samples from different lakes, but also for samples from the same lake depending on the cultivation medium. Table 3 presents the number of isolated colonies formed.

Table 3. Colonies formed on media M_I and M_{II}, representing the number of living cells/ml of water

Lake	**Number of cells (cells/ml)**		
	M_I	**M_{II}**	**Total (M_I + M_{II})**
Brâncoveanu	23	350	373
Black	9	181	190
Red (with Island)	0	406	406
Mud	66	372	438
Ocnița	2	236	238
Swallow (Sf. Ioan)	2	14	16
Mine Entrance (Gura Minei)	14	165	179
Flax (Pânzelor)	18	310	328
Cats (Mâțelor)	0	55	55
Without Bottom	89	403	492

The results indicate that:

- A much higher number of extremely halophylic microorganisms grow on the medium M_{II} than on M_I. The difference is due to the influence of the Mg^{2+}. The solid medium M_I allows the growth of a smaller number of microorganisms, the result indicating the limiting action of magnesium on the microorganisms isolated from the lakes.

Because other results of the research group (Mironescu et al., 2005) indicated that magnesium influences positively the growth of halophylic archaeae, it can be supposed that the microorganisms growing on the medium M_I are halophylic archaeae (*Halobacteriaceaea*). This supposition needs more investigations to be proven.

- A relatively high number of microorganisms are found in the lakes Without Bottom and Mud Lake, both on M_I and M_{II}. The explanation could be that in these lakes the concentration of sodium ions is near the concentration of media used for isolation (125 g/l) and pH is 6.5, favouring the grow of a large number of halotolerant and halophylic microorganisms. Compared with the other lakes, a very high number of colonies appears on M_I , indicating the existence of a different group of microorganisms in these two lakes.
- A high number of microorganisms is found in Brancoveanu Lake (growing both on M_I and M_{II}) and Red lake (growing only on M_{II}), even the water is saturated with salt.
- The smallest number of salt-loving microorganisms is found in the Swallow Lake. As Table 1 shows, the ions have very low concentrations and pH is basic (7.3). As the results in Table 3 show, these conditions are not favourable for the halotolerant or halophylic microorganisms.
- The analysed lakes can be classified depending on the total number of microorganisms as follows: Without Bottom>Mud>Red>Brâncoveanu> Flax>Ocnita>Black>Mine Entrance>Cats>Swallow.

On the solid media, most of the colonies have these characteristics: small dimension, lenticular or round, beige colour. Only few colonies appeared pigmented. In order to identify some microorganisms producers of red pigments, the colonies which presented red, pink or even orange colour were analysed. For a better identification, they were encoded, the code containing the abbreviation of the lake and a number. The characteristics on solid and liquid media of the pigments-producer microorganisms are presented in Tables 4 and 5. The results evidence the presence of pigment-producing microorganisms in quite all the analysed lakes. Only 17 types of orange or pink colonies were found.

Table 4. Characteristics of the microorganisms producing red pigmentation on solid media

No.	**Lake**	**Sym bol**	**Cultivation medium**	**No. colonies**	**Characteristics (, colour, shape, dimension, edge, Gram stain, other observations)**
1.	Brâncoveanu	B1	M_{II}	2	Orange, round, small,
2.	Red	R1	M_{II}	1	Orange, round, small
3.	Red	R2	M_{II}	1	Orange, round, small
4.	Red	R3	M_{I}	1	Pink, round, middle
5.	Red	R4	M_{I}	2	Orange, round, middle, convex
6.	Mud	M1	M_{I}	1	Pink-red, round, large, mucilaginous, with disordered margins
7.	Mud	M2	M_{II}	2	Pink, round
8.	Ocnita	O1	M_{II}	2	Orange, round
9.	Ocnita	O2	M_{II}	1	Pink, round
10.	Ocnita	O3	M_{II}	1	Orange, round
11.	Swallow	S1	M_{I}	1	Orange, round
12.	Swallow	S2	M_{II}	2	Orange, round
13.	Flax	F1	M_{II}	1	Orange, round
14.	Cats	C1	M_{II}	2	Pink, round
15.	Cats	C2	M_{II}	1	Orange, round
16.	Cats	C3	M_{I}	1	Orange, round
17.	Without Bottom	WB1	M_{II}	3	Orange, round, small

