OPTIMALIZATION OF DNA ISOLATION PROCESS IN FRESHWATER MICROALGAE USING HOMOGENIZER

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Abstract: Last decades microalgae are gaining much interest due to high-valued biomolecules content. Increasing number of genetic studies and modifications require quality input material and short time analysis at the same time. Appropriate DNA extraction is one of the crucial and time limiting steps. Therefore in this study we set up optimum parameters for automatic homogenizer Precellys® 24 Evolution (using bead mills technology) for DNA extraction from selected species of green microalgae. Speed parameter 4 500 rpm, 6 800 rpm and 10 000 rpm was tested to obtain the best ratio quality/quantity/purity of DNA material. There were 3 protocols set up due to different sensitivity of microalgae cell walls on bead mills power, otherwise universal protocol needs to be a compromise between quality and quantity.

Key Words: green microalgae, DNA isolation, homogenizer

INRODUCTION

Microalgae are abounding in exceptional ability to adapt to extreme conditions. It means they can quickly reprogram their growth, metabolism and physiology (Weber et al. 2007). Some of mechanisms able to manage these changes may be induced by epigenetic aberrations in genetic code. People started to realize these special algal features and exponential growth of publications in Science Direct database confirms that high-valued products obtained from microalgae became hot topic during last decade. Especially genetic engineering became necessary for achieving new and economically viable products (Enzing et al. 2012) and has made significant progress towards biofuel production (Dunahay et al. 1992, Roessler et al. 1994), pharmaceutical (Soetaert et Vandamme 2010), feed (Yaakob et al. 2014) and food industries (Johanningmeier et Fischer 2010). And the basis of all genetic analysis is a quality input DNA material.

Nowadays, isolation of DNA is well managed and established process, ordinarily simplified by commercial kits. Anyway, obsolete sample extraction technique, like mortar and pestle, are still being used (Bhau et al. 2016). Even though it is established and well proved extraction, on the other hand, it is time consuming process with considerable losses of sample and therefore there is an effort to replace this technique for equipment much more effective. One of the options for grinding samples prior to DNA analysis is automatic homogenizer based on bead beating technology. There have been many protocols created for DNA extraction from different plant (Ramos et al. 2014) and animal tissues and microorganisms (Lopez et al. 2017) available, anyway there are still predicaments you can face in terms of green microalgae material.

While plant or animal cells are part of tissues and organs and these higher structures are further protected for example by cuticle, waxes, hair etc., microalgae are unicellular microorganisms, which need protection of the single cell against environment (Skaloud et al. 2013). In this regard, microalgae are protected by rigid, thick cell wall, which can be difficult to disrupt and extract (Kim et al. 2016). In this study we decided to optimize parameters of extraction in automatic homogenizer with the aim

to obtain optimal quality and quantity DNA material from microalgae for sensitive downstream applications including PCR, qPCR, genotyping, sequencing and more.

MATERIAL AND METHODS

Microalgae species and cultivation

For this study, five species of microalgae from different family were chosen (see Table 1). Algae samples were purchased from UTEX Culture Collection of Algae (Austin, USA) and subsequently re-cultivated in our laboratory of Plant Metabolomics and Epigenetics in Mendel University in Brno (Brno, Czech Republic). Inorganic liquid Tris Acetate Phosphate (TAP) medium, containing only inorganic salts and trace elements for algal growth (Purkayastha et al. 2017), was used.

Cultivation proceeded in Erlenmayer flasks, under sterile and well defined conditions:

- light: 70 µmol m^2/s
- temperature: 23 °C
- photoperiod: 12 h light/12 h dark

Figure 1. Classification of selected green microalgae species

Empire	Eukaryota				
Kingdom	Plantae				
Subkingdom	Viridiplantae				
Phylum	Chlorophyta				
Subphylum	Chlorophytina				
Class	Trebouxiophyceae			Chlorophyceae	
Order	Trebouxiophyceae	Chlorellales		Sphaeropleales	
Family	Coccomyxaceae	Chlorellaceae		Scenedesmaceae	
Genus	Coccomyxa	Chlorella	Parachlorella	Scenedesmus	
Species	*Coccomyxa subellipsoidea*	*Chlorella vulgaris*	*Parachlorella kessleri*	*Scenedesmus obliquus*	*Scenedesmus quadricauda*

DNA extraction

Many different physical parameters can affect DNA extraction step: liquid nitrogen treatment, bead mills type/size, rotor speed, number of cycles and also DNA isolation method. There are many variations of DNA isolation kits, specialized at animal/plant tissues with different kit patents. In this study we used PowerPlant® Pro DNA Isolation Kit (Quiagen, Germany).

