JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION OF PHYSICO-CHEMICAL CONDITIONS FOR GROWTH AND ANTI-ARCHAEAL PRODUCTION BY *Haloarcula* sp. SWO25

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doi: 10.15414/jmbfs.2018.7.5.524-531

ARTICLE INFO

Received 4. 10. 2017
Revised 7. 12. 2017
Accepted 10. 1. 2018
Published 1. 4. 2018

Regular article

OPEN ACCESS

ABSTRACT

An extremely halophilic archeon SWO25, affiliated to *Haloarcula* sp., isolated from sebkha of Ouargla (South Algeria) was screened for secretion of antagonistic substance. To enhance the growth and antagonistic activity, physicochemical conditions were optimized by using a full factorial design. The selected factors were NaCl, $MgSO_4$ and temperature. At first, the antagonistic activity was partially characterized and suitability of Gompertz and Baranyi equations were evaluated for describing archaeal growth curves. The results show that the secreted antagonistic activity has broad spectrum, highest activity was obtained against *Halobacterium salinarum* and was completely abolished by proteinase K and pronase, suggesting that it is a halocin. The modified Gompertz equation was statistically sufficient to describe the growth data of strain SWO25. Secondary models for Growth rate (μ), lag-time (λ), $LnOD_{max}$ and antagonistic activity (Ac) followed a linear trend with lowest R^2 greater than 80% and lack of fit not significant (>0.05). A clear interaction effect between NaCl concentration and temperature on growth rate and anti-archaeal activity was shown; this interaction was affected by MgSO4 concentration in the case of antagonistic activity.

Optimal conditions for growth parameters and anti-archaeal activity were different; they were simultaneously optimized at 20% NaCl, 7.5% $MgSO_4$ and a temperature of 45°C. Under these conditions 1.6 and 1.4 fold increase in the specific growth rate and the maximal biomass respectively were reached but there was no further increase on anti-archaeal production. This study contributes to a large-scale biomass and anti-archaeal production for fundamental studies or potential applications.

Keywords: *Haloarcula* sp. SWO25; Halophiles; Halocin; growth; Modeling; Optimization; salt*Temperature Interaction

INTRODUCTION

Halophilic archaea (*Haloarchaea, Halobacteria*) are salt-loving microorganisms, flourishing in hypersaline environments, as they can grow easily at 20-25% NaCl. Modern interest in *Halobacteria* is due to their unique characteristics at genetic, biochemical, physiological and evolutionary levels (**Oren, 2002**). Products derived from *Haloarchaea* have wide applications in biotechnology; they offer real and largely unexploited opportunities in industrial demands mainly for their catalysis under high salt and non-aqueous reaction conditions (**Litchfield, 2011**). The *Haloarchaea* are also known for the production bacteriocin-like proteins called halocins (**Torreblanca *et al.*, 1994**), but research in this field is just rising; very few halocins have been partially or fully characterized (**Shand, and Leyva 2007**). The genus *Haloarcula* is the most and best studied and a great number of reports were carried out on enzymes that function optimally under extreme conditions rendering them robust biocatalysts with potential applications in harsh industrial processes (**Fukushima *et al.*, 2005**; **Camacho *et al., 2009***; **Li and Yu 2013**; **Ogan *et al.*, 2012**; **Li and Yu 2014**). Reports on halocins from *Haloarcula* species were very limited (**Torreblanca *et al.*, 1994**; **Atanasova *et al.*, 2013**). Many bacteriocins are known and widely studied, so far, nisin is the only bacteriocin approved by both United States FDA and the WHO for use as a food preservative (***Hwanhlem et al.*, 2015**). *Haloarchaea* may be a new source of such bioactive compounds.

Mathematical modeling of microbial growth is a useful tool for a variety of purposes, as, in the estimation of parameters required to study the growth under different physical and chemical conditions, or else in the formulation of appropriate microbiological media. Microbial responses are tested under controlled conditions and the results are then expressed as a mathematical equation that will allow prediction of untested combinations of conditions (**Fakruddin *et al.*, 2011**). The only statistical optimization statement available was done on growth rate and enzymes from *Haloarcula marismortui* (**Camacho *et al.*, 2009)** and biomass production of *Haloarcula* sp. IRU1 on textile wastewater (**Taran *et al.*,2011**).

An extremely halophilic archeon SWO25, affiliated to *Haloarcula* sp. was isolated from sebkha of Ouargla (South Algeria) and screened for the secretion of anti-archaeal substance. The objective of this survey was firstly to characterize the antagonistic activity and to determine the suitability and usefulness of two growth models; Gompertz and Baranyi. In a second stage, growth temperature, sodium chloride and magnesium sulfate concentrations were statistically optimized for growth and antimicrobial activity by the selected strain. The application of a complete factorial design, and the evaluation of the growth parameters through the Gompertz and Baranyi models, enabled a rapid and simultaneous exploration of the influence of cited factors on both growth and halocin production, an attempt not made so far in *Haloarchaea*.

MATERIAL AND METHODS

Microorganisms

The *Haloarchaeal* strain SWO25 used throughout this work was recovered from Sebkha of Ouargla located in south Algeria and was affiliated to *Haloarcula* sp. by 16S rRNA sequencing (accession number HQ844527/HQ641747).The Haloarchaea used for the inhibitory tests were the isolates: *Haloarcula* sp. Strain *SWO32* (HQ641749/HQ641748), *Halorubrum tebenquichense strain* SWO33 (HQ641750), *Halorubrum chaoviator* strain SWO40 (JN873333), *Natrinema* sp strain SI14 (JN873317) and the reference strain *Halobacterium salinarum* DSM 3754.

Culture media and growth conditions

All microorganisms were grown in Brown medium (**Tindall, 1992**) containing (gL^{-1}): 250 NaCl; 20 $MgSO_4$, $7H_2O$; 2 KCl; 3 Na_3-citrate and 5 yeast extract, pH 7.2, at 40°C with constant shaking in liquid media, or with the addition of 2% (w/v) agar plates prepared with the same medium in plastic containers.

