

Selective Extraction of Carotenoids from the Microalga *Dunaliella salina* with Retention of Viability

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Received 14 June 2001; accepted 16 January 2002

DOI: 10.1002/bit.10270

Abstract: Simultaneous production and selective extraction of β -carotene from living cells of *Dunaliella salina* in a two-phase system of aqueous and organic phases has been investigated. Solvents with values of $\log P_{\text{octanol}}$, which denotes hydrophobicity of a compound, ranging from 3 to 9 were used as organic phase. Viability and activity of *Dunaliella salina* in the presence of organic solvents were checked by microscopic observation and photosynthetic oxygen-production-rate measurements, respectively. Extraction ability of different solvents for both β -carotene and chlorophyll was determined spectrophotometrically. In addition, β -carotene contents of the cells growing in the aqueous phase and extracted β -carotene by the different organic phases were quantified by the same method. Results showed that solvents having $\log P_{\text{octanol}} > 6$ can be considered biocompatible for this alga. Moreover, pigment extraction ability of a solvent is inversely dependent on its $\log P_{\text{octanol}}$ value. By increasing the degenerative hydrophobicity the extraction ability for both chlorophyll and β -carotene, decreases. However, this decrease is more profound for chlorophyll. Therefore, selective extraction of β -carotene becomes feasible. Comparison of the total β -carotene produced in the presence and in the absence of solvents shows that the presence of a second phase of biocompatible solvents in the culture media may induce the β -carotene production pathway. The β -carotene productivity per cell in a two-phase system with dodecane was the highest observed. Extraction ability of the biocompatible solvents dodecane, tetradecane, and hexadecane was similar. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 79: 29–36, 2002.

Keywords: β -carotene; *Dunaliella salina*; two-phase system and selective extraction

INTRODUCTION

β -Carotene and other carotenoids are naturally occurring pigments that have important nutritional and

biological properties. β -Carotene plays an important role in the human body because of its pro-vitamin A activity (Chen et al., 1993). Carotenoids are also strong antioxidants, scavenging potentially harmful oxy radicals, which are commonly associated with the induction of certain cancers (Leach et al., 1998). Therefore, carotenoids, mainly β -carotene, are widely used by the food, pharmaceutical, and cosmetic industries. Increasing demand for β -carotene, mostly natural β -carotene, has resulted in growing interest in extracting β -carotene from different natural sources (Vega et al., 1996) such as vegetable and fruit wastes (Favati et al., 1988; Keat et al., 1991; Sadler et al., 1990; Spanos et al., 1993; Vega et al., 1996). However, by far the highest concentrations of β -carotene are found in the halotolerant microalga *Dunaliella salina*, reaching levels of up to 100 g kg^{-1} on a dry weight basis (Ben-Amotz, 1993).

β -Carotene can be prepared by spray-drying of algal biomass and sold in the form of β -carotene-rich biomass tablets or capsules. It can also be separated from the algal cells by extraction with organic solvents or edible oils (Leach et al., 1998).

Regarding the extraction of β -carotene from algae several methods have been described (Table I). β -Carotene and chlorophyll are extracted by contacting the cells with organic solvents or edible oils. To increase the extraction yield, the cells are destroyed before adding organic solvents or during the extraction process by strong solvents with high polarity. After separation of organic phase the raffinate separation and purification of β -carotene can be done by using several methods.

The fermentative extraction of β -carotene, using two-phase systems and without affecting the viability of *Dunaliella salina* has not been reported yet. Whole-cell biocatalysis in a two-phase system is used for the production of metabolites with a greater affinity to another phase, immiscible with the aqueous cell phase. In such a system the extraction rate is often less than in conven-

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Contract grant sponsor: Iranian Ministry of Agriculture Jihad

Table I. Methods for β -carotene extraction from *Dunaliella salina*.

