

COMPARISON OF ASTAXANTHIN EXTRACTABILITY YIELDS FOR DISRUPTED *HAEMATOCOCCUS* CYSTS

(PARÇALANMIŞ *HAEMATOCOCCUS* KİSTLERİ İÇİN ASTAKSANTİN
EKSTRAKT VERİMLERİNİN KARŞILAŞTIRILMASI)

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ABSTRACT

This comprehensive study focusing on the extractability of astaxanthin from *Haematococcus* cysts was carried out to compare the effectiveness of the cell disruption procedures. Both single step and combined cell disruption procedures were evaluated. A physical, chemical, enzymatic or mechanical disruption process was applied prior to mechanical disruption (bead vortex homogenizer or tissue homogenizer) for the combined cell disruption procedures. In this study the best result was obtained with the consecutive mechanical disruption processes using the combined procedure of tissue homogenizer and bead vortex homogenizer, yielding 68.32 mg Ast/g DW which was 31% higher than that obtained with the single step bead vortex homogenizer treatment. It was concluded that the combined disruption procedures were more effective than the single step disruption procedures with respect to the extractability of astaxanthin from *Haematococcus* cysts.

Keywords: Astaxanthin, Cell disruption, Extraction, *Haematococcus pluvialis*

ÖZ

Bu çalışmanın ana amacı, minimum hücre içi metabolit kaybı ile maksimum ürün elde edilmesidir. Hücre parçalama teknikleri karşılaştırılarak Haematococcus kistlerinden astaksantin ekstrakte edilebilirliği araştırılmıştır. Astaksantin eldesi üzerine fiziksel, kimyasal ve mekanik hücre parçalama işlemleri değerlendirilmiştir. Sonuç olarak, 68.32 mg Ast/g DW astaksantin verimi ile kombine prosedür olan doku homojenizatörü takiben bilyalı vorteks homojenizatör uygulamasında en iyi verime ulaşılmıştır. Bu çalışmada kombine hücre parçalama işleminde tek basamaklı hücre parçalama işlemine göre daha başarılı olunmuştur.

Anahtar Kelimeler: Astaksantin, Hücre parçalama, Ekstraksiyon, *Haematococcus pluvialis*

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1. INTRODUCTION

Astaxanthin is a valuable carotenoid pigment with applications in the nutraceutical, cosmetic, food and feed industries [1]. Astaxanthin sells for approximately \$2500 kg⁻¹ and the annual worldwide aquaculture market estimated at \$200 million. Although over 95% of this market consumes synthetically derived astaxanthin, consumer demand for natural products over the synthetic pigments provides an opportunity for the production of natural astaxanthin by *Haematococcus* [2]. *Haematococcus pluvialis* can accumulate astaxanthin in cytoplasmic lipid vesicles, up to 8% of its dry weight in the form of 3*S*, 3'*S* enantiomers, as a mixture of mono and di-esters [3].

Under optimal conditions the cells are spherical to ellipsoid and are enclosed by a cell wall, which is separated from the protoplast by a region filled with a watery jelly and traversed by cytoplasmic threads [4, 5]. There are a lot of known factors that affect the astaxanthin accumulation in *H. pluvialis*; such as nutrient limitation or supplement [6], oxygen stress [7] and high light intensity [8]. Under stress conditions, the transformation of the vegetative cells into the aplanospore is accompanied by several morphological features. The cell wall becomes considerably thicker, immobilizing the flagellae which are eventually shed [5].

Haematococcus cysts have to be broken in order to recover astaxanthin. Cell disruption is one of the important steps in the downstream processing of intracellular products from microorganisms [9, 10]. There are several methods such as homogenization [11, 12], sonication [13] and chemical extraction [14] which have been used to release intracellular components. The cell disruption techniques have an important effect on the yields and quality of the intracellular compounds and the scale of operation restrict the type of applications. Intracellular product recovery is still accomplished mainly by mechanical methods, such as high-pressure homogenization and bead mill disintegration which are used at industrial scale [15].

The main goal of this study was to obtain the maximum cell disruption with minimum loss of intracellular metabolites. The extractability of astaxanthin from *Haematococcus* cysts was compared using different cell disruption procedures in the single stage and the combined processes. A physical, chemical or enzymatic disruption process was applied prior to mechanical disruption (bead vortex homogenizer or tissue homogenizer) for the combined cell disruption procedures. Furthermore, the optimum disruption parameters including disruption time and rotation speed were determined for the mechanical disruption processes.

