Användning av algbiomassa producerad på avfallsmaterial som substrat för bioetanol produktion med jäst

The use of algal biomass produced on waste material as substrate for bioethanol production with yeast

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Summary

In this project, we have investigated the possible use of microalgal biomass as substrate in ethanol producing fermentations. For growth and ethanol production the fermenting microorganism (yeast) need sugars, which are today commonly provided from agricultural and forestry products but these have at some points disadvantages. Algal biomass have here been investigated as an alternative sustainable source of raw material since; i) cultivations can be set up in places where food crops cannot grow thus not interfering with food production or leading to increased deforestation, ii) most microalgae do not contain lignin and therefore pretreatment will result in low or no formation of inhibitors, iii) microalgal cultivations can contribute to environmental benefits by capturing carbon dioxide from flue gas and utilize nutrients such as nitrogen and phosphorus from municipal waste water, resulting in purification of such waste streams together with production of energy-rich biomass.
From the investigations made in this project it was seen that the algal biomass cultivated on waste material, flue gas and waste water, can be used to provide fermentable sugars as substrates in fermentation. The most promising pretreatment method seemed to be weak acid hydrolysis in a thermal pretreatment process, which released up to 83 % sugars of the total content of carbohydrates. Pretreatment did not lead to formation of the inhibitors furfural or HMF, which is a major issue when dealing with pretreatment of lignocellulosic raw materials. Variations in species composition of the microalgal community in the production plant was investigated by terminal restriction fragment length polymorphism (T-RFLP) giving a finger print of the composition of the algal community. The T-RFLP analysis showed clear variations of species composition over cultivation season (May to October/November.

Sammanfattning

I detta projekt har vi undersökt potentialen för att använda mikroalgsbiomassa som substrat i etanolfermenteringar. För tillväxt och etanolproduktion med mikroorganismer (jäst) krävs en sockerkälla, vilken vanligtvis kommer från jord- eller skogsbruksprodukter men dessa har vissa nackdelar. Algbiomassa har här utretts som ett mer hållbart alternativ till dagens substrat då det visar på flera fördelar; i) odlingsanläggningarna kan upprättas på platser där de inte konkurrerar med odling av livsmedelsprodukter eller kräver ökad skogsavverkning, ii) de flesta mikroalger innehåller inget lignin, vilket medför att förbehandlingen resulterar i låg eller ingen bildning av inhiberande föreningar, iii) odling av mikroalgsbiomassa kan ge till flera positiva miljöeffekter genom att algerna kan utnyttja koldioxid från rökgaser samt näringsämnena kväve och fosfor från avloppsvatten, något som leder till en upprening av dessa avfallsströmmar samtidigt som det bildas energirik biomassa.
Från undersökningarna i detta projekt har vi sett att algbiomassa odlad på rökgas och avloppsvatten kan ge fermenterbara socker som substrat för etanolproduktion. Den mest lovande förbehandlingen var svag syrahydrolys i kombination med termisk förbehandling vilket frigjorde upp till ca 83 % socker av de totala kolhydraterna. Förbehandlingen gav inte upphov till bildande av inhibitorerna furfural eller HMF, vilket är ett stort problem vid förbehandling av lignocellulosa material. Variationer i artsammansättningen hos mikroalgspopulationen i produktionsanläggningen undersöktes med T-RFLP (terminal restriction fragment length polymorphism) vilken ger ett fingeravtryck av artsammansättningen. T-RFLP analysen visade tydliga variationer av artsammansättningen under odlingssäsongen (maj till oktober/november).

Background

This project was made in collaboration with dr Francesco Gentili at the department of Wildlife, Fish and Environmental Studies at the Swedish University of Agricultural Sciences (SLU) in Umeå, who is running the algal cultivation plant.
Microalgal biomass was cultivated in a development plant system on the rooftop of the heat and power plant Umeå Energi. Placing the development plant on the rooftop is beneficial since the energy source for microalgae is sunlight. Since algae capture carbon directly from carbon dioxide in the air, flue gas from the heat and power plant was used as carbon source and led into the algal cultivation. This results in reduced levels of carbon dioxide in the outgoing flue gas. To provide the algae with nutrients such as nitrogen and phosphorus, the system was continuously fed with municipal waste water (from the waste water treatment plant Umeva), which is rich in those nutrients. Thus, microalgal cultivation could be used as alternative purification steps for outgoing waste streams, with concomitant production of microalgal biomass. The aim of this project was to investigate whether this biomass is suitable in production of bioethanol.
The municipal waste naturally contains several algal species and microorganisms other than the alga used as initial inoculum in the plant, hence a continuous inflow will lead to a mixed and continuously variable population in the system. To assess this, part of the project was focused on investigating the variations in the species composition. We also aimed to investigate if there are any correlations between the species variations, the macromolecular content and the fermentability of the biomass. The experiments are performed on biomass samples harvested during three different cultivation seasons (2010, 2011 and 2012).