For growth evaluation and antimicrobial production, 25 mL cultures of strain SWO25 were grown in 100 mL flasks, shaking at 120 rpm in a thermostatic orbital shaker (HS-B20 digital, IKA Labortechnik, Germany). The growth was monitored spectrophotometrically (UV mini 1240, Shimadzu, Japan) at 610 nm.

Activity assays

The inhibitory activity produced by strain SWO25 was determined by using *Halobacterium salinarum* DSM 3754 as a target strain. Cell-free culture supernatant was obtained by centrifugation at 10.000 rpm (7379×g) for 10 min. 5-10 µL cell-free supernatants from the stain SWO25 were spotted onto top-agar laws of target strain prepared as described by **Shand** (**2006**). The assays were based on the formation of clear zones on double layer agar plates. The activity (Ac) was expressed in mm inhibition diameter.

Antagonism studies

Cell-free culture supernatants from strain SWO25 were spotted onto top-agar lawns of the haloarchaea isolates and the reference strain. Fresh media used to grow each culture were also spotted onto plates as a control. The plates were incubated and inspected for the presence of zones of inhibition in the indicator lawn.

Effect of enzymes, heat treatments, salt concentration, on halocin-like activity

Stability of the antagonistic activity to heat was determined by heating aliquots of cell-free culture supernatants at 80°C for 10, 30 min and 100°C for 10, 30 and 60 min.
Samples of active cell-free supernatants were examined for susceptibility to proteolytic enzymes. The following enzymes at 1, 4, 10 mg·mL^{-1} and respective buffers were employed: pronase, trypsin, in 0.01 mol·L^{-1} sodium phosphate (pH 7.0); proteinase-K in 0.1 mol·L^{-1} sodium acetate, 0.005 mol·L^{-1} calcium acetate (pH 7.5). Enzyme solutions (10 µL) were added to 75 µL of active cell-free supernatant, and the mixture was incubated at 37°C for 1 h, after which, the enzymes were heat-inactivated (100°C for 10 min). As positive controls, active cell-free supernatant with added respective buffer solutions were treated in the same manner as the test preparations. Heat-inactivated enzyme solutions served as negative controls. The salt dependence of the antagonistic activity was studied by recursive dilution and replacement with distilled water at different ionic concentrations. After each treatment, the remaining activity was determined against *H. salinarum* DSM 3754 by the agar diffusion method described previously.

Experimental design and statistical analysis

A full factorial design was employed to study the effect of three factors (independent variables): NaCl (X_1), $MgSO_4$ (X_2) concentrations and incubation temperature (X_3) on growth parameters and antagonistic activity (Ac). The selected factors were studied at two levels noted -1 and +1. Level 0 (the mean domain) was used in three repetitions to determine experimental error. The levels were as follows: NaCl [-1:20%], [0:25%], [+1:30%]; MgSO4.7H2O [-1:2.5%], [0:5%], [+1:7.5%]; temperature [-1:35°C], [0:40°C], [+1:45°C]. All experiments were performed in duplicate (except for the run 1). As response variables : cell concentration of *Haloarcula* sp. SWO25 was measured as optic density obtained after every 6 h of incubation and the maximum antagonistic activity detected in the supernatants between 17 and 41 h incubation time.

Primary models

To estimate the growth parameters, growth curves were described using the modified Gompertz model (Eq(1)) (**Zwietering *et al.*, 1990**) and the Baranyi and Roberts model (Eq(2) (**Lopez *et al.*, 2004**). The growth curves were defined as the logarithm of the relative population size in terms of optical density units against time.
At time t, the reparametrized Gompertz and the Baranyi models are expressed by the following functions:

$$\ln N = \ln N_0 + Ae^{-e^{\left(\frac{\mu \max . e}{A}(\lambda - t)+1\right)}}$$

(Eq(1))

$$\ln N = \ln N_0 + \mu \max . t + \ln[e^{-\mu \max . t} - e^{-\mu \max (t+T)} + e^{-\mu \max . T}] - \ln\left[1 + \frac{e^{\mu \max (t-T)} + e^{-\mu \max . T}}{e^{\ln N \max - \ln No}}\right]$$

(Eq(2)

Where N denotes the microbial biomass as the O.D. measured at time t; N_0 and N_{max} O.D. measured at time t =0 and at maximum microbial population size respectively.

A is the asymptotic level ($\ln(N_{max}/N_0)$), μ_{max} is the maximum specific growth rate (per hour), is defined as the tangent to a point of inflection of the growth curve, λ or T is the lag phase duration (hours), t is time (hours), and e is exp(1).

Evaluation of the growth models

The growth parameters (Lag time, µmax; ln N_{max}) were determined for each of the 18 runs of the full factorial design using the two growth equations. The choice of the best primary model was based on statistical analysis.

Model fitting

The two models were fitted to the data by non-linear regression based on Levenberg Marquart algorithm using MINITAB 16. To determine which of the models Baranyi and Gompertz, described best the data, the *p*-value of the lack of fit, the root mean square error (RMSE) and the adjusted coefficient of determination (R^2adj) were calculated. The primary criterion used to choose the best model was the capacity of the model to describe the data well for all runs and conditions. If the two models fitted the data well for all conditions, the model with the best statistical parameter fits was chosen (highest R^2adj, lowest RMSE). If these first two criteria were equally met, the number of parameters of the model and the biological meaning of the model parameters were considered (***den* Besten *et al.*, 2006; Santillana Farakos *et al.*, 2013**).