Breaking method	Extracting solvent	Reference
Osmotic shock	Ethanol, hexane, cyclohexane, and benzene	Avron & Ben-Amotz (1980)
Thermal treatment	Halogenated, aliphatic or aromatic hydrocarbon	Ruegg (1984)
Homogenization	Edible oil	Nonomuro (1987a, 1987b)
No additional breaking	Super-critical CO ₂	Leorenzo (1991)
Osmotic shock and mechanical methods	Hexane, cyclohexane, and petroleum ether	Haigh (1994)
Thermal treatment	Edible oil	Sasol-Chem. Co. (1994)
Strong solvent (methanol)	Methylene chloride and ethanol	Mitsubishi-oil Co. (1994) ^a
Strong solvents	Acetone, methanol, and di-ethylether	Liang & Pang (1997) ^b
Thermal treatment and organic solvents	Mixture of one acid ester and oil	Heidlas et al. (1998) ^c

^aFrom yeast (*Rhodotorula glutinis*).

^bFrom alga *Spirulina*.

^cFrom different natural sources including algae.

tional ones but growth of microorganisms as well as production and separation of metabolites occur simultaneously and continuously (Vermuë and Tramper, 1995). Therefore, overall productivity in nonconventional systems can be higher and downstream processing is often easier. Toxicity of the second organic phase for microorganisms depends on its hydrophobicity. The log P_{octanol} is a measure of the hydrophobicity and is used to predict the activity retention of a biocatalyst in organic media. It is defined as the logarithm of the partition coefficient of the solvent in a two-phase system of octanol and water. In general, retention of the biocatalyst-activity in organic solvents is low in solvents having log $P_{\text{octanol}} < 2$, cannot be predicted in solvents having a log P_{octanol} between 2 and 4, and is high in apolar solvents having log $P_{\text{octanol}} > 4$ (Laane et al., 1987; Laane and Tramper, 1990). In this article we describe the effect of organic solvents with different log P_{octanol} on the viability and β -carotene production by *Dunaliella salina*.

In addition, it has been already reported that chlorophyll is heterogeneously bound to other compounds in the chloroplast and at least two or even three fractions of chlorophyll exist in the chloroplast. Therefore, different solvents having different polarities can extract different types of chlorophyll (Deroche and Briantais, 1974; Oquist and Samulsson, 1980). This then is the reason to investigate the possibility of selective extraction of carotenoids from *Dunaliella salina* by nonpolar organic solvents.

MATERIALS AND METHODS

Organism and Medium

Dunaliella salina (CCAP 19/18) was grown in the culture medium containing 1M NaCl, 10 mM KNO₃, 1 mM NaH₂PO₄ · 2H₂O and 5 mM NaHCO₃ · 5 mL l⁻¹ of trace elements stock with 12.3 mM Na₂EDTA · 2H₂O, 4.66 mM FeCl₃ · 6H₂O, 42.0 mM CuSO₄ · 7H₂O, 60.6 mM ZnSO₄ · 7H₂O, 17.0 mM CoCl₂ · 6H₂O, 366 mM MnCl₂ · 4H₂O and 1.04 mM Na₂MoO₄ was also added.

The pH of the medium after addition of 50 mmol of Tris-buffer was adjusted to 7.5 by some drops of a 3M HCl. The medium was sterilized at 121°C for 40 min before inoculation. To avoid precipitation, the phosphorous and carbon sources (NaHCO₃) were autoclaved separately.

Growth Conditions

A 1-L bottle with 75 mL of culture medium was inoculated with 3 mL of a 3-week-old pure culture, containing about 150 cell/ μ L. After 24 hours, growth and activity of the cells were measured. Then, 10 mL of different solvents (Table II), with different log P_{octanol} values, were added. Bottles were illuminated by fluorescent lamps (SYLVANIA CF-EL 55W/840) from the bottom side ($D = 8.5$ cm). The average light intensity was 180 μ mol m⁻² s⁻¹. The light intensity was determined by a Quantum/Radiometer/Photometer [2- π Quantum sensor, Li SA-190, (Li Cor, USA)]. The temperature was 26.5 \pm 0.5°C and the two liquid phases in each bottle were stirred at 70 rpm.

Experiment Design

Experiments were done in two steps. In the first step solvents with log P_{octanol} values ranging from 2.9 to 8.8, were used. In this step the effect of different solvents on the viability of the cells was investigated. To avoid evaporation of the solvents closed bottles were used. In

Table II. Physical properties of the used organic solvents.

