AlgaePARC Biorefinery

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1 Samenvatting

1.1 Achtergrond en doel van het project

Aquatische biomassa (waaronder microalgen en zeewier) is een interessante nieuwe grondstof voor bioraffinage en vormt een belangrijke aanvulling op het bestaande biomassa aanbod. Microalgen worden geteeld in waterig milieu en hebben een unieke samenstelling. De biomassa bevat een scala van waardevolle componenten zoals oliën, eiwitten, suikers en bioactieve stoffen. Ze zijn daardoor uitermate geschikt voor bioraffinage, waarmee meerdere waardevolle producten uit de biomassa kunnen worden gewonnen. Er zijn echter nog weinig concepten beschikbaar voor de extractie en raffinage van lipiden, eiwitten en koolhydraten uit algen. Hiervoor moeten nieuwe, duurzame technieken worden ontwikkeld, geoptimaliseerd en geïntegreerd voor zowel teelt als aansluitende bioraffinage en product opwerking. Dit is de aanleiding voor het onderhavige AlgaePARC Biorefinery TKI-project (2013-2018), dat is geïnitieerd door Wageningen Universiteit en Research in samenwerking met tien industriële partners en de Universiteit Twente. Het project is uitgevoerd met financiële steun van het Nederlandse Ministerie van Economische Zaken in het kader van de TKI BioBased Economy onder contractnr. TKIBE01009.

Microalgen zijn een potentiële grondstof voor voeding, diervoeders, chemicaliën en biobrandstoffen. Algen kunnen worden geteeld in zeewater in open of gesloten kweeksystemen op marginale grond. De teelt legt daarmee geen beslag op zoetwater voorraden en vruchtbare landbouwgrond. Bovendien hebben microalgen een veel hogere olie- en eiwit productie per hectare grondoppervlak dan traditionele gewassen zoals palm, soja, koolzaad en maïs. Ondanks dit potentieel is de huidige wereld productie en toepassing van algen nog beperkt tot ca. 15.000 ton droge stof per jaar. Algen worden tot dusver vrijwel uitsluitend toegepast in nichemarkten voor specialties in voeding en voedingssupplementen (o.a. ω-3-vetzuren, β-caroteen), diervoeders (met name voor aquacultuur) en als ingrediënt (kleurstoffen, anti-oxidanten) in cosmetica. Succesvolle groei van de algenindustrie vereist reductie van de productiekosten en verhoging van de economische opbrengsten. Dit kan worden gerealiseerd door verbetering van de kosteneffectiviteit van algenproductie en -verwerking door technologieontwikkeling en -optimalisatie, ontwikkeling van bioraffinage concepten die meerdere waardevolle producten opleveren en ontwikkeling van nieuwe producten en toepassingen. Langs deze weg kan de algensector zich ontwikkelen van de huidige niche toepassingen tot een industriële activiteit voor bulkproducten zoals biobrandstoffen.

De technologie is nog onvolgroeid, wereldwijd lopen programma's om de biomassaproductie te verbeteren. De industrie die hierin investeert is zich ervan bewust dat de ontwikkeling van commerciële microalgen grondstoffen voor bulkproducten tijd vergt (10-15 jaar). Vergelijkbare inspanningen moeten worden geleverd om effectieve verwerking van microalgen te realiseren. Valorisatie van alle biomassa componenten via bioraffinage is daarbij een voorwaarde voor economische haalbaarheid en duurzaamheid.

Er zijn wereldwijd onderzoeksprogramma's gestart om technologie te ontwikkelen voor de productie van biodiesel uit microalgen. Voorbeelden zijn onder meer drie EU FP-7 demonstratieprojecten: BIOFAT[1], ALL GAS[2] en InteSusAl[3], die als doel hebben om algenbrandstofproductie op een schaal van 10 hectare te demonstreren. Daarnaast lopen er grote projecten in de VS, zoals de alliantie tussen Synthetic Genomics en Exxon Mobil (600 m €) en startups zoals Sapphire Energy (nu Qualitas), Solazyme (nu Corbion), Algenol en vele anderen. De focus in veel projecten ligt op verbeterde productie van algen biomassa en de omzetting daarvan in één hoofdproduct met relatief laagwaardige residuen. Om het proces economisch haalbaar te maken, moeten alle biomassacomponenten optimaal worden gevaloriseerd. Voor dit doel is een bioraffinageproces noodzakelijk. Algen olie (tot 40 a 50% van de biomassa) kan worden gebruikt voor de productie van brandstof, terwijl de resterende eiwitten en koolhydraten kunnen worden gebruikt in de voedingsmiddelen- en diervoederindustrie en voor non-food toepassingen zoals bioplastics.
Bioraffinage van microalgen bestond niet bij de start van dit project en de onderzoeksinspanningen waren toen nog zeer beperkt. Het is een nieuw terrein waarop Nederland pionierswerk verricht. Door de uitvoering van dit project zijn onze leidende positie en innovatieve reputatie op het gebied van microalgen verder geconsolideerd

[1] http://www.biofat-project.eu/
[2] http://www.all-gas.eu/
[3] http://intesusal-algae.eu/2/

Het hoofddoel van dit project was om duurzame concepten te ontwerpen voor bioraffinage van microalgen door ontwikkeling en integratie van opschaalbare, energiezuinige technieken voor extractie en raffinage van grondstoffen (oliën, eiwitten, koolhydraten) uit microalgen biomassa. Om dit projectdoel te bereiken, werden drie onderling samenhangende werkpakketten gedefinieerd:

WP1: Technologieontwikkeling en -optimalisatie

Het doel van dit deelproject was om nieuwe, opschaalbare technologieën te ontwikkelen en te verbeteren voor de bioraffinage van microalgen. Deze technieken dienen mild, efficiënt, kosteneffectief en duurzaam te zijn. De focus lag op milde technieken voor celdisruptie, en extractieprocessen die winning van alle biomassafracties mogelijk maken met optimaal behoud van structuur en functionaliteit. De hiervoor benodigde algenbiomassa werd gekweekt op AlgaePARC (http://www.algaeparc.com/). Geselecteerde technologieën werden opgeschaald en gevalideerd op pilot schaal. Daarnaast werd gewerkt aan ontwikkeling en modellering van energie- en kosteneffectieve oogstmethoden geïntegreerd met de opwerking van producten via bioraffinage.

WP2: Systeemanalyse, techno-economische en duurzaamheidsevaluatie en flexibiliteit

Het doel van dit deelproject was het uitvoeren van duurzaamheids gedreven ontwerp van integrale algen bioraffinaderijen inclusief verwerkingscondities, productrouting en (terug)winning van hulpstoffen. Hiervoor werden data uit het project gebruikt, gegevens van projectpartners en aanvullende data waar nodig. Daarnaast werd de integratie van algenbioraffinage in de toeleveringsketen bestudeerd en gemodelleerd. Een belangrijk resultaat is de ontwikkeling van een geïntegreerd techno-economisch model dat modules voor microalgenproductie, oogst, bioraffinage en logistiek combineert. Dit model maakt ontwerp, analyse en selectie mogelijk van haalbare microalgen waardeketens om commerciële kansen te beoordelen en nieuwe onderzoekslijnen te definiëren.

WP3: Ontwikkeling van basis technieken en basis kennis

Voor de ontwikkeling van bioraffinage-technieken is gedetailleerde kennis nodig van de samenstelling van de celwand als functie van de algen soort/stam[4], de kweekcondities en het stadium in de cel cyclus. Ook moeten basis technieken ("enabling technologies"[5]) worden ontwikkeld. Dit was de hoofddoelstelling van WP3. Een gedetailleerde analyse werd uitgevoerd van de samenstelling van de celwanden en de variabiliteit ervan onder verschillende procesomstandigheden (volledig medium, stikstofbeperking, kweek in zeewater en zoet water) en gedurende de celcyclus. Daarnaast zijn cellulaire mechanismen en genen geïdentificeerd die verantwoordelijk zijn voor de biosynthese van de celwand. Dit opent de deur voor verder onderzoek naar het induceren van cel disruptie en genetische modificatie.

1.2 Technologieontwikkeling en -optimalisatie voor microalgen bioraffinage (WP1)

Om producten uit algen optimaal te valoriseren en exploiteren moeten technieken worden ontwikkeld voor efficiënte oogst en ontwatering, extractie en scheiding van verschillende fracties met behoud van de kwaliteit van alle producten. Hiertoe moeten technieken mild zijn, bij voorkeur toepasbaar voor een verscheidenheid aan eindproducten, zo weinig mogelijk hulpstoffen en energie gebruiken en lage kosten hebben. De eerste stap na de oogst en ontwatering is disruptie van de algen celwanden om de inhoudstoffen toegankelijk te maken. Vervolgens moeten extractie- en scheidingstechnieken worden ontwikkeld voor opwerking en zuivering van de producten. Deze technieken moeten flexibel zijn en toepasbaar voor verschillende algensoorten. Bovendien kunnen de kweekomstandigheden (zoet water, zeewater, beperking van voedingsstoffen etc.) leiden tot veranderingen in de celwand en de biomassa samenstelling. Met deze verschillen moet rekening worden gehouden bij het selecteren van de juiste

[4] Engels: Strain

[5] Een *enabling technology* is een uitvinding of een innovatie, die kan worden toegepast om radicale veranderingen in de mogelijkheden van een gebruiker of cultuur te stimuleren. Dergelijke 'Activerende' technologieën worden gekenmerkt door een snelle ontwikkeling van opeenvolgende afgeleide technologieën, vaak op verschillende gebieden. Vertaling Wikipedia, https://en.wikipedia.org/wiki/Enabling_technology. Laatst gezien dd 13 juni 2019.

technologie. In dit project werd technologie ontwikkeld op lab schaal en deels opgeschaald en gevalideerd op pilot schaal.

1.2.1 Selectie van algensoort en biomassaproductie

Om representatieve algen voor het project te selecteren, werden drie algensoorten *Phaeodactylum tricornutum, Neochloris oleoabundans en Chloroccocum litoralle* gekarakteriseerd voor wat betreft groei, productiviteit en biomassa samenstelling. Ook werden benchmarks ontwikkeld voor celdisruptie van deze algensoorten, met behulp van standaard technieken. Op basis van de resultaten werd de technologie ontwikkeling in het project grotendeels gericht op *Neochloris oleoabundans*, een soort die goed groeit in zowel zoet als zout water en in staat is om olie op te hopen (tot 40 a 50%) bij kweek onder nutrient beperkte condities.

In het project is algenbiomassa geproduceerd onder verschillende teeltomstandigheden. Het toepassen van nutriëntlimitatie door beperking van de stikstof (N) toevoer aan de groeiende algen gedurende een bepaalde tijdsperiode resulteerde in ophoping van olie in de biomassa. De procesvoering en het effect van verschillende kweekomstandigheden en hun impact op de samenstelling van de algenbiomassa en de celwand en de consequenties voor bioraffinage zijn onderzocht. Batches van *N. oleoabundans* werden geproduceerd op laboratorium- en pilotschaal in verschillende binnen en buiten geplaatste fotobioreactoren op AlgaePARC en aan partners aangeleverd in de vorm van verse of bevroren pasta voor nader onderzoek. In totaal werd ca. 200 kg *Neochloris*-biomassa geproduceerd. Ongeveer 165 kg werd gekweekt in (kunstmatig) zeewater en de rest in zoet water. Ongeveer 150 kg *Neochloris*-biomassa werd geproduceerd onder N-beperkte omstandigheden. Deze biomassa werd voornamelijk gebruikt door partners voor de extractie van olie.

1.2.2 Evaluatie van technieken voor het oogsten van algen

Momenteel wordt de oogst/ontwatering van microalgen veelal uitgevoerd met behulp van mechanische vast/vloeistof scheidingstechnieken zoals centrifugatie, flotatie of membraan filtratie. Deze technieken zijn efficiënt maar ook energie-intensief en zijn relatief duur. Studies hebben aangetoond dat 20 tot 30% van de totale verwerkingskosten kan worden toegeschreven aan het oogsten wanneer centrifugatie wordt gebruikt in combinatie met een open teeltsysteem. Om de verwerkingskosten te verlagen, is een (pre-) concentratiestap of een alternatieve oogsttechnologie gewenst. Het toepassen van een (pre-) concentratie stap zoals flocculatie-sedimentatie resulteert in een lager energieverbruik.

In dit project werd flocculatie van microalgen onderzocht als alternatieve, milde technologie voor het (pre)concentreren van biomassa gekweekt in zout water. Bij flocculatie worden afzonderlijke cellen geaggregeerd tot grotere deeltjes of 'vlokken' die kunnen bezinken. Kationische polymeren, en in het bijzonder poly-acrylamiden, veroorzaakten met succes flocculatie van mariene microalgen. Het gebruik van deze kationische polymeren resulteerde in biomassa winning van meer dan 90% en een concentratiefactor van 5 - 10 na 2 uur sedimentatie. Een positief effect van toenemende flocculant doseringen op de biomassa terugwinning werd waargenomen met de microalg *N. oleoabundans*. Na het bereiken van een optimum in de winning van biomassa (> 95%), gaven hogere flocculant doseringen geen verdere verhoging maar juist een afname te zien. Deze waarnemingen werden vertaald in een wiskundig model. De kosten van flocculatie met polymeren zijn relatief hoog. Er is dus nog steeds behoefte aan alternatieve, goedkope flocculanten voor toepassing bij hoge(re) zoutconcentratie.

1.2.3 Ontwikkeling en optimalisatie van technologieën voor celdisruptie

Na de oogst/onwatering is de volgende stap in het bioraffinage schema het openbreken (disruptie) van de algencellen. Deze stap is nodig om de celwanden te breken en zo extractie van de producten die zich in de cellen bevinden mogelijk te maken. Verschillende technologieën zijn met succes toegepast voor disruptie van microalgen. Voorbeelden zijn mechanische technieken zoals hogedruk homogenisatie of kogel malen, chemische technieken zoals alkali-warmte behandelingen en fysische technieken zoals microgolven of ultrasound. Mechanische en fysische technologieën gebruiken relatief veel energie. Dit hoge verbruik en de kosten kunnen de economische haalbaarheid negatief beïnvloeden. Dit project richtte zich daarom

vooral op milde celdisruptie met behulp van enzymen en optimalisatie van kogel malen en homogenisatie. Daarnaast werd gewerkt aan Pulsed Electric Field (PEF) -technologie en Controlled Instantaneous Pressure Drop (CIPD) –techniek.

Toepassing van enzymen voor celdisruptie

Een van onze doelen was het identificeren van enzymen die bruikbaar zijn voor milde disruptie van algen cellen. Daartoe zijn experimenten uitgevoerd met de algensoorten *Neochloris oleoabundans* en *Phaeodactylum tricornutum*. Bij behandeling met cellulases en hemicellulases kwamen geen suikers uit de celwand vrij. Ook andere koolhydraatafbrekende enzymen bleken niet of slechts in geringe mate effectief. Dit impliceert dat de doelbestanddelen van deze enzymen (koolhydraten) niet aanwezig zijn in de algen celwanden ofwel niet toegankelijk zijn in de celmatrix. Omdat (ook) eiwitten belangrijke componenten zijn in algencelwanden werd de werking van 10 verschillende proteasen (eiwitafbrekende enzymen) geanalyseerd. Over het algemeen bleken alkalische proteasen het meest effectief. Deze enzymen kunnen een aanzienlijk deel van de celwand van de algen *Neochloris oleoabundans* en *Phaeodactylum tricornutum* afbreken. Dit resultaat biedt een gunstig perspectief voor milde verwerking van algen. Een volgende stap is om te onderzoeken of toepassing van uitsluitend proteasen het meest effectief is of dat combinatie met mechanische disruptietechnieken (zoals bijv. kogel malen) effectiever is.

Celdisruptie met behulp van kogel malen en hogedruk homogenisatie

Kogel malen en hoge druk homogenisatie zijn efficiënte methoden voor het openbreken van de cel. Na optimalisatie vertoonden beide methoden een hoge desintegratie van de cellen (± 90%) en een snelle afgifte van hydrofiele componenten (eiwitten en koolhydraten). Echter het bleek niet mogelijk (vrijwel) alle eiwitten en koolhydraten aanwezig in de cellen van *N. oleoabundans* vrij te maken, veel onoplosbaar eiwit bleef achter in het celmateriaal. Groeiomstandigheden (gestrest of niet-gestrest, kweek in zout of zoet water) leken de efficiëntie van celdisruptie te beïnvloeden als gevolg van veranderingen in de dikte en samenstelling van de celwand.

Celdisruptie met Pulsed Electric Field (PEF)

Pulsed Electric Field (PEF) wordt gezien als een veelbelovende technologie voor cel disruptie in een microalgen bioraffinage proces. Bij de eerste testen werd de hoogste opbrengst aan eiwitten (13%) verkregen met *N. oleoabundans* gekweekt in zoutwater medium. De hoge afgifte van ionen wijst erop dat de toepassing van PEF leidt tot de vorming van poriën in de celmembraan. In vergelijking echter met de mechanische benchmark (kogel malen), werden met PEF veel lagere eiwit opbrengsten verkregen. Bovendien was de vereiste energie-input voor PEF hoger dan voor kogel malen.

1.2.4 Integratie van processtappen

Om de productie van bulkproducten op grote schaal mogelijk te maken moet bioraffinage minder complex en goedkoper worden. Om dit te realiseren moeten we ons niet alleen richten op de individuele processtappen (oogst/ontwatering, celdisruptie, extractie en zuivering), maar ook op het integrale procesontwerp. Door het vereiste aantal stappen en het gebruik van chemische hulpstoffen te verminderen, kunnen we het proces als geheel vereenvoudigen en de (economische) haalbaarheid van bioraffinage verbeteren. In dit project onderzochten we diverse opties om dit te bereiken waaronder de integratie van verschillende bewerkingen (oogsten, celdisruptie en extractie) in één enkele stap en reductie van het chemicaliëngebruik. Chemicaliën (bijvoorbeeld flocculanten, oplosmiddelen, enz.) zijn niet alleen een verbruiksartikel met bijbehorende kosten, maar ze moeten ook worden teruggewonnen door extra processtappen.
Een interessante benadering voor geïntegreerde bioraffinage met meerdere producten is het gebruik van een waterig bifasisch systeem (Aqueous Biphasic System; ABS) op basis van ionische vloeistoffen. Ionische vloeistoffen of Ionic Liquids (IL's) zijn vloeibare zouten met specifieke oplosmiddeleigenschappen. IL's kunnen bijvoorbeeld worden gebruikt om in één stap lipiden uit natte, niet-gebroken algenbiomassa te extraheren. Directe productextractie met ABS op basis van IL kan zo een aparte celdisruptie stap overbodig maken en is daarmee een veelbelovende optie om het ontwerp van een bioraffinageproces te vereenvoudigen.

Naast de kosten en het integrale procesontwerp, is mildheid van processen een sleutelcriterium in onderzoek en ontwikkeling van microalgen bioraffinage, omdat hierdoor behoud van functionaliteit en kwaliteit van alle componenten gewaarborgd is en de verschillende producten optimaal kunnen worden

gevaloriseerd. Volledige valorisatie van alle componenten door bioraffinage zal de overgang vergemakkelijken van de huidige kleinschalige productie van specialiteiten uit microalgen naar toekomstige grootschalige productie van bulkproducten. Door ontwikkeling van geïntegreerde processtappen en het vermijden van gebruik van moeilijk terug te winnen chemicaliën zal de bioraffinageketen minder complex worden. De ontwikkeling van vereenvoudigde processen is essentieel om multiproduct bioraffinage van microalgen economisch haalbaar te maken.

1.2.5 Ontwikkeling van productopwerking: extractie en fractionering

Verschillende technologieën werden geëvalueerd voor productextractie en -fractionering, in het bijzonder membraanfiltratie en waterige tweefasen systemen, die hieronder worden besproken. Op het gebied van productextractie lag de nadruk op de ontwikkeling van een nieuw type extractieproces en onderzoek waarbij het gebruik van conventionele oplosmiddelen op pilot schaal werd geëvalueerd.

Ultrafiltratie / diafiltratie als fractioneringstechniek

De vloeibare fractie verkregen na hogedruk homogenisatie en centrifugatie van de alg *N. oleoabundans* werd onderworpen aan membraanfiltratie via ultrafiltratie en diafiltratie met verschillende typen membranen met verschillende Molecular Weight Cut-Off (MWCO) waarden en poriegrootte [6]. Doel was om de wateroplosbare moleculen van elkaar te scheiden en met name de hoeveelheid eiwitten in het filtraat te verhogen. De resultaten toonden dat het filtraat geen polysacchariden en pigmenten bevatte en dat de grootste hoeveelheid eiwitten in het filtraat werd verkregen met de lagere MCWO waarden. Ultrafiltratie gevolgd door diafiltratie verhoogde de hoeveelheid eiwitten in het filtraat.

Extractie en fractionering met behulp van waterige tweefasensystemen (Aqueous Two-Phase Systems; ATPS)

Waterige tweefasensystemen (ATPS) werden getest als een nieuwe benadering voor het scheiden (fractioneren) van hydrofiele van hydrofobe componenten. Bovendien werden stabiliteitsstudies uitgevoerd om te zien of dergelijke ATPS de natieve eiwitstructuur behouden. Iolilyte 221PG-citraat bleek de meest efficiënte ATPS te zijn voor scheiding van het enzym Rubisco. Uit stabiliteitsstudies bleek echter dat ATP's op basis van Poly Ethyleen Glycol (PEG) beter presteren gelet op het behouden van de eiwit integriteit. In het project hebben we het potentieel van ATPS aangetoond om verschillende biomoleculen tegelijkertijd te scheiden en te valoriseren, ten behoeve van duurzame multiproduct bioraffinage van algen gekweekt in zowel zout water als zoet water. Gebruik van zout water om microalgen te kweken, kan de kosten en het beslag op zoetwater voorraden verlagen, wat een voordeel is in het kader van duurzaamheid.

Oliewinning uit natte algen biomassa met behulp van nieuwe oplosmiddelen

In deze taak werd een nieuw, effectief, schakelbaar oplosmiddelsysteem voor directe extractie van lipiden uit niet gebroken microalgen in (geconcentreerde) waterige oplossingen ontwikkeld. Het betreft hier een bijzondere categorie 'schakelbare' oplosmiddelen ('switchable solvents'), waarvan de eigenschappen als oplosmiddel kunnen worden 'geschakeld' door toe- resp. afvoer van CO_2 of door verhoging of verlaging van de temperatuur. N-ethylbutylamine (EBA) werd geïdentificeerd als meest belovende, schakelbare oplosmiddel voor gecombineerde extractie van lipiden uit natte algenbiomassa en aansluitende fasescheiding. In vergelijking met de Bligh & Dyer standaard laboratoriummethode voor lipidenbepaling, kan EBA alle lipiden, neutraal en polair, uit zoetwater microalgen extraheren zonder celdisruptie en zonder voorafgaande droging van het materiaal. De organische extractiefase, die de lipiden en EBA bevat, kan worden gescheiden van het (geconcentreerde) microalgen kweekmedium door fasescheiding. Vervolgens kan de EBA worden gescheiden van de lipiden door water toe te voegen en in contact te brengen met CO_2 of door de temperatuur van het water-organische mengsel te verlagen. In beide gevallen wordt de EBA overgebracht naar de waterige fase, waardoor het lipiden product als afzonderlijke fase achterblijft. Na een eenvoudige fasescheiding om dit product te winnen, kan het oplosmiddel EBA worden teruggewonnen als afzonderlijke organische fase door de CO_2 te verwijderen of de temperatuur te verhogen.
De studie bevestigt dat zowel het gebruik van kweek technieken die het lipiden gehalte verhogen als het toepassen van EBA als 'schakelbaar' oplosmiddel in meerdere extractiestadia veelbelovend is voor de ontwikkeling van een efficiënte, directe lipide-extractietechnologie toepasbaar op niet-gebroken, natte

[6] Molecular Weight Cutt Off (MWCO); Molecuul gewicht waarbij 90% van de betreffende, opgeloste stof wordt tegengehouden door het membraan. De poriegrootte kan worden afgeleid uit de MWCO. Een hogere MWCO impliceert een grotere poriegrootte van het membraan

microalgen. De resultaten tonen dat toepassing van EBA als 'schakelbaar' oplosmiddel voor lipide-extractie uit microalgen uit energieoogpunt veelbelovend is, omdat hoge opbrengsten kunnen worden verkregen, de verwerkingscondities vrij mild zijn (en dus de apparatuurkosten laag zijn) en niet-gebroken, natte microalgen-slurries zonder voorbehandeling kunnen worden verwerkt.

Olie-extractie op pilot schaal

Enzym- en pH-gebaseerde technologieën werden onderzocht voor extractie van olie uit natte *Neochloris oleoabundans* biomassa. De bereikte rendementen waren echter relatief laag, wat mede veroorzaakt werd door het relatief lage oliegehalte in de gebruikte biomassa. Extractie met ethanol en in het bijzonder iso-propylalcohol (IPA) bleken goede opties te zijn. Waardevolle co-producten die verder kunnen worden onderzocht bij deze extractie routes zijn eiwitten en fosfo/glyco-lipiden. De resultaten bevestigden dat de procescondities van celdisruptie door kogel malen zorgvuldig moeten worden gecontroleerd wanneer olie wordt gewonnen uit natte biomassa. Bovendien zijn lange enzymatische behandelingstijden vereist.

1.3 Systeemanalyse, techno-economische en duurzaamheids beoordeling en flexibiliteit (WP2)

Algen zijn een potentiële bron voor een breed spectrum van producten. De focus in een algen bioraffinaderij ligt dan ook niet op een of enkele producten, maar op een efficiënte productmix. Om deze mix te verkrijgen, moeten de processtappen ('unit operations') in de keten van algengrondstof tot eindproduct efficiënt worden gecombineerd en zo mogelijk geïntegreerd. De keuze en operationele omstandigheden voor de 'unit-operations' in de keten moeten goed uitgebalanceerd zijn om het meest efficiënte bioraffinage schema te verkrijgen. In de beschikbare literatuur wordt de keuze voor de processtappen meestal gegeven in tamelijk kwalitatieve in plaats van kwantitatieve termen, en is sterk gericht op afzonderlijke stappen in plaats van een reeks van processtappen. Het werk in dit project richtte zich daarom op de ontwikkeling van simulaties voor een systematische analyse om een goed gebalanceerde en kwantitatieve organisatie van de productieketen te (kunnen) definiëren.

Verwerking in algen bioraffinaderijen op gecentraliseerde locaties resulteert in een efficiënter gebruik van kapitaal en gerelateerde kosten en een efficiënter gebruik van arbeid. Aan de andere kant vereist een gecentraliseerde, grootschalige bioraffinaderij toevoer van algenmateriaal uit meerdere kweekeenheden, waardoor de transportkosten en het transportbrandstofverbruik en de emissies stijgen. Volgens Bruins en Sanders [1][7] kunnen lokale bioraffinaderijen aantrekkelijker zijn dan grootschalige, gecentraliseerde bioraffinaderijen door lagere transportbehoefte. Het werk in dit project had daarom ook tot doel om de balans te vinden tussen de schaal van een algenbioraffinaderij en de impact van de transportlogistiek.
De doelstellingen van WP2 waren:

- *Economische en duurzame bioraffinaderijen:* definiëren van bioraffinaderijen die voldoen aan economische en duurzaamheidscriteria door de beste keuzes voor processtappen, en ontwikkeling van een flexibel hulpmiddel voor evaluatie van verschillende bioraffinageketens;
- *Bioraffinaderijen en de toeleveringsketen:* definiëren welke typen bioraffinaderijen lokale processing vereisen en voor welke typen bioraffinaderijen gecentraliseerde verwerking (meer) geschikt is, en ontwikkeling van een flexibel hulpmiddel om verschillende opties voor gecentraliseerde of lokale verwerking te evalueren.

1.3.1 Economische en duurzame bioraffinaderijen

Een model gebaseerde benadering werd toegepast als basis voor de systeemevaluatie. Voor elke processtap werd een simulatiemodel gedefinieerd. Deze modellen omvatten de in– en uitgaande massa en energiestromen van de hoofd- en nevenstromen in elke processtap. Aanvullende relaties zijn opgenomen om de energievraag en productopbrengst te koppelen aan economische parameters. De modellen voor de unit operations zijn geprogrammeerd in Excel-spreadsheets. Een flexibele structuur wordt gebruikt om alle unit operations in elke combinatie met elkaar te kunnen verbinden.

