INFLUENCE OF CULTIVATION MEDIA ON HALOBACTERIA

II. POLYSACCHARIDES PRODUCTION

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Abstract: The production of extracellular polysaccharide (EPS) at the cultivation in flasks of halobacterium *Haloferax mediterranei* is investigated. The influence of concentration of three compounds (glucose, Mg^{2+}, PO_4^{3-}) is analysed by following a factorial experimental design of cultivation media. The model obtained for the EPS production fits well with the experiments. The EPS formation is influenced principally by glucose and all three compounds combined. The graphically analysis of the model shows that for the tested concentrations of the three variables the maximal yield is 3.4 mg/l EPS.

Keywords: *Haloferax mediterranei*, polysaccharides, experimental design

INTRODUCTION

H. mediterranei is an extreme halophilic archaeum (halobacterium) that produces interesting metabolites:

- polyhydroxybutyrate, an intracellular polymer that can be used to produce biodegradable packaging as biological substitute for oil-derived plastics [Rodriguez-Valera et al.,1991],
- proteins with antibacterial activity [Meseguer et Rodriguez-Valera, 1986],
- enzymes with halophilic properties [Rodriguez-Valera 1991] [Oren 2002],
- carotenoidic pigments [Oren and Rodriguez-Valera, 2001] and
- extracellular polysaccharide (EPS) [Anton et al, 1988].

EPS from *H. mediterranei* are the first reported polysaccharides produced by halobacteria. Rheological studies showed that it exhibits pseudoplastic behaviour [Boan et al, 1998]. The viscosity remains relatively constant in a large temperature and pH interval; due to the adaptation to high salt concentrations, the EPS are resistant to salinities up to 40% NaCl [Anton et al, 1988]. All these properties make the EPS interesting for applications in media with high salts concentration or variable pH or temperature.

Because of the relative new discovery of this microorganism and of his potential, the researches on the optimal cultivation conditions for the EPS production are only at the beginning, few studies being available. So, [Anton et al., 1988] shows that at the cultivation of *H. mediterranei* in optimal physical conditions (control and optimal values of pH, temperature, aeration and agitation) 1% glucose in the growth medium, combined with 0.1% NH_4Cl as nitrogen source and 0.015% KH_2PO_4 as phosphate source a yield of 3g/l EPS is achieved.

[Severina et al., 1989] studied the influence of various compound on the growth of microorganism and biopolymer formation at the cultivation in flasks, the study concluding that (1) the growth and the EPS production are not associated processes, (2) glucose is the best metabolised carbon source and (3) higher glucose concentrations (in this case 10 g/l) together with the addition of organic substrates (yeast extract) favour the EPS production.

The objective of this research is to study the influence of concentration of some chemical factors on the EPS production in order to define the cultivation conditions of the microorganism for optimal biopolymer production. 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 microorganism, the media and the cultivation condition were the same as in the precedent paper [Mironescu et al., 2003]. The experiments were conducted as before ([Mironescu et al., 2003]).

After 5 days the cultivation was stopped and the medium (containing EPS) was separated from cells by centrifugation at 11000 rpm for 30 min. and salts were removed through dialysis (24h against water). EPS was precipitated with ethanol 96% in the proportion dialysate : ethanol = 1 : 2. The concentration of EPS was determined gravimetrical in the precipitate, after drying at 80^{o}C to constant weight (app. 24 h).

RESULTS AND DISCUSIONS

In order to analyse the influence of the three chemical factors on the EPS production, the results of the experimental cultivations are statistically analysed using the algorithm of [Kafarow, 1973] for the first order orthogonal design. The regression coefficients and the results of the Student *t*-test for the analysis of coefficients significance are presented in table 2.