2. MATERIALS AND METHODS

2.1. Preparation of Algal Biomass

Haematococcus pluvialis Flotow EGE MACC-35 was obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. Stock culture of *H. pluvialis* was grown photoautotrophically in BG11 medium [16] at 25 °C under continuous illumination (100 μmol photons m⁻² s⁻¹) in 15-L vertical panel photobioreactor (60 cm L x 8 cm W x 55 cm H, thickness of glass: 1 cm). The gas spargers were placed from both opposite corners of the reactor to supply air-CO₂ gas mixture (3% CO₂/air v/v) of 1 vvm to the culture. The diameter of the single nozzle sparger was 0.5 cm. The cells from the stock culture were harvested and concentrated by centrifugation at 2,000 x g for 5 min and the supernatant was removed. The sedimented cells were transferred, incubated aseptically in a 70 L panel

photobioreactor (108 cm L x 8 cm W x 126 cm H, thickness of glass: 1.5 cm) containing 50 L of N:P:K 20:20:20 fertilizer [17] under the light intensity of $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, at 25 °C for 14 days of growth period. Air was supplied to the culture at a flow rate of 50 L min^{-1} (1.4 vvm). To induce astaxanthin accumulation, 14-day old culture (approximately 2×10^6 cells ml^{-1}) was incubated in a 70 L panel photobioreactor containing 50 L of tap water at $1400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 14 days of induction period. The temperature was maintained at 29 ± 2 °C due to the heat dissipation from the fluorescent lamps, and was controlled with digital thermocouple inside the reactor.

Following the induction period, the encysted cell biomass was harvested by centrifugation at $2,000 \times g$, for 5 min, the pellet was frozen at -20 °C and then lyophilized. The resulting freeze-dried biomass was used as raw material for the cell disruption process.

2.2. Disruption of Microalgal Biomass

2.2.1. Tissue homogenizer

10 mg freeze-dried biomass was placed in a 20 ml tube containing 5 mL acetone and then homogenized with a tissue homogenizer (Yellow line DI25, IKA -Works, Germany) at different motor speeds. Three rotation speeds: 8000 rpm, 13500 rpm, and 20500 rpm were tested at two different disruption time: 1 and 2 min, resulting in a total of 6 different conditions to maximize the extractability of astaxanthin from *Haematococcus* cysts.

2.2.2. Autoclave treatment

10 mg freeze-dried biomass was autoclaved at 121 °C for 30 min under the pressure of 1.5 atm.

2.2.3. Bead vortex homogenizer

The bead mill was simulated using a bead vortex homogenizer for small scale studies. 10 mg freeze-dried biomass was placed in a 20 ml tube containing 5 ml acetone and charged with 50% (w/v) of glass beads (1 mm diameter). The suspension was agitated for different periods (1 and 2 min) with vortex homogenizer (Heidolph Reax Top) at different rotation speeds (825 and 1650 rpm), and then separated from glass beads.

2.2.4. Sonication

10 mg freeze-dried biomass was placed in a 20 ml tube containing 5 ml acetone and was sonicated using a sonicator (Bandelin Electronic UW 2070, Berlin, Germany) for 10 min at 0.8 sec intervals. The dismembrator had a maximum power output of 180 W and was operated at a constant frequency of 20 kHz. The horn was placed at the center of the tube. Sonication was carried out in an ice-water bath to avoid the increase of temperature that could affect the cell viability and the product quality.

2.2.5. Alkaline hydrolysis

10 mg freeze-dried biomass was treated in 0.1 M NaOH for 30 min. The supernatant was removed by centrifugation ($2,000 \times g$, 5 min).

2.2.6. Enzyme treatment

The mixtures of 10 mg freeze-dried biomass, 10 mg cellulase (Sigma Chemical Co., St Louis, MO, USA) and 10 mg distilled water were prepared to obtain enzyme reaction mixtures and were incubated for 24 h at 37 °C. Enzyme-treated biomass was harvested by centrifugation at $2,000 \times g$, for 5 min [18].

