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Development and optimization of a metabolite extraction process for the high throughput screening of microalgal chimiodiversity

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Background

Taxonomy of aquatic photosynthetic microorganisms

Biodiversity of microalgae is a vast field of investigation for seeking new and original metabolites. However for some species their extraction remains a challenge.

Biodiversity → Chimiodiversity

■ Usual extraction process :

Recalcitrant microalgae matrix → Grinding method e.g. sonication → Bad chimiodiversity extraction yield

Causes :
- Strong wall
- Highly hidden
- Highly bounded
- Numerous exopolymeric substances

Objective & Methodology

The aim of this study is to identify new and original metabolites from microalgae avoiding the risk to isolate molecules already known.

Microalgae constitute an untapped source of metabolites. This chimiodiversity is a considerable field to investigate. Polyketides, sterols, toxins, terpenes, alkaloids, oxilipins, non ribosomal peptides, mycosporines-like can be cited (Shimizu, 1996). Among them, some molecules are strongly bounded to thylakoid membranes like pigments, galactolipids (*e.g.* mono- and digalactosyldiacylglycerols) (Frassanito *et al.*, 2005).

The strategy adopted in this study to extract almost all the metabolites was grinding the cells with the greatest efficiency possible to foster contacts between solvent and metabolite. Overall, the optimized grinding process can be applied to studies involving simultaneously high-throughput screening of biological activities and dereplication procedures. So, the requirement parameters are :

- High extractibility yield
- Fidelity of analytes (no modification of metabolites)
- Miniaturization of samples (cultures, total extract)
- Compatibility with analysis and bioassays (solvent)
- Precision, reproductibility of total extract
- Simplicity of implementation
- Solvent green, clean
- Safety for the experimenter

The study was achieved as following :

Part 1 : Comparison and selection of the best cell disruption method among 9 laboratory techniques. The response variable was the grinding yield determined by cell counting.

Part 2 : Optimization of the process by response surface design.

Part 3 : Integration of the proposed protocol in the context of high throughput screening.

Two complex biological models were chosen :

A: *Phaeodactylum tricornutum* (PT)
Diatoms, silica frustule

B: *Porphyridium purpureum* (PP)
Rhodophytae, spherical, strong cell wall, exopolymeric substances

Central composite design with center and star points to study the influence of factors and their interaction followed by optimization of the method.

Image analysis :
Pictures taken under microscope with Ipx4 software v4.21 were processed using Matlab 7.0.

Specific particle size groups were selected :
[1-10 pixels] small fragments
[10-49 pixels] whole cells PT
[25-49 pixels] whole cells PP
[100-1000 pixels] aggregates

Complementary informations :
Samples were stained with Lugol before analysis

HPLC analysis were achieved according to Van Heukelem & Thomas (2001) modified

Results (1) : comparison & selection of disruption techniques

Soaking	50 x10⁶ cells, 500µL EtOH, 24 hours, Ambient temperature	Disruption yield PT: 5%, Disruption yield PP: 0%
Cryogrinding	50 x10⁶ cells, Filtration GF/C filter, 20mL H_2O +200mL liquid N_2	Grinding yield : 1%
Potter homogeniser	300 x10⁶ cells, 3mL EtOH, F1 : potter volume [10-15]mL, F2 : grinding time [1-5]min	Disruption yield PT: 49 to 79%, Disruption yield PP: 10 to 42%
Bead beater	300 x10⁶ cells, 15mL EtOH, F1 : diameter glass beads [2-4]mm, F2 : grinding time [1-4]min	Disruption yield PT: 86 to 94%, Disruption yield PP: 21 to 85%
Homogeniser PT3100	3 x10⁶ cells, 3mL EtOH, 18000 rpm, F1 : cycle number [2-4], F2 : disruption time [10-180]s	Disruption yield PT: 35 to 65%, Disruption yield PP: 0 to 56% → Rejected
Planetary micro mill	3 x10⁶ cells, F1 : grinding time 2 x4min, F2 : beads [ZrO_2 20mm (15u.) - Glass beads 2-3mm (200g)]	Disruption yield PT: 100% in each case, Disruption yield PP: 100% in each case → Validated → Evaluation of influential factors
Sonication	300 x10⁶ cells, 3mL EtOH, F1 : cycle number [10-20]min, F2 : disruption time [2-4] cycles	Disruption yield PT: 85 to 99%, PP: 0%
Mixer mill Retsch MM400	300 x10⁶ cells, 5mL EtOH, F1 : stainless steel balls [2], F2 : liquid N_2 bath	Disruption yield PT: 98%, PP: 89%
Mixer mill Retsch MM400	300 x10⁶ cells, 500µL EtOH, F1 : Glass beads 500µm [0.9]g, F2 : grinding time [5-32]min	Disruption yield PT: 97 to 100%, PP: 76 to 99%