Table 2: Estimation of the regression coefficients and results of the Student *t*-test for analysis of coefficients significance

	REGRESSION COEFFICIENTS							
	b0	*b1*	*b2*	*b3*	*b12*	*b13*	*b23*	*b123*
ACTION	0,01556 ↑	0,00577 ↗	-0,00139 ↙	0,00182 →	-0,00051 **0**	-0,00112 **0**	0,00562 ↗	0,00720 ↗
	Significance of coefficients							
	t0	*t1*	*t2*	*t3*	*t12*	*t13*	*t23*	*t123*
	48,79	18,09	4,37	5,72	1,59	3,53	17,64	22,58

The higher influence is attributed to the free coefficient, suggesting that other chemical or physical agents have a big importance on the synthesis of EPS, also. Experimental results obtained at the cultivation in bioreactor (unpublished) and data from the literature ([Rodriguez-Valera et al., 1991]) show the high influence of NaCl concentration and aeration on the EPS production.

The action of glucose, together with the combined action of phosphate and magnesium and the combined action of all three components seems to have also a high influence on the production of this biopolymer.

Phosphate shows a limiting effect on the synthesis of EPS, a high concentration inhibiting the production. This result is similar with the experiments of [Anton et al., 1988].

The regression equation obtained is:

$$\begin{aligned} y &= 0.01556 + 0.00577 \cdot x_1 - 0.00139 \cdot x_2 + 0.00182 \cdot x_3 + \\ &+ 0.00562 \cdot x_2 \cdot x_3 + 0.0072 \cdot x_1 \cdot x_2 \cdot x_3 \end{aligned}$$

where x_1 is the glucose, x_2 is KH_2PO_4 and x_3 is $MgCl_2 \cdot 6H_2O$.

By the calculation for the adequacy of fit of the estimated regression equation using the Fisher *F*-function [Kafarow, 1969] the value of F is 2,135657. This value is smaller as the tabulated value for F for the significance level 0.05, this results meaning that the model fits well with the experimental data.

Using the model, the EPS production as function of glucose, phosphate and magnesium concentrations can be estimated. Graphical 3-D estimations of the influence of two variables at the maintenance of the third one at constant value are presented in figure 1, 2 and 3.