Goals/Introduction

This project investigates the potential of using microalgal biomass produced on waste streams as substrate in ethanol production. In the project we investigated i) macromolecular composition of biomass, ii) different pretreatment processes to yield fermentable sugars from the biomass, iii) fermentability and ethanol production in algal hydrolysates, vi) variations in species composition during the cultivation seasons.
For macromolecular composition we investigated total protein (modified Biuret method[1]) and total carbohydrate (acidic-phenol method[2]) contents. As pretreatment methods we investigated enzymatic pretreatment with amylases (Alphastar and Glucostar), cellulases/β-glucosidases (Novozyme188 and Celluclast) and an acid pretreatment with 5 % H_2SO_4 in a thermal process at 121˚C for 60 min. The total sugar release and the sugar composition in the hydrolysates as well as sugar consumption during fermentation were analyzed by ion chromatography (IC with high pH elution). The possible need for homogenization/mechanical pretreatment of the pre-dried biomass samples was also assessed. Fermentations in different hydrolysates were done to analyze the fermentability. The content of glycerol and some acids from acid pretreatment as well as the ethanol production during fermentations was monitored by HPLC. To investigate variations in species compositions of the samples a method called

[1] Lowry O, Rosebrough N, Farr A and Randall R, (1951), *DC Protein Assay Introduction Manual - Protein Measurement with the Folin Phenol Reagent,* Journal of Biological Chemistry, vol. 193, pp. 265-275
[2] Herbert D, Phipps PJ & Strange RE, (1971), *Chemical analysis of microbial cells*, Methods in Microbiology, vol 5B, pp. 209-344, Eds. JR Norris & DW Ribons. Academic Press, London.

terminal restriction fragment length polymorphism (T-RFLP) [3, 4] was used. The method is based on species variations in the 18S rRNA gene, which results in each sample getting a unique series of peaks, a “fingerprint”. The fingerprint corresponds to the species present in the sample and their relative amount. T-RFLP fingerprints of different samples from Umeå were compared both to each other as well as with those of monocultures of algal lab strains, to try to identify some of the present species.

Results

The samples used in this project were harvested during the cultivation seasons of 2010, 2011 and 2012; a total of 8 samples from each season were analyzed (numbered subsequently 1-8). The samples had a slightly different appearance from green to black/brown (see Appendix A: date of harvest, notation for each sample and a short description of each sample is presented).

Determination of macromolecular composition in biomass

The total content of proteins and carbohydrates of the different algae samples were analyzed and the results are presented in Figure 1, where the changes in content can be followed over the cultivation seasons. The pre-drying of the algal biomass samples from Umeå seem to have affected the biomass to become slightly hydrophobic, why homogenization/mechanical pretreatment by bead milling (in FastPrep) was needed to be done prior the measurements of total proteins. During seasons of 2011 and 2012 the variations in content were found to be small within a range of 0.1-0.2 g/g dry weight biomass for both total proteins and carbohydrates. During season 2010 the protein content was initially high at above 0.4 g/g, probably caused by the inoculum of *Scenedesmus dimorphus*. As the cultivation proceeded and other microorganisms entered the plant by the waste water the protein content decreased to the same level as found during the other seasons.