Solvent	log P_{octanol}	Density (g L ⁻¹)
Toluene	2.9	865
Hexane	3.5	660
Octane	4.5	702
Decane	5.6	730
Dodecane	6.6	750
Tetradecane	7.6	760
Hexadecane	8.8	770

the following step, activity of the cells in the presence of nonvolatile solvents with $\log P_{\text{octanol}} > 4$, were determined. To allow gas exchange with the atmosphere, an open-bottle system was applied. In principle, even nonvolatile solvents evaporate in time, resulting in changes of experimental conditions. For that reason, bottles with a small opening ($D = 18$ mm) with cotton plugs were used. Evaporation was negligible in those systems.

Determination of the Viability and the Physiological Activity of the Cells

Viability of the cells was checked by microscopic observations. Living cells move by their flagella. Cells that are destroyed or that do not move are considered dead.

For determination of the physiological activity of the cells the oxygen evolution rate and the increase of cell number was determined as a function of time. The oxygen evolution rate was determined by oxyLab-3 equipped with DW3 liquid phase oxygen electrode unit and LS2 Tungsten-halogen light source (Hansatech Co., UK). For each measurement an 8-mL sample was used consisting of 4 mL 25 mM Tris-buffer of pH 7.5 and 4 mL of culture suspension. The light intensity was about $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ [Light intensity was measured by QRT1 Quantitherm light/temperature meter (Hansatech Co., UK)] and temperature was 25°C . The light intensity was adjusted by using natural density filters for LS2 light source. The oxygen activity in the buffer was lowered by purging the sample with a mixture of air and nitrogen gas; then extra carbon dioxide, in the form of sodium bicarbonate (25 μL , 0.56M), and algal sample were added.

The cell number was determined by counting the cells using a 0.1 mm deep counting chamber (Neubauer improved). The optical density of the culture media was also determined spectrophotometrically (Spectronic, 20 GENE SYS). Calibration equation for the relation between the cell number in the exponential phase and the optical density at 530 nm was calculated.

Determination of cell number before adding of solvents to culture media, was done by determination of OD. Cell number was obtained via a calibration curve. Because of the influence of solvent on the OD, the cell number after addition of the solvents was determined by direct cell counting.

Absorbance measurements were done in duplicate and counting of the cells was carried out 4 times for each sample.

Determination of the Biocompatibility of the Solvents

The oxygen production rate and the cell number were determined for each sample before adding solvents, and 24 and 96 h after addition of the solvents. Then, the

oxygen production rate per cell was determined for each sample. By taking the average from the data for each treatment and determination of confidence interval ($\alpha = 0.05$), physiological activities of cells before and after adding the solvents were compared. Biocompatibility of different solvents was determined by dividing oxygen evolution rate per cell after adding solvent to oxygen evolution rate per cell before adding solvent.

Sample Preparation for Determination of the β -Carotene Content

According to Craft and Soares (1992) the solubility of β -carotene in tetrahydrofuran (THF) is higher than in other solvents. Our tests also confirmed the higher ability of THF for β -carotene extraction over other usual solvents, such as acetone and ethanol. Therefore, THF was chosen as a solvent for all-trans β -carotene (Sigma, type I) and as an extracting agent for β -carotene from the cells.

A sample of 2 mL was taken from the aqueous phase of each culture medium that had been mixed thoroughly. After 5-min centrifugation at 5000 rpm the upper phase was decanted and 2 mL of THF was added to the biomass. Each sample was mixed by vortex for 1–2 min to reach complete extraction. Samples then were centrifuged again for 5 min at 5000 rpm for separation of the biomass (now colorless) and the solvent phase. The extracted pigments in the solvent phase were quantified by the spectrophotometric method described below. From organic phases 2-mL samples were also taken and β -carotene concentrations directly determined by the spectrophotometric method (see below).

