

# Enzymatic extraction of laminarin from brown seaweed *Ecklonia maxima*

*by*

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## Abstract

Response surface methodology was used to investigate the enzymatic extraction of  $\beta$ -glucan laminarin from *Ecklonia maxima*, a South African kelp. A commercial cellulase (Celluclast® 1.5L, “Celluclast”) was used to hydrolyse the seaweed material in the range of 0 to 4.0% (*v/dw*) enzyme dosage, pH 3.0 to 6.0, and 40 to 60 °C. Samples were taken at five evenly-spaced points over six-hour hydrolysis experiments. A spectrophotometric endo-1,3- $\beta$ -D-glucanase assay for laminarin was developed, and samples were quantified for this response and various others. Response surfaces were regressed for solubilised yield (including supernatant dissolved solids, supernatant mass fraction, and pellet-solids loading), and the concentrations of laminarin, reducing sugars, inorganic sulfates, total phenolics, and antioxidant capacity in the hydrolysate supernatant.

Response surfaces were validated, with laminarin extraction shown to be significantly influenced by pH and temperature linear and quadratic terms, but not by enzyme dosage. Reducing sugar and inorganic sulfate concentrations, solubilised yield, supernatant mass fraction, and pellet-solids loading showed the significant effect of the linear enzyme-to-substrate term. Total phenolics and antioxidant capacity, in contrast, were significantly influenced by temperature and pH only. Comparisons to alternate carbohydrase extraction (Accellerase® 1500, “Accellerase”) showed Celluclast to perform similarly to Accellerase in all responses. Further comparison to dilute-acid thermal (“conventional”) hydrolysis (pH 1.0 and 70 °C) showed that enzymatic extraction methods were superior for the release of reducing sugars, inorganic sulfate, and solubilised yield (after 4.5 hours). Conventional extraction was shown to be superior to enzymatic extraction methods for laminarin (when measured with the developed spectrophotometric assay) and antioxidant capacity.

Comparison between kelp batches (May 2018 and June 2019 harvests) showed laminarin to be present in higher amounts in May 2018. Solubilised yield, reducing sugars, and supernatant mass fraction also measured significantly higher in May 2018 while inorganic sulfates, total phenolics, and antioxidant capacity were higher in June 2019.

Differences were found between the spectrophotometric results of the developed laminarin enzymatic assay and the HPLC quantification of glucose in the ethanol-precipitated polysaccharide-rich fractions of the conventional, Celluclast, and Accellerase hydrolysed samples. These differences were theorised to be caused by either the inhibition of the 1,3- $\beta$ -D-endoglucanase enzyme by various bioactive components in the enzymatic extracts (polyphenols, phlorotannins, and alginate), or the contamination of the HPLC results with cellulose-derived glucose. The assays showed similar readings when samples



were conventionally hydrolysed (27 to 39 mgLE·gDW<sup>-1</sup> and 36 to 39 mgGIE·gDW<sup>-1</sup> for spectrophotometric and HPLC respectively) but enzymatic and raw extracts measured with the developed spectrophotometric assay were lower in comparison. The laminarin content of the raw supernatant was determined as  $54.7 \pm 12.3$  mg·gDW<sup>-1</sup>(n = 3), compared to the spectrophotometric measurement of  $6.7 \pm 0.1$  mgLE·gDW<sup>-1</sup>(n = 3). Enzymatic extraction showed no significant changes from the readings for raw material with either analysis technique, and HPLC measurement showed conventional extraction to decrease laminarin content. None of the treatment types tested increased the yield of laminarin over that found in the raw material.

Further work on additional batches of *E. maxima* is required to ascertain the effect of enzymatic extraction on laminarin, and an additional 1,6-β-D-glucanase should be included in the analytical enzyme for spectrophotometric laminarin measurement. Inhibition of the 1,3-β-D-glucanase enzyme should be studied, and HPLC columns capable of polysaccharide separation and quantification should be considered.