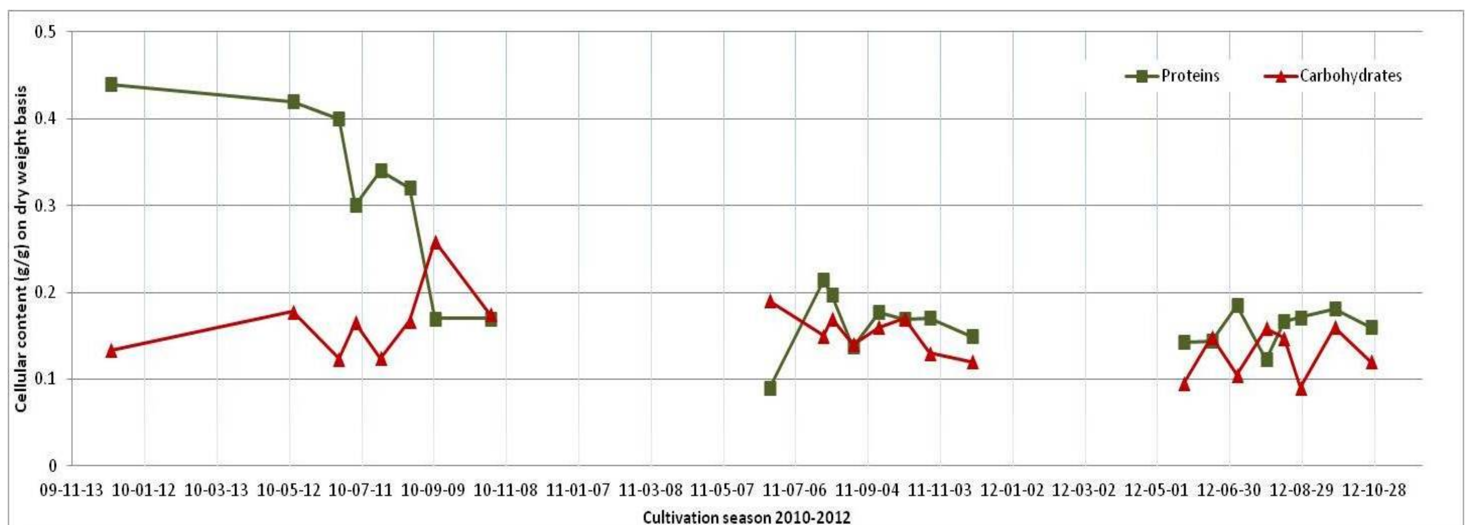

Figure 1. Variation in protein and carbohydrate content in algal biomass samples harvested at different time points over the cultivation seasons of 2010-2012.

[3] Joo S, Lee S-R & Park S (2010*) Monitoring of phytoplankton community structure using terminal restriction fragment length polymorphism (T-RFLP)*. J Microbiol Methods **81***:* 61–68

[4] A. Bérard, U. Dorigo, J.F. Humbert, F. Martin-Laurent (2005) *Microalgae community structure analysis based on 18 S rDNA amplification from DNA extracted directly from soil as a potential soil bioindicator*, Agronomie, 25 (2005), pp. 1–7

Investigation of different biomass pretreatment processes to yield fermentable sugars

Both enzymatic and acid pretreatment methods were investigated. Pretreatment with amylases gave very low sugar release, around 2-4 % of total carbohydrates, and was thus excluded from the further investigations. On the other hand, acid thermal pretreatment and treatment with cellulases/β-glucosidases resulted in a substantial release of sugars (Table 1 and 2). The results are presented as total sugar release per total carbohydrate and the proportion of C6 and C5 sugars in the released sugars.

Table 1 Sugars released from acid pretreatment and the composition of C5 and C6 sugars.

Sample	Total sugar release per total carbohydrate (g/g)	Proportion of C6 sugars in released sugars (g/g)	Proportion of C5 sugars in released sugars (g/g)
2010-1	0.39	0.90	0.10
2010-2	0.58	0.76	0.24
2010-3	0.46	0.82	0.19
2010-4	0.37	0.75	0.25
2010-5	0.38	0.84	0.16
2010-6	0.55	0.73	0.27
2010-7	0.39	0.76	0.24
2010-8	0.67	0.78	0.22
2011-1	0.48	0.79	0.22
2011-2	0.66	0.86	0.14
2011-3	0.65	0.86	0.14
2011-4	0.70	0.84	0.16
2011-5	0.55	0.80	0.20
2011-6	0.42	0.78	0.23
2011-7	0.56	0.81	0.19
2011-8	0.62	0.76	0.24
2012-1	*	0.84	0.16
2012-2	0.64	0.81	0.19
2012-3	0.83	0.82	0.18
2012-4	0.60	0.80	0.20
2012-5	0.60	0.77	0.23
2012-6	0.77	0.80	0.20
2012-7	0.34	0.86	0.14
2012-8	0.52	0.87	0.13

*higher sugar release from acid pretreatment were measured than from total carbohydrates measurement per dry weight biomass, why the value of released sugars/available carbohydrates for this sample cannot be determined

The sugars release during acid pretreatment varied between 35-83 % of the total available carbohydrates, and contained about 75-85 % of C6 sugar units. Of the C6 sugars there were approx. the same level of glucose, mannose and galactose. Also rhamnose was found in the hydrolysate. There were no detectable levels of furfural or HMF in any of the acid pretreated hydrolysates.

Table 2 Sugar released from enzymatic pretreatment and the composition of C5 and C6 sugars.

Sample	Total sugar release per total carbohydrate (g/g)	Proportion of C6 sugars in released sugars (g/g)	Proportion of C5 sugars in released sugars (g/g)
2010-1	0.33	0.99	0.01
2010-2	0.33	0.94	0.06
2010-3	0.34	0.95	0.05
2010-4	0.29	0.93	0.07
2010-5	0.20	0.96	0.04
2010-6	0.22	0.96	0.05
2010-7	n.a	n.a	n.a
2010-8	0.12	0.96	0.04
2011-1	0.18	0.95	0.06
2011-2	0.37	0.98	0.02
2011-3	0.39	0.99	0.01
2011-4	0.42	0.99	0.01
2011-5	0.51	0.95	0.05
2011-6	0.34	0.94	0.06
2011-7	0.38	0.95	0.05
2011-8	0.46	0.93	0.07
2012-1	0.68	0.96	0.04
2012-2	n.a	n.a	n.a
2012-3	n.a	n.a	n.a
2012-4	n.a	n.a	n.a
2012-5	n.a	n.a	n.a
2012-6	0.47	0.94	0.06
2012-7	0.28	0.97	0.03
2012-8	0.45	0.98	0.02

n.a - data not available

The sugar release during enzymatic pretreatment varied between 12-68 % of the total available carbohydrates, and contained 90-99 % of C6 sugar units, which was compared to acid treatment a smaller release with a higher proportion of C6 sugars.

