ACTA
PHYSIOLOGIAE
PLANTARUM
Vol. 24. No. 3. 2002: 323-329

Effect of culture conditions on growth of green alga - *Haematococcus pluvialis* and astaxanthin production

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Key words: Haematococcus pluvialis, light intensity, effective culture ratio, C/N ratio, temperature, biomass, astaxanthin, total carotenoid, chlorophyll

Abstract

Influence of culture conditions such as light, temperature and C/N ratio was studied on growth of *Haematococcus pluvialis* and astaxanthin production. Light had significant effect on astaxanthin production and it varied with its intensity and direction of illumination and effective culture ratio (ECR, volume of culture medium/volume of flask). A 6-fold increase in astaxanthin production (37 mg/L) was achieved with $5.1468 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity (high light, HL) at effective culture ratio of 0.13 compared to that at 0.52 ECR, while the difference in the astaxanthin production was less than 2 - fold between the effective culture ratios at $1.6175 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity (low light, LL). Multidirectional (three-directional) light illumination considerably enhanced the astaxanthin production (4-fold) compared to unidirectional illumination. Cell count was high at low temperature (25 °C) while astaxanthin content was high at 35 °C in both autotrophic and heterotrophic media. In a heterotrophic medium at low C/N ratio *H. pluvialis* growth was higher with prolonged vegetative phase, while high C/N ratio favoured early encystment and higher astaxanthin formation.

List of abbreviations: BBM: Bold basal medium, ECR: effective culture ratio, KM1: heterotrophic medium, HL: high light, LL: low light, ML: multidirectional light, TC: total carotenoids

Introduction

Haematococcus pluvialis a green alga, has gained importance in recent years for its ability to accumulate astaxanthin. Astaxanthin, a ketocarotenoid, is extensively used as a pigmentation source in feeds of farmed salmon and trout (Johnson and Schroeder 1995). Recently *Haematococcus* algae received US Food and Drug Administration clearance and several European countries approval its marketing as dietary-supplement ingredient for human consumption (Lorenz and Cysewski 2000). *Haematococcus* accumulates astaxanthin under unfavourable growth conditions such as nitrogen, phosphorus starvation (Boussiba and Vonshak 1991, Boussiba *et al.* 1999), high temperature (Tjahono *et al.* 1994), oxidative stress (Kobayashi *et al.* 1993), salt stress (Cordero *et al.* 1996, Sarada *et al.* 2002), C/N ratio (Kakizono *et al.* 1992, Boussiba and Vonshak 1991) and light (Kobayashi *et al.* (1992). However, there are many differences in the experimental design and mode, therefore the present study foccused on how light intensity effected astaxanthin formation at different culture volumes and also showed how the direction of light illumination played a significant role on astaxanthin production in *H. pluvialis*. The effect of temperature and C/N ratio

on *H. pluvialis* growth and astaxanthin production were also studied. The results obtained may be useful for both open cultivation and closed photobioreactor systems.

Materials and Methods

Maintenance of culture

Haematococcus pluvialis was obtained from Algal Culture Centre, Plant Physiology Institute, University of Gottingen, Germany and was maintained in heterotrophic as well as autotrophic conditions in both liquid and semisolid media (Usha et al. 1999). The axenic cultures were incubated at 25±1 °C temperature under continuous light intensity of $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1}. A 4 day old culture was used as inoculum for all the experiments.

Effect of light intensity

The effect of light intensity was studied at effective culture ratio (volume of culture medium/volume of flask) of 0.13, 0.26, 0.4, 0.52 using 150 ml conical flask containing corresponding volume of heterotrophic medium containing (g/L) sodium acetate, 1.2; L-asparagine, 0.4; yeast extract, 2.0; $MgCl_2.6H_20$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.02 and pH adjusted to 6.8 (KM1, Usha *et al.* 1999). The flasks were closed with cotton plugs and the media flasks were sterilized in an autoclave at 1.3 Kg·cm^{-2} for 20 minutes. The culture flask were inoculated with 10 % inoculum. One set of culture flasks were incubated at low light, LL ($1.6175 \cdot 10^7$ erg·m^{-2}·s^{-1}) and another set at high light, HL ($5.1468 \cdot 10^7$ erg·m^{-2}·s^{-1}) at 25 °C temperature for 20 days. The cultures were harvested after the incubation period to determine the content of biomass, chlorophyll and carotenoids.