7 Bruins M.E., Sanders J.P.M. Small-scale processing of biomass for biorefinery. Biofuels, Bioprod. Bioref. 2012. 6:135–145

De input van energie en hulpstoffen (bijvoorbeeld oplosmiddelen) is gebaseerd op gegevens voor het specifieke energieverbruik van elke unit operation, die gekoppeld is aan de hoeveelheid materiaal die wordt verwerkt. Apparatuur gegevens (capaciteit, kosten, schalingsregels etc.) zijn afgeleid uit gegevens van projectpartners die industriële apparatuur leveren (GEA, WAB, Evodos), procestechnologische partners (Bodec), met aanvullende data uit literatuur en engineering databases (DACE, NETL Matche.com). Voor elk scenario werden de investeringskosten berekend op basis van de aanschafkosten van de apparatuur plus kosten voor piping, installatie van pompen, constructie, automatisering, enz. Daarbij werd een algemene, overall Lang factor[8] gebruikt die de bijdrage van individuele aspecten samenvat. Voor standaardapparatuur was de toegepaste Lang-factor 3,5. Voor units die in ATEX[9]-omgeving worden gebruikt om explosiegevaar te voorkomen, wordt een hogere Lang factor gebruikt. De beschouwde operationele kosten omvatten energie, verbruiksgoederen, arbeidskosten en kosten als gevolg van verlies van biomassa tijdens de verwerking.

Voor oogst en ontwatering werden 28 combinaties van unit operations geëvalueerd voor wat betreft operationele kosten en energieverbruik. Flocculatie van algen geproduceerd in open vijvers bleek een laag energieverbruik te hebben (<0,1 kWh.kg^{-1} droge algen) met kosten tussen 0,4-1,4 €.kg^{-1}. Andere methoden zoals druk, vacuüm- en membraanfiltratie, centrifugatie en combinaties resulteren voor open-vijverkweeksystemen in een energieverbruik tussen 1,0-4,5 kWh.kg^{-1} en kosten beneden 2 €.kg^{-1}. Voor kweeksystemen met hogere productiviteit dan open vijvers (paneelreactoren, buisvormige fotobioreactoren) dalen de kosten en het energieverbruik met een factor 3 tot 4.

Voor cel disruptie in grootschalige algen bioraffinaderijen heeft homogenisatie de voorkeur gezien de (lagere) operationele kosten. De simulaties voor de lipiden-extractiemethoden, experimenteel onderzocht in het kader van WP1 (met IPA of ethanol als oplosmiddel), tonen aan dat de scheiding van oplosmiddel en water (gericht op de terugwinning van het oplosmiddel via destillatie of verdamping), hoge kosten en een hoog energieverbruik tot gevolg heeft. Deze methoden bleken duurder en energie-intensiever te zijn dan traditionele lipiden-extractie met hexaan. In een bioraffinaderij primair gericht op eiwit winning met centrifugatie en ultrafiltratiestappen werd 20-25% van het eiwit teruggewonnen in de natieve, werkzame vorm. Het grootste deel van het natieve eiwit ging echter verloren in het residu van de centrifugatiestap. Dit deel kan alsnog worden gewonnen door alkalische of zure extractie, maar tijdens deze behandeling gaan de natieve eiwiteigenschappen verloren.

Verschillende specifieke types algen bioraffinaderijen werden geanalyseerd. Excel-modellen werden ook ontwikkeld voor andere traditionele methoden voor extractie en scheiding van algencomponenten. Vervolgens is een Excel-model ontwikkeld, opgebouwd uit verschillende modulen, voor evaluatie van de integrale keten van kweek in combinatie met oogsten, ontwatering en productextractie en –scheiding, en logistiek. Dit model maakt ontwerp, analyse en selectie mogelijk van haalbare microalgen waardeketens om commerciële kansen te beoordelen en verbeterpunten en nieuwe onderzoekslijnen te definiëren.

1.3.2 Bioraffinaderijen en de logistieke keten

Met betrekking tot bioraffinaderijen en de logistieke keten zijn twee aspecten onderzocht:

- De impact van transport op de keuze voor gecentraliseerde versus gedecentraliseerde verwerking van geconcentreerde algenbiomassa.
- Opslag. Algenpasta die wordt verwerkt in gecentraliseerde bioraffinaderijen moet vóór en na het transport worden opgeslagen, terwijl transport ook een vorm van opslag is. De mogelijke opslagtijd werd experimenteel onderzocht.

De bijdrage van transport aan de totale kosten van algenproducten is gering, zelfs over grote afstand. De voordelen van grootschalige verwerking zijn groter dan de transportkosten over lange afstand en daarom heeft gecentraliseerde verwerking de voorkeur. Zo zijn de kostenvoordelen van grootschalige lipide- en

[8] De Lang factor is een geschatte verhouding van de totale kosten voor het maken van een proces in een fabriek, tot de kosten van alle belangrijke technische componenten. Deze factor wordt veel gebruikt in de industriële engineering om de kapitaals- en operationele kosten van een fabriek te berekenen. Vertaling van https://en.wikipedia.org/wiki/Lang_factor dd. 27-06-2019

[9] De term ATEX is afgeleid van de Franse titel van EC Directive 94/9/EC: *Appareils destinés à être utilisés en ATmosphères EXplosives.*

eiwit bioraffinaderijen altijd groter dan kosten voor transport over meer dan 2000 km vanuit kweeksystemen met lage productiviteit naar een gecentraliseerde bioraffinage installatie. Voor kweeksystemen met hoge productiviteit (zoals tubulaire en vlakke paneel-PBR's) die ca. 45 ton algenbiomassa.ha^{-1}.jaar^{--1} produceren ligt het break-even punt op ongeveer 750 km transportafstand.

De resultaten van de experimenten met opslag van algenbiomassa resulteerden in een aanbeveling van maximaal 3 dagen opslagtijd (in afwezigheid van lucht) voor algen gekweekt onder optimale condities en maximaal 7 dagen voor algen gekweekt onder nutriënten limitatie voor accumulatie van olie in de biomassa. Na deze opslagperioden wordt het verlies aan koolhydraten en eiwitten aanzienlijk, terwijl lipiden in deze periode stabiel blijven.

1.4 Ontwikkeling van hulpmiddelen, basiskennis over celwandsamenstelling (WP3)

Neochloris oleoabundans wordt beschouwd als een van de meest geschikte microalgen soorten, gezien het industriële potentieel en de hulpmiddelen die voor deze soort beschikbaar zijn. De eerste stap die moet worden gezet na het oogsten van *N. oleoabundans* en vóór de winning van meerdere producten uit de biomassa, is het doorbreken van de fysieke barrière van de cel om toegang te krijgen tot de componenten die zich in de cel bevinden. De intracellulaire componenten van *N. oleoabundans* worden omsloten door de celwand, die zich op het buitenste deel van de celmembraan bevindt. Succesvolle valorisatie van de waardevolle componenten vereist behoud van de integriteit en functionaliteit van de cellulaire componenten bij disruptie van de celwand en de daarop volgende extractie en product opwerking.

In tegenstelling tot land gebonden gewassen, waarvoor gedurende vele decennia methoden zijn ontwikkeld om eigenschappen van de celwand te karakteriseren, is voor microalgen zoals *N. oleoabundans* een beperkte 'gereedschapskist' beschikbaar. Daardoor is er weinig informatie voorhanden over het type celwand bouwstenen en de manier waarop deze geassembleerd zijn tot complexe structuren. Bovendien zijn de specifieke functies van de afzonderlijke bouwstenen en hun biosyntheseroutes grotendeels onbekend. Voorafgaand aan de bewerking en inzet van celwandbestanddelen en -structuren voor specifieke toepassingen is het essentieel om de biochemische samenstelling, de morfologische structuur en hun onderliggende genetica, evenals hun biosynthetische routes op te helderen.

Algencelwanden vertonen over het algemeen een grotere biologische diversiteit en metabolische plasticiteit in vergelijking met landgebonden planten. Celwanden van soorten behorend tot het phylum Chlorophyta vertonen zowel unieke als gemeenschappelijke kenmerken vergeleken met de celwand van (hogere) planten en schimmels. Vergeleken met hogere plantencelwanden, bevatten microalgen een aanzienlijke hoeveelheid polysacchariden in hun celwand. Bovendien kan de celwand van microalgen die behoren tot de Chlorophyta een chitine- of chitineachtige structuur bevatten, een kenmerk van schimmelcelwanden.

Afgezien van de diversiteit van de celwand tussen verschillende soorten, kunnen structurele eigenschappen van de celwand ook veranderen als reactie op omgevingsfactoren of gedurende de levenscyclus. Stikstofgebrek en zoutgehalte zijn twee omgevingsfactoren die de koolstof verdeling binnen cellen kunnen beïnvloeden. Veranderingen in de koolstofbalans en de verdeling daarvan kunnen een significante verandering in de samenstelling en morfologie van de celwanden veroorzaken. Ook de ontwikkeling gedurende de celcyclus kan de celwandkenmerken veranderen.

Soort-specifieke celwandkenmerken samen met hernieuwde vorming ('remodelling') van de celwand als gevolg van omgevingsfactoren of groeifasen kunnen een verschillende mate van gevoeligheid veroorzaken voor de methoden die beschikbaar zijn voor de disruptie van algen celwanden. Veelal richten deze zich op disruptie van de gehele celwand, waardoor een complex mengsel ontstaat van componenten die relatief lastig te scheiden zijn. De ontwikkeling van op maat gemaakte, milde en niet-disruptieve methoden vereist gedegen kennis van de celwanden, inclusief de biochemische samenstelling en morfologische kenmerken. Deze fundamentele kennis is een vereiste voor het ontwikkelen van een milde, selectieve voorbehandelingsstrategie en optimalisering van extractieprocessen.

Het doel van dit project was om de celwandsamenstelling en -morfologie van de algensoort *Neochloris oleoabundans* te onderzoeken onder optimale groeicondities, en de hernieuwde vorming van de celwand te bestuderen bij blootstelling aan verschillende omgevingsfactoren. Daarnaast onderzochten we de morfologische kenmerken en remodelling van de celwand gedurende de celcyclus. Om de bestaande kennis te vergroten, hebben we de biosynthese en modificatie van de *N. oleoabundans*-celwanden gedurende de levenscyclus bestudeerd en de moleculaire determinanten van de metabolische routes gekarakteriseerd.

In dit project hebben we met succes een robuust protocol ontwikkeld dat de isolatie en verdere karakterisering mogelijk maakt van de celwanden van *N. oleoabundans*. We hebben vastgesteld dat de celwand een gevarieerd assortiment bestanddelen bevat en over het algemeen unieke kenmerken vertoont. De celwandbouwstenen van *N. oleoabundans* zijn een combinatie van koolhydraten (veelal niet-cellulosic); eiwitten, lipiden en anorganische materialen. Een vergelijkende transcriptomics studie gevolgd door biochemische karakterisering en morfologische observatie onthulden de moleculaire mechanismen die ten grondslag liggen aan de celwandontwikkeling gedurende de gehele celcyclus.

Toegang tot de intracellulaire componenten kan alleen worden verkregen na disruptie van de celwanden. In dit project hebben we aangetoond dat de celwand van *N. oleoabundans* wezenlijk verschilt van (hogere) plantencelwanden. We hebben indicaties verkregen dat de celwand van *N. oleoabundans* niet uitsluitend een rigide barrière vormt. In tegendeel, deze dynamische ultrastructuur reguleert de celmorfogenese tijdens de celcyclus en moduleert de groei en interactie met de omgeving. De opgedane kennis over de samenstelling van de celwand van *N. oleoabundans* biedt mogelijkheden voor de ontwikkeling van enzymatische (voor)behandelingen om toegang te krijgen tot de waardevolle producten in de cel. Door de aanwezigheid van specifieke polysacchariden in de celwand kan behandeling met enzymen die polysacchariden afbreken een interessante optie zijn. Ook de aanwezigheid van eiwitten in de celwand biedt de mogelijkheid om proteasen te gebruiken om de celwand te breken of te verzwakken, zoals reeds is aangetoond in dit project.

1.5 Conclusies en aanbevelingen

Alle projectdoelstellingen zijn bereikt. De algemene resultaten van het project omvatten

- energie-efficiënte, nieuwe en geoptimaliseerde technieken die op laboratoriumschaal zijn ontwikkeld en opgeschaald kunnen worden; enkele technieken zijn gevalideerd op pilot schaal;
- inzicht in hoe bioraffinage van algen moet worden georganiseerd, op basis van economische prestaties en duurzaamheid;
- kennis over hoe bioraffinage van microalgen kan worden gekoppeld aan de algensoort, groeiomstandigheden en celcyclus;
- inzicht in bioraffinagekosten voor verschillende technologieën en concepten.

Er zijn diverse mogelijkheden voor spin-off en vervolgactiviteiten, variërend van follow-up en aanverwante projecten door de R&D instellingen en commerciële partners tot meer algemene, commerciële voordelen en start-ups.

Het project heeft waardevolle resultaten opgeleverd voor concrete product leads en toepassingen van algen producten waaronder voeders voor aquacultuur, bioplastics en ingrediënten voor voeding. Daarnaast kunnen ze als (co) producten de techno-economische haalbaarheid van de productie van algen biobrandstoffen verbeteren.

Het project heeft nieuwe wetenschappelijke, technologische en economische inzichten opgeleverd die de algenkweek en bioraffinage ten goede zullen komen voor productontwikkeling in de voedings-, diervoeder- en non-food sector. In het bijzonder heeft het project waardevolle, commercieel relevante informatie opgeleverd over

- Technologie voor oogst en ontwatering van algen
- Mogelijkheden van bioraffinage-technologieën voor celdisruptie, -extractie en -fractionering, inclusief hun integratie in (multiproduct) processchema's;
- Samenstelling en variabiliteit van celwanden van algen ten behoeve van optimale verwerking;
- Fysiologische gegevens met betrekking tot de teelt en verwerking van algen;
- Strategieën en protocollen voor algenopslag en logistiek;

- Gedetailleerde techno-economische evaluatie op basis van massabalansen, stroomschema's gebaseerd op verkregen experimentele data;
- Geïntegreerd model dat ontwerp, analyse en selectie van haalbare microalgen waardeketens mogelijk maakt, van teelt tot verwerking, gekoppeld aan logistiek;
- Aanknopingspunten voor kosten / energiereductie van algenkweek en bioraffinage;
- Beoordeling van milieueffecten met focus op energieverbruik / CO_2-emissies.

Resultaten zijn gedocumenteerd in rapporten en publicaties, beschikbaar voor projectpartners, RVO, industrie en het brede publiek. Met name de ontwikkeling van een geïntegreerd techno-economisch model dat modules combineert voor microalgen productie, oogst, bioraffinage en logistiek is een waardevol instrument om verdere R&D te begeleiden en voor evaluatie van commerciële kansen.

De projectresultaten zullen aanzienlijke impact hebben voor de Biobased Economy door promotie en uitbreiding van de ontwikkeling van algen bioraffinage voor de vastlegging en benutting van CO_2 en als bron van biobased producten en biobrandstoffen. Het wordt aanbevolen om vervolgprojecten in TKI-kader te ontwikkelen in samenwerking met de industrie, naast nieuwe projecten in internationale programma's incl. H2020 en BBI. Van bijzonder belang zijn vervolgprojecten op het gebied van voedsel en diervoeders, biotechnologie en biobrandstoffen op basis van de multiproduct-bioraffinage benadering, hulpmiddelen en technologieën die in dit project zijn ontwikkeld. Daarnaast zou een vervolgproject prototypen van nieuwe producten met algeningrediënten dienen te omvatten om expansie naar nieuwe markten te stimuleren.

1.6 Implementatie van het project en disseminatie van resultaten

Tijdens het project werden verschillende technische en organisatorische uitdagingen met succes aangepakt, incl. veranderingen in het partnerschap en daaruit voortvloeiende wijzigingen in het werkplan. Verder deden zich enkele heroriëntaties en uitbreidingen voor in de projecttaken en -aanpak die de resultaten en de impact van het project verbeterd hebben.

Projectresultaten staan ter beschikking van de partners en RVO via (vertrouwelijke) periodieke halfjaarlijkse rapporten en aanvullende interne documenten. Daarnaast worden de projectresultaten op grote schaal gedissemineerd via meer dan 30 publicaties in wetenschappelijke tijdschriften, conferentie documenten, de openbare rapportage (o.a. via de website http://www.algaeparc.com/) en andere communicatie uitingen.

1.7 Gebruikte afkortingen

ASW	Artificial Sea Water; Kunstmatig zeewater
ATEX	ontleent zijn naam aan de Franse titel van de richtlijn 94/9 / EG: Appareils destinés à être utilisés en ATmosphères EXplosives
ATPS	Aqueous Two Phase Systems; waterige tweefasen systemen.
ABS	Aqueous Biphasic System; waterig bifasisch systeem.
DSP	downstream-processing; product opwerking
EBA	N-ethyl butylamine
FW	Fresh Water (gekweekt in zoet water medium)
IL(s)	Ionic Liquid (s)
N-gelimiteerde	stikstof beperkt; toevoer van stikstofvoedingsstoffen in het kweekmedium is beperkt
PEG	Poly Ethylene Glycol
PEF	Pulsed Electric Field
Rubisco	Ribulose-1,5-bisfosfaatcarboxylase / oxygenase

2 Summary

2.1 Aim of the project

Aquatic biomass is an interesting new feedstock for biorefining and is an important addition to the existing biomass supply. Algae have a unique composition and consist of a wide range of valuable components such as oils, fatty acids, proteins and sugars. They are therefore extremely suitable for biorefining. However, unlike terrestrial biomass, much is still unknown about biorefinery of aquatic biomass. There are currently few concepts for the extraction and refining of lipids, proteins and carbohydrates from algae for application in the fuel, chemical and food industries. To develop these concepts in the form of new technologies and sustainable and competitive processes, both cultivation and biorefining processes must be developed, optimized and integrated in a sustainable manner. This has been done in this project.

The AlgaePARC Biorefinery TKI project (2013-2018) has been initiated by Wageningen University and Research Centre in cooperation with ten industrial partners and University of Twente. The project was performed with financial support from the Netherlands' Ministry of Economic Affairs in the framework of the TKI BioBased Economy under contract nr. TKIBE01009.

Microalgae are a potential feedstock for biofuels, feed, food and chemicals. They can grow on seawater, having therefore a low water footprint, and they do not require agricultural land. In addition, microalgae have much higher oil and protein productivities per ground area than higher plants such as palm, soybean, rapeseed and corn. Interestingly, in contrast to terrestrial crops hardly any production capacity for microalgae exists at this stage and production is done in niche markets for specialty products (e.g. carotenoids, pigments, functional substances, food products (proteins and carbohydrates) and ω-3-fatty acids). The current world production capacity is limited to ca 15,000 ton dry matter per year. Successful scale-up of the algae industry requires reduction of production costs and enhancement of the (economic) output. This can be achieved by improving the cost-effectiveness of algae production and processing through technology development, development of multiple-product biorefinery for specialties from algae and development of new products.

Time is required to develop from an activity operating in niche markets, as the algae industry is today, to an industrial activity for bulk products such as biofuels. The technology is still immature, worldwide large programs have been started devoted to improve biomass production. The industry investing in running research programs is aware that development of commercial microalgae feedstock for bulk products requires time (10-15 years). In order to bring this process into the market, similar efforts need to be done in the establishment of a microalgae biorefinery. The usage and valorization of all biomass is a required step to reach economic feasibility and develop a sustainable process to produce commodities from microalgae. Most of the lipids (up to 50 % of the biomass) can be used for fuel while the remaining proteins and carbohydrates can be used in the chemical, food and feed industry.

Worldwide research programs were initiated to develop technologies for production of biodiesel from microalgae. Examples are amongst others three EU FP-7 Demonstrations projects BIOFAT, ALL GAS and InteSusAl, which have as overall goal to demonstrate algae-fuel production at 10-ha scale. Or larger projects in the US such as the alliance between Synthetic Genomics and Exxon Mobil (600 m€) or startups such as Sapphire Energy , now Qualitas, Solazyme (now Corbion), Algenol and many others. Focus within many of these projects mostly lies on improved production of biomass; and the conversion of biomass into one single product. However, to make the process economically feasible it is necessary to valorise all biomass components. For this purpose a biorefinery process is necessary.

Biorefinery of microalgae did not exist at the start of this project and research efforts were still very limited then. It is therefore a new field in which the Netherlands pioneered. Our world leading position and innovative reputation in the field of microalgae has been further consolidated with this project. International industry invested in Dutch innovation.

The overarching objective of the project was to develop a sustainable microalgae biorefinery concept through development and integration of scalable technologies for biorefinery of commodities (lipids,

proteins, carbohydrates) from microalgae. Several developed technologies were implemented at pilot scale. To achieve the overall goal three interrelated Work Packages were defined:

WP1: Technology Development/Optimization (Lab-Pilot Plant)
The aim of work in this WP was to develop new, scalable technologies for the biorefinery of microalgae cultivated at AlgaePARC whereby these technologies should be mild, efficient, cost effective and sustainable. The focus was on mild cell disruption techniques, enzyme-assisted disruption and mild extraction processes that enable recovery of all biomass fractions while fully retaining their structure and functionality. Selected technologies were scaled up to pilot scale. Furthermore the work aimed at development and modelling of energy and cost effective harvesting methods integrated with further downstream processing of algae via biorefinery.

WP2: Systems Analysis and sustainability assessment
The main aim of this work was to perform Life Cycle Assessment driven design of integral algae biorefineries including processing conditions and product routing and resource recovery based on data from the project partners. Furthermore the work addressed integration of the algae biorefinery within the supply chain. A major result is the development of an integrated techno-economic model combining modules for microalgae production, harvesting, biorefinery and logistics to assess business opportunities and define new research lines with impact on the overall process.

WP3: Enabling Technologies[10] – Basic Knowledge
For the development of biorefinery techniques detailed knowledge on the cell wall composition as a function of algal strain and cell stage is vital. The main aim of WP3 was to generate this knowledge. Detailed analysis of cell wall composition and its variability under different process conditions (replenished medium, nitrogen starvation, cultivation in seawater and freshwater) and throughout the cell cycle was done. In addition cellular mechanism and genes responsible for cell wall biosynthesis have been identified which opens doors to further research on inducing cell disruption and genetic engineering.

2.2 Technology development and optimization for the microalgae biorefinery (WP1)

Biorefinery techniques are necessary to exploit all products produced by microalgae. Technologies to efficiently harvest algae and to separate the different fractions without damaging one or more of the products need to be developed, and they should be applicable for a variety of end products of sufficient quality at large quantities. To achieve this, the developed techniques should be mild, inexpensive and low in energy consumption. First focus in obtaining the products should be on cell disruption to release the products or to make them available for extraction. When the products are released from the cells, different extraction and separation techniques need to be used for purification. It is important to keep in mind that the different technologies should be tailored to the different species of interest and should therefore be flexible. Some algal species lack a cell wall, making their cell disruption less energy intensive. In addition, different cultivation conditions (freshwater, seawater, nutrient limitation or starvation) could lead to changes in the cell wall. These differences need to be taken into account when selecting the cell disruption and separation approach for specific algae species cultivated under specific conditions. The work was done on lab and pilot scale.

2.2.1 Selection of algal strain and biomass production

In order to select the most relevant algae for extensive use in this project, three model algae strains *Phaeodactylum tricornutum*, *Neochloris oleoabundans*, *Chloroccocum litoralle* were characterized.

[10] An enabling technology is an invention or innovation, that can be applied to drive radical change in the capabilities of a user or culture. Enabling technologies are characterized by rapid development of subsequent derivative technologies, often in diverse fields. Vertaling Wikipedia, https://en.wikipedia.org/wiki/Enabling_technology. Dd 27-06-2019.

Additional benchmarks for mild cell disruption for these strains, by using a number of standard disruption technologies, were developed. Based on the results, technology development was largely focused on *Neochloris oleoabundans*, an algal species that can grow well in both fresh water and seawater and is able to accumulate oils under specific cultivation conditions.
In the framework of the project, algae biomass has been produced under different cultivation conditions; applying nitrogen stress for a certain period of time resulted in accumulation of oil. The challenges of different cultivation conditions, and their impact on algal biomass composition, cell wall composition as well as downstream processes have been investigated. Batches of *N. oleoabundans* were produced at lab and pilot scale in various indoor and outdoor photobioreactors at AlgaePARC facilities and supplied to partners as fresh or frozen paste. The algal biomass was concentrated using Evodos type 10 and 25 separators, supplied by partner Evodos. In total ca. 200 kg paste of *Neochloris* biomass was produced. About 165 kg was cultivated in (artificial) seawater and the remainder in fresh water. About 150 kg *Neochloris* biomass was produced under N-limited conditions. This biomass was mainly used by partners for extraction of oil.

2.2.2 Evaluation of algae harvesting technologies

Currently, harvesting of microalgae is done by mechanical solid-liquid separation technologies such as filtration and centrifugation. Although these conventional techniques are proven to be very efficient, they are energy-intensive. Studies have shown that 20 to 30% of the total processing costs can be attributed to harvesting when centrifugation is used in combination with an open pond type of cultivation system. To reduce processing costs, either a (pre-)concentration step, or an alternative harvesting technology is desired. Instead of using merely technologies such as filtration or centrifugation, applying a (pre-)concentration technology like flocculation-sedimentation can result in a lower energy consumption. With flocculation, single cells become aggregated into larger particles or 'flocs' which settle to the bottom of a harvesting tank. Although flocculation followed by sedimentation is already a proven- and often applied technology in wastewater treatment it is still challenging to harvest microalgae grown in seawater.

Within this project, flocculation of marine microalgae was investigated as an alternative potential low-cost and mild technology for (pre)concentrating the biomass. Although flocculation is already a widely applied technology, there were still challenges to induce flocculation of microalgae at seawater salinity. Cationic polymers, and in particular polyacrylamides, successfully induced flocculation of marine microalgae. The use of those cationic polymers resulted in biomass recoveries higher than 90% and a concentration factor ranging between 5 and 10 after 2 hours of sedimentation. A positive effect of increasing flocculant dosages on the biomass recovery was observed with the marine cultivated microalga *N. oleoabundans*. After reaching an optimum in biomass recovery (>95%), higher flocculant dosages did not enhance the biomass recovery. Further increasing the flocculant dosage even resulted in decreasing biomass recoveries. This mechanism of charge attraction at low flocculant dosage and repulsion at high flocculant dosage was translated into a mathematical model. This dosage in combination with the cost price of the flocculants makes polymeric flocculation rather expensive. There is thus still a need for alternative and low-cost flocculants for successful flocculation at high salinities.

2.2.3 Development and optimization of cell disruption technologies

After harvesting, the next step in the algae biorefinery scheme is cell disruption. This step is needed to break the cell walls to enable extraction of the products that are located inside the algal cells. Several technologies have proven to be successful to disrupt microalgae. Examples are mechanical technologies such as high pressure homogenization or bead milling, chemical technologies such as alkali-heat treatments and physical technologies such as microwave cell disruption. Mechanical and physical technologies are regarded as energy intensive. This high energy consumption as well as costs may limit the economic feasibility of a microalgae biorefinery. Work in the project focused mainly on mild cell disruption using enzymes and optimization of bead milling and homogenization. Furthermore work was done on Pulsed Electric Field (PEF) technology and Controlled Instantaneous Pressure Drop (CIPD) technologies.

Usage of enzymes for cell disruption

One of our aims was to find enzymes applicable for mild cell disruption of algae. Therefore, three different types of experiments have been performed on the algae species *Neochloris oleoabundans* and *Phaeodactylum tricornutum*. Initially, experiments were performed by mixing algae with cellulases and hemicellulases. Such treatments did not release any sugars from the cell wall. A range of other enzymes that can act on plant carbohydrates were also evaluated. Overall, the tested carbohydrate degrading enzymes were not or only slightly effective in cell wall degradation of the selected algae. Thus either the target components of these enzymes are not present in algal cells or they are not accessible within the cell matrix. Results from both within and outside the project indicate that proteins are important components of algal cell walls. Thus the degradation of cell wall proteins by suitable enzymes could be a method for mild degradation of cell walls to facilitate easier lipid extraction and/or mechanical cell wall disruption. Thus the action of 10 different proteases (protein degrading enzymes) on algal cell walls was analysed. Overall, alkaline proteases were found to be most effective on algal cells. These enzymes can degrade a significant part of the cell wall of the algae *Phaeodactylum* and *Neochloris.* This finding represents an important discovery for the mild processing of algae. A next step could be to investigate if proteases will be most effective for cell wall degradation on their own or in combination with mechanical disruption techniques such as bead milling.