For the samples 2010-7 and 2012-2 to 2012-5 the sugar release from enzymatic pretreatment was very low. Those samples contained some Penicillin-G resistant microorganisms (microscopic investigation, data not shown) that probably consumed the released sugars during the enzymatic pretreatment. Analysis of the enzymatic hydrolysate from those 5 samples showed high levels of lactic acid which further strengthen the belief that the present microorganisms consumed the released sugars. The other samples had no detectable content of lactic acid.

There were no detectable levels of furfural or HMF in any of the enzymatically or acid pretreated hydrolysates. Enzymatic pretreatment results in higher relative content of fermentable C6 sugar units, but the acid pretreatment results in much higher overall sugar release. From HPLC analysis of the acid pretreated hydrolysate there is glycerol present at around 2-2.7 g/l (corresponding to 0.02-0.024 g glycerol /g dry weight biomass) and acetate around 0.4-1.6 g/l (corresponding to 0.004-0.014 g acetate /g dry weight biomass).

Analysis of fermentability and ethanol production in algal hydrolysates

The acid hydrolysates were pH adjusted before fermentation, whereas the enzymatically pretreated hydrolysates were used untreated. For the fermentation the yeast *Saccharomyces cerevisiae*, strain Ethanol red, was used, which is an industrial yeast strain used for ethanol production. As all natural *S. cerevisiae* strains the strain used can only consume C6 sugars (glucose, mannose and galactose) but not the C5 sugars (xylose and arabinose). The fermentations were done in cultures of 2-4 ml for the acid pretreated hydrolysates and 0.8-1.3 ml for the enzymatically pretreated hydrolysates at 30°C and followed for a total time of 95 h. As control, fermentation was also performed in 2.5 ml of YPD (yeast extract, peptone, dextrose) media with a glucose concentration of 5 g/l, which was the same approx. concentration as in the hydrolysates. All yields were calculated based on the C6 consumption (presented in Table 3). In general, fermenations in enzymatic treated hydrolysates gave higher ethanol yields than when the same biomass was acid treated. Most ethanol yields were close or higher that the fermentation in YPD indicating no or very little inhibiting effect of the hydrolysates.

Table 3 Ethanol yields in the different hydrolysates after fermentation with *S. cerevisiae* Ethanol Red. Cultivation in YPD with 5g/l glucose was used as reference and gave a yield of 0.27 g ethanol /g consumed C6 sugars.

Sample	Ethanol yield on consumed C6 sugars in acid hydrolysate (g/g)	Ethanol yield on consumed C6 sugars in enzymatic hydrolysate (g/g)
2010-1	0.18	n.a.
2010-2	0.16	n.a.
2010-3	0.27	n.a.
2010-4	0.37	n.a.
2010-5	0.32	n.a.
2010-6	0.21	n.a.
2010-7	0.13	n.m.
2010-8	0.27	n.a.
2011-1	*	0.40
2011-2	0.31	0.35
2011-3	0.30	0.34
2011-4	0.33	0.38
2011-5	0.32	0.33
2011-6	0.14	0.43
2011-7	*	0.35
2011-8	0.25	n.a.
2012-1	0.29	0.37
2012-2	0.32	n.m.
2012-3	*	n.m.
2012-4	0.30	n.m.
2012-5	0.20	n.m.
2012-6	0.24	0.43
2012-7	0.22	0.41
2012-8	0.36	0.38

n.a - Data not available, not enough material of samples to perform fermentation, n.m. – Data not available, unsuccessful enzymatic hydrolysis, see also section about enzymatic treatment, * Yields could not be determined due to low sugar consumption during fermentation

Investigation of changes in species composition during the cultivation season

The variations in species composition of samples from cultivation season 2011 and 2012 were investigated using the T-RLFP method. Two different sets of primers were used. Primer pair 1 (PP1) is a universal eukaryote pair consisting of the forward and reverse primers called P45 and P47, respectively in work by Bérard et al 2005[4]. Primer pair 2 (PP2) is an algae-specific primer pair consisting of the forward and reverse primers called P73 and P47 respectively by Bérard et al 2005[4].

The forward primers were labeled with 6-FAM fluorescence marker. Frozen liquid biomass samples were used for DNA extraction, followed by amplification and purification from 1.5 % agarose gels and finally enzymatic digested using the FastDigest MspI (Fermentas). The resulting DNA fragments were separated by capillary electrophoresis (separation performed by KI, Solna) where the fragments containing the fluorescence dye was detected. The method is based on species variations in the 18S rRNA gene, which results in each sample getting a unique series of peaks, a "fingerprint".