**Keywords:** Laminarin, enzymatic hydrolysis, *Ecklonia maxima*, brown seaweed, solubilisation, response surface methodology

## Opsomming

Respons oppervlak metodologie is gebruik om die ensimatiese ekstrahering van  $\beta$ -glukaan laminarien uit *Ecklonia maxima*, 'n Suid-Afrikaans kelp, te ondersoek. 'n Kommersiële sellulase (Celluclast® 1.5L, “Celluclast”) is gebruik om seewiermateriaal te hidroliseer in die bestek van 0 tot 4.0% (v/dw) ensiemdosering, pH 3.0 tot 6.0, en 40 to 60 °C. Steekproewe is by elke vyf gelyk-gespasieerde punte oor ses-uur hidrolise eksperimente geneem. 'n Spektrofotometriese endo-1,3- $\beta$ -D-glukanase proef vir laminarien is ontwikkel en steekproewe is gekwantifiseer vir hierdie respons en verskillende ander. Respons oppervlaktes is met regressie gepas vir opgeloste opbrengs (insluitend bodrywende opgeloste vaste stowwe, bodrywende massafraksie, en pellet-vaste-stof-lading), en die konsentrasies van laminarien, vermindering van suikers, anorganiese sulfate, totale fenoliese komponente, en antioksidantkapasiteit in die hidrolisaat bodrywende stof.

Respons oppervlaktes is gevalideer, waar aangetoon is dat laminarienekstrahering beduidend deur pH en temperatuur liniêre en kwadratiese terme beïnvloed word, maar nie deur ensiemdosering nie. Reduserende suikers en anorganiese sulfaatkonsentrasies, opgeloste opbrengs, bodrywende massafraksie, en pellet-vaste-stof-lading het die beduidende effek van die liniêre ensiem-tot-substraat term aangetoon. Totale fenoliese komponente en antioksidantkapasiteit, in kontras, is beduidend beïnvloed deur slegs temperatuur en pH. Vergelykings met alternatiewe koolhidrase ekstrahering (Accellerase® 1500, “Accellerase”) het aangetoon dat Celluclast soortgelyk aan Accellerase in alle response optree. Verdere vergelyking met verdunde-suur termiese (“konvensionele”) hidroliese (pH 1.0 en 70 °C) het aangetoon dat ensimatiese ekstraheringmetodes superieur is vir die vrystelling van gereduseerde suikers, anorganiese sulfaat, en opgeloste opbrengs (na 4.5 ure). Konvensionele ekstrahering is bewys om superieur tot ensimatiese ekstraheringmetodes vir laminarien (as gemeet word met die ontwikkelde spektrofotometriese toets) en antioksidantkapasiteit te wees.

Vergelyking tussen kelplote (Mei 2018- en Junie 2019-oeste) het aangetoon dat laminarien teenwoordig was in hoër hoeveelhede in Mei 2018. Opgeloste opbrengs, gereduseerde suikers, en bodrywende massafraksie is ook beduidend hoër gemeet in Mei 2018, terwyl anorganiese sulfate, totale fenoliese komponente, en antioksidantkapasiteit hoër was in Junie 2019.

Verskille is gevind tussen die spektrofotometriese resultate van die ontwikkelde laminarien ensimatiese toets en die HPLC-kwantifisering van glukose in die etanol gepresipiteerde polisakkariedryke fraksies van die konvensionele, Celluclast en Accellerase gehidroliseerde steekproewe. Hierdie verskille is getooritiseer om veroorsaak te word deur of die inhibisie van die 1,3- $\beta$ -D-endoglukanase ensiem by verskeie bio-aktiewe komponente in die ensimatiese ekstrakte (polifenole, florotanniene en alginaat), of

die kontaminasie van die HPLC-resultate met sellulose-afgeleide glukose. Die toetse het soortgelyke lesings aangetoon as steekproewe konvensioneel gehidroliseer is ( $27\text{--}39 \text{ mgLE}\cdot\text{gDW}^{-1}$  en  $36\text{--}39 \text{ mgGLE}\cdot\text{gDW}^{-1}$  vir spektrofotometrie en HPLC onderskeidelik) maar ensimatiese en rou ekstrakte gemeet met die ontwikkelde spektrofotometriese toets was laer in vergelyking. Die laminarieninhoud van die rou bodrywende stof is bepaal as  $54.7 \pm 12.3 \text{ mg}\cdot\text{gDW}^{-1}$  ( $n=3$ ), in vergelyking met die spektrofotometriese mate van  $6.7 \pm 0.1 \text{ mgLE}\cdot\text{gDW}^{-1}$  ( $n=3$ ). Ensimatiese ekstrahering het geen beduidende veranderinge uit die lesings vir rou materiaal met beide analisetegnieke aangetoon nie, en HPLC-meting het gewys dat konvensionele ekstrahering laminarieninhoud verminder. Geen behandelingstipes getoets het die opbrengs van laminarien verhoog in vergelyking met die in die rou materiaal gevind nie.