Secondary models

The effects of NaCl (X1), MgSO4(X2) concentrations and incubation temperature (X3) on λ, µmax, N_{max} and antimicrobial activity were studied in secondary model. The data were analyzed using the analysis of variance ANOVA to study the individual and interaction effects of selected factors.
The correlation of the independent variables and the response 'y' was estimated by a first order polynomial equation (3), using the least-square method as shown below:

$$y = a_0 + \sum_{i=1}^{3} a_i . x_i + \sum_{\substack{i,j=1 \\ i \neq j}}^{3} a_{ij} . x_i x_j + \varepsilon$$

(**Eq(3)**)

Where 'y' represents the response: growth parameters or the ant-archaeal activity; a_0 the value of fitted response at the center point of design and represents the global mean, a_i and a_{ij} are the regression coefficients corresponding to the main factors (linear term) and interactions effects respectively.
x_i are the dimensionless variables related to the standardized natural variables X_i defined above:

$$x_i = \frac{X_i - X_{oi}}{\Delta X_i} \qquad i = 1,2,3$$

Where X_{oi} is the value of X_i at the centre point and ΔX_i is the step change of the variable X_i. The residual error ε that supposed to have Gaussian distribution was estimated by the difference between the predicted $\hat{y}$ and the observed y value of the response.

RESULTS AND DISCUSSION

Inhibitory activity and spectrum

The haloarchaeon SWO25 used throughout this work was isolated from the athalassic environment of Sebkha of Ouargla and was affiliated to the genus *Haloarcula* (**Imadalou-Idres *et al.*, 2013**). It was characterized by the production of a potent anti-archaeal substance, which was found in the cell free supernatant. The activity spectrum of the inhibitory substance appears to be fairly broad since the active cell-free supernatant inhibited the growth of all the indicator strains used and the highest activity was obtained against *H. salinarum*. In most cases, halocins have broad spectrum and *H.salinarum* is known for its sensitivity to the majority of characterized halocins (**Kis-Papo, and Oren 2000**).

Physicochemical stability of the halocin-like activity

In order to confirm the protein nature of the anti-archaeal substance produced, the active cell-free culture supernatant was treated with different amounts of proteases. Consequently, the antagonistic activity was completely abolished by protease treatment (proteinase K and pronase) (Fig. 1), indicating that an intact protein is required for activity. This observation suggests that the SWO25 antagonistic activity must be a secreted protein; it is most likely about halocin.

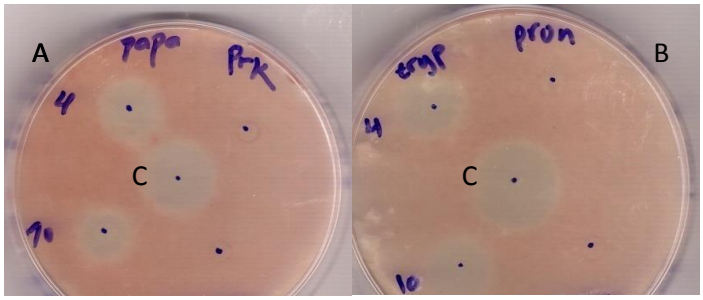


Figure 1 The anti-archaeal activity of the strain SWO25 on *H. salinarum* as test microorganism. (A) Culture supernatants with, papain (papa), proteinase K (Prk) and without protease (C). (B) Culture supernatants with trypsin (tryp), pronase (pron) and without proteases (C). Proteases were used at 4 and 10 mgL^{-1}

The action of the proteinase K, trypsin and the pronase on the active substance produced by strain SWO25 (Table 1) was similar to that obtained on halocins H6, C8, and S8. Indeed, the three halocins are resistant to trypsin, S8 and C8 are sensitive to protéinase K whereas H6 is sensitive to pronase (**Torreblanca *et al., 1989***; **Li *et al.,* 2003**; **Price and Shand 2000**). In a recent report; halocins from seven genera of *Haloarchaea* were divided into three groups based on their sensitivity to proteinase K or to trypsin and similarly to our results, a strain of *Haloarcula* sp. SS5-2 was shown to be sensitive to proteinase K and resistant to trypsin (**Atanasova *et al.,* 2013**). The antimicrobial activity was stable after heat treatment at 100°C for 60 min and remained stable during storage at 4°C for more than 1 year. To evaluate the salt dependence of the halocin-like activity, the active cell-free supernatant was diluted at different ionic concentrations. Antimicrobial activity of the sample was not affected in solutions with ionic concentrations higher than 15.6%, below this value a reduction in the antimicrobial activity was noticed (Table 1). Halocin already characterized differ in their thermal stability and salt dependence. Halocins A4, C8, H6, R1 and S8 are salt-independent, while H4 and H1 are respectively partially and completely salt-dependant (**Torreblanca *et al., 1989***; **Li *et al., 2003***; **Platas *et al.,* 2002**; **Shand, and Leyva 2007**).

Table 1 Physicochemical stability of the antimicrobial activity of cell-free culture supernatant from strain SWO25

Treatment	Halocin-like activity[a]
Heat	
30 min at 80°C	+
60 min at 100°C	+
Enzymes	
Pronase	-
Trypsin	25-35%[b]
Proteinase K	-
Salt concentration	
25%	16 mm
21.8%	16 mm
18.7%	16 mm
15.6%	16 mm
12.5%	14 mm

Legend : a) Correspond to the presence (+) or the absence (-) of activity observed when H.salinarum was used as test microorganism in diffusion agar method,
b) residual activity when 4 and 10 mg / ml of enzymes were tested

Primary models

The data fits obtained with the two models were evaluated statistically taking into consideration fitting behavior, examination of residuals, and statistics for goodness-of-fit. The results show that all experimental growth data fit well with the two models as shown with the three replicates of the center points (Fig. 2).