The results of the T-RFLP from the mixed population samples from Umeå were compared to pure monocultures of selected algae species. The yeast *Saccharomyces cerevisiae* was used as a reference for a eukaryotic microorganism not belonging to the algal families to compare the specificity of the universal PP1 with the algae specific PP2.

The different primer pairs gave different peak patterns, where some peaks could be correlated to the reference algae peaks while some peaks remain unidentified. Table 4 and 5 presents the *identified* peaks from the different mixed cultures with primer pair 1 (PP1) and primer pair 2 (PP2) respectively, presented as the percentage of the total peak areas in the sample. All peaks with an area below 100 were excluded from calculations, since this is in the range of the background. The results are based on the peak *area* and not the peak height since others have previously used this procedure[3]. Sample 2012-6 was not sent for analysis since the amplification was unsuccessful with both primer pairs.

Table 4. Presence in percentage (%) of some algae species in different samples harvested during the cultivation seasons 2011 and 2012, analyzed by the T-RFLP method using the general eukaryotic primer pair 1 (PP1).

Primer pair 1	*Dictyosphaerium pulchellum*	*Scenedesmus obliquus + dimorphus*	Yeast	Total area units
2011-2	98			20255
2011-3				26321
2011-4	100			2284
2011-6	4			3934
2011-7				833
2011-8				612
2012-1			24	3584
2012-2			3	4862
2012-3	36	23		5096
2012-4	46	7		3789
2012-5				4660
2012-7		70	6	5832
2012-8		60		1266

Table 5. Presence in percentage (%) of some algae species in different samples harvested during the cultivation seasons 2011 and 2012, analyzed by the T-RFLP method using the algae specific primer pair 2 (PP2).

Primer pair 2	*Dictyosphaerium pulchellum*	*Scenedesmus obliquus + dimorphus*	*Chlorella emersonii*	*Chlorella sorokiniana*	Total area of peaks
2011-2	97	1	2		19328
2011-3				99	17139
2011-4	72				401
2011-5	18		82		624
2011-6	76		20	5*	6151
2011-7	100				145
2011-8			100		113
2012-1	31		19	32*	618
2012-2	34		35	31*	1681
2012-3	75	8	11	6*	4998
2012-4	85	6	10		2080
2012-5	21	32	33	14*	1880
2012-7		84	17		885
2012-8		100			384

* the mixed samples show peaks that correspond to one of the two peaks that are in the profile for *C. sorokiniana*

PP1 gave clear profiles for the *Chlorella* species, *Ankistrodesmus falcatus*, *Nitzschia epithymoides* and *Navicula pelliculosa*, but none of the mixed samples showed any of those peaks. For PP2 there were no profiles for *N. epithymoides*, *N. pelliculosa* or yeast. PP2 showed a clear fingerprint for *A. falcatus* but none of the mixed samples contained those peaks. Sample 2011-1 gave no peaks with either PP1 or PP2 and sample 2011-5 gave no peaks with PP1.

Primer pair 1 resulted in a higher overall DNA concentration compared to primer pair 2 for all samples. The peaks from using PP1 show presence of several peaks that cannot be explained by the chosen algae standards. Those peaks can correspond either to algae species other than the ones used as standards, but also to eukaryote microorganisms other than algae.

PP2 did, as expected, not amplify the DNA from the diatoms *N. epithymoides* and *N. pelliculosa* or the yeast *Saccharomyces cerevisiae*. The analysis with PP2 shows more or less no presence of unidentifiable peaks, which is related to the fact that this primer pair do not amplify many other eukaryotes than algae[4], resulting in lower total number of peaks. All algae peaks that were identified for PP1 were also identified for PP2, confirming peak correlation results. Using PP2 also gives separate peak patterns for the two *Chlorella* species (which PP1 did not) and allows separate identification of those two.

There is presence of the inoculum species *S. dimorphus* in several samples, even if it often seems to be outgrown by *D. pulchellum* to a large extent, and occasionally also by *C. emersonii* (Table 5).

The similarity in peak patterns for species of the same genus (e.g. for *Scenedesmus* species with PP1 and PP2 and *Chlorella* species for PP1) indicates that the peaks that are identified here could correspond to an additional species within the genus. To visualize how the T-RFLP results for the different primers and standards may look, some of the peak charts are presented in Appendix B.

Conclusions

Microalgal biomass is a promising alternative/complement to provide substrates for ethanol production since

- Pretreatment of the algal biomass result in no formation of the inhibiting compounds HMF and furfural
- Ethanol yields from algal hydrolysate is in the same range or above compared to YPD reference of 5g/l glucose
- Production of algal biomass is in many ways more sustainable and environmental friendly compared to production of conventional lignocellulosic substrates.