Table 5. Characteristics of the microorganisms producing red pigmentation on liquid media

No.	Sym bol	Cultivation medium	Colour	Oxygen requirement			
				Aerobic		Facultative anaerobic	Anaerobic
				Pellicle	Ring	Turbidity	Sediment
1.	B1	M_{IV}	orange	-	-	+	-
2.	R1	M_{IV}	orange	-	-	+	-

3.	R2	M_{IV}	orange	-	-	+	-
4.	R3	M_{III}	pink	-	-	++	+++
5.	R4	M_{III}	orange	++	-	+++	+++
6.	M1	M_{III}	pink-red	+++	-	-	-
7.	M2	M_{IV}	pink	+	-	-	-
8.	O1	M_{IV}	orange	-	-	-	+
9.	O2	M_{IV}	pink	-	-	++	+
10.	O3	M_{IV}	orange	-	-	+	-
11.	S1	M_{III}	orange	-	-	+++	+++
12.	S2	M_{IV}	orange	-	-	-	-
13.	F1	M_{IV}	orange	-	-	+	-
14.	C1	M_{IV}	pink	-	-	++	-
15.	C2	M_{IV}	orange	-	-	+	-
16.	C3	M_{III}	orange	-	-	+++	+++
17.	WB1	M_{IV}	orange	+	-	-	-

Symbols represent: - absence, + small, ++ abundant, +++ very abundant growth.

One of the extreme halophilic microorganisms isolated from the Lake Without Bottom, M1, is producing a pink pigment with very intense colour. Also, the microorganism has the fastest grow (compared with the other strain isolated). Taking into account these two characteristics, M1 was chosen as model microorganism for the cultivation in bioreactor and for the analysis of pigments formation. The biomass and total pigments production during cultivation are presented in Figure 1.

After a lag phase (around 24 h - figure 1), the cell population begin to grow and, after a small quantity of biomass is produced, pigments synthesis begins, too. The biomass and pigments synthesis occur in the exponential growth phase. The maximal colour intensity is obtained after approximately 90 hours of the cultivation. When the microorganism passes into the stationary phase, the colour intensity begins to decrease until it is almost totally vanished.

The results show that only in the lag phase and exponential growing phases the formed biomass and the quantity of the synthesised pigments are proportional. The stationary phase is characterized by the decrease of the pigment quantity, although the concentration of biomass remains constant.

The variation of the absorption spectrum at the three wavelengths is presented in figure 2. Every absorption spectrum corresponds to a fraction from the carotenoidic pigments synthesised by the microorganism M1.

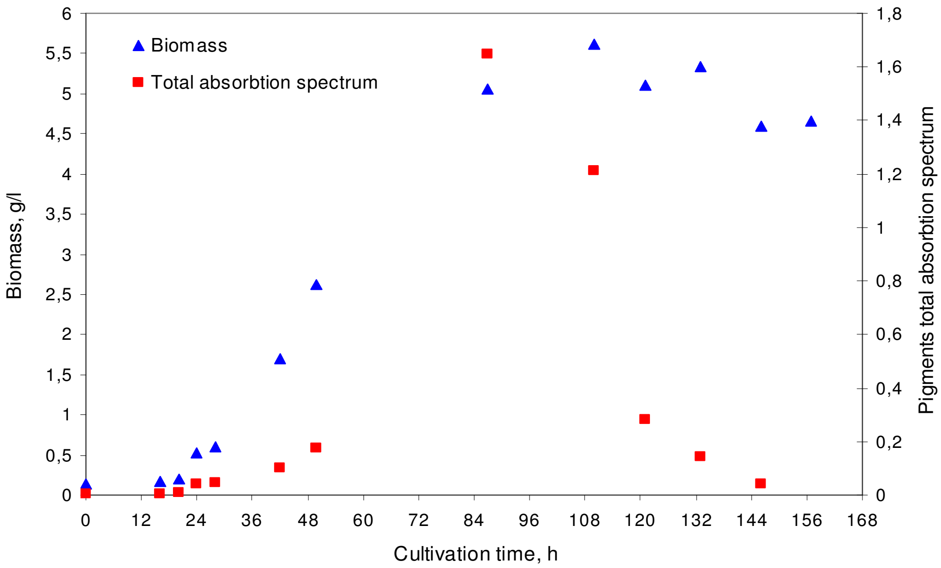

Figure 1: The evolution of biomass and the carotenoidic pigments at the cultivation in the batch system in bioreactor of the microorganism M1