Verdere werk op addisionele lotte van *E. maxima* word benodig om die effek van ensimatiese ekstrahering op laminarien vas te stel, en 'n addisionele 1,6- $\beta$ -D-glukanase ensiem moet by die analitiese ensiem vir spektrofotometriese laminarienaafmeting ingesluit word. Inhibisie van die 1,3- $\beta$ -D-glukanase ensiem moet bestudeer word en HPLC-kolomme geskik vir polisakkarieskeiding en kwantifisering moet oorweeg word.

**Keywords:** Laminarien, ensimatiese hidrolise, *Ecklonia maxima*, kelp, ontbinding, Respons oppervlak metodologie

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## Glossary

**AEC** – Anion-exchange chromatography

**CCD** – central composite design

**Chlorophyte/Chlorophyta** – Green seaweed

**DNS** – Dinitrosalicylic acid

**EAE** – Enzyme-assisted extraction

***Ecklonia maxima*** – Endemic South African kelp

**HPLC** – High performance liquid chromatography

**HSD** – Honest significant difference

**IEC** – Ion-exchange chromatography

**LC-MS** – Liquid chromatography – mass spectrometry

**MAE** – Microwave-assisted extraction

**MWCO** – Molecular weight cut-off

**Phaeophyte/Phaeophyta** – Brown seaweed

**PLE** – Pressurised liquid extraction

**Rhodophyte/Rhodophyta** – Red seaweed

**RSM** – Response surface methodology

**SEC** – Size-exclusion chromatography

**SFE** – Supercritical-fluid extraction

**TCA** – Trichloroacetic acid

**UAE** – Ultrasound-assisted extraction

**UAEH** – Ultrasound-assisted enzymatic hydrolysis

## Nomenclature

Variable	Symbol	Unit
Antioxidant capacity	$AO$	$\text{gTE}\cdot\text{l}^{-1}$
Inorganic sulfates	$S$	$\text{gSE}\cdot\text{l}^{-1}$
Laminarin	$L$	$\text{gLE}\cdot\text{l}^{-1}$
Reducing sugars	$RS$	$\text{gGLE}\cdot\text{l}^{-1}$
Pellet-solids loading	$SL_P$	%
Supernatant dissolved solids	$DS$	%
Solubilised yield	$SY$	%
Supernatant fraction	$F_{SN}$	%
Total phenolics	$TP$	$\text{gGAE}\cdot\text{l}^{-1}$





# Chapter 1

## Introduction

### 1.1. Nature and scope of project

Marine macroalgae (seaweeds) contain unique compounds not found in terrestrial plants (Pulz and Gross, 2004). Different extraction techniques exist to obtain these compounds in economically viable amounts, and the exploitation of macroalgae-derived products extends to industries including foods, pharmaceuticals, cosmetics, etc. (Wijesinghe and Jeon, 2012b).

Phaeophytes (brown seaweeds) have their own unique compounds. The three main polysaccharides unique to the brown seaweeds are fucoidan, alginic acid, and laminarin. All three have been isolated and shown to have various bioactive properties (Kadam et al., 2015b; Wijesinghe and Jeon, 2012b). *Ecklonia maxima*, a fast-growing endemic South African giant kelp, is of particular economic interest due to its abundance and ease of harvest (Branch et al., 2007). Agricultural biostimulants derived from *E. maxima* (Kelpak®) have been produced in South Africa since 1978 (Amosu et al., 2015).