Effect of direction of illumination

The cultures were incubated under unidirectional LL ($1.6175 \cdot 10^7$ erg·m^{-2}·s^{-1}) and HL ($5.1468 \cdot 10^7$ erg·m^{-2}·s^{-1}) where lamps were fixed above the flasks. Another set were incubated under multidirectional light, ML ($2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1}) where culture flasks were kept on glass plates on the culture racks to enable the light illumination from sides ($2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} from either side) as well as from bottom ($1.6175 \cdot 10^7$ erg·m^{-2}·s^{-1}). One set of flasks was kept under dark conditions. Analysis was done after 20 days. The experiments were carried out at 0.13 and 0.26 effective culture ratios.

Effect of salinity

A set of culture flasks were inoculated with effective culture ratio of 0.13 and incubated under $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity at 25 °C for a period of 5 days for growth and the cultured cells were subjected to stress by adding sodium chloride at 0.25 % along with 4.4 mM sodium acetate. Flasks were distributed under unidirectional LL, HL, ML and dark conditions. Culture flasks without sodium chloride served as control.

Effect of temperature

Effect of temperature on *H. pluvialis* growth and astaxanthin production was studied at 25±1 °C, 30±1 °C and 34±1 °C in both autotrophic (BBM, Kanz and Bold 1969) and heterotrophic (KM1, Kobayashi *et al.* 1991) media at 0.26 ECR and $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity. The cell count was recorded at 2-day interval for a period of 10 days and the biomass, chlorophyll and carotenoid contents were analysed at the end of 15 days experimental period.

Effect of carbon/nitrogen ratio (C/N)

The effect of C/N ratio on *H. pluvialis* growth and astaxanthin production was studied at 1.4, 2.8, 5.6 and 11.2 in heterotrophic medium (KM!) at 0.26 ECR and $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity keeping sodium acetate concentration constant. L-asparagine was the nitrogen source. If the carbon content in L-asparagine is considered, then the corresponding C/N ratio are 2.25, 4.5, 9.0 and 18, respectively. Growth was monitored at different intervals and the cultures were harvested after 15 days and analyzed for biomass and carotenoids. The influence of C/N ratio was also studied on astaxanthin production in two stage cultivation mode along with sodium chloride stress (0.25 %). All the experiments were carried out in triplicates and twice repeated and the average values are presented.

Analyses

Growth measurement

Growth was monitored both in terms of cell count per mL and on dry weight basis. Cell count was done in a haemocytometer. Dry weight of the cells was carried out at 60 °C till constant weight was attained.

Chlorophylls content

Known quantity of the cells were extracted in acetone and the chlorophylls content was estimated spectrophotometrically (Shimadzu 160A) by taking absorbance at 645 and 661.5 nm using Lichtenthaler (1987) method.

Total carotenoids and astaxanthin content

Carotenoids were extracted with acetone and analyzed spectrophotometrically by the method of Lichtenthaler (1987) by taking absorbance at 450, 470 and 480 nm. Astaxanthin was determined at 480 nm using an absorption coefficient, A 1 % of 2500 as per the method of Davies (1976).

Soluble protein content

One mL of culture was centrifuged and the pellet was suspended in 2 M sodium hydroxide for 1 hr in ice as described by whitelam and Codd (1982) and the alkaline soluble protein was determined by Lowry *et al.* (1951) method.

Results

Influence of intensity of light

Results obtained for the effect of light intensity on astaxanthin production at different effective culture ratios is presented in Fig. 1. Total carotenoid and astaxanthin production were found to be high at low ECR, *i.e.* low culture volumes under both HL and LL intensities. At HL the increase in astaxanthin production was 6-fold higher with low ECR than with high ECR. Whereas, under low light intensity the difference in astaxanthin production at high ECR was only 1.3-fold higher than at low ECR. The highest astaxanthin content (2.28 % w/w) was obtained at 0.13 ECR under high light intensity. Astaxanthin content decreased with increase in ECR. In contrast the chlorophylls (a + b) content increased with increase in ECR under both high and low light intensities to the extent of 2.6 and 2.2-fold, respectively (data not shown). The biomass yields were around 2-fold less at low light intensity (Fig. 1A and 1B).

