INFLUENCE OF CULTIVATION MEDIA ON HALOBACTERIA
I. GROWTH AND BIOMASS FORMATION

MIRONESCU Monica *, MIRONESCU Ion Dan *, JÂŞCANU Vasile *, POSTEN Clemens **

*"Lucian Blaga" University of Sibiu
*University "Fridericiana" Karlsruhe

Abstract: The growth and production of biomass by an extremely halophilic archaeum named *Haloferax mediterranei* is analysed. The methods for the biomass quantification are optimised. Cultivations in flasks are performed and the influence of concentration of three compounds (glucose, Mg^{2+}, PO_4^{3-}) is investigated. The culture growth is positively influenced by glucose and magnesium, phosphate showing not a clear influence.

Keywords: *Haloferax mediterranei*, biomass quantification, cultivation conditions

INTRODUCTION

Halophilic archaea (halobacteria) are salt-loving microorganisms, growing optimally at NaCl concentrations between 2.5 and 4.5 M. Modern interest in halobacteria is due to their unique characteristics at the genetic, biochemical, physiological and evolutionary levels [Oren, 2002]. In addition, they offer genuine and largely untapped opportunities in biotechnology. The main directions in this realm are the use of retinal proteins, which are transformed by light between two alternative states, for optical "biocomputer chips and for information storage [DasSharma, 1995], the synthesis of poly-hydroxyalcanoates produced in very large amounts in some species for use as biodegradable plastics [Fernandez-Castillo et al., 1986], the synthesis of extracellular polysaccharides resistant at high salt concentrations [Anton et al., 1988], as source of stable enzymes for processes requiring extreme conditions or organic solvents [Rodriguez-Valera ,1991] or as producers of stabilisers for biomolecules [Schiraldi et al, 2002].

The extreme halophile *Haloferax mediterranei* belongs to the family *Halobacteriaceae*, order *Halobacteriales*, domain *Archaea*, growing at extremely high concentrations of NaCl (between 18% and 25%), at neutral pH (7.2-7.4) and temperatures between 35 and 45°C. Magnesium requirement is also high, ranging from 0.01 to 0.04 M. They are typical aerobically chemoorganotrophic microorganisms [Torreblanca et al., 1986].
Due to his extreme salt tolerance, the microorganism can be grown without sterile precautions, which can obviously reduce production costs and make the high-cell cultivation of this archaebacterium attractive for the industry.
The methods for the cell growth measurement presented in the literature don't take into account all factors influencing this special microorganism. The first goal of this study is to find the optimal procedure for the biomass quantification.
The growth of microorganism varies with the chemical and physical conditions during the cultivation. The second objective of this research is to study the influence of some chemical factors on the growth of *H. mediterranei* in order to define the conditions of cultivation of the microorganism for optimal production of biomass. The most important chemical agents that can influence the cells growth are the carbon, nitrogen, phosphate, magnesium sources and concentrations [Rodriguez-Valera et al., 1991]. By using a factorial experimental design, three nutrients are investigated: glucose, KH_2PO_4 (as phosphate source) and $MgCl_2$ (as magnesium source).

MATERIALS AND METHODS

The strain *Haloferax mediterranei* ATCC 13500 was used. For the inoculum preparation, a sterile solution containing NaCl 125g/l; K_2SO_4 5g/l; yeast extract 5g/l; peptone 5g/l; $CaCl_2 \cdot 2H2O$ 0.134g/l; glucose 5g/l was used. A small quantity of pure culture of *H. mediterranei* was inoculated in the medium and maintained for 72h at 38^{o}C with agitation.
For all cultivations of halobacteria, a basis growth medium containing NaCl 125g/l; K_2SO_4 5g/l; yeast extract 5g/l; peptone 5g/l; $CaCl_2 \cdot 2H2O$ 0.134g/l was used. For the three variables analysed (glucose, Mg^{2+} and PO_4^{3-}) three various concentrations (minimum, medium and maximum) of glucose monohydrate, KH_2PO_4 and $MgCl_2 \cdot 6H_2O$ were tested during nine experiments, as presented in table 1. The analysed components were added to the basis growth media following a first order orthogonal design [Kafarow, 1973].