Green algal cells were harvested by pipetting algal stock solution into 2 ml tubes, centrifuged (13 000 rpm/1 min) and culturing medium was removed. Glass beads (0.5 mm in diameter) were added to the weighed algal fresh sample and tubes were transferred into liquid nitrogen for a minute to disrupt cell walls. Algal cells were mixed with lysis buffer, phenolic separation solution, precipitation solution and RNase A solution from PowerPlant® DNA isolation kit. Tubes with samples were inserted into automatic homogenizer Precellys® 24 Evolution. This equipment is based on high-speed rotation, which is able to disrupt cell walls from high variety of samples. Multi-directional movement (3D motion) of sample holder transmits high level of energy to the beads inside each tube, and can grind up to 24 samples at one time. This ensures equal homogenization for all processed samples. The speed can range from 4 000 up to 10 000 rpm and whole process takes proportionally seconds (30 s up to 90 s), where also number of cycles can be set (up to 3). Another advantage of this equipment is also avoiding cross contamination comparing mortar and pestle. We were monitoring three different homogenizer speed set up: 4 500 rpm, 6 800 rpm and 10 000 rpm at a constant 20 s extraction time in 2 cycles. After homogenization, DNA isolation further proceeded according to kit manual. Samples were centrifuged at 13 000 x g for 10 minutes. Avoiding pellet, supernatants were transferred to a clean collection tube and treated with ethanol. DNA was captured on a spin filter and washed two times. Finally 75 µl of 10 mM Tris (pH 8.0) was loaded onto filter, incubated 2 minutes at room temperature and centrifuged 30 seconds at 10 000 × g. DNA material flowed through and was stored frozen (-20 °C). Microalgae samples were tested in triplicates.