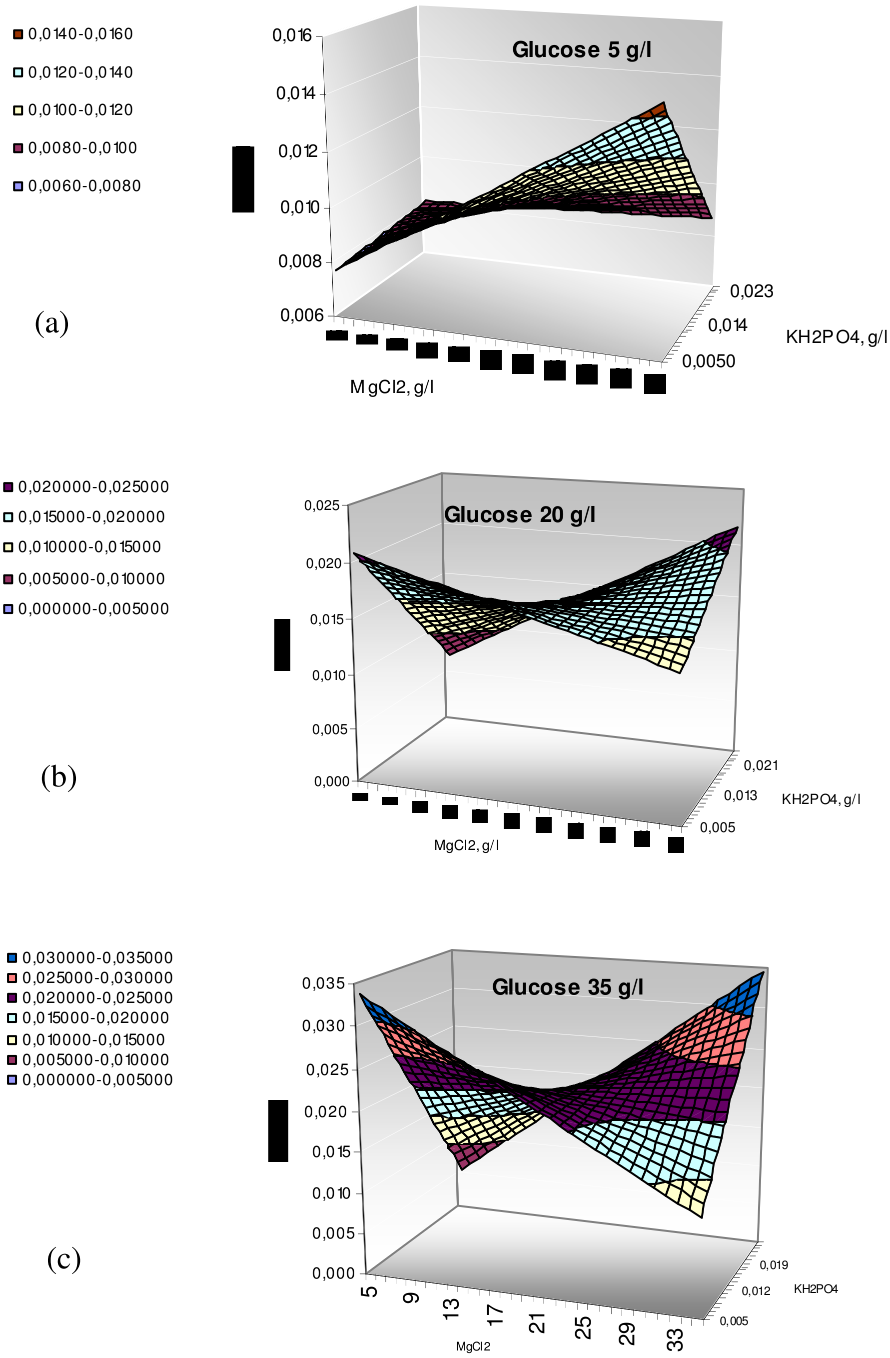

Figure 1: Evolution of EPS production at constant glucose concentration and various Mg^{2+} and PO_4^{3-} concentrations in the growth medium