Table 1: Concentrations of glucose monohydrate, KH_2PO_4 and $MgCl_2 \cdot 6H_2O$ in the cultivation media in the experimental cultivations

Experiment	Glucose [g/l]	KH_2PO_4 [g/l]	$MgCl_2*6H_2O$[g/l]
1	5	0,005	5
2	35	0,005	5
3	5	0,02	5
4	35	0,02	5
5	5	0,005	35
6	35	0,005	35
7	5	0,02	35
8	35	0,02	35
9	20	0,015	20

In order to avoid the Maillard reactions during the media sterilisation, two solutions were prepared separately: a saline solution (containing all the salts, yeast extract and peptone) and a glucose solution. pH of all solutions was adjusted to 7.2 with 1N KOH and the saline and glucose solutions were sterilised at 120^{o}C for 20 min. and after that sterile mixed.
100 ml each cultivation medium and 5 ml inoculum were added in 250 ml sterile flasks. During the cultivation the temperature was maintained at 38^{o}C and the flasks were stirred at 150 rpm. No pH control and aeration were made. For each experiment three identical cultivations in flasks were made. After 5 days the cultivation was stopped.
The optical density OD of *H. mediterranei* cells was measured in a UV2 Unicam UV/VIS spectrophotometer, after the dilution of broth culture samples at appropriate dilution to be in the linear response range of absorbance. The dilutions were made with 1% NaCl solutions. The measurements were made at 600 nm in order to avoid the linear part of the absorption spectrum of pigments produced by the microorganism [D'Souza et al., 1997] [Oren and Rodriguez-Valera, 2001].
For the dry cell weight (DCW) determination, sample broth was centrifuged at 11000 rpm, 4^{o}C for 30 minutes. In order to remove the salts without lysis of the cells, the residue was washed twice with NaCl 1% solution. After washing, samples were centrifuged (11000 rpm, 4^{o}C for 20 minutes) and dried at 80^{o}C for 24 h, the salt crystals at the surface removed with distilled water and dried again to constant weight. DCW was determined gravimetrically using a Sartorius balance.
The spectral analysis was made with a Cary 50 Varian spectrometer.

RESULTS AND DISCUSIONS

Generally, the biotechnologists measure the optical density (OD) of the media containing microorganisms, a rapid and accurate indicator for the biomass concentration.
Because of the adaptation of the halobacterium to very high salts concentrations, a high risk of cells lysis in media having less than 3% salts (1% NaCl) occurs [Toreblanca et al., 1986]. In order to study how high this risk is, three experiments where samples are diluted with various NaCl concentrations are made. The results are presented in figure 1.

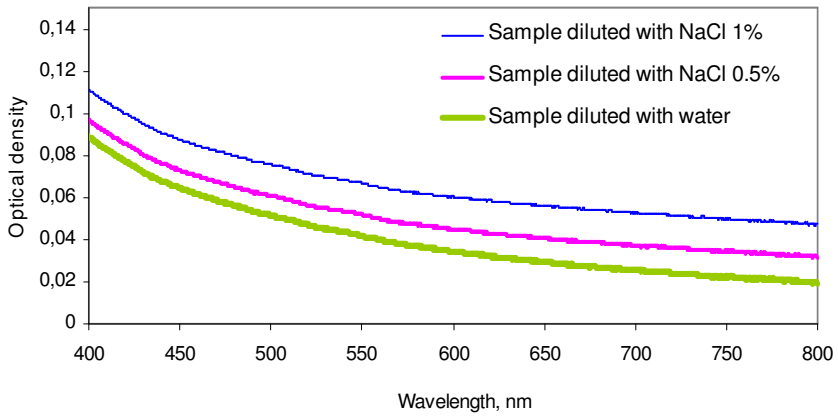

Figure1: Variation of the absorption spectra of a sample diluted with solutions having various salt concentrations; for each measurement, the spectrometer was calibrated with the solution used for dilution

A clear difference between the absorption spectra of the sample diluted with NaCl 1%, NaCl 0.5% and water is observed. A first cause of this change can be the cells lysis having as result the reduction of OD. The modification of the structure and/or conformation of the extracellular polysaccharides produced by *H. mediterranei* must be also tacked into account and in this direction more studies are necessary.
Anyway, in order to avoid the cell disruption in media with less than the minimal NaCl concentration needed for the cell integrity maintenance and in order to avoid unnecessary NaCl consumption, it can be considered that the optimal concentration for the samples dilutions is 1% NaCl. The1% NaCl

solution is also used at the cell wash for the removal of salts without cell lysis during the method elaborated for the DCW measurement.
In order to correlate the OD value measured spectrofotometrically with the biomass concentration, a relation must be derived. In figure 2 the both OD and DCW values are presented. At small cell concentrations the correlation is different from bigger cell concentrations (corresponding to OD higher as 2.5).