2.3. Analytical Procedure

The cell disruption performance was determined using a modified method from Mendes-Pinto et al. [19]. 10 mg disrupted cells were mixed with 5 ml acetone (Carlo Erba, 99%). The suspension was agitated for 3-4 min in a vortex and was left for 16 hours at the room temperature. The cell debris was removed by centrifugation prior to analysis. The absorbance of the combined extracts was measured at 480 nm. Astaxanthin concentration was determined using a calibration curve obtained with pure astaxanthin (Acros Co., New Jersey, USA) as a standard ($E_{1cm}^{1\%}$ 2500 in acetone). All data represent the means of three independent experiments.

3. RESULTS

The effects of 6 different cell disruption procedures on the extractability of astaxanthin from *Haematococcus* cysts were investigated. Both single step and combined cell disruption procedures were evaluated. A physical, chemical, enzymatic or mechanical disruption process was applied prior to mechanical disruption (bead vortex homogenizer or tissue homogenizer) for the combined cell disruption procedures. Furthermore, the optimum disruption parameters including disruption time and rotation speed were determined for the mechanical disruption processes.

3.1. Determination of Optimum Disruption Parameters for the Mechanical Disruption Processes

As shown in Figure 1A, when the cells were treated in the tissue homogenizer at 13500 rpm for 2 min, the release of astaxanthin was 32.15 mg Ast/g DW which was 1.1 fold higher than for 1 min at the same rotation speed. The release of astaxanthin was significantly lower (21.74 mg Ast/g DW) at 8000 rpm for 1 min. The best result (39.44 mg Ast/g DW) was obtained at the rotation speed of 20500 rpm for 2 min in the process of tissue homogenizer. In Figure 1B, the lowest astaxanthin recovery, 21.22 mg Ast/g DW, was obtained at 825 rpm for 1 min whereas the release of astaxanthin was 38.64 mg Ast/g DW at 1650 rpm for the same disruption time in the process of bead vortex homogenizer.

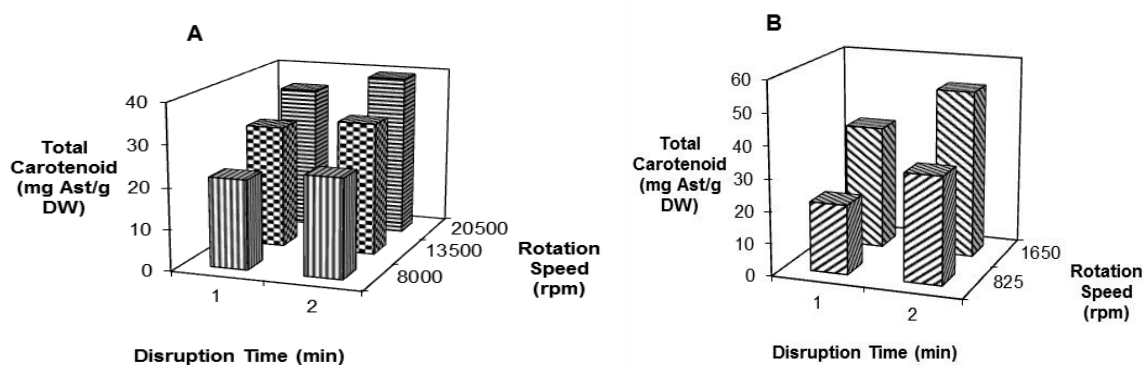


Figure 1. Determination of optimum disruption parameters for the mechanical disruption processes:

A. Tissue homogenizer, B. Bead vortex homogenizer

3.2. Single Stage Cell Disruption Procedures

When single step cell disruption procedures were applied, the best recoveries of astaxanthin were found in bead vortex homogenizer at 1650 rpm for 2 min and tissue homogenizer at 20500 rpm for 2 min with the values of 52.36 and 39.44 mg Ast/g DW, respectively (Figure 2). The release of astaxanthin was 29.29 mg Ast/g DW with the alkaline hydrolysis procedure employed. Similar yields were observed for cells that were exposed to sonication (27.84 mg Ast/g DW). The astaxanthin obtained by autoclave treatment was 1.02 times higher than that obtained by enzyme treatment.