Laminarin is the main storage polysaccharide of brown seaweeds. It is classified as a (1,3;1,6)- $\beta$ -glucan, and is mainly found intracellularly in the vacuoles of seaweed cells. It is linear along the 1,3- $\beta$ -bonded polysaccharide, with occasional 1,6- $\beta$ -branching determining its solubility. Less frequently branched forms are only soluble at increased temperatures (50 to 90 °C) (Shin et al., 2009). These molecules play a role in the mannitol metabolism of brown seaweeds and exhibit various physiological effects in mammals, including anti-tumour, anti-inflammatory and prebiotic action (Groisillier et al., 2014; Kadam et al., 2015b). As a product, it may show potential in the sphere of nutritive and functional food additives and co-extraction alongside fucoidan could create a valuable seaweed extract for the same markets.

### 1.2. Motivation

Traditional chemical extraction of polysaccharides relied on mineral acids or bases. Temperatures up to 90 °C have also been used to obtain biological compounds in solution. High temperature and extreme pH are both considered to be “harsh” conditions. Harsh chemical extraction methods have the potential to degrade biological compounds, and the biological functionalities for which they are valued (Li et al., 2006). From a commercial perspective, a move to milder extraction conditions (if economically viable)

would increase the biological integrity of extracted compounds (and therefore compound value). Increased research of alternative extraction techniques such as enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) has taken place for general bioactive compounds (Kadam et al., 2013). These methods do not rely solely on mineral acids and bases and their increased use stems from a conscious move towards safer, more efficient, and more environmentally conscious extraction and production methods.

EAE has shown promise with the production of enhanced biofunctional hydrolysates from macroalgae (and therefore more valuable) (Wijesinghe and Jeon, 2012a). The high specificity of enzymatic hydrolysis allowed the integrity of non-targeted molecules to be preserved. Cellulases should exhibit no enzymatic action towards the 1,3- and 1,6- $\beta$ -D-glucan links in laminarin, making co-extraction of laminarin from brown seaweed alongside cell-wall-entrapped molecules (particularly fucoidan) theoretically possible when this enzyme class is used. Such an approach would make it possible to extract higher worth extracts from brown seaweed species, where laminarin conserves its biological activity.

### **1.3. Method of investigation**

Enzymatic hydrolysis of seaweed with a commercial cellulase enzyme was studied in order to optimise laminarin extraction. Response surface methodology was used as the statistical tool of choice when constructing the experimental design (central-composite design). The measurement of fucoidan in this study was considered an additional response. The method of investigation is shown in Figure 1-1 following.

