BIOTECHNOLOGY LETTERS
Volume 18 No.2 (February 1996) p.213-218
Received as revised 2nd January.

ASTAXANTHIN PRODUCTION FROM THE GREEN ALGA *Haematococcus pluvialis* WITH DIFFERENT STRESS CONDITIONS

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SUMMARY

Haematococcus pluvialis was induced to produce the astaxanthin pigment. A factorial design was carried out with three sodium acetate concentrations, 0.025, 0.05, 0.1 (g/l), and three NaCl concentrations (0.1, 0.2 and 0.4 %).The best conditions in algal culture for astaxanthin production were 0.2 % NaCl, 0.025 g/l sodium acetate and 0.05 g/l sodium acetate, each a 3.0, 1.83 and 1.78 % of astaxanthin, production in base to total dry weight, respectively. The higher astaxanthin production by bioreactor was 18.6 mg/l in the condition with 0.2 % NaCl.

INTRODUCTION

The green alga *Haematococcus pluvialis* Flotow (Chlorophyceae, order Volvocales), is well known for its ability to accumulate large amounts of a ketocarotenoid astaxanthin (3,3-dihydroxy- ß,ß-carotene-4,4-dione) (Goodwin, 1986; Fan *et al.*, 1994). Astaxanthin has been used as a pigmentation source for crustacean and fish aquaculture when added to artificial feed sources because if gives an attractive pigmentation to their eggs, flesh and skin (Davies, 1976; No and Storebakken, 1991; Sommer *et al.*, 1992; Choubert and Heinrich, 1993).

The massive accumulation of this pigment in *H. pluvialis* under extreme enviromental conditions (nitrogen limitation, high light intensity, and high temperatures) has been investigate for several autors (Borowitzka *et al.*, 1991; Boussiba *et al.*, 1992; Kobayashi *et al.*, 1993; Lee and Ding, 1994; Tjahjono *et al.*, 1994), but not is known the effect multivariate of NaCl by sodium acetate in different conditions. Further research is also necesary in fundamental aspects of carotenogenesis, a research area in biotechnology that at present deserves considerable attention.

The aim of this work was to determine the effect of a factorial design of sodium acetate concentrations (0.025, 0.05, and 0.1 g/l), and NaCl concentrations (0.1, 0.2 and 0.4 %), for optimizing the induction to astaxanthin production from *Haematococcus pluvialis*.

MATERIALS AND METHODS

Haematococcus pluvialis, was obtained from the CCAP, strain 34/7,.FBA Ambleside Cumbria, United Kindom.
This microalga was grown in 5000 ml in medium with 1.0 g/l $NaNO_3$, 0.025 g/l $CaCl_2 \cdot 2H_2O$, 0.075 g/l $MgSO_4 \cdot 7H_2O$, 0.075 g/l K_2HPO_4, 0.175 g/l KH_2PO_4, 0.025 g/l NaCl., the oligoelements were those used in ALGAL medium (Fábregas *et al.*, 1984).
The cells were maintained in semi-continuos culture at a dilution rate 20%/d, in regime cyclostate with dark/light cycle (12:12 h). The culture conditions were: submited aereation of 6 l/min., temp 25 °C, and pH 7.5, that was controled by bubbling CO_2 with impulse of 150 ml for 10 sec every 10 min. Ilumination was provided by cool white fluorescent lamps at an irradiance of 26 $\mu E\ m^{-2}\ s^{-1}$, its was measured with a Neurtex luxmeter HD 8366, the values were converted to $\mu E\ m^{-2}\ s^{-1}$ .These conditions supported growth of a green vegetative culture.

