

Milking of Microalgae:
Production and selective extraction of β -carotene in two-
phase bioreactors

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bioreactors**

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Chapter 1

General introduction

General introduction

Microalgae are photosynthetic microorganisms which utilize light energy and CO₂ for the production of high-value compounds. Although microalgae are a unique source for high-value compounds, their commercial application is still limited (Borowitzka, 1999). A major bottleneck for the application of most microalgae is the low productivity of the processes used up till now. One fundamental reason for this is the relatively low growth rate, mainly because of inefficient use of light (usually from solar illumination). Therefore, it takes a long time to just produce the biomass.

Dunaliella salina is a halotolerant unicellular microalga and has no rigid cell wall. Under stress conditions (e.g. high light intensity, high salinity and nutrient deficiency) it can produce and accumulate high concentrations of β -carotene (up to 10% of the dry weight) in oil globules in the cells (Ben-Amotz, 1995). β -Carotene is one of the important members of the family of carotenoids; a group of natural fat-soluble stereoisometric pigments. β -Carotene shows pro-vitamin A activity and as such it plays an important role in the human body (Chen et al., 1993). In addition, β -carotene (like other carotenoids) is a strong antioxidant, scavenging potentially harmful oxy radicals, which are commonly associated with the induction of certain cancers (Leach et al., 1998) and there is an inverse relation between the consumption of certain carotenoids and the risk of cancer (Chen et al., 1993). β -Carotene can be also used as a coloring agent. Therefore, β -carotene has several applications in food, pharmaceuticals and cosmetics. The great demand of β -carotene has been met by industry, mainly by synthetic production. Increasing demand for natural carotenoids has resulted in growing interest in extracting β -carotene from different natural sources (Vega et al., 1996). *Dunaliella salina* is the main source for the natural β -carotene in the market. The estimated market size for natural β -carotene is 10-100 tonnes.year⁻¹ and its price is >750 €.Kg⁻¹ (Pulz et al., 2001).

In a commercial production process, the cells of *Dunaliella salina* are first grown and then stressed to produce β -carotene. The orange cells which are rich in β -carotene are harvested, concentrated and subsequently disrupted. After that, an extraction process takes place using organic solvents. The extracted pigment is finally purified. It is also possible to use the concentrated biomass as a final product. In this case

drying is applied to produce β -carotene rich biomass, which can be utilized in the form of capsules or tablets (Schlipalius, 1991, Leach et al., 1998). Figure 1.1 shows a large commercial pond for cultivation of *Dunaliella salina* in Australia.



Figure 1.1: Large commercial ponds for cultivation of *Dunaliella salina* in Australia (Cognis Ltd.)

Most of the microalgal products are secondary metabolites which are produced when cell growth is limited. If a method can be applied in which the produced biomass is reused for production of metabolites, this can make the process more beneficial and can be a solution to overcome the low productivity. This method in which both production and extraction occur simultaneously can be called “milking” of microalgae. The aim of this thesis is to investigate the possible application of a milking process using two-phase bioreactors in microalgal biotechnology. Two-phase bioreactors consist of one aqueous phase in which cells are grown, and the second phase in which the product is extracted. Two-phase bioreactors consist of either two aqueous phases or an aqueous and an organic phase. When the extracted product is polar or has both polar and nonpolar parts the aqueous-aqueous two-phase bioreactors are applied (Zijlstra et al., 1996). When the target product is a nonpolar compound aqueous-organic two-phase bioreactors are applied (Vermuë and Tramper, 1995). In

both cases it is possible to keep the cells viable and extract the target product during the fermentation process.

By using two-phase bioreactors it would be possible to reach our goals: continuous simultaneous production and selective extraction of product from the microalgae cells which leads to higher productivity. In this technique the cells would stay viable and keep their product formation ability for a long time.

We have chosen β -carotene extraction from *Dunaliella salina* in a two-phase bioreactor with an aqueous phase and an organic solvent phase as our model system. This will give us a proof of principle for the production of a commercially interesting compound. The goal is to develop an alternative and more efficient process than the commercial production process of β -carotene.

Since β -carotene is a nonpolar compound an aqueous-organic two-phase bioreactor has to be used. Obviously the choice of the organic solvent is important. The applied organic solvent should be biocompatible for the cells and has to be able to extract β -carotene from the cells. Results of these investigations are shown in Chapter 2. The next step is to study the parameters which may influence the extraction rates. In Chapter 3 the effect of mixing rate on the extraction process is discussed while Chapter 4 focuses on the effect of light intensity on the extraction. The results of Chapter 2, 3 and 4 are combined in the milking process described in Chapter 5. In this chapter the two different stages for cell growth and β -carotene production are discussed and the productivity of milking process is also evaluated. Chapter 6 describes the mechanisms behind the selective extraction. Formation and location of β -carotene containing globules in the cells as well as the effect of solvent on the cell membrane are discussed in this chapter. In chapter 7 we highlight the opportunities of the “milking” process and we discuss the approaches which are helpful in answering two following fundamental questions: is it possible to milk any compound from any type of microalgae? And is it possible to produce enough product by means of this technique?

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Chapter 2

Selective extraction of carotenoids from the microalga *Dunaliella salina* with retention of viability

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 *D. 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.*

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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 *D. 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 2.1). 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 and 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 *D. 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 conventional 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 $\log P_{\text{octanol}}$ 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 $\log P_{\text{octanol}}$ 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 $\log P_{\text{octanol}}$ on the viability and β -carotene production by *D. salina*.

Table 2.1: Methods for β -carotene extraction from *D. salina*

<i>Breaking method</i>	<i>Extracting solvent</i>	<i>Reference</i>
Osmotic shock	Ethanol, Hexane, Cyclohexane and Benzene	Avron & Ben-Amotz (1980)
Thermal treatment	Halogenated , aliphatic or aromatic hydrocarbon	Ruegg (1984)
Homogenisation	Edible oil	Nonomuro (1987, (a),(b))
No additional breaking	Super-critical CO ₂	Leorenzo (1991)
Osmotic shock and mechanical method	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) ¹
Strong solvents	Acetone, Methanol and di-ethylether	Liang & Pang (1997) ²
Thermal treatment and organic solvent	Mixture of one acid ester and oil	Heidlas et al. (1998) ³

¹ From yeast (*Rhodotorula glutinis*)

² From alga *Spirulina*

³ From different natural sources including algae

In addition, it has been already reported that chlorophyll is heterogenically 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 *D. salina* by nonpolar organic solvents.

Materials and methods

Organism and medium

D. 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 3 M 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-litre bottle with 75 ml of culture medium was inoculated with 3 ml of a 3-weeks-old pure culture, containing about 150 cell / µl. After 24 hours, growth and activity of the cells were measured. Then, 10 ml of different solvents (Table 2.2), 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 µmol 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 ± 0.5°C and the two liquid phases in each bottle were stirred at 70 rpm.

Table 2.2: Physical properties of the used organic solvents

Solvent	$\log P_{\text{octanol}}$	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

Experiment design

Experiments were done in two steps. In the first step solvents with $\log P_{\text{octanol}}$ 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 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 non-volatile solvents evaporate in time, resulting in changes of experimental conditions. For that reason, bottles with a small opening ($D = 18 \text{ mm}$) 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 μ l, 0.56 M), and algal sample were added.

The cell number was determined by counting the cells using 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 (α = 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 μ L 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 $P_{octanol}$ 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 2.2. 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_{octanol} values of β -carotene and chlorophyll

Estimations of the log P_{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 colour of the culture media including the solvents with the log P_{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_{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 2.3).

Table 2.3: Viability of cells of *D. 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	+	+	+
Tetradecan	7.6	+	+	+
Hexadecan	8.8	+	+	+

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 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 sample 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 (Figure 2.1). According to Figure 2.1 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 method and by the slowing the physiological activity of the cells by the time.

In Figure 2.2 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 *D. 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 *D. salina* is shifted to $\log P = 6$.

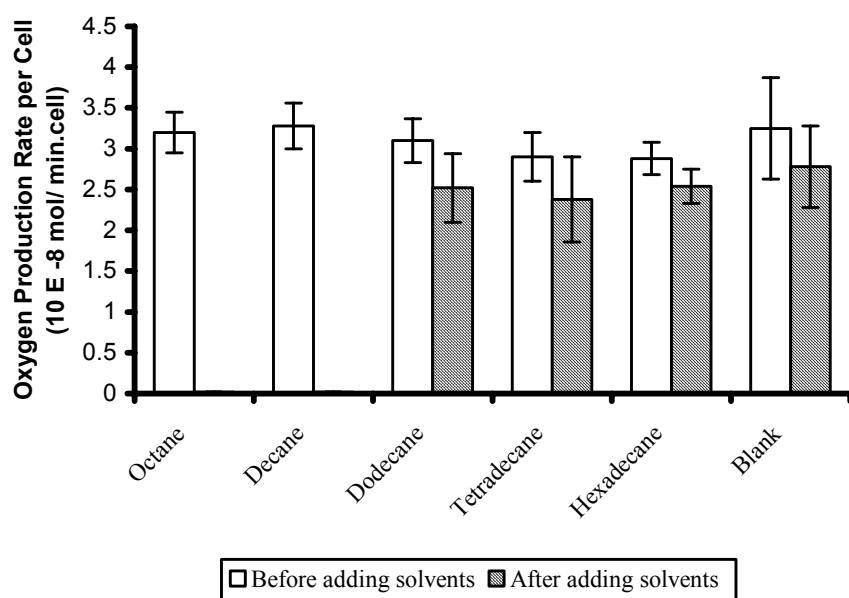


Figure 2.1: Oxygen production rate per cell for *D. salina* cells growing in the presence and absence of different organic solvents at different stages. Error bars show 95% confidence interval

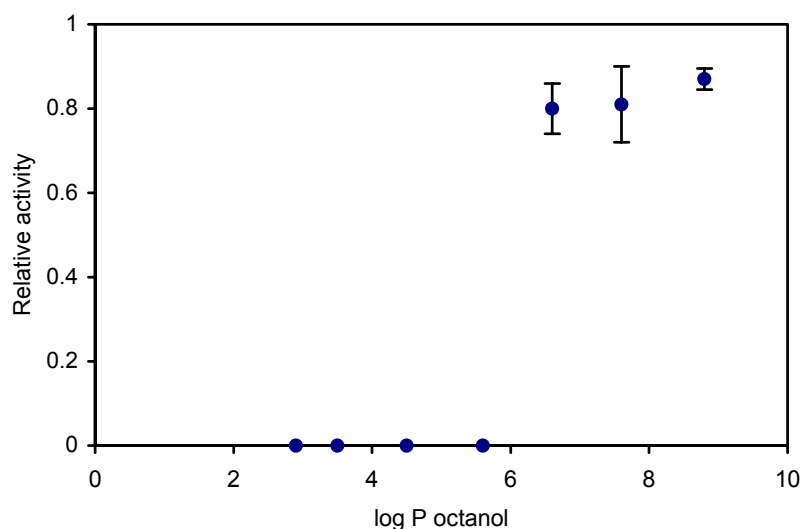


Figure 2.2: Activity of *D. salina* as a function of $\log P_{\text{octanol}}$ (Relative activity of the cells in the presence of the solvents was calculated by dividing the average activity of cells after addition of solvents by their average activity before addition of the solvents). Error bars show 95% confidence interval

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 *D. salina*, growing in aqueous phase in the different samples, are shown in Figure 2.3. 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 (Figure 2.4) are not comparable with the mentioned spectra of the cells in aqueous phase (Figure 2.3). 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 respectively 17.2 and 17.6, respectively.

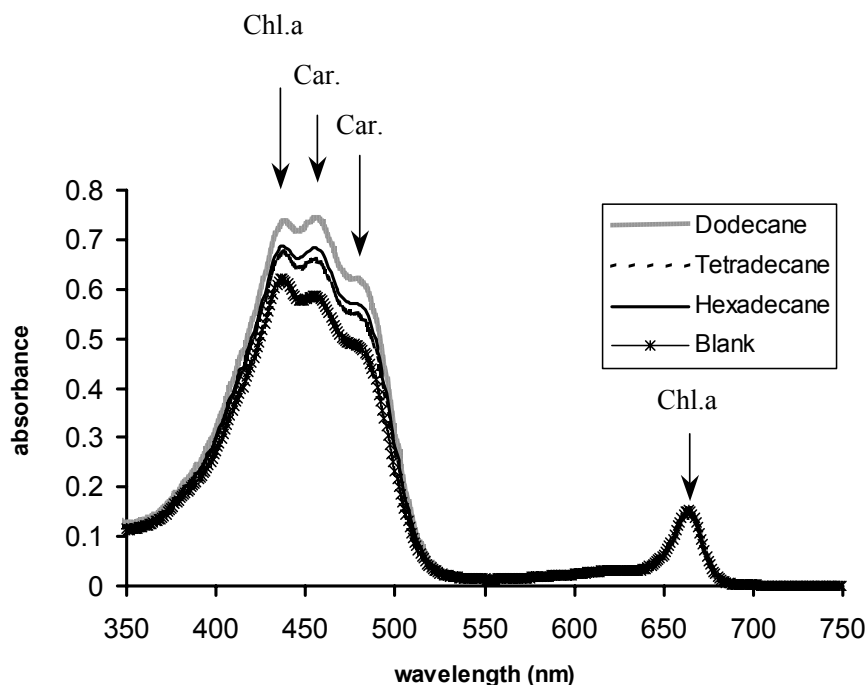


Figure 2.3: Spectra of the extracted pigments in THF from *D. salina* whole cells growing in the presence and absence of organic solvents

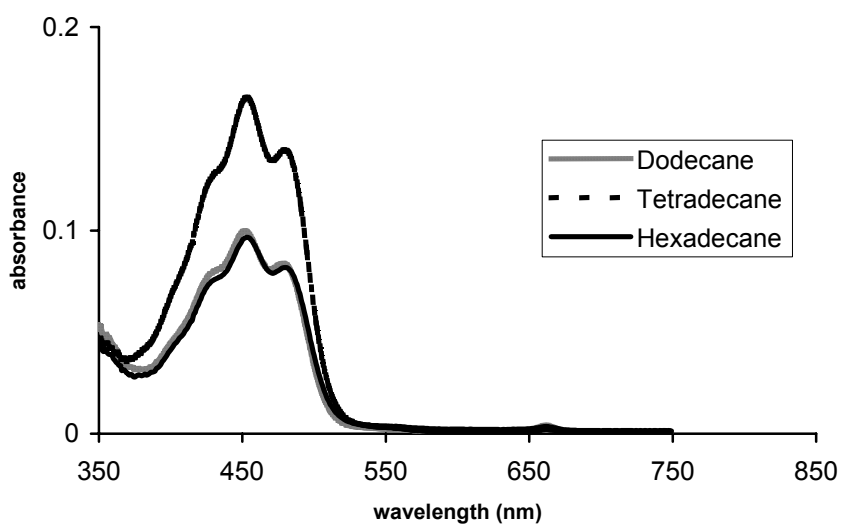


Figure 2.4: Spectra of the pigments extracted by biocompatible solvents in biphasic systems

According to the literature chlorophyll of plant cells is heterogenically 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 lyophilised wheat chloroplasts preferentially removed β -carotene and the far-red chlorophyll *a* forms (chlorophyll with weak hydrophobic bonds). Oquist and Samulsson (1980) reported that petroleum ether could extract only 3% of total chlorophyll of lyophilised 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 2.2 from the whole algal cells was investigated. Figure 2.5 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 4 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 2.6 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.

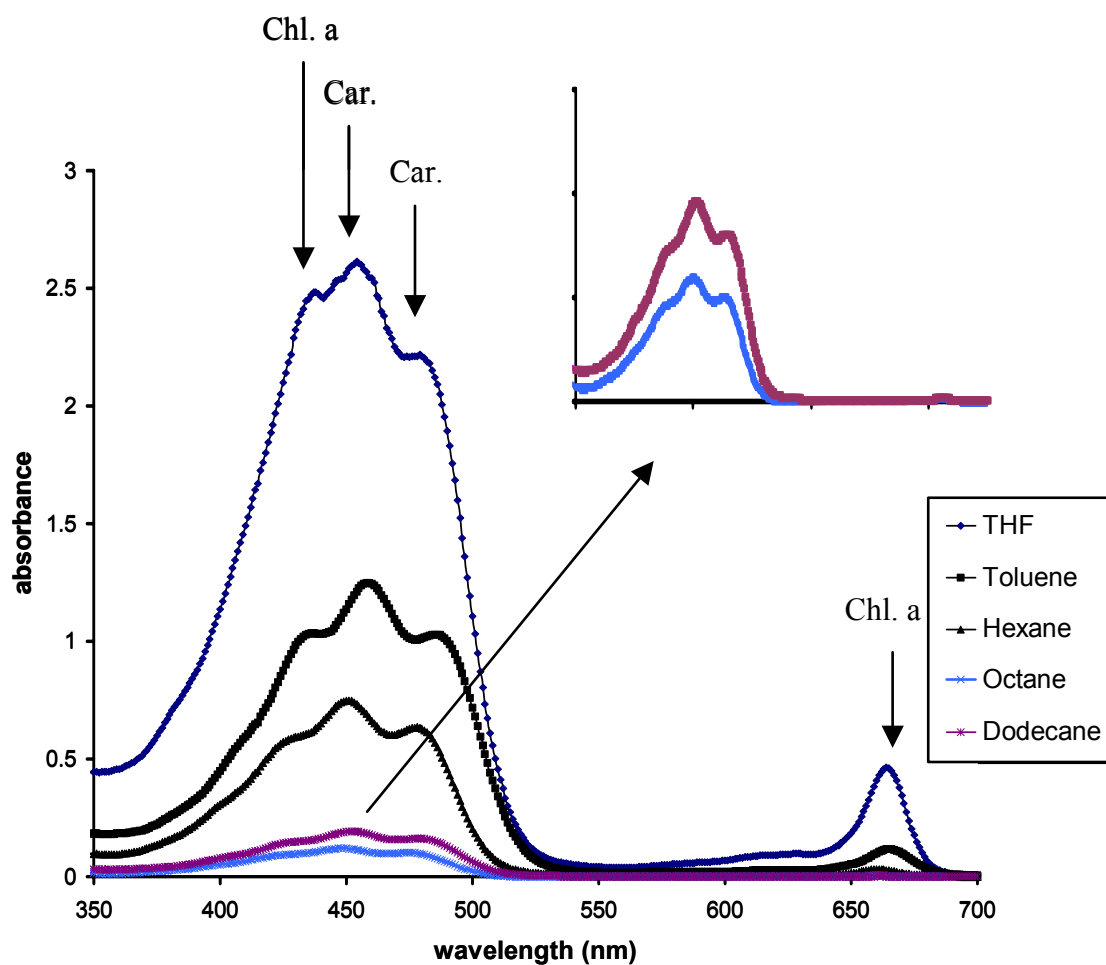


Figure 2.5: Spectra of extracted pigments from *D. salina* by different solvents

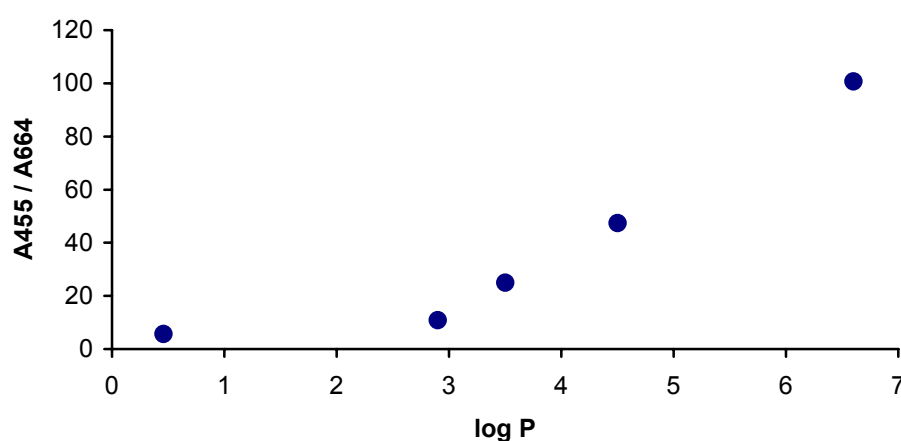


Figure 2.6: Ratio between absorbance at λ_{\max} of β -carotene over absorbance at λ_{\max} of chlorophyll in the spectra of extracted pigments from *D. salina* by different solvents

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 inactivation and breaking down of the cell membrane (Sikkema et al., 1995; Bassetti and Tramper, 1994). These solvents can break down the cell membrane and release more intracellular compounds 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 dodecane (Figure 2.7-A). Figure 2.4 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 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 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 *D. 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 *D. 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 2.7-B 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 (Figure 2.7-A).

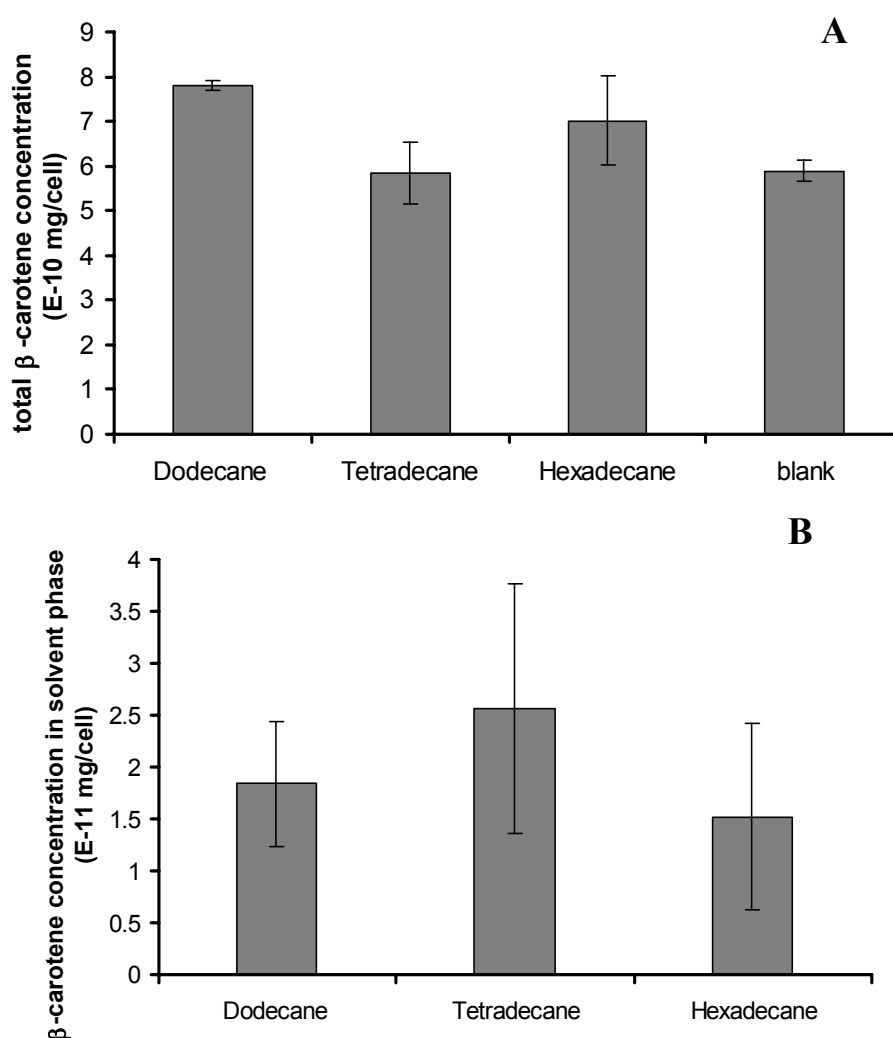


Figure 2.7: Total β -carotene produced by *D. salina* with different solvents under treatment (A) and extracted part of β -carotene by the organic phases (B). Error bars show 95% confidence interval

Conclusion

Screening the viability and activity of *D. 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 *D. salina*. It can further be concluded that the biocompatible organic phase has no negative effect on the production of β -carotene by *D. salina*. An appropriate solvent even could stimulate production.