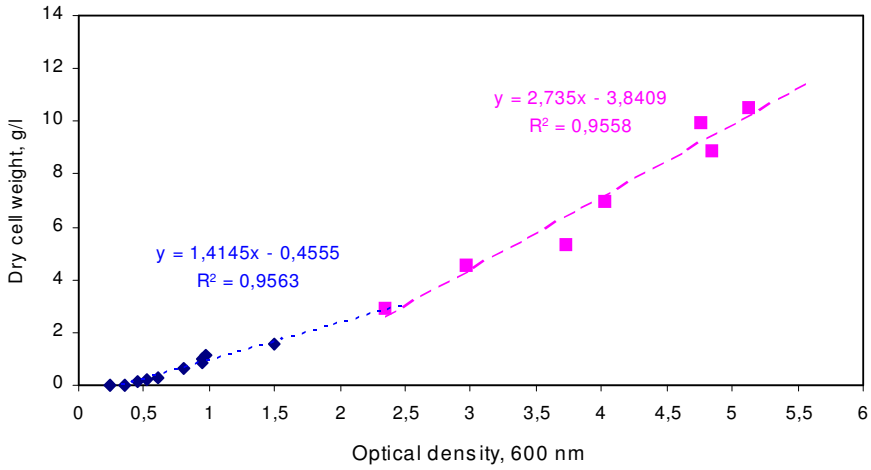

Figure 2: The correlation functions between OD and DCW for small and high biomass concentration

The obtained relations

$$y_1 = 1{,}4145 \cdot x - 0{,}4555 \quad \text{for small biomass concentrations}$$

and

$$y_2 = 2{,}735 \cdot x - 3{,}8409 \quad \text{for high biomass concentrations}$$

are used for the calculation of biomass concentration ***y*** expressed as DCW in g/l as function of OD (***x*** in the two functions).

The evolution of samples biomass during the growth in flasks is represented graphically in figure 3. The results are expressed as media between the three parallel experimental values, the precision of the measurements being 0.2%.
The variation of the biomass concentration in the nine experiments indicates that the cultures have different growths. In the first 4 cultivations (Experiments 1 to 4) the microorganisms attire faster the stationary phase (approx. after 72h) with the smaller biomass concentration and more, they begin to be destroyed (the decrease of the OD value in the experiments 2 to

4). A different situation is registered for the experiments 5 to 9, in witch the cultures are still in the exponential growth phase after 5 days. The major difference between the cultivation media is the magnesium concentration, much bigger for the experiments 5 to 8 as for the experiments 1 to 4.

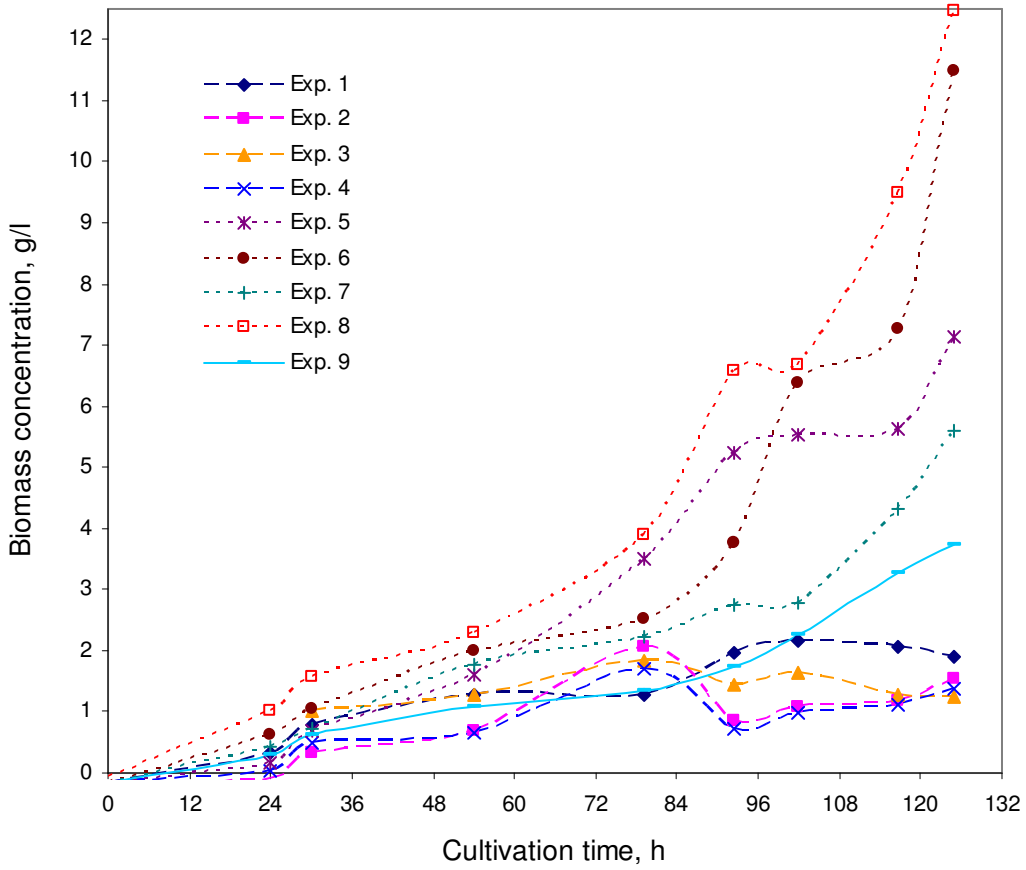

Figure 3: The evolution of biomass formation at the cultivation in flasks of *H. mediterranei*