Induction to astaxanthin production To induce astaxanthin production from *H. pluvialis*, a volume of the green culture was transferred, after being centrifugated at 1500 rpm for 5 min, to a nitrogen-deficient medium containing no organic sources, except sodium acetate (0.025, 0.05, and 0.1 g/l), and NaCl (0.1, 0.2 and 0.4 %) and different combinations of them. (Table 1).
The cultures were kept in 60 ml glass tube for triplicate in a cyclostate regimen with dark/light cycle (12:12 h). They were submited with aereation of 250 ml/min, temp 25 °C, Illumination was provided by cool white fluorescent lamps at the irradiance of 140 $\mu E\ m^{-2}s^{-1}$
After five days, the total and organic dry weight were obtained by filtering an appropiate volume of culture through a precalibrated Whatman GF/C glass fiber filter, after washed with destiled water. The samples were dried in a oven at 60 °C for 12 h, weighed to the nearest 0.1 mg, ashed in a muffle furnace at 500 °C for 12 h and reweighed to obtain ash content. The organic dry weight was obtained by difference.
Finally the culture were centrifugated at 3000 rpm. for 5 min. and freeze-dry in a lyophilyser Labconco. The samples were stored at -20 °C until their analysis.

Analyses Cell pigments were extracted with dimethyl sulfoxide (DMSO) (Boussiba and Vonshak, 1991). In the supernatant obtained, ethyl acetate and hexan was added to remove the DMSO, and the epiphase was recovered after centrifugation and dried with N_2. The residue was resuspended in 150 µl chloroform and analysed in high performance layer chromatography (HPLC). All extraction processes were conducted in the dark ..
The HPLC used was a Hewlet Packard series 1050, the column was reverse fase ODS-Hypersil, 5 µm and 250 x 4 mm I.D. Separation was achieved with a mobile phase with mixture of methanol 100 % / Acetronitile:water (90:10) / Ethylacetate 100% / run at a flow rate 1 $ml \cdot min^{-1}$.
The wavelenght selected was 474 nm in base to spectral analysis acquired from UV-Visible diode-array detector (HPLC ^{3D} ChemStation) for optimum detection wavelenght.
The astaxanthin pigment was identified by comparison with available standard (F. Hoffmann. La Roche LTD, Madrid, Spain Lot. 210031), and its quantification was performed by using the calibration curve obtained with astaxanthin standard.
The statistical analysis was made with SPSS program, and non parametric analysis was selected.

RESULTS

The *H. pluvialis* culture, in flagellate form, was stable in semicontinuos culture at a renewal rate of 20 %/d The average cell density was $0.25x10^6$ cell/ml.

The encystment was induced with NaCl and sodium acetate in separate culture. The flagella of most algal cells disappeared during 24 h incubation in each condition.

The assay finished in the fifth day, at the moment in which one of the cultures was almost totally in the red aplanospore form.

The highest astaxanthin productions (Table 1) were 3.0, 1.83 and 1.78 % by total dry weight, these percentages were obtained in the cultures with 0.2 % NaCl and with 0.025, and 0.05 g/l of sodium acetate respectivelly, and significant differences among them were found (Duncan´s multiple range test). The highest astaxanthin productions expresed in %·organic dry weight were 3.15, 1.93 and 1.81 in the cultures with 0.2 % NaCl, 0.025 and 0.05 g/l of sodium acetate, respectivelly.

The lower values were obtained in NaCl 0.4 %+sodium acetate 0.1 g/l, with 0.47 and 0.50 % astaxanthin production/ total dry weight and organic dry weight respectively.

In other induction conditions with NaCl and sodium acetate, the astaxanthin accumulation for *H. pluvialis* was between 0.80 to 1.7 %·total dry weight, and 0.82 to 1.73 % by organic dry weight.

The highest astaxanthin production by bioreactor was 18.6 mg/l in the condition with 0.2 % NaCl.

The ash content was 10.5% in the condition with 0.4% NaCl, it was the highest, but it was not very different from that of the control (9.1%) nor the algal culture with 0.1% NaCl+0.1 g/l sodium acetate (9.34%) (Duncan's multiple range test).

We decided not quantificate the astaxanthin content for each cell due the different sizes found in the cultures of *H. pluvialis* (Fig.1).

DISCUSSION

The astaxanthin production from *Haematococcus pluvialis* obtained by for many autors are dificult to compare, due to the fact that culture conditions for this algae have been different.

The percentages of astaxanthin production, obtained (between 0.5 to 3 %) are highest to those reported for Boussiba and Vonshak (1991) with *H. pluvialis* cultivated under different growth conditions.