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Chapter 3

Effect of mixing rate on β -carotene production and extraction from *Dunaliella salina* in two-phase bioreactors

Abstract:

*β -Carotene is applied in food, cosmetic, and pharmaceutical industries. *Dunaliella salina* is the main source for natural β -carotene in the market. The effect of mixing rate, which is supposed to lead to the facilitated release of β -carotene from the cells of *D. salina* in two-phase bioreactors, has been investigated. Three pairs of bioreactors were inoculated at the same time, operated at 100, 150 and 170 rounds per minute, respectively and illuminated with a light intensity of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each pair consisted of one bioreactor containing only aqueous phase for the blank and one containing the water phase together with dodecane, which is biocompatible for the cells. Comparison of the viability and growth of the cells grown under different agitation rates show that 170 rpm and 150 rpm are just as good as 100 rpm. Presence and absence of the organic phase has also no influence on the viability and growth of the cells. In Contrast to the growth rate, the extraction rate of β -carotene is influenced by the stirrer speed. The extraction rate increases at higher stirring rate. The effectiveness of extraction respect to the power in-put is comparable for all the applied mixing rates, even so it is slightly lower for 100 rpm than the others. The chlorophyll concentration in the organic phase remained very low during the experiment, although at higher mixing rates, chlorophyll impurity increased up to 3% (w/w) of the total extracted pigments. Comparison of carotenoid and chlorophyll shows that at 170 rpm, which shows the highest extraction for both pigments 0.5% of the chlorophyll and 6% of the carotenoid is extracted.*

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Introduction

β -Carotene is also known as pro-vitamin A. Besides obvious applications such as in vitamin pills, β -carotene is applied in food, cosmetic, and pharmaceutical industries as e.g. colorant, antioxidant, and anti-cancer agent (Leach et al., 1998). *D. salina* is the main source for natural β -carotene that is in the market. This unicellular green microalga can produce and accumulate β -carotene under stress conditions such as high light intensity, high salinity and nutrient deficiency. β -Carotene is a lipophilic product, which accumulates in oil droplets inside the chloroplast of the cells under stress conditions up to a concentration of 10% of its dry weight (Ben-Amotz, 1995).

Application of two-phase water organic bioreactors for extractive fermentation has shown promising results (Leon et al., 1998). For successful application of these two-phase bioreactors, several factors have to be considered. Obviously, not only accumulation inside the cells is required but also preferably intra-cellular compound should be excreted by the cells in order to facilitate product recovery (Buitelaar et al., 1991). It should be also taken in to the account that extraction of intra-cellular products, like β -carotene, should not adversely affect the culture's metabolic activity (Payne et al., 1987).

In our previous work, we investigated the effect of various organic solvents on *D. salina* and found that *D. salina* stays active and viable in the presence of organic solvents with logP values > 6. Further, for dodecane (logP = 6.6) we found that phase separation was rapid. In addition, β -carotene of high purity was obtained; chlorophyll was hardly extracted to dodecane. We found that most of the β -carotene stayed in the cells and was not extracted (Hejazi et al., 2002). The overall productivity in the two-phase bioreactor can be increased considerably if the produced β -carotene is also extracted. Therefore, we investigated the factors that could lead to facilitated release of β -carotene to the organic phase in more detail.

Different mechanisms can be responsible for extraction of intra-cellular products like carotenoid from the cells. Croughan and co-workers (1987) investigated shear caused by mixing and reported that shear did influence extraction. Sikkema and co-workers (1995) found that the organic phase penetrated through the lipid bilayer of the cell membrane by diffusion. The product was released from the cells to the media

as a result of changes in the cell membrane in the presence of the organic phase (Sikkema et al., 1995). In our case, solubilities of dodecane, the oil droplets, and β -carotene in water are very low. Therefore, it seems probable that direct contact between the cells and organic phase is required for faster extraction of β -carotene. Mixing will apply some shear stress to the cells. At the same time, mixing of the media can improve contact of the cells with the organic phase. Therefore, we investigated cell growth and viability of *D. salina* in the absence and presence of an organic phase. Further, we varied stirrer speed in order to enhance extraction. The data that we obtained for the various experiments were related to the product quality (extraction of β -carotene versus chlorophyll extraction)

Materials and methods

Organism and medium

D. salina (CCAP 19/18) was grown in a culture medium containing 1M NaCl, 10 mM KNO₃, 1 mM NaH₂PO₄·2H₂O. Further, 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 added. The pH of the medium after addition of 100 mmol of Tris-buffer was adjusted to 7.5 with 3 M HCl. The medium was sterilized at 121°C for 40 minutes before inoculation. To avoid precipitation, the phosphate was autoclaved separately. Daily, 5 mM of carbon source (NaHCO₃) was added in order to avoid limitation of carbon source. For sterilization, solid NaHCO₃ was put in an oven over night at 120 °C and then mixed with sterilized water.

Experiment set up

One-liter bottles containing 300 ml of culture media have been inoculated with 20 ml of three-week-old pure cultures. After 24 hours, the cell number and viability was determined. Subsequently, the second organic phase (dodecane) was added. The bioreactor contained 80% of aqueous phase and 20% of organic phase. The bottles

were illuminated with fluorescent lamps (SYLVANIA CF-EL 55W/840) with a light intensity of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ from the bottom side. Mixing of the culture media took place with two different mixers that were fixed on one axis. One magnetic stirrer (diameter 6.0 cm, bar width 1.0 cm) was placed at about 0.6 cm from the bottom of the vessel in the aqueous phase. The other six-bladed turbine stirrer (diameter 3 cm, paddle width and height 0.5 cm) is placed in the interface between the water phase and the dodecane phase. Three pairs of bioreactors were inoculated at the same time and operated at 100, 150 and 170 rounds per minute, respectively. Each pair consisted of one bioreactor containing only aqueous phase for the blank and one containing the water phase together with dodecane. The reaction temperature was 26 ± 1 °C. The pH of the culture media was monitored on-line and controlled by addition of 3 M HCl. The pH remained between 7.5 and 8 throughout the experiments.

Calculation of hydrodynamic stress parameters

Three important parameters to quantify hydrodynamic stress generated by an impeller are the tip speed, the volumetric power input, and the shear rate. All the equations required for the calculation of these parameters are given in the appendix. We calculated the aforementioned parameters for our own experiment and for an experiment performed by Yang and co-workers (1992) with another type of fragile alga, *Ochromonas malhamensis*.

Cell number

The cell number was determined by direct counting, using a light microscope (magnification $\times 400$) with an 0.1 mm deep counting chamber (Neubauer improved). More details can be found in Hejazi et al.(2002).

Viability of the cells

Viability of the cells was determined by flowcytometry (FACSCAN, BECTON DICKINSON BV). The algal cells that contain chlorophyll show red fluorescence. When the cells are treated with FDA (Fluorescein Diacetate: in the membrane of the living cells FDA is cleaved by nonspecific estereases into a fluorescein) fluorescence green is formed and two acetates (Jochem, 1999). Therefore, the living cells show green fluorescence upon exposure to FDA and the dead cells remain red. We found that the incubation time for FDA to enter the cells of *D. salina* is 5 minutes and after this measurement could take place. We added 50 μ l of FDA solution (1 mg FDA per ml of acetone) to 1 ml of cell suspension and fluorescence was measured after 5 minutes.

β -Carotene and chlorophyll concentrations

β -Carotene and chlorophyll concentrations were determined spectrophotometrically using standard curves. Sample preparation before detection was carried out as described in our previous publication (Hejazi et al., 2002). A sample of 1 ml was taken from the aqueous phase that had been mixed thoroughly. After 5 minutes centrifugation at 4000 rpm the upper phase was decanted and 2 ml of THF (tetrahydrofuran) was added to the biomass. Each sample was mixed by vortex for 1-2 minutes to reach complete extraction. Samples then were centrifuged again for 5 minutes at 4000 rpm for separation of the biomass (now colourless) and the solvent phase. The extracted pigments in the solvent phase were quantified by the spectrophotometric method.

From organic phase 2 ml samples were also taken and pigments concentrations directly determined by the spectrophotometer.

β -Carotene degradation rate

We filled two series of 15-ml glass tubes with 10 ml of dodecane with known amounts of β -carotene, and closed them. The first set of the tubes was covered with aluminum

foil and the second set was left without any cover. The tubes were illuminated ($700 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 26 ± 1 °C and the β -carotene concentration was measured as a function of time. Degradation of β -carotene was followed for up to 14 days.

Results and discussions

Cell growth and viability in the presence and absence of the second phase

To investigate the effect of mixing rate on the cells, their viability and growth was followed regularly. Figure 3.1 shows an example of the flowcytometry results. Figure 3.1A shows the natural fluorescence of the cells grown under the highest mixing rate (170 rpm) at the first day of the experiment and before addition of the dodecane phase. Only one population of cells with high FL3 (auto-fluorescence) and low FL1 (FDA related fluorescence) was detected. After addition of FDA (Figure 3.1B) most of the cells (>95%) show high FL1, which means that the cells are viable. A low percentage of the cells (<2%) showed high FL3 but low FL1, which indicates that these cells are dead. After two-weeks of incubation, still more than 90% of the cells was viable (Figure 3.1C). Comparison of the viability of the cells grown under different agitation rates show that 170 rpm and 150 rpm are just as good as 100 rpm (the results are not shown). Therefore, dodecane and agitation rate in the applied range have no detrimental influence on the cells.

We tested the effect of stirring speed and the presence of dodecane on cell growth (Figure 3.2) and we found no difference. Clearly, these stirring speeds and the resulting shear rates in the absence and presence of dodecane are not thus high that cell growth is negatively influenced. Some data are available from literature with respect to shear sensitivity of algal cells. Markl and co-workers (1991) cultivated *Chlamydomonas reinhardtii* in tank reactors at stirrer speeds of 300 – 2400 rpm. They found that the growth rate of wild-type cells was not affected but the cell-wall-lacking cells could not be cultivated at speeds greater than 1200 rpm. Inactivation of the cells of a fragile alga *Ochromonas malhamensis* in the presence of mechanical agitation was also investigated (Yang et al., 1992). They found out that the critical mixing rate

for the alga is 350 rpm, and at lower mixing rates, they did not observe any negative shear effect on the growth of the algal cells.

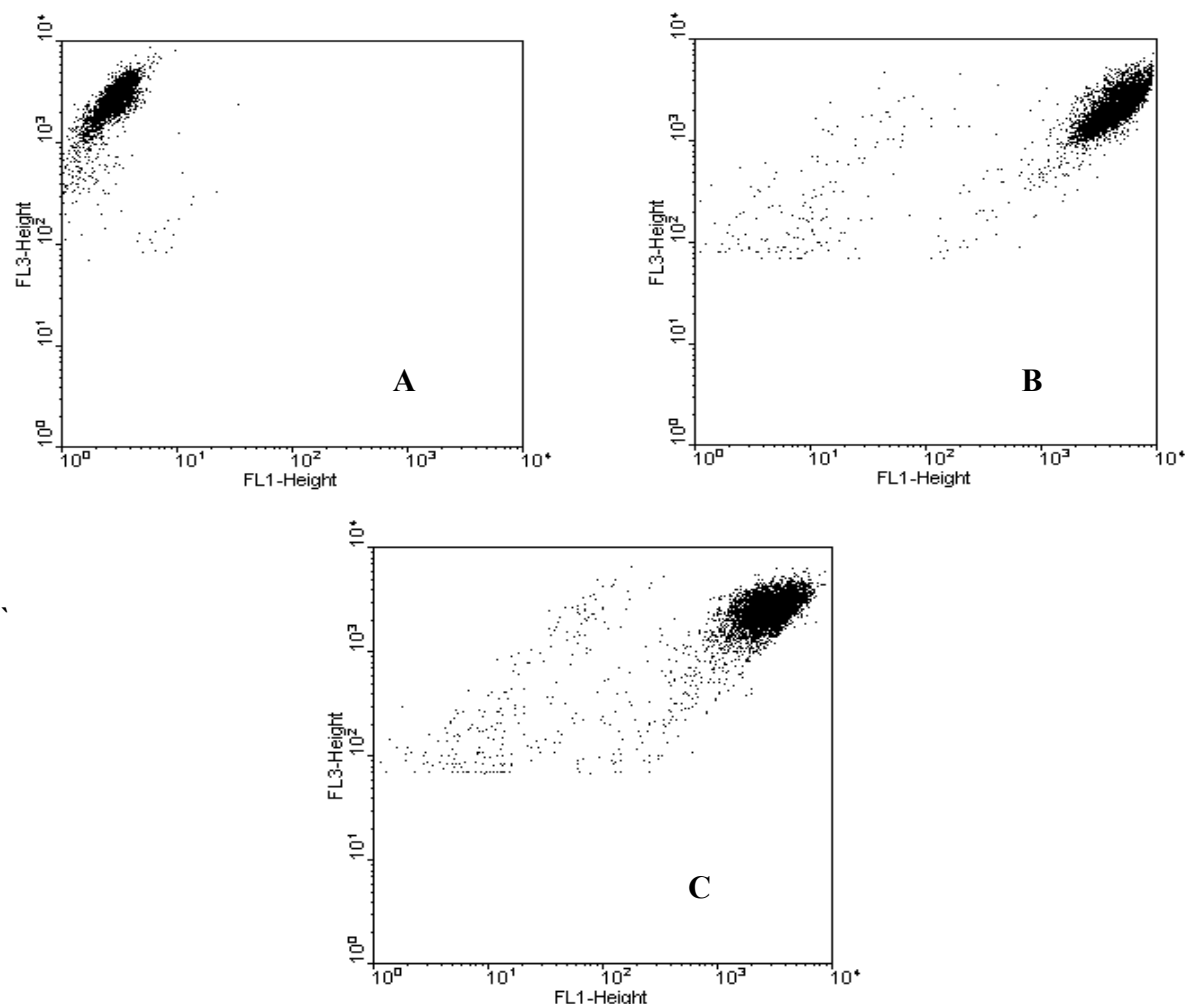


Figure 3.1: Examples of the FCM analysis of the viability of the cells of *D. salina* grown in a two-phase bioreactor under the highest mixing rate (170 rpm), A: natural fluorescence of the cells at the first day, B: fluorescence of the cells at the first day after addition of FDA, C: fluorescence of the cells at the last day after addition of FDA

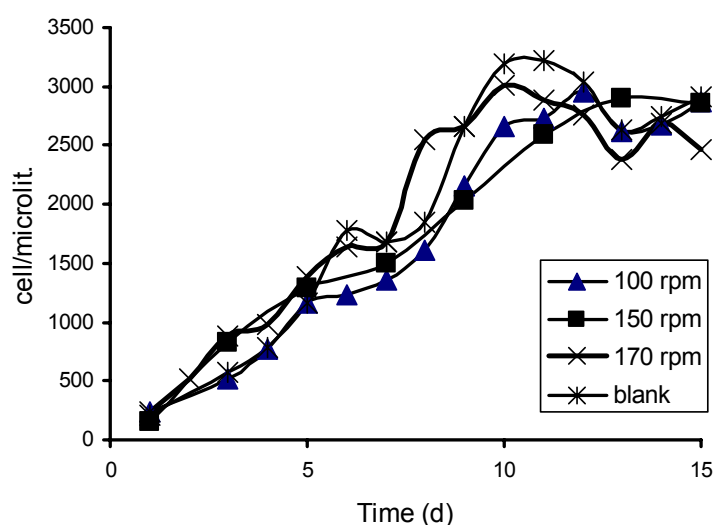


Figure 3.2: Growth of *D. salina* in two-phase bioreactors under different mixing rates. The cells in the blank bioreactor were grown in the absence of the organic phase and stirred at 100 rpm

We calculated the hydrodynamic stress factors for the bioreactors that were used in Yang et al (1992) and compared those with our study (Table 3.1 in the appendix). We found that the factors that we calculated for our experiments were considerably lower than the ones calculated for the literature data. This indicates that the shear stresses that we applied were considerably less than those reported to have negative effects on the cells. Although comparison is difficult because different alga were used, it seems reasonable to assume that we chose our stirrer speeds in such a way that we did not influence the cells in a negative way. This could also be concluded from the β -carotene concentration of the aqueous phase. For all mixing rates, the same concentration was found, this would not be expected if cell break-up occurred.

β -Carotene extraction

Figure 3.3 shows that the extraction rate of β -carotene was influenced by the stirrer speed. The extraction rate increased at higher stirring rates. As stated before the viability and growth of the cells was not influenced by the increase in stirring rate. Therefore, the higher extraction of β -carotene was not due to the cell death. The extracted amount of β -carotene per amount of the cell in the last day of the

experiment was: 0.7, 0.4, and 0.1 pg for 170, 150 and 100 rpm respectively. This effect can be caused either by the solvent molecules, dissolved in water-phase, or by the direct contact between the cells and organic phase. According to Sikkema and co-workers (1995) apolar compounds such as cyclic hydrocarbons can easily penetrate the lipid bilayer of the cell membrane. These lipophilic compounds may accumulate in the lipid bilayer resulting in expansion of the membrane, which helps the liberation of intra-cellular compounds. However, the solubility of the β -carotene in the aqueous phase is very low and in non mixed media the extraction rate of β -carotene is limited. When mixing is applied the cells are directly contacted with the organic phase and a bridge between the lipophilic compounds inside the cells and organic phase can be made. This phenomenon may explain the results that we obtained.

Mixing also facilitates extraction because it causes shear stress to the cells. Although excessive fluid shear can be detrimental, moderate fluid shear can lead to increased cell permeability (Croughan et al., 1987). This could also explain our results.

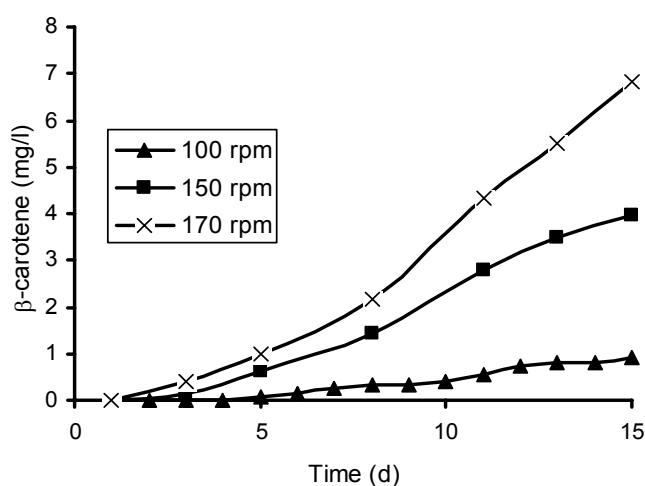


Figure 3.3: β -carotene extraction from *D. salina* in two-phase bioreactors in response to the mixing rate

According to literature, light is an important factor in β -carotene degradation (deMan, 1990) and we applied relatively strong light for illumination of the bioreactor. We determined the degradation rate of the β -carotene for our experiment conditions. The

results (Figure 3.4) indicate that after seven days all the β -carotene was degraded inside the tubes without any cover. For the tubes with the cover, the degradation rate was very low and after two weeks 90% of the β -carotene remained. This means that the actual extracted concentration of β -carotene was higher than the concentrations shown in Figure 3.3.

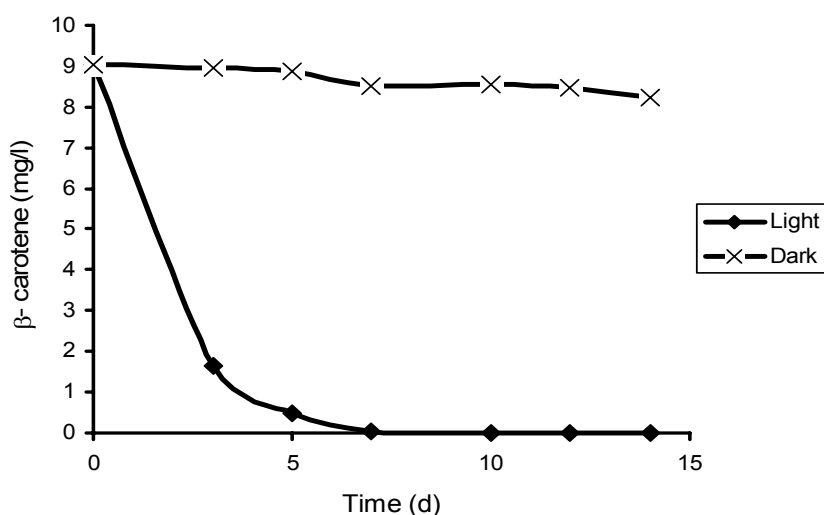


Figure 3.4: β -carotene degradation as a function of time

Relation between power in-put and β -carotene extraction

If we want to optimize the amount of energy required for extraction, the data in Figure 3.3 have to be divided by the amount of energy that was put in (Figure 3.5). The effectiveness of extraction respect to the power in-put is comparable for all the applied mixing rates, even so it is slightly lower for 100 rpm than the others. This could be because of the higher β -carotene production by the cells for higher mixing rates (see the next paragraph). These results show that increasing the stirrer speed within the range where viability is not influenced leads to a more effective extraction.

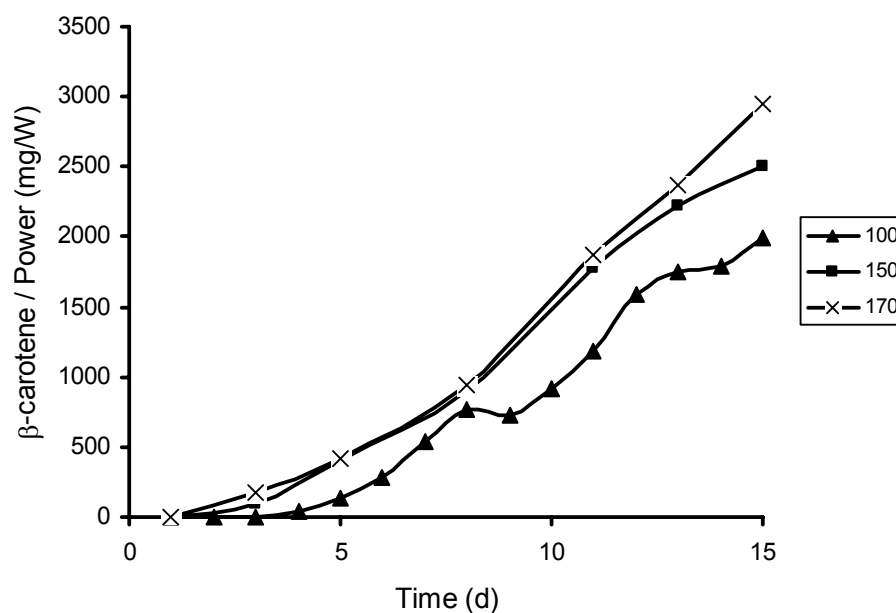


Figure 3,5: β -carotene extraction from *D. salina* in two-phase bioreactors per unit of power in put

β -Carotene versus chlorophyll concentration in aqueous phase

To determine the effect of extraction on the pigment composition of the cells, we investigated the concentration of β -carotene and chlorophyll (Figure 3.6). For each mixing rate, the relative concentration of β -carotene over chlorophyll was higher for the cells growing in the presence of organic phase than for the cells growing in the absence of organic phase. The chlorophyll production by the cells was not affected by the presence of organic phase; therefore, it can be concluded that more β -carotene is produced in the presence of the organic phase.