Table 1. Duration of vegetative and encystment phases in *H. pluvialis* at different C/N ratio

Carbon /Nitrogen (C/N) ratio	Days to achieve maximum vegetative growth and encystment from the inoculation of the culture		Astaxanthin content (%)
	vegetative	encystment	
1.4	10	13	31.3
2.8	9	10	55.5
5.6	6	7	67.4
11.2	4	5	75

Effect of direction of illumination

In this experiment carotenoid formation was compared among the cultures exposed to unidirectional HL and LL intensities, multidirectional light sources and dark conditions. Since astaxanthin production was high at low ECR, the cultures were inoculated at 0.13 and 0.26 ECR ratios only. The results are presented in Fig. 2. Total carotenoid and astaxanthin production was found to be 9 - 10 % more under multidirectional light illumination than at high light intensity. At 0.26 ECR the increase in astaxanthin production between HL and LL intensities was 5-fold while it was 10-fold high at 0.13

Table 2. Effect of C/N ratio on *H. pluvialis* cell number and cell enlargement

Duration (days)	Cell number		
	C/N ratio 1.4	C/N ratio 5.6	C/N ratio 11.2
5 days	$2.45 \cdot 10^5$	$2.55 \cdot 10^5$	$2.8 \cdot 10^5$
8 days	$4.25 \cdot 10^5$	$4.25 \cdot 10^5$	$2.4 \cdot 10^5$
15 days	$2.7 \cdot 10^5$	$2.35 \cdot 10^5$	$1.0 \cdot 10^5$
	Cell enlargement (%) of total cell number*		
after 15 days	23.4	24	70

* The total cells observed in the field of the microscope

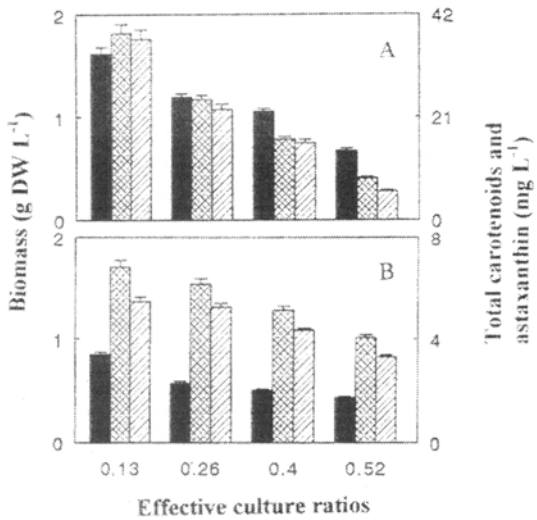

Fig. 1. Effect of light intensity on astaxanthin production at different effective culture ratios
A. High light B. Low light
from the left: Biomass; Total carotenoids; Astaxanthin
Data represent an average of 3 replicates. Bars indicate ±SD.

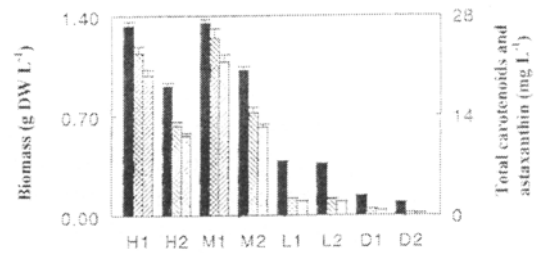

Fig. 2. Effect of light intensity and direction on astaxanthin at 0.13 & 0.26 effective culture ratio
H1, M1, L1, D1- Cultures with 0.13 ECR at high light, multidirectional light, low light, and dark conditions
H2, M2, L2, D2- Cultures with 0.26 ECR at high light, multidirectional light, low light, and dark conditions
from the left: Biomass; Total carotenoids; Astaxanthin
Data represent an average of 3 replicates. Bars indicate ±SD.