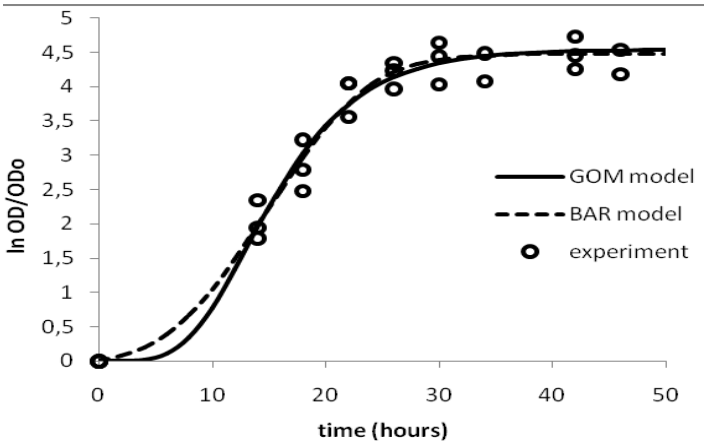


Figure 2 Growth curve of SWO25 at 40°C fitted with the Gompertz (Gom) and Baranyi (Bar) models

A typical sigmoid growth trend involving a lag phase, a logarithmic phase and a stationary phase was observed. Nevertheless, statistical analysis shows that the Gompertz model was more satisfactory than the Baranyi one. Indeed, on the nine experiments of the factorial design, eight and only six showed a good fitting respectively with Gompertz and Baranyi models (Table 2).

Table 2 Analysis of Variance for the primary models

Model		**Gompertz**	**Baranyi**
Run 1:NaCl=20%, MgSO4=2,5,T=35°C			
	RMSE	**0,1414**	**0,1304**
	Lack of Fit: p value	-	-
Run 2:NaCl=30, MgSO4=2,5, T=35			
	RMSE	**0,1059**	**0,1059**
	Lack of Fit	0,902	0,8950
Run 3:NaCl=20, MgSO4=7,5, T=35			
	RMSE	**0,0607**	**0,0607**
	Lack of Fit	0,3920	0,3940
Run 4:NaCl=30, MgSO4=7,5, T=35			
	RMSE	**0,1308**	**0,1316**
	Lack of Fit	0,5340	0,5070
Run 5:NaCl=20, MgSO4=2,5, T=45			
	RMSE	**0,0498**	**0,0797**
	Lack of Fit	0,1860	0,0140
Run 6:NaCl=30, MgSO4=2,5, T=45			
	RMSE	**0,1090**	**0,1825**
	Lack of Fit	0,0030	0,0000
Run 7:NaCl=20, MgSO4=7,5, T=45			
	RMSE	**0,2743**	**0,2888**
	Lack of Fit	0,8030	0,4670
Run 8:NaCl=30, MgSO4=7,5, T=45			
	RMSE	**0,1339**	**0,2619**
	Lack of Fit	0,0610	0,0020
Run 0:NaCl=25, MgSO4=5, T=40			
	RMSE	**0,2356**	**0,2257**
	Lack of Fit	0,6230	0,8480
Goodness of fit		**7/9**	4/9

Legend: a : one replicate, b : two replicates of the experiment, c : three replicates of experiment For the goodness of fit, 0/9 indicates that the model was accepted for none of the nine experimental conditions, and 9/9 indicates that the model was accepted for all experimental conditions, Not determined

An over-estimate of μ_{max} and λ was observed in the case of Gompertz model compared to Baranyi's model (data not shown). Similar results were reported by **Gil *et al.,* (2011**). We have than chosen the growth parameters of Gompertz equation in the secondary models.

Secondary models

The influence of NaCl, $MgSO_4$ concentrations and incubation temperature on growth parameters and halocin-like activity was studied using a full factorial design. In accordance with their predicted values (Table 3), the experimental run

responses showed that the maximal growth rate and high diameter of antimicrobial activity were expected in conditions in which the variables X_1 (NaCl), X_3 (temperature) were at +1 level and any level of the variable X_2 ($MgSO_4$) (runs 6 and 8*)*. However, these conditions do not support maximum values of optical density or shorter lag time (Table 3). An optimal growth must necessarily take account of the three parameters of growth μ_{max}, λ and ln N_{max}.

Table 3 Predicted values of the responses of the secondary models. The experiment values are fitted by the primary Gompertz model

	Growth rate µmax (h^{-1})		Ln Nmax		Lag time λ (h)		Activity Ac (mm)	
Runs	Exper.	Predi.	Exper.	Predi.	Exper.	Predi.	Exper.	Predi.
1	0,2350	0,3223	0,7353	0,7985	3,144	6,650	16,0	15,8
2	0,2588	0,2448	0,7079	0,6996	7,194	6,561	18.0	17,6
3	0,3459	0,3223	0,8017	0,7985	7,858	6,650	15,0	15,1
4	0,1911	0,2448	0,6763	0,6996	2,905	6,561	17,0	16,9
5	0,3535	0,3583	0,9776	0,9468	10,003	10,536	14,0	12,9
6	0,5320	0,5201	0,4371	0,4381	15,1881	16,025	17,0	17,6
7	0,4028	0,3583	0,9403	0,9468	13,064	10,536	14,0	14,1
8	0,5558	0,5201	0,4873	0,4381	17,196	16,025	20,0	18,9
10	0,3191	0,2448	0,6494	0,6996	10,158	6,561	17,0	17,6
11	0,3859	0,3223	0,8586	0,7985	8,947	6,650	15,0	15,1
12	0,2101	0,2448	0,7648	0,6996	5,987	6,561	17,0	16,9
13	0,3806	0,3583	0,8813	0,9468	10,581	10,536	13,0	12,9
14	0,4985	0,5201	0,4320	0,4381	14,772	16,025	17,0	17,6
15	0,2965	0,3583	0,9881	0,9468	8,494	10,536	13,0	14,1
16	0,4941	0,5201	0,3959	0,4381	16,944	16,025	19,0	18,9
17	0,4058	0,3252	0,9066	0,9314	9,235	7,897	16,0	17,0
18	0,3215	0,3252	0,8656	0,9314	7,686	7,897	17,0	17,0
19	0,2481	0,3252	1,0221	0,9314	6,768	7,897	18,0	17,0

The *p*-value serves as a tool for checking the significance of each of the variables. The *p*-value (α=5% of signification) for the parameter $MgSO_4$ (X_2) in the 4 models is greater than 0.05, indicating that the level of $MgSO_4$ concentration has no effect on these responses (Table 4). However its influence could not be totally overruled because of its interactive effect with other variables. In fact, the two ways interaction NaCl*T and $MgSO_4$*T have positive effects on halocin activity (Table 4).