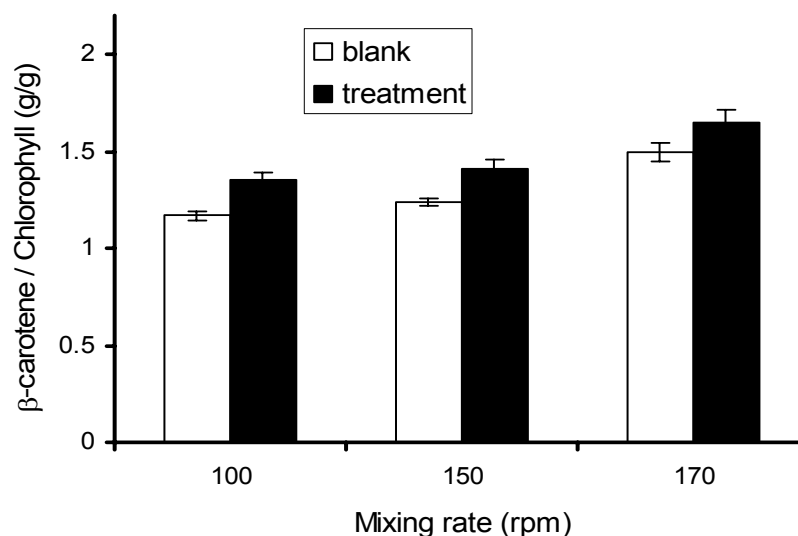


Figure 3.6: Relative concentration of β -carotene over chlorophyll in the cells of *D. salina* grown in two-phase bioreactors under different mixing rates. Error bars show 95% confidence interval of triplicate samples taken from the bioreactors

It is known that when the cells are under stress (e.g. high light intensity) the size of the photosystem, and therewith, the chlorophyll concentration of the cells decreases. This causes the higher ratio of β -carotene over chlorophyll compared to normal growth conditions. The results of our experiments (growth and viability, and chlorophyll and β -carotene production by the cells under different situations) suggest that higher production of β -carotene is caused by the continuous extraction of the β -carotene. The presence of the biocompatible solvents is not considered a stress factor. The former conclusion is confirmed by the fact that the total amount of β -carotene produced (β -carotene content of the cells + extracted part to the organic phase) is highest for the highest mixing rate (170 rpm). For 100 and 150 rpm these concentrations are significantly lower (Figure 3.7).

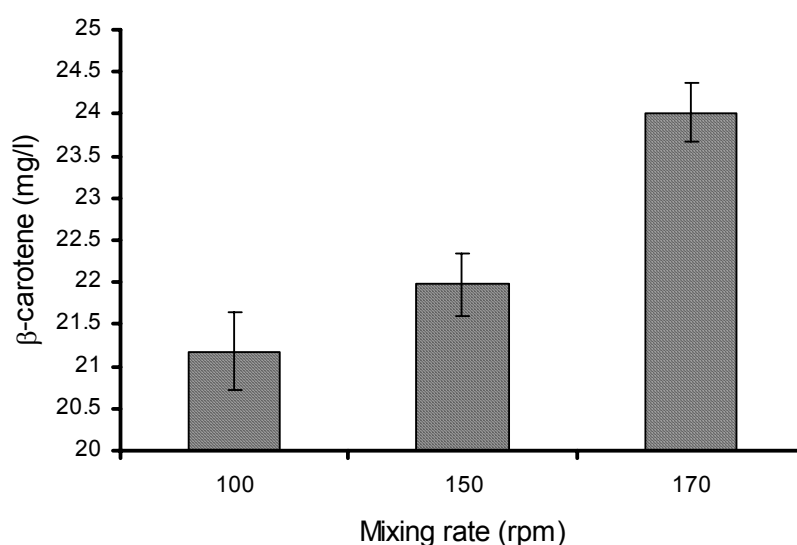


Figure 3.7: Total β -carotene produced by the cells of *D. salina* in two-phase bioreactors after 15 d of inoculation. Error bars show 95% confidence interval of triplicate samples taken from the bioreactors

β -Carotene versus chlorophyll concentration in organic phase

The chlorophyll concentration in the organic phase remained very low during the experiment although at higher mixing rates, chlorophyll impurity increased up to 3% (w/w) of the total extracted pigments. Comparison of β -carotene and chlorophyll showed that at 170 rpm, which showed the highest extraction for both pigments 0.5% of the chlorophyll and 6% of the β -carotene was extracted. This amount could be increased considerably by increasing the surface area that is available for extraction.

Conclusion

It is concluded from the results that an increase in mixing rate, in the applied range, does not have any negative influence on the cell growth and activity. The increase in mixing rate, however, enhances the extraction and production rate of the β -carotene. Increase in the β -carotene extraction rate and efficiency was accompanied by a slight increase in chlorophyll extraction rate but the maximum impurity was 3% of W/W total extracted pigments.

Acknowledgement

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Appendix

Three important parameters to quantify hydrodynamic stress generated by an impeller are the tip speed, the volumetric power input, and the shear rate.

The impeller tip speed v_i can be calculated from the agitation speed N and the impeller diameter D_i :

$$v_i = \pi \cdot N \cdot D_i$$

The volumetric power input P/V of a non-gassed stirred system is represented by:

$$P/V = \rho_L \cdot N_P \cdot N^3 \cdot D_i^5 / V_L$$

In which ρ_L is the fluid density, N_P is the power number, and V_L is the liquid volume. The power number is dependent on the type of impeller, system geometry, and Reynolds number Re :

$$N_P = f(Re), Re = D_i^2 \cdot N \cdot \rho_L / \mu_L$$

In which μ_L is the liquid dynamic viscosity. N_P for a six-blade turbine impeller can be calculated using the standard graph of power number of impeller as a function of Reynolds number (Janssen et al., 1987).

The time-averaged average and maximum shear rate (γ_{ave} , γ_{max}) generated by a six-blade turbine impeller in a Newtonian fluid can be calculated by:

$$\gamma_{ave} = 4.2 \cdot N \cdot (D_i/T)^{0.3} \cdot (D_i/W)$$

$$\gamma_{max} = 9.7 \cdot N \cdot (D_i/T)^{0.3} \cdot (D_i/W)$$

in which T is the vessel diameter and W is the impeller blade width. The resulting shear stress σ can be calculated as:

$$\sigma = \gamma \cdot \mu_L$$

Through the motion of turbulent eddies, hydrodynamic forces with a duration on the order of milliseconds arise which generate much higher stresses than time-averaged shear. The microscale stress rate γ_{ms} can be estimated as:

$$\gamma_{ms} = v_e / l_e = (P/V)^{0.5} \cdot (\rho_L / \mu_L)^{0.5}$$

in which v_e is the eddy velocity and l_e is the smallest eddy size.

Table I shows the calculated shear parameters for our own experiment and the experiment of Yang & Wang (1992).

Table 3.1: calculated shear factors for our experiment and the experiment of Yang & Wang (1992)

Experiment	Mixing rate (rpm)	$\gamma \text{ (s}^{-1}\text{)}$		$\sigma \text{ (N m}^{-2}\text{)} \times 10^{-2}$	
		ave	max	ave	max
Our experiment	100	1.56	3.61	1.34	3.10
	150	2.35	5.42	2.01	4.65
	170	2.66	6.14	2.28	5.26
Yang & Wang (1992)	300	8.28		7.10	
	350	19.13		16.40	
		9.67		8.28	
		22.32		19.10	

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Chapter 4

Effect of light intensity on β -carotene production and extraction from *Dunaliella salina* in two-phase bioreactors

Abstract:

*Application of two-phase bioreactors is a useful technique for improvement of the productivity of fermentations. Fermentative extraction of the products in situ is performed in this technique. The effect of light intensity on the extraction of β -carotene from *Dunaliella salina*, in the fermentative extraction, has been investigated. Three different average light exposures were applied: $1.5 \cdot 10^{-8}$ (low), $2.7 \cdot 10^{-8}$ (intermediate) and $4.5 \cdot 10^{-8}$ (high) $\mu\text{mol s}^{-1}\text{cell}^{-1}$. Results show that β -carotene content of the cells increases by increasing the light exposure. Increase in the β -carotene content of the cells is not necessarily coupled with an increase in the volumetric production of β -carotene. Final volumetric production is about the same for the three bioreactors. β -Carotene extraction rate is enhanced by the increase in the light exposure. The results suggest that extraction rate is related to β -carotene content of the cells and is not essentially related to the volumetric production of β -carotene. Although the effectiveness of extraction with respect to the light input is comparable for all light intensities applied, increasing the light input per cell leads to a higher volumetric extraction rate. Moreover extracted β -carotene stays very pure even so the extraction increased by the increase of light intensity.*

This chapter has been accepted for publication in Biomolecular Engineering as: Hejazi M A, and Wijffels R. H., Effect of light intensity on β -carotene production and extraction from *Dunaliella salina* in two-phase bioreactors

Introduction

Application of fermentative extraction is a useful technique for improvement of the productivity of fermentations (Leon et al., 1998). For successful application of two-phase bioreactors however, several requirements should be met. In order to facilitate product recovery high production of the target product by the cells and rapid release of the product into the organic phase are required (Buitelaar et al., 1991).

β -Carotene is a lipophilic high-value compound. It is considered as pro-vitamin A, and has also different applications in food, cosmetic, and pharmaceutical industries as a colorant, antioxidant, and anti-cancer agent (Leach et al., 1998). β -Carotene is accumulated in oil droplets inside the cells of *Dunaliella salina* under stress conditions up to the concentration of 10% of dry weight (Ben-Amotz, 1995).

Leon and co-workers (2001) studied organic solvent toxicity in photoautotrophic unicellular microorganisms. They reported that activity of *D. salina* in the presence of organic solvents having log P value higher than 5.6 is similar to the activity in the absence of solvent after 15 min. The parameter log P is a measure for hydrophobicity of a compound. In our previous work, we investigated the possible application of two-phase bioreactors in fermentative extraction of β -carotene by *D. salina*. We studied the effect of various organic solvents, having different log P values, for longer time (7 days). It was shown that *D. salina* stays active and viable in the presence of organic solvents with log P > 6 (Hejazi et al., 2002). Furthermore, we studied the effect of the mixing rate on the extraction of β -carotene and showed that increase of the mixing rate in the applied range enhances the extraction with no harmful effect on the cells (Hejazi et al., in press).

As we mentioned in the first paragraph for successful application of the fermentative extraction the extraction rate should be high. In addition to the mixing rate the extraction can be influenced by some other factors. The interface of aqueous/organic phases (geometry of the bioreactor), the cell concentration, and β -carotene concentration of the cells are important variables. A change in the amount of light per amount of cells results in different concentrations of β -carotene per cell. The cells which receive more light produce more β -carotene (Ben-Amotz, 1993). Changes in

biomass concentration and β -carotene content of the cells may also influence the volumetric β -carotene production.

Therefore, it is important to investigate the effect of light intensity per cell and monitor its effect on β -carotene content of cells of *D. salina* and the volumetric production as well as the extraction rate.

This paper reveals the effect of changes in the light intensity per cell on above-mentioned parameters.

Materials and methods

Organism and medium

D. salina (CCAP 19/18) was grown in a culture medium containing 1M NaCl, 10 mM KNO₃, 1 mM NaH₂PO₄·2H₂O. Further, 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 added. The pH of the medium after addition of 100 mmol of Tris-buffer was adjusted to 7.5 with 3 M HCl. The medium was sterilized at 121°C for 40 minutes before inoculation. To avoid precipitation, the phosphate was autoclaved separately. Daily, 5 mM of carbon source (NaHCO₃) was added in order to avoid carbon limitation. For sterilization, solid NaHCO₃ was put in an oven over night at 120 °C and then mixed with sterilized water.

Experimental setup

One-liter bottles (ϕ =7.8 cm) containing 300 ml of culture media have been inoculated with pure cultures. After 24 hour, the cell number and viability was determined. Subsequently, the organic phase (dodecane) was added. The bioreactor contained 80% of aqueous phase and 20% of organic solvent phase. The organic solvent phase was protected against the light by covering it with the aluminum foil. The bottles were illuminated with fluorescent lamps (SYLVANIA CF-EL 55W/840) with three different light intensities ranging from 850 to 1150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The light

intensity was determined with a Li SA-190, 2- π Quantum sensor (Li Cor, USA). Mixing of the cultures took place with two different mixers that were fixed on one axis. One magnetic stirrer (diameter 6.0 cm, bar width 1.0 cm) was placed at about 0.6 cm from the bottom of the vessel in the aqueous phase. The other six-blade-turbine stirrer (diameter 3 cm, paddle width and height 0.5 cm) was placed in the interface between the water phase and the dodecane phase. Three pairs of bioreactors were inoculated at the same time. Each pair consisted of one bioreactor containing only aqueous phase for the blank and one containing the water phase together with dodecane. The blank samples were used as control and data from these samples are not shown. The reaction temperature was 26 ± 1 °C. The pH of the culture media was monitored on-line and controlled by addition of 3 M HCl. The pH remained between 7.5 and 8 throughout the experiments.

Cell number

The cell number was determined by direct counting, using a light microscope (magnification $\times 400$) with a 0.1 mm deep counting chamber (Neubauer improved). More details can be found in Hejazi et al. (2002).

Calculation of light intensity per amount of cells

The light intensity ($\mu\text{mol s}^{-1}\text{m}^{-2}$) was measured regularly using above-mentioned light meter. The illuminated surface of the bioreactor was calculated using geometrical parameters of the bioreactors. Then, the total amount of light falling on the bioreactor surface was computed by multiplying the surface area by the measured light intensities ($\mu\text{mol s}^{-1}$). By dividing the total amount of light falling on the bioreactor by the cell concentration the amount of available light per amount of cells was determined ($\mu\text{mol s}^{-1}\text{cell}^{-1}$). During incubation the average light intensity per cell for each bioreactor was calculated. Three different average light exposures were applied: 1.5×10^{-8} (low), 2.7×10^{-8} (intermediate) and 4.5×10^{-8} (high) $\mu\text{mol s}^{-1}\text{cell}^{-1}$.

β -Carotene and chlorophyll concentrations

β -Carotene and chlorophyll concentrations were determined spectrophotometrically using standard curves. Sample preparation before detection was carried out as previously described (Hejazi et al., 2002). A sample of 1 ml was taken from the aqueous phase that had been mixed thoroughly. After 5 minutes centrifugation at 4000 rpm the upper phase was decanted and 2 ml of THF (tetrahydrofuran) was added to the biomass. Each sample was mixed by vortex for 1-2 minutes to reach complete extraction. Samples then were centrifuged again for 5 minutes at 4000 rpm for separation of the biomass (now colourless) and the solvent phase. The extracted pigments in the solvent phase were quantified by the spectrophotometric method. From the organic phase 2 ml samples were also taken and pigments concentrations directly determined by the spectrophotometer.

Results and discussion

β -Carotene production by the cells

Figure 4.1 shows the volumetric production of β -carotene in the three bioreactors at three different light intensities as a function of time. The volumetric production for the intermediate and high light intensities followed about the same pattern. Volumetric production for the low light intensity from day 3 till day 15 was higher than the others but on the last day of the experiment it became even lower.

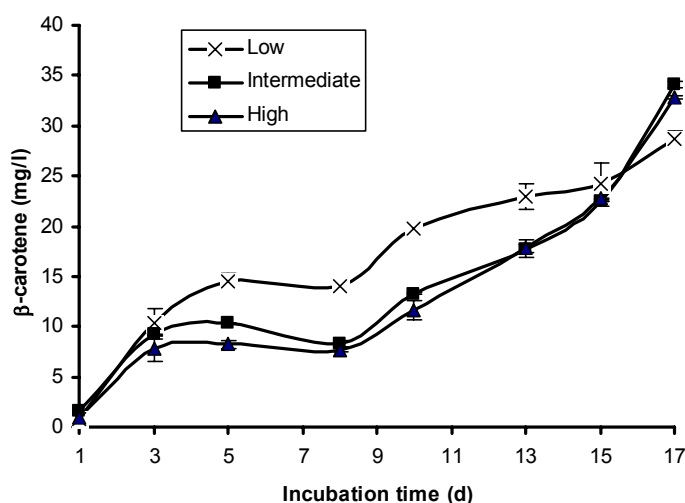


Figure 4.1: Volumetric β -carotene production (based on total volume, aqueous phase + organic phase) in the cultures of *D. salina* as a function of time under three different light intensities. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

The β -carotene content of the cells increased by increasing the light exposure (Figure 4.2). During the first 3 days the cells produced more β -carotene as a result of low cell concentration and therefore high light intensity per cell. Later on, when the cells started to grow, the β -carotene content of the cells declined until day 9 and again started to increase for the intermediate and high light intensities afterwards. At low light intensity no increase of β -carotene content of the cells was observed after day 9. The volumetric production and β -carotene content of the cells followed different patterns due to the differences in the cell growth rate at three different light exposures. Growth rate was the highest at low light intensity (data are not shown).

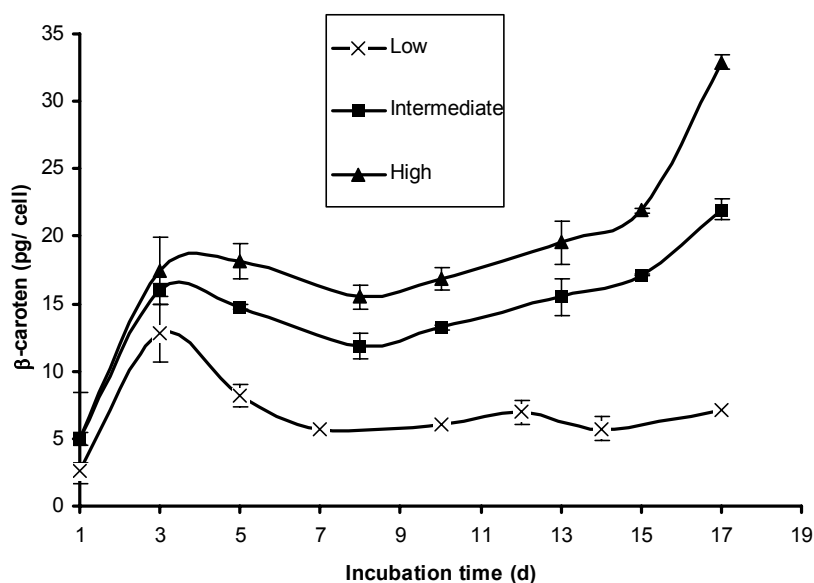


Figure 4.2: β -carotene production per cell of *D. salina* as a function of under three different light intensities. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

Ben-Amotz (1995) studied the properties of *Dunaliella bardawil* grown outdoors in a two-stage system. He reported that volumetric productivity of β -carotene in the production stage (containing stressed cells) is lower than during the growth stage (containing non-stressed cells). The β -carotene productivity per cell, however, is higher in the production stage than the productivity during growth stage.

It can be concluded that an increase in β -carotene content of the cells, which is obtained under stress conditions, is not necessarily coupled with an increase in the volumetric production of β -carotene. Other parameters such as biomass concentration are also important.

Chlorophyll production by the cells

Figure 4.3 shows the volumetric production of chlorophyll in the three bioreactors at three different light intensities as a function of time. In contrast to β -carotene production, chlorophyll production in the bioreactor illuminated with low light intensity is higher than in the other bioreactors. It is already known that the size of the

photosystem and chlorophyll production by the cells is influenced by the light intensity. The cells produce more chlorophyll to capture sufficient light energy when the light intensity is lower (Ben-Amotz, 1987, and Masuda et al., 2002).

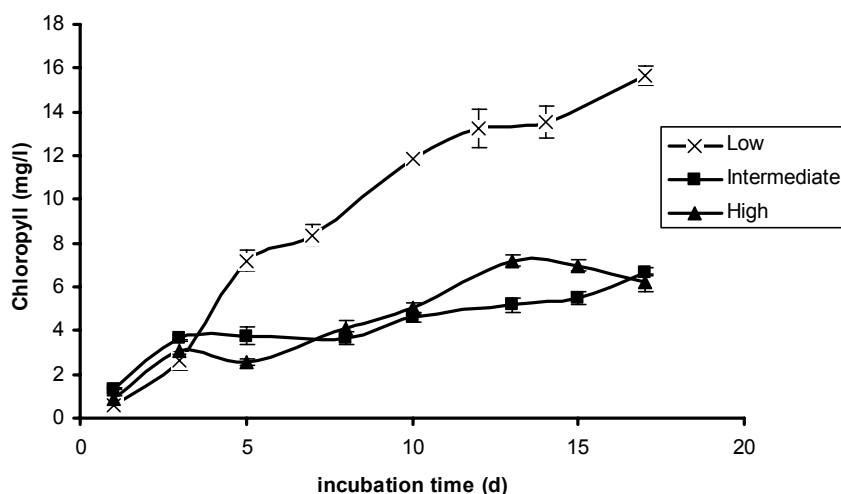


Figure 4.3: Volumetric chlorophyll production (based on aqueous phase) in the cultures of *D. salina* as a function of time under three different light intensities. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

β -Carotene extraction

Figure 4.4 shows the β -carotene content of the organic phase vs. the incubation time in above-mentioned bioreactors. As we can see the extraction rate was increased by the increase in the amount of light per amount of the cells. Since the volumetric production was about the same for all the bioreactors (Figure 4.1) the same amount of extraction was expected. Higher extraction from the bioreactors which were illuminated at higher light intensities can be explained by the function and location of β -carotene. β -Carotene in the cells of *D. salina* has two different functions. The first function is related to its energy transfer to the chlorophyll in the photosystem of the cells (Tanada, 1951) and second function is protection of the cells against oxidation. Green cells, which are grown under normal growth conditions, have low β -carotene contents. The β -carotene is mostly involved in the photosynthesis inside the

photosystem. When the cells are stressed they produce more β -carotene, which is typically accumulated in the oil globules (Lers et al., 1990). According to the literature there is a relation between chlorophyll content of the cells and β -carotene which is involved in the photosystem (Pineau et al., 2001). Taking this in to the account and comparing the volumetric β -carotene and chlorophyll productions (Figure 4.1 and 4.3) together with β -carotene per cell (Figure 4.2) it appears that the amount of β -carotene which is not involved in photo system was increased when light intensity increased. Therefore, increase in the extraction seems to be due to increase in the amount of β -carotene which is not involved in the photo system and accumulated in the oil globules.

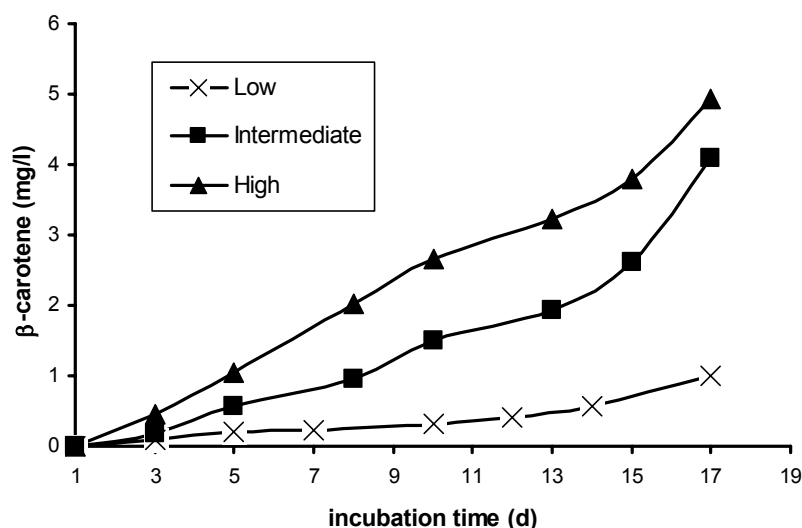


Figure 4.4: β -Carotene concentration of organic phase during incubations of *D. salina* under three different light intensities. The error bars were very small and are not given

Relation between power input and β -carotene extraction

Illumination of the photo bioreactors is an energy consuming process. To calculate the amount of consumed energy or power-input (W cell^{-1}) the light per amount of cells ($\mu\text{mol s}^{-1}\text{cell}^{-1}$) was divided by a constant value. The amount of this constant value depends on the type of the light source. For the cool-white fluorescent lamp, which was used in our experiment, this amount is $4.59 (\mu\text{mol s}^{-1} \text{W}^{-1})$ (Thimijn et al., 1983).