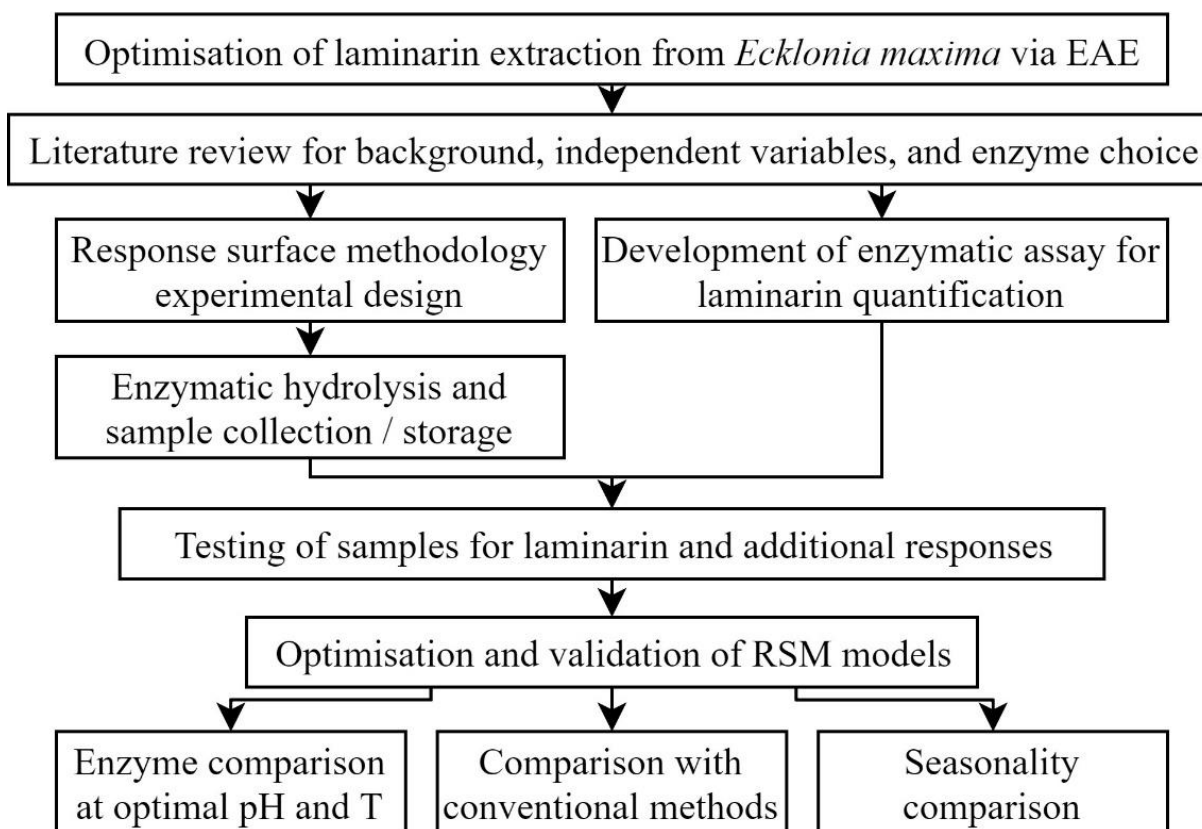


Figure 1-1 Flow diagram illustrating the method of investigation

## 1.4. Thesis structure

This document is structured as a series of nine chapters. Chapter 2 presents background and a literature review pertaining to the proposed problem. Chapter 3 covers the aims, objectives and deliverables of the project. Experimental sampling methodologies and protocols are presented in Chapter 4, with project objectives presented one-by-one from Chapter 5 to 8. Conclusions and recommendations are presented last (Chapter 9).



# Chapter 2

## *Literature Review*

### **2.1. Background and history of seaweed as nutrition**

Consumption of certain edible seaweeds has been a part of eastern culture for many years, both as a culinary ingredient, and as traditional medicine (Norziah and Ching, 2000). There has been a drive towards the use of macroalgae in biofuel production due to their agricultural advantages over terrestrial feedstocks (Smith et al., 2010). The large scale required for profitability in such endeavours serves as the impetus for higher worth, more profitable products from the seaweed. In recent years, a focussed line of inquiry was directed towards the specific bioactive ingredients found in these algae, along with their potential as functional foods or “nutraceuticals” (Freile-Pelegrin et al., 2008)

Algae are classed as non-complex, photosynthesizing organisms. There are both microscopic and macroscopic members of this group (Garson, 1989). Seaweeds (marine macroalgae) can be divided into three taxonomic groups: Chlorophyta (green algae), Rhodophyta (red algae), and Phaeophyta (brown algae), all with unique bioactive compounds of their own (Dawczynski et al., 2007). Polysaccharides are one subset of the bioactive compounds available from seaweeds, with some exhibiting hydrocolloid properties, and many (like laminarin) exhibiting various bioactivities (Mohamed et al., 2012).

Found in the cell vacuoles of brown seaweeds, the laminarin molecule plays a storage role. It is reported to constitute up to 35% of the dry weight of some Phaeophytes, with a heavy dependence on season and environmental factors (Kadam et al., 2015b). Reported bioactivities include anti-inflammatory, antitumour, anticoagulant, antioxidant and anti-apoptotic functions (Choi et al., 2012; Lee et al., 2012; Rioux et al., 2010). The relative abundance and reported biological and physiological activities of laminarin may have the potential to push the substance to wider commercialisation. As with any product, commercialisation is likely to be dependent on economic factors such as legislation, feedstock pricing, and processing costs. Focus must first be put on extraction studies and clinical trials before commercialisation prospects become clear (Okolie et al., 2017).

The shortage of agar in Britain during the Second World War catalysed the birth of the South African seaweed industry (Anderson et al., 1989). The industry exported raw materials initially and did not

produce higher value products. Symbiosis with abalone farming is likely to increase the farming of marine macroalgae resource in the future (Rothman et al., 2006)

## **2.2. Global seaweed markets**

High worth compounds such as unique polysaccharides are among the more attractive, higher value products available from seaweeds, although crude extracts are also in high demand. Local markets must provide higher worth products in order to compete in the global economy – this includes both local harvesting and local processing.