Table 4 Estimated Coefficients (coded units) and their p-values

	LnNmax		**µmax (h^{-1})**		**Lag time (h)**		**Activity (mm)**	
Term	Coef	P	Coef	P	Coef	P	Coef	P
Constant	0.7172	0.000	0.35933	0.000	9.878	0.000	16.1528	0.000
NaCl	-0.1483	0.000	0.02310	0.181	1.415	0.039	1.5972	0.000
MgSO4	0.0220	0.162	0.00093	0.955	0.297	0.629	0.0972	0.609
T	-0.0247	0.121	0.07988	0.001	3.403	0.000	-0.2778	0.163
NaCl*MgSO4	-0.0097	0.518	-0,0206	0.229	-0.832	0.192	0.4028	0.054
NaCL*T	-0.1061	0.000	0.05778	0.005	1.330	0.049	0.7778	0.002
MgSO4*T	-0.0115	0.447	-0.00286	0.862	0.348	0.572	0.5278	0.017

Legend: Underlined values of the p-value indicate that the corresponding coefficient is not significant

NaCl concentration (X_1) had marked effects on, lnN_{max}, Lag-time (negative effects) and on antimicrobial activity (positive effect). Incubation temperature(X_3) alone was only significant on the growth rate µmax and the lag-time, and was greatly dependent on NaCl concentration (X_1), since the four responses were influenced by the interaction effect NaCl*Temperature ($X_1 \times X_3$).

The equations for the four responses in uncoded units are given:

ln Nmax = -2,912 + 0,1439 NaCl + 0,0651 MgSO4 + 0,1057 T - 0,00078 NaCl*MgSO4 - 0,004243 NaCl*T - 0,00092 MgSO4*T

lag time = 25,4 - 1,51 NaCl + 0,67 MgSO4 - 0,788 T - 0,0665 NaCl*MgSO4 + 0,0532 NaCl*T + 0,0278 MgSO4*T

µ = 1,662 - 0,0796 NaCl + 0,0507 MgSO4 - 0,0407 T - 0,00165 NaCl*MgSO4 + 0,002311 NaCl*T - 0,00023 MgSO4*T

Ac = 53,78 - 1,086 NaCl - 2,456 MgSO4 - 1,044 T + 0,0322 NaCl*MgSO4 + 0,03111 NaCl*T + 0,0422 MgSO4*T

The Pareto charts of the figure 3 display the absolute value of the effects and the reference line is drawn at **t** (t is the (1–0.05/2) quantile of a t-distribution with degrees of freedom equal to the degrees of freedom for the error term (here 10)). Any effect that extends past this reference line is statistically significant. These plots show that MgSO4 main effect is statiscally not significant for the majority of the responses.