The amount of extracted β -carotene per unit of power per cell was calculated by dividing the extraction by the power-input (Figure 4.5). Although the effectiveness of extraction with respect to the light input is comparable for all light intensities applied, increasing the light input per cell leads to a higher volumetric extraction rate (higher concentration of β -carotene in the solvent phase).

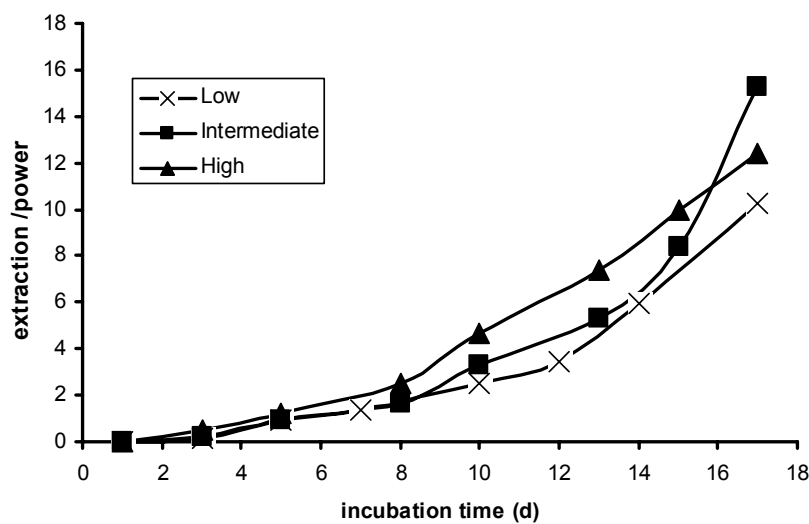


Figure 4.5: Extraction of β -carotene per unit of power input from cells of *D. salina* ($\text{mg cell W}^{-1} \text{l}^{-1} 10^{-8}$). There is no significant difference between the samples at the 0.05 level; therefore error bars are not given

Purity of extracted β -carotene

One of the important advantages of the *in situ* extraction of β -carotene is selectivity of its extraction over chlorophyll (Hejazi et al., 2002). Detection of chlorophyll in the organic phase showed very low chlorophyll content on all three bioreactors (results are not shown). In our previous work we showed that by increasing the mixing rate and β -carotene extraction, chlorophyll impurity increased up to 3% (Hejazi et al., in press). We can conclude that extracted β -carotene stays very pure when the increase in the extraction rate is due to enhancement of β -carotene content of the cells.

Conclusions

It is concluded that β -carotene extraction is enhanced by an increase in the amount of light per amount of cells. This increase is not coupled with the increase in volumetric β -carotene production but is due to enhancement of β -carotene content of the cells. Furthermore, it is concluded that increasing the light intensity per cell within the applied range leads to a more effective extraction. Moreover extracted β -carotene stays very pure even so the extraction increased.

Acknowledgement

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Chapter 5

Milking microalga *Dunaliella salina* for β -carotene production in two-phase bioreactors

Abstract

*A new method for production of β -carotene from *Dunaliella salina* was developed. Cells were grown in low light intensity after which the cells were transferred to the production bioreactor, illuminated at a higher light intensity. It was a two-phase bioreactor consisting of an aqueous and a biocompatible organic phase. In this bioreactor mixing of the cells and extraction were performed by re-circulation of the organic phase.*

Two sets of experiments were performed. In the first experiment bioreactors were operated at two different solvent re-circulation rates of 150 and 200 ml.min⁻¹. The results indicated that the β -carotene extraction rate significantly increased at the higher re-circulation rate, without exerting any influence on cell number and viability.

*A second experiment was carried out with the appropriate re-circulation rate (200 ml.min⁻¹) to study long-term production of β -carotene. The results showed that *D. salina* at high light intensity stayed viable for a long period (>47 d) in the presence of a biocompatible organic phase, in which cell growth, however, was very slow. β -Carotene could be continuously extracted to the organic phase; the cells kept producing β -carotene and the extracted molecules were continuously re-produced. As a result β -carotene was continuously removed (milked) from the cells. The β -carotene extraction efficiency in this system was more than 55%, and productivity of β -carotene is 2.45 mg. m⁻².d⁻¹, much higher than obtained in commercial plants.*

This Chapter has been submitted for publication as: Hejazi M A, Holverda E., and Wijffels R H. Milking microalga *Dunaliella salina* for β -carotene production in two-phase bioreactors.

Introduction:

Dunaliella salina is a unicellular green microalga that can produce and accumulate β -carotene in response to stress conditions. β -Carotene is widely used in food, pharmaceutical and cosmetic industries as pro-vitamin A, antioxidant and a colorant agent. Recently, the great demand for β -carotene has been answered by industry, both by synthetic production and by preparation from natural sources. Increasing demand for natural carotenoids has resulted in growing interest in extracting β -carotene from different sources (Vega et al., 1996). The estimated market size for β -carotene is 10-100 tonnes.year⁻¹ and the estimated price >750 €.Kg⁻¹ (Pulz et al., 2001).

Commercial production of β -carotene from *D. salina* takes place in two steps. The first step in which the cells are grown is followed by a step wherein cells are stressed. Due to stress β -carotene accumulates, after which the cells are harvested, and β -carotene extracted and purified. The productivity of this system is very low about 0.1 mg.l⁻¹.d⁻¹ (Borowitzka, 1999).

Several methods have been suggested to improve the productivity of fermentation systems. One of the methods suggested is production in two-phase bioreactors, in which *in situ* product removal is feasible.

“Milking” products from bacteria has been reported by Sauer and Galinski (1998). They applied this technique for production of ectoines, a group of osmo-regulating solutes, from the halophilic bacterium *Halomonas elongata*. Ectoines were biosynthesized when the bacteria were incubated in media with high salinity. Following the cells were transferred to a medium with low salinity to which ectoines were extracted. Subsequent re-incubation in a medium with higher salt concentration resulted in re-synthesis of these compounds. By repeatedly performing this bacterial milking process (at least nine times), they were able to produce large amount of the products.

Milking products from microalgae has been reported by Frenz and co-workers (1989). They collected a substantial fraction of *Botryococcus braunii* hydrocarbons (hydrophobic hydrocarbons) via a short contact between the cells and hexane. In their method the cells were harvested after growth, separated by filtration and then

contacted with the organic phase for extraction. Due to the short extraction time the cells were still viable after extraction and were returned to the bioreactor. They showed that growth and hydrocarbon production in subsequent cultures were not impaired, even after repeated extractions.

In both cases, it was claimed that milking products from bacteria and microalgae, resulted in enhanced productivity. They did not however compare the productivity of milking process with the existing processes.

Our previous studies showed that selective extraction of β -carotene from *D. salina* with retention of viability in two-phase bioreactors was feasible. Organic phases with a log P value higher than 6 are considered biocompatible for *D. salina* and can be applied in this process (Hejazi et al., 2002). It was also shown that the extraction rate of β -carotene was a function of the mixing rate of the culture and the β -carotene content of the cells (Hejazi et al., in press).

We assume that because of the essential function of β -carotene in the cells of *D. salina*, β -carotene will be continuously produced, substituting the extracted materials. The objective of this paper is to develop a method for milking β -carotene from the microalga efficiently and continuously for a long periods of time. Preliminary experiments indicated the possibility of continuous milking of β -carotene from the cells in a two-phase bioreactor. To obtain higher productivity the growth phase and production and extraction phase (milking phase) were separated. In the milking phase the organic phase was re-circulated through the aqueous phase for mixing and extraction purposes. In this fashion we were able to improve the extraction rate by increasing the contact between the cells and organic phase.

Two sets of experiments were performed. First, the effect of solvent re-circulation rate on the cell growth and viability as well as extraction was determined. Second, long-term milking of cells for β -carotene production was studied.

Materials and methods

Organism and medium

Dunaliella salina (CCAP 19/18) was grown in a culture medium containing 1M NaCl, 10 mM KNO₃ and 1 mM NaH₂PO₄.2H₂O (Hejazi et al., 2002). The pH of the medium was adjusted to 7.5 by 3 M HCl. The medium was sterilized at 121°C for 40 minutes before inoculation. To avoid precipitation, the phosphate-salt solutions were autoclaved separately. Daily, 5 mM of carbon source (NaHCO₃) was added in order to avoid limitation of carbon. For sterilization, solid NaHCO₃ was put in an oven over night at 120 °C and then mixed with sterilized water.

Experimental setup

Growth and β -carotene production by *D. salina* were carried out in two different bioreactors. Growth was carried out in a 450 ml bubble column bioreactor at low light intensity (250 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). The cells were transferred to the second bioreactor when the concentration reached $1.6 \cdot 10^9 \text{ cell.l}^{-1}$. In the second bioreactor, which was a one-liter flat panel bioreactor with an optical path of 2.5 cm, the cells were diluted two times by fresh culture media and reached a volume of 700 ml. β -Carotene production and extraction were performed in the second bioreactor at higher light intensity (1200 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). Dodecane, which is biocompatible for *Dunaliella salina* (Hejazi et al., 2002), was used as organic phase in the second bioreactor. The bioreactor contained 80% of aqueous phase and 20% of organic phase. The production bioreactor was a liquid impelled loop reactor and both mixing of the culture media and β -carotene extraction were carried out by re-circulation of the organic solvent through the aqueous phase. Continuous re-circulation of the organic phase through the aqueous phase increased the contact area between the organic phase and the cells and thus improved mass transfer. The high light intensity together with the short light path enhanced stressing the cells. The temperature was controlled at 26 ± 1 °C. Two sets of experiments were performed. First, the effect of the solvent-recirculation rate on extraction and cell viability was determined. It was not our purpose to study

the effect of shear force on the cell viability. However, it was useful to study the effect of at least one re-circulation rate above the minimum required re-circulation rate for keeping the cells in suspension. The first set of experiments was run for one week and cell growth, β -carotene production and extraction rates were followed in the bioreactors run with two different solvent re-circulation rates. The applied solvent re-circulation rates were 150 (minimum required re-circulation rate) and 200 ml.min⁻¹. The second set of experiments was performed to investigate long-term milking of the cells for β -carotene production, designed on the basis of the results of the first set of experiments. During the experiment the organic phase was replaced three times when the concentration of β -carotene was about 110 mg.l⁻¹, to avoid saturation of the solvent (the solubility of β -carotene in dodecane is about 180 mg.l⁻¹). In order to keep the volume of media constant a total amount of 150 ml of fresh culture media was added as a replacement for the samples taken during the experiment.

Viability of the cells

Viability of the cells was determined by light microscopy and flowcytometry (Facsan, Becton Dickinson BV). Flowcytometry permitted distinction of viable from dead cells due to differences in fluorescence. The algal cells containing chlorophyll show red fluorescence. When the cells are treated with FDA (Fluorescein Diacetate: in the membrane of the living cells FDA is cleaved by nonspecific esterases into a fluorescein), fluorescence green is formed as well as two acetates (Jochem, 1999). The living cells therefore, show green fluorescence upon exposure to FDA and the dead cells remain red. We found the incubation time for FDA to enter the cells of *D. salina* to be 5 minutes. 50 μ l of FDA solution (1 mg FDA per ml of acetone) were added to 1 ml of cell suspension and fluorescence was measured after 5 minutes. Two different populations (dead and living cells) were observed.

While light-microscopic observations showed large amounts of viable and active cells during the experiment, fluorescence behavior of the cells showed some changes during the experimental period which became very obvious in the last three weeks of the experiment. Both auto-fluorescence and FDA related fluorescence of the cells decreased. Decrease of auto-fluorescence was due to decrease in chlorophyll

concentration of the cell. Decrease of the green or FDA related fluorescence was perhaps due to some changes in the composition of the cell membrane either by physiological changes, which happen during carotenogeneses or by the effect of the solvent phase (Sikkema et al., 1996). Due to the former change the incubation time for FDA was increased to 30 min in the last three weeks of the experiment.

Cell counting

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).

β -Carotene and chlorophyll determination

β -Carotene and chlorophyll concentrations were determined spectrophotometrically using standard curves (Hejazi et al., 2002). In addition, pigments were detected by HPLC analysis. The sample was injected on the HPLC column (Prevail C18, 5 μ m particle size, 250 x 4.6 mm), which was kept at 30°C. The gradient was started with acetonitril:iso-propanol 85:15, gradually changing min to 50: 50 in 18. It changed again to the initial composition of 85:15 in 2 min. To stabilize the column we run the column with the same composition for more 5 min. The flow rate was 1.0 ml.min⁻¹. Detection was by a UV3000 photo-diode array detector set at 435 nm for chlorophyll and 455 nm for β -carotene.

Calculation of hydrodynamic stress parameters

Any kind of biocatalyst, when placed in a moving fluid with velocity gradients, experiences a shear force (τ) which magnitude depends on the dynamic viscosity of the fluid (μ), the fluid velocity gradient (dv) and the size of the pertinent biocatalyst (dx) (van't Riet et al., 1991). The general formula for shear calculation is:

$$\tau = \mu \, dv/dx$$

It is assumed that three main different regimes of solvent flow exist in the flat panel bioreactor. The first exists in the outlet of the sparger. The second flow is called “jet flow” and appears when a liquid is injected to another liquid at high injection velocities (Meister et al., 1967). The third flow is the flow of the dispersed-phase drops in the riser. All three flows and related shear forces were calculated (see appendix) and the results compared with the literature.

Results and discussions

First set of experiments: Effect of solvent re-circulation rate on the growth and extraction

Growth of algal cells

Study of cell growth was performed in the bubble column bioreactor from which the cells was transferred to the production bioreactor after reaching the concentration of $1.6 \cdot 10^9 \text{ cell.l}^{-1}$. Cell number and viability was followed in the production bioreactor at different solvent re-circulation rates. Results indicated that application of different re-circulation rates, 150 and 200 ml.min^{-1} , did not have a significant effect on the cell number (Figure 5.1).

The viability of the cells was not influenced by the increase in re-circulation rate and stayed higher than 90% during the duration of the experiment (results are not shown). We calculated the velocity of the solvent phase and related shear force in different regions in the bioreactor (Table 5.1 of the appendix). Calculation of the superficial flow rates and shear forces showed that the highest superficial flow and shear force appear at the outlet of the sparger when the solvent flow is 200 ml.min^{-1} . According to Barbosa and co-workers (Barbosa et al., in press), the critical superficial flow rate for *D. salina*, in a bubble column bioreactor sparged with air is about 30 m.s^{-1} . This flow rate is considerably higher than the maximum applied superficial flow rate in our experiment (4.2 m.s^{-1}). We compared our results with the result from our previous experiment, which was performed in stirred bioreactors (Hejazi et al., in press) as well. The calculated shear force for the highest applied stirring rate in our

previous research was 5.26 N.m^{-2} . This value is considerably lower than the maximum shear force in the outlet of the sparger (200 N.m^{-2}) but it is at the same magnitude of the maximum shear force in the riser of the bioreactor (6.8 N.m^{-2}).

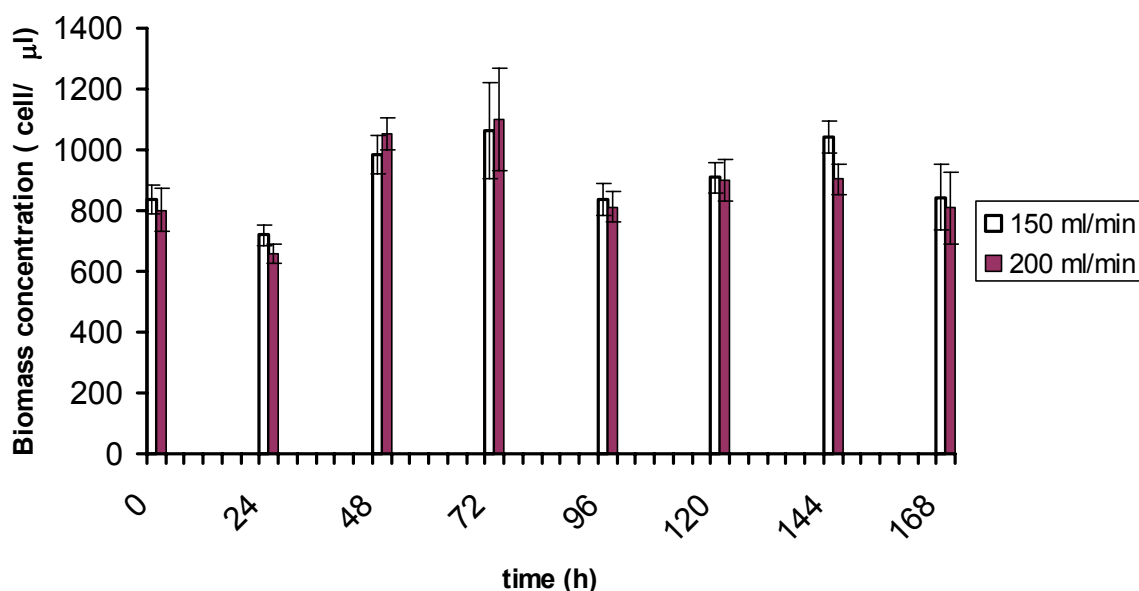


Figure 5.1: The effect of mixing rates on cell population in the cultures of *D. salina* cultivated in two-phase bioreactors. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

β -Carotene production and extraction

The β -carotene content of the cells (aqueous phase) and organic phase was determined in the bioreactors with the two different solvent re-circulation rates. Results showed no significant effect of re-circulation rate on β -carotene concentration inside the cells. The β -carotene content of the organic phases however was significantly different. The concentration of β -carotene in the solvent phase after one week was 3 times higher with the higher (200 ml.min^{-1}) re-circulation rate (Figure 5.2), in agreement with our previous results which showing β -carotene extraction was a function of contact area between the cells and the organic solvent (Hejazi et al., in press).

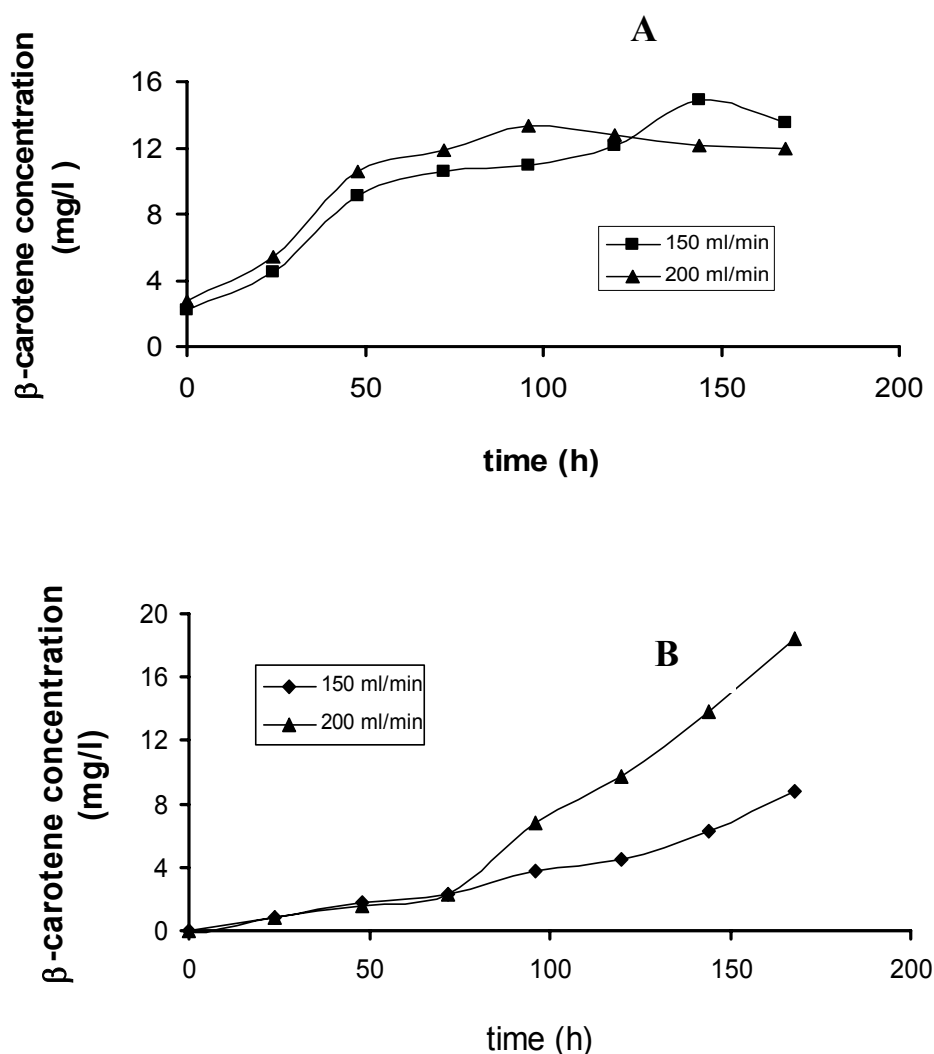


Figure 5.2: β -Carotene content of the aqueous (A) and organic (B) phases in response to different mixing rates in the cultures of *D. salina* cultivated in two-phase bioreactors

Second set of experiments: Long-term milking of the cells for β -carotene production

The second experiment was performed on the basis of the results from the first experiment. The cells were cultivated first in the growth bioreactor and then transferred to the milking bioreactor. All conditions were the same as in the first experiment and 200 ml.min⁻¹ was chosen as solvent re-circulation rate.

Growth and viability of the algal cells

The cells were transferred from the growth bioreactor to the milking bioreactor when the concentration reached $1.6 \times 10^9 \text{ cell.l}^{-1}$. Cell growth in the milking bioreactor was observed by regular sampling and direct counting of the cells under the microscope. In the first week of the experiment fluctuation in the cell concentration was observed. After that, the cells showed very slow growth and in the last week of the experiment, cell population was almost constant (Figure 5.3). It is already known that stressing conditions limit growth (Ben-Amotz, 1993).

Cell viability was followed by light microscopic observations and flowcytometry, the former showing large amounts of active and viable cells during the experiment. Flowcytomery results were comparable, showing above 90% viability (results are not shown). This was interpreted to indicate that long term milking of the cells does not have any harmful effect on cell viability.

 β -Carotene production in the presence of organic phase

Figure 5.4 shows the total volumetric production of β -carotene in the second bioreactor (both aqueous and organic phases) as a function of time. β -Carotene production was shown to be carried on for a long period of time in the presence of organic phase. The β -carotene content of the cells reached 51 pg.cell^{-1} (Figure 5.5). This is about two times higher than the concentration reported for commercial production (Ben-Amotz, 1995).