### **2.2.1. International seaweed markets**

In 2013, the global industry for macroalgae was worth approximately US\$ 7 billion (Lorbeer et al., 2013) and in 2014 was growing at an annual rate of 7.5 % a year (Mazarrasa et al., 2014). The majority of total economic activity for both production and consumption of seaweeds globally is reliant on Asia. In 2017, global seaweed export material was of 95 % Asian origin and 72 % of the global seaweed export value was reported from Asia. In the same year Asian average price per ton wet weight was US\$ 1090, compared to nearest pricewise competitor Africa (US\$ 1250). In 2017, China and Japan made up 99.6 % of production (92.4 % and 7.2 % respectively), with annual global production at 685 kilotons wet weight (FAO, 2019). The laminarin market was estimated at US\$ 2 million in 2019, with a compound annual growth rate of 8 % forecast until 2024. The Asian market is by far the most dominant and the sectors of food, beverages, feeds, and pharmaceuticals constitute the majority market share (More, 2019). Seaweed has also been identified as the main 3<sup>rd</sup> generation biofuel source, but the ethanol produced would be a lower worth product than nutraceuticals or functional foods, therefore economically unattractive in comparison (Offei et al., 2018).

It is clear from Figure 2-1 that Asia serves as the backbone of global production. The value of these exports (Figure 2-2), where the total does not so closely resemble Asian trends, indicates a lower cost for seaweed exports from Asia when compared to their closest overall competitor Europe.

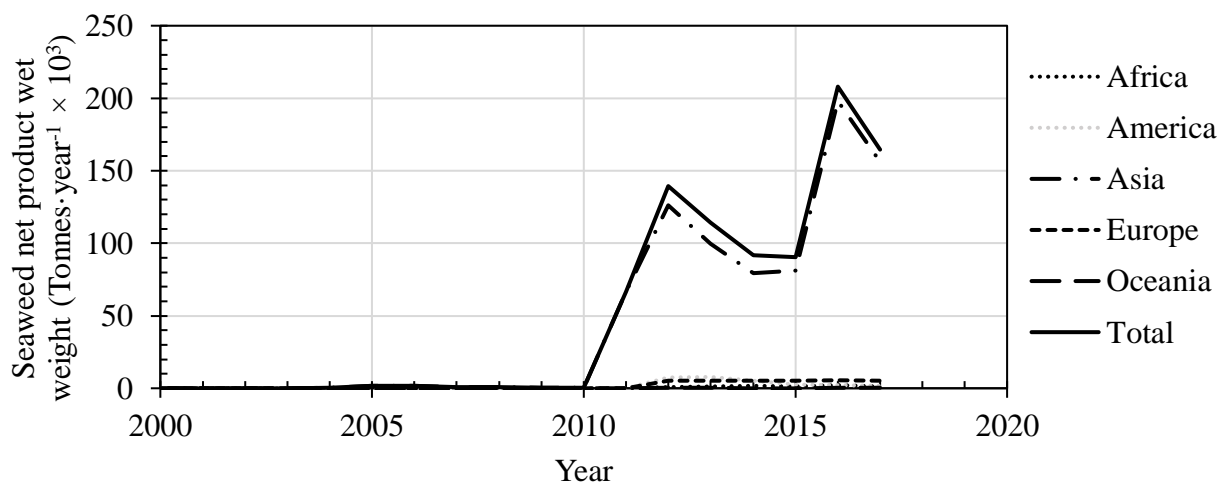


Figure 2-1 Seaweed product (fit for human consumption) export weight by continent (2000-2017) (FAO, 2019)