ECR. The encysted cells were bright red color in cultures grown under HL and ML while the cells were brownish red under LL and greenish yellow under dark conditions. The chlorophylls content was significantly high in cultures grown under low light and dark conditions compared to HL and ML. The biomass yields were significantly low under dark conditions and 3 - fold less under low light intensities. However, a three fold increase in biomass and 2-fold increase in astaxanthin production was achieved under dark stress conditions compared to that of control dark culture.

Effect of salinity

The results obtained for salinity stress under LL, HL, ML and dark conditions are shown in Fig. 3. The effect of salinity was studied at 0.13 effective culture ratio only. The results indicated that the total carotenoid and astaxanthin production were similar in stress and control cultures under all the experimental conditions studied. There was a 2-fold increase in astaxanthin content under NaCl treatment compared to untreated control cultures grown in dark. However, astaxanthin content was high in salt stress conditions. Also the biomass yields were the same under LL, HL, ML in stress and control cultures. (Fig. 3). As observed earlier, there was a significant increase in astaxanthin production at HL and ML compared to that at LL in both control and stress given cultures.

Effect of temperature

Of the different incubation temperatures studied the cell number was high ($4.8 \cdot 10^5$ cells/mL) in heterotrophic media at 25 °C on 6th day in comparison to 30 and 35 °C. Maximum cell number was obtained on 10th day in autotrophic medium ($1.8 \cdot 10^5$ cells/mL), however, the cell number was considerably low compared to heterotrophic medium (Fig. 4). The biomass yield registered an increase with increase in temperature in both autotrophic and heterotrophic media. Decrease in chlorophylls content was recorded with increase in temperature (Fig. 5A) and in contrast the carotenoids content increased with increase in temperature and maximum carotenoids production (14 mg/L and 11 mg/L) with high astaxanthin content (1.41 % w/w and 1.6

Table 3. Effect of C/N ratio and sodium chloride (salinity) stress on total carotenoids and astaxanthin production

Carbon/Nitrogen (C/N) ratio	Total carotenoids (w/w)	Astaxanthin (w/w)	Astaxanthin (%)
5.6	1.36	0.78	56
5.6+	1.84	1.6	87.5
11.2	0.62	0.4	65
11.2+	1.76	1.56	90

+ indicates stress coupled with C/N ratio

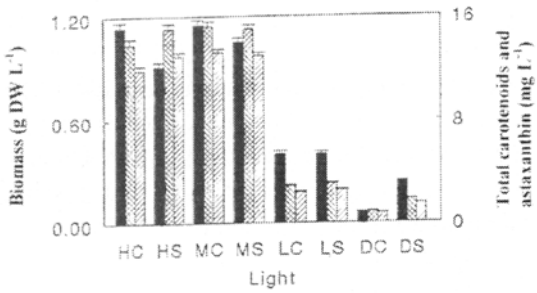

Fig. 3. Astaxanthin production in stress induced and control cultures at high light, low light, multidirectional light and dark conditions
HC,MC,LC,DC - Control cultures at high light, multi directional light, low light and dark conditions
HS, MS,LS,DS - Stress induced cultures at high light, multi directional light, low light and dark conditions
from the left: Biomass; Total carotenoids; Astaxanthin
Data represent an average of 3 replicates. Bars indicate ±SD.

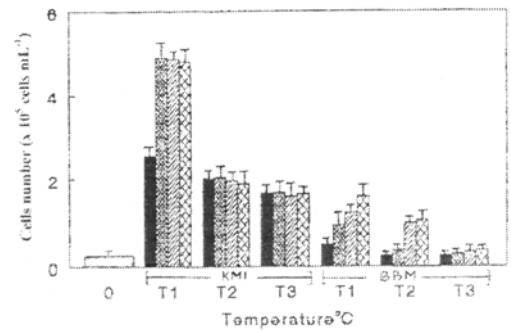

Fig. 4. Growth pattern of *H. pluvialis* grown at different temperatures (T1 - 25 °C, T2 - 30 °C, T3 - 35 °C) in heterotrophic (KM1), autotrophic (BBM) media incubated at $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity.
from the left: 4th day; 6th day; 8th day; 10th day;
Data represent an average of 3 replicates. Bars indicate ±SD
'O' Initial cell count at the start of experiment in all the temperature flasks.