Cell disruption using bead milling and high-pressure homogenization

Bead milling and high-pressure homogenization are efficient cell disruption methods. After optimization, both methods showed high cell disintegration (± 90%), with a fast release of hydrophilic components (proteins and carbohydrates). Nevertheless, despite their efficiency they did not lead to release of total proteins and carbohydrates (insoluble protein remained in the pellet) present in *N. oleoabundans*. Growth conditions (stressed or non-stressed, marine or fresh water) seemed to impact the efficiency of cell disruption due to changes in cell wall thickness and composition.

Cell disruption using Pulsed Electric Field (PEF)

Pulsed Electric Field (PEF) has been considered as a promising technology to disrupt microalgae in a biorefinery framework. In initial tests, the highest yield of proteins (13%) was obtained with N. *oleoabundans* cultivated in seawater medium in a batch mode PEF. The high release of ions illustrated that the application of PEF for the disruption of fresh and marine cultivated microalgae, resulted in a weakening of the cell membrane suggesting the formation of pores. Nevertheless, in comparison to the mechanical benchmark, no sufficient amounts of protein were liberated by the application of PEF and much lower yields were obtained with this technology. Moreover, the required energy input for PEF was higher than the mechanical benchmark. It should be considered that the mode of PEF operation is different from bead milling. Where bead milling causes a complete cell disintegration, PEF merely makes pores in the cell walls.

2.2.4 Integration of unit operations

Biorefinery should become less complex and less expensive for production of bulk-products at large scale. To do so, we should not only focus on the specific DSP-unit operations (i.e. harvesting, cell disruption and extraction) but rather on the overall process design as well. By reducing the required number of unit operations and preventing the use of chemical additives we can simplify the process and enhance the (economic) feasibility of a biorefinery. We investigated two possible approaches to reduce the number of unit operations. In the first approach the number of main DSP-unit operations (i.e. harvesting, cell disruption and extraction) were reduced by integration in a single step. The second approach is to avoid the use of chemicals. Chemicals (e.g. flocculants, solvents, etc.) are not only a consumable with associated costs, but they need to be recovered by using additional unit operations as well. These additional unit operations result in a complex process. By using external fields different phases can be separated, resulting in a solvent-free and more simple product separation.

An interesting approach for such an integrated and multi-product process biorefinery is the use of an ionic-liquid based aqueous biphasic system (ABS). Ionic-Liquids (ILs) are liquid salts with specific, interesting solvent properties. ILs are already used to extract lipids from wet and non-broken biomass in a single step.

A main challenge in developing IL-based ABS for direct product extraction is the ability to extract the hydrophilic proteins from intact biomass. Proteins are typically larger molecules than the lipid molecules

and are thus difficult to extract from whole cells. As extracting the large proteins from intact cells is difficult, an alternative could be to first completely dissolve the cell walls instead of merely weakening them, followed by extraction. As dissolving cell walls can take place in aqueous systems at room temperature, it will become possible to extract native proteins. Direct product extraction using IL-based ABS could make cell disruption redundant, and this makes it a very promising route to simplify a biorefinery process design.

Besides the costs and the overall process design, mildness should remain a key-criterion in microalgal biorefinery research and development. Complete valorisation of all components in a biorefinery will facilitate the transition from current small-scale production of specialties from microalgae towards future large-scale production of bulk commodities. By developing unit operations in which different steps in the process chain are integrated, or by avoiding the use of chemicals that are difficult to recycle, the biorefinery chain will become less complex. Development of such simplified processes is essential to bring the multi-product microalgae biorefinery towards economic feasibility.

2.2.5 Development of Down Stream Processing: Extraction and Fractionation

Several technologies were evaluated for product extraction and fractionation, in particular membrane filtration and aqueous two-phase system, which are discussed below. Additionally, R&D was performed on product extraction, focussing on developing a new extraction based process as well as a trial where the use of conventional solvents (ethanol) was evaluated at pilot scale.

Ultrafiltration/diafiltration as a fractionation technique

The supernatant obtained after high-pressure homogenization and centrifugation of *N. oleoabundans* was submitted to ultrafiltration and diafiltration using low, medium and high kDa membrane cut-offs in order to separate the hydrophilic molecules from each other and increase the amounts of proteins in the filtrate. Results showed that the permeate did not contain polysaccharides and pigments, and that the highest amount of proteins was obtained with the low kDa membrane cut-off. The process of Ultrafiltration followed by diafiltration increased the quantity of proteins in the filtrate.

Extraction and Fractionation by Aqueous Two-Phase Systems (ATPS)

Aqueous two-phase systems (ATPS) were adopted as a new approach to fractionate hydrophilic from hydrophobic components. Additionally, stability studies were performed to see if ATPS retain the native protein structure. Iolilyte 221PG-Citrate was found to be the most efficient ATPS in Rubisco separation. However, stability studies indicated that PEG-based ATPSs have a better performance in retaining the Rubisco integrity.
In the project we demonstrated the potential of ATPS to separate different biomolecules simultaneously, giving value to different microalgae components for a sustainable multiproduct biorefinery. Furthermore, components from microalgae cultivated in saline water and fresh water can be selectively fractionated using ATPS. Using salt water to cultivate microalgae can reduce costs and the fresh water footprint for large scale production, which is an advantage in view of a sustainable processing.

Oil extraction of wet biomass with ethanol

Neochloris oleoabundans (stored frozen and defrosted) grown under stressed conditions in fresh water was disrupted by bead milling. Ethanol was added to the disrupted cells and these were subsequently centrifuged. An ethanol concentration of 20% (v/v) final concentration did not result in a three phase system with a clear top layer (containing a dark green oil/lipid layer) that could be easily separated. Unfrozen and salt water cultivated algae showed a slight, green oily layer on top. It is not clear if the storage condition (frozen), the composition of the microalgae and/or salt play a role in the separation of the oil.

Oil extraction of wet biomass using novel solvents

In this task a novel, effective, switchable solvent system for direct lipid extraction from unbroken microalgae in (concentrated) aqueous solutions was developed.
Among other candidates, N-ethyl butyl amine (EBA) was identified as most promising switchable solvent for both wet lipid extraction as well as phase separation by hydrophilicity switching. Compared to the Bligh

& Dyer standard lab test for lipids determination, EBA is able to extract all lipids, neutral and polar, from freshwater microalgae (as tested for esp. non-stressed *Neochloris oleoabundans*) without cell disruption and without drying. The organic extraction phase, containing the lipids and EBA, can be separated from the (concentrated) microalgae culture medium by phase separation. Subsequently, the EBA can be separated from the lipids by adding water and contacting with CO_2 or decreasing the water-organic mixture temperature. In both cases the EBA transfers to the aqueous phase, leaving the lipid product as separate phase behind. After a simple phase separation to harvest this product, the solvent EBA can be recovered as separate organic phase by removing the CO_2 or increase the temperature.

The study confirmed that by using both algae stressing techniques and applying EBA as solvent in multiple stages of extraction is very promising in the development of an efficient lipid extraction technology targeting non-broken, wet microalgae. Overall, it was found that applying EBA as switchable solvent for lipid extraction from microalgae is from an energy point of view very promising, as high yields can be obtained, processing conditions are quite mild (and hence equipment costs are low) and non-broken, wet microalgae slurries can be used without pretreatment.

Oil extraction at pilot scale

Enzyme and pH-based technologies were explored to produce algae oil from *Neochloris oleoabundans* under aqueous conditions. Yields, however, were relatively low. Aqueous technology would work more efficiently if algal biomass contained significantly higher oil contents than the material used in this project. Ethanol and especially Iso Propyl Alcohol (IPA) based extraction showed to be good options. Valuable co-products that could be further explored are proteins and phospho-/glyco-lipids. Results confirmed that bead milling conditions have to be carefully controlled when producing oil through aqueous extraction. Furthermore, long enzymatic treatment times are required.

2.3 System analysis, techno-economic and sustainability assessment, and flexibility (WP2)

Algae have potential for a broad spectrum of products. The focus in an algae biorefinery is therefore not on one or a few products, but on an efficient product mix. To obtain this mix, unit operations in the chain from algal feedstock to end product have to be combined efficiently.

The choice and operational conditions for the unit operations in the chain should therefore be well balanced to obtain the most efficient biorefinery. In the current literature the choice of unit operations is mostly given in rather qualitative instead of quantitative terms, and is strongly focussed on single operations instead of a chain of operations. The work in this project therefore focussed on development of simulations for a systematic analysis to define a balanced and quantitative organisation of the production chain.

Processing in algae biorefineries at centralized locations results in more efficient use of capital and related costs, and more efficient use of labour. On the other hand a centralized large scale refinery requires the feedstock supply of multiple cultivation units, which increases the transport costs and transport fuel consumption and emissions. Bruins and Sanders [1][11] state that application of local biorefineries can be more attractive than centralized biorefineries, by reduced efforts for transport. The work in the project had therefore also the challenge to find the best trade-off for the scale of an algae biorefinery and the impact of the transport logistics.

The objectives of WP2 were:

- Economic and sustainable biorefineries: to define biorefineries that meet economic and sustainability criteria by the best choices for unit operations, and to develop a flexible tool to evaluate different biorefinery chain options.

11 Bruins M.E., Sanders J.P.M. Small-scale processing of biomass for biorefinery. Biofuels, Bioprod. Bioref. 2012. 6:135–145

- Biorefineries and the supply chain: to define which biorefineries request for local and which biorefineries request for centralized processing, and to develop a flexible tool to evaluate different options for centralized or local processing.

2.3.1 Economic and sustainable biorefineries

A model-based approach was applied as basis for the system evaluation. For each unit operation a simulation model was defined. The models concern the input-output component mass and energy balances of main and co-streams for each unit operation. Additional relations are included to connect the energy demand and product yield to economic estimation elements. The models for the unit operations were programmed in Excel spreadsheets. A flexible structure is used to connect all unit operations with each other in any combination.

The energy input and co-stream input (for example solvents) are based on data for the specific energy consumption of each unit operations, which is linked to the amount of material being processed. Equipment data (dimensions, costs, scaling rules etc.) was derived from data of industrial equipment suppliers (GEA, WAB, Evodos), engineering companies (Bodec), literature and engineering databases (DACE, NETL Matche.com). For each scenario the investment costs were calculated from the purchase cost of the equipment plus costs due to piping, installation of pumps, construction, automation etc. A general overall Lang factor[12] that sums the contribution of individual aspects is used. For standard equipment the applied Lang factor was 3.5. For units that are operated in ATEX-environment to avoid explosion risk a higher Lang factor is used. The considered operational costs included energy, consumables, labour and costs related to the loss of biomass.

For harvesting and dewatering 28 combinations of unit operations were evaluated with respect to operational costs and energy consumption. Flocculation of open pond cultivated algae proved to be low in energy consumption (<0.1 $kWh.kg^{-1}$) while the costs are in the range of 0.4-1.4 $€.kg^{-1}$. The effect of flocculant in the water recycle to the cultivation unit, and subsequent processing steps is, however, not yet well described in the literature. Other methods, pressure, vacuum and membrane filtration, centrifugation, and their combinations result for open pond cultivation systems in energy consumption ranging between 1.0-4.5 $kWh.kg^{-1}$ and costs below $2€.kg^{-1}$. For high productivity systems (flat plate, tubular cultivation or beneficial climate), the costs and energy consumption drop by a factor 3-4.

For cell disruption in large scale algae biorefineries homogenisation is preferred with respect to the operational costs. The simulations for the lipid extraction methods, experimentally investigated by WP1 (with IPA or ethanol as solvent), showed that the water-solvent separation (distillation or evaporation) results in high costs and high energy consumption. These methods proved to be more costly and energy intensive than traditional lipid extraction with hexane. In the protein biorefinery with centrifugation and ultrafiltration steps 20-25% of the protein was recovered as native protein. The majority of the native protein was lost in the residue from the centrifugation step. This part can be recovered by alkaline or acid extraction, but then the native protein properties are lost during this treatment.

Some specific algae biorefineries were analyzed. Excel models were also developed for other traditional methods for extraction and separation of algal components. Next, an Excel model was developed to evaluate the total chain of cultivation in combination with harvesting, dewatering and product extraction and separation. The Excel model allows simulations using a large range of combinations of cultivation units and conditions, and harvesting and extraction methods.

2.3.2 Biorefineries and the supply chain

In the work addressing biorefineries and the supply chain two aspects have been investigated:

- The impact of transport on decision making for centralized versus decentralized processing of concentrated algal biomass.

[12] The Lang Factor is an estimated ratio of the total cost of creating a process within a plant, to the cost of all major technical components. It is widely used in industrial engineering to calculate the capital and operating costs of a plant. Source: https://en.wikipedia.org/wiki/Lang_factor dd. 27-06-2019.

- Algal paste processed in centralized biorefineries needs to be stored before and after transport, while transport is also a form of storage. The feasible storage time was investigated experimentally.

The benefits of large scale processing surmount the costs of transport over long distances and therefore centralized processing is preferred. The break-even point for transport distance is at lower distance for high productivity cultivation units (tubular and flat panel PBRs or beneficial climatic conditions).

The contribution of transport to the total cost of algal products is small, even over large transport distances. The costs benefits of large scale lipid and protein biorefineries always surmount the costs for transport over 2000 km from low productivity cultivation plants to a centralized biorefinery. For high productivity cultivation plants there is a break-even point in distance. For cultivation plants with 45 tonne algal biomass.ha^{-1}.year^{-1} the break-even point is at about 750 km.

The storage experiments in absence of air resulted in a recommendation of maximal 3 days storage time (in absence of air) for non-stressed algae and maximal 7 days for stressed algae. After these storage periods the loss of carbohydrates and proteins becomes significant, while lipids remain stable over this period.

2.4 Enabling tools, basic knowledge on cell wall composition (WP3)

Neochloris oleoabundans is considered as one of the most suitable microalgae, given its industrial potential and the tools available for this species. The first step to be taken after harvesting the *N. oleoabundans* biomass and prior to generating multiple products is to overcome the physical barrier of the cell in order to access its components. The intracellular components of *N. oleoabundans* are enclosed within the cell wall, which is considered as a rigid layer located on the outer part of the cell membrane. A successful valorisation of the multiple products depends on the kept integrity and functionalities of the cellular components upon disruption of the cell wall.

In contrast to terrestrial plants, for which tools and methods to characterize cell wall properties have been developed over many decades, microalgae such as *N. oleoabundans* have a limited toolbox available. Hence, there is little information available on the type of cell wall monomers and their assembly to higher complex structures. Moreover, a specific function of the individual cell wall building blocks and their coordinated biosynthesis pathways still remain unidentified on the whole. Prior to engineering the cell wall constituents and structures, with different purposes for particular applications, it is considered vital to identify the biochemical composition, morphological structure and their underlying genetics as well as their biosynthetic pathways.

Algae cell walls, in general, display a greater biological diversity and metabolic plasticity compared to terrestrial plants. Cell walls of species belonging to the phylum Chlorophyta display both unique and common features with the cell wall of (higher) plants and fungi. Similar to higher plant cell walls, microalgae contain a substantial amount of polysaccharides in their cell wall. Additionally, the cell wall of microalgae belonging to the Chlorophyta might contain chitin or chitin-like structure, a feature of fungal cell walls.

Apart from the diversity of the cell wall among different species, structural properties of the cell wall might also change in response to environmental factors or throughout the life cycle. Nitrogen deficiency and salinity are two environmental factors that can influence cell carbon partitioning. Changes in carbon balance and its partitioning can make a significant alteration in cell wall composition and morphology. Cell development throughout the cell cycle can also change the cell wall characteristics.

Species-specific cell wall characteristics together with remodelling of the cell wall due to environmental factors or growth phases can cause a different level of susceptibility to the various disruption methods. Several studies have reported on the development of methods for microalgae cell walls disruption. These methods consist mostly of harsh treatments that target the cell wall as a whole, but the development of tailor-made mild and non-disruptive methods requires a better knowledge of the cell walls, including

biochemical composition and morphological features. The fundamental knowledge to uncover the biochemical composition and configuration of the cell wall is a prerequisite to reveal its imperceptible rigidity and further design an applicable and selective disruption strategy.

The work in this project aimed to examine the *N. oleoabundans* cell wall composition and morphology under optimum growth conditions, and to study its cell wall remodelling when exposed to different environments. Additionally, we unveiled the morphologic characteristics and remodelling of the cell wall throughout the cell cycle. In order to enlarge the existing knowledge, we studied the biosynthesis and modification of the *N. oleoabundans* cell walls throughout the life cycle and characterized the molecular determinants of the metabolic pathways.

Understanding the composition and structure of the cell wall is crucial to optimize the extraction process during algae biorefinery. Access to all intracellular components can only be obtained after disruption of the cell walls. In this project we successfully developed a robust protocol that allows the isolation and further characterization of *Neochloris oleoabundans* cell walls, a microalga species belonging to the phylum Chlorophyta. We have observed that the *N. oleoabundans* cell wall comprises a diverse assortment of constituents and overall displays unique features. The cell wall building blocks of *N. oleoabundans* is a combination of carbohydrates, frequently non-cellulosic; and proteins, lipids and inorganic materials. A comparative transcriptomics study followed by biochemical characterization and morphological observation revealed the molecular mechanisms underlying the cell wall development throughout the cell cycle.
As a key message of this project, we have shown that the *N. oleoabundans* cell wall is substantially different from (higher) plant cell walls. We indicated that the cell wall in *N. oleoabundans* is not just a rigid barrier of the cell. In contrast, this dynamic ultrastructure regulates the cell morphogenesis during the cell cycle and modulates the growth and interaction with the environment. With respect to cell wall polysaccharide composition, enzymatic treatments with carbohydrate hydrolases can be considered a promising approach to weaken the cell wall prior to reaching the internal products of interest. In the same vein, the protein-rich cell wall of *N. oleoabundans* suggests the possibility of using proteases to break or weaken the cell wall.

2.5 Conclusions and recommendations

All project objectives have been achieved. The overall results of the project include

- low-cost, energy-efficient, novel and optimized techniques developed at laboratory scale that can be scaled-up; partly validated at pilot scale;
- insight into how biorefinery of algae should be organized, based on economic performance and sustainability;
- knowledge on how biorefinery of microalgae can be linked to the strain, growing conditions and cell cycle;
- insight in biorefinery costs for different technologies and concepts.

There are many opportunities for spin-off and follow up activities ranging from follow-up and related projects by R&D and commercial partners to more general commercial benefits and start-ups.

The project has generated valuable results for concrete product leads and application areas including aquaculture feeds, bioplastics, and food ingredients, that may lead to concrete market opportunities. In addition, as (co) products they will enhance the techno-economic feasibility of algae biofuels production. The project has generated new, scientific, technological and economic insights that will benefit algae cultivation and biorefinery for product formulation in the food, feed and non-food sectors. In particular the project generated valuable, commercially relevant information on

- Algae harvesting technologies;
- Opportunities and challenges of biorefinery technologies for cell disruption, extraction and fractionation including their integration in (multiproduct) flowsheets;
- Composition and variability of algae cell walls relating to optimum processing;
- Physiological data related to algae cultivation and processing;
- Strategies and protocols for algae storage and logistics;
- Detailed techno-economic evaluation based on mass balances, flowsheets built on acquired experimental data;

- Integrated model enabling design, analysis and selection of viable microalgae value chains from cultivation through processing, coupled to logistics;
- Leads for cost/energy reduction of algae cultivation and biorefinery;
- Assessment of environmental impact with focus on energy use/CO2 emissions.

Results are documented in reports and publications, available for project partners, RVO and parties outside the consortium in industry, R&D and the general public. In particular the development of an integrated techno-economic model combining modules for microalgae production, harvesting, biorefinery and logistics to guide further R&D is a highly valuable project result for both industry and academia, to assess business opportunities and define new research lines with impact on the overall process, respectively .

The project results will have significant impact for the Biobased Economy by promotion and expansion of the development of algae biorefinery for Carbon Capture and Utilisation, and as a source of biobased products and biofuels. It is recommended to develop follow up projects in the TKI framework in close collaboration with industry in addition to new projects in international programs incl. H2020 and BBI. Of particular interest are follow up projects in the area of food and feed, biotech and biofuels based on the multiproduct biorefinery approach, tools and technologies developed in this project. In addition, these follow up project should include prototyping new products with algae ingredients to stimulate expansion to new markets.

2.6 Implementation and dissemination of the project

During the project several technical and organizational challenges were successfully addressed incl. changes in the partnership and consequent changes in the work plan. Furthermore several major reorientations and expansions occurred in the project tasks and approach that enhanced the successful outcomes and the impact of the project.

Project results are disseminated to the partners and RVO via (confidential) periodic 6-monthly reports and additional, internal documents. Furthermore the project results are widely disseminated via a range of publications in scientific journals as well as conference papers, the public report and other communications

3 Abbreviations

ASW	Artificial Sea Water
ATEX	derives its name from the French title of the 94/9/EC directive: Appareils destinés à être utilisés en ATmosphères EXplosives
ATPS	Aqueous Two Phase Systems
ABS	Aqueous Biphasic System
DSP	Down Stream Processing
EBA	N-ethyl butyl amine
FW	Fresh Water (cultivated)
IL(s)	Ionic Liquid(s)
kDa	kiloDalton
N-limited	Nitrogen limited; supply of nitrogen nutrients in the cultivation medium is limited
PEG	PolyEthylene Glycol
PEF	Pulsed Electric Field
Rubisco	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
TAG	Tri Acyl Glycerols

4 Starting points and objectives of the project

Microalgae are a potential feedstock for biofuels, feed, food and chemicals. They can grow on seawater, having therefore a low water footprint and they do not require agricultural land. Furthermore microalgae have much higher oil and protein productivities per ground area than higher plants such as palm, soybean, rapeseed and corn. Interestingly, in contrast to terrestrial crops hardly any production capacity for microalgae exists at this stage and production is done in niche markets for specialty products (e.g. carotenoids, pigments, functional substances, food products (proteins and carbohydrates) and ω-3-fatty acids) with a total world production capacity of ca >10,000 ton dry matter per year. Successful scale-up of the algae industry requires reduction of production costs and enhancement of the (economic) output. This can be achieved by improving the cost-effectiveness of algae production and processing through technology development, development of multiple-product biorefinery for specialties from algae and development of new products.

Time is required to develop from an activity operating in niche markets, as the algae industry is today, to an industrial activity for bulk products. The technology is still immature, worldwide large programs have been started devoted to improve biomass production. The industry investing in running research programs is aware that development of commercial microalgae feedstock for bulk products requires time (10-15 years). In order to bring this process into the market, similar efforts need to be done in the establishment of a microalgae biorefinery. The usage and valorization of all biomass is a required step to reach economic feasibility and develop a sustainable process to produce commodities from microalgae. Most of the lipids (up to 50 % of the biomass) can be used for fuel while the remaining proteins and carbohydrates can be used in the chemical, food, feed and pharma industry.

Worldwide research programs were initiated to develop technologies for production of biodiesel from microalgae. Examples are amongst others three EU FP-7 Demonstrations projects BIOFAT, ALL GAS and InteSusAl, which have as overall goal to demonstrate algae-fuel production at 10-ha scale. Or larger projects in the US such as the alliance between Synthetic Genomics and Exxon Mobil (600 m€) or startups such as Sapphire Energy , now Qualitas, Solazyme (now Corbion), Algenol and many others. Focus within many of these projects mostly lies on improved production of biomass; and the conversion of biomass into one single product. However, to make the process economically feasible it is necessary to valorise all biomass components. To achieve this a biorefinery process that valorises all fractions of the algal biomass producing multiple products is necessary.

Biorefinery of microalgae did not exist at the start of this project and research efforts were still very limited then. It is therefore a new field in which the Netherlands pioneered. Our world leading position and innovative reputation in the field of microalgae has been further consolidated with this program. International industry invested in Dutch innovation.

In the 5-year research program (2010-2015) which ran partly in parallel at AlgaePARC there was a focus on biomass production. Similar efforts needed to be done and were done for biorefinery in the current program AlgaePARC biorefinery. These efforts on both production and biorefinery took place partially in parallel because production and biorefinery need to be integrated due to the need for biomass supply and variability of biomass properties with the strain and process used.

The overarching objective of the project was to develop a sustainable microalgae biorefinery concept through development and integration of scalable technologies for biorefinery of commodities (lipids, proteins, carbohydrates) from microalgae at pilot scale. To achieve this goal three interrelated sub-projects or Work Packages were defined:

WP1: Technology Development/Optimization (Lab-Pilot Scale)

The aim of work in this WP was to develop new, scalable technologies for the biorefinery of microalgae cultivated at AlgaePARC whereby these technologies should be mild, efficient, cost effective and sustainable. The focus was on mild cell disruption techniques, enzyme-assisted disruption and mild extraction processes that enable recovery of all biomass fractions while fully retaining their structure and functionality. Selected technologies were scaled up to pilot scale. Furthermore the work aimed at

development and modelling of energy and cost effective harvesting methods integrated with further downstream processing of algae via biorefinery.

WP2: Systems Analysis and sustainability assessment

The aim of this work was to perform Life Cycle Assessment driven design of integral algae biorefineries including processing conditions and product routing and resource recovery based on input of data by the partners. Furthermore the work addressed integration of the algae biorefinery within the supply chain.

WP3: Enabling Technologies – Basic Knowledge

For the development of algal biorefinery technologies detailed knowledge about the cell wall composition as a function of algal strain and cell stage is vital. To acquire this knowledge was the main aim of WP3. Detailed analyses of cell wall composition and its variability under different process conditions (replenished medium, nitrogen starvation, cultivation in seawater and freshwater) and throughout the cell cycle was done. In addition cellular mechanism and genes responsible for cell wall biosynthesis have been identified which opens doors to further research on inducing cell disruption and genetic engineering.

Project consortium

The AlgaePARC Biorefinery project (2013-2018) was initiated by Wageningen University and Research Centre in cooperation with ten industrial partners and University Twente. The project was performed with financial support from the Netherlands' Ministry of Economic Affairs in the framework of the TKI BioBased Economy under contract nr. TKIBE01009. Partners in the consortium were

- Wageningen Food & Biobased Research (WFBR)
- Wageningen University Bioprocess Engineering (BPE)
- Wageningen University Plant Breeding (PB)
- Wageningen University Biobased Chemistry and Technology (BCT)
- University of Twente – UTwente
- Evodos Algae Technologies BV
- Bodec BV
- EWOS Innovation AS, now Cargill
- POS BioSciences
- DSM Innovation Center
- TOTAL Marketing Services S.A.
- BASF GmbH
- GEA
- WAB
- Dyadic Nederland BV (until October 2016)

Coordinator: Wageningen University, BPE
Budget € 5,202,097.17
Committed subsidy € 3,073,919.74
Project duration 1 October 2013 – 30 November 2018
Websites:

- RVO: https://www.rvo.nl/subsidies-regelingen/projecten/algae-parc-biorefinery-0
- WUR: http://www.algaeparc.com/project/2/algaeparc-biorefinery

5 Technology development for the microalgae biorefinery (WP1)

Biorefinery techniques are necessary to exploit all products produced by microalgae after cultivation. Technologies to efficiently harvest and separate the different fractions without damaging one or more of the products need to be developed, and they should be applicable for a variety of end products of sufficient quality at large quantities. To achieve this, the developed techniques should be mild, inexpensive and low in energy consumption. Typical process steps in an algae biorefinery are presented in Figure 1 below.

Figure 1 Process steps in the microalgae biorefinery

First focus in obtaining the products should be on cell disruption to release the products or to make them available for extraction. When the products are released from the cells, different extraction and separation techniques need to be used for purification. It is important to keep in mind that the different technologies should be tailored to the different species of interest and should therefore be flexible. Some algal species lack a cell wall, making their cell disruption less energy intensive. These differences need to be taken into account when selecting the cell disruption and separation approach for specific algae species. The focus of the work described is on the selected algae *Neochloris oleoabundans*, an algal species that can grow well in both fresh and seawater and is able to accumulate oils under specific cultivation conditions.

5.1 Algae selection, raw algae supply and technological benchmark

Algae selection

In order to select the most relevant algae for extensive use in this project, three model algae strains *Phaeodactylum tricornutum, Neochloris oleoabundans, Chloroccocum litoralle* were characterized. Additional benchmarks for mild cell disruption for these strains, by using a number of standard disruption technologies were developed.

Supply and harvesting of microalgae.