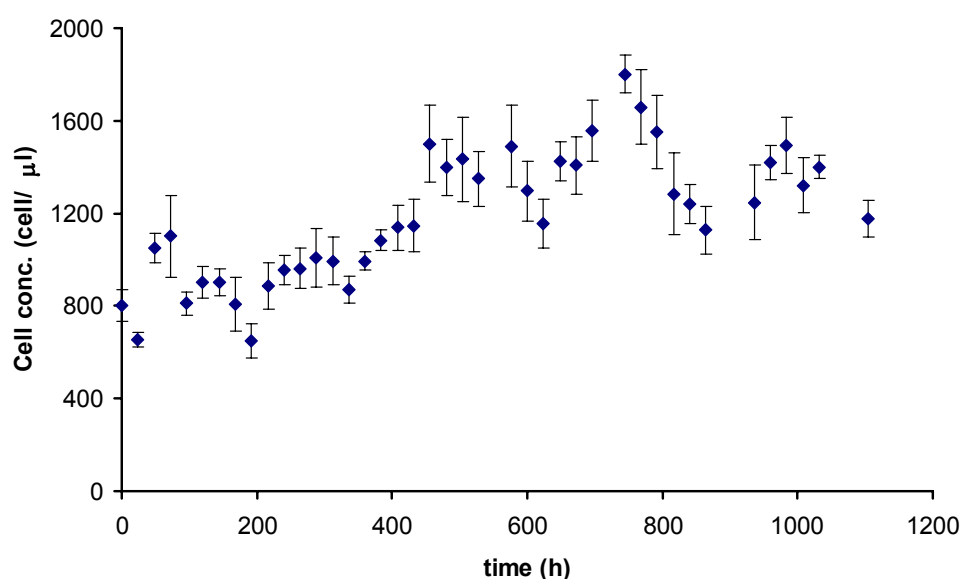


Figure 5.3: Growth of *D. salina* in the presence of organic biocompatible solvent. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

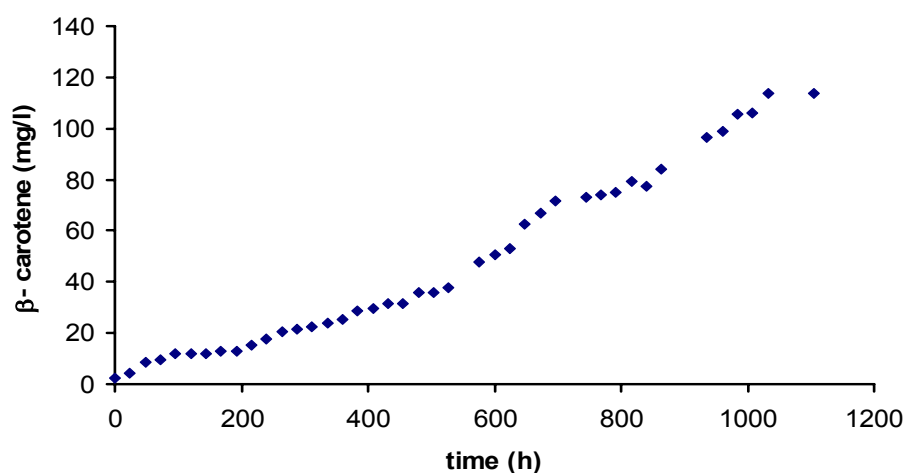


Figure 5.4: Total volumetric production of β -carotene by *D. salina* in the presence of organic phase

Chlorophyll production by the cells

In contrast to the increase in β -carotene concentration during the extraction period chlorophyll concentration decreased to a value of $1.43 \text{ pg.cell}^{-1}$ on the last day of experiment (Figure 5.5). The ratio of β -carotene over chlorophyll changed from 0.6 to

35 (Figure 5.6). Ben-Amotz (1995) reported that in his work, this ratio was 10, indicating the relative concentration of β -carotene over chlorophyll in our experiments is 3.5 times higher than the ratio reported by Ben-Amotz.

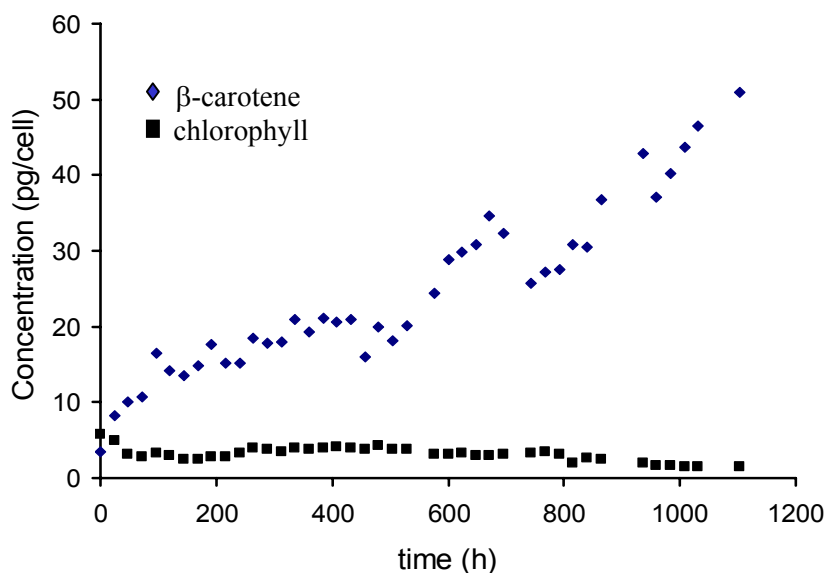


Figure 5.5: β -Carotene and chlorophyll content of the cells of *D. salina*, cultivated in the presence of organic phase, as a function of time

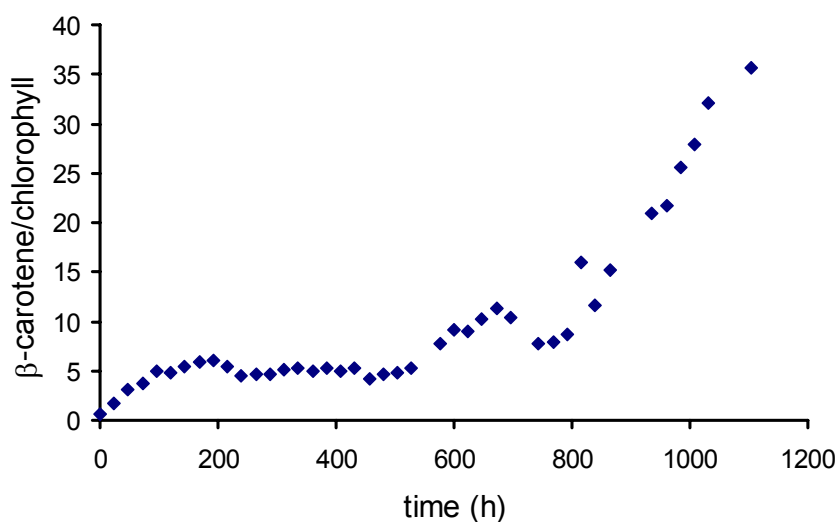


Figure 5.6: β -Carotene / chlorophyll in *D. salina*, cultivated in the presence of organic phase, as a function of time

β -Carotene extraction to the organic phase

Figure 5.7 shows the actual concentration of extracted β -carotene in the organic phase, total β -carotene production being 115 mg.l^{-1} (Figure 5.4). Since the total volume of culture media and the organic phase in the production bioreactor was 875 ml, the actual amount of β -carotene produced in the bioreactor was 101 mg, yielding more than 55% in extraction efficiency.

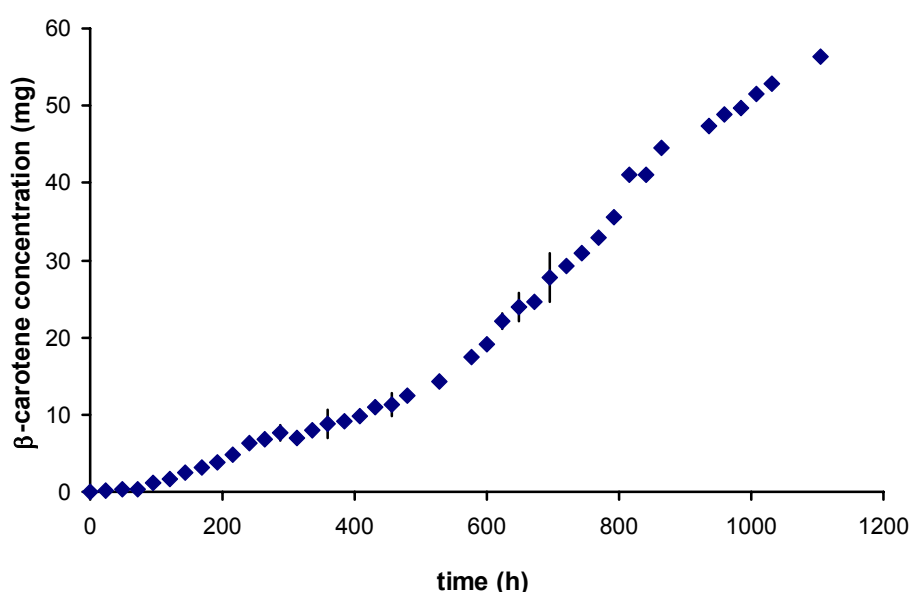


Figure 5.7: β -Carotene concentration in the organic phase. Error bars show 95% confidence interval of triplicate samples taken from the bioreactor

Selectivity of extraction

The Pigment content of the organic solvent was determined regularly by HPLC analysis in addition to spectrophotometric analysis. Figure 5.8 shows an example of the several HPLC diagrams, all showing that the extracted β -carotene to be very high purity. Thereby the extraction is selective even at the higher contact rate between the cells and the organic solvent for a very long time.

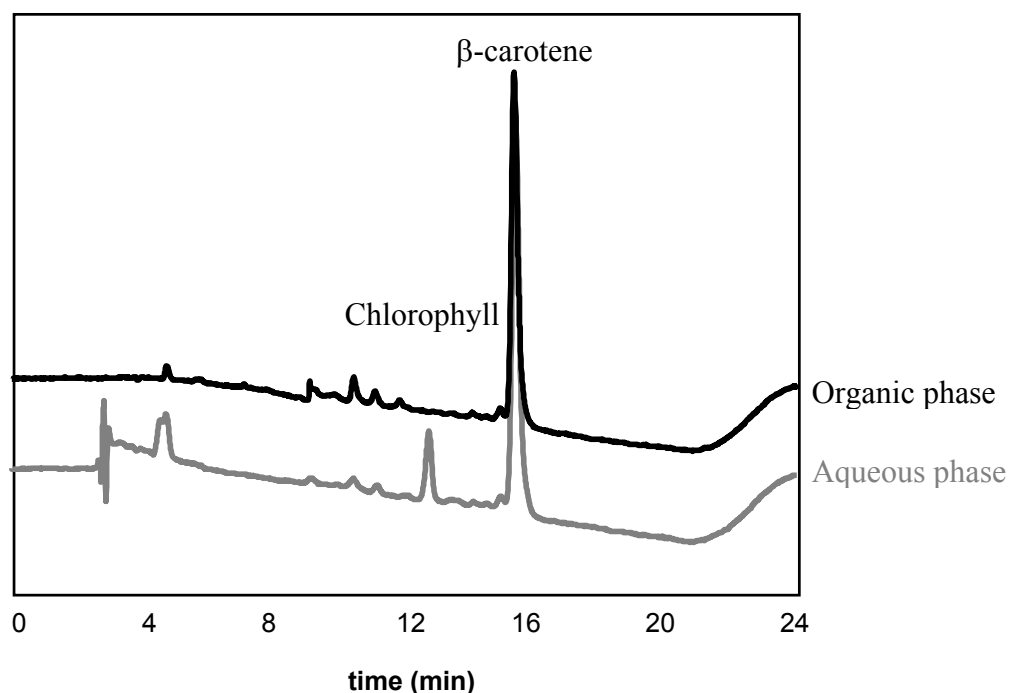


Figure 5.8: HPLC diagram of the pigment contents of the organic and the aqueous (the cells of *D. salina*) phases. Detection carried out by a UV3000 photo-diode array detector set at 435 nm for chlorophyll and 455 nm for β -carotene

Milking process

Although cell growth was very slow, β -carotene content of the cells and organic phase increased with time (Figure 5.3, 5.4 and 5.7). Figure 5.6 shows that β -carotene ratio to chlorophyll inside the cells was even higher than was reported before (Ben-Amotz, 1995). This means that the cells kept producing β -carotene while extraction was taking place and extracted molecules being substituted with newly produced molecules. Hence we view β -carotene as being milked from the cells. We run the milking process for 47 days and stopped it just because of time limitation. Since the cells were still viable on the last day of experiment we concluded that it was possible to run the processes for longer times.

Productivity of the system

The volumetric productivity of the system was $2.5 \text{ mg.l}^{-1}.\text{d}^{-1}$ of β -carotene. For comparison the largest commercial plant for β -carotene production is located in Australia, where very large ponds (250 ha) are used. In the ponds, $1 \text{ ton DM.ha}^{-1}.\text{year}^{-1}$ of algal biomass is produced containing 10% β -carotene (Borowitzka, 1999). The volumetric productivity in this system is thus $0.1 \text{ mg.l}^{-1}.\text{d}^{-1}$. A more productive system was developed by Ben-Amotz, in which growth and production steps were separated. The system consisted of smaller ponds (0.3 to 100 m^2 for nursery ponds and 3000 to 4000 m^2 for the production ponds), the areal productivity being $300 \pm 50 \text{ mg } \beta\text{-carotene m}^{-2}.\text{d}^{-1}$ (Ben-Amotz, 1995), which results in a volumetric productivity of $1.5 \text{ mg.l}^{-1}.\text{d}^{-1}$.

This comparison shows that the volumetric productivity of the system we developed is 25 times higher than commercial production site in Australia and is 70% higher than the system introduced by Ben-Amotz (1995). Our production system, however, is in lab scale, and this comparison is ment to focus on the commercial potential of our system.

Conclusion

All the present methods for β -carotene production are based on harvesting the cells, applying a multi-step extraction processes to extract and refine the β -carotene. We developed a new method in which we can milk the cells for very long time ($>47 \text{ d}$) for β -carotene production. The cells stay viable and keep producing β -carotene in the presence of a biocompatible organic phase. In this process β -carotene is extracted selectively and the volumetric productivity of this system is very high in comparison to existing systems. The advantages of our system can be summarized as follows: (a) elimination of cell harvesting and concentration; (b) elimination of cell destruction, having viable and productive culture for long periods; (c) simplicity of purification due to selectivity of extraction; and (e) all together a higher productivity.

Acknowledgement:

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Appendix

Injection of a fluid through another fluid causes shear. The amount of shear force depends on the dynamic viscosity of the fluid, the fluid velocity gradient and the size of the pertinent biocatalyst. Dynamic viscosity of the medium and the size of the biocatalyst are considered constant inside a bioreactor. The flow regime of the fluid, however, is not constant. Three different flow regimes of a solvent in water phase were considered. The first one is superficial flow (v_s), which exists in the outlet of the sparger, is calculated by:

$$v_s = F_g / 0.25\pi d_i^2$$

F_g is the volumetric solvent flow rate and d_i is the inner diameter of the sparger.

The second one is the flow of the jet part. It is assumed that the velocity and diameter of the jet are constant and equal to the average nozzle velocity and diameter (Meister and Scheels, 1969). Then the shear in the jet will be equal with the shear in the nozzle outlet.

The third flow is the solvent flow in the riser. The velocity of the solvent in the riser can be calculated by the following correlation:

$$V_d = C (V_{sd} + V_{sc}) + V_{d\infty}$$

C is a distribution parameter which is 1.5 when the concentration of the dispersed phase at the wall of the tube is zero and the velocity profile is pronounced parabolic. $V_{d\infty}$ is terminal velocity which is considered to be 0.14 m.s^{-1} (Van Sonsbeek et al., 1990). V_{sc} which is the superficial velocity of the continuous phase, is considered zero. V_{sd} , the superficial velocity of the dispersed phase can be calculated using equation 1. A in the equation is the area of the riser of the bioreactor.

Table I shows the calculated solvent velocities and related shear forces in different regions inside the bioreactor when the solvent re-circulation rate is 200 ml.min⁻¹.

Table 5.1: Solvent velocity and related shear force in different regions inside the bioreactor when the solvent re-circulation rate is 200 ml. Min⁻¹

<i>Region</i>	<i>Velocity (m.s⁻¹)</i>	<i>Shear force (N. m⁻²)</i>
<i>Sparger outlet and jet</i>	<i>4.2</i>	<i>200</i>
<i>Riser</i>	<i>0.1</i>	<i>6.8</i>

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Chapter 6

Mechanism of extraction of β -carotene from microalga *Dunaliella salina* in two-phase bioreactors

Abstract

*We have shown that it is possible to extract β -carotene selectively from *Dunaliella salina* in two-phase bioreactors. The cells continue to produce β -carotene and the extracted part is substituted by newly produced molecules. This process is called “milking”. The exact mechanism of the extraction process is not known. We performed several studies in macro-scale (bioreactors) and micro-scale (using microscopic techniques) for better understanding this mechanism. Macro-scale experiments showed that direct contact between the cells and the organic solvent was not a requirement for the extraction but it accelerated the extraction. It was also shown that dodecane was taken up by the cells. Results from electron microscopy observations showed that dodecane uptake led to more activity in the cell membrane and more active endo-exo-cytosis. In the cells growing in the presence of the organic solvent extra-chloroplast β -carotene containing globules were observed. It seems that one of the mechanisms of extraction is transport of the globules from the chloroplast to the space between the cell and the chloroplast membranes and subsequently from there to outside by exo-cytosis. Another possible mode for the extraction could be release of β -carotene from the globules as a result of alterations in the membrane of globules. β -Carotene molecules diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exo-cytosis after accumulation in the vesicles.*

This chapter has been submitted as : Hejazi M A, Kleinegris D., and Wijffels R H. Mechanism of extraction of β -carotene from microalga *Dunaliella salina* in two-phase bioreactors.

Introduction

Dunaliella salina is a unicellular microalga with the ability to produce and accumulate β -carotene up to 10% of its dry weight (Ben Amotz, 1995). β -Carotene is an orange pigment with several applications in food, pharmaceuticals, and cosmetic industry. β -Carotene is one of the photosynthetic pigments and is produced in the thylakoid membranes in the chloroplast. It has two different functions in the cells: (i) it has a role in energy transfer in the photosystem of the cells (Tanada, 1951) and (ii) a role in the protection of the cells against oxidation (Lers et al., 1989). The production of β -carotene is enhanced in by stress factors (e.g. high light intensity, high salinity and nutrient deficiency) and over produced β -carotene is accumulated in oil globules in the cells (Rabbani et al., 1998).

We have shown that it is possible to extract β -carotene selectively from *D. salina* in two-phase bioreactors (Hejazi et al., 2002). In this process the cells are first grown and then stressed to produce larger amounts of β -carotene. In the stress stage the biocompatible organic phase is added and β -carotene is extracted selectively. The solvent is continuously re-circulated through the aqueous phase containing the cells. The cells continue to produce β -carotene and the extracted part is substituted by newly produced molecules. Due to the substitution process it is possible to produce larger amounts of β -carotene than in the commercial production process. This process is called 'milking' (Hejazi et al., submitted for publication). The exact mechanism of this extraction process is not known.

Our previous results showed that the extraction rate was affected at least by two parameters: contact area between the cells and the organic solvent (Hejazi et al., in press) and β -carotene content of the cells (Hejazi et al., 2003). The previous results indicated that interaction between the solvent and the cell membrane as well as β -carotene function and location inside the cells are important parameters in the extraction.

The membrane of *D. salina* is continuously active in an endo- and exocytosis process (Ginzburg et al., 1999). During this process the medium in which the algae grow is taken up in small vesicles. After a few seconds the vesicles are released again. We

think this physiological property of the cells might play a role in the extraction process.

In this paper we will look in more detail to the mechanism of the extraction process. The effect of the presence of the organic phase on the distribution and location of β -carotene globules was determined. We studied whether direct contact between the cells and the organic solvent is important for extraction. In addition we looked at the effect of the solvent on the cells and more specifically the cell membrane.

For this study we performed experiments in bioreactors and analyzed the cells with microscopic techniques.

Materials and methods

Cell pre- culture

D. salina (19/18, CCAP) was cultivated in three erlenmeyers of 50 ml each. The alga was grown in a climate cabinet at a temperature of 20°C and a light intensity of 50-80 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. Every week new erlenmeyers were inoculated with 1 ml of the old cultures. The medium contained 1 M NaCl, 5 mM KNO_3 , 0.5 mM $\text{NaH}_2\text{PO}_4.2\text{H}_2\text{O}$, trace elements (5 ml l^{-1} of stock solution containing 12.3 mM $\text{Na}_2\text{EDTA}.2\text{H}_2\text{O}$, 4.66 mM $\text{FeCl}_3.6\text{H}_2\text{O}$, 42.0 mM $\text{CuSO}_4.7\text{H}_2\text{O}$, 60.6 mM $\text{ZnSO}_4.7\text{H}_2\text{O}$, 17.0 mM $\text{CoCl}_2.6\text{H}_2\text{O}$, 366 mM $\text{MnCl}_2.4\text{H}_2\text{O}$ and 1.04 mM Na_2MoO_4), and 25 mmol tris buffer. The pH of the medium was adjusted to 7.5 by 3 M HCl. Before inoculation the medium was autoclaved for 40 minutes at 121°C. To avoid precipitation, the phosphate was autoclaved separately.

Macro-scale experiment

This experiment was performed to investigate whether direct contact between the cells and the organic phase is a requirement for the extraction. Four small bottles (100 ml) were filled with 40 ml of cells (suspended or immobilized). The cell suspension was immobilized in a layer of 0.5% agarose (ultra low gelling temperature). On top of the layer with cells either 25 ml artificial seawater and 20 ml

dodecane or only 20 ml dodecane was added to bring the cells in indirect or direct contact with organic solvent. Bottles containing suspended cells were mixed to bring the cells in contact with the organic phase or left to sink to the bottom to prevent them from direct contact with the dodecane. The bottles were illuminated from the side and the upper part of the bottles (organic phase) was covered with aluminium foil to prevent the extracted β -carotene from degradation. Figure 6.1 shows the set-up of the reactors.

Every day samples were taken from the organic phase to measure the β -carotene and chlorophyll concentration with a spectrophotometer. At the end of the experiment microscopic samples were prepared and observed by light microscope.

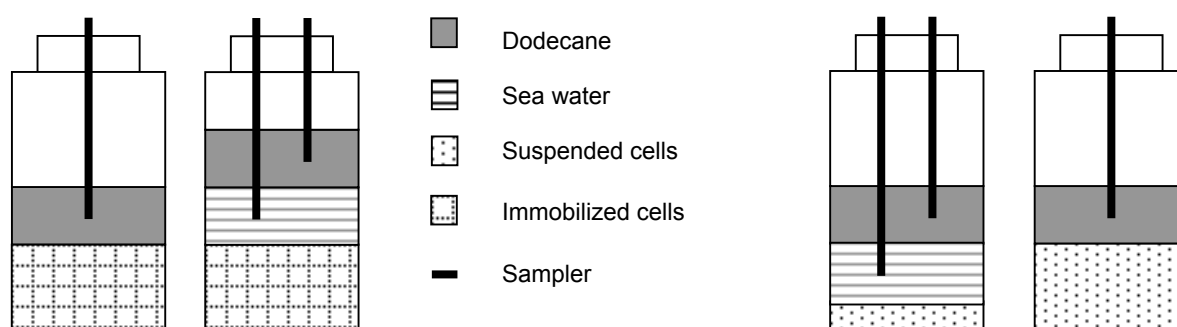


Figure 6.1: set-up of the small reactors to investigate the involvement of direct and indirect contact between the cells and the organic phase in the extraction

Electron microscopy

The aim of this experiment was to determine the molecular and phase effects of the solvent on ultra-structure of the cells. Three one-liter bottles containing 300 ml of culture media were inoculated with 20 ml cell suspension of a pre-culture. This medium had about the same composition as used for the pre-cultures. However, to obtain faster cell growth phosphate and nitrate concentrations were doubled (10 mM KNO_3 and 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$). In addition 3 ml of sterilized carbon source (0.5 M NaHCO_3) was added to avoid any carbon limitation. The solid carbon source (NaHCO_3) was sterilized in an oven at 120°C overnight and then dissolved in sterilized water.

The cells in each bioreactor were grown under different circumstances. The cells in the first reactor were grown in only aqueous phase (medium). In two other reactors dodecane was added. To separate molecular and phase effects of the organic solvent on the cells the second and third bioreactor were run at two different conditions. In the second reactor the aqueous phase was saturated with biocompatible organic solvent (1 ml). The third reactor contained 80% aqueous phase and 20% organic phase (75 ml). Dodecane was used as biocompatible organic phase. The temperature was controlled using a water bath and kept at 25°C. The bottles were illuminated with fluorescent lamps (SYLVANIA CF-EL 55W/840) with an average light intensity of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ (measured with a lightmeter: Licor model Li-250). Mixing of the culture media and the organic phase was done with two different mixers, fixed on one axis. A magnetic stirrer (diameter 6.0 cm, bar width 1.0 cm) was placed 0.6 cm from the bottom. The second stirrer was placed in the interface between the water phase and the dodecane. This was a six-bladed turbine stirrer (diameter 3 cm, paddle width and height 0.5 cm).