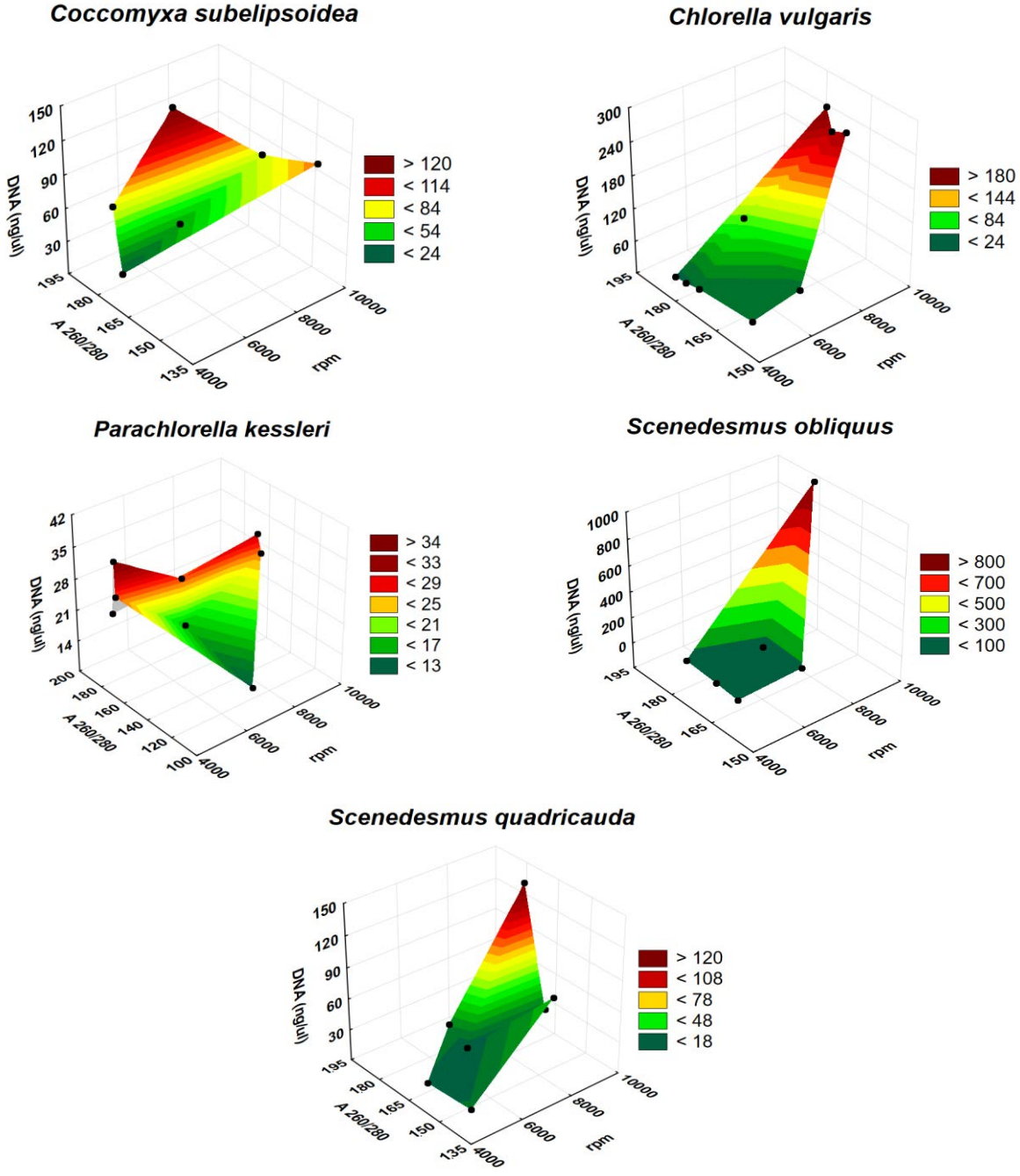
DNA quality

After DNA isolation, the concentration and purity of obtained material was determined by Tecan Infinite® 200 PRO spectrophotometer using NanoQuant plate, and calculated from absorbance ratio A 260/280. The integrity of obtained DNA was verified by agarose gel electrophoresis. DNA samples were loaded directly onto an agarose gel of viscosity 0.8% which was made in TAE and was colored with ethidium bromide dye. The electrophoretic process was running 1.5 h using 60 V.

RESULTS AND DISCUSSION

Quality and quantity of isolated DNA

Figure 2. 3D histogram shows a plot of DNA concentration vs. rpm and vs. A 260/280 ratio



Legend: DNA (ng/µl) - concentration of DNA (ng/µl), A 260/280 - absorbance purity ratio, rpm - homogenization speed; greens colour symbolize lower DNA concentration and red colours increasing DNA concentration

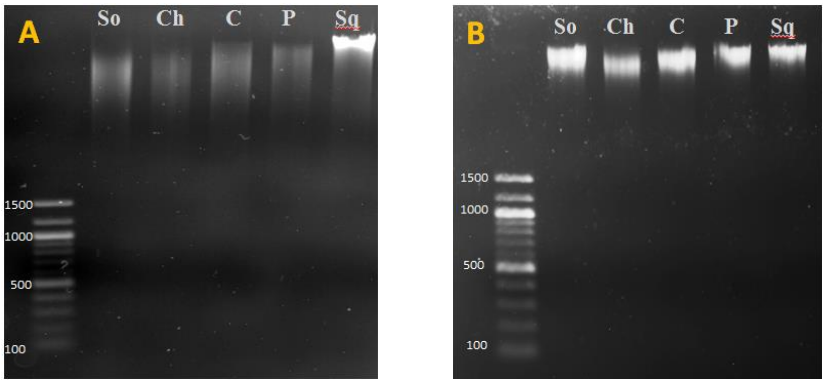
Testing three different homogenizer speed modes (rpm), DNA concentration ranged between 20–900 ng/µl within five different algae species. Considering the highest yield of DNA material obtained, we got the best results from the highest speed mode (10 000 rpm) in *Scenedesmus obliquus* (946.4 ± 14.8 ng/ul), Chlorella vulgaris (236.1 ± 37.4 ng/µl) and *Scenedesmus quadricauda* (130.0 ± 4.9 ng/µl). The higher speed, the more efficient cell damage and DNA release. However, exceptions were *Coccomyxa subelipsoidea* with the best obtained DNA yield (141.8 ± 12.3 ng/µl), using speed 6 800 rpm and *Parachlorella kessleri*, where we obtained the highest DNA concentration (34.6 ± 4.4 ng / µl) using the lowest speed 4 500 rpm. This could be caused by huge variety and different cell wall characteristics in each microalga species, where disruption and extraction methods can largely differ (Praveenkumar et al. 2015).