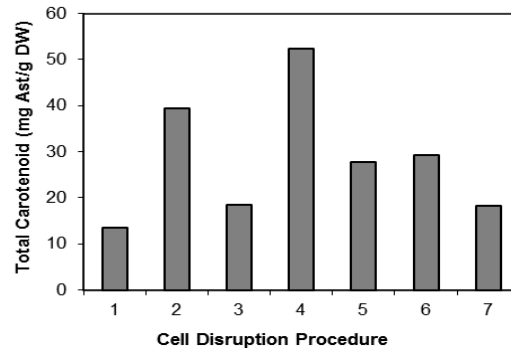


Figure 2. Comparison of the single step cell disruption procedures for the release of astaxanthin from *Haematococcus* cysts: 1. Control; 2. Tissue Homogenizer; 3. Autoclave treatment; 4. Bead vortex homogenizer; 5. Sonication; 6. Alkaline hydrolysis; 7. Enzyme treatment

3.3. Combined Cell Disruption Procedures

The intact cyst cells were treated with the combination of two different disruption processes in order to increase the efficiency of astaxanthin extractability from *Haematococcus* cysts (Table 1). A physical, chemical, enzymatic or mechanical disruption process was applied prior to mechanical disruption (bead vortex homogenizer or tissue homogenizer). When the cells were treated in the tissue homogenizer following bead vortex homogenizer treatment, the release of astaxanthin was 68.32 mg Ast/g DW which was 1.3 fold higher than the single step bead vortex homogenizer treatment. The release of astaxanthin was 57.42 mg Ast/g DW with the combination of sonication and tissue homogenizer. The astaxanthin recovery increased by 76% with the combination of alkaline hydrolysis and bead vortex homogenizer in comparison with the single step alkaline hydrolysis procedure. The release of astaxanthin (43.50 mg Ast/g DW) obtained with the combined procedure of enzyme treatment and bead vortex homogenizer was 21% higher than that obtained with the combined procedure of enzyme treatment and tissue homogenizer. The lowest yield was attained with the combination procedure of autoclave treatment and tissue homogenizer (34.22 mg Ast/g DW).

The best result was obtained with the consecutive mechanical disruption processes using the combined procedure of tissue homogenizer and bead vortex homogenizer yielding 68.32 mg Ast/g DW which was 31% higher than that obtained with the single step bead vortex homogenizer treatment. It was concluded that the combined disruption procedures on the extractability of astaxanthin from *Haematococcus* cysts were more effective than the single step disruption procedures.

Table 1. Comparison of the combined cell disruption procedures for the release of astaxanthin from *Haematococcus* cysts: 1. Control; 2. Tissue Homogenizer; 3. Autoclave treatment; 4. Bead vortex homogenizer; 5. Sonication; 6. Alkaline hydrolysis; 7. Enzyme treatment. (+): Applied, (-): Unapplied

Cell disruption procedures							mg Ast/g DW
1	2	3	4	5	6	7	
-	+	+	-	-	-	-	34.22
-	+	-	+	-	-	-	68.32
-	+	-	-	+	-	-	57.42
-	+	-	-	-	+	-	47.62
-	+	-	-	-	-	+	36.09
-	-	+	+	-	-	-	43.21
-	-	-	+	+	-	-	60.32
-	-	-	+	-	+	-	51.68
-	-	-	+	-	-	+	43.50

These results are an average of 3 different determinations with a standard error of $\pm 5\%$

4. DISCUSSION

Several experiments on the cell disruption procedures have been carried out by different authors. It is rather difficult to compare the results due to the differences in cultivation and disruption conditions. In some previous work, the algal cells were disrupted by using the tissue homogenizer [8], grinding with mortar and pestle [20- 22], heating at 60 °C [23] and 70 °C [24, 25], or enzymatic disruption [26, 27]. As reported by Kobayashi et al. [18], the percentage of astaxanthin extractability from *Haematococcus* cysts was 13% with the enzyme (cellulase) treatment while this value was 38% in the combination procedure of heat-acetone and enzyme treatment.

Haematococcus pluvialis shows low growth rates and low final cell densities under optimal growth conditions [28]. Cells grown at a higher specific growth rate are probably easier to disrupt than cells grown at a lower specific growth rate, since they direct the available energy towards reproduction rather than towards synthesis or strengthening the wall structure [29, 30].