Determination of β -Carotene

β -Carotene concentrations were determined spectrophotometrically. For that purpose, standard curves for β -carotene concentrations in different solvents were made. Approximately 30 mg of all-trans β -carotene (Sigma, type I) was dissolved in 10 mL of THF (Merck, 99.5% purity) containing butylated hydroxytoluene (BHT) as an antioxidant. Thirty μL of this concentrated stock solution was added to 30 mL of each solvent (THF, dodecane, tetradecane, and hexadecane) and then solutions were diluted several times by the same solvent. These solutions were prepared in duplicate and subsequently absorbance measurements were conducted in duplicate, using a spectrophotometer (Beckman DU640 Spectrophotometer) in the wavelength maxima for each solvent. Wavelength maxima for β -carotene in the different solvents were from 455 to 458 nm. β -Carotene concentrations were plotted as a function of absorbance and calibration equations were determined by linear regression.

Relation Between Solvent $\log P_{\text{octanol}}$ and Its Pigment Extraction Ability From the Cells

Sample preparation for this purpose was done using the same procedure as already described for β -carotene determination. Except that, THF was replaced by the solvents listed in Table II. Again, by the same spectrophotometric method spectra of pigments extracted by the different solvents were identified. The shape of the spectra ranging from 350 to 700 nm was qualitatively compared with the spectra of the pigments extracted by THF as a reference solvent.

Estimation of $\log P_{\text{octanol}}$ Values of β -Carotene and Chlorophyll

Estimations of the $\log P_{\text{octanol}}$ values for both chlorophyll and β -carotene were done using the internet site: <http://esc.syrres.com/interkow/kowdemo.htm>. This on-line demo is a working version of SRC's LOGKOW/KOWWIN program (Meylan and Howard, 1995). For calculation of $\log P$ value the chemical structures of chlorophyll and β -carotene were introduced as a SMILES (Simplified Molecular Input Line Entry System) notation.

RESULTS AND DISCUSSION

Viability of Cells in the Presence of Different Organic Solvents

The viability of the cells in the presence of different organic solvents was judged by the appearance of the culture media and microscopic observations. The color of the culture media including the solvents with the $\log P_{\text{octanol}}$ values lower than 4 changed from green to white or milky after 24 h. Furthermore, microscopic observations showed only dead and destroyed cells. For octane and decane with $\log P_{\text{octanol}}$ values of 4.5 and 5.6, respectively, the color of the culture media was still green after 24 h. Microscopic observations showed both living and dead cells. The living cells appeared to be not as active and mobile as the cells in the blank samples. After more than 24 h, the color of both culture media became white. Microscopic observations did not show any living cells either. Samples without solvents and samples with solvents having $\log P_{\text{octanol}} > 6$ contained viable cells even after 7 d of inoculation (Table III).

Activity of Cells in the Presence of Organic Solvents

Activity of cells in the presence of solvents with $\log P_{\text{octanol}} > 4$ was followed by evaluation of photosynthetic oxygen evolution rate per cell. For investigation about the effect of solvents on the activity of the cells this parameter was determined before addition of the

Table III. Viability of cells of *Dunaliella salina* in the presence of 12% (v/v) organic solvents.

Solvents	$\log P_{\text{octanol}}$	Time (days)		
		1	3	7
Toluene	2.9	–	–	–
Hexane	3.5	–	–	–
Octane	4.5	+	–	–
Decane	5.6	+	–	–
Dodecane	6.6	+	+	+
Tetradecane	7.6	+	+	+
Hexadecane	8.8	+	+	+

solvents and also 24 and 96 h after addition of the solvents. Two samples were also taken as blank samples, without any solvents, and the same measurements were carried out for them.

Microscopic observation 24 h after addition of solvents for all samples treated in the different conditions showed the same results as in the viability experiment. At the same time, for the cells growing in the presence of octane and decane photosynthetic oxygen production rate was almost zero. However, oxygen production rate per cell for the cells growing in the presence of the solvents with $\log P_{\text{octanol}} > 6$ was about the same as in the blank samples in all the measurements.