The accumulation of astaxanthin was associated with a change in the cell stage from biflagellate vegetative green cells to aplanospores red cells, it depended from the culture conditions applicated. This form change has been reported to occur at nitrogen, phosphate or magnesium defficiences (Droop, 1954; Zlotnik *et al.*, 1993).

The best salinity, for the astaxanthin production, was the intermedia assay (0.2%). Although it has been mentioned that higher salinities like 1% can be lethal to *H. pluvialis* (Borowitzka *et al.*, 1991), in other case, it was observed that 0.8% salinities caused the dead of many cells, and when 0.4% NaCl was used, the ash content was higher (10.5 %), for the presence of and many cell residues in the culture, probably due to the negative effect over the cells, moreover it enhanced the aplanospore forms. Boussiba and Vonshak (1991) reported that exposing *H. pluvialis* to stress by adding salt 0.8 % of NaCl, caused complete cessation of growth, but induced a massive accumulation of astaxanthin.

The conditions of 0.2 % NaCl, 0.025, and 0.05 g/l of sodium acetate, used in this assay, were the best sources for astaxanthin production in *H. pluvialis*.

Several companies have been investigating astaxanthin production from different natural sources, but up to this moment, between the most promising and recognized natural source of this pigment is the alga *H. pluvialis* (Johnson and An,1991).

ACKNOWLEDGMENTS

We are grateful to Dr. Ephraim Cohen from Ben-Gurion University of the Negev, for providing us the strain of *Haematococcus pluvialis.*, and to F. Hoffmann La Roche, Madrid,Spain, for providing us the astaxanthin standard.

This work was supported by the proyect AIR2-CT94-1283, of the European Union.

REFERENCES.

Borowitzka, M.A, Huisman J.M, and Osborn, A. (1991) J. Applied Phycology, **3**,295-304.

Boussiba, S and Vonshak A (1991) Plant Cell Physiol., **32**, 1077-1082.

Boussiba, S, Fan L. and Vonshak A. (1992) Meth. Enzimology, **213**, 386-391.

Davies, B. H. (1976) Carotenoids. In: Chemistry and biochemistry of plant pigments. T. W. Goodwin, ed. vol. 2, pp 38-165, Academic Press Inc. London,

Droop, M. R. (1954) Arch. Mikrobiol., **20**,391-397.

Choubert, G. and Heinrich, O. (1993) Aquaculture, **112**,217-226.

Fabregas, J., Abalde, J., Herrero, C., Cabezas, B. and Veiga, M. (1984) Aquaculture, **42**, 207-215.

Fan, L., Vonshak, A. and Boussiba, S. (1994) J. Phycol., **30**, 829-833.

Goodwin, T. W. (1986) Ann. Rev. Nutr. **6**, 273-297.

Johnson, E. A. and An, G. H. (1991) Critical Reviews in Biotechnology, **11**, 297-326.

Kobayashi, M. K., Kakizono, T. and Nagai, S. (1993) Applied and Environmental Microbiology, **59**, 867-873.

Lee, Y. K. and Ding, S. Y. (1994) J. Phycol., **30**, 445-449.

No, H. K. and Storebakken, T. (1991) Aquaculture, **97**, 203-216.

Sommer, T. R., D'Souza, F. M. L. and Morrissy, N. M. (1992) Aquaculture, **106**, 63-74.

Tjahjono, A. E., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N. and Nagai, S. (1994) Biotechnology Letters, **16**, 133-138.

Zlotnik , I. S., Sukenik , A. and Dubinsky, Z. (1993) J. Phycol., **29**, 463-469.

Table 1. Average and standard error of astaxanthin production in Haematococcus pluvialis in %/ total dry weight, %/organic dry weight, in mg/bioreactor, and ash content (%), at different conditions.