The cells, which were obtained from the three bioreactors, were concentrated by centrifugation and fixed for 30 min in 0.2% glutaraldehyde. Further fixation with aldehydes and osmium tetroxide, dehydration and embedding in LR White (London Resins Company) was essentially done as described by van Lent and co-workers (1990). Ultra-thin sections of approximately 60 nm were cut and mounted on 150 mesh copper grids covered with a formvar film. Sections were stained for 5 min with 2% (w/v) uranyl acetate and lead citrate according to Reynolds (1963). Sections were observed and photographed with a Philips CM12 transmission electron microscope.

To observe the effect of direct contact of pure dodecane on the cells a part of the harvested cells from the two-phase bioreactor, was centrifuged and the pellets were suspended in pure dodecane for 5 min. Afterwards, electron microscopy samples were prepared using above-mentioned procedure.

Confocal laser scanning microscopy (CLSM)

Although carotenoids have been considered as non-fluorescent for a long time, weak fluorescence from all-trans- β -carotene in fluid solution at ambient temperature has

been shown (Jorgensen et al., 1992). Chlorophyll fluorescence is very well known. Therefore, in addition to electron microscopy the CLSM technique was applied to investigate the location and distribution of the cell pigments (chlorophyll and β -carotene) in green and orange (stressed) cells of *D. salina*. The cells were stabilized using lugal solution. The argon ion laser was excited at wavelengths of 450 and 480 nm, since the absorption spectrum of β -carotene has peaks at these wavelengths. At these wavelengths chlorophyll is auto-fluorescent as well. Green and red fluorescence, being from β -carotene and chlorophyll respectively, was detected with a photomultiplier tube. From these data three-dimensional pictures were formed with the computer and software packages LSM Image Browser (Zeiss) and 3D Viewer (Zeiss).

Determination of dodecane concentration in the medium

In order to determine whether dodecane is taken up by the cells 50 ml cell suspension samples from a two-phase bioreactor and a bioreactor without dodecane (as a control) were taken. To determine the solubility of dodecane in the fresh medium, dodecane was also added to a bottle containing fresh medium. It was left for 24 hours to saturate. Then samples were taken from the lower part of bottle (saturated fresh medium) and analyzed for dodecane concentration.

The concentration of dodecane in the medium was determined with a gas chromatograph (GC), based on the method described by Kropp and co-workers (2000). Extraction of dodecane from the samples was performed by addition of 15 ml of dichloromethane to the samples three times. The samples then centrifuged (3' 1500 rpm) and decanted. 10 μ l Tetradecane was added as an internal standard to each sample. The extraction samples were pooled and concentrated in a rotary evaporator (40°C, no vacuum) to volumes of about 2 ml. The analysis were performed using a HP-5 column and a flame ionization detector. The temperatures of the injector and detector were 200°C and 250°C, respectively. The oven was held at 90°C for 2 minutes, before the temperature was increased with 4°C /minute to a temperature of 200°C. Helium was used as the carrier gas.

β -Carotene and chlorophyll analysis

β -Carotene and chlorophyll concentrations were determined spectrophotometrically. A sample of 1 ml was taken from the organic phase and the absorbance was measured by spectrophotometer at the wavelength maxima for β -carotene and chlorophyll and compared with standard curves (Hejazi et al., 2002).

Fatty acids analysis

The fatty acid composition of the cells growing under different circumstances was determined using the method described by Rodriguez-Ruiz and co-workers (1998). A 150 ml of cell suspension was harvested from each of the reactors and centrifuged (10', 10.000 rpm). The pellet was re-suspended in 5 ml of the supernatant, moved to reagent tubes and centrifuged again (8' 2500 rpm). A 3 ml solution of methanol/: acetyl chloride in a ratio of 20 : 1 (v/v) and 1.5 ml hexane were added to the pellet and mixed. The total solution was heated in a heating block at 100°C for 30 minutes. Every 10 minutes the solution was carefully shaken. After 30 minutes the reagent tubes were cooled down to ambient temperature. Two phases were separated from each other. The top layer was hexane containing the fatty methyl esters. This layer was moved to gas chromatography tubes and analyzed overnight with a GC using a CP-SIL-88 column, and a flame ionization detector. The injector and detector temperature were 250°C and 270°C, respectively, and the oven was held at 160°C for 4 minutes before its temperature was increased by 4°C/minute to 220. Hydrogen (125 Pa) was used as carrier gas.

Results and discussions

Macro-scale experiment

This experiment was performed to investigate whether direct contact between the cells and organic phase is a requirement for the extraction.

We conducted a series of experiments in small reactors with suspended and immobilized cells in direct and indirect contact with the organic phase. Immobilized cells were used to prevent dead or living cells from floating upward to the organic phase, resulting in unwanted extraction. To prove that the extraction was not a result of the contact between dead cells and organic phase the amount of chlorophyll present in the solvent phase was measured as well.

As shown in Figure 6.2 in all bioreactors extraction took place. However, the extraction rate in direct contact was about two times higher than in indirect contact. This means that direct contact between the cells and the organic solvent accelerates the extraction rate and thus is an important parameter in an efficient extraction process. The results are in agreement with our previous results, which showed that the extraction rate was enhanced by enhancement of mixing rate (Hejazi et al., in press). No reasonable amounts of chlorophyll were detected in any of the samples, showing almost no damage of cells during the extraction.

On the basis of aforementioned results we think that the dissolved molecules of dodecane in the water phase play role in the extraction process. The mass transfer is, however, improved by creating direct contact between the cells and the organic phase due to mixing. Where as in the case of indirect contact the extraction process goes much slower because of low solubility of β -carotene in the aqueous phase.

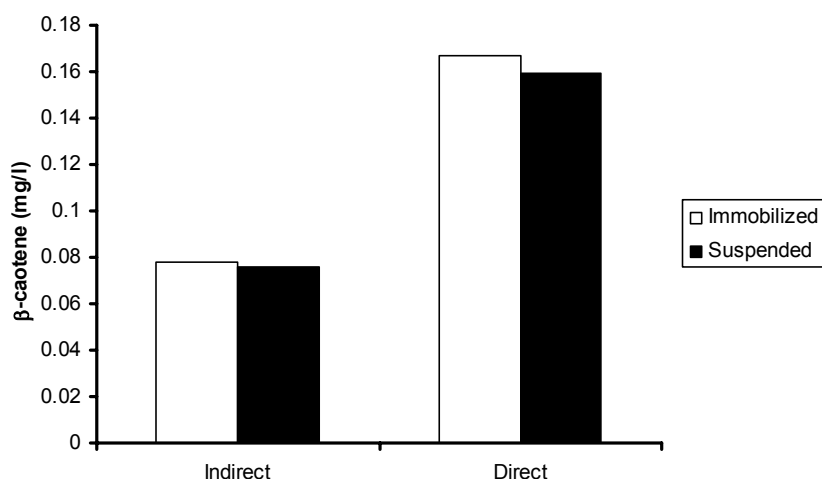


Figure 6.2: β -carotene extraction from *D. salina* in direct and indirect contact with the organic phase (dodecane)

Dodecane uptake by the cells

On the basis of aforementioned results we think that the extraction process is most probably accompanied by up take of the solvent molecules by the cells and subsequent alterations in the cell membrane helping β -carotene molecules being extracted (Sikkema et al., 1995). When the cells are in direct contact with the organic phase from one hand the solvent molecules can be easily taken up by the cells and from the other hand the extracted β -carotene is immediately taken up by the organic phase. To prove this hypothesis a set of experiments was carried out. Fresh medium (without any cells) was saturated with dodecane to determine the solubility of dodecane in pure medium. The cells grown in the presence of dodecane were centrifuged to see whether the centripetal force influenced the amount of dodecane up take by the cells, as well.

As can be seen in the Figure 6.3, the amount of dodecane in the fresh medium saturated with dodecane was not detectable where as in medium containing the cells and dodecane detectable amounts of dodecane was present. This indicates that molecules of dodecane are absorbed by the cells. Literature studies suggest that the cell membrane would be a first place that dodecane can accumulate in (McIntash et al., 1980).

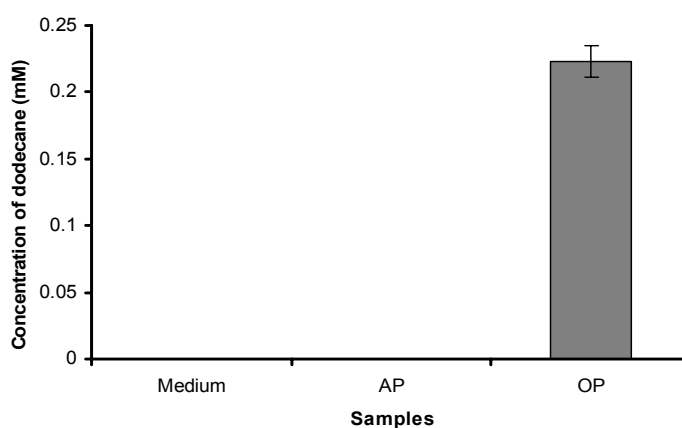


Figure 6.3: Dodecane content of the fresh medium without cells saturated with dodecane (Medium), The cells growing in the absence of dodecane (AP), and the cells growing in the presence of dodecane (OP).

Cell characteristics

To investigate ultra-structural features of the cells growing under different circumstances cells were observed with electron microscope. The effect of both carotenogenesis and the organic phase on the accumulation and location of β -carotene containing globules in the cells were investigated. We also studied the effect of the organic phase on the cell membrane. The cells growing in the three bioreactors in the presence of organic phase, in the saturated aqueous phase, and in the absence of organic solvent were studied.

-Effect of carotenogenesis on the ultra-structure of the cells

Obvious differences were visible between green and orange cells. Green cells in all cases showed a big chloroplast and a very little amount of β -carotene containing lipid globules inside the chloroplast (Figure 6.4). When carotenogenesis started, in all cells (in presence and absence of dodecane) more of these globules appeared. This effect has also been described in literature (Ben-Amotz, 199; Lers et al., 1990; and Rabbani et al., 1998). The globules containing β -carotene were located around thylakoid membranes and around the outer membrane of the chloroplast.

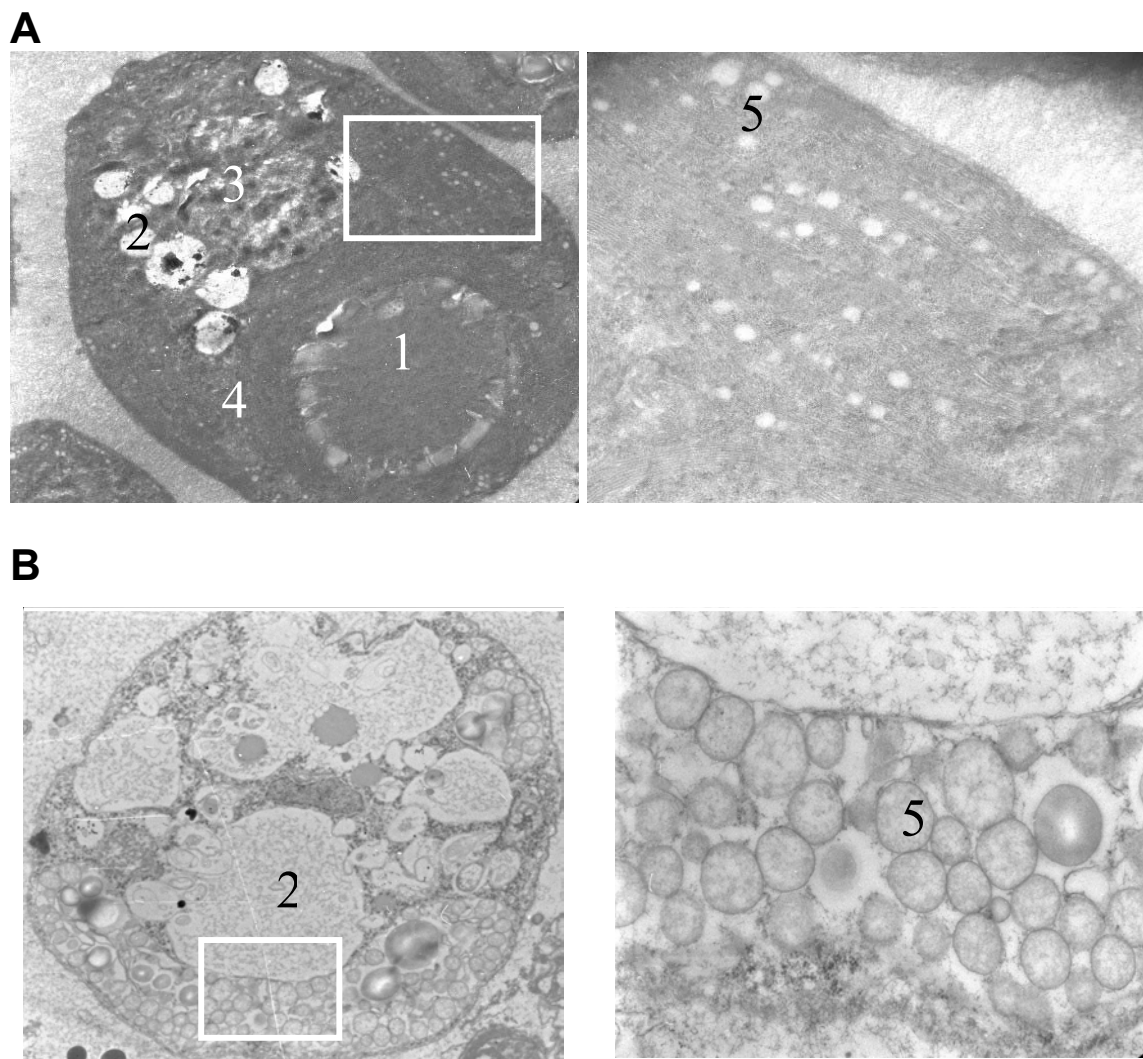


Figure 6.4: Electron microscopy picture of non-stressed (A) and stressed (B) cells, grown in absence of dodecane. Left: whole cell, Right: enlarged part (white rectangle in whole cell) 1: pyrenoid with surrounding starch molecules, 2: vacuoles 3: nucleus, 4: chloroplast, 5: globules between thylakoid membranes of chloroplast.

We determined the fluorescence of the cells by CLSM. With CLSM it is possible to follow the changes in distribution of the cell pigments in different physiological stages (non-stressed and stressed cells). The cells were excited by a laser source of the CLSM at 450 and 480 nm, and green and red fluorescence could be detected. Green fluorescence was related to β -carotene and red fluorescence was from chlorophyll. The non-stressed cells showed very strong red fluorescence which covered almost the whole cell (*D. salina* has a very big chloroplast) and a little green fluorescence. In

the stressed cells, however, the red fluorescence was not distributed homogenously in the chloroplast anymore. It was seen as spots in different places in the chloroplast. In the stressed cells strong green fluorescence around the cell membrane was detected (Figure 6.5). Since β -carotene is fluorescent in lipophilic environments (Jorgensen et al., 1992) we assumed the fluorescence came from the β -carotene accumulated in the oil globules. This together with electron microscopy pictures showed that in stressed cells most of the β -carotene containing globules were located in outer part of the chloroplast. This part of β -carotene seems to be available for the extraction. The changes in red fluorescence of chlorophyll after stressing the cells confirmed the damage in cell photosystem which has been reported in literature (Ben-Amotz 1995; Lers et al., 1990; and Rabbani et al., 1998).

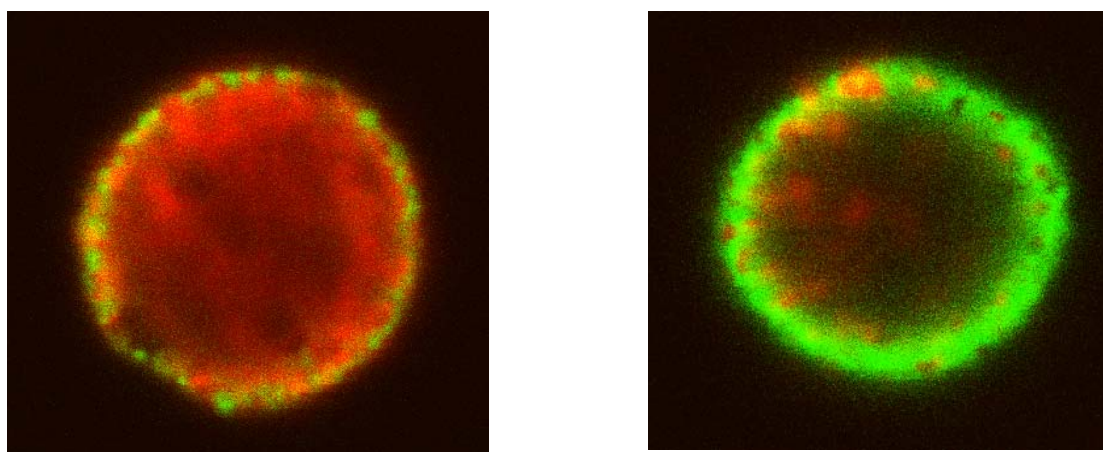


Figure 6.5: CLSM of non-stressed (left) and stressed (right) cells of *D. salina*. Green and red fluorescence indicate β -carotene and chlorophyll respectively.

Electron microscopy pictures showed that both gray and white globules were presented. This means that the globules are probably filled with different compounds. There are three possible explanations for the color difference: uptake of dodecane by the cells, a difference in the composition of fatty acids and difference in β -carotene concentration. Since white globules were also seen in the cells growing in the absence of the organic phase, we do not think the effect was caused by dodecane uptake.

It is known that the production of fatty acids by *D. salina* is related to the cultivation conditions such as salt concentration and light intensity (Azachi et al., 2002; Mendoza et al., 1999). The fatty acid composition of the cells grown under different circumstances was determined to investigate possible effect of the presence of dodecane on the fatty acid composition. The results showed that all the samples contain about the same composition of fatty acids (results are not shown). We therefore think that the difference in the color is because of difference in β -carotene concentration in the globules.

-Effect of the organic solvent on the ultra-structure of the cells

Two main differences were noticed between the stressed cells growing in the presence and absence of dodecane. The first difference was observed in the cell membrane. The membrane of the cells growing in the presence of the organic phase and in the saturated phase showed an undulated shape. A larger space between the cell and the chloroplast membranes was observed in these types of the cells. This indicates that the cell membrane is more active when the cells are treated with the organic solvent. This activity seems to be higher for the cells growing in the presence of the organic phase than the cells growing in the saturated phase (Figure 6.6).

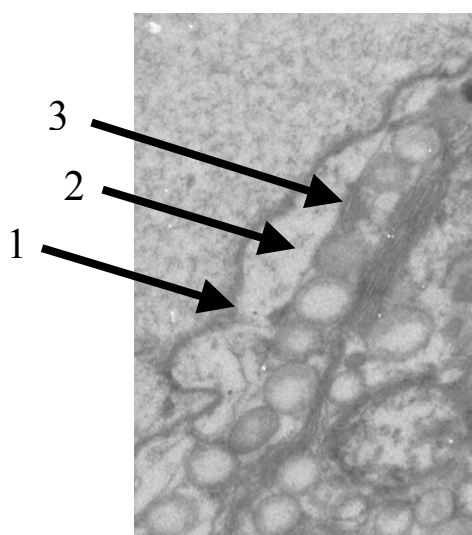


Figure 6.6: Parts of a cell growing in the presence of dodecane (a biocompatible organic solvent) which shows active cell membrane (1) and wider space between the cell membrane and chloroplast membrane (2). 3 Shows chloroplast membrane

The second observation was the location of the β -carotene globules. In the cells growing in the absence of the organic phase all the β -carotene globules were located inside the chloroplast next to the thylakoid membrane (Figure 6.4). In the cells growing in the presence of the organic phase and in the saturated phase however, some globules were observed in the space between the cell and the chloroplast membranes which became wider as a result of presence of the solvent. These globules were in contact with the cell membrane and looked as being extracted. When the cells were brought in contact with pure dodecane the movement of the globules towards the outside of the chloroplast and in the direction of the outer cell membrane was observed more clearly (Figure 6.7).

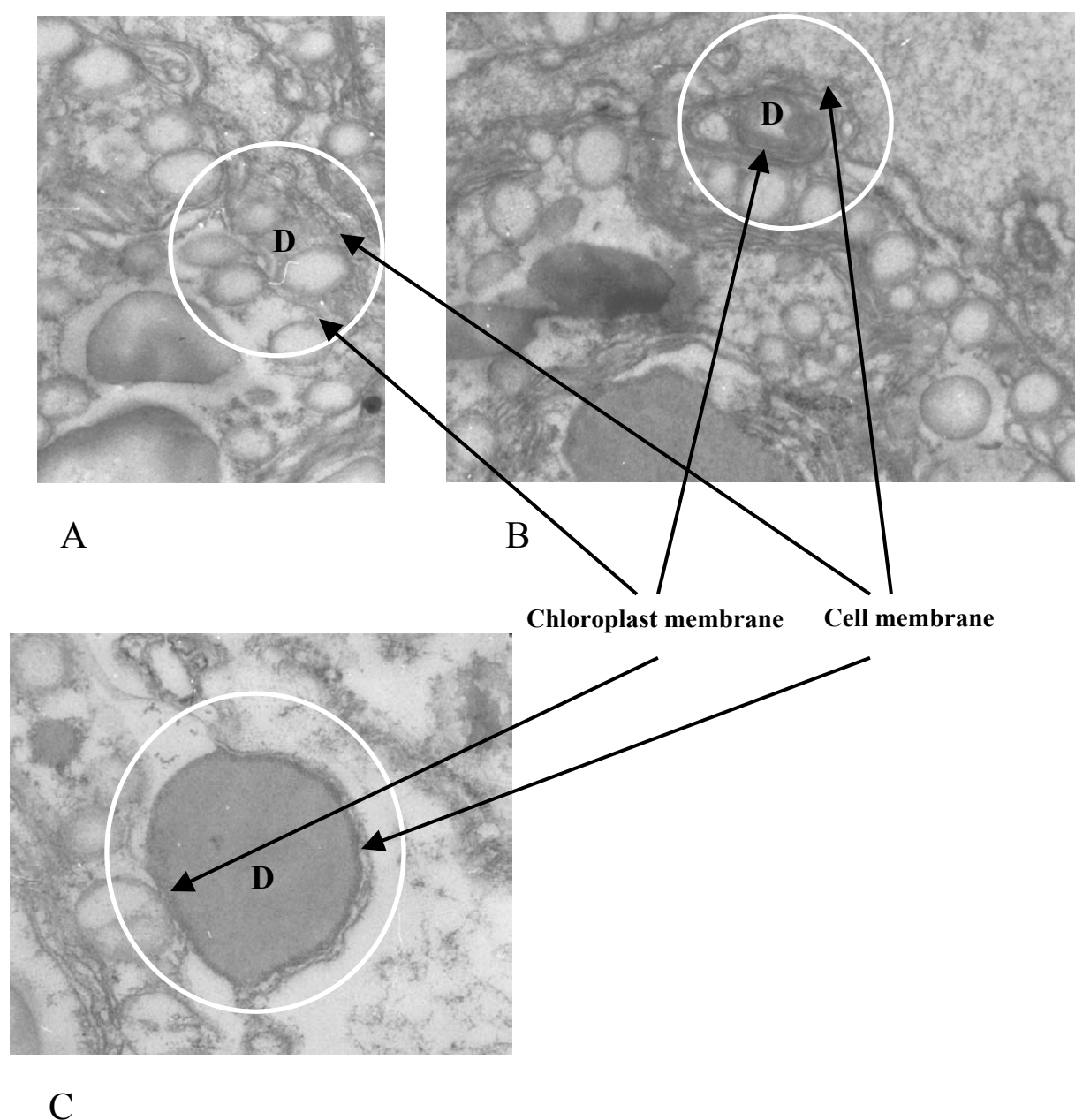
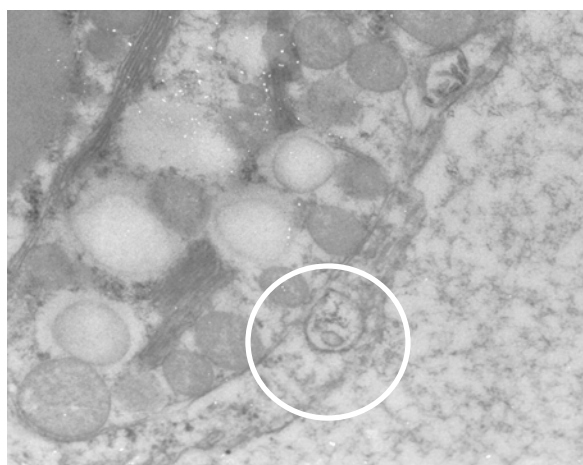


Figure 6.7: The space between the cell and chloroplast membrane in the cells of *D. salina* in which β -Carotene globules (D) are observed. part of cells: growing in saturated phase (A), in the presence of dodecane phase (B), and in the presence of dodecane phase brought to direct contact with dodecane (C) are shown

The cells growing in the presence of the organic phase and the saturated phase showed also some smaller vesicles in the space between the chloroplast and the cell

membranes(Figure 6.8). This could indicate that endo-and exo-cytosis of the cells is more active in the presence of dodecane and it could play a role in the extraction process. It was also shown by Zhang and co-workers (1993) that vesicle formation and endo- and exo-cytosis is affected by the presence of some molecules.