By taking the average of the measurements at 24 and 96 h after solvent addition, activity of cells in the presence of solvents with $\log P_{\text{octanol}} > 6$ was calculated. Activity of the cells after addition of the solvents was a little lower than their activity before addition of the solvents, but it was not significant (Fig. 1). According to Figure 1 the activity of cells in the blank samples shows a similar decrease. It seems that this decrease is not caused by the solvents, but by the batch-wise cultivation

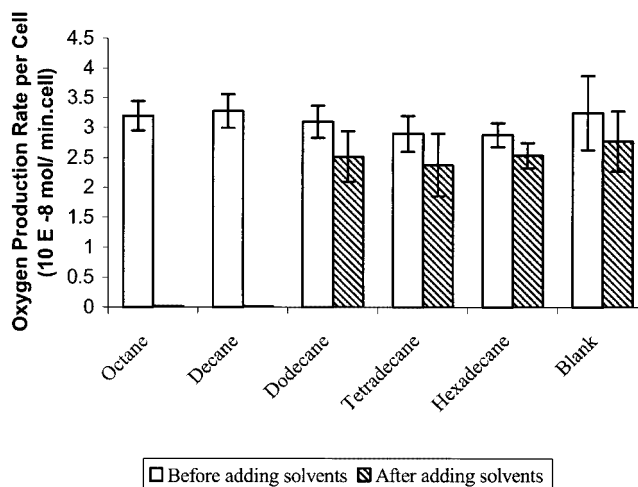


Figure 1. Oxygen production rate per cell for *Dunaliella salina* cells growing in the presence and absence of different organic solvents at different stages. Error bars show 95% confidence interval.

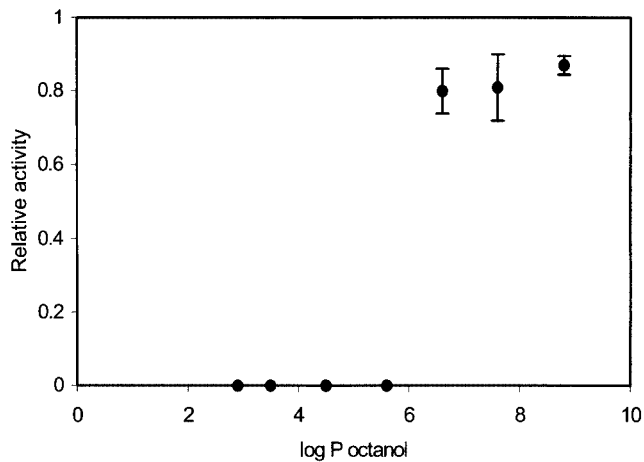


Figure 2. Activity of *Dunaliella salina* as a function of $\log P_{\text{octanol}}$ (Relative activity of the cells in the presence of the solvents was calculated by dividing the average activity of cells after addition of solvents by their average activity before addition of the solvents.). Error bars show 95% confidence interval.

method and by the slowing of the physiological activity of the cells by time.

In Figure 2 the relative activity of cells in the presence of different organic solvents has been plotted as a function of solvents $\log P_{\text{octanol}}$ values. It shows that the relative activity of cells in the presence of the solvents with $\log P_{\text{octanol}} < 6$ is (almost) zero but for the solvents with $\log P_{\text{octanol}} > 6$ is higher than 70%. Therefore, solvents having $\log P_{\text{octanol}} < 6$ are toxic for the cells and solvents with $\log P_{\text{octanol}} > 6$ can be considered as biocompatible solvents. Comparison of these results with former results obtained for other types of cells, show that *Dunaliella salina* is more sensitive to solvents than other types of cells. The break point for most bacterial cells is $\log P = 4$ (Laane et al., 1987) and for plant cells is $\log P = 5$ (Bassetti and Tramper, 1994; Buitelaar et al., 1990), this point for *Dunaliella salina* is shifted to $\log P = 6$.

Selective Extraction of β -Carotene by Biocompatible Solvents

Our spectrophotometric analysis showed that pure all-trans β -carotene in THF has two peaks at 458 and 485 nm and a shoulder around 437 nm. According to the literature the spectrum of chlorophyll *a* also has two peaks but at 420 and 660 nm in ether and they shift to right or left in other organic solvents (Hall and Rao, 1988). The spectra of pigments of *Dunaliella salina*, growing in aqueous phase in the different samples, are shown in Figure 3. Four peaks can be observed, respectively at 437, 455, 483, and 664 nm. It indicates that the cells contain both β -carotene and chlorophyll *a*. However, the spectra of the pigments extracted to biocompatible solvent phases (Fig. 4) are not comparable with the mentioned spectra of the cells in aqueous phase