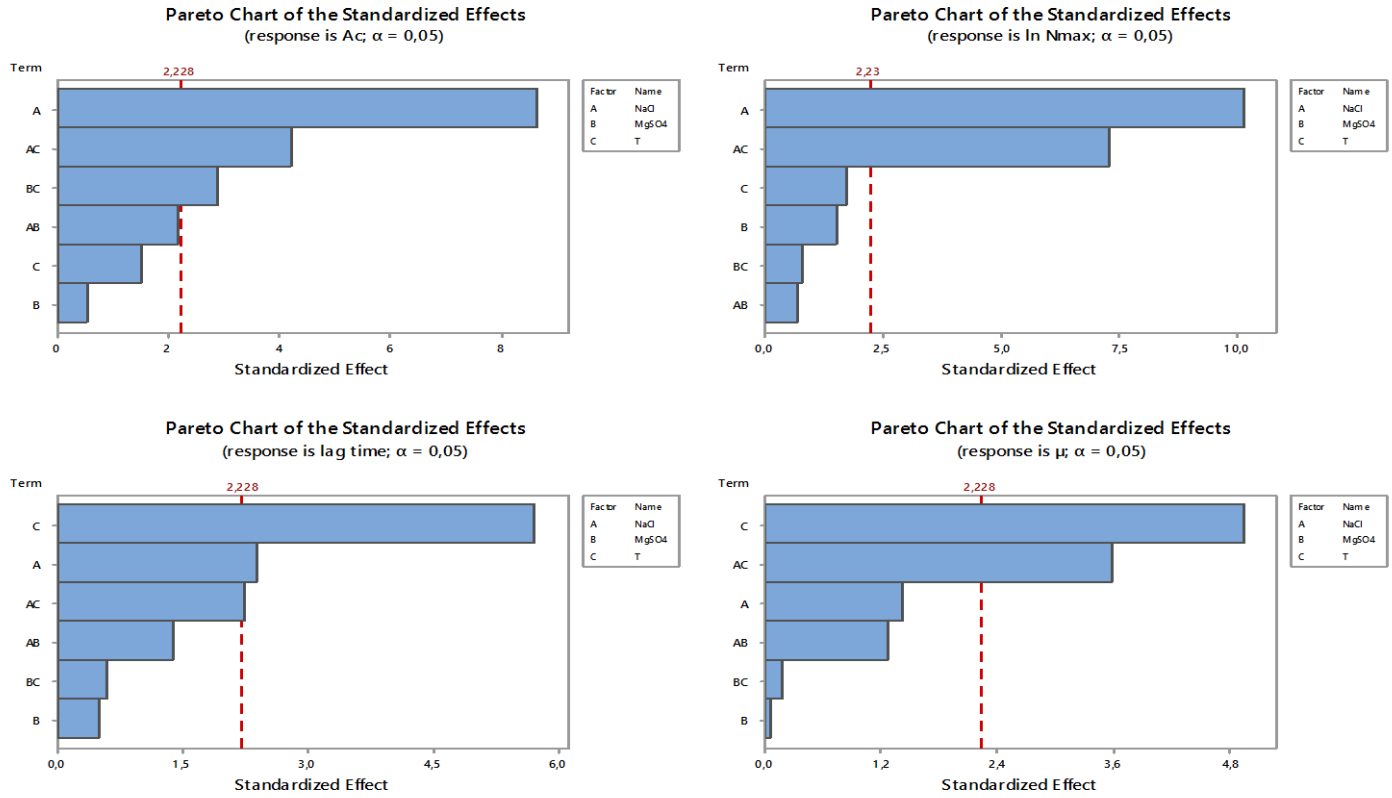


Figure 3 Pareto charts for the four responses**.**

The following table 5 of analysis of variance shows the goodness of fit for ¾ models (lack of fit >0.05) and the lowest R^2 was greater than 80% indicating that most of variation of these responses are explicated by those linear models. However, the model for lnN_{max} exhibits a significant effect of curvature (*p*-value less than 0.05) i.e., that the influence of NaCl and temperature on the maximum quantity of biomass formed is nonlinear.

Table 5 Analysis of variance for the secondary models (the first three responses are based on the Gompertz primary model)

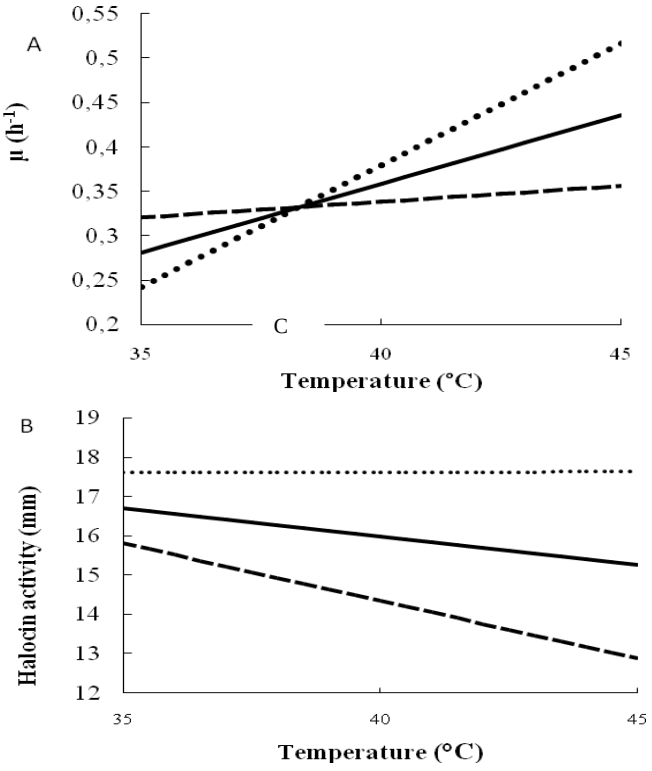
Source of variation	lnNmax	μmax (h^{-1})	Lag time (h)	Activity Ac (mm)
	P-value			
Model	0.000	0.004	0.001	0.000
Main Effects	0.000	0.004	0.001	0.000
2-Way Interaction.	0.000	0.019	0.107	0.001
Curvature	0.000	0.398	0.197	0.085
Lack of Fit	0.842	0.050	0.010	0.191
R^2 %	95.64	82.00	83.27	92.55

Effect of temperature and NaCl interaction on growth rate

The lowest growth rate was achieved at 30% of NaCl (0.24 h^{-1} at 35°C). These results show evidence of the extreme halophilicity of the strain SWO25. A clear positive interaction effect between NaCl and temperature on growth rate was observed. Indeed, regardless of $MgSO_4$, at approximately 38°C, no significant difference (α= 0.05) on growth rate μ_{max} (0.3 h^{-1}) was identified when NaCl concentration increased from 20 to 30% NaCl. At temperatures higher than 38°C, the growth rate μ_{max} was almost stable at 20% of NaCl (from 0.32 h^{-1} at 35°C to 0.35 h^{-1} at 45°C), while above 20% NaCl , the growth rate increased from 0.33 h^{-1} at 38°C to 0.43 h^{-1} at 45°C and from 0.32 h^{-1} at 38°C to 0.51 h^{-1} at 45°C respectively for 25 and 30% NaCl. However, below 38°C, μ_{max} varies in opposite way to NaCl concentration (Fig. 4A), suggesting an effect of thermal protection of NaCl on growth of *Haloarcula* sp. SWO25. The same interaction was observed on growth rate of the archaeon *Halobacterium* sp. NRC-1 (**Camacho *et al., 2010***).

Effect of temperature and NaCl interaction on halocin-like activity

For antimicrobial production, the interaction of NaCl with temperature was influenced by the amount of $MgSO_4$. In fact, at 2.5% $MgSO_4$ and increasing temperature from 35 to 45°C the activity was stable at 30% of NaCl, but a reduction of 19 and 8.6% of the activity was noticed at 20 and 25% NaCl respectively (Fig. 4B). At 7.5% $MgSO_4$ and increasing temperature from 35 to 45°C the activity was nearly steady at 25% of NaCl. It decreases from 15.1 mm at 35°C to 14.1 mm at 45°C for 20% NaCl, however it increases from 16.9 mm at 35°C to 18.8 at 45°C for 30% NaCl (Fig. 4C).