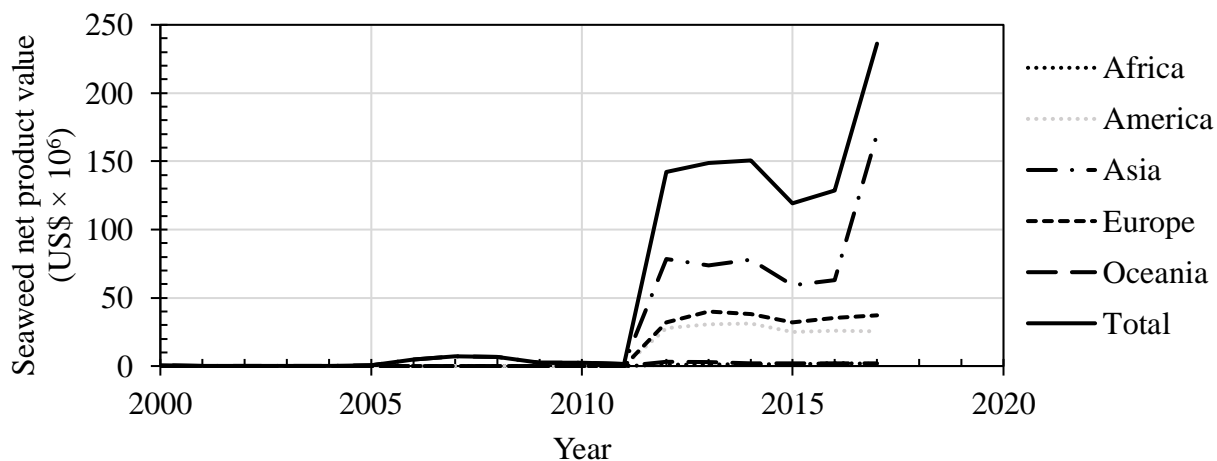


Figure 2-2 Seaweed product (fit for human consumption) export value by continent (2000-2017) (FAO, 2019)

A shift has taken place from the harvest of wild seaweed to controlled cultivation (farming) on an industrial scale. Cultivation allows more certainty regarding growth conditions and would, therefore, reduce some fluctuation of raw material supply. This reduction in fluctuation in turn allows producers and downstream beneficiation operations to make more accurate estimates of profit margins and production rate requirements (Cardozo et al., 2007). The prevalence of cultivation does not however negate the benefit of sustainable harvesting of wild kelps, which grow naturally in colonies, and do not require the approach of aquaculture as much as free-floating red and green seaweeds.

A large source of raw material is available globally, and more useful biological functions are being associated with macroalgae-derived compounds. The health potentials of these compounds adds monetary value and marketability to seaweed-derived products. Market growth is also likely to be

bolstered by increasing extraction efficiencies from newer technologies, as well as negative public perception towards synthetic drugs. Anthropological pressures, stemming from the increase in global population density, may also push the seaweed market forward as a whole. This makes the further commercialisation of laminarin a likely prospect, dampened only by its dependence on season, salinity, frond age, and available nutritive salts (Anderson et al., 1981; Chizhov et al., 1998).

### **2.2.2. South African seaweed market**

In 2017 the seaweed production of South Africa was reported as 862 tonnes wet weight, and a value of approximately US\$ 175 000. This came as an approximate 65 % decrease both in terms of value and weight from 2016 (FAO, 2019). This amounts to 0.03 % of the global production value (halving from approximately 0.07 % in 2016).

South Africa harvests and exports kelp for phycocolloid production (South African kelps constituting 22 to 40 % alginic acid by weight) and abalone feed production, as well as commercial agricultural biostimulants Kelpak® and Afrikelp®. As an underutilised resource, kelp has the potential to supplement many industrial roles in South Africa – whether it be as nutritive additives, vitamin sources, fertilisers, and higher worth biostimulants (Amosu et al., 2015).

In South Africa, *Laminaria pallida* (“split-fan kelp”) and *Ecklonia maxima* (“sea bamboo”) constitute a portion of the sea-plant industry. Their increased population densities above that of red and green seaweeds provides ease of harvest (Branch et al., 2007). The economic importance of *E. maxima* stems from its use as agricultural feedstock and fertiliser supplement, as well as the role it plays as the feedstock for farmed abalone. This latter relationship has led to the experimentation of degrading the kelp with enzymes isolated from abalone gut (Robertson-Andersson et al., 2006). *Laminaria* in general, constituting 31 species, has found use in medicine, food industry, and agriculture. *Ecklonia maxima* has not reached this level of utilisation, partially due to the worldwide availability of *Laminaria* and *Saccharina* species.

The potential for increased kelp-product manufacturing is present in South Africa. Concession areas are in place, indicating sustainable planning, with licenses allocated to commercial operators. Kelps can be found in fourteen of the twenty-three concession areas. The Marine Living Resources Act of 1998 prevents overharvesting of wild seaweeds (RSA, 1998).