At the start of the project three model strains were collected and distributed. A commercial sample of freeze dried *Phaeodactylum tricornutum* was acquired for the project from the company Necton. Secondly, a 300 L indoor reactor at AlgaePARC was used to produce 100g quantities of *Neochloris oleoabundans*. Thirdly, a 20 L indoor reactor, as well as the 300 L reactor were also used to produce 100 g quantities of *Chloroccocum litoralle*. The algae cultures were concentrated with the existing centrifuge at AlgaePARC pilot facility (target dry matter concentration approx. 7% on total weight), as well as a batch type Evodos centrifuge (dry matter concentrations approx. 15 – 20 % on total weight basis). At laboratory scale, additional algae samples were also produced by different partners. Batches of algae were supplied to the various research partners. At the start of the project, installation of the 1500L indoor algae photobioreactor at the AlgaePARC facility was ongoing and completed in 2015. This photobioreactor was destined to produce kg quantities of algae biomass. In total over 2000 hours run time were executed to test the reactor and to provide the project partners with fresh or frozen algae paste.

Supply of *Neochloris oleoabundans* for research and industrial applications

Within the framework of the project, biomass has been produced under different cultivation conditions; applying nitrogen stress (i.e. a limited supply of nitrogen nutrients to the culture) for a certain period of time resulted in accumulation of oil in the cells. The challenges of different cultivation conditions, and their impact on algal biomass composition as well as downstream processes have been investigated.

Production at Pilot scale

For production of sufficient amounts of specific biomass for both academic and industrial partners, in first instance, a 1500L indoor reactor airlift tubular pilot algae reactor, which is destined to produce kg quantities

of algae biomass was developed and installed (in 2015). In total over 2000 hours run time were executed in order to test the reactor and to provide the project partners with fresh or frozen algal paste. Although kg's of biomass were produced and harvested using the new Evodos type 10 and 25 separators under non-stressed conditions, it appeared that for N-limited cultures this reactor was less suitable (e.g more contamination occurred). Therefore, a second - commercial- reactor, the LGEM tubular photobioreactor, was purchased and commissioned in order to ensure more reliable supply of material.
The required biomass was produced in a process as depicted below. First small shake flasks were inoculated with seed algae and this process was further scaled up to about 1500 L indoors (***Figure 2***) and outdoors.

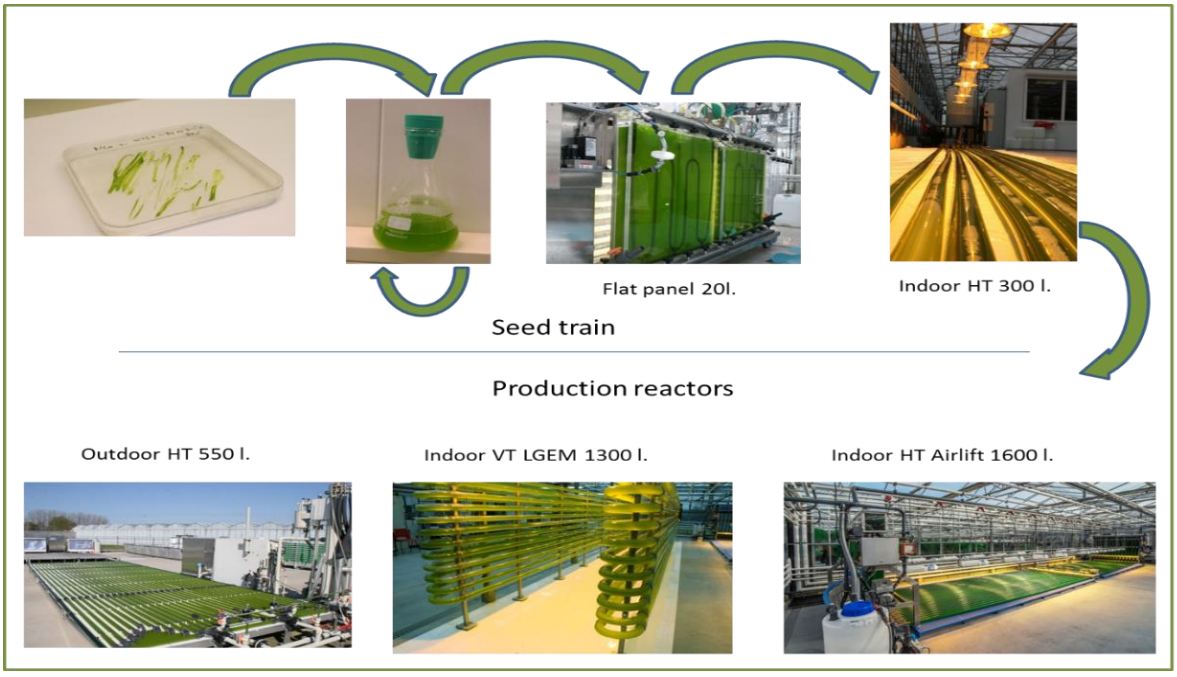

Figure 2. Neochloris oleoabundans seed & pilot scale production systems

Biomass production

In total, over 200 kg paste of *Neochloris* biomass was produced in 2016-2017. About 165 kg was cultivated in (artificial) seawater and the other part in fresh water. About 150 kg *Neochloris* biomass was produced under N-limited conditions. This biomass was mainly used for extractions of oil.

5.2 Evaluation of algae harvesting technologies

Currently, harvesting of microalgae is done by mechanical solid-liquid separation technologies such as filtration and centrifugation. Although these conventional techniques are proven to be very efficient, they are energy-intensive. Studies have shown that 20 to 30% of the total 10 processing costs can be attributed to harvesting when centrifugation is used in combination with an open pond type of cultivation system. To reduce processing costs, either a (pre-)concentration step, or an alternative harvesting technology is desired. Instead of using merely technologies such as filtration or centrifugation, applying a (pre-)concentration technology like flocculation-sedimentation can result in a lower energy consumption. With flocculation, single cells become aggregated into larger particles or 'flocs' which settle to the bottom of a harvesting tank. Although flocculation followed by sedimentation is already a proven- and often applied technology in wastewater treatment and in microalgae-biotechnology, it is still challenging to harvest microalgae grown in seawater.

Due to the high salt concentration present in seawater, however, most types of flocculants cannot be applied, or require high flocculant dosages. At the same time, there is little knowledge available on the flocculation mechanisms involved at high salinities. This makes it difficult to understand and to develop methods to overcome the current limitations in flocculating marine microalgae. There is thus a need for a

better mechanistic understanding, which may lead to alternative flocculants and towards the ability to design general applicable harvesting processes.

Results flocculation

Our results show that cationic polymeric flocculants can be successfully applied for harvesting two different marine microalgae. With the diatom *P. tricornutum* as well as with the relatively small green microalga *N. oleoabundans* high biomass recoveries were obtained after 2 hours of sedimentation. Flocs are formed because the cationic groups of the polymeric flocculant adsorb to the negative charged wall of stable cells. The final effect is the destabilisation of the cell suspension. Based on experimentally obtained results, a mathematical model is developed to predict the optimal flocculant dosage required at different biomass concentrations. To test and validate the model three different commercially available cationic polymeric flocculants were used to flocculate *N. oleoabundans* cultivated under marine conditions.

Although it is possible to harvest marine microalgae using flocculation, it is not known yet if flocculation can contribute in lowering the cost price of algal harvesting. When the optimal flocculant dosage of 43 to 109 mg flocculant/g biomass is taken into account, the price of 1 kg of harvested biomass would range between 0.15 $/kg biomass and 0.49 $/kg biomass. In this estimation only the flocculant usage is taken into account. To decrease the flocculant demand and belonging costs of the flocculants, more efforts should be taken to further understand the role of the flocculant in inducing flocculation. When this role is known, optimized flocculants can be designed that are suited to the requirements of microalgal flocculation.

The decrease in net cationic charge in elevated salinities incites decreased functionality of cationic polymers and induces flocculation of *Neochloris oleoabundans*. In high salinities, the resulting lower charge caused diminished efficiency in forming polymeric bridges between individual cells. This insight resulted in the conclusion that the cationic charge is an important criterion in selecting cationic polymers as a flocculant for marine applications where the apparent polymer length is of minor significance. This study also revealed that, in both brackish and marine conditions, polymeric bridging is a dominant mechanism in floc formation for cationic polymers.

5.3 Development of Cell disruption technologies

5.3.1 Usage of enzymes for cell disruption

After harvesting, the next step in the algae biorefinery scheme is the cell disruption. There are already a great variety of technologies present that, in general, are proven to be successful to disrupt microalgae. Examples are mechanical technologies such as high pressure homogenization or bead milling, chemical technologies such as alkali-heat treatments and physical technologies such as microwave cell disruption. Mechanical and physical technologies are regarded as energy intensive.

This high energy consumption may limit the economic feasibility of a microalgae biorefinery. Optimization studies on mild cell disruption of microalgae by bead milling resulted in required energy inputs lower than 0.5 kWh/kgDW. Although the energy input of bead milling is already strongly reduced, mechanical disruption typically has a high degree of disruption, resulting in the presence of fine particles that are difficult to separate afterwards. In addition, the high degree of disruption is also associated with a non-selective release of hydrophobic (e.g. pigments) and hydrophilic (e.g. proteins) components. In addition to sufficient cell disruption, preventing the production of ultra-small fines is therefore important. A possible technology that is regarded as highly potential to disrupt cells is 'Pulsed Electric Field' (PEF). At large scale, PEF is already applied in the food industry for mild pasteurization of juices as it inactivates bacterial contaminations. Moreover, with PEF only short electrical pulses are applied and therefore no undesired side effects such as high temperature increases are expected, making it a potential mild technology as well. Additionally, enzymatic cell wall degradation can be applied as a mild method, envisaging the enzymes can be found and used. In this task we developed and tested conventional as well as new cell disruption technologies for *Neochloris oleoabundance* and other algal species if relevant.

Furthermore, enzymatic treatment is a plausible approach, and could be an efficient method to create a protoplast or to weaken the integrity of the rigid cell wall of *N. oleoabundans* with low energy input. Indeed, a large selection of enzymes already exists in the market, and therefore before applying this method, a thorough understanding of the cell wall composition is required in order to select the appropriate enzyme that breakdown these components.

Results

Saccharification of algal samples

We analyzed the hydrolysis of *P. tricornutum* and *N. oleoabundans* cells using such a cellulase and hemicellulase mixture. The results reveal that these enzymes release very little glucose or xylose from algal cell walls, this suggests that either the cellulose in these cell walls is inaccessible or there is little cellulose present. Thus, a different approach for cell wall degradation is needed as compared to land-plant cell wall degradation.

Other carbohydrate active enzymes

A wide array of other enzymes from *Myceliophthora thermophila* that can act on plant carbohydrates, for example mannases, galactanases, pectinases, laminarinases and xylanases, mainly acting as endo-enzymes were evaluated. To test whether these enzymes would be active on algae, they we incubated with algal cells and the amount of reducing sugars in the supernatant was measured. The selection of enzymes applied on algae was based on information provided in WP3. This setup would identify all enzymes that would release sugars from cell walls, both those are able to partially degrade polymers into oligomers as well as enzymes that would release mono-sugars from polymers.

The experiments with carbohydrate degrading enzymes did not reveal a significant release of sugars by the action of any of the selected enzymes as compared to the control. Thus either the target components of these enzymes are not present in algal cells or they are not accessible within the cell wall matrix.

The action of proteases on algal cell walls

Several publications suggest that proteins are important components of algal cell walls, which is confirmed by experiments performed in WP3. Thus, the degradation of cell-wall proteins could be an important method for mild degradation. Such method could also facilitate easier lipid extraction and/or mechanical cell wall degradation of algae. We thus also analyzed the action of proteases on algal cell walls. This was performed using a similar setup as described for the sugar degrading enzymes. For proteases, we used the ninhydrine assay to determine the activity of the enzymes.

Ten different proteases were applied on freeze dried algae and their action was analyzed. These experiments revealed that alkaline proteases are the most effective in the release of amino acids. This can be an important indication for cell wall weakening. For *Phaeodactylum*, it has already had been established that weakening the cell walls by proteases can be an important method to enhance extractability and it is expected a similar result can be obtained with *Neochloris*. Thus based on the results performed so far, alkaline proteases are the most effective enzymes for the cell wall weakening of algae.

Conclusion

The experiments performed showed that cellulases and hemicellulases were not, or only slightly, active on the selected algae. Furthermore, none of the investigated carbohydrate degrading enzymes are active on freeze dried algal cells. Thus it is very likely that the carbohydrates in the cell walls of the selected algal cell wall are not accessible for the enzymes. Possibly, these sugars have different linkages compared to land-plant cell wall polysaccharides, the sugars are protected by modifications such as sulfation or are embedded in a matrix of other resilient polymers.

The enzymatic treatment by using Alcalase has released 90% of the proteins (measurements on N-basis), which suggests that this method did not simply affect solubilize soluble, but also increase solubilization of insoluble proteins without the need of a mechanical treatment. Nevertheless, the protein fraction obtained in the supernatant after enzymatic treatment cannot be used in all applications in the market because

Alcalase could have adverse health effects if the recommended exposure limit to this enzyme is not respected.

Alkaline proteases were found to be most effective on algal cells. These enzymes can degrade a significant part of the cell wall, both of *Phaeodactylum* and *Neochloris*. This finding represent an important discovery for the mild processing of algae. A next step could be to investigate if proteases can be used for cell wall degradation by themselves or if a combined mechanical/enzymatic treatment will be most effective.

5.3.2 Cell disruption via bead milling and high-pressure homogenization

Bead milling and high-pressure homogenization are mechanical treatments that efficiently break cell wall of resistant species. Many studies were already conducted and proved the efficiency of both techniques, but the major drawback was the high-energy input required to operate them. For instance, the study of Montalescot et al. concluded that 55.10^3 kWh/kg is required to break the cell wall of *N. oculata* by bead milling, and 7.5 kWh/kg was required to break the cell wall of *C. vulgaris* by high-pressure homogenization after 2 passages (Safi et al. 2014 [13]). Therefore, to make this unit operation viable in the biorefinery process, the energy input should be significantly reduced for both techniques. The following results tackle the issue of energy input, and the main objective is to show that by modifying some parameters there is a possibility to reduce the energy input of these mechanical treatments.

Bead Milling

Previously, it was concluded that by decreasing the diameters of the beads, the disintegration rate and the release of hydrophilic molecules increased. Indeed, the results below show the continuation of this study by screening multiple beads diameters and check their efficiency on breaking the cell wall of *N. oleoabundans*. In addition, the results will show a screening of different cell disruption methods and help predict the efficiency in terms of disintegration, release of soluble molecules, and energy input.

The disintegration level was assessed by flow cytometry after bead milling. It was possible to reach over 99% disintegration with all types of beads. However, by comparing all bead sizes it can be observed that the speed of disintegration was four times faster with the 0.3 mm beads where 99 ± 0.2% disintegration was reached after 20 min. Whereas 99 ± 0.1% was reached with 1 mm beads, but after 60 min. The first order mathematical model followed the same trend as the experimental data, and the kinetics showed a threefold increase in the disintegration rate from $k_{dis\ (1mm)}$ of 0.009 s^{-1} and $k_{dis\ (0.3\ mm)}$ of 0.026 s^{-1}.
The disintegration efficiency results of 1 mm beads correspond to the results obtained on *Chlorella vulgaris* by Postma et al. 2015 [14] and that is due to the similarities in the structural characteristics of both species. Nevertheless, the specific energy input obtained in this study is 5.9 kWh/kg after 1h of disruption (97% disruption efficiency), whereas 1 kWh/kg was obtained with 0.3 mm after 15 min of disruption (99% disruption efficiency). Furthermore, *N. oleoabundans* has a spherical shape with a diameter of 3-6 um, and therefore with 0.3 mm beads, the surface contact between the beads and the cells is increased due to the size and the number of beads in the milling chamber, which leads to a faster disintegration rate.

Proteins and carbohydrates

Given the efficiency of cell disintegration, the protein release was enhanced after reducing the beads diameter. Thus, the concentration of proteins in the aqueous phase increased from 12 mg/mL with 1 mm beads after 20 min to 16 mg/mL with 0.3 mm beads after 15 min, which accounts for 36 to 48.5% of total proteins present in *N. oleoabundans* respectively. Furthermore, similarly to disintegration, the kinetics rate of protein release increased three fold with $k_{prot\ (1mm)}$ of 0.009 s^{-1} and $k_{prot\ (0.3\ mm)}$ of 0.03 s^{-1}, which implies that by decreasing the beads diameter the release of proteins is also improved. Nonetheless, the amount

[13] Carl SAFI, Alina VIOLETA URSU, Céline LAROCHE, Bachar ZEBIB, Othmane MERAH, Pierre-Yves PONTALIER, Carlos VACA-GARCIA. Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. Algal Research 2014.
[14] Postma, P.R., Miron, T.L., Olivieri, G., Barbosa, M.J., Wijffels, R.H., Eppink, M.H., 2015. Mild disintegration of the green microalgae Chlorella vulgaris using bead milling. Bioresour. Technol. 184, 297–304.

of proteins released with 0.4 mm beads was similar with 0.3 mm beads, but the release kinetics increased from 0.025 to 0.03 s^{-1} respectively.

Decreasing the beads diameter also increased the release of carbohydrates. The highest release of carbohydrates was 9.1 mg/mL for 0.65 mm beads after 25 min, which accounts to a yield of 60% per total carbohydrates. Indeed, the carbohydrates release was lower after using 0.4 and 0.3 mm beads compared to 0.65 mm beads, with 5 to 6 mg/mL obtained after 20 min in the aqueous phase respectively.

High-pressure homogenization

Flow cytometry showed that fresh *N. oleoabundans* was completely broken (99%) after applying 1 passage at 1000 bar. The highest release of proteins was around 20 mg/mL (52% of total proteins) after 1000 bar and the release of carbohydrates was statistically the same in terms of concentrations after applying 500 to 2000 bar. The highest concentration obtained was 4.5 mg/100 mL. The specific energy input at 1000 bar is 0.27 kWh/kg, which is significantly lower than the energy input obtained in the study of Safi et al. 2014 (7.5 kWh/kg). During the process, the temperature did not exceed 30 °C due to the cooling system integrated to the machine. Moreover, two passages were also tested without any improvement in terms of disintegration rate or release of hydrophilic components, which suggests that 1 passage is sufficient for a total disintegration. Nonetheless, it can be also implied that not all the proteins and carbohydrates were solubilized, and that is because the remaining are insoluble in water.

High-pressure homogenization was also tested on *N. oleoabundans* grown under different growth conditions (stress or non-stressed, fresh or marine water). It was shown that growth conditions not only affect the composition of the cells, but also the cell wall rigidity. Thus, it can be observed that *N. oleoabundans* grown in fresh water and with non-stressed conditions has the weaker cell wall and the higher release of proteins. On the other hand, when it is grown in marine water, the cell wall showed more resistance and resulted in a 2-fold decrease in protein release (10.4 mg/mL). Furthermore, when it is grown with marine water and stressed, the rigidity of the cell wall is even stronger, and a 4-fold decrease in protein release (4.5 mg/mL) was obtained compared to *N. oleoabundans* grown in fresh water non-stressed conditions. It is noteworthy that with stressed conditions, a very strong formation of gel or emulsion was obtained after homogenization and it was very difficult to recover the supernatant for further analysis.

Conclusions

The results of the present work showed promising improvements in terms of cell disruption and specific energy input. However, the mechanical treatments are still incapable of releasing total proteins from the cells despite the high disintegration rate. Therefore, additional efforts are required to find the best compromise to solubilize the remained proteins.

The growth conditions (fresh or marine water, stressed or non-stressed) have a significant impact on the rigidity of the cell wall. The toughest cell wall was for *N. oleoabundans* grown in marine stressed conditions, and the weakest was for *N. oleoabundans* grown in fresh non-stressed conditions. A plausible explanation (or hypothesis) is that when *N. oleoabundans* is grown under marine stressed conditions, the cell wall becomes thicker and triggers a defense mechanism to protect the integrity of the cells and to regulate the excess minerals exposure. In addition, when some species (like *Haematococcus pluvialis*) are submitted to stress conditions they develop a layer of sporopollenin, which is an extremely resistant layer and would require high energy input to be broken. The presence of sporopollenin is not yet proven for stressed *N. oleoabundans* but could be a possibility.

5.3.3 Cell disruption using pulsed electric field

Pulsed Electric Field (PEF) has been considered as a promising technology to disrupt microalgae in a biorefinery framework. In initial tests, the highest yield of proteins of 13% was obtained with *N. oleoabundans* cultivated in seawater medium in a batch mode PEF. Despite the effect of an osmotic shock that *N. oleoabundans* suffered during the washing treatment, no yields similar to bead milling were obtained. The high release of ions illustrated that the application of PEF for the disruption of fresh and marine cultivated microalgae, resulted in a weakening of the cell membrane suggesting the formation of

pores. Nevertheless, with respect to the mechanical benchmark, no sufficient amounts of protein were liberated by the application of PEF. Moreover, the required energy input for PEF was higher than the mechanical benchmark. It should be considered that the mode of PEF operation is different from bead milling. Where bead milling causes a complete cell disintegration REF, PEF merely electroporates the cell.

The hypothesis that PEF treatment of microalgae is hindered by the rigid outer cell wall, was investigated by subjecting the cell wall containing microalgae *C. reinhardtii* (cc-124) and its cell wall deficient mutant (cc-400) to PEF. By assuming that the cell wall deficient mutant (cc-400) mimics pre-treated microalgal biomass, we could characterize the effect of the operating conditions on treated cell-wall deficient microalgae.

The study of 't Lam c.s. [15] even showed that PEF released at least a 4 fold lower protein release than bead milling. It is therefore reasonable that in general, protein release by means of PEF is limited by the presence of an outer cell wall. Compared to bead beating as a positive control, PEF yielded only up to 5% chlorophyll release in the aqueous phase. These results indicate that the cells were not complete disintegrated into fine particles.

5.3.4 Controlled Instantaneous Pressure Drop (CIPD)

By CIPD equipment pressure is released in a controlled way (i.e. by time to release the pressure) from the operational pressure (0 – 6 bar) down to app. 40 mbar. Due to this pressure shock, cells can open and the cell content can be released. Bringing fresh algae into the CIPD unit and exposing it to a pressure drop will show if this technology can serve as an alternative for cell structure opening technique.

Bringing fresh algae into the CIPD unit and exposing it to a pressure drop will show if this technology can serve as an alternative for cell structure opening technique. The experiments in the reported period are executed with 20 g paste of algae (non-stressed Neochloris) per experiment. Unfortunately, results of the tests executed with the CIPD do not show significant differences between treatments. Our conclusion is that the CIPD does not result in the release of additional protein as a result of the lack of disruption of algae (non-stressed *Neochloris*). We recommend not continuing with this technology for cell disruption of algae.

5.3.5 Integration of unit operations

If the aim is to produce bulk-products at large scale, the biorefinery should become less complex and less expensive. To do so, we should not only focus on the specific DSP-unit operations (i.e. harvesting, cell disruption and extraction) but rather on the overall process design as well. By reducing the required number of unit operations and preventing the use of chemical additives we can simplify the process and enhance the (economic) feasibility of a biorefinery. We discussed two possible approaches to reduce the number of unit operations. In the first approach the number of main DSP-unit operations (i.e. harvesting, cell disruption and extraction) were reduced by integration in a single step. The second approach is to avoid the use of chemicals. Chemicals (e.g. flocculants, solvents, etc.) are not only a consumable with associated costs, but they need to be recovered by using additional unit operations as well. These additional unit operations result in a complex process. By using external fields different phases can be separated, resulting in a solvent-free and more simple product separation.

An interesting approach for such an integrated and multi-product process biorefinery is the use of an ionic-liquid based aqueous biphasic system (ABS). Ionic-Liquids (ILs) are already used to extract lipids from wet

[15] Lam, Gerard 't; Postma, P.R.; Fernandes, D.A.; Timmermans, R.A.H.; Vermuë, M.H.; Barbosa, M.J.; Eppink, M.H.; Wijffels, R.H.; Olivieri, G., 2017. Pulsed Electric Field for protein release of the microalgae Chlorella vulgaris and Neochloris oleoabundans. Algal Res 24, 181 - 187

and non-broken biomass in a single step. They were also used to weaken- and promote extraction of astaxanthin from non-broken *Haematococcus pluvialis*.

A main challenge in developing IL-based ABS for direct product extraction is the ability to extract the hydrophilic proteins from intact biomass. Proteins are typically larger molecules than the lipid molecules and are thus difficult to extract from whole cells. As extracting the large proteins from intact cells is difficult, an alternative could be to first completely dissolve the cell walls instead of merely weakening them, followed by extraction. As dissolving cell walls can take place in aqueous systems at room temperature, it will become possible to extract native proteins.
The direct product extraction using IL-based ABS could make cell disruption redundant, and this makes it a very promising route to simplify a biorefinery process design. However, it still involves the use of costly solvents, which need to be recovered. External fields could be applied as alternative for application of chemicals. Some external fields were already proposed for mild and low cost cell disruption of microalgae, such as electrical fields and ultrasound treatment.

Besides cell disruption, external fields may also simplify extraction (***Figure 3***). In other areas, external fields were already studied for separation of the lipids from an aqueous phase.

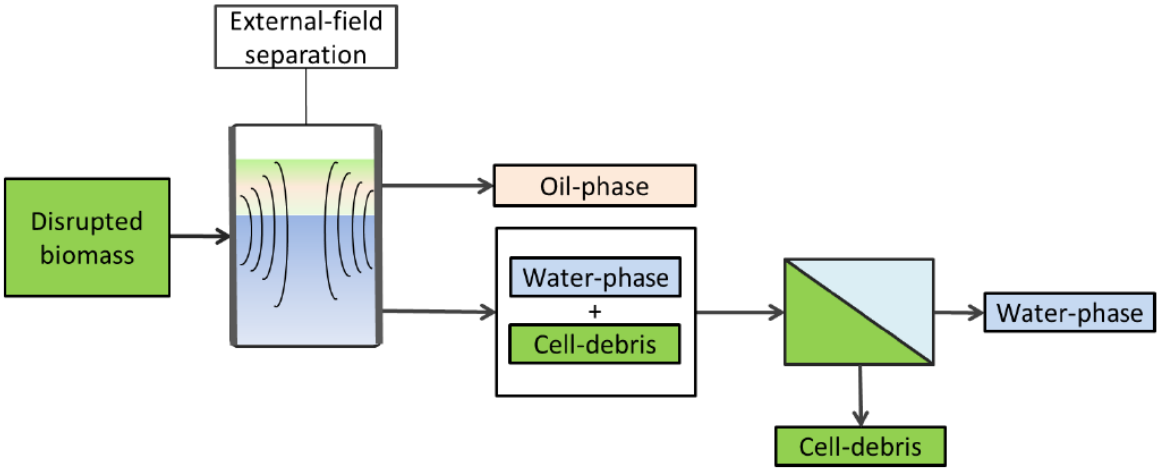

Figure 3. Illustration of separation of the hydrophobic (lipid) phase from the aqueous phase using external fields.

Besides the costs and the overall process design, mildness should remain a key-criterion in microalgal biorefinery research. Complete valorisation of all components in a biorefinery will facilitate the transition from current small-scale production of specialties from microalgae towards future large-scale production of bulk commodities. By developing unit operations in which different steps in the biorefinery chain are integrated, or by avoiding the use of chemicals that are difficult to recycle, the biorefinery chain will become less complex. Development of such simplified processes is essential to bring the multi-product microalgae biorefinery towards economic feasibility.

5.4 Development of Down Stream Processing: Extraction and Fractionation

Several technologies were evaluated within the extraction and fractionation task, being the membrane filtrations and aqueous two-phase system specifically, which are discussed below. Additionally, also R&D was performed on extraction, where there was a focus on developing a new extraction based process as well as a trial where conventional solvents (ethanol) were evaluated on pilot scale.

5.4.1 Ultrafiltration/diafiltration as a fractionation technique

Separation of components based on chemical-free, low energy and mild operating conditions is possible through ultrafiltration; a method that could be scaled-up to an industrial level as known, for example, in the dairy industry. The integration of membrane technology in such a context, however, is not highly

developed for microalgae. It has been used mainly for harvesting cells, and its use in separating microalgal biomass components in an integrated process is hardly explored. Only a limited number of studies have investigated this technique to purify components like polysaccharides from *Porphyridium cruentum Spirulina platensis* and *Chlorella pyrenoidosa*, or to concentrate proteins from *Chlorella vulgaris* and *Haematococcus pluvialis* in the retentate.