Standard Pareto chart for fine particle yield

Method	Effectiveness (disruption yield)	Reproducibility	Operating cost	Temperature stability	Easy to use	Diluted samples	Other considerations
Soaking	PP: PT:	-	-	+	-	-	Lung processing
Cryogrinding	PP: PT:	-	-	+	-	-	Liquid nitrogen handling required
Potter homogeniser	PP: PT:	-	+	-	-	-	n.r.
Bead Beater	PP+ PT+	+	+	-	-	-	Particle release
Homogeniser	PP+ PT-	-	+	-	+	+	Blade blunted diatoms
Planetary micro mill	PP+ PT+	+	+	-	+	+	Particle release
Sonication	PP- PT+	+	+	-	-	-	ROS release, hot spots
Mixer mill – stainless steel jars	PP- PT+	+	+	-	+	-	n.r.
Mixer mill – grinding tube	PP+ PT+	+	+	-	+	-	n.r.

(+) Interesting advantage of the method (-) Downside of the method (n.r.) Nothing to report

Results (2) : Optimisation of the grinding method

Optimisation of the method with mixer mill Retsch MM400

Optimal point with best optimization

3 responses superposition : Particle fineness (max), Whole cells (min), Aggregates (min)

Single response : particle fineness (max)

Optimal point PT : 17 min, 0.92 g beads
Optimal point PP : 23 min, 0.30 g beads

Optimal point PT : 28 min, 0.92 g beads
Optimal point PP : 30 min, 0.30 g beads

Porphyridium purpureum chromatogram subplot

#	Pigment	Extinction λ in 30min
1	Zeaxanthin	+47%
2	Canthaxanthin like	+98%
3	Derivative product 1 from chlorophyll a	+113%
4	Derivative product 2 from chlorophyll a	+33%
5	Chlorophyll a like 1	+39%
6	Chlorophyll a like 2	+34%
7	Derivative product 3 from chlorophyll a	+94%
8	Chlorophyll a	+29%
9	β,β-carotene	-88%

Triboelectric phenomenon expected

For *Phaeodactylum tricornutum*, Student's T test showed no extraction differences between $A_{Control}$ and A_{Lugol}, meaning these metabolites are easy accessible.

Optimized grinding → Depleted matrix

On-line identification of original metabolites in microalgae

Dereplication

Isoplot absorbance UV-DAD

ELSD

Mass spectrometry

Molecule X

Molecule Y

Comparison to database

Molecule X = Unknown

Molecule Y = Chlorophyll a

Without interest for the targeted application

Biochemical assay or bioassay on cell lines

Phaeodactylum tricornutum total extract :
[1100 µg.mL⁻¹] CV= 9% n=11
Abs_{560nm} (d/4) = 0.656

Porphyridium purpureum total extract :
[6700 µg.mL⁻¹] CV= 4% n=11
Abs_{560nm} (d/8) = 0.853

Reading

C1 to C4 : controls (e.g. fluorescent labels, cell control)
PT_{1-10} to PT_{2-11} : range of concentrations (µg.mL⁻¹)
PP_{min} to PP_{max} : range of concentrations (µg.mL⁻¹)

IC_{50} determination :

If low → hit to purify in this species
If high → not a good candidate

Shimizu (1996). *Ann. Rev. Microbiol.* 50: 431-465 ; Frassanito *et al.* (2005) *J. Chrom. A* 1082: 33-42 ; Van Heukelem & Thomas (2001) *J. Chrom. A* 910(1): 31-49

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