Lers and co-workers (1990) showed that β -carotene is the main pigment of the globules and they have almost no chlorophyll. Furthermore our results suggest that the β -carotene extracted from the cells comes from the globules. This explains why the extraction is selective as well as why the extraction rate is a function of β -carotene content of the cells as was shown in our previous papers (Hejazi et al., 2002, Hejazi et al., 2003).



A



B

Figure 8: Part of cells growing in saturated phase. A vesicles which is located between the cell and the chloroplast membrane (**A**) and another vesicles which seems being released from the cells of *D. salina* (**B**) are shown

Hypothesis for the extraction process

Based on the results the following hypotheses for the extraction can be made: Dodecane dissolves in the cell membrane and causes some alterations there. Subsequently, it is taken up by the cells into the space between cell membrane and chloroplast membrane by diffusion. This leads to more activity in the membranes and to more active endo- and exo-cytosis. As a result the globules are moved from the chloroplast to the space between the cell and chloroplast membranes and afterwards released from there to the medium. Another hypothesis suggests that molecules of β -carotene are released from the globules as a result of alterations in the membranes of the globules. The molecules diffuse from the chloroplast to the space between the chloroplast and cell membranes. From there the molecules either directly diffuse to the medium or first are accumulated inside the vesicles and then released from the cells by exo-cytosis.

Conclusions

Direct contact between the cells and organic phase is an important parameter for the extraction. Dodecane is taken up by the cells and has some influence on the ultra-structure of the cells. The cell membrane is more active in the presence of the biocompatible organic phase. We think that one of the mechanisms of the extraction of β -carotene is movement of the globules from the chloroplast to the space between the cell and the chloroplast membranes and subsequently from there to outside by exo-cytosis. Another possible mode for the extraction could be release of β -carotene from the globules as a result of alterations in the membrane of the globules. β -Carotene molecules subsequently diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exo-cytosis after accumulation in vesicles.

Acknowledgement:

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Chapter 7

Milking of microalgae

Abstract

*The low productivity of algal cultures in producing high-value compounds is the largest bottleneck in commercialization. Milking of microalgae for their content of high-value compounds using cultures in which cell mass is reused for continuous production is proposed as a solution to overcome this bottleneck. Having successfully developed a method in which we can milk β -carotene from the microalga *Dunaliella salina* in two-phase bioreactors passed the question whether it would be possible to milk other species of microalgae? Would this technique be suitable for mass production of secondary metabolites? To answer these questions, the mechanism of extraction in relation to the production pathway of the target product should be well understood. Our previous results as well as the results of other researches suggest that the chemical behaviour and molecular structure of the solvent, the chemical properties of the product and its location in the cells and finally, physiological behaviour of cells and particularly the nature of cell membrane are important parameters for a successful milking process. This paper discusses approaches which are helpful in understanding the mechanism of the milking process and its relation to the product-formation pathway. Some other products which may perhaps be milked from various species of microalgae (astaxanthin, neurotoxins and DHA) are discussed.*

This chapter has been submitted as: Hejazi M A, and Wijffels R H. Milking of microalgae.

Introduction

Milking is in an essence efficient process because cows (the biomass) are continuously reused for milk production. Not all cows however are efficient milk producers and production of milk from cows may not always be cost effective.

Although microalgae are a unique source of high-value compounds their commercial application is still limited (Borowitzka, 1999a). A major bottleneck for the application of microalgae is the low culture productivity. One fundamental reason for this is that the growth rate of cells is slow because of inefficient use of strong light. Furthermore, most of the microalgal products are secondary metabolites, produced when cell growth is limited. Milking of secondary metabolites from microalgae, i. e. continuously removing the secondary metabolites from the cells, for the production of high-value compounds, in which the culture biomass is reused for continuous production may therefore be a solution to overcome this bottleneck.

Milking products from microorganisms has been reported before. Frenz and co-workers (1989) collected a substantial fraction of hydrocarbons (hydrophobic hydrocarbons) from the microalga, *Botryococcus braunii*, establishing a short contact between the cells and hexane. In their method the cells were harvested, separated then contacted with the organic phase for extraction. After extraction the cells were returned to the bioreactor. Sauer and Galinski (1998) applied a milking technique for production of ectoines, a group of osmo-regulating solutes, from the halophilic bacterium *Halomonas elongata*. Ectoines were biosynthesized when the bacteria were incubated in media with higher salinity. After that cells were transferred to a medium with low salinity to which ectoines were extracted. Subsequent reincubation in a medium with higher salt concentration resulted in resynthesis of these compounds. Both reports claimed it is possible to produce large amounts of product using these processes. However, they did not compare the productivity of the milking process with existing processes.

We developed a new method in which we can milk β -carotene from *Dunaliella salina* in two-phase bioreactors (Figure 7.1). In this technique the cells first are grown under normal growth conditions and afterwards are stressed (by excess light) to produce larger amounts of β -carotene. In this stage the second phase (biocompatible

organic phase) is added and the β -carotene is extracted selectively via continuous re-circulation of a biocompatible organic solvent through the aqueous phase containing the cells. The cells continue to produce β -carotene and thus the extracted part is substituted by newly produced molecules. By 'milking' β -carotene from the cells, the cells are continuously reused and need not to be grown again. In contrast to the commercial process this method does not involve harvesting, concentrating and destroying of the cells for extraction of the favorable product. Furthermore, the purification of the product is very simple because of the selectivity of the extraction process. Finally, productivity of the system is about 25 times higher than that of commercially applied systems (Hejazi et al., submitted for publication). We therefore propose that application of this process in a general way would be very useful in commercialization of microalgal biotechnology and development of markets for microalgal products. Is it possible however to milk all microalgae? And is it possible to mass produce the products by means of this technique?

To answer these questions the mechanism of the milking process at cellular level should be understood. Due to the fact that the milking process is directly related to the production pathway related to the stress factors which enhance production of β -carotene, this inter-relation should also be studied. In this paper the processing approaches helpful for understanding the mechanism of milking and its relation with stress factors will be discussed. In addition, some microalgal products that could be produced by milking processes will be discussed.

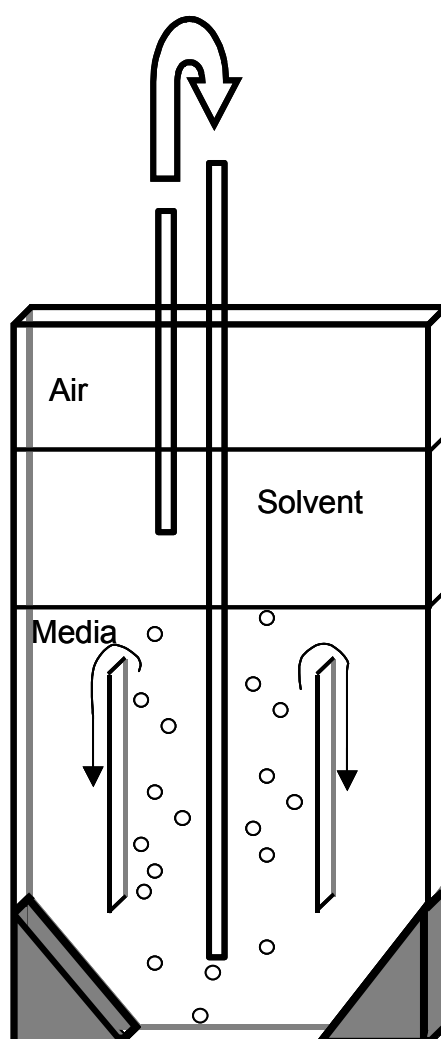


Figure 7.1: scheme of a flat panel two-phase bioreactor used in milking process of *Dunaliella salina* for β -carotene production

Mechanism of extraction

Several hypotheses were made concerning the mode of β -carotene extraction from cells of *D. salina*. We performed some experiments to test these hypotheses. Our results gave an indication for the location and distribution of β -carotene globules inside the cells and mode of extraction of β -carotene (Figure 7.2): when the cells are grown in the presence of a biocompatible organic solvent, the organic solvent is dissolved in the cell membrane, in which it causes some changes in the membranes. Some globules are extracted from the chloroplast to the space between the

chloroplast and cell membranes and subsequently from there to the outside of the cell. Another possible mode for the extraction could be release of β -carotene from the globules as a result of alterations in the membranes of the globules. β -Carotene molecules diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exocytosis after accumulation in the vesicles (Hejazi et al., Submitted for publication). Ginzburg and co-workers (1999) showed that the cells of *D. salina* have an ability for continuous endo- and exo-cytosis. During this process the cell membrane makes small vesicles and in this way medium components are taken up and subsequently released.

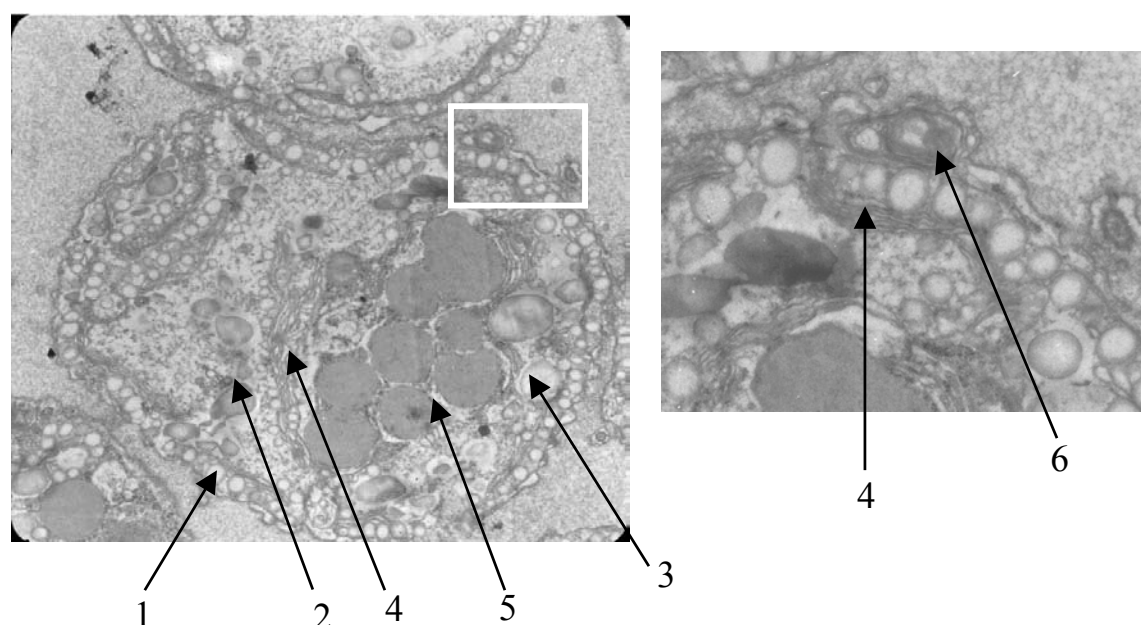


Figure 7.2: Electron microscopy picture of an stressed cell, grown in two-phase system with medium and dodecane. Left: whole cell, Right: enlarged part 1: β -carotene globules, 2: starch molecules, 3: vacuoles, 4: thylakoid membranes, 4: large lipid globules, 5: a globule in the space between the chloroplast and cell membranes that seems being extracted

Our results as well as results obtained by other researchers suggest that several parameters are involved in the successful milking of an intracellular product from the cells (Figure 7.3). Contact between product and solvent is the first step in the extraction process and properties of the cell membrane are important in this contact.

The other important factor is the location and the way in which the product is accumulated inside the cells (Hejazi et al., submitted for publication). Direct contact between lipophilic compounds and hydrophobic

parts of the cell membrane may be prevented by the presence of cell wall and/or the hydrophilic parts of the outer membrane (Sikkema et al., 1995). This could most probably prevent the solvents from making contact with the product.

The solvent chosen needs not only to be biocompatible for the cells but must also be a good solvent for extraction of the product (Buitelaar et al., 1991). Reaction of different solvents with the cell membrane depends on their molecular structures, e. g. the reaction of cyclic hydrocarbons with the cell membrane is different than the reaction of alkanes with the cell membrane (Sikkema et al., 1995 and McIntash et al., 1980). Therefore this parameter should also be taken into account.

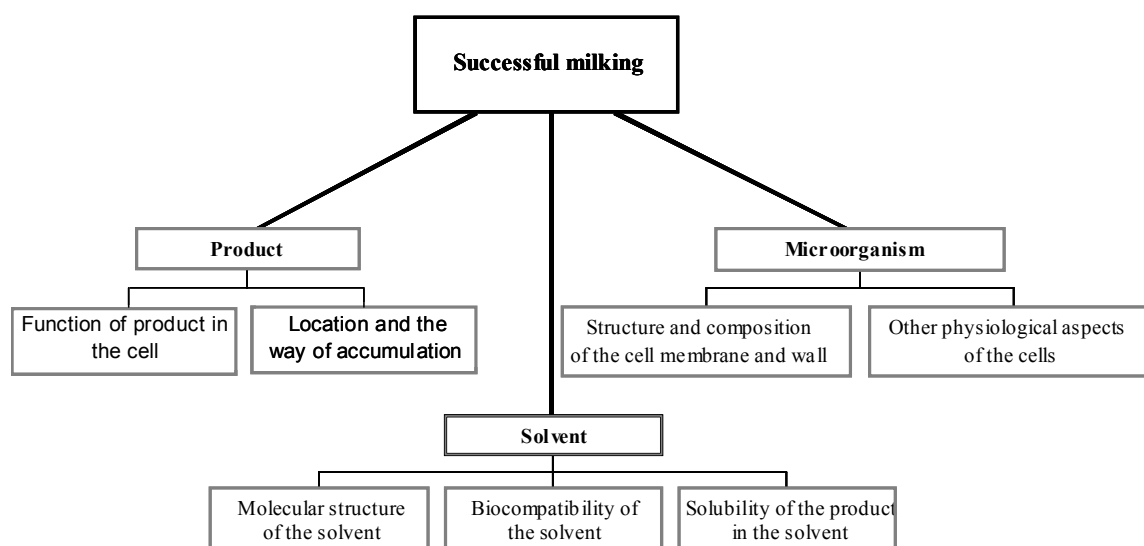


Figure 7.3: Important parameters in the milking process

Our results also suggested that the physiological properties of the cells and their ability for continuous endo- and exo-cytosis most probably play a role in this process. Since the ability for end- and exo-cytosis is not specific for *D. salina* and is found in most eukaryots (Zhang et al., 1993), determination of the involvement of this process in the extraction is very important.

An approach for studying the details of the extraction process could be development of a method for monitoring it in micro-scale bioreactors. It would be possible then to monitor the changes, for instance, in fluorescence signals of the living cells with microscopic techniques. On-line monitoring of changes in fluorescence in the cells will help to understand exactly what is happening at the cell level. It might be even possible to show the extraction process in motion photography. It would be possible to see where the main changes occur (in the membranes, inside the chloroplast or somewhere else). Another approach would be following the kinetics of the uptake of the organic solvent and β -carotene release from the cells. Using analytical and electron microscopy methods it would be possible to determine changes in the composition and structure of whole cells and different cell organelles (cell membrane, chloroplast and β -carotene globules) in time.

Understanding the mechanism of extraction is not only useful for the design of the process and its scale-up but would be also useful in applying the process for other products. By this information we can select a solvent (not only on the basis of their biocompatibility but also on the basis of other chemical properties), we can predict what kind of microalga can be used in the milking process (on the basis of cell wall and cell membrane properties) and what kind of products can be milked from the cells (on the basis of its location and way of accumulation).

Understanding the mechanism of stress and its relation with the milking process

D. salina has an ability for production and accumulation of large amounts of β -carotene under stress conditions such as high light intensity, high salinity and nutrient deficiency (Ben Amotz, 1995). Some studies have been performed on the mechanism involved in stressing cells for β -carotene production. Lers and co-workers (1990) studied massive β -carotene accumulation by *D. bardawil*. They suggested that β -carotene accumulation occurs as a result of photo-induced activation of the gene(s) expressing the β -carotene biosynthetic enzyme(s), or controlling factor(s). They further suggested that, possibly, a similarity might exist between the mechanism of β -carotene overproduction in high-illuminated, stationary phase cells and low-

illuminated cells of which the growth was limited by nutrient deficiencies, or other environmental stresses. Rabbani and co-workers (1998) also studied the mechanism of β -carotene accumulation by *D. bardawil* and its relation to lipid production. They found that β -carotene and lipid accumulation in the cells are inter-dependant processes. They suggested that accumulation of β -carotene in the lipid globules prevents inhibitory effects of β -carotene over-production under stress conditions. When the synthesis of triacylglycerol is blocked, the overproduction of β -carotene is also inhibited. Therefore, activation of the lipid production pathway is enough for carotenoid accumulation.

Our previous experiments showed that by using solvents a higher cell concentration of β -carotene can be obtained. This is due to substitution of extracted molecules by newly produced molecules by the cells (Hejazi et al., submitted for publication). The mechanism of replacement of extracted molecules of β -carotene in the cell and the interaction between this mechanism and other stress factors is not known. Previous studies described in the previous paragraphs, suggest that during the milking process continuous extraction of β -carotene containing globules enhances the lipid production pathway. Subsequently, new globules with the capacity to accumulate β -carotene molecules appeared. These globules absorb more β -carotene molecules from the production site and the inhibitory effect of β -carotene on over-production is prevented, thus continuous production of β -carotene follows.

The interaction between milking process and stress factors should be studied in a more systematic way at molecular level. By better understanding the mechanism of stress and its relation to the milking process it would be possible to design an efficient milking process.

Application of the concept of 'milking' to other processes

The main aim of our research project was to investigate the milking process as an alternative for the common processes used in production of microalgal products. We chose *D. salina* and β -carotene respectively, as models for microalgae and products. We will discuss in this part three different microalgal products as examples of high

value compounds which we think can be produced using the milking process. These products have different features and are obtained from microalgae of different properties. The first example is astaxanthin production by the autotrophic microalga *Haematococcus pluvialis* in an aqueous – organic two-phase bioreactor. In contrast with *D. salina*, *H. pluvialis* has a rigid cell wall. The second process is autotrophic production of neurotoxins by *Alexandrium tamarense*. These products are less lipophilic and their process should be carried out in an aqueous – aqueous two-phase bioreactor. The third example is production of docosahexaenoic acid (DHA) by *Cryptocodinium cohnii*. This product is a lipophilic compound produced heterotrophically and its process should be done in an aqueous – organic two-phase bioreactor.

1. Astaxanthin

Astaxanthin is a pigment of the group of carotenoids which has several applications. Astaxanthin is used in aquaculture e.g. to give salmon a pink color. Recent studies have also described nutraceutical importance of this carotenoid such as free-radical scavenging, immunomodulation and cancer prevention (Margalith, 1999). *Haematococcus pluvialis* is a microalga with the ability for production and accumulation of astaxanthin. According to Borowitzka (1992) *H. pluvialis* can accumulate astaxanthin up to 1% of dry weight. It was also reported that under proper conditions it can reach up to 6-8% of dry weight (Lee and Zhang, 1999). Average concentration of astaxanthin in commercially produced *H. pluvialis* is about 3% of dry weight.

Application of two-phase bioreactors for milking *H. pluvialis* for astaxanthin production would be interesting for a few reasons. The market value of astaxanthin is 2500 € /kg (Choi et al., 2002) which is higher than the value of β –carotene (750 € /kg) (Pulz et al., 2001). However, cultivation *H. pluvialis* is more complex than cultivation of *D. salina* and the productivity of the cultivation system is lower (Lee and Zhang, 1999). Since the final product cost is sensitive to the algal productivity and the length of the

growth period (Borowitzka, 1992) the process is at present hardly economically feasible.

According to Borowitzka (1992) extraction, purification and concentration are a heavy burden on the production cost. Since the concentration of the product in the cells is obviously important, thus milking is expected to be more successful for *H. pluvialis* than for *D. salina*, which can accumulate relatively high amounts of desired product (β -carotene).

To study this process the same systematic approach used for *Dunaliella salina* has to be applied: select biocompatible solvents, test the possibility of extraction, and the effect of the organic solvent on accumulation and on location in the cell. *H. pluvialis* has a rigid cell wall and accumulation of astaxanthin in this cell is taken place in a different mode than β -carotene in *D. salina*. Under optimal conditions the cells of *H. pluvialis* are enclosed by a cell wall which is separated from the protoplast by a watery jelly region traversed by cytoplasmic threads. It has been verified that the astaxanthin accumulation occurs in the cytoplasm of *Haematococcus* (Lee and Zahng, 1999). It has been demonstrated that astaxanthin accumulation occurs when the cells are transformed from vegetative cells into the aplanospore. This transformation accompanied by several morphological feature changes. The cell wall becomes considerably thicker and the volume of the cell increases dramatically. Some authors reported that pigment production is not exclusive to aplanospores but may also be demonstrated in growing flagellates (Margalith, 1999). Extraction of astaxanthin from *H. pluvialis* would therefore be different from the extraction of β -carotene from *D. salina*. Possible solutions can be found in natural mutants of the algae without a rigid cell wall or select altogether other microalgal species with the ability of astaxanthin accumulation (Lee and Zahng, 1999).

2. Neurotoxins

Microalgae are a unique source of some bioactive metabolites, which have great potential

applications in pharmaceuticals. Neurotoxins serving as a good example of these metabolites. Neurotoxins form a varied group of compounds. They vary in chemical

structures and mechanisms of action and produce very distinct biological effects (Shimizu, 2000). In general, these toxins have been shown to block the influx of sodium through excitable nerve membranes, thus preventing formation of action potentials (Borowitzka, 1999b). Okadaic acid, a diarrhetic shellfish toxin and tumor promoter found in many dinoflagellates of the genera *Dinophysis* and *Prorocentrum*, is used for studies of cellular regulation. Saxitoxin and its analogues; the gonyautoxins, neosaxitoxin and C toxins (the paralytic shellfish toxins (PSTc) produced by *Gymnodinium catenatum* and many species of *Alexandrium* including *A.minutum*), along with the brevetoxins (neurotoxin shellfish toxins produced by *Gymnodinium breve* and other red tide dinoflagellates), are used to investigate the structure and function of ion channels in cell membranes. These compounds are therefore important tools to study the effect of various agents on neuromuscular systems. The production of toxins for toxin standards is also essential for seafood and water monitoring programs (Parker et al., 2002). Saxitoxin has also a similar effect as tetrodotoxin (Borowitzka, 1999b). Tetrodotoxin, is isolated from puffer fish (Nishikawa et al., 2003) and is used for production of a drug called tetrodin, used for treating heroin addiction (<http://www.wextech.ca/page/products/tetrodin.htm>).