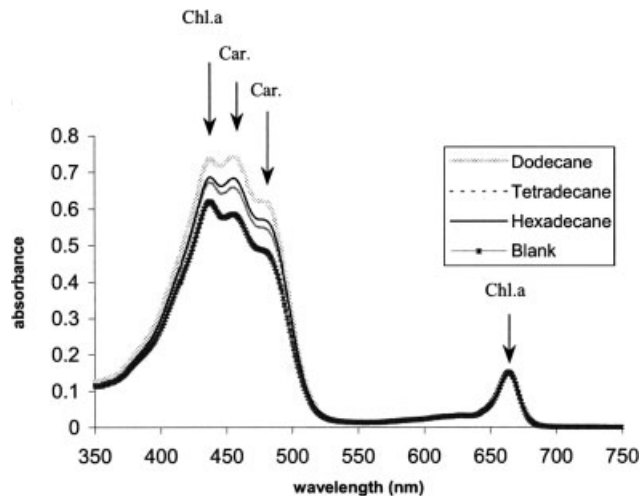


Figure 3. Spectra of the extracted pigments in THF from *Dunaliella salina* whole cells growing in the presence and absence of organic solvents.

(Fig. 3). Spectra of solvent phases have only two peaks at 455 and 483 nm and the peaks for chlorophyll are almost absent. It seems that there is a preference for the extraction of β -carotene over chlorophyll by the biocompatible solvents, even though our estimations show that both chlorophyll and β -carotene are very hydrophobe and have almost same $\log P_{\text{octanol}}$ values. The estimated $\log P_{\text{octanol}}$ values for chlorophyll and β -carotene are 17.2 and 17.6, respectively.

According to the literature chlorophyll of plant cells is heterogeneously bound to other compounds in the chloroplast and most of these bonds are strong hydrophilic bonds. Highly polar solvents are needed for breaking down these strong chemical bonds (Costes and Bazier, 1979; Sestak, 1977). Deroche and Briantais (1974) showed petroleum ether ($\log P_{\text{octanol}} < 3.5$) extraction of lyophilized wheat chloroplasts preferentially removed β -carotene and the far-red chlorophyll *a* forms (chlorophyll with weak hydrophobic bonds). Oquist and

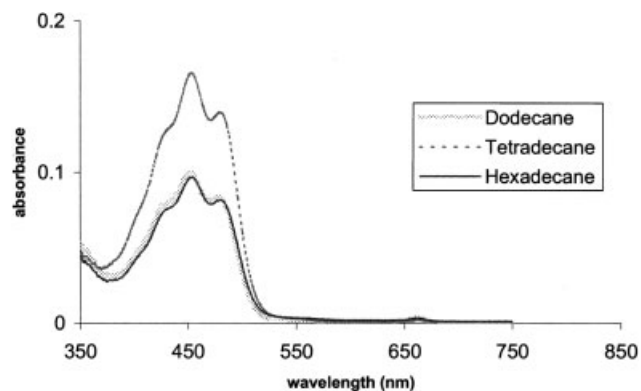


Figure 4. Spectra of the pigments extracted by biocompatible solvents in biphasic systems.