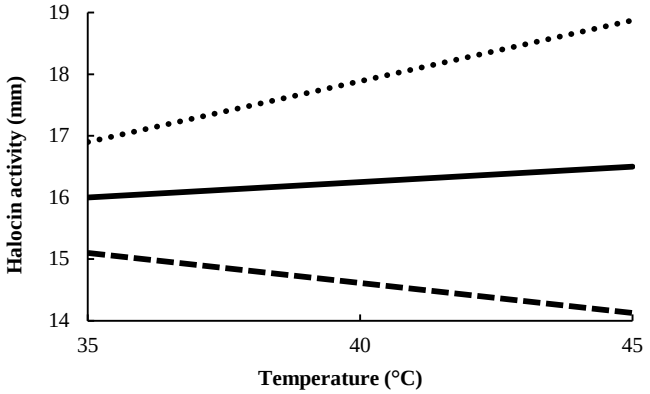


Figure 4: Effect of NaCl*Temperature Interaction on growth rate of SWO25(A), on halocin activity at 2.5 %$MgSO_4$ (B) and 7.5% $MgSO_4$ (C); 30% (⋯⋯); 25 % (▬) and 20% NaCl (▬ ▬).

The thermal protective effect of salt was observed on halophilic proteins (**Martinez-Espinosa *et al.*, 2001**; **Tehei, *et al.*,2002; Zhang *et al.*,. 2012**). $MgSO_4$ interaction with temperature influences the activity and the requirements for NaCl varied as a function of $MgSO_4$ concentration. Low $MgSO_4$ and high NaCl or high $MgSO_4$ and low NaCl concentrations were necessary for high halocin activity and stability during the increase of temperature. Similar effect has been reported for haloarchaeal amylase (**Enache *et al.*, 2009**). Magnesium ions are in general required for structure and stability of halophilic proteins (**Eichler,2001; Lanyi, 1974**). Moreover, our results show a probable thermal protective effect of MgSO4 on the halocin-like activity.

Optimization

Optimal conditions were calculated for each of the four models; it was observed that optimal values of the factors studied were different for each growth parameter and for antimicrobial activity, with the exception of the growth rate which could have similar optimal values to those of halocin-like activity (Fig. 5 A, B, C, D).

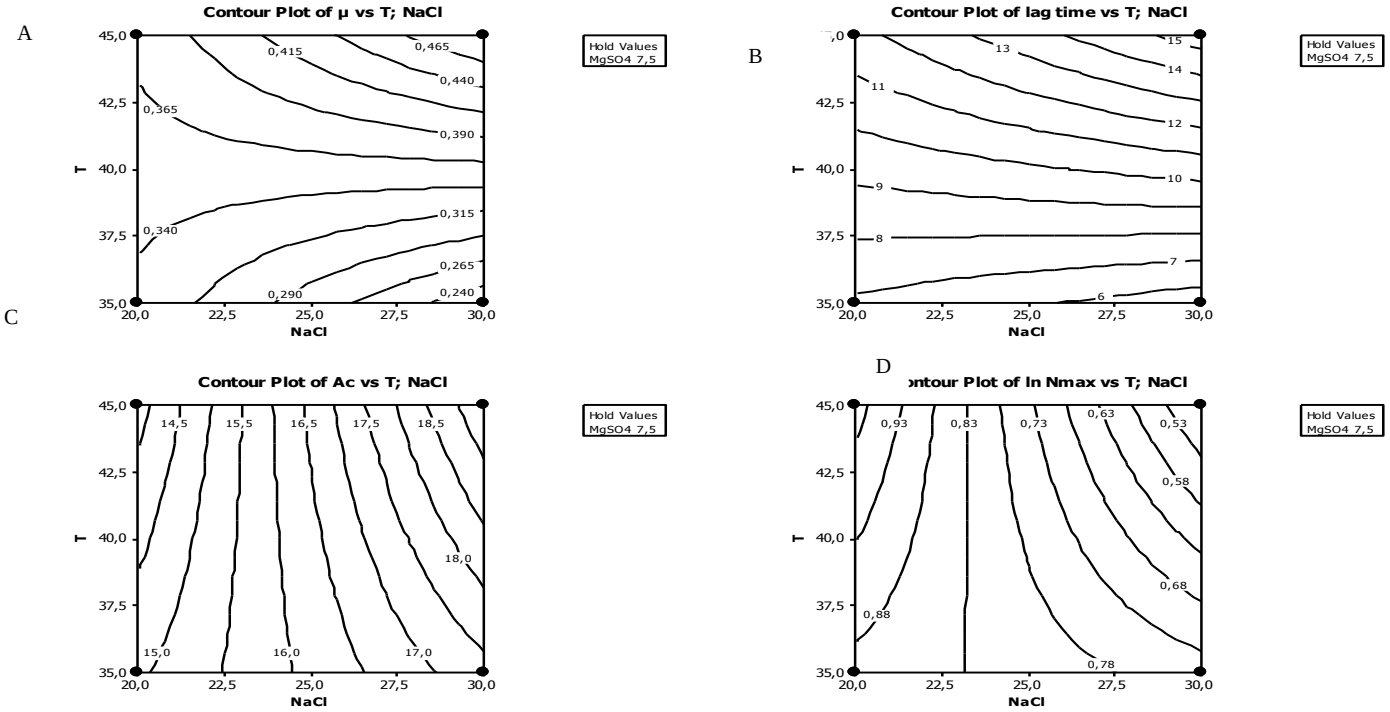


Figure 5 2D contour plot showing the effect of temperature and NaCl on growth rate μ (A), lag time (B), halocin activity (C) and Ln Nmax (D).

In the purpose to provide the optimal conditions for the four responses at the same time, superposition of the contour plots was drawn in the T*NaCl plan for the four responses. A number of numerical solutions were suggested by Minitab 16 within the experimental range of parameters for maximum growth and halocin production (Fig.6). The white area represents the intersection of the contour lines corresponding to the desired maxima for the four responses simultaneously. The suggested solution revealed that the parameters were optimized at 20% NaCl, 7.5% $MgSO_4$ and a temperature of 45°C.