Mechanical methods have several disadvantages, such as the absence of biological specificity, generation of high temperature in cell suspensions and high shear stress high capital investment cost, and long processing times. Chemical extraction is a possible substitute for mechanical disruption owing to its simplicity, relative speed and low cost [31, 32].

The addition of NaOH was necessary for the hydrolysis of astaxanthin esters, but NaOH could result in significant degradation of astaxanthins, especially when the reaction temperature was high. The results of Yuan and Chen [33] indicated that, although high temperature favoured the hydrolysis of astaxanthin esters, the degradation of astaxanthins was also promoted markedly at the same time. Therefore, the saponification should be carried out at a low temperature to minimize the degradation of astaxanthins during the hydrolysis of astaxanthin esters [27].

The results of this study indicate that tissue homogenizer has drawbacks, such as foaming and oxygen permeability except the above-mentioned. The reaction temperature also increases at high rotation speeds for the long disruption time due to the heat energy given off by the

friction forces. The high temperature could lead to metabolite degradation. To overcome these problems, tissue homogenizer should be operated at the optimum rotation speed within the shortest possible time. The mechanical tissue homogenizer is applied under the effects of both the frictional forces and the shear stress whereas the thermal autoclave treatment is only achieved at the high temperature.

Disruption involving rapid agitation of microbial cell suspension with glass beads is also a powerful technique. This procedure is particularly efficient in the disruption of yeasts and filamentous fungi, which have rigid cell walls mainly constituted by polysaccharides (80–90%) [29, 34]. The degree of disruption increases with bead loading due to increased bead-to-bead interaction; however, heating and power consumption also increase, because of this interaction [35].

Under the stress conditions *Haematococcus* cell is surrounded by a heavy resistant cellulose wall while astaxanthin is accumulated within oil droplets. This cellulosic structure naturally protects metabolites inside the cell. In this study, the cellulase enzyme was selected for the cell disruption process due to the cellulosic cell wall of *Haematococcus* cysts.

Sonication is normally carried out in bursts to reduce the heating effect and only used on a small scale. The mechanism of the cell disruption is associated with the cavitation phenomena, i.e. shear stress developed by viscous dissipative eddies arising from shock waves produced by imploding cavitation bubbles. Shear forces creating eddies larger than the cells are more likely to move the cells rather than disrupt them, whereas eddies smaller than the cells are capable of generating disruptive shear stresses. Thus, larger cells will experience more disruptive eddies than smaller cells. Increasing the power (intensity) will shift the size distribution towards smaller eddies, which in turn, will increase the number of disruptive eddies acting on the cells resulting in greater disruption [36]. It is also worth noting that the sonication process is more efficient for the microorganisms enclosed by less rigid cell wall. No experimental studies had been encountered concerning the sonication process on the extractability of astaxanthin from *Haematococcus* cysts in the scientific literature.

The ideal process involves the minimum number of downstream steps with high recovery and minimum effort for the adequate quality of the desired product. Increasing the number of process steps will normally decrease the productivity as well as increase in cost for downstream processing. The cell disruption processes have been receiving special attention in recent years for the combination of process steps to increase yield, to reduce of process time and to cut down in capital expenditure.

The effectiveness of the various disruption methods differs for different microbial species, depending on the properties of valuable intracellular products. It is also interesting to note that the grade of cell sticking on the chamber wall was significantly higher for the recovery of astaxanthin from *Haematococcus* cysts. Transferring the sample from one apparatus to another was a big problem due to the remaining cell debris for the combined disruption process. Therefore, a physical, chemical, enzymatic or mechanical disruption process was applied prior to mechanical disruption in order to minimize the loss of intracellular metabolites.

5. CONCLUSION

In this work, the best result was obtained with the consecutive mechanical disruption processes using the combined procedure of tissue homogenizer and bead vortex homogenizer yielding 68.32 mg Ast/g DW which was 31% higher than that obtained with the single step bead vortex homogenizer treatment. It was concluded that the combined disruption procedures on the extractability of astaxanthin from *Haematococcus* cysts were more effective than the single step disruption procedures. The main point is whether the increasing of 31% yield will meet the expectations concerning the capital expenditure for the combined disruption process, or not.

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