A 260/280 purity value of measured samples fluctuated between 1.4–1.9. Value of ~ 1.8 is generally accepted as pure for DNA material, therefore values suitable for further analysis were set up to 1.7–1.9. Inaccurate ratios may indicate contamination by residual phenols, guanidine or other reagent used in protocol (Wilfinger et al. 1997). In case of *Coccomyxa* and *Parachlorella*, the higher speed, the worse purity was obtained. In case of *Chlorella*, and both *Scenedesmus* species the purity had an opposite trend. The lower speed, the purity of DNA was getting worse.

Integrity of DNA material

The goal was not only to get the best yield, but also to get intact DNA material and therefore samples with the highest DNA concentration were subsequently run on gel electrophoresis to verify DNA integrity. Gel electrophoresis in the Figure 3A showed the integrity of the DNA in each microalgae. Four of five samples were not acceptable.

Figure 3 Agarose gel electrophoresis of A: the highest yields protocols in different microalgae, B: optimized protocols in different microalgae



Legend: So - Scenedesmus obliquus, Ch - Chlorella vulgaris, C - Coccomyxa subelipsoidea, P - Parachlorella kessleri, Sq - Scenedesmus quadricauda; 100, 500, 1000, 1500 - DNA marker in base pairs

Limiting step was to break microalgae cell wall and at the same time obtain intact gDNA. Only *Scenedesmus quadricauda* appears to endure such a bead mills power in regards to be able to keep DNA integrity. *Scenedesmus quadricauda* has probably the strongest cell wall and the highest speed 10 000 rpm did not affect the DNA integrity with the best yield at the same time.

Table 1 Optimal speed settings for DNA quality and quantity

	Speed (rpm)	Yield (ng/µl)	Purity (A 260/280)	DNA integrity
Coccomyxa subelipsoidea	4 500	58.60	1.82	↑
Chlorella vulgaris	4 500	26.20	1.85	↑
Parachlorella kessleri	6 800	26.90	1.76	↑
Scenedesmus obligus	4 500	42.00	1.75	↑
Scenedesmus quadricauda	10 000	130.00	1.81	↑

Therefore for the rest of microalgae we needed to find a compromise between quality and quantity and we chose samples with the best ratio yield : quality : purity (see Figure 3B) and according this we set up 3 different protocols for obtaining suitable, full length genome DNA and passable DNA concentration and purity (see Table 1). DNA marker with upper most band of 1 500 bp is proving there is no shearing in this area.

CONCLUSION

In the present work we carried out with the objective of optimizing an automatic homogenizer extraction method of genomic DNA in five species of fresh green microalgae. Speed parameter was tested in regards to concentration, purity and integrity of DNA material. This article points on high variability in microalgae species. The highest rotor speed (10 000 rpm) led to highest yields only in three species (*Chlorella vulgaris, Scenedesmus obliquus* and *Scenedesmus quadricauda*) with the fact, only DNA of *Scenedesmus quadricauda* stayed intact after homogenization. The highest DNA concentration of *Coccomyxa subelipsoidea* was obtained with 6800 rpm and of *Parachlorella kessleri* with 4 500 rpm speed mode also with unsatisfactory DNA integrity. With the optimized protocols we obtained lower yields but better quality of DNA material and integrity. A protocol of 4 500 rpm × 20 s × 2 cycles gave the best results for *Coccomyxa subelipsoidea, Chlorella vulgaris and Scenedesmus obliquus* homogenization, 6 800 rpm × 20 s × 2 cycles was the best option for *Parachlorella kessleri* specie and finally 10 000 rpm × 20 s × 2 cycles has showed as the best set up for *Scenedesmus quadricauda*. It is impossible to focus extraction protocol for each microalgae species separately, anyway it necessary to take into account algal morphology before extraction. In this study we set optimum extraction parameters for selected species of green microalgae to obtain high quality DNA material for further research.

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