According to T-RFLP results, samples 2011-2, 2011-4 and 2012-4 show high content of *D. pulchellum*, those are also some of the samples with high ethanol yields from fermentation in the algal hydrolysates.

Publications from the project

- Poster: Qvirist L, Gentili F, Albers E (2012) *Bioethanol production using algal biomass produced on waste material as substrate*, Presented on Life Science Engineering Area of Advance Conference "From Human Health to Biosustainability – Future Challenges for Life Science at Chalmers" the 19th of November 2012, Gothenburg

Appendix A

Table A1. Samples of algal biomass that has been used in this project, as well as the date when they were harvested, a notation to be used in the text and a short description of the appearance of the sample.

Sample harvest date	Sample notation	Sample appearance information
2009-12-15	2010-1	Green
2010-05-15	2010-2	Brownish
2010-06-22	2010-3	Brownish
2010-07-06	2010-4	Brownish
2010-07-27	2010-5	Brownish
2010-08-20	2010-6	Brownish
2010-09-10	2010-7	Brownish
2010-10-26	2010-8	Brownish
2011-06-14	2011-1	Brown
2011-07-29	2011-2	Black, dense and "heavy"
2011-08-05	2011-3	Black, dense and "heavy"
2011-08-23	2011-4	Black, dense and "heavy"
2011-09-13	2011-5	Black
2011-10-04	2011-6	Black
2011-10-25	2011-7	Black
2011-11-29	2011-8	Black
2012-05-23	2012-1	Grey, hard
2012-06-15	2012-2	Brown/black
2012-07-06	2012-3	Brown/black
2012-07-30	2012-4	Brown/black
2012-08-14	2012-5	Green, very fluffy, fibrous and cotton-like
2012-08-28	2012-6	Black, little fluffy
2012-09-25	2012-7	Green
2012-10-25	2012-8	Brown/green

All samples were dried and homogenized in a grinder. Large variations in color, density and texture indicate presence of different microorganisms over the cultivation season.

Appendix B

Figures 1a-d show the results for sample 2011-2, 2012-3, *D. pulchellum* and *Scenedesmus sp.* amplified with primer pair 1.

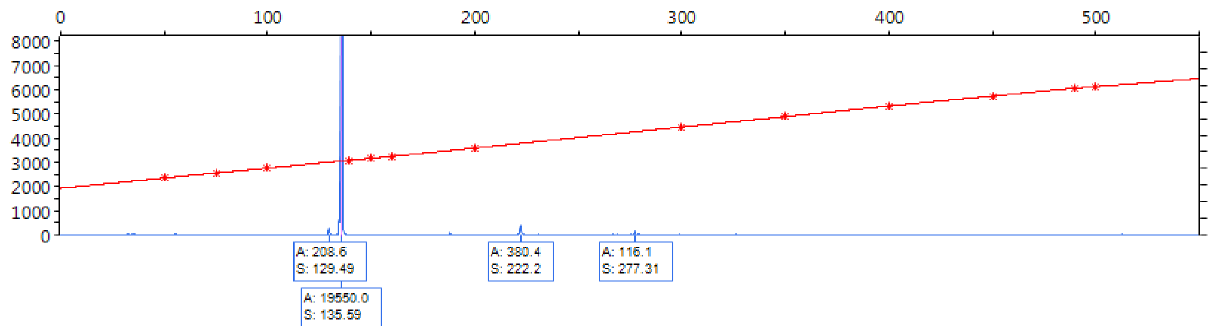

Figure 1a showing the T-RFLP result for sample 2011-2 using primer pair 1.

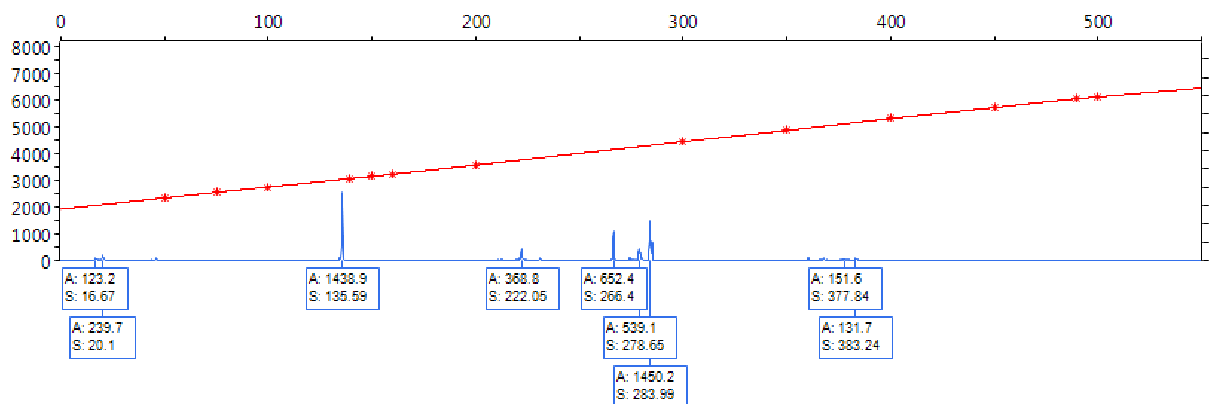

Figure 1b showing the T-RFLP result for sample 2012-3 using primer pair 1.