% w/w) was obtained at 35 °C in KM1 and BBM media, respectively (Fig. 5B).

Effect of carbon/nitrogen ratio

It was evident from the results that the initial cell number was found to be similar at different C/N ratios in the initial period while the cell number decreased significantly at higher C/N ratios in the later period (Fig. 6C). The chlorophyll content was considerably high (12.64 mg/L) at C/N ratio of 1.4, while it was less at higher ratios (Fig. 6A). The protein content was found to be high at C/N ratios of 1.4 and 2.8 whereas a drastic decrease in protein content was observed at higher ratios (Fig. 6B). The total carotenoid and astaxanthin contents were found to be maximum (7.82 and 4.54 mg/L) at C/N ratio of 2.8 - 5.6. It was observed that the percentage of astaxanthin increased with the increase in C/N ratio and the highest percentage (80 % of total carotenoids) was obtained at 11.2 C/N ratio (Fig.

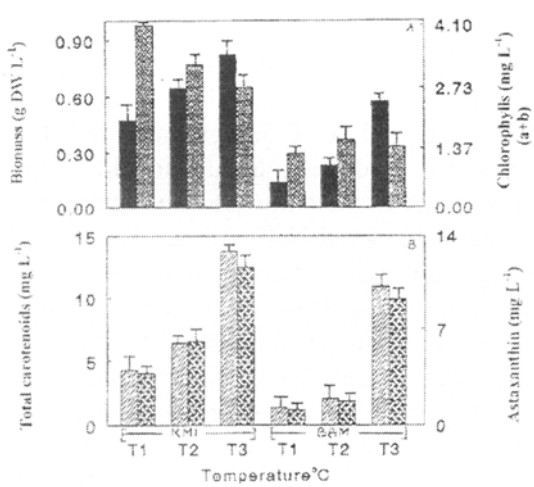

Fig. 5. Effect of different temperatures (T1 - 25 °C, T2 - 30 °C, T3 - 35 °C) on (from the left): biomass; chlorophylls; total carotenoids; astaxanthin accumulation in *H. pluvialis* cultures grown in heterotrophic (KM1), autotrophic (BBM) media incubated at $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity.
Data represent an average of 3 replicates. Bars indicate ±SD.

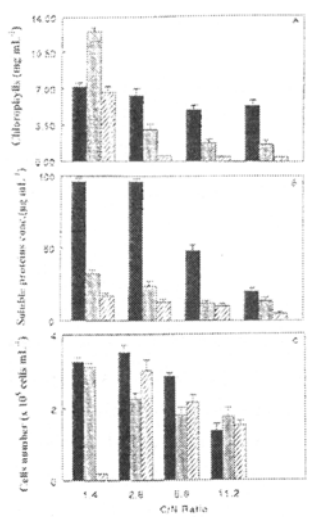

Fig. 6. Influence of carbon/nitrogen ratio on growth (A), chlorophylls content (B); Soluble proteins content; (C) cell number in *H. pluvialis*. grown in heterotrophic (KM1) medium at $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity.
from the left: 4th day; 7th day; 10th day;
Data represent an average of 3 replicates. Bars indicate ±SD.

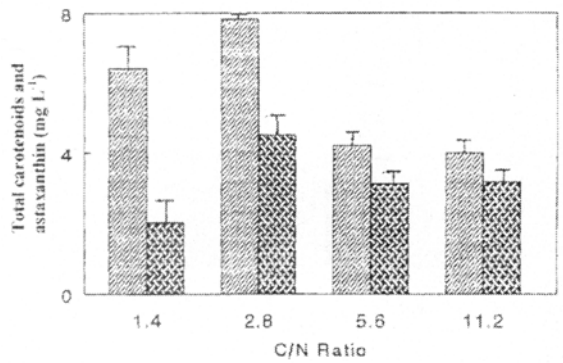

Fig. 7. Influence of carbon/nitrogen ratio on (from the left): total carotenoids; and astaxanthin production in *H. pluvialis* grown in heterotrophic (KM1) medium at $2.2058 \cdot 10^7$ erg·m^{-2}·s^{-1} light intensity
Data represent an average of 3 replicates. Bars indicate ±SD.