The objective of this study was to conduct a process that releases relatively high amounts of proteins in the supernatant after cell disruption, and that apply a two-step filtration in order to obtain a protein rich fraction in the filtrate, free from polysaccharides and chlorophyll. The process has been tested on different membranes pore size, and supernatants obtained after applying high-pressure homogenization on a 100 $g.L^{-1}$ *Neochloris oleoabundans* solution.

In our study, a concentrated solution of *N. oleoabundans* was prepared and then submitted to cell disruption by high-pressure homogenization. Afterwards, the solution was centrifuged, then the collected supernatant was analysed for proteins and carbohydrates content. The recovered supernatant was then submitted to ultrafiltration and diafiltration using several different kDa membrane cut-offs.

Furthermore, the permeate flow rates differed significantly ($p < 0.05$) between the three membranes tested. Tukeys' honest significance test showed that all trials tested differed from each other in terms of permeate flow rate. Moreover, among all membranes tested, the highest permeate flow rate obtained was for the medium kDa cut-off and the lowest permeate flow rate was observed for the highest kDa membrane. Therefore, the results are in contrast with the hypothesis that increasing the molecular weight of the membrane would lead to a higher permeate flow rate. The behaviour of the membranes was also tested by evaluating their permeability (Lp) during the process. Hence, similar to the permeate flow rate, the permeability followed the same trends due to the formation of the small polarisation layer that reduces the permeability of the membranes.

The main objective of this study was to carry out a mild process with concentrated microalgae to obtain a protein fraction in the filtrate after cell disruption. The results demonstrated that diafiltration increased the amount of proteins in the filtrate after applying the step of ultrafiltration. However, the filtration process revealed equal efficiency for the samples treated with low or medium kDa membrane cut-off in terms of protein yield, although the flow rate was higher with the low kDa membrane cut-off. Moreover, the study also concluded that increasing the cut off of the membrane does not necessarily improve the performance of the process and the yield of proteins in the filtrate given that the lowest protein yield and flow rate were obtained with the high kDa membrane cut-off.

5.4.2 Extraction and Fractionation by Aqueous two-phase system

5.4.2.1 Selective & Mild separation of Rubisco

Two ionic liquids were selected based on their mild interaction with proteins: Iolilyte 221PG and Cholinium dihydrogen phosphate (Ch DHp). Biocompatible components: Potassium citrate and PEG 400 were selected to replace commonly used inorganic salts. Finally, the systems are: (PEG 400-Potassium citrate), (Iolilyte 221PG-Potassium citrate) and (PEG 400-Ch DHp). After the named systems were characterized, the partition studies were completed with a model protein (Rubisco) that was selected for being the most common biomarker protein present in microalgae and an interesting food ingredient.

Rubisco was selected as a model protein to evaluate the performance of three ATPS in a microalgae biorefinery framework. Partition coefficient values demonstrate the preference of Rubisco for the top phase in the three cases. The best partitioning was obtained by the ionic liquid based-ATPS (Iolilyte 221PG-Citrate) with a maximal extraction efficiency of 98.8% by a single extraction step.

Choline dihydrogen phosphate (Ch DHp) was identified as one of the most promising media for the stabilization of proteins and other biomolecules. The fact that Rubisco prefers to separate in the top phase in the PEG-based ATPSs is beneficial for the stability of the protein due to PEG which is the main component of that phase and stabilizes the protein.

5.4.2.2 Fractionation of proteins and pigments using ATPS

Aqueous two-phase systems can generate interfacial partitioning of different molecules, including proteins, and it has gained attention in the last years for large scale-processes. In the traditional ATPS (polymer–salt), protein partitioning is governed by hydrophobic interactions and the salting out effect. Similarly, the interfacial concentration of proteins in polymer–salt ATPSs was described based on a protein solubility model, in which protein precipitation was a result of increasing the salt concentration in the bottom phase. Ionic liquid-based three phase partitioning (ILTPP) has been investigated because it combines the advantages of the IL-ATPS and TPP for the concentration and recovery of different molecules including proteins. ILTPP is capable of inducing the formation of a dense and stable protein layer in the middle with IL-ATPS phase-forming components.

PEG400-Ch DHp partition behaviour is clearly beneficial for the partitioning of the molecules in different phases (***Figure 4***). Besides that, proteins recovered in the interface seem to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. Recovery of the proteins in the bottom phase and recycle of the cholinium dihydrogen phosphate for reuse is possible by ultrafiltration as described by Ramalho et al [16]. This type of ATPS and their recyclability will need to be studied further separate microalgae biomolecules in an integrated biorefinery approach.

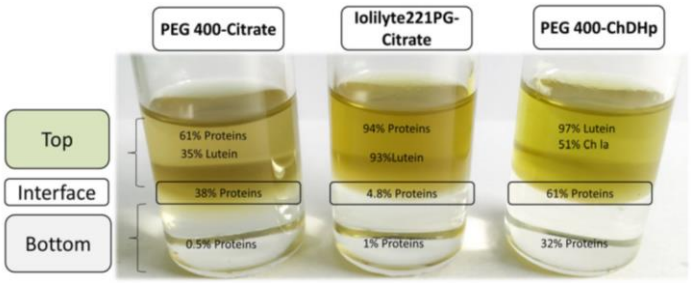

Figure 4. Extraction performance of three different extraction systems for the separation of carotenoids from proteins.

The partitioning behaviour of other molecules of industrial interest such as lipids and carbohydrates from microalgae should be investigated to design a correct biorefinery approach.

Conclusions

In this research, biocompatible ionic liquid-based ATPSs were investigated and compared with a traditional PEG-salt ATPS. We selected biocompatible phase-forming components and ionic liquids suitable for protein separation. The potential of the ATPSs was demonstrated based on the characterization of the systems. Phase equilibrium data of the three systems were here reported for the first time and the parameters evaluated were useful to understand the phenomena behind each ATPS. Iolilyte 221PGCitrate had the highest phase-forming ability and was the most efficient ATPS in Rubisco separation. Rubisco prefers to separate into the polymer-rich (PEG400) phase, which is a nice environment for Rubisco. Cholinium-based ATPS could be very promising in the separation of microalgae components as well.

As a first step towards the development of a multiproduct microalgae biorefinery, three kind of ATPSs were investigated to separate proteins and pigments from microalgae extract. The traditional polymer–salt (PEG400-citrate), and two IL-ATPSs: IL–citrate (Iolilyte221PG-citrate) and polymer–IL (PEG400-Ch DHp). Although Iolilyte221PG-citrate showed outstanding partition coefficient for pigments, a high amount of proteins also moved to the top phase. This behaviour resulted in low selectivity between pigments and proteins. The quality of pigments and proteins separated by Iolilyte 221PG-citrate were considered low as pigments suffered degradation (oxidation) and the proteins did not retain their native form. Thus, proteins in the interface can be directly recovered and do not need to be extracted from the phase forming components. This simplifies the recycling of phase forming components. Besides that, proteins recovered

[16] Ramalho CC, Neves CM, Quental MV, Coutinho JA and Freire MG, Separation of immunoglobulin G using aqueous biphasic systems composed of cholinium-based ionic liquids and poly(propylene glycol). J Chem Technol Biotechnol 93:1931–1939 (2018).

in the interface seem to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. We demonstrated the potential of ATPS to separate different biomolecules simultaneously, giving value to different microalgae components for a sustainable multiproduct biorefinery. Furthermore, components from microalgae cultivated in saline water and freshwater can be selectively fractionated using ATPS. Using salt water to cultivate microalgae can reduce costs and the freshwater footprint for large scale production, which is an advantage in view of a sustainable process.

The use of cationic tensioactive compounds allows the extraction of four biomolecules with different nature (hydrophobic and hydrophilic) corresponding to a multiproduct biorefinery approach. The selective fractionation of the biomolecules extracted was achieved by polymeric-based ABS using electrolytes. These systems are shown to have the ability to fractionate proteins, carbohydrates and pigments from the complex matrix.

5.4.3 Oil extraction of wet biomass with ethanol

Neochloris oleoabundans (stored frozen and defrosted) grown under stressed conditions in fresh water was disrupted by bead milling. Ethanol was added to the disrupted cells and these were subsequently centrifuged. An ethanol concentration (20% (v/v) final concentration) did not result in a three phase system with a clear top layer (containing a dark green oil/lipid layer) that could be easily separated. Unfrozen and salt water cultivated algae showed a slight, green oily layer on top.
The bead milled material was centrifuged. After centrifugation only a small oil layer at the top and at the inner side of the centrifuge tube was visible. It was very difficult to separate the oil from the water/ethanol and biomass. This is in contrast with stressed *N. oleoabundans* cultivated in saline water which was not frozen for storage. To investigate the effect of salt on the separation of oil and water, the oil layer (oil enriched fraction) was isolated as good as possible from the remaining part. Both the oil enriched fraction and the remaining cell debris were divided into two portions. To one part of the portions salt was added (1% w/v NaCl) to investigate if salt increases the separation of oil and liquid. With the addition of salt, the fraction with the oil enriched portion showed a slightly better separation of oil. However, only a very low amount of oil was obtained. The portion without salt enrichment did not show the presence of oil. In conclusion only minor amounts of oil were obtained from frozen stressed *N. oleoabundans* cultivated in fresh water. Addition of salt during the centrifugation step improved the separation only to a small extent. It is not clear if the storage condition (frozen), the composition of the microalgae and/or salt play a role in the separation of oil.

It was very difficult to separate the oil phase from the water/ethanol and biomass after centrifugation. This is in contrast with stressed *N. oleoabundans,* cultivated in saline water, which was not frozen for storage and where some kind of a green, oily top-layer was present. It is not clear if the storage condition (frozen), the composition of the microalgae and/or salt play a role in the bad separation of oil.

5.4.4 Extraction of algal lipids using novel solvents

In this task a novel, effective, switchable solvent system for direct lipid extraction from unbroken microalgae in (concentrated) aqueous solutions was developed.

From various candidates, N-ethyl butyl amine (EBA) was identified as most promising switchable solvent for both wet lipid extraction as well as phase separation by hydrophilicity switching. Compared to the Bligh & Dyer standard lab test for lipids determination, EBA is able to extract all lipids, neutral and polar, from freshwater microalgae (as tested for esp. non-stressed *Neochloris oleoabundans sp.*) without cell disruption and without drying. The organic extraction phase, containing the lipids and EBA, can be separated from the (concentrated) microalgae culture medium by phase separation. Subsequently, the EBA can be separated from the lipids by adding water and contacting with CO_2 or decreasing the water-organic mixture temperature. In both cases the EBA transfers to the aqueous phase, leaving the lipid product as separate phase behind. After a simple phase separation to harvest this product, the solvent EBA can be recovered as separate organic phase by removing the CO_2 or increase the temperature.

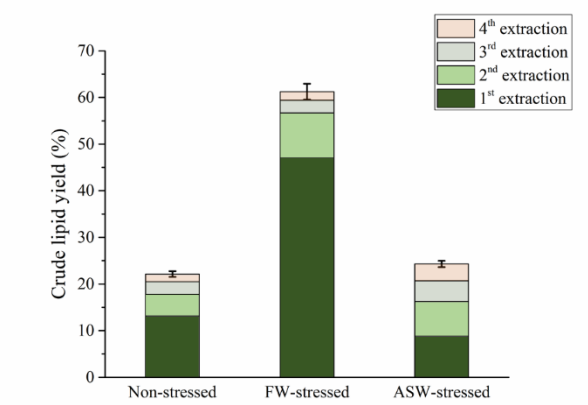

Figure 5. Crude lipid yield of non-stressed, FW-stressed and ASW-stressed Neochloris oleoabundans extracted by EBA method for multistage extractions.

From the lipid yields in multistep extraction experiments, at different EBA solvent to Algae feed ratios and the visual observations during lipid extraction (***Figure 5*** and ***Figure 6***), it was concluded that (i) the solvent penetrates complete the algae cell with destroying the cell wall; (ii) the solvent is able to extract the lipids out of the algae cell. It was hypothesized that a simple, cell volume-based extraction model.

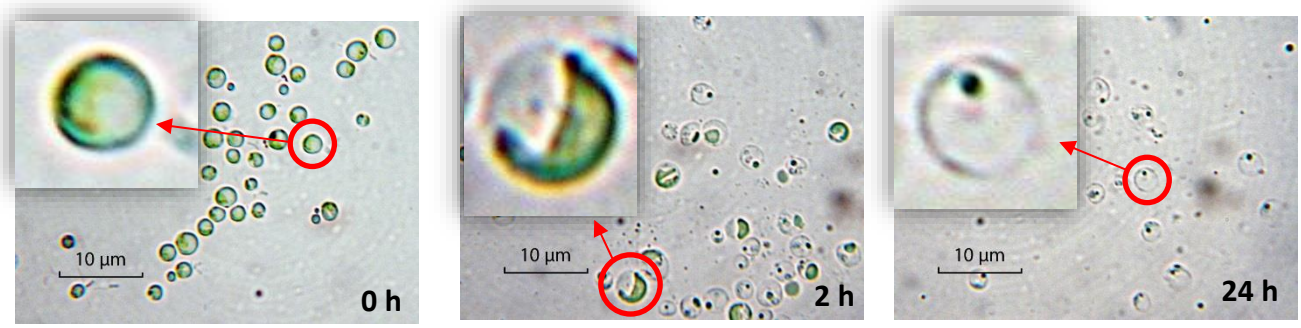

Figure 6. Visual observations of the EBA extraction process (FW-stressed Neochloris oleoabundans)

In addition, the study confirms that by using both algae stressing techniques and applying EBA as solvent in multiple stages of extraction is very promising in the development of an efficient lipid extraction technology targeting non-broken, wet microalgae. Overall, it was found that applying EBA as switchable solvent for lipid extraction from microalgae is from an energy point of view very promising, as high yields can be obtained, processing conditions are quite mild (and hence equipment costs are low) and non-broken, wet microalgae slurries can be used without pretreatment. Mini-plant demonstration with continuous processing and attention for complete solvent recovery (in view of emissions and lipid product contamination) are areas for further development, using the selected solvent.

5.4.5 Valorization of *Neochloris* biomass with a focus on oil extraction at pilot scale

Based on previous work performed at POS on high-oil biomass, the use of Iso Propyl Alcohol (IPA) was explored to produce oil. A process was tested to extract the lipids, winterize the oil and produce a protein rich fraction from wet algal biomass of stressed *Neochloris oleoabundans.*

The crude oil was very viscous, and its dark color was very similar to the oil extracted using ethanol. Approximately, 21% solids (i.e., impurities) were removed during solvent winterization. The oil fraction

after solvent winterization was better in color and less viscous. The crude algal oil had 55.5% (w/w) of neutral oil before winterization and improved the neutral oil content after solvent winterization to 67.7%, w/w. The Phospholipids and glycolipids of crude oil were 1.26% and 2.22% (w/w), respectively.

The defatted biomass was very light in color. The main component of the cell debris is carbohydrates, about 47% w/w, while it still contains 2.8% residual oil and 34% protein. Until this phase of the project bead milling for cell wall disruption was encouraged and the use of any organic solvent during the extraction process was discouraged. However, POS has gotten good results during past work using Micro fluidization for cell wall disruption and ethanol for extraction. The extraction development was expanded to include these technologies. The first set of experiments was conducted using water as the only solvent to recover oil. The diluted biomass was passed four times through the laboratory scale microfluidizer and achieved >98% cell lysis. The algae cells seem to be tough to rupture even with the high pressure applied on these trials. After centrifuging the cell lysate, it was very difficult to clearly identify the oil and aqueous phases due to dark green color and consequently, the recovery of the oil was not successful. After several attempts the decision was made to stop aqueous extraction trials and move forward with solvent extraction using 95% ethanol. First the wet biomass was de-watered and mixed with 95% ethanol at 1:5 w/w biomass-to-ethanol ratio. Like the aqueous process, the mixture was passed four times through the microfluidizer and was able to achieve >98% cell lysis. After removing cell debris (centrifugation), the supernatant contained oil, water, and ethanol as well as soluble materials in diluted ethanol (pigments, sugars, salts, etc.). The colour of this fraction was very dark, close to black color and consequently, the oil recovered was darker in color as well. The oil is very viscous (semi-pourable) and approximately 1.5 kg of oil recovered from ~20 kg of wet biomass. To remove the darker color, few bleaching trials were conducted using carbon and clay. The bleached oil was lighter in color and less viscous (pourable) compared to the previous crude oil. However, the oil yield was very poor as oil also adsorbed to the bleaching clay.

In conclusion, the medium bead milled slurry showed to be the best in recovering oil using a non-solvent based approach. About 58-60% oil could be recovered using a hot water treatment of the medium bead milled slurry biomass, whereas about 66% oil could be extracted using an enzyme treatment of Viscozyme followed by Alcalase. Further optimization can be done using this enzyme combination to explore the possibility of improving the oil yield. Oil extracted through enzyme-assisted process had similar fatty acid composition to the oil extracted with the use of petroleum ether.

The oil is semi solid at Room temperature, likely due to the large quantity (~50%) of non-oil substances**.** Different groups of fatty acids are present the recovered algae oil using 95% ethanol. Furthermore, it contains small amounts of phospholipids. The most abundant phospholipid was Phosphatidylcholine, which accounted for almost all phospholipids in the final algae oil. The algae oil contained 0.15% Phosphatidylinositol and the rest (N-acylphosphatidylethanolamine, phosphatidyl ethanolamine, phosphatidic acid and lysophosphatidylcholine) was <0.1%. Similarly, Digalactosyl diacylglycerol was the major glycolipid found in the final oil, whereas Monogalatosyl diacylglycerol and Steryl Glucoside contents were <0.1%. These phospholipids will have to be removed as part of the refining process but could possibly form another co-product.

Enzyme and pH-based technologies were explored to produce the algae oil from *Neochloris oleoabundans* under aqueous conditions. Yields, however, were relatively low and water usage was high. Aqueous technology could work if algal biomass is processed that contained significantly higher oil contents than the AlgaeParc material. Ethanol and IPA base extraction showed to be good options, whereby the latter was the best. Valuable co-products that could be further explored are proteins and phospho-/glyco-lipids. Results confirmed that medium bead milled slurry was the most appropriate in recovering oil, followed by the continued bead milled slurry. The continued bead milled slurry was very pasty/gel-like even at 10% solids content and it was hard to recover water/oil layer from the biomass resulting in lower oil recovery from continued bead milled slurry compared to medium bead milled slurry. It is shown that bead milling conditions have to be carefully controlled when producing oil through aqueous extraction. Furthermore, long enzymatic treatment times are required.

6 System analysis, techno-economic and sustainability assessment and flexibility (WP 2)

6.1 Objectives

Algae have potential for a broad spectrum of products. The focus in an algae biorefinery is therefore not on one or a few products, but on an efficient mix. To obtain this mix, unit operations in the chain from algal feedstock to end product have to be combined efficiently.

The choice and operational conditions for the unit operations in the chain should therefore be well balanced to obtain the most efficient biorefinery. In the current literature the choice of unit operations is mostly given in rather qualitative instead quantitative terms, and is strongly focussed on single operations instead of a chain of operations. WP2 is therefore focussed on the development of simulations for a systematic analysis to define a balanced and quantitative organisation of the production chain.

Processing in algae biorefineries at centralized locations results in more efficient use of capital and related costs, and more efficient use of labour. At the other hand a centralized large scale refinery needs the supply of multiple cultivation units, which increases the transport costs and transport fuel consumption and emissions. Bruins and Sanders [1] state that application of local biorefineries can be more attractive than centralized biorefineries, by reduced efforts for transport. WP2 has therefore also the challenge to find the best trade-off for the scale of algae biorefinery and the impact of the transport logistics.

The objectives of WP2 are as follows:

- Economic and sustainable biorefineries: to define biorefineries that meet economic and sustainability criteria by the best choices for unit operations, and to develop a flexible tool to evaluate different biorefinery chain options.
- Biorefineries and the supply chain: to define which biorefineries request for local and which biorefineries request for centralized processing, and to develop a flexible tool to evaluate different options for centralized or local processing.

6.2 Economic and sustainable biorefineries

6.2.1 Simulation models

A model-based approach was applied as basis for the system evaluation. For each unit operation a simulation model was defined. The models concern the input-output component mass and energy balances of main and co-streams for each unit operation (see Figure 7). Additional relations are included to connect the energy demand and product yield to economic estimation elements. The models for the unit operations were programmed in Excel spreadsheets. A flexible structure is used to connect all unit operations with each other in any combination.

The energy input and co-stream input (for example solvents) are based on data for the specific energy consumption of each unit operations, which is linked to the amount of material being processed. Equipment data (dimensions, costs, scaling rules etc.) was derived from data of industrial equipment suppliers (GEA, WAB, Evodos), engineering companies (Bodec), literature and engineering databases (DACE, NETL Matche.com).

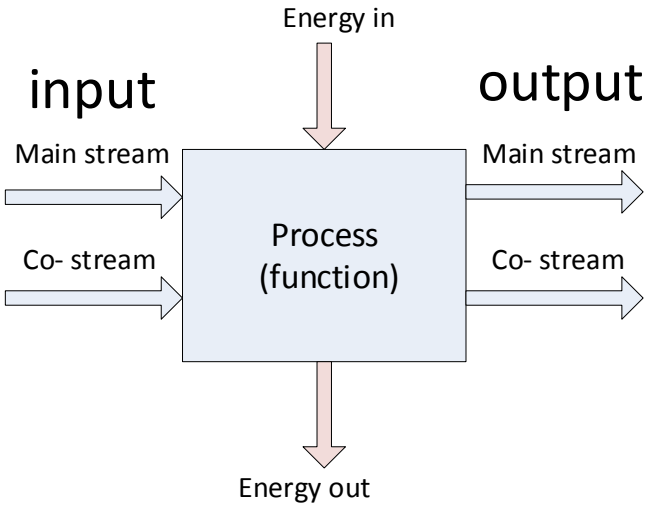

Figure 7 Representation of the process units and properties of energy and mass streams into and out of the system. The main stream contains the algal biomass or the aimed components, the co-stream the required additional components or emitted products.

6.2.2 Cost estimation

For each scenario the investment cost are calculated from the purchase cost of equipment. The total investment cost per unit are higher than purchase cost due to piping, installation of pumps, construction, automation etc. A general overall Lang factor that sums the contribution of individual aspects is used. For standard equipment the applied Lang factor was 3.5. For units that are operated in ATEX-environment to avoid explosion risk a higher Lang factor is used

Extrapolation of the purchase cost to other capacities and scales is based on the scaling factor rule:

$$\frac{costB}{costA} = \left(\frac{size\ B}{size\ A}\right)^{n} \quad (3)$$

Where $costA$ and $costB$ represent the purchase cost of a unit operation with size or capacity $size\ A$ and $size\ B$. n is the corresponding scaling factor for the equipment, which differs between equipment types and is derived from the used data bases.

The operational cost include energy, consumables, labour and cost related to the loss of biomass. The energy cost follow directly from the energy uptake of the installations. These were specified according to the equipment suppliers data and literature. Electricity price and the cost for direct heating by natural gas are based on EuroStat information for 2014 [2].The price for steam used for the drying operations is based on a 80% efficiency from steam generation by gas heating.

The costs for labour are based on the work load of one operator, who can supervise 5 medium level automated continuous unit operations and is working in a 4-shift system. A supervisor coordinates 4 operators and the plant manager is responsible for 20 operators. The salaries of the operators, supervisor and plant manager are obtained from the basic salary in the Netherlands multiplied with a factor for educational level and responsibility. Moreover, overhead cost of 20% are taken into account for administration, laboratory etc.

The costs of operations and the required resources in the biorefinery are assigned to the mass of the specific product processed in each unit operation. Products which do not pass an operation are not charged with the costs or resources for this operation. Therefore, products with a short processing route are in general cheaper than products that need a long processing route.
In the harvesting section all components follow the same path. Cost and resource allocation corresponds then to the amount of biomass being processed.

6.2.3 Results harvesting and dewatering

The analysis for harvesting and dewatering is performed for all combinations of harvesting and dewatering operations as given in Figure 8. Drying is an extension to these operations to cover the option for a dried algae powder.

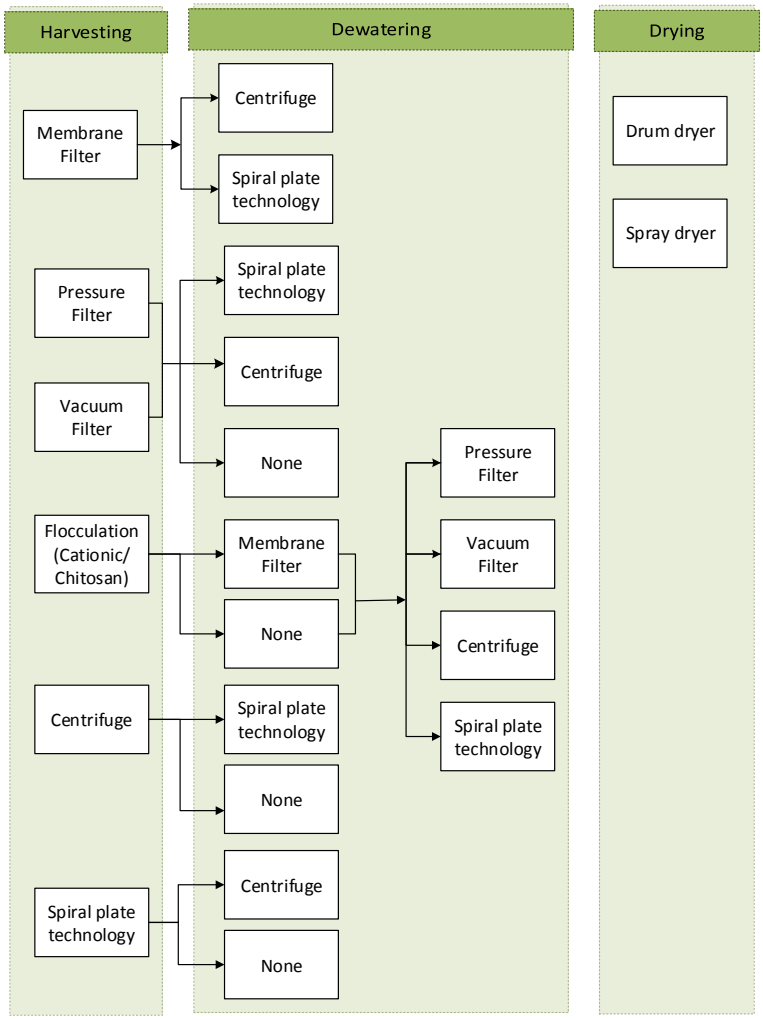

Figure 8 Combination of operations for harvesting and dewatering of algal biomass at large scale.

The combinations of unit operations are presented in Table 1 on the following page.

Table 1. Combinations of unit operations for harvesting and dewatering steps.