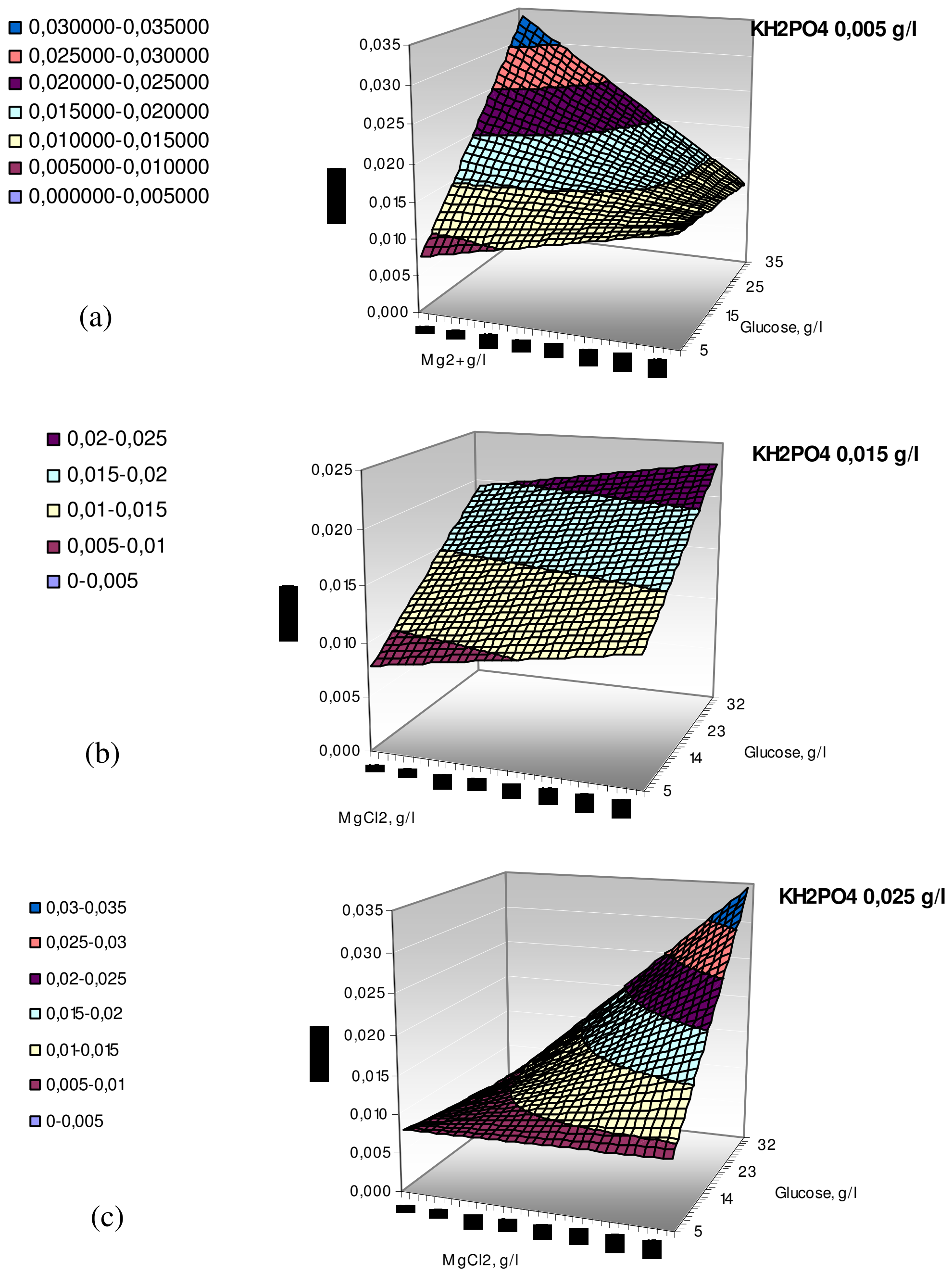

Figure 2: Evolution of EPS production at constant phosphate concentration and various Mg^{2+} and glucose concentrations in the growth medium