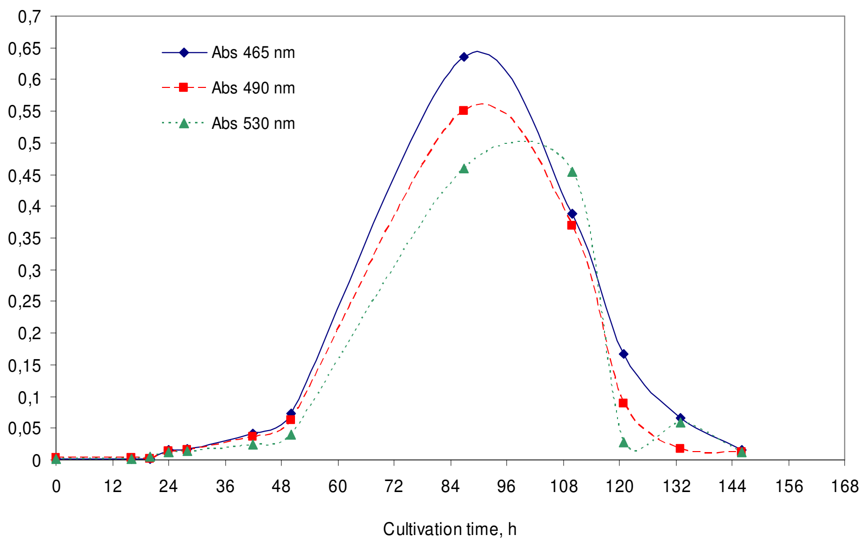

Figure 2: The evolution of carotenoidic pigments formation during the cultivation in bioreactor.

The formation of the three fractions is different in the exponential growing phase. As Figure 2 shows, the fraction that absorbs at 465 nm has the highest absorption value most probably due to the production of this fraction in a bigger quantity, while the fraction that absorbs at 530 nm is formed in a smaller quantity. All fractions are degraded very fast in the stationary phase.
Further investigations are necessary to identify exactly the pigments types and their quantity.

CONCLUSIONS AND PERSPECTIVES

The salty lakes from Ocna Sibiului are rich in microorganisms growing at 125 g/l NaCl, which can be considered as extremely halophilic. Depending on the total number of microorganisms, the 10 analysed lakes can be classified as follows: Without Bottom > Mud > Red > Brâncoveanu > Flax > Ocnita > Black > Mine Entrance > Cats > Swallow. Taking into account the large number of microorganisms which can inhabit these environments (Ventosa et al., 1998) (Oren, 1999), it is very difficult to identify the microorganisms producers of pigments without genetic studies.
In this research, two media with different magnesium concentrations (25 and 50 g/l) were tested. The results show that the microorganisms are strongly influenced by the magnesium concentration in the substrate; the increase of magnesium content in the cultivation medium determines the decrease of types and number of microorganisms.
The physical and chemical characteristics of the analysed lakes (salts content and pH) influence the number and types of microorganisms isolated. Low ions concentrations and basic pH determines the decrease of number and types of strains isolated.
From the microorganisms isolated, the strains producing red pigmentation on solid media were encoded and further analysed on solid and liquid media.
A procedure for the extraction and analysis of red pigments was developed.
Excepting the strains M1, M2 and WB1 (which are aerobic), the halophilic microorganisms are facultative anaerobic or anaerobic.
M1 was considered the best producer of red pigments and was cultivated in bioreactor in batch system. The results show that the microorganism forms carotenoidic pigments during the cultivation, the production of pigments being related to the formation of biomass. In the cultivation conditions used in this work, the maximal colour intensity of pigments it is obtained at the end of the exponential phase (after 90 hours from the beginning of cultivation).

The main pigment fractions were measured spectrophometrically at 465, 490 and 530 nm. The results show that the fractions are produced in a differentiate way, the fraction that absorbs at 465 nm having the higher absorption spectrum at the end of the exponential growth phase.
As perspective, the identification of the strain M1 and of pigments is intended.

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