Neurotoxins are in general large molecules with hydrophobic and hydrophilic areas. Extraction with hydrophobic solvents would not work in this case and aqueous two-phase systems have to be utilized. The latter can be made by dissolving two incompatible polymers or a polymer and salt in water (Zijlstra et al., 1996). For neurotoxins, an aqueous two-phase system consisting of an aqueous phase with high salt concentrations where the microalgae is present and a phase with a polymer to which the molecules are extracted, can be used. The microalga to be used is a marine alga, for which there is no difficulty to use higher salt concentrations. The most important issues in designing technically and economically feasible processes are the partitioning of the cells and their products in aqueous two-phase systems, and the activity and stability of the cells in aqueous two-phase systems (Zijlstra et al., 1998). Therefore design of a two-phase-system in which simultaneous production and extraction is possible, is suggested as the initial step. The research should be systematically continued by studying viability of cells and extraction capacity separately in the presence of the phases that can be possibly used. The second

aqueous phase containing polyethylene glycol, can be the ideal phase to which the neurotoxins are extracted.

3. Docosahexaenoic acid (DHA)

The ω -3 long chain polyunsaturated fatty acids (PUFAs) are valuable ingredients of food and pharmaceutical products due to their beneficial influence on human health. PUFAs, particularly DHA, are important in the development and functioning of brain, retina and reproductive tissues for both adults and infants (Jiang and Chen, 2000). PUFAs can also be used in the treatment of various diseases and disorders, including cardiovascular problems, a variety of cancers and inflammatory disease. PUFAs are currently produced commercially from fish oil, which is an insufficient source for these products (Cohen, 1994). The heterotrophic marine microalga *Cryptocodinium cohnii* is known for its ability to accumulate fatty acids with a high fraction (30-50%) of DHA.

Lipids are important components of algal cell membranes (Jiang and Chen, 2000) but are also accumulating in globules in other parts of the cells (Ratledge, 2002). Sijtsma and co-workers (1998) performed economic feasibility calculations for DHA production from microorganisms. They compared the DHA production costs of three different microorganisms: a fungus *Thraustochytrium aureum*, an alga *C. cohnii* and an oleaginous yeast *Cryptococcus curvatus*. They showed that the product costs largely depended on the productivity of the microorganisms (biomass concentration and lipid production).

Growth and fatty acid formation by micralgae are affected by medium composition and environmental conditions. Lipid accumulation in an oleaginous microorganism often begins when it exhausts a nutrient, usually nitrogen, from the medium (Ratledge, 2002). Jiang and Chen (2000) studied the effect of medium glucose concentration on DHA content of *C. cohnii*: low glucose concentrations enhance the degree of fatty acid instauration and DHA formation but was accompanied by a slow growth rate. de Swaaf and co-workers (2003) also studied DHA production by *C. cohnii*, investigating effect of different carbon sources on biomass, lipid, and DHA concentrations. It was shown by them that when growth is exponential, lipid

concentration decreases. Afterwards when the growth become linear as a result of an undefined nutrient limitation, lipid concentration increases exponentially. It may be concluded from this that lipid production like β -carotene production is taking place under growth limiting conditions. In the linear growth phase the cells are stressed due to nutrient limitation and produce more lipid. From their results it can further be concluded that the quality of the lipid (%DHA) is also affected by the growth rate and lipid concentration. The concentration of DHA is negatively affected by increase in the lipid concentration. The highest lipid quality was obtained with the cells grown on glucose as carbon source, under which conditions cell concentration and lipid content of the cells were the lowest.

These results show that growth and DHA accumulation by the cells require different medium compositions. A similar process which is used for *D. Salina* can be used for *C. cohnii* to enhance DHA productivity. In this process the cells are first grown under proper conditions for growth and afterwards they are stressed to produce higher concentrations of DHA. In the DHA production stage, a biocompatible organic solvent is added to perform the extraction. By application of this process, high quality lipid will be produced on one hand which reduces the extraction and purification costs. On the other hand, higher amounts of DHA will be produced due to substitution of extracted lipids by newly synthesized lipid. As a result the productivity of the system is increased

Conclusions

A great potential for application of a newly developed method called “ microalgal milking” is described. It is assumed that by applying this technique in a general way the main problem in commercialization of microalgal products which is the low productivity, will be solved.

In order to design an efficient milking process, the mechanism of extraction in relation to the production pathway of the target product should be well understood. for a successful milking process the chemical behaviour and molecular structure of the solvent, the chemical properties of the product and its location in the cells and finally, physiological behaviour of cells and particularly the nature of cell membrane are

important parameters. This paper discussed approaches which are helpful in understanding the mechanism of the milking process and its relation to the product formation pathway. We also introduced a few examples of interesting opportunities for microalgal products (astaxanthin, neurotoxins and DHA) which can be continuously milked from their cells.

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Summary

The low productivity of photobioreactors used for production of high-value compounds from microalgae is a big bottleneck in commercialization. “Milking” of microalgae for the production of high-value compounds in which the produced biomass is reused for production can be a solution to overcome this bottleneck.

As it was described in **Chapter 1** our main aim was to investigate the possible application of a “milking” process using two-phase bioreactors in microalgal biotechnology. We chose β -carotene extraction from *Dunaliella salina* in a two-phase bioreactor with an aqueous phase and an organic solvent phase as our model system. The goal was to develop an alternative and more efficient process than the commercial production process of β -carotene.

In **chapter 2** biocompatibility of different solvents with values of $\log P_{\text{octanol}}$ ranging from 3 to 9 for the cells of *Dunaliella salina* was investigated. Extraction ability of different solvents for both β -carotene and chlorophyll was determined as well. 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, decreased. However, this decrease was more pronounced for chlorophyll. Therefore, selective extraction of β -carotene becomes feasible. 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.

Effect of mixing rate which is supposed to lead to the facilitated release of β -carotene from the cells of *Dunaliella salina* in two-phase bioreactors, was investigated in **chapter 3**. Three pairs of bioreactors were inoculated at the same time, operated at 100, 150 and 170 rounds per minute, respectively and illuminated with a light intensity of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each pair consisted of one bioreactor containing only aqueous phase for the blank and one containing the water phase together with the

biocompatible solvent (dodecane). Comparison of the viability and growth of the cells grown under different agitation rates showed that 170 rpm and 150 rpm were just as good as 100 rpm. Presence and absence of the organic phase had also no influence on the viability and growth of the cells. In contrast to the growth rate, the extraction rate of β -carotene was influenced by the stirrer speed. The extraction rate increases at higher stirring rate. The effectiveness of extraction per amount of power in-put was comparable for all the applied mixing rates.

In **chapter 4** the effect of light intensity on the extraction of β -carotene from *Dunaliella salina*, in the fermentative extraction, was investigated. Three different average light exposures were applied: $1.5 \cdot 10^{-8}$ (low), $2.7 \cdot 10^{-8}$ (intermediate) and $4.5 \cdot 10^{-8}$ (high) $\mu\text{mol s}^{-1}\text{cell}^{-1}$. Results showed that β -carotene content of the cells increases by increasing the light exposure. Increase in the β -carotene content of the cells was not necessarily coupled with an increase in the volumetric production of β -carotene. β -Carotene extraction rate was enhanced by the increase in the light exposure. The results suggest that extraction rate was related to β -carotene content of the cells and was not essentially related to the volumetric productivity of β -carotene. Although the effectiveness of extraction with respect to the light input was comparable for all light intensities applied, increasing the light input per cell led to a higher volumetric extraction rate.

On the basis of the previous results the “milking” process for β -carotene production was developed and introduced in **Chapter 5**. Growth of the cells was performed at low light intensity after which the cells were transferred to the production bioreactor, which was illuminated at a higher light intensity. The second bioreactor was a two-phase bioreactor consisting out of an aqueous and a biocompatible organic phase. In this bioreactor mixing and extraction were performed by re-circulation of the organic phase. The results showed that *D. salina* stayed viable for a long period (>47 d) in the presence of a biocompatible organic phase at high light intensity. The cell growth, however, was very slow in this situation. β -Carotene could be continuously extracted to the organic phase. The cells kept producing β -carotene and the extracted molecules were substituted by the cells. As a result β -carotene was continuously

milking from the cells. The β -carotene extraction efficiency in this system was more than 55%. The productivity of the system was $2.45 \text{ mg. m}^{-2}.\text{d}^{-1}$ which is much higher than obtained in commercial plants for β -carotene production.

Several studies at macro-scale (bioreactors) and micro-scale (using microscopic techniques) were performed for better understanding the mechanism of the extraction process. The results are presented in **Chapter 6**. Based on the results two hypothesis were made for the extraction: one of the mechanisms of extraction is transport of the β -carotene globules from the chloroplast to the space between the cell and the chloroplast membranes and subsequently from there to the outside by exo-cytosis. Another possible mode for the extraction could be release of β -carotene from the globules as a result of alterations in the membrane of globules. β -Carotene molecules diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exo-cytosis after accumulation in the vesicles.

In the last chapter, **Chapter 7**, we discuss the approaches which are helpful in answering two following fundamental questions: is it possible to milk all microalgae? And would this technique be suitable for mass production of high-value secondary metabolites? We think to answer these questions the mechanism of extraction and its relation with the production pathway of the target product should be exactly understood. Our previous results and the results of other researches suggest that chemical behavior and molecular structure of the solvent, chemical properties of the product and its location inside the cells and finally physiological behavior of the cell membrane and the cells by themselves are important parameters in a successful milking process. In this chapter we also discuss some other products (astaxanthin, neurotoxins and DHA) which we think can be milked from microalgae.

Samenvatting

De lage productiviteit van fotobioreactoren die worden gebruikt tijdens de productie van hoogwaardige verbindingen uit microalgen is een belangrijke bottleneck in het commercieel levensvatbaar maken van dergelijke processen. Het 'melken' van microalgen tijdens de productie van deze hoogwaardige verbindingen, waarbij de geproduceerde biomassa wordt hergebruikt, is mogelijkwerijs een oplossing voor deze bottleneck.

Zoals in **Hoofdstuk 1** is beschreven is ons hoofddoel het onderzoeken van de mogelijkheden voor de toepassing van een 'melk-proces' in de microalgen biotechnologie, waarbij gebruik wordt gemaakt van tweefasen bioreactoren. Als model systeem is gekozen voor β -caroteen extractie uit *Dunaliella salina* in een tweefasen bioreactor met een waterfase en een organisch oplosmiddel fase. Het doel was het ontwikkelen van een alternatief proces dat efficiënter is dan het huidige commerciële productieproces voor β -caroteen uit microalgen.

In **Hoofdstuk 2** is de biocompatibiliteit van diverse oplosmiddelen met een $\log P_{\text{octanol}}$ waarde in de range van 3 tot 9 voor *Dunaliella salina* cellen bestudeerd. Tevens is de extractiecapaciteit van de diverse oplosmiddelen voor zowel β -caroteen als chlorofyl bepaald. De resultaten laten zien dat oplosmiddelen met een $\log P_{\text{octanol}}$ waarde van groter dan 6 als biocompatibel voor deze alg kunnen worden beschouwd. Verder is de pigment extractiecapaciteit van een oplosmiddel omgekeerd evenredig met zijn $\log P_{\text{octanol}}$ waarde. Naarmate de hydrofobiciteit van het oplosmiddel hoger was, nam de extractiecapaciteit voor zowel chlorofyl als β -caroteen af. Deze afname was echter duidelijker voor chlorofyl. Hiermee is selectieve extractie van β -caroteen mogelijk. De hoogste β -caroteen productiviteit per cel in een 2-fasensysteem werd met dodecaan als oplosmiddel gemeten. De extractiecapaciteit van dodecaan, tetradecaan en hexadecaan was vergelijkbaar.

In **Hoofdstuk 3** werd het effect van de mengsnelheid bekeken, dat moet leiden tot het gemakkelijker vrijkomen van β -caroteen uit *Dunaliella salina* cellen in tweefasen

bioreactoren. Drie paar bioreactoren, geopereerd bij 100, 150 en 170 toeren per minuut en verlicht met een lichtintensiteit van $700 \mu\text{mol fotonen m}^{-2} \text{ s}^{-1}$, werden tegelijkertijd aangeënt. Elk paar bestond uit een bioreactor met alleen waterfase, als blanco, en een bioreactor die zowel een waterfase als een biocompatibele oplosmiddelfase bevat. Een vergelijking van de levensvatbaarheid en de groei van de cellen bij verschillende mengsnelheden liet zien dat een mengsnelheid van 170 en 150 rpm even goed waren als een mengsnelheid van 100 tpm. De aan- of afwezigheid van het organische oplosmiddel had ook geen invloed op de levensvatbaarheid en de groei van de cellen. In tegenstelling tot de groeisnelheid werd de extractiesnelheid van β -caroteen beïnvloed door de roersnelheid. De extractiesnelheid nam toe met de roersnelheid. De effectiviteit van extractie per hoeveelheid ingevoerd vermogen was vergelijkbaar voor alle mengsnelheden.

In **Hoofdstuk 4** werd het effect van de lichtintensiteit op de extractie van β -caroteen uit *Dunaliella salina*, tijdens de fermentatieve extractie, bestudeerd. Drie verschillende lichtintensiteiten werden toegepast: $1.5 \cdot 10^{-8}$ (laag), $2.7 \cdot 10^{-8}$ (gemiddeld) en $4.5 \cdot 10^{-8}$ (hoog) $\mu\text{mol s}^{-1} \text{ cel}^{-1}$. De resultaten laten zien dat het β -caroteen gehalte in de cellen toeneemt door de blootstelling aan licht te verhogen. Een toename in het β -caroteen gehalte van de cellen was niet noodzakelijkerwijs gekoppeld aan een toename in de volumetrische productie van β -caroteen. De β -caroteen extractiesnelheid werd verbeterd door de toename in de blootstelling aan licht. De resultaten suggereren dat de extractiesnelheid gerelateerd is aan het β -caroteen gehalte van de cellen en niet aan de volumetrische productiviteit van β -caroteen. Ook al was de effectiviteit van extractie met betrekking tot de lichtinput vergelijkbaar voor alle toegepaste lichtintensiteiten, leidde het verhogen van de lichtinput per cel toch tot een hogere volumetrische extractiesnelheid.

Op basis van de voorgaande resultaten is het 'melk-proces' voor β -caroteen productie ontwikkeld en geïntroduceerd in **Hoofdstuk 5**. De groei van de cellen werd uitgevoerd bij lage lichtintensiteit waarna de cellen werden overgebracht naar de productiereactor, die werd verlicht bij een hogere lichtintensiteit. De tweede bioreactor was een tweefasen bioreactor bestaande uit een waterfase en een

biocompatibele organische fase. In deze bioreactor werden menging en extractie uitgevoerd door de organische fase te recirculeren. De resultaten laten zien dat *D. salina* gedurende langere tijd bleef leven (> 47 dagen) in aanwezigheid van een biocompatibele organische fase bij hoge lichtintensiteit. De groei van cellen, echter, was in deze situatie erg traag. Het was mogelijk om β -caroteen continu te extraheren naar de organische fase. De cellen bleven β -caroteen produceren en de geëxtraheerde moleculen werden door de cellen vervangen. Het resultaat was dat β -caroteen continu uit de cellen werd gemolken. De β -caroteen extractie efficiëntie in dit systeem was hoger dan 55%. De productiviteit van het systeem was $2.45 \text{ mg m}^{-2} \text{ d}^{-1}$, hetgeen een stuk hoger ligt dan hetgeen in een commerciële installatie voor de productie van β -caroteen kan worden bereikt.

Verscheidene studies op macro-schaal (bioreactoren) en micro-schaal (gebruik makende van microscopische technieken) zijn uitgevoerd om een beter begrip te krijgen van het mechanisme van het extractieproces. De resultaten hiervan worden in **Hoofdstuk 6** gepresenteerd. Op basis van de resultaten zijn twee hypothesen opgesteld voor de extractie: een van de mechanismen van extractie is transport van de bolletjes van de chloroplast naar de ruimte tussen de cel en de chloroplast membranen en vervolgens van daar naar de buitenkant van de cel via exocytose.

Een andere mogelijke manier van extractie kan het vrijkomen van β -caroteen uit de bolletjes ten gevolge van een verandering van het membraan van de bolletjes zijn. β -caroteen moleculen diffunderen uit de chloroplast naar de ruimte tussen de cel en de chloroplast membranen en vanaf daar naar het medium door diffusie of exocytose na accumulatie in de zakjes.

In het laatste hoofdstuk, **Hoofdstuk 7**, worden de benaderingswijzen behandeld die van pas kunnen komen bij het beantwoorden van de volgende fundamentele vragen: is het mogelijk om alle microalgen te melken? En zou deze techniek geschikt zijn voor de grootschalige productie van waardevolle secundaire metabolieten? We denken dat we, om deze vragen te beantwoorden, het mechanisme van extractie en de relatie met de productie route van het doelproduct precies moeten begrijpen. Onze eerdere resultaten en de resultaten van andere onderzoekers suggereren dat

het chemische gedrag en de moleculaire structuur van het oplosmiddel, de chemische eigenschappen van het product en de locatie van het product in de cellen en als laatste het fysiologische gedrag van het celmembraan en de cellen zelf belangrijke parameters zijn in een succesvol melkproces. In dit hoofdstuk bediscussiëren we enkele andere producten (astaxantine, neurotoxines en meervoudig onverzadigde vetzuren) waarvan we het idee hebben dat die ook uit microalgen kunnen worden gemolken.

Curriculum Vitae

Mohammad Amin Hejazi was born in Tabriz, Iran, on 27 March 1970. He obtained his BSc and MSc in Food Science and Technology from respectively Tabriz University in September 1993 and Tarbiat Modarress University, Tehran in May 1997.

He then worked in the Agriculture Engineering Division of the Agriculture Research Department in the province of East-Azerbaijan where he started the Food Technology Laboratory. One of the research projects he executed was destined as distinguished research by the governor general of the province of East-Azerbaijan in 1999.

He was awarded a scholarship from the Ministry of Science, Research and Technology of Iran, allowing him to do a PhD abroad, financed by the Iranian Ministry of Agriculture Jihad. He started this PhD in the Food and Bioprocess Engineering Group of Wageningen University in May 1999. From 1 October 2003 he will continue his research in the field of marine biotechnology as a Postdoc at the same group.

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Patent

Hejazi M. A., Wijffels R. H., Holwerda E., and Tramper J., Process for continuous production and extraction of carotenoids from natural sources, EP Application. No. 02076803.2 and PCT/NL03/0034.

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می‌تواند در حضور حلال زیست-سازگار و تحت شرایط شدت نور بالا زنده بماند ولی در این شرایط شدت رشد سلولها پائین است. سلولها همچنین توانایی تولید بتاکاروتن خود را حفظ می‌کنند و مولکولهای استخراج شده بتاکاروتن توسط مولکولهای جدیدی که تولید می‌شوند جایگزین می‌گردند. در نتیجه بتاکاروتن بصورت مداوم از سلولها دوشیده می‌شود. ضریب کارایی استخراج بتاکاروتن در این سیستم 55% است. بهره‌وری این سیستم خیلی بیشتر از مقدار تعیین شده برای واحدهای صنعتی رایج بوده و 2.45 میلی‌گرم در روز است.

ما همچنین آزمایشات متعددی برای درک مکانیزم فرآیند استخراج انجام دادیم. دو فرضیه براساس نتایج حاصله برای این مکانیزم پیشنهاد می‌شود. یکی از این مکانیزم‌ها می‌تواند انتقال گلبولهای حاوی بتاکاروتن از کلروپلاست به فضای مابین غشاء سلولی و غشاء کلروپلاستی و از آنجا به بیرون سلول توسط پدیده اگزو-سایتوزیز (exa-cytosis) باشد. مکانیزم دیگر می‌تواند آزاد شدن مولکولهای بتاکاروتن از گلبولها در نتیجه تغییرات انجام یافته در غشاء گلبولها باشد. مولکولهای بتاکاروتن از کلروپلاست به فضای مابین غشاء سلولی و غشاء کلروپلاستی نشر (diffusion) پیدا کرده و از آنجا توسط پدیده نشر ویا توسط پدیده اگزو-سایتوزیز و پس از تجمع در داخل وزیکولها به محیط بیرونی استخراج می‌گردند.

خلاصه :

بهره‌وری پائین بیوراکتورهای نوری مورد استفاده برای تولید ترکیبات با ارزش از جلبکهای میکروسکوپی یک تنگنای عمده در تجارتي کردن این محصولات است. "دوشیدن" جلبکهای میکروسکوپی برای تولید ترکیبات با ارزش که در آن بیومس تولید شده مجدداً جهت تولید بکار گرفته می‌شود، می‌تواند راه‌حلی برای این تنگنا باشد.

منظور اصلی این تحقیق بررسی امکان بکارگیری فرآیند "دوشیدن" در بیوتکنولوژی جلبکهای میکروسکوپی با بکارگیری بیوراکتورهای دوفازه بود. استخراج بتاکاروتن از دونالیا سالیانا در بیوراکتورهای دوفازه بعنوان سیستم مدل برای این تحقیق انتخاب گردید. هدف توسعه یک روش جایگزین و کاراتر نسبت به روش صنعتی جاری برای تولید بتاکاروتن بود.

ابتدا قابلیت زیست-سازگاری حلالهای مختلف آلی با مقادیر $\log P_{\text{octanol}}$ از 3 تا 9 برای دونالیا سالیانا مورد بررسی قرار گرفت. نتایج نشان داد که حلالهای دارای $\log P_{\text{octanol}} > 6$ می‌توانند بعنوان حلال زیست-سازگار در نظر گرفته شوند. همچنین نتایج نشان داد که استخراج بتاکاروتن توسط حلالهای زیست-سازگار یک استخراج انتخابی است و علیرغم وجود کلروفیل و بتاکاروتن در داخل سلولها، تنها بتاکاروتن استخراج می‌گردد. آزمایشات بعدی نشان داد که سطح تماس بین سلولها و حلال زیست-سازگار و همچنین محتوای بتاکاروتن سلولها عوامل موثر در سرعت استخراج هستند.

براساس این آزمایشات فرآیند دوشیدن بتاکاروتن از دونالیا سالیانا طراحی گردید. این فرآیند دارای دومرحله است: مرحله رشد که در آن سلولها تحت شرایط شدت نور پائین رشد داده می‌شوند و مرحله تولید و استخراج که در آن سلولها تحت شرایط شدت نور بالا قرار داده می‌شوند تا تولید بتاکاروتن افزایش یابد. در مرحله دوم فاز آلی نیز افزوده می‌شود و عمل بهم‌زدن و استخراج با سیرکولاسیون مداوم حلال در داخل بیوراکتور انجام می‌گیرد. نتایج نشان می‌دهد که دونالیا سالیانا بمدت طولانی

باريشيق

انسانلاريق ، انسانليغي خوشلايين
بىر ملتىك ، بىرلشمە يە باشلايين
بو خان خانلىق حكومتىن بوشلايين
بوگون گرەك بشراولسون بىر ملت
بىر ملتە اولارمى يوزحكومت ؟!

استاد شەريار

Peace

*We are all human being
Let us have a good living .
We are all one nation ,
Let us leave dissipation .
We are not enemies ,
Let us be families .*

M. Eshraghi (free translation)

تقدیم بہ :

روح بلند پدرم

مادر فداکارم

ہمہ محرابانم

سلوا و مریم عزیزم

و ہمہ آہنایی کہ دوستان می دارم

ای نام تو بهترین
سراغاز

محمد امین حجازی