Combinations	Harvesting	Dewatering	
1	Membrane Filter	Centrifuge	----
2	Membrane Filter	Spiral Plate Technology	----
3	Pressure Filter	Centrifuge	----
4	Pressure Filter	Spiral Plate Technology	----
5	Vacuum Filter	Centrifuge	----
6	Vacuum Filter	Spiral Plate Technology	----
7	Cationic Flocculation	Membrane Filter	Pressure Filter
8	Cationic Flocculation	Membrane Filter	Vacuum Filter
9	Cationic Flocculation	Membrane Filter	Centrifuge
10	Cationic Flocculation	Membrane Filter	Spiral Plate Technology
11	Cationic Flocculation	Pressure Filter	----
12	Cationic Flocculation	Vacuum Filter	----
13	Cationic Flocculation	Centrifuge	----
14	Cationic Flocculation	Spiral Plate Technology	----
15	Chitosan Flocculation	Membrane Filter	Pressure Filter
16	Chitosan Flocculation	Membrane Filter	Vacuum Filter
17	Chitosan Flocculation	Membrane Filter	Centrifuge
18	Chitosan Flocculation	Membrane Filter	Spiral Plate Technology
19	Chitosan Flocculation	Pressure Filter	----
20	Chitosan Flocculation	Vacuum Filter	----
21	Chitosan Flocculation	Centrifuge	----
22	Chitosan Flocculation	Spiral Plate Technology	----
23	Centrifuge	----	----
24	Centrifuge	Spiral Plate Technology	----
25	Spiral Plate Technology	----	----
26	Spiral Plate Technology	Centrifuge	----
27	Pressure Filter	----	----
28	Vacuum Filter	----	----

Figure 9A shows the results of the different combinations of unit operations for an 100 ha open pond cultivation system. Harvesting and dewatering systems that start with spiral plate technology are out of the costs range of this graph. For large scale processing a high number of spiral plate units is required and the investment and labour costs increase significantly. For the use of spiral plate technology, pre-concentration (flocculation, filtration) is a realistic option to reduce the costs. For small scale processing a few of these unit operations are required and the operating and investment-related costs are more balanced. The strongest benefit of spiral plate technology compared to the other unit operations is the high final dry matter content, which limits the costs of further processing. Therefore, this operation is considered as a strong (>150 $kg.m^{-3}$) finishing step

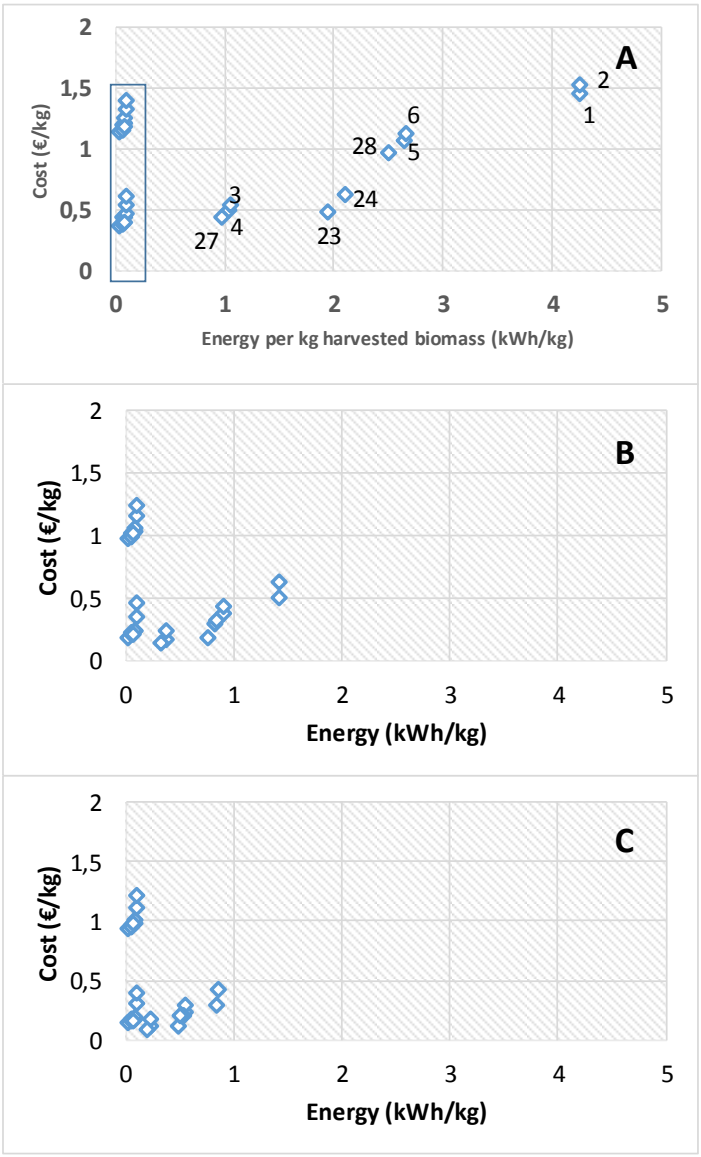

Figure 9 Combined graphs of energy demand and total costs for the harvesting and dewatering systems. A) Application for an open pond system and concentrating a feed rate of 400 $m^3.hr^{-1}$ from 0.5 to 150 $kg.m^{-3}$, B) Application for a tubular cultivation system and concentrating a feed rate of 280 $m^3.hr^{-1}$ from 1.5 to 150 $kg.m^{-3}$. C) Application for a flat plate cultivation system and concentrating a feed rate of 250 m^3.hr from 2.5 to 150 $kg.m^{-3}$

Single-step filtration methods are the lowest in costs and attractive due to the simplicity of the operations. However, there is a risk of fouling or equipment blockage leading to a lower performance in higher product concentrations such as 150 $kg.m^{-3}$ dry matter. As an alternative, filtration can be done to a lower level of dry matter to reduce the risk of fouling (intermediate concentration of 100 $kg.m^{-3}$ dry matter) in combination with an effective finishing step like centrifugation or spiral plate technology. A single harvesting step with centrifugation is also economically attractive. Such a single operation results in a relative simple processing system. However, two step operations offer better possibilities for extension of production capacity.The boxed area in Figure 9A concerns chemical flocculation (cationic and chitosan) for harvesting. Flocculation is followed either by one-step or two-step dewatering. Flocculation is energy efficient and with 25 $kg.m^{-3}$ dry matter in the concentrated stream, a significant volume reduction. As a consequence, the required dimensions and energy consumption of the downstream equipment are significantly smaller.

Combinations of flocculants with other operations are, from an economic and energy consumption points of view, competitive to other combinations of operations. However, there are some important remarks to the application of flocculants:

- The recovery with flocculants is below that of other methods 70-90%. The co- product stream from flocculation contains a significant amount of algae, and has to be recycled to the cultivation unit to recover the remainder of algae. The flocculants attached to the recycled algae can affect the cultivation.
- If the side product stream cannot be reused for cultivation, a significant value loss of biomass occurs, thus, the costs of this operation will increase.
- The flocculants are also attached to the microalgae in the concentrated stream. The presence of flocculants and their interaction with microalgae may affect the performance of the following extraction and fractionation steps.

Figure 9B and Figure 9C give the harvesting and dewatering characteristics for 100 ha tubular and flat plate systems. he biomass concentration and yearly productivity increase from pond, to tubular, to plates, while the average flow rate towards the harvesting and dewatering system decreases. The costs and energy consumption shift in Figure 9B and Figure 9C towards the lower-left corner. The processing costs fall below 1.0 €.kg^{-1} algae for nearly all scenarios, and the energy consumption below 1.0 kWh.kg^{-1} algae. This trend is due to the 3-5 fold increase in the amount of biomass in the feed from the closed photobioreactors. Simultaneously, there is a decrease in volumetric flow rate of feed, which results in smaller equipment dimensions and a lower energy consumption.

The harvesting and dewatering costs for the combination of pressure filtration/centrifugation for 1, 10 and 100 ha are 9.45, 1.24 and 0.50 €.kg^{-1} respectively. These results indicate an important benefit for large scale processing. The same trend was found for other combinations of unit operations. Labour is the major contributor to the total costs and reduction of labour costs is essential to improve the total costs. The costs for labour can be reduced by applying harvesting systems with large capacities (flocculation, centrifuges) or by reducing the labour by implementing a high degree of automation.

The results above concern harvesting and dewatering for a cultivation system in Northwest Europe. Production capacity of cultivation systems, labour costs and energy prices in South Europe differ from the applied values. For harvesting-dewatering with pressure filtration and centrifugation in South Europe the results are graphically presented in Figure 10. The total costs in Northwest Europe are higher than those in South Europe. This result is mainly due to the higher labour costs. The higher production rate in South Europe requires more and larger equipment and, thus, more investments. The investment costs per kg dry mass, however, remains about the same. On this scale of harvesting and dewatering, the maximum size of equipment is used and, then, the benefits from scaling are only small.

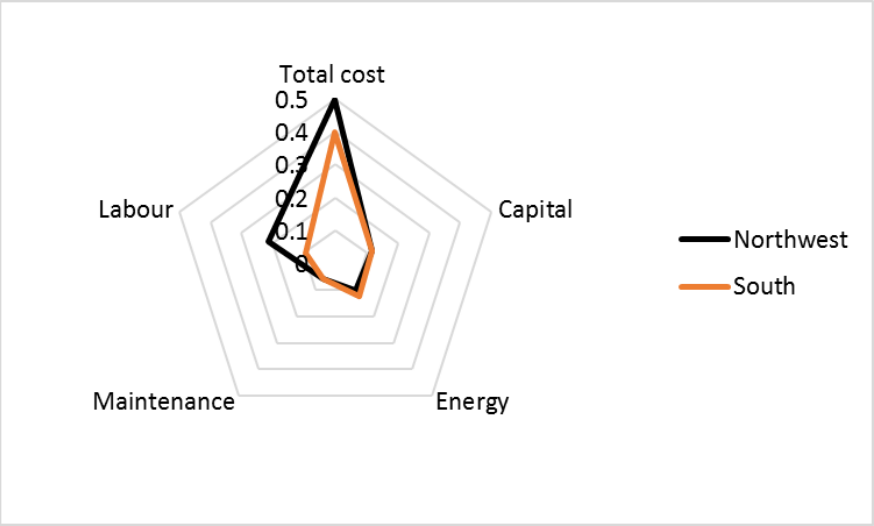

Figure 10 Comparison of costs for harvesting by pressure filtration and centrifugation of an open pond algae broth in Northwest and South Europe.

6.2.4 Biorefinery

6.2.4.1 Bead milling and high pressure homogenization

Bead milling experiments performed for *N. oleoabundans* showed for non-stressed algae after 10 minutes a degree of disruption>90%, a protein release in the range of 50-60%, and lipid release of 60%. For stressed algae, with a stronger cell wall, the same level of disruption and protein release was achieved after 30 minutes.
The total costs for an industrial beadmil with a volume of 600 litre with 25% bead filling are about 0.24€.kg^{-1} dry algae for non-stressed algae and, due to the longer residence time, 0.47€.kg^{-1} dry algae for stressed algae. The energy uptake is 0.95 and 1.92 kWh.kg^{-1} dry algae respectively. Upscaling for higher capacities (from 30 to 50 ton.ha^{-1}.year^{-1}, 100 ha cultivation site) reduces the costs by 35%.

Experimental results for pressure homogenization at 1500 bar resulted in 90% disruption for non-stressed algae and 65% for stressed algae. To achieve 90% disruption for stressed algae three passes at 1500 bar are required.
The costs are 0.08 €.kg^{-1} for non-stressed algae and 0.20 €.kg^{-1} stressed algae. The energy uptake is respectively 0.31 and 0.93 kWh.kg^{-1} dry algae. Upscaling from a cultivation unit producing 30 ton.ha^{-1}.year^{-1} to 50 ton.ha^{-1}.year^{-1} reduces the costs with 20%.
An enzymatic pretreatment (50°C and 2 hour) showed for stressed and non-stressed algae disruption at 500 bar for both stressed and non-stressed algae. These homogenisation conditions result, nevertheless, in doubling of the costs for the non-stressed algae and a 12% reduction of costs for the stressed algae. The costs for the treatment are dominated by the price of used enzyme.
At large scale, homogenisation is for *N. oleoabundans* more attractive than bead milling. The main reason is the relative long residence time needed to achieve sufficient disruption in a bead mill.

6.2.4.2 Drying of algal paste

The costs for drum drying and spray drying are similar and in the range 0.40-0.45€.kg^{-1} dry matter and the energy uptake 5-6 kWh heat.kg^{-1} dry matter. As the maximal capacity of a drum drier is about 1000kg water evaporation per hour, drum drying needs more units which raises investment and labour costs, but drum dryers are 20-30% more energy efficient than spray dryers. The effect of upscaling is not strong and the costs remain in the range 0.40-0.45€.kg^{-1} dry matter. The costs can be further reduced by realizing the highest possible dry matter content at harvesting.
The costs of drying and also the energy uptake are rather high. The benefits of dry processing instead of wet processing of algal paste (higher product yield, storability, decoupling processing operations in the chain) should compensate these extra costs.

6.2.4.3 Lipid biorefinery

Based on the experiments of WP1 two new procedures using solvent extraction are compared to traditional solvent extraction with hexane [3,4]. These two methods are: 1) disruption (bead milling or homogenisation) followed by lipid extraction with 22% ethanol, and 2) homogenisation followed by lipid extraction with isopropylalcohol. The processing and solvent recovery routes are given in ***Figure 11***. The systems were evaluated for three scenarios. Scenario 1 concerns a feed of stressed algae, 1000 kg.hr^{-1} with 33% of dry matter. Scenario 2, same feed of stressed algae, 3000 kg.hr^{-1} and 33% dry matter, scenario 3 for 3000 kg.hr^{-1} with 15% of dry matter. The feed rate in scenario 3 corresponds to the averaged flow of an algae cultivation system of 100 ha with 30 ton.ha^{-1}.year^{-1}.

The results are summarized in Table 2 where the costs are expressed in kg end product, i.e. lipids and residue. The new procedures result in higher production costs than the traditional extraction method. There is a potential to reduce the costs by processing concentrated solutions (33% instead of 15% dry matter) of algal paste (for example by using spiral plate technology).
In the procedure with 22% ethanol, disruption with the bead mill is the main bottleneck. The limited volume of the beadmills in combination with the residence time make that a large set of bead mills have to be operated in parallel, and the units are expensive. For small capacities it is still an option, but for large scale the homogenizer is to be preferred. The 22% ethanol mixture has as main advantage that the distillation is simple, the top product needs a concentration of 33% ethanol. The distillation is actually not more than evaporation.

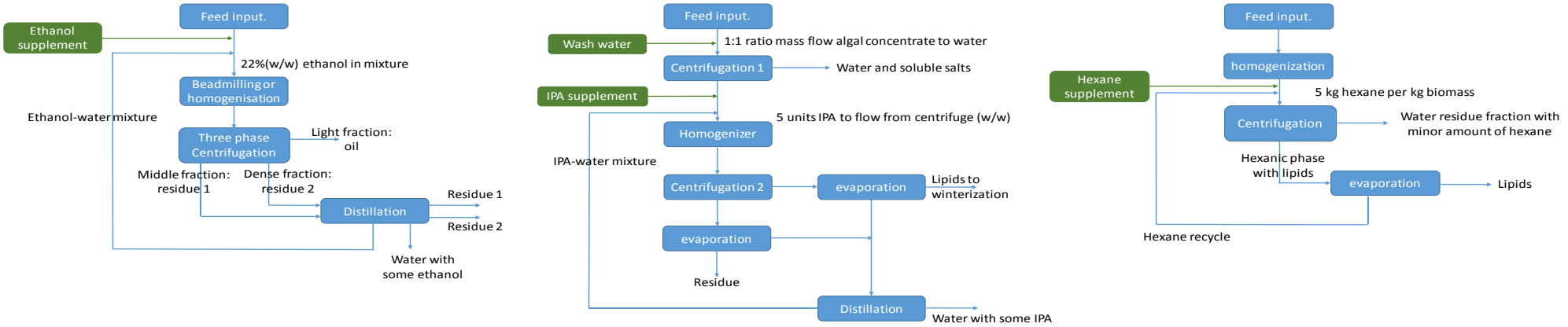

Figure 11 Processing routes for lipid extraction with 22% ethanol (left), with isopropylalcohol (middle) and hexane (right).

In the procedure with isopropylalcohol (IPA), the applied evaporation of IPA and water to separate the solvent phase from the lipids is the main bottleneck. Hereby, evaporation of water accounts for high energy costs.
In hexane extraction water is also present in the system, but here the hexanic phase with lipids is easily separated from the water or water-alcohol phase by centrifugation instead of evaporation or distillation. These results show that evaporation or distillation to separate the lipids and solvent phases should be avoided. Moreover, the hexane treatment has less extraction steps which makes the system simpler and cost effective.

Table 2 Comparison of costs (€/kg end product) for lipid extraction according FBR-POS experiments to traditional extraction with hexane. Scenario 1: feed rate 1000 kg.hr^{-1} with 33% dry matter. Scenario 2: feed rate 3000 kg.hr^{-1}, 33% dry matter. Scenario 3: feed rate 3000 kg.hr^{-1}, 15% dry matter.

	Scenario 1 €.kg^{-1}	Scenario 2 €.kg^{-1}	Scenario 3 €.kg^{-1}
HPH-hexane wet extraction	0.21	0.12	0.21
Extraction with 22% ethanol, bead milling	1.06	0.84	1.84
Extraction with 22% ethanol, homogenization	0.38	0.28	0.59
Extraction with IPA	1.50	1.34	1.45

Hexane extraction can also be applied to dried disrupted product [5]. The absence of water in this dried product to be processed results in smaller flows and therefore lower costs. The costs for the hexane extraction on the dried product are 10% below those of a wet product. This reduction does not compensate the extra costs for drying. Drying is only meaningful if the algal paste has to be stored for a longer time.

The potential of other methods from the literature was also investigated. Good extraction yields were reached with ethanol-hexane mixtures [6,7]. The benefit of this method is that the hexane with lipids is mechanically separated from the water-ethanol phase. For dry processing of (disrupted) algal paste the amount of water in the system is very low and the method competes with hexane extraction. For wet extraction, however, the separation of ethanol and water by distillation the same limitations of water-ethanol separation as the new methods explored in WP1.

6.2.4.4 Protein biorefinery

The protein biorefinery is given in Figure 12. In this system chemical solvents are absent and the operation is performed at low temperatures, by which the protein remain in their native stage. Figure 12 gives for non-stressed *N. oleoabundans*) the flows and the mass balances for the 3-phase separator in combination with the two step ultrafiltration system. The allocated costs and energy consumption for each product are also included in Figure 12 (italic numbers).

The mass balances show that only a minor amount of TAG is separated in the 3 phase separator. This low amount is, at one hand, result of the low lipid content in the feed of non-stressed algae. At the other hand, not all lipids are released at homogenization and therefore TAG is also in the debris. For stressed algae the TAG content will increase to about 17.5 kg.hr^{-1}. If the small flow of TAG from the non-stressed algae is not considered as a meaningful product, 2-phase separation can be applied at comparable costs.

The release of protein and carbohydrates at homogenization is in the range of 50-60%. Therefore the amounts of the non-released protein, carbohydrates, lipids and others in the debris is considerable. The solvent content in the debris is in the range of 70-80% and contains also significant amounts of released components. The major part of the proteins is thus in the residue from the centrifugation system. The protein from this stream can be recovered by alkaline-acid treatments. This treatment results in denatured protein and amino acids, which still can have a significant value.

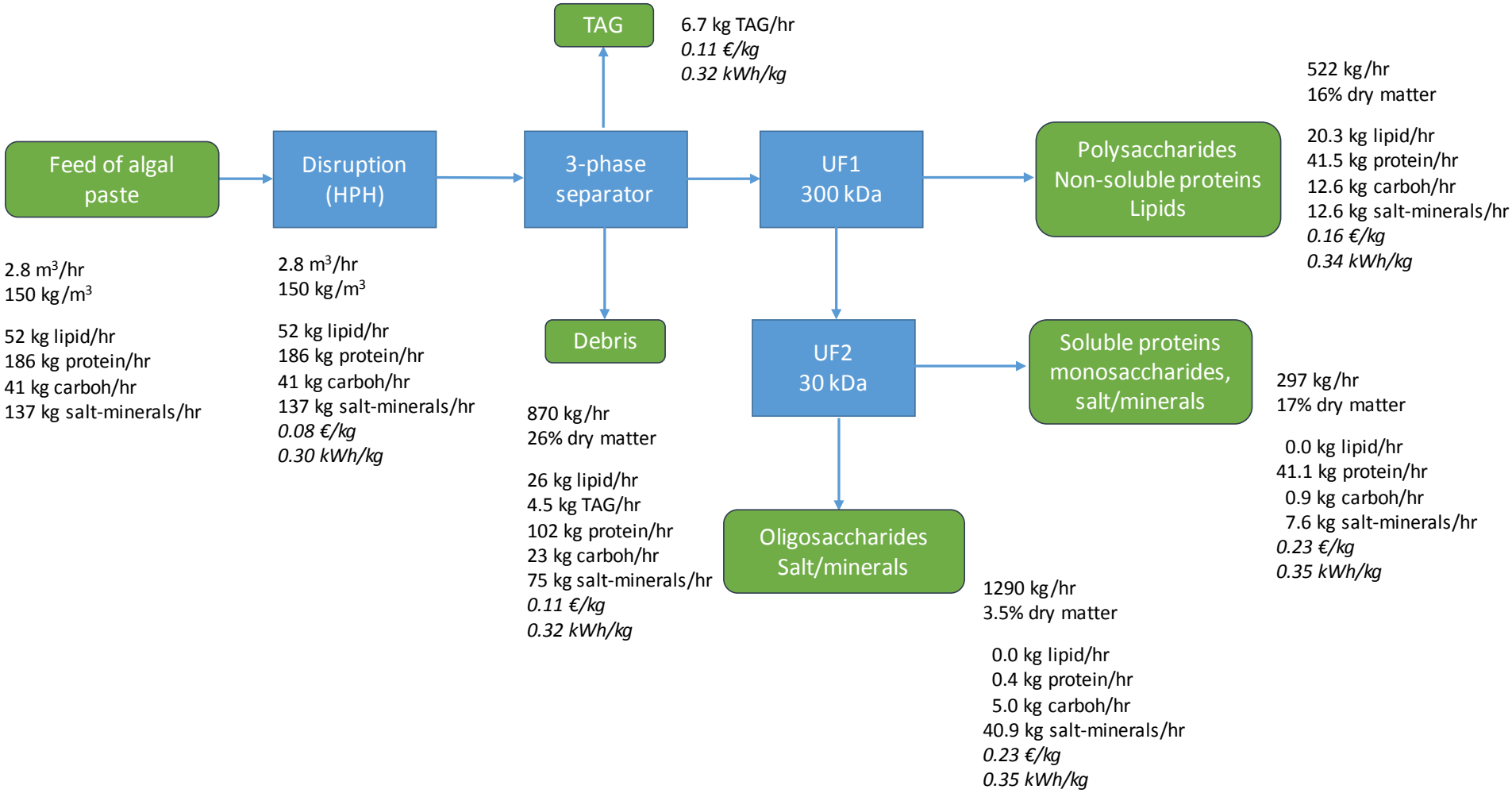

Figure 12 Soluble component fractionation from 3-phase centrifugal separation for homogenized non-stressed algae with component mass balances and estimated allocated costs and energy consumption for each product (italic)

The first UF system retains the large carbohydrates, the non-soluble proteins, and the remaining lipids. This stream is a mixture of components from which 50% protein. The stream can be best further processed by an alkaline-acid treatment to recover protein in non-native state. The product of this treatment is dissolved and can be further concentrated and dried. The costs for finishing steps are estimated at 0.10 €.kg^{-1} from which 50% is related to energy costs.

The second UF separates the proteins from the other components and concentrates the stream. Also the majority of the salt and minerals (others) are separated from the protein. Salt and mineral content can be reduced further by diafiltration. The permeate flow is very dilute and has hardly valuable components. The total allocated costs over all operational steps for both product streams from the second UF are 0.23 €.kg^{-1}.
From the total amount of proteins 22% is harvested as native protein in the retentate from UF2. This value corresponds to the values measured by WP1. The main sources for the low yield are:

1. the partial release of proteins at homogenization or bead milling,
2. the amount of proteins that join the debris in the solvent, and
3. the ratio soluble/non-soluble proteins.

The distribution of costs over the categories capital, energy, maintenance, consumables and labour is given in Figure 13. Labour has a high contribution to the costs and can be reduced by applying a high level of automation which to limit the handling activities of an operator. The simulations are based on continuous processing during 24 hours a day. Concentrating the processing activities in one shift during day time will also reduce the contribution of labour to the costs.

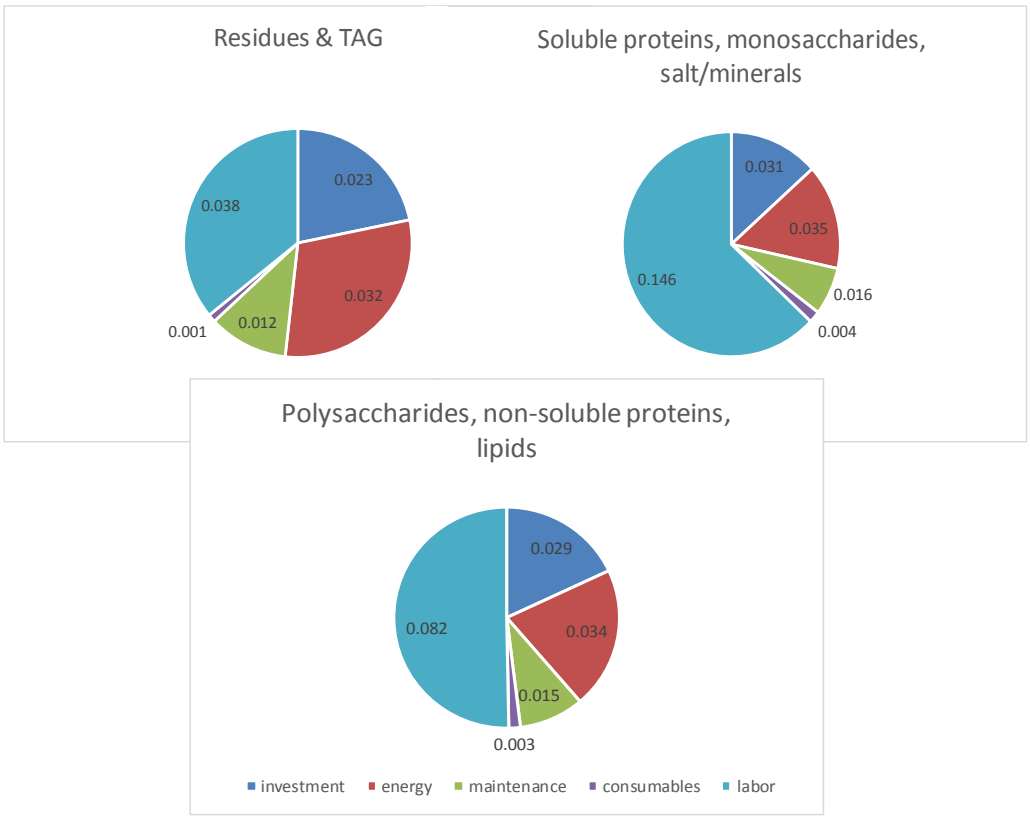

Figure 13 Distribution of costs for the different products from the protein biorefinery

6.2.4.5 Total chain simulation

In the previous phase of AlgaePARC, the focus was on the cultivation technologies. The research concerned amongst others the comparison of open-pond, horizontal and vertical tubular and flat plate photobioreactors. The performance of the systems was evaluated under various conditions on laboratory and pilot plant scale. In an Excel worksheet cultivation simulation tool, developed during the previous AlgaePARC-project, the results were projected on large scale production at several locations and climatic conditions. In that study the production in the Netherlands and South of Spain were estimated among other locations (see Ruiz et al. [8]).

The mentioned cultivation worksheet is in the current project integrated with Excel worksheets for harvesting and dewatering, and the biorefinery, see Figure 14. In this sheet the user can define the desired value chain by making choices. The options for the simulation of the cultivation are extensively reported in the previous AlgaePARC project and are discussed by Ruiz et al. [8] and not within the scope of the current project. Examples of choices that can be made are:

- Production location, which impacts on the climatic conditions, local labour costs, costs of resources like energy, etc.,
- Algae species, with impacts on biomass composition and yields,
- Stressed or non-stressed cultivation,
- Photosynthetic efficiencies (aka productivity),
- All kind of specific data.

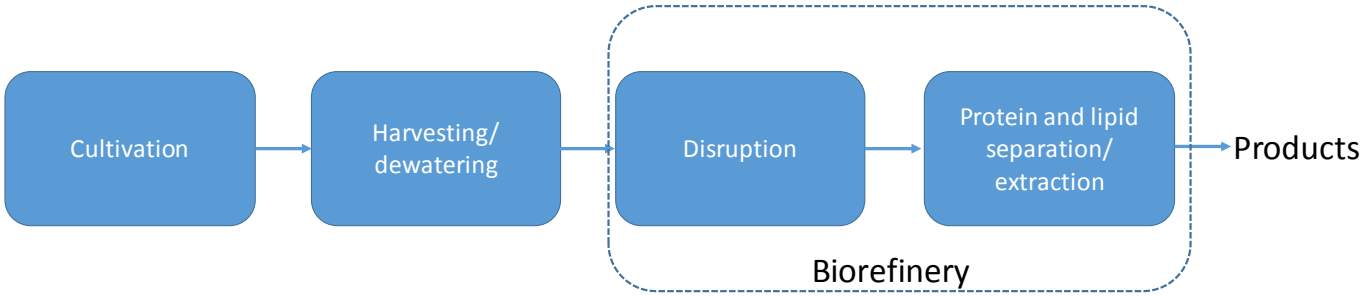

Figure 14 Overview of the integrated Excel models for simulation of the total chain.