CONDITION	ASTAXANTHIN PRODUCTION			ASH
	%/total dry weight	%/organic.dry weight	mg/l	%
Control	0.90 ±0.04	1.10 ± 0.01	6.7 ± 0.3	9.10
Acetate, 0.025 $g.l^{-1}$	1.83 ± 0.02	1.93 ± 0.35	13.6 ± 0.8	1.00
Acetate 0.05 $g.l^{-1}$	1.78 ± 0.20	1.81 ± 0.42	13.3 ± 0.8	5.16
Acetate 0.1 $g.l^{-1}$	1.33 ± 0.18	1..39 ± 0.30	6.4 ± 0.2	1.95
NaCl 0.1 %	0.93 ± 0.14	0.99 ± 0.28	5.7 ± 0.5	2.85
NaCl 0.2 %	3.00 ± 0.50	3.15 ± 0.87	18.6 ± 0.3	4.66
NaCl 0.4 %	1.27 ± 0.08	1.44 ± 0.20	7.0 ± 0.6	10.50
NaCl 0.1+Acetate 0.025	1.35 ± 0.17	1.89 ± 0.43	9.7 ± 0.3	5.72
NaCl 0.1+Acetate 0.05	1.35 ± 0.05	1.43 ± 0.07	10.4 ± 0.3	4.71
NaCl 0.1+Acetate 0.1	1.40 ± 0.15	1.57 ± 0.28	15.0 ± 0.8	9.34
NaCl 0.2+Acetate 0.025	1.37 ± 0.16	1.41 ± 0.32	9.0 ± 0.3	4.55
NaCl 0.2+Acetate 0.05	0.80 ± 0.05	0.82 ± 0.09	6.3 ± 0.4	1.93
NaCl 0.2+Acetate 0.1	1.70 ± 0.01	1.73 ± 0.02	11.7 ± 0.1	1.88
NaCl 0.4+Acetate 0.025	1.35 ± 0.05	1.38 ± 0.08	7.3 ± 0.2	0.74
NaCl 0.4+Acetate 0.05	1.20 ± 0.10	1.29 ± 0.19	7.61 ± 0.3	4.12
NaCl 0.4+Acetate 0.1	0.47 ± 0.07	0.50 ± 0.08	3.0 ± 0.1	2.00

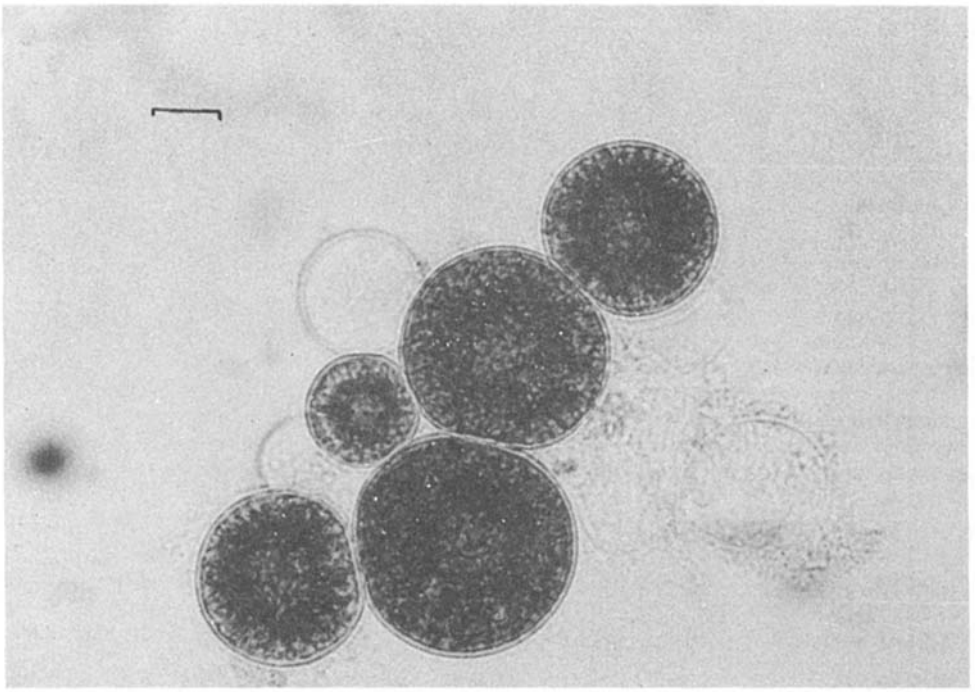

Fig. 1 *Haematococcus pluvialis* cells . grown under 0.2% NaCl conditions, the sizes are different in the same stress condition. Bar represent 12 μm.