7). As shown in Table 1, encystment was faster at high C/N ratios with higher astaxanthin content.

As shown in Table 2, when the 9 day grown cells were transferred to media containing different C/N ratios, the cell number increased only in low C/N ratio. In cultures with high C/N ratio there was no increase in cell count. The proportion of cells that showed enlargement was significant at high C/N ratio (70 % of cells) while only 23 % of cells showed enlargement at low C/N ratios (Table 2).

The data obtained for different C/N ratios coupled to salinity stress is presented in Table 3. Under these conditions considerable increase in astaxanthin content was observed. Moreover cultures at 11.2 C/N ratio produced 3.7-fold more astaxanthin than the cultures at low C/N (2-fold) compared to their respective controls (cultures without stress).

Discussion

The enhanced astaxanthin accumulation obtained at HL intensity under all ECR compared to low light intensity indicates photodependence of carotenoids biosynthesis. Kobayashi *et al.* (1992) reported that light quantity defined as the multiplication of light intensity by the net illumination time is an important parameter in carotenoids biosynthesis than light intensity alone. In the present study, the results also showed that under the same light regime if the effective culture ratio was increased by 4-fold, the carotenoids production decreased proportionately. In other words carotenoids production increased 6 - fold when the ECR was low (0.13). After growth phase *Haematococcus* cells tend to settle at the bottom of the culture flask and undergo encystment followed by carotenoids accumulation. Under these conditions the quantum of light that reaches the cells is more important then the light intensity and quantity. The increase in astaxanthin production at low effective culture ratio in the present study is in accordance with the above statement. The increase in astaxanthin production under ML further substantiated higher exposure of cultures to light is one of the essential parameters that influence the astaxanthin formation in *H. pluvialis*. Similar productivities of astaxanthin under HL, ML and salinity conditions suggest that photoinduced and stress induced carotenoids formation may act through a common induction pathway. The two fold increase in astaxanthin production under stress in dark culture compared to control culture in dark conditions indicates light –independent astaxanthin formation which was also reported by Kobayashi *et al.* (1997). Stress induces carotenoid accumulation in both light and dark conditions (to a lesser extent) therefore due to cyst formation and carotenoid accumulation the cell weight increases along with biomass increase while in control there was no cyst formation and carotenoid induction thus results in less biomass.

The results showed that a temperature of 25±1 °C were suitable for growth and 30 °C to 35 °C for astaxanthin production, while Tjahjono *et al.* (1994) reported 20 °C for growth and 30 °C for astaxanthin production. In our study low C/N ratio favored growth and prolonged the vegetative phase while high C/N ratio induced encystment and cell enlargement leading to astaxanthin accumulation. The significant increase in astaxanthin content (2.0 - 3.7-fold) obtained at different C/N ratios with sodium chloride stress shows the synergistic or cumulative effect of the two factors. Similarly Tjahjono *et al.* 1994 reported hyper accumulation of astaxanthin at 30 °C with supplementation of acetate, Fe^{2+} and H_2O_2. They also reported that light was not so critical at this elevated temperature for astaxanthin induction. In the present study, the results showed that cultures subjected to high light intensity coupled with salinity stress did not show any

further increase in astaxanthin production while the cultures at high C/N ratio coupled with salinity stress resulted in enhanced astaxanthin production. Therefore information on different cultural and environmental studies and their cumulative / synergistic effects will help to improve the overall growth and astaxanthin yields of this potential organism. Thus the results obtained have implications for scale up studies.

Acknowledgements

The authors thank Dr. V. Prakash, Director, CFTRI for his support to carry out this study. The award of the Senior Research Fellowship to Usha Tripathi by the Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged.

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Received September 21, 2002; accepted April 12, 2002