Studies on *H. mediterranei* show that glucose is the better metabolised sugar [Anton et al., 1988] and glucose in small concentrations (between 2 and 10 g/l) shows a better biomass and EPS formation at the cultivation in flasks at 10 g/l [Severina et al., 1989]. No studies are made at bigger concentrations. The results presented in figure 1 show that a big glucose concentration (35 g/l) have a positive influence on the biomass formation. The microorganism

adapts very well to high glucose concentrations, passing through the lag phase faster (cultivations 6 and 8) as in the other experiments.
[Anton et al., 1988] shows that the phosphate plays a very important role in the growth of *H. mediterranei*, the optimal phosphate concentration being 0.015 g/l. The results obtained in the cultivations presented in figure 1 show a very unclear influence of phosphate on the biomass production. One of the causes could be the different cultivation conditions (different media composition and physical conditions). Anyway, the better growing of biomass is observed for a higher phosphate concentration (0.02 g/l) as that reported in the literature.

CONCLUSIONS

The analysis method for the biomass concentration determination at the cultivation of the halobacteria *Haloferax mediterranei* must be adapted at the specificity of the microorganism. In order to avoid cell lysis and conformational modifications of the polysaccharide excreted into the medium, solutions with 1% or more NaCl are recommended to be used for the dilution of samples.
The analysed compounds (glucose, Mg^{2+} and PO_4^{3-}) have a different influence on the biomass and EPS formation. The major factor influencing the biomass formation is magnesium, followed by glucose, a bigger quantity of them having a positive action on the biomass growth. Phosphate shows a variable influence depending on the other compounds.

REFERENCES

1. Anton J., Meseguer, I. and F. Rodriguez-Valera. (1988). Production of an Extracellular Polysaccharide by *Haloferax mediterranei*. *Applied and Environmental Microbiology*, 54, 2381-2386
2. DasSharma, S., (1995), Halophilic archaea: An overview, in Robb, F.T., Place, A.R., Sowers, K.R., Schreier, H.J., DasSharma, S., Fleischmann, E.M. (ed.), Archaea, a laboratory manual, Cold Spring Harbor Laboratory Press
3. D'Souza, S.E., Altekar, W., D'Souza, S.F., (1997). Adaptive response of Haloferax mediterranei to low concentrations of NaCl (<20%) in the growth medium, *Archives of Microbiology*, 168, 68-71
4. Fernandez-Castillo R., Rodriguez-Valera F., Gonzalez-Ramos J, Ruiz-Berraquero F., 1986, Accumulation of Poly(ß-Hydroxybutyrate) by Halobacteria, Applied and Environmental Microbiology, 51 (1), 214-216
5. Kafarow, S.V., 1973, Cybernetic methods in chemistry and chemical engineering, Mir Publishers, Moscow

6. Oren, A., Rodriguez-Valera, F. (2001) The contribution of halophilic Bacteria to the red coloration of saltern crystallizer ponds, *FEMS Microbiology Ecology*, 36, 123-130
7. Oren, A, (2002), *Diversity of halophilic microorganisms: Environments, phylogeny, physiology and applications*, Journal of Industrial Microbiology and Biotechnology, 28, 56-63
8. Rodriguez-Valera F. (1991). Biotechnological potential of halobacteria. *Biochem.Soc. Symp*. 58. 135-147
9. Rodriguez-Valera, F., J.A. Garcia Lillo, Anton, J., Meseguer, I. (1991). Biopolymer production by *Haloferax mediterranei*. In: *General and Applied Aspects of Halophilic Microorganisms* (F. Rodriguez- Valera, Ed.) , 373-380, New York
10. Schiraldi, C., Giuliano, M., De Rosa, M., (2002), *Perspectives on biotechnological applications of archaea*, Archaea, 1, 75-86
11. Severina, L.O., Usenko, I.A., Plakunov, V.K., (1988), *Biosynthesis of an exopolysaccharide by the extreme halophilic Halobacterium mediterranei*, Mikrobiologiya, 58 (4), p. 557-561
12. Toreblanca, M., Rodriguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M., Kates, M., (1986). Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov., *System. Appl. Microbiology*, 8, 89-99