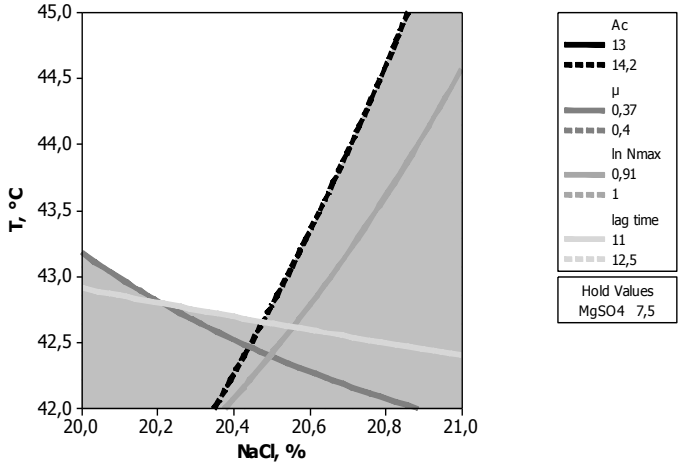


Figure 6 Overlaid contour plot for the four responses in the Temperature*NaCl plane at high $MgSO_4$ concentration. Ac corresponds to the halocin activity.

Validation

In order to verify the optimization results and to validate the four developed linear models, values calculated for optimal growth and halocin production were

carried out in experimental conditions and as control, cells were cultivated in Brown medium (materials and methods) at 40°C incubation temperature. The experimental values (λ: 11.19 h ±0,000018, μ_{max}: 0.3526h^{-1} ± 0.056807, N_{max}: 2.6145 ± 0.3311, Ac: 13.5 mm ± 0.7071) were considerably close to the predicted values calculated by minitab (λ:12.0; μ_{max}: 0.377; N_{max}: 2.63; Ac: 13.72 mm), and were higher compared to those reached in the control conditions for the growth parameters (μ_{max}: 0.224 h^{-1} ± 0.006; N_{max}: 1.81 ± 1.078), but the values remain the same for the halocin activity . Consequently, 1.6 and 1.4 fold increase in the specific growth rate and the N_{max} respectively were reached in optimized conditions (Fig.7). However, the lag time was lacking in the control conditions and was about 11.19 h in the optimized conditions. This could be explained by the inoculum used which was under the same conditions as those of the control.

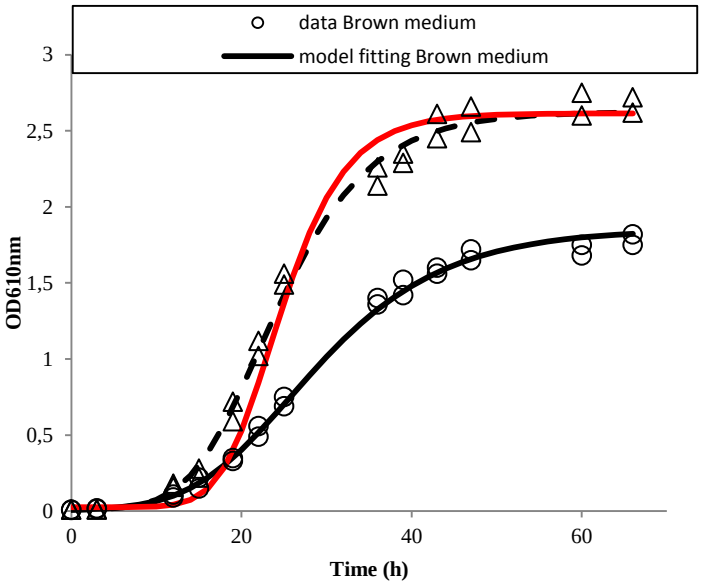


Figure 7 Growth curve of the strain SWO25 in Brown medium at 40°C incubation temperature, NaCl: 25% and MgSO4: 2% (o: data; —— model (RMSE=0,0918)) and in Brown at optimized conditions(T=45°C, MgSO4=7.5% and NaCl=20%) (Δ: data; – – – - model (RMSE=0,1248)) fitted with the Gompertz model. The red curve is obtained by applying the growth parameters predicted by the secondary model and corresponding to the optimal conditions (T=45°C, MgSO4=7.5% and NaCl=20%)

The optimal NaCl concentration for growth of most *Haloarcula* strains was shown at 2.8-4.3 mol l^{-1} (16-25%) and 0.005-0.1 mol l^{-1}(0.01-0.24%) magnesium concentration (**Bowers and Wiegel 2011**) except for *Hrr valismortis and Hrr quadrata* whose optimal growth occur at 4.5 mol l^{-1} (25%) NaCl, and 0.1-0.5 mol l^{-1}(0.24-1.21%) magnesium for *Hrr quadrata* (**Oren, 2014**). The large amounts of magnesium tolerated by the strain SWO25 could be attributed to the nature of halophilic habitats where the $MgSO_4$ concentration can be very high (34.1 gl^{-1}) as measured in Sebkha of Ouargla.

A large range of high temperature for optimal growth of *Haloarchaea* was reported (**Robinson *et al.*, 2005**). Optimal antimicrobial activity of halocins H1 and Sech7a were obtained at 20% salt concentration, while optimal temperature for H1 was between 37-42°C and at 45°C for Sech7a (**Platas *et al.*,1996** ; **Pasic *et al.*,2008**).

CONCLUSION

The present study is the first report on statistical optimization of physicochemical conditions for *Haloarcula sp* SWO25 growth and antimicrobial production. The inhibitory substance produced by *Haloarcula* sp strain SWO25 was shown to be a halocin and was partially characterized. The results provide growth model for *Haloarcula* sp. SWO25 and suitable conditions of salinity and temperature for improved growth and halocin activity. Remarkably, halocin showed the highest activity in extremely saline conditions (5.2 mol l^{-1} NaCl and 0.62 mol l^{-1} $MgSO_4$) and high temperature (45°C) and resist up to 100°C. This finding makes it interesting for future investigations in industry under those hard conditions.

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