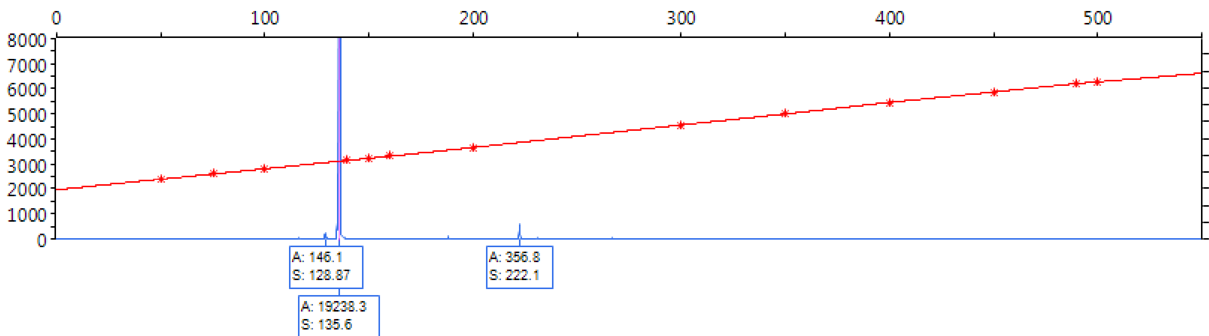

Figure 1c showing the T-RFLP result for *D.pulchellum* using primer pair 1.

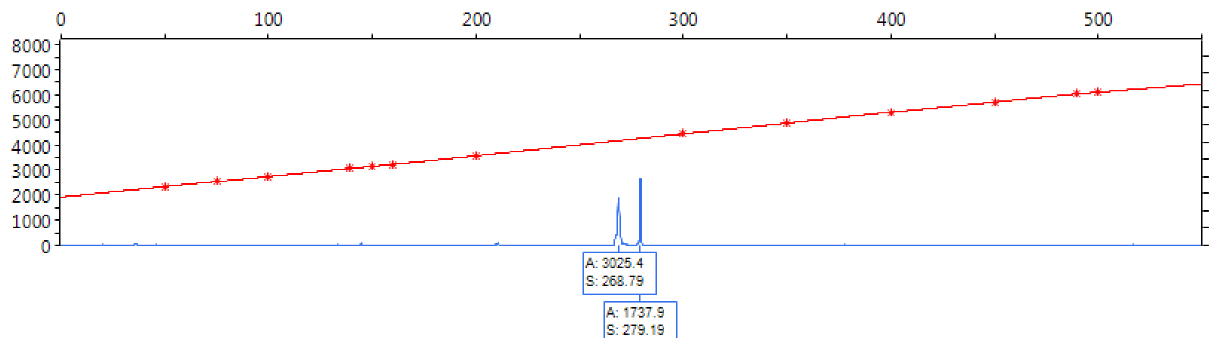

Figure 1d showing the T-RFLP result for *Scenedesmus sp.* using primer pair 1.

Figures 2a-d show the results for sample 2011-2, 2012-3, *D. pulchellum* and *Scenedesmus sp.* amplified with primer pair 2

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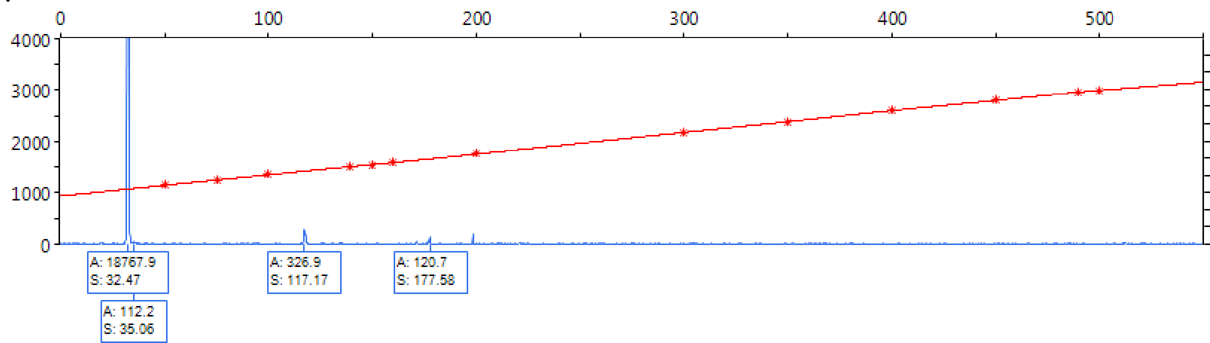

Figure 2a showing the T-RFLP result for sample 2011-2 using primer pair 2.