For harvesting/dewatering the unit operations from Figure 8 can be selected, while for disruption a choice can be made between homogenisation and bead milling. The resulting decision tree for the disruption and the algal biorefinery is given in
Figure ***15***. The lipid extraction in this decision tree is based on the traditional methods from the literature for wet and dry processing with hexane and ethanol-hexane mixtures. The results for extraction with IPA and 22% ethanol came available after finishing this work sheet and consequently were not implemented.

The decision tree in
Figure ***15*** includes the alkaline treatment and hexane extraction for respectively protein and lipid extraction from the residual streams. Also post processing of the protein solutions to a dry product is included.

The total chain simulation tool makes use of an own data bank for production and equipment costs which is based on experimental results from the project and information from the project partners. The equipment and resource costs are based on information from the suppliers for equipment and engineering companies participating in this project.

By making choices the user can select his pathway through the decision tree for harvesting/dewatering and the biorefinery. For the selected pathway an overview of the costs (total costs, CAPEX, OPEX), the use of resources (energy, chemicals) and labour is produced. By projecting different scenarios the user can compare pathways, unit operations and options for production.

Conclusion

The costs for concentrating algal streams from 0.5 to 150 kg.m^{-3} dry matter (open pond system), are in the range of 0.3 and 2.0 €.kg^{-1} algae and the energy consumption up to 4.5 kWh.kg^{-1} algae. For algal broth from closed cultivation systems with a higher dry matter content the production costs and energy consumption fall to below 0.5€.kg^{-1} algae and below 0.5 kWh.kg^{-1} algae.

Flocculation for harvesting followed in combination with a second unit operation requires the lowest energy demand but takes additional costs for flocculants and the relatively low biomass recovery. The impact of flocculants in the water recycle stream to cultivation units, and on fractionation and extraction steps, however, may limit the use of flocculants.

Figure 15 Decision tree for disruption, protein and lipid separation/extraction. Dark cells refer to the process of each step, grey cells to the possible unit operations, and the italic cells to the products treated at each stage.

For large scale processing homogenisation is preferred for disruption. The investigated lipid extraction methods with organic solvents (22% ethanol or isopropylalcohol) were compared to a traditional lipid extraction with hexane. The processing costs of using ispropylalcohol are dominated by the costs for IPA-water separation and IPA recovery. As a result the costs are much higher than the traditional method for hexane extraction. The method with 22% ethanol requires energy for ethanol-water separation. The costs are below those with IPA extraction and approach the costs of traditional hexane extraction.

In the protein biorefinery the yield of native protein is around 20-25%. The main loss of native protein is with the residue of the centrifugation unit.

With a flexible simulation tool a range of biorefineries can be evaluated in combination with a range of cultivation units, climatic and regional conditions.

6.3 Biorefineries and the supply chain

In the biorefineries and the supply chain two aspects have been investigated:

- The impact of transport on decision making for centralized and decentralized processing of concentrated algal biomass.
- Algal paste processed in centralized biorefineries need to be stored before and after transport, while transport is also a form of storage. The feasible storage time is investigated experimentally.

6.3.1 Centralized versus decentralized processing: impact of transport

Methods

Processing at large scale with high capacities can have the "benefits of scale" with lower capital costs per unit of product, effective use of resources and lower labour costs. In contrast the products need to be transported to a centralized location which need extra costs and which bring extra emissions.
Figure 16 illustrates the flow of material in the logistic chain. Due to the large volumes of cultivation broth and the water needed at the cultivation sites it is always beneficial to concentrate the broth at the cultivation site. The concentrated algal paste from a number of cultivation sites is transported to a biorefinery. The main decision variables in this system are: 1) the number and size of cultivation sites, 2) the distance to the biorefinery, and 3) the capacity/scale of the biorefinery.

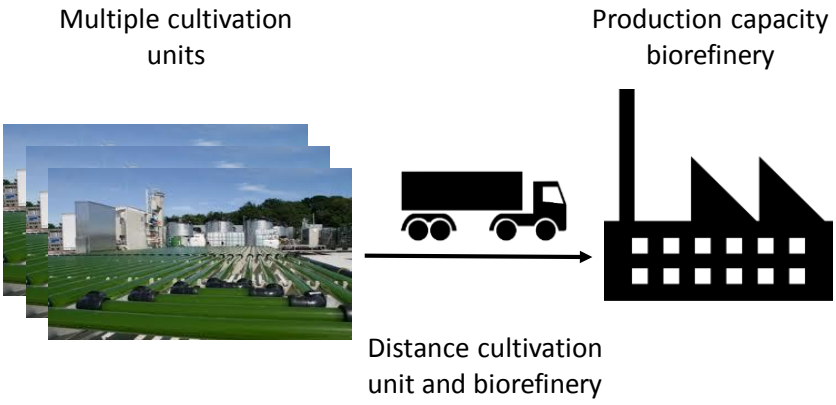

Figure 16. Schematic representation of the approach for the logistics model.

This task was performed with an Excel model. By repeating the simulations for different options the effect and role of the decision variables is evaluated. The most important settings for these simulations were:

- Different cultivation methods in units of 100 ha.
- Harvesting at the cultivation site with pressure filtration followed by centrifugation.
- Transport of biomass at a concentration of 150kg/m^3.
- Costs for cooled transport in the Netherlands.
- Applied to a lipid and protein biorefinery, both with disruption by homogenisation.

Results

Figure 17 gives the costs for biomass transport. The costs range from 0.00-0.03€.kg^{-1} for nearby refineries to 0.17€.kg^{-1} at large distances. Comparing to the costs of cultivation (11-12 €.kg^{-1} in the Netherlands), harvesting (0.5-1.5 €.kg^{-1}), disruption (0.10 €.kg^{-1}) and biorefining of algal paste (0.20-0.60€.kg^{-1}), the costs for transport have only a minor contribution (below 1%) to the total costs. Even if the costs of cultivation decrease by cultivation under favourable climatic conditions, the contribution of the costs of transport in the total chain is low.

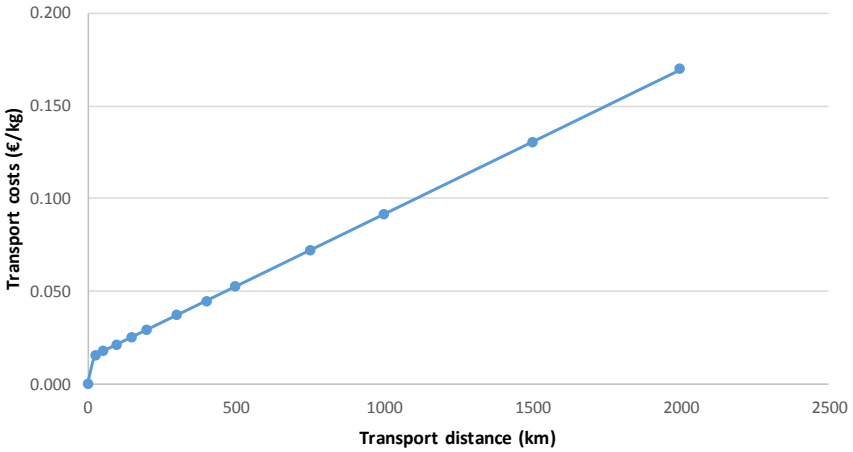

Figure 17 Costs of cooled transport of algal paste (150 kg.m^{-3}) from a cultivation location to a biorefinery as a function of transport distance.

However, for decision making on transport versus planning biorefinery locations the benefits of large scale processing have to be evaluated and compared to the transport costs.
Figure ***18*** illustrates the processing costs per kg feed rate of a protein a lipid biorefinery for different scales of input capacity.
The production capacity of an open pond system in the Netherlands with 15 tonne.ha^{-1}.year^{-1} corresponds after harvesting and concentration to 150 kg.m^{-3} to an inflow of 1.4 m^3.hr^{-1}, which is the left point in the graph. The right point in the graph corresponds to the processing capacity of 100 of these open pond systems. Both lines in
Figure ***18*** show that processing the feed of 100 open ponds gives a significant benefit of scale. The difference between the low and high capacities is for both types of biorefineries about 0.20-0.22€.kg^{-1} product.

From Figure 17 follows that the costs for transport are below 0.20€.kg^{-1} product, which implies that for open pond systems with the given capacities the costs of transport to a centralized biorefinery are below the benefits of large scale processing. So, centralized processing is thus always beneficial.
Cultivation units with a higher productivity (other photobioreactors, or other favourable climatic conditions) deliver more biomass. Then, the amount of biomass to be transported increases. Although more transport movements, the transport costs expressed per kg algal biomass remain the same. The benefits of processing scale, however, decrease. The processing costs for a lipid biorefinery processing the algal biomass of a 100 ha cultivation unit with 45 tonne.ha^{-1}.year^{-1} are about 0.48€.kg^{-1} and for the protein biorefinery about 0.27€.kg^{-1} (second point in
Figure ***18***). With increasing scale of the biorefineries, the benefit of large scale processing reduces to 0.08€.kg^{-1} for the lipid and 0.07€.kg^{-1} for the protein biorefinery. The break-even point on distance is then around 750km. For higher production capacities of cultivation units the break-even point shifts further to lower transport distances.

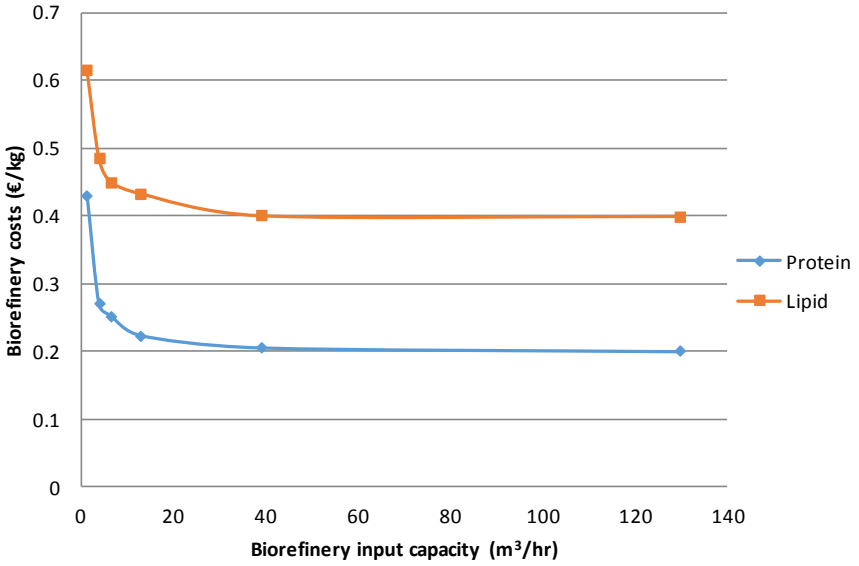

Figure 18 Effect of processing scale on the costs for a lipid and a protein biorefinery

Conclusion

The contribution of transport to the total cost of algal products is small, even over large transport distances. The costs benefits of large scale lipid and protein biorefineries always surmount the costs for transport over 2000 km from low productivity cultivation plants to a centralized biorefinery. For high productivity cultivation plants there is a break-even point in distance. For cultivation plants with 45 tonne algal biomass.ha^{-1}.year^{-1} the break-even point is at about 750 km.

6.3.2 Storage experiments

6.3.2.1 Materials and methods

N. oleoabundans was cultured in artificial seawater medium at AlgaePARC (Wageningen, The Netherlands). Two cultivation conditions were applied to obtain both stressed (N-) and non-stressed (N+) biomass. The algae broth was concentrated by centrifugation. The wet paste was stored for 0, 3, 7, 10, 14, 21 and 28 days under air-deprived conditions in separate ziplock bags at 4°C and 20°C. During storage the color and structural changes of the algal paste were noted. After each storage period samples were freeze dried. The freeze dried samples were crushed to powder and stored at room temperature under nitrogen gas until analysis.

The samples were analyzed with respect to

- CFU-counts
- Dry weight (oven drying at 105°C)
- Lipid content (gas chromatography, quick colorimetric method)
- Protein content (Bradford)
- Carbohydrates (HPAEC)
- Volatiles (HPLC)
- Ash content (TGA/DSC1 analyzer)

The analysis were complemented with qualitative visual observations.

6.3.2.2 Results

Qualitative visual observations

At harvesting the algal paste was dark green for the N+ paste (non-stressed) and bright green for the N- paste (stressed) (

Figure 19). At 20°C, both pastes changed to brown within a week. At 4°C the N+ paste turned brown after 14 days, while the N- paste remained green but not bright after 28 days of storage
Figure 19). In the brown colored algal samples gas with a strong odor developed. The odor is result of the production of volatile metabolites, which were detected by HPLC analysis.

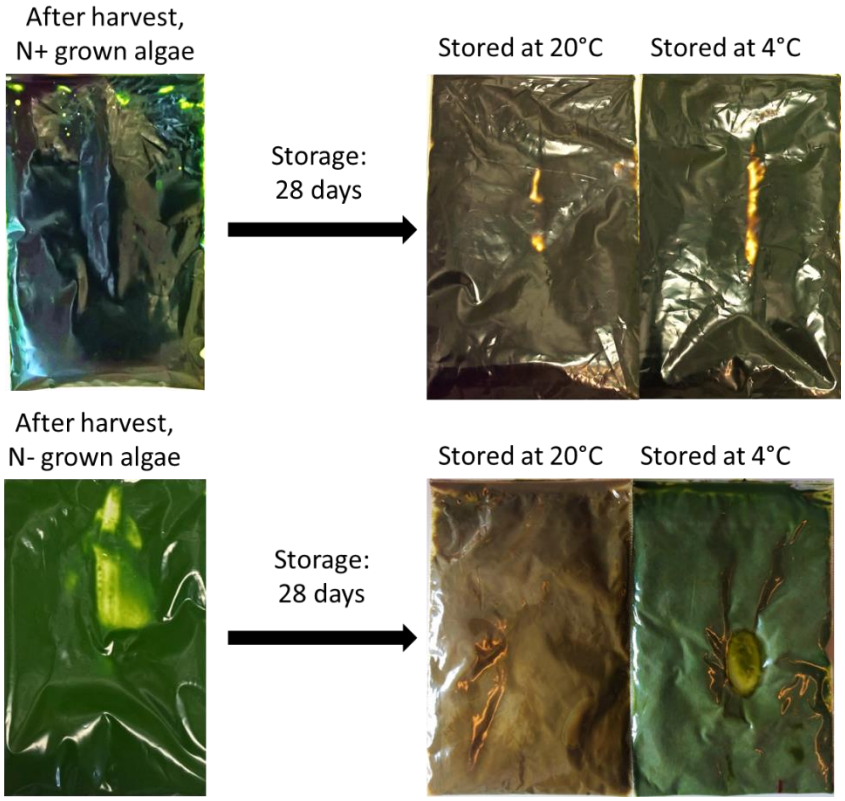

Figure 19: Impression of color changes for N+ (top) and N- (bottom) paste of Neochloris oleoabundans during storage.

Microbial infection

The changes in CFU count over the total period is negligible and indicates no strong accumulation of aerobic microbes during storage. Fungal growth was regularly seen for N- paste and incidentally for N+ paste.

Lipids

The total fatty acid (TFA) content measured showed for N+ and N- paste a minor change in total fatty acid content. During storage at 4°C the fatty acid profiles did not change. At 20°C the relative amounts of C18:1 and C16:0 increased, while C16:3, C18:2 and C18:3 are decreasing.

Proteins

The soluble protein in *N. oleoabundans* N+ paste has at the start of the storage period a rapid and strong breakdown of soluble proteins (see Figure 20). At 4°C the breakdown of soluble protein starts after seven days. At the end of the storage period the protein content is the same as for the N+ paste stored at 20°C (3-4%). Stressed algae (N- paste) have a lower soluble protein content (7.5%). Figure 20 shows a steady increase on soluble protein content to around 11.5% of the dry mass at the end of storage. The increase is consequence of soluble protein release by death cells releasing and the loss of volatiles during freeze drying used in the experimental procedure.

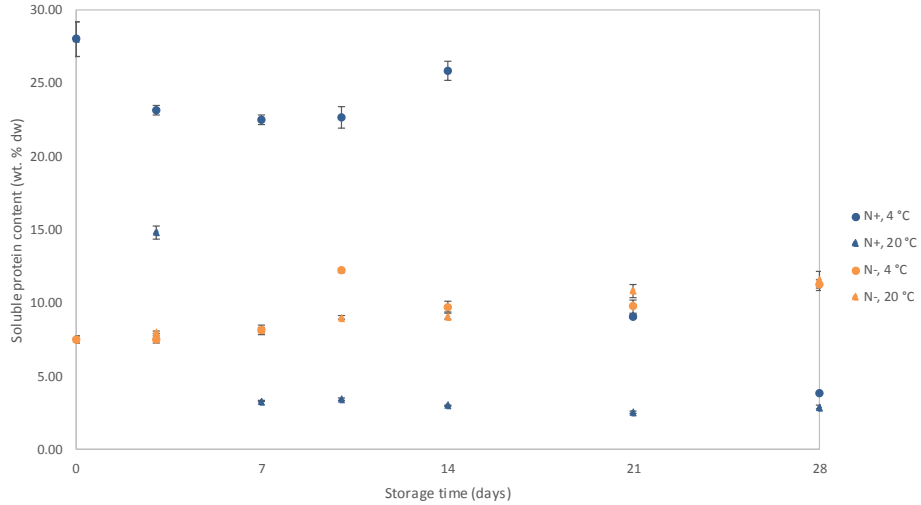

Figure 20: Soluble protein content per dry weight of algal paste of Neochloris oleoabundans during storage for N+ paste (blue) and N- paste (orange) stored at either 4°C (circles) and 20°C (triangles).

Carbohydrates

The carbohydrate content in *N. oleoabundans* N+ paste decreases steadily from 8.5% to 5.4% (at 4°C) and 3.9% (at 20°C) (see ***Figure 21***). The decline is result of glucose conversion which occurred during storage at 20°C in the first week, and started for 4°C in the second week.

N- paste at 4°C shows hardly any decrease of glucose during storage. The decrease of the glucose content in the N- paste stored at 20°C started after day 10. Loss of glucose can be expected as this carbohydrate can be used easily used by viable micro-algae for respiration processes, as well as consumed as carbon source by contaminating micro-organisms. In the N- paste stored at 4°C the total amount of carbohydrates remains constant over time.

Volatile formation and loss of dry weight

Anaerobic degradation of sugars and proteins/peptides by mesophilic bacteria results in the formation of fermentation products. The HPLC measurements indicate the presence of acetic acid, propionic acid, butyric acid, oxalic acid, glycolic acid and ethanol. For both N- and N+ paste of *N. oleoabundans* volatile production is strongest at 20°C.

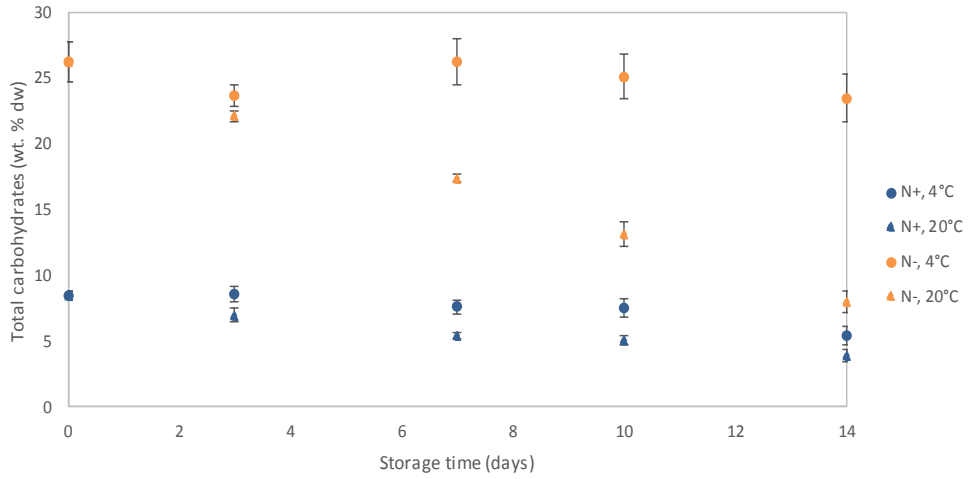

Figure 21: Total amount of carbohydrates per dry weight of algal paste during storage for N+ paste (blue) and N- paste (orange) of Neochloris oleoabundans stored at 4°C (circles) and 20°C (triangles).

Conclusions

During storage time the changes in composition are stronger and faster for the paste of non-stressed *N. oleoabundans* than for the paste of stressed *N. oleoabundans*. The stress conditions result in a changed physiological structure and composition. The stressed marine *N. oleoabundans* proved to be more robust for storage conditions.

Based on the experiments, we advise to store algal paste of marine *N. oleoabundans* at 4°C in absence of air for a limited period in the range of 3-7 days. Although stressed algae are most robust during storage we recommend a maximum storage time of 7 days because of fungal growth in the paste. For non-stressed algae a maximum of 3 days storage is recommended.

6.3.3 Summary

For harvesting and dewatering 28 combinations of unit operations were evaluated with respect to operational costs and energy consumption. Flocculation of open pond cultivated algae proved to be low in energy consumption (<0.1 $kWh.kg^{-1}$) while the costs are in the range of 0.4-1.4 $€.kg^{-1}$. The effect of flocculant in the water recycle to the cultivation unit, and subsequent processing steps is, however, not yet well described in the literature. Other methods, pressure, vacuum and membrane filtration, centrifugation, and their combinations result for open pond cultivation systems in energy consumption ranging between 1.0-4.5 $kWh.kg^{-1}$ and costs below $2€.kg^{-1}$. For high productivity systems (flat plate, tubular cultivation or beneficial climate), the costs and energy consumption drop by a factor 3-4.

For disruption in large scale algae biorefineries homogenisation is preferred with respect to the operational costs. The simulations for the lipid extraction methods, experimentally investigated by WP1 (with IPA or ethanol as solvent), showed that the water-solvent separation (distillation or evaporation) results in high costs and high energy consumption. These methods proved to be more costly and energy intensive than traditional lipid extraction with hexane. In the protein biorefinery with centrifugation and ultrafiltration steps 20-25% of the protein was recovered as native protein. The majority of the native protein was lost in the residue from the centrifugation step. This part can be recovered by alkaline or acid extraction, but then the native protein properties are lost during this treatment.

Some specific algae biorefineries were analysed. Excel models were also developed for other traditional methods for extraction and separation of algal components. Next, an Excel model is developed to evaluate the total chain of cultivation in combination with harvesting, dewatering and product extraction and separation. The Excel model allows a large range of combinations of cultivation units and conditions, and harvesting and extraction methods.

The benefits of large scale processing surmount the costs of transport over long distances and therefore centralized processing is preferred. The break-even point for transport distance is at lower distance for high productivity cultivation units (tubular, flat plate or beneficial climatic conditions). The storage experiments in absence of air resulted in a recommendation of maximal 3 days storage time for non-stressed algae and maximal 7 days for stressed algae. After these storage period the loss of carbohydrates and protein becomes significant, lipids remain stable over this period.

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7 Enabling tools, basic knowledge on cell wall composition (Results WP3)

7.1 Summary

Understanding the composition and structure of the cell wall is crucial to optimize the extraction process during algae biorefinery. Accessing to the all cytoplasmic component can only be done after disruption of cell walls. Within WP3 we were successful to develop a robust protocol that allows the isolation and further characterization of *Neochloris oleoabundans* cell wall, a microalga species belonging to Chlorophyta phylum. We have observed that *N. oleoabundans* cell wall comprises a diverse assortment of constituents and overall displays unique features. The cell wall building blocks of *N. oleoabundans* is a combination of carbohydrates, frequently non-cellulosic; and proteins, lipids and inorganic materials. A comparative transcriptomics study followed by biochemical characterization and morphological observation revealed the molecular mechanisms underlying the cell wall development throughout the cell cycle.

7.2 Introduction

Neochloris oleoabundans is considered as one of the most suitable microalgae, given its industrial potential and the tools available for this species. The first step to be taken after harvesting the *N. oleoabundans* biomass and prior to generating multiple cytoplasmic products is to overcome the physical barrier of the cell in order to access its components. The intracellular components of *N. oleoabundans* are enclosed within the cell wall, which is considered as a rigid layer located on the outer part of the cell membrane. A successful valorisation of the multiple products depends on the kept integrity and functionalities of the cellular components upon disruption of the cell wall.
In contrast to terrestrial plants, for which tools and methods to characterize cell wall properties have been developed over many decades, microalgae such as *N. oleoabundans* have a limited toolbox available. Hence, there is little information available on the type of cell wall monomers and their assembly to higher complex structures. Moreover, a specific function of the individual cell wall building blocks and their coordinated biosynthesis pathways still remain unidentified on the whole. Prior to engineering the cell wall constituents and structures, with different purposes for particular applications, it is considered vital to identify the biochemical composition, morphological structure and their underlying genetics as well as their biosynthetic pathways.

Algae cell walls, in general, display a greater biological diversity and metabolic plasticity compared to terrestrial plants (Bioenergy, 2017; Domozych et al., 2012). Cell walls of species belonging to the Chlorophyta phylum display both unique and common features with the cell wall of (higher) plants and fungi. Similar to the higher plant cell walls, microalgae contain a substantial amount of polysaccharides in their cell wall. Additionally, the cell wall of microalgae belonging to Chlorophyta phylum might contain chitin or chitin-like structure, a feature of fungal cell walls.

Apart from the diversity of the cell wall among different species, structural properties of the cell wall might also change in response to environmental factors or throughout the life cycle. Nitrogen deficiency and salinity are two environmental factors that can influence cell carbon partitioning (Chen et al., 2017; Chu, 2017; Garibay-Hernández et al., 2013). Changes in carbon balance and its partitioning can make a significant alteration in cell wall composition and morphology. Cell development throughout the cell cycle can also change the cell wall characteristics. Formation of the daughter cell together with the development of the maternal cell requires biosynthesis and cell wall component modification. Some of these modifications have been observed on the cell wall of *Chlorella ellipsoidea* (Takeda & Hirokawa, 1978; Takeda & Hirokawa, 1979). Previous reports described an increase in hemicellulose content of the cell wall during the cell-growth phase, while the rigid-cell wall, chitin-like components, remained nearly unchanged. Species-specific cell wall characteristics together with remodelling of the cell wall due to the environmental factors or growth phases can cause a different level of susceptibility to the various disruption methods. Several studies have reported on the development of methods for microalgae cell walls disruption. These methods consist mostly of harsh treatments that target cell wall as a whole, but the development of tailor-made mild and non-disruptive methods requires a better knowledge of the cell walls, including biochemical

composition and morphological features. The fundamental knowledge to uncover the biochemical composition and configuration of the cell wall is a prerequisite to reveal its imperceptible rigidity and further design an applicable and selective disruption strategy.

7.3 Objectives

This project aims to examine the *N. oleoabundans* cell wall compositions and morphology under the optimum growth condition, and study its cell wall remodelling when it is exposed to different environments. Additionally, we unveiled the morphologic characteristics and remodelling of the cell wall throughout the cell cycle. In order to enlarge the existent knowledge, we studied the biosynthesis and modification of the *N. oleoabundans* cell walls throughout the life cycle and characterized the molecular determinants of the metabolic pathways.

7.4 Method/Approach

Chemistry-based analytical techniques such as Gas Chromatography (GC) or High Performance Anion Exchange Chromatography (HPAEC), Ion Chromatography (IC), together with high-resolution microscopy such as Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM) and confocal microscope; and molecular and bioinformatic tools are some of the approaches assisted in characterizing and further mapping of the cell wall composition.

7.5 Results

The results from WP3 indicate that the cell wall of *N. oleoabundans* has 31.5% proteins, 8% ash and 22% lipids in addition to the 32% of carbohydrates (***Figure 22***).