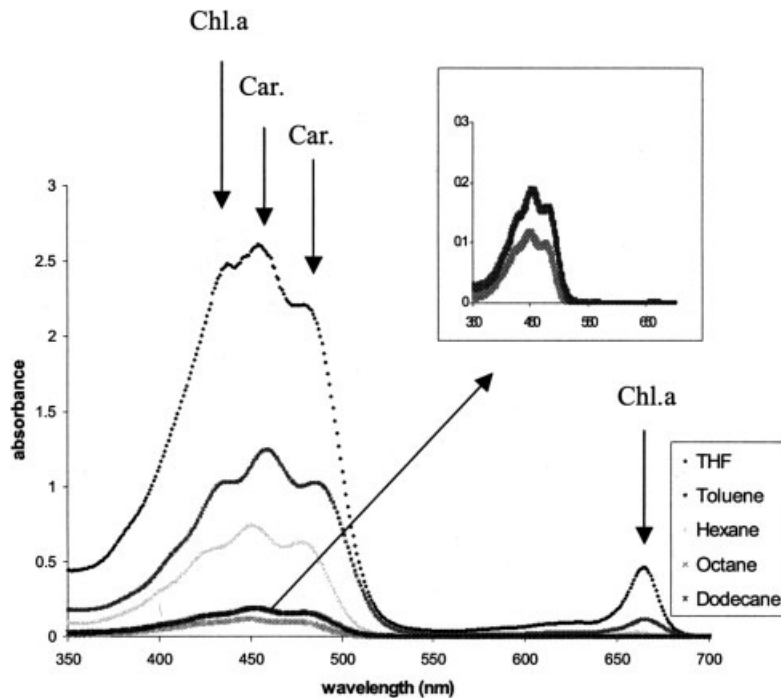


Figure 5. Spectra of extracted pigments from *Dunaliella salina* by different solvents.

Samulsson (1980) reported that petroleum ether could extract only 3% of total chlorophyll of lyophilized pea chloroplast thylakoids; by adding of 1% of ethanol its extraction capacity increases to 63%. Complete chlorophyll extraction is only possible by using polar solvents. Meanwhile, β -carotene can be easily extracted by the solvents with lower polarity.

For getting more information about the former phenomena pigment extraction ability of solvents listed in Table II from the whole algal cells was investigated. Figure 5 shows that the pigment extraction ability of a solvent from whole algal cells is dependent on the solvent hydrophobicity. By increasing solvent hydrophobicity its ability for pigment extraction decreases. Furthermore, in the spectra of THF ($\log P_{\text{octanol}} = 0.46$), reference solvent, and toluene the four peaks of β -carotene and chlorophyll are clearly distinct and in the spectrum of hexane the peaks of chlorophyll are visible. However, in the spectra of octane and decane peaks of chlorophyll are almost missing. Figure 6 also shows that the ratio of absorbance at 455 (λ_{max} of β -carotene) over absorbance at 664 (λ_{max} of chlorophyll), in the spectra of extracted pigments by different solvents, increases by increasing the $\log P_{\text{octanol}}$ of the solvents.

It is obvious that the effect of the solvents on the cell membrane has an important role on the extraction of intracellular compounds by the solvents. Solvents with lower hydrophobicity reach critical concentrations more easily, necessary for the inactivation and breaking down of the cell membrane (Bassetti and Tramper, 1994; Sikkema et al., 1995). These solvents can break down the cell membrane and release more intracellular com-

pounds such as pigments. By increasing the hydrophobicity the effect of solvents on the cell membrane decreases and the extraction ability for both chlorophyll and β -carotene decreases, as well. However, this decrease is stronger for chlorophyll. The results are in agreement with the results of previous researchers. It seems that selective extraction of β -carotene might be because of the strong bonds between chlorophyll and other cell components.

β -Carotene Production in Biphasic Systems

The β -carotene content of the aqueous and the organic phases for different treatments was determined spectrophotometrically. Evaluation of the total β -carotene showed an increase for the β -carotene production in biphasic systems, e.g., in the biphasic system with

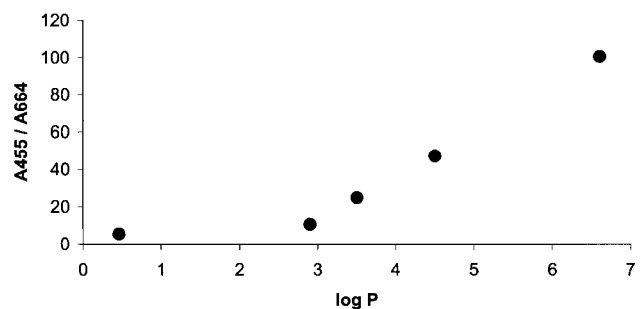


Figure 6. Ratio between absorbance at λ_{max} of β -carotene over absorbance at λ_{max} of chlorophyll in the spectra of extracted pigments from *Dunaliella salina* by different solvents.