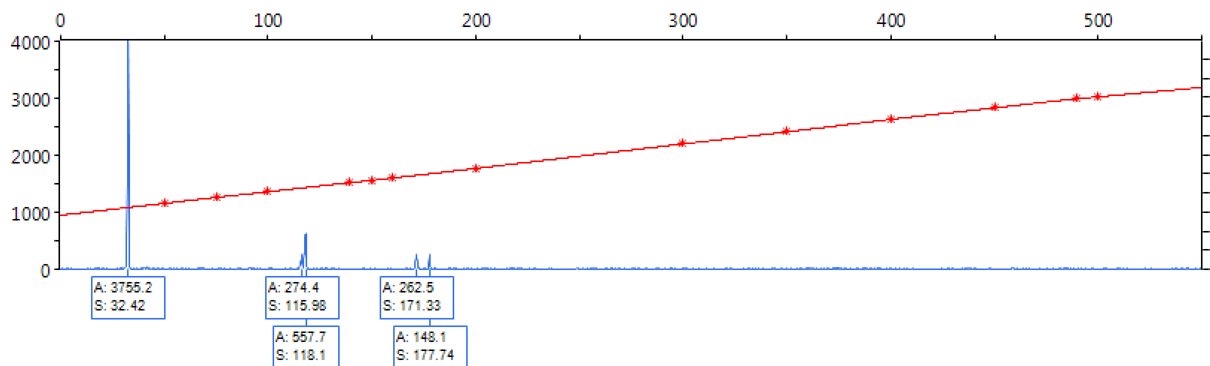

Figure 2a showing the T-RFLP result for sample 2012-3 using primer pair 2.

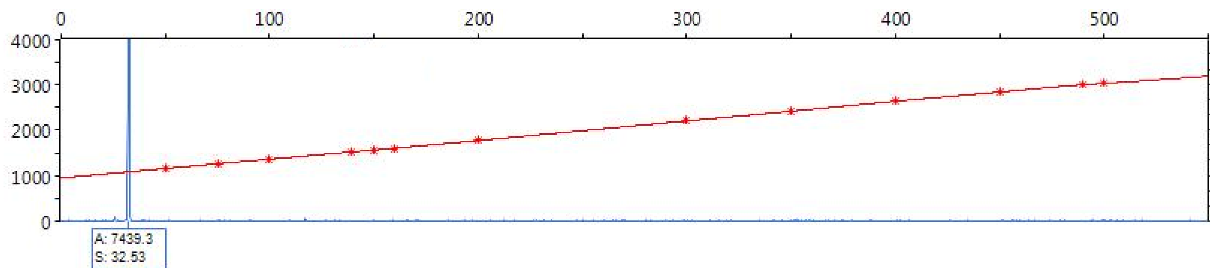

Figure 2a showing the T-RFLP result for *D.pulchellum* using primer pair 2.

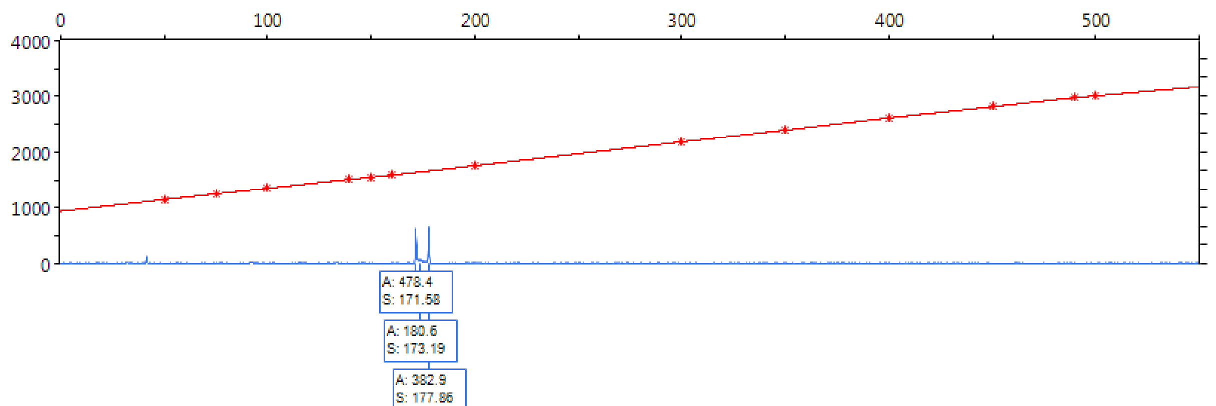

Figure 2a showing the T-RFLP result for *Scenedesmus sp.* using primer pair 2.