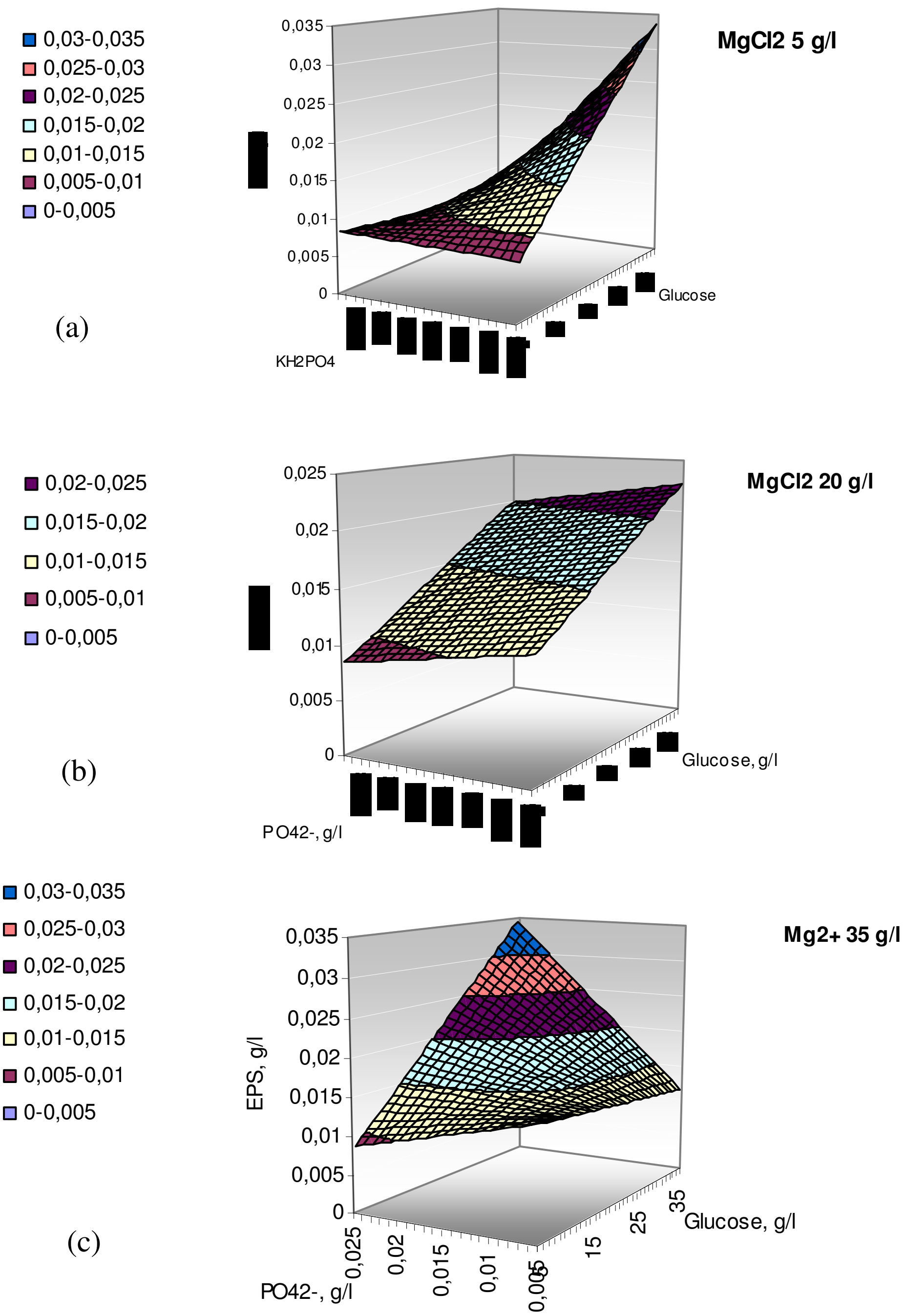

Figure 3: Evolution of EPS production at constant magnesium concentration and various phosphate and glucose concentrations in the growth medium

The plots obtained with the model allow the study of the influence of the three variables on the EPS synthesis in the domain of tested concentrations:

- Glucose concentration between 5 and 35 g/l;
- $MgCl_2$ concentration between 5 and 35 g/l;
- KH_2PO_4 concentration between 0.005 and 0.025 g/l.

At glucose concentration maintained constant at 5g/l (figure 1a), PO_4^{3-} shows no influence on yield how long the Mg^{2+} concentration is small. When $MgCl_2$ quantity attire values higher as 17 g/l, the combined action of more magnesium and little phosphate gives higher yield on EPS.

The response surfaces obtained in figures 1b and 1c have an interesting form, showing the similar action of other two variables on the EPS production for constant glucose concentration in a big domain (for 20g/l to 35g/l). It seems that high quantity of each variable combined with small concentrations of the other gives very small EPS yield. Inverse, concentrations at the same level of both components ($MgCl_2$ and KH_2PO_4) have a positive influence on the EPS synthesis. A median value is registered for middle concentrations of the two variables.

In figure 2a the EPS production for small phosphate concentrations is presented. A maximal yield is obtained for 5g/l $MgCl_2$ and 35 g/l glucose and then the yield decreases for all other values of glucose or magnesium.

The evolution of the EPS yield for constant middle and high phosphate concentration (figure 2b and 2c) and for constant small and middle magnesium concentration (figure 3a and 3b) is similar, only glucose showing a influence, higher glucose quantities stimulating the EPS synthesis.

For high magnesium concentrations, the maximal yield is obtained for big quantities of glucose and phosphate (figure 3c).

The bigger EPS concentration is obtained for 35 g/l glucose, 0.025 g/l KH_2PO_4 and 35 g/l $MgCl_2$ (figure 2c and 3c).

CONCLUSIONS

For the analysis of influence of concentration of three chemical compounds (glucose, $MgCl_2$ and KH_2PO_4) on the EPS production by the archaebacterium *Haloferax mediterranei*, a model based on a regression equation is obtained. The model fits well with the experimental data and shows that the EPS formation is basically influenced from the free coefficient, the three combined compounds and the glucose concentration.

The plots in 3D obtained with the model show the evolution of EPS production. For the analysed concentrations of the three variables, no maxim is obtained, researches in smaller and in bigger domains being necessary to find the maximally possible concentration of EPS that can be obtained in the conditions of these experiments.

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