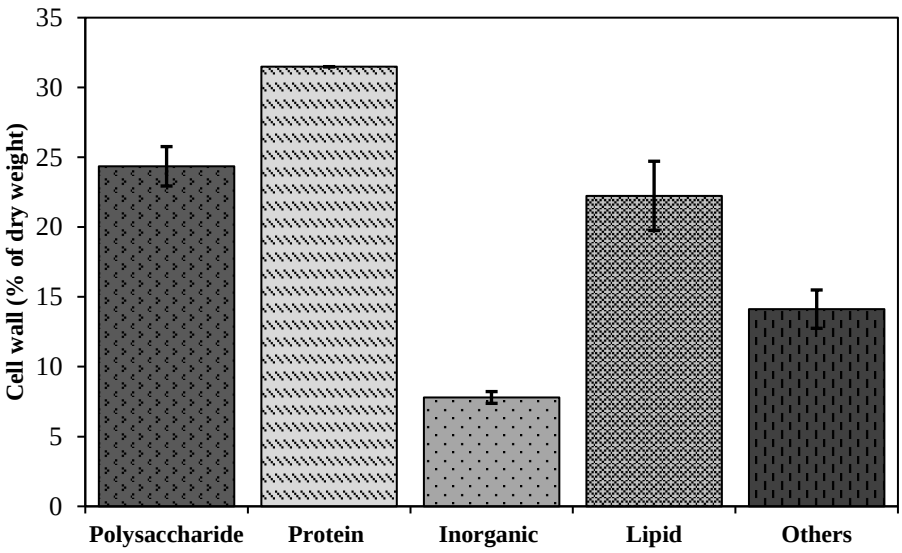

Figure 22. Percentage of components in the cell wall of N. oleoabundans. Values are the average of four replicates (two biological replicates, each measured in two technical replicates) and error bars represent the standard deviation (SD). "Others" fraction (~14%) stand for the portion of the cell wall composition that remained uncharacterized, most likely due to losses during the chemical analysis procedure. Acid-treatment is an essential step for the characterization of the cell wall, but inevitably destroys some components.

Cell wall carbohydrates of *N. oleoabundans* are composed of rhamnose, arabinose, glucosamine, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid ().

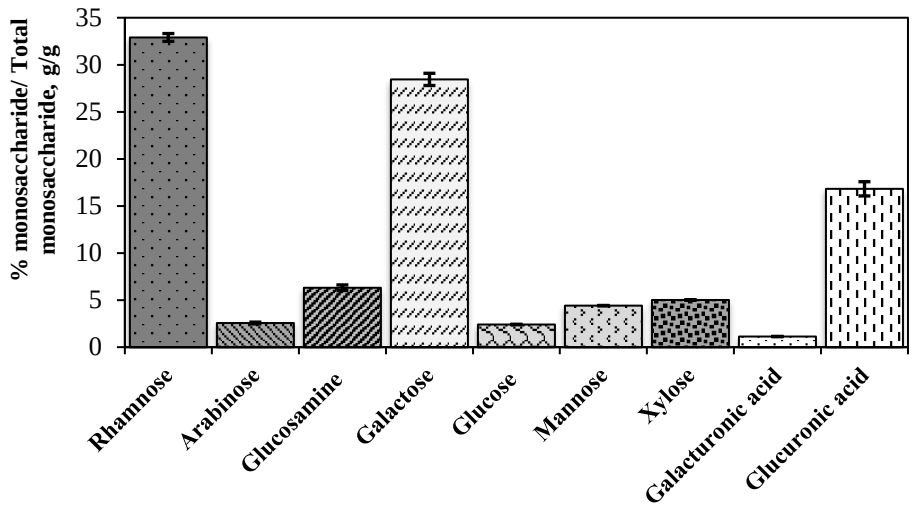

Figure 23. Monosaccharides composition of N. oleoabundans cell wall polysaccharides. Bars represent the amount of xylose, mannose, glucose and galacturonic acid are corrected based on TFA hydrolysis, while quantification of the other sugars is based on H2SO4 hydrolysis. Values are the average of four replicates (two biological replicates, each measured in two technical replicates) and error bars represent the standard deviation (SD).

Characterization of amino acids profiles of the cell wall indicated that valine is the main amino acid presented in *N. oleoabundans* grow under freshwater nitrogen-replete condition (Figure 3). According to the amino acid classification based on the side chain structure, the *N. oleoabundans* cell wall is mainly composed of non-polar amino acids.

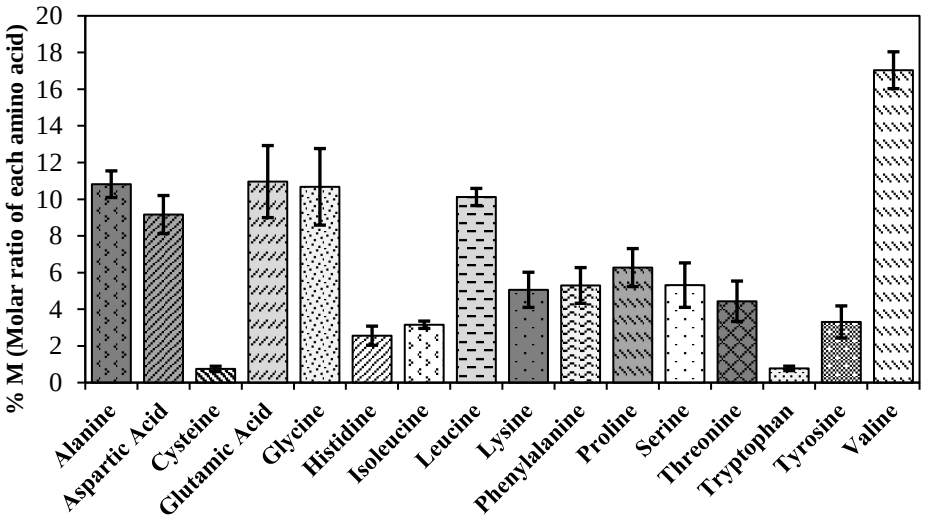

Figure 24. Detected amino acids composition of N. oleoabundans cell walls using EZ:faast™ method. Bars represent the percentage of molarity (%M) of each amino acid to the total identified amino acid content. Values are the average of four replicates (two biological replicates, each measured in two technical replicates) and error bars represent the standard deviation (SD). Using the EZ:faast™ method, we were able to disclose 16 amino acids in the cell wall. Due to the intrinsic limitation of the method we were unable to detect asparagine, glutamine and arginine. Asparagine and glutamine are quantitatively converted to aspartic acid and glutamic acid during acid hydrolysis. Therefore, the absolute value of these two amino acids might be overrepresented. Methionine was not detected in the cell wall, which is most probably due to degradation during the HCl hydrolysis.

Identification of the lipids with GC analysis and further comparison of the mass spectra with a NIST library (National Institute of Standards and Technology) indicated that *N. oleoabundans* cell wall contains long-chain fatty acid (with more than 20 carbon atoms) along with saturated fatty acids C16-C18.
Results of the chemical composition of *N. oleoabundans* cell walls suggested the presence of complex ultrastructure which probably comprises glycoproteins and/or glycolipids. We have measured that approximately eight percent of *N. oleoabundans* cell wall was made up of inorganic ions, among which sulphate and sodium were the major components.
The High Pressure Freezing and Freeze Substitution (HPF FS) method allows the preservation of the cell morphology better than conventional fixation methods. In this method, cell organelles are visualized sharper and have enhanced quality relative to the conventional method. More specifically, the HPF FS is a robust method which allows the better visualization of the cell wall structure. Microscopic visualization of the *N. oleoabundans* indicates that the cell wall consists of two layers. The external outer layer (O) which has more electron-dense structure and the internal layer (I) of the cell wall which has less electron-dense structure. The outer layer of the cell wall covers with the hair-liked assembly (Figure 4)

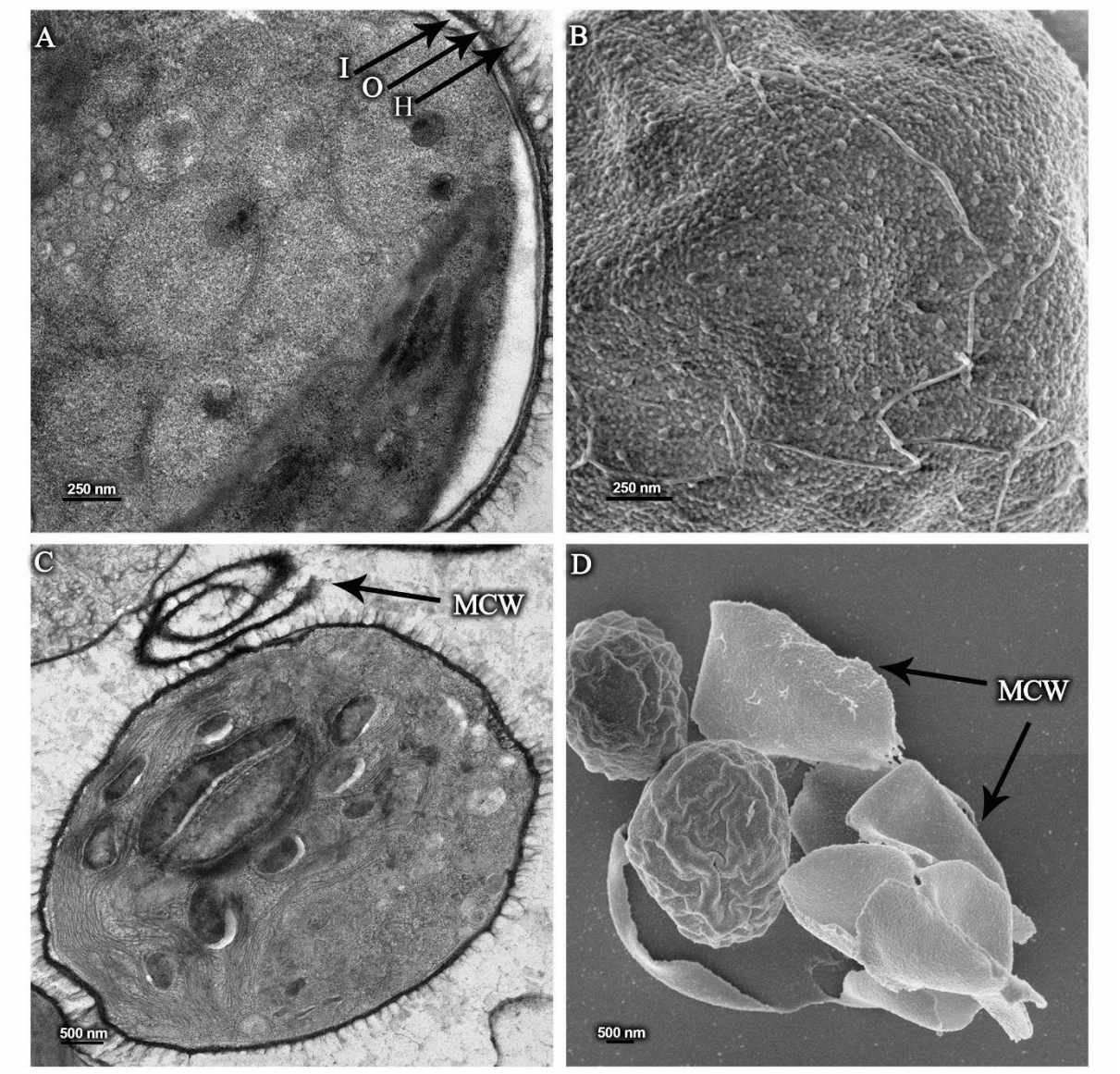

Figure 4. Electron microscopy images of N. oleoabundans cells. A, doubled-layered cell wall with hair-like structures (TEM). B, cell wall surface with hair-like structures (SEM). C and D, accumulation of maternal cell wall in the medium (TEM and SEM, respectively). I: internal-inner-layer of cell wall, O: external-outer-layer of cell wall, H: hair-like structures, MCW: maternal cell wall.

Characterization of the cell wall compositions, in particular proteins and carbohydrates, cultivated either in freshwater or seawater with or without sufficient nitrogen was performed. The results revealed that the cell wall composition varied in that the modifications were different in the four cultivation media: Freshwater nitrogen-replete (control culture) and -depleted conditions, and seawater nitrogen-replete and -depleted conditions. Nitrogen deficiency in freshwater cultivation was the only condition that significantly ($p<0.05$) increased the total carbohydrates of the cell wall. Salinity and nitrogen deficiency also had an impact on the nitrogenous components of the cell wall. Under such stress conditions we observed a decrease in glucosamine in the cell wall.

7.5.1 Discussion

The *N. oleoabundans* cell wall as a tenacious barrier of the cell needs to be removed before the intracellular content can be reached. Biological and biochemical insights are required to deconstruct the cell wall effectively while keeping the functionality of the internal components. In this project, a range of biochemical, microscopy and molecular experiments were performed in order to reveal the *N. oleoabundans* cell wall composition, morphology and development throughout the cell cycle.

We have shown that *N. oleoabundans* cell wall is deprived of polysaccharides in the cell wall (Figure 1). Carbohydrate characterization revealed a very low amount of glucose (<1%), implying lack or a rather scarce amount of cellulosic polymer in the *N. oleoabundans* cell wall (Figure 2). Rhamnose is the most abundant monosaccharide of the *N. oleoabundans* cell wall. A high amount of rhamnose-containing polysaccharides may be responsible for the rigidity of *N. oleoabundans* cell walls, which is supported by results reported for other Chlorophyta algae (Russell, 1995). The existence of chitin-like structure in *N. oleoabundans* cell wall might contribute to the cell wall rigidity as well.

Unlike most of the cell walls in the plant kingdom, which are polysaccharide-rich, in *N. oleoabundans* proteins/ glycoproteins are the major components of the cell wall. We have shown the presence of arabinogalactan proteins (AGPs) in *N. oleoabundans* cell wall and these differ depending on the growing media

We demonstrated that due to the nature of cell division in *N. oleoabundans*, the cell wall throughout the cell cycle is mostly doubled. The outer electron-dense layer is the original maternal cell wall, which is carpeted with hair-like structures (Figure 4).

Monosaccharides profile of the cell wall was significantly different in the different growing conditions or throughout the life cycle. We observed that glucosamine, glucose, galactose and rhamnose were the main components of cell wall polysaccharides that their contents changed throughout the cell wall development. Nitrogen deficiency in the culture medium substantially lowered the protein content of the cell wall and alter the abundance of the constitutive amino acids.

7.5.2 Conclusion and recommendations

As a key message of this project, we showed that the *N. oleoabundans* cell wall is substantially different from (higher) plant cell walls. We indicated that the cell wall in *N. oleoabundans* is not just a rigid barrier of the cell. In contrast, this dynamic ultrastructure regulates the cell morphogenesis during the cell cycle and modulates the growth and interaction with the environment.

In reliance on cell wall polysaccharide composition, enzymatic treatments with carbohydrate hydrolases can be considered as a promising approach to weaken the cell wall prior to reaching the internal commodities of interest. In the same vein, the protein-rich cell wall of *N. oleoabundans* insinuates the possibility of using proteases to break/weaken the cell wall.

A breakthrough cost-effective cell wall disruption method to substitute the traditional energy-consuming approaches is an urgent need for the successful downstream processing of microalgae. Based on the results from this project, we venture to suggest the existence of autolysis enzymes as a cell-cycle regulated mechanism in *N. oleoabundans*. These specific autolysis enzymes are capable of degrading the cell wall at a particular site and a certain phase of the cell cycle. In reliance on these results, we are eager to recommend future research in order to demonstrate the potential application of autolysis enzyme as a specific and cost-effective tool for cell wall deconstruction.

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8 Conclusions and recommendations

The project objectives have been successfully achieved. Overall we can conclude that the project has generated numerous new, scientific, technological and economic insights that will benefit algae cultivation and biorefinery for product formulation in the food, feed and non-food sectors. In particular the project generated valuable, commercially relevant information on

- Algae cultivation in fresh water and sea water on pilot scale in industrial PBRs, that can be directly extrapolated to full scale, incl. procedures for stressing of algae to optimize lipids production, practical management of cultures;
- Algae harvesting technologies incl. a model to design, evaluate and select suitable harvesting trains;
- Opportunities and challenges of biorefinery technologies for cell disruption, extraction and fractionation including their integration in (multiproduct) flowsheets;
- Composition and variability of algae cell walls relating to optimum processing;
- Physiological data related to algae cultivation and processing;
- Strategies and protocols for algae storage and logistics;
- Detailed techno-economic evaluation based on mass balances, flowsheets built on acquired experimental data;
- Integrated model, enabling design, analysis and selection of viable microalgae value chains from cultivation through processing, coupled to logistics;
- Assessment of environmental impact.

Results are documented in a range of reports and publications, available for project partners and RVO and parties outside the consortium in industry, the R&D sector and the general public. In particular the development of an integrated techno-economic model combining modules for microalgae production, biorefinery and logistic for use by industry and to guide further R&D is a highly valuable project result.

The project has also generated valuable results for concrete product leads and application areas, including aquaculture feeds, bioplastics, and ingredients for food. These potential products may lead to concrete market opportunities In addition as (co) products they will enhance the techno-economic feasibility of algae biofuels production. The project results will be implemented by the partners in follow up projects and commercial developments initiated and led by the industrial partners and academic/knowledge partners.

9 Contribution to the objectives of the TKI program

The project outcomes and follow up activities will have significant impact for the mission of the Top sector Biobased Economy by promotion and expansion of the development of algae biotechnology incl. biorefinery and logistics for Carbon Capture and Utilisation, and as a rich source of biobased products and biofuels. The project results thus contribute to sustainable energy management and the energy transition. Furthermore the project results contribute substantially to the international knowledge position of the R&D and commercial project partners in the area of algae biotechnology. Through the collaboration in the project a strong network has been established for further development e.g. in ongoing and new international projects with involvement by WU and other partners.

It is recommended to develop follow up projects in the TKI framework in close collaboration with industry in addition to new projects in international programs incl. H2020 and BBI. Of particular interest are follow up projects in the area of food and feed, biotech and biofuels based on the multiproduct biorefinery approach and technologies developed in this project.

Contribution to the Dutch Economy

The project has a strong connection with recognized transition paths. The energy transition comprises a balanced set of transitions in different sectors of Dutch society, which (will) result in a significant reduction of CO_2 emissions and energy consumption. However, biomass production with algae is a new industry sector and teaming up with existing industry sectors is a critical factor for success. The Netherlands has a strong position in horticulture, chemistry, industrial biotechnology and maritime industries, all of which can contribute to building production chains with algae.

The Netherlands currently has a strong position in the valorisation of biomass and the development of biorefinery concepts. With regard to the production of bulk products (fuels and base chemicals) from algal systems, it had a much weaker position: Biorefinery of microalgae did not exist prior to this project and research efforts were very limited. It is therefore a new field in which Netherlands pioneered. Our world leading position and innovative reputation in the field of microalgae has been further consolidated with this program. International industry nvested in Dutch innovation.

Improvement of the Netherlands' knowledge position is mandatory to develop a true sustainable production of biomass based fuels, chemicals and energy avoiding competition between food and non-food uses of biomass.
This consortium has strengthen the knowledge position of the Netherlands in this topic. by improving the "whole crop biorefinery" concept of algal systems to such a level that implementation of a microalgae biorefinery can be brought to a demonstration level for the economic production of chemicals, fuel and energy from algal systems.

Contribution to Wageningen UR

Wageningen UR became a leading institute in the field of microalgae in Europe. One of our strongest points is the fact that we to cover the entire chain. A multidisciplinary research program was developed. Projects are financed from different sources via NWO, PPS constructions and bilateral. In these projects we presently collaborate with 40 industrial companies covering the entire value chain.

Our ambition is to be an innovative and high quality research and education organization in the algae field; more specific:

- to be the leading algal research and education institute in Europe;
- to belong to the top 5 research and education institutes in the world;
- to be the preferred institute for students to become professionals in the field;
- to establish partnerships with key institutes on complementary expertise areas;
- to establish public-private-partnerships for precompetitive research, research networks, to build up know how and to develop bilateral industrial collaborations;
- to be a preferred partner for industry;
- to be involved as consultant in strategic decisions in respect to algae production chains for leading companies;
- to diversify sources of income and establish research projects funded by NWO, EU, public-private partnerships and industry;
- to establish a publication track record in high impact journals.

We have integrated microalgae production with biorefinery concepts, which is an essential step for implementation of this technology in our society. This project focused on this integration and has strengthened our position in the global microalgal research field.
This project has contributed to reach most of the ambitions referred above. We are presently number 1 research institution in this field in Europe and third worldwide

Contribution to University Twente

At the University of Twente (motto: 'High Tech, Human Touch'), 'Green Energy' is one of the five leading themes. The research group Thermo-Chemical Conversion of Biomass (TCCB, recently renamed to Sustainable Process Technology (SPT)) of the University of Twente (with the motto: High Tech, Human Touch) is the leading research group on thermo-chemical conversion of biomass in the Netherlands. The

High Pressure laboratory and experimental facilities, complete with staff of skilled technicians, enable the development of novel technologies over a wide range of process conditions. Since 2007 activities have started on the production of renewable fuels from microalgae, initially focusing on gasification and hydrothermal liquefaction for fuels production. In recent years the activities have broadened and now include activities in the field of biomass fractionation to recover food/feed ingredients and recycling of nutrients and minerals. These biorefinery activities are done in close cooperation with partners inside- and outside the Netherlands, among which the Universities of Wageningen and Bologna and with companies interested in biofuels or algae production (e.g. Feyecon in the Netherlands). The projects have been funded so far by the University of Twente (Impact-Strategic Research project), local SME's (BEON), co-funded by national funds (EOS-LT) and the province of Overijssel (BE2.O).

We have made a significant contribution to the development of technologies enabling the establishment of a standalone, integrated Algae Biorefinery concept, which is seen as an important biomass opportunity for the Netherlands and a 'showcase' of the biobased economy and serves (to students and society) as good example of a sustainable production system of food/feed, fuels and (bio-)chemicals. In this project the focus of the work by UT was put on energy and resource-efficient extraction of lipids from wet algal biomass using switchable solvents.

Contribution to industrial partners

Microalgae have the potential to be a sustainable biobased feedstock for the supply for food, feed, chemicals, materials and fuels because they can be grown on arable land at high productivity. In the Netherlands public private partnership innovation programs such as AlgaePARC for production of microalgae are in place and need to be followed up by biorefinery programs. This is especially important for industrial end users and technology suppliers. For industrial partners involved in technology development (Evodos, POS Bioscience, WAB, GEA – Westfalia, BODEC) this project allowed further development of their technology and expansion of their current business to new markets and applications. For intermediate and end users (TOTAL, EWOS (now Cargill), DSM, BASF) this program represented a further step towards using a new and sustainable feedstock. Important for all participating companies is the competitive advantage they got in an emerging new field with a potential future role in a biobased economy.

Spin off inside and outside the sector

The project results offer numerous opportunities for spin-off and follow up activities ranging from follow-up and related projects by R&D partners and companies to more general commercial benefits and start-ups.

10 Overview of publications about the project

The project results are widely disseminated via a range of publications in scientific journals as well as conference papers. A cumulative list of publications (as per April 2019) is presented below.

1. Open workshop / poster session 5th June 2014 + and accompanying publications/ interviews
2. Items in AlgaePARC website/AlgaePARC newsletter
3. G.P. 't Lam , M.H. Vermuë, G. Olivieri, L.A.M. van den Broek, M.J. Barbosa , M.H.M. Eppink , R.H. Wijffels, D.M.M. Kleinegris. 2014. Cationic polymers for successful flocculation of marine microalgae, *Bioresource Technology* 169: 804–807.
4. Y. Du, B. Schuur, D.W.F. Brilman, 2014. Switchable solvents for extracting lipids from *Phaeodactylum tricornutum.* Poster presentation NPS 2014.
5. G.P. 't Lam et al, 2014. Harvesting marine microalgae using flocculation, May 2014, presentation ICAB (Lyngby, Denmark) and ESBES+IFIBIOP (Lille, France).
6. G.P. 't Lam, E.K. Zegeye, M.H. Vermuë, D.M.M. Kleinegris, M.H.M. Eppink, R.H. Wijffels, G. Olivieri 2015. Dosage effect of cationic polymers on the flocculation efficiency of marine microalgae. Bioresource Technology. 198: 797-802

7. G.P. 't Lam, J.B. Giraldo, M.H. Vermuë, G. Olivieri, M.H.M. Eppink, R.H. Wijffels. (2016) Understanding the salinity effect on cationic polymers in inducing flocculation of the microalga *Neochloris oleoabundans*. Journal of Biotechnology. 225C: 10-17.
8. G.P. 't Lam, P.R. Postma, D.A. Fernandes, R.A.H. Timmermans, M.H. Vermuë, M. Barbosa, M.H.M. Eppink, R.H. Wijffels, G. Olivieri. (2017) Pulsed Electric Field for protein release of the microalgae *Chlorella vulgaris* and *Neochloris oleoabundans. Algal Research* 24:181-187
9. G.P. 't Lam, M.H. Vermuë, G. Olivieri, M.H.M. Eppink, R.H. Wijffels. Towards a better understanding of marine microalgae flocculation. Wageningen, the Netherlands: 16th Dutch National Biotechnology Conference (NBC-16), 2016. Oral presentation.
10. G.P. 't Lam, M.H. Vermuë, G. Olivieri, M.H.M. Eppink, R.H. Wijffels. Towards a better understanding of marine microalgae flocculation. Gent, Belgium, 12th Renewables Resources and Biorefineries (RRB-12), Poster presentation.
11. 't Lam G.P., Vermuë M.H., Olivieri G., Eppink M.H.M., Wijffels R.H. (2017) Pulsed Electric Field for Microalgae Cell Disruption. Oral presentation at the Netherlands Biotechnology Conference (NBC-2017) 'Biotechnology creating opportunities'.
12. 't Lam G.P., van der Kolk A.J., Chordia A., Vermuë M.H., Olivieri G., Eppink M.H.M., Wijffels R.H. (2017) Mild and selective protein release of cell wall deficient microalgae using pulsed electric field. *ACS Sustainable Chemistry & Engineering* 5:6046-6053
13. 't Lam G.P., Vermuë M.H., Eppink M.H.M., Wijffels R.H., van den Berg C. (2018) Multi-Product Microalgae Biorefineries: From Concept Towards Reality. Trends in Biotechnology 36:216-227
14. G.P. 't Lam (2017) Harvesting and Cell Disruption of Microalgae. PhD Thesis. Wageningen University. ISBN 978-94-6343-173-6
15. A.J.B. van Boxtel et al, 2014. Combining process design and LCA for algae production systems. Oral presentation at European workshop "LCA of Algal based biofuels and biomaterials" (2nd edition), 24 April 2014 in Brussels Belgium.
16. A.J.B. van Boxtel, P. Perez-Lopez, E. Breitmayer, P.M. Slegers, 2015. The potential of optimized process design to advance LCA performance of algae production systems. Applied Energy 154 (2015) 1122-1127.
17. F. Fasaei , J.H. Bitter, P.M. Slegers, A.J.B van Boxtel Techno-economic evaluation of microalgae harvesting and dewatering systems. Algal Research 31 (2018) 347–362
18. I. Houthoff, W. de Koster, L. van den Broek, A. van Boxtel, E. Slegers. Robustness of marine *Neochloris oleoabundans* paste during storage. Submitted.
19. Ying Du, Boelo Schuur, Sascha R. A. Kersten, Derk W. F. Brilman. 2015. Opportunities for switchable solvents for lipid extraction from wet algal biomass: an energy evaluation, Algal Research 11 (2015) 271–283
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24. Y. Du, B. Schuur, D.W.F. Brilman, Swing strategies for solvent recovery in wet lipid extraction processes 2019 (manuscript in preparation).
25. P.R. Postma, G.P. 't Lam, M.J. Barbosa, R.H. Wijffels, M.H.M Eppink, G. Olivieri. Microalgal biorefinery for bulk and high added value products: design and bottlenecks in product extraction within cell disintegration. Handbook of Electroporation, pp 1-20. D. Miklavčič (Ed.). Springer International Publishing, Switzerland.
26. P.R. Postma, E. Suarez Garcia, C. Safi, K. Yonathan, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink. Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates. Bioresource Technology, 2016
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PR for the project has been realized via the above mentioned publications and papers, WUR and RVO website, websites and other communications by project partners. Further PR opportunities can be realized via additional papers, and websites that offer the public report, partner communications and other activities.

Website AlgaePARC: http://www.algaeparc.com/project/2/algaeparc-biorefinery

Website RVO: https://www.rvo.nl/subsidies-regelingen/projecten/algae-parc-biorefinery-0

11 Availability of report, contacts

The public report is available free of charge as a pdf file from the coordinator Wageningen University, Chair group Bio Process Engineering at the address listed below. It can also be downloaded via the AlgaePARC website http://www.algaeparc.com/project/2/algaeparc-biorefinery
http://www.algaeparc.com/project/2/algaeparc-biorefinery

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