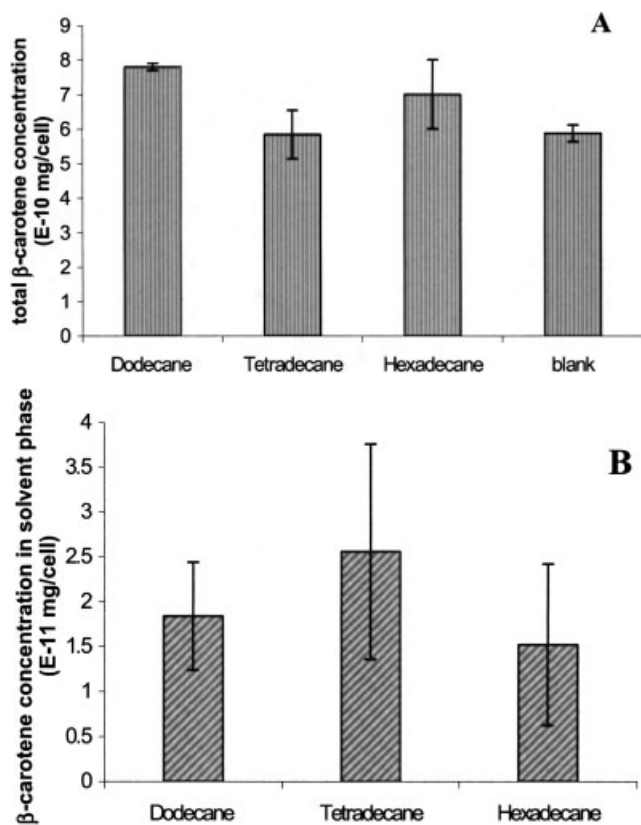


Figure 7. Total β -carotene produced by *Dunaliella salina* with different solvents under treatment (A) and extracted part of β -carotene by the organic phases (B). Error bars show 95% confidence interval.

dodecane (Fig. 7A). Figure 4 obviously shows that the chlorophyll content of all the samples is almost the same. But the ratio of absorbance at 455 (λ_{\max} of β -carotene) over absorbance at 664 (λ_{\max} of chlorophyll) for the cells in two-phase system is higher than in the blank samples. The ratio is 5.01, 4.48, 4.70, and 3.85 for dodecane, tetradecane, hexadecane, and blank samples, respectively. These data indicate that the relative concentration of β -carotene over chlorophyll for the cells growing in biphasic systems is higher than for the cells growing in blank samples. On the other hand, in the samples with second-organic phase, a part of the intracellular pigments, which mainly consist of β -carotene is extracted to the organic phase. Therefore, it can be concluded that the presence of a biocompatible solvent has no negative effect on the production of β -carotene by *Dunaliella salina* and even seems to induce β -carotene production.

It has been previously described that several parameters such as high salinity, high light intensity, and limitation of nutrients in the culture media can act as stress factors and induce carotenoid production by *Dunaliella salina* (Ben-Amotz, 1987; Cowan et al., 1992; Orset and Young, 2000). In fact, those stress factors have negative effects on the normal growth rate of the alga but it seems that biocompatible solvents may

induce carotenoids production without having significant effects on the growth of the alga.

Figure 7B shows the extracted part of β -carotene by the biocompatible solvents. It indicates that there is no significant difference between extraction ability of the different biocompatible solvents. However, relative β -carotene concentration of the cells and total β -carotene produced in a two-phase system with dodecane were significantly higher than blank samples (Fig. 7A).

CONCLUSION

Screening the viability and activity of *Dunaliella salina* in the presence of different organic phases indicates that cells remain viable and active in the presence of organic solvents with $\log P_{\text{octanol}} > 6$. This alga is thus more sensitive to the presence of organic solvents than mammalian, bacterial, and plant cells.

β -Carotene can be extracted more easily than chlorophyll by biocompatible solvents. Therefore, by using a biphasic system β -carotene of high purity can be produced from green active *Dunaliella salina*. It can further be concluded that the biocompatible organic phase has no negative effect on the production of β -carotene by *Dunaliella salina*. An appropriate solvent even could stimulate production.

We thank Dr. Carlos Vilchez, Dept. of Chemistry, University of Huelva, Spain, and Ir. Marcel Janssen for useful discussions.

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