Uncertainties regarding composition, seasonality, processing, and other factors must be taken into account with large scale laminarin production. If kelp were harvested in correct intervals and seasons



then South African operators and processing operations could compete in a global market as small as that of laminarin (US\$ 2 million).

### 2.3. Brown seaweeds (Phaeophyta)

Common genera of the Phaeophyta class include the genera *Chordariopsis*, *Splachnidium*, *Ecklonia*, *Laminaria*, *Macrocystis*, and *Saccharina*. Pigments such as xanthophylls, fucoxanthin, chlorophyll a, chlorophyll c, and  $\beta$ -carotenes provide the “brown” hue. Structural polysaccharides present in the algae include fucoidan, alginate, and cellulose. Laminarin polysaccharide and mannitol perform the role of carbon storage (Jung et al., 2013). Reported bioactivities from brown seaweed compounds include cytotoxic, anti-tumour, ichthyotoxic, feed-deterring, nematocidal, anti-fungal, anti-inflammatory, algicidal, hepatoprotective, anti-viral, free-radical scavenging, anti-oxidant, anti-diabetic, and anti-hypertensive actions (Charoensiddhi et al., 2017; El Gamal, 2011; Mišurcová, 2011)

*Ecklonia maxima* and other kelps have a root-like structure called the “holdfast” that anchors the seaweed to other kelps or rocky outcrops on the ocean floor (Figure 2-3). A hollow, bulb-like structure at the apex of the seaweed serves as a float, meaning that sea bamboo can often break the water-surface to become visible. Lethal and non-lethal harvesting techniques on *E. maxima* have been investigated and found to not affect the canopy growth. The species is endemic to the shallow, temperate water of the southern Atlantic coast of Africa. Plants are found in depths up to 8 m and growth (stipe elongation) can reach 57 mm / week in sub-canopy plants. Larger plants grow at greater rates when compared to smaller plants due to the increase in photosynthetic tissue (Rothman et al., 2006).

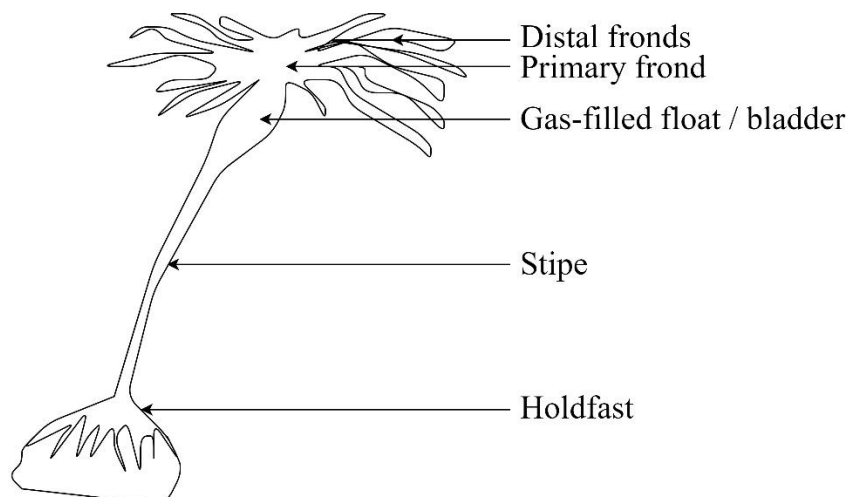


Figure 2-3 Simplified structural diagram of *Ecklonia maxima* kelp (adapted and redrawn from Branch et al. (2007) and Rothman et al. (2006))

## 2.4. Brown seaweed polysaccharides

Brown seaweeds utilise laminarin for food storage, and cellulose, hemicellulose, fucoidan, and alginic acid for structure and support (along with proteins). Various polysaccharides, proteins and ions interact in the structure of the phaeophyte cell wall (Figure 2-4). Sulfated fucans (fucoidans) link closely with cellulose microfibrils. These microfibrils are embedded in an alginate matrix. Polyphenolics associate with alginates and have the potential to form high molecular weight complexes (Deniaud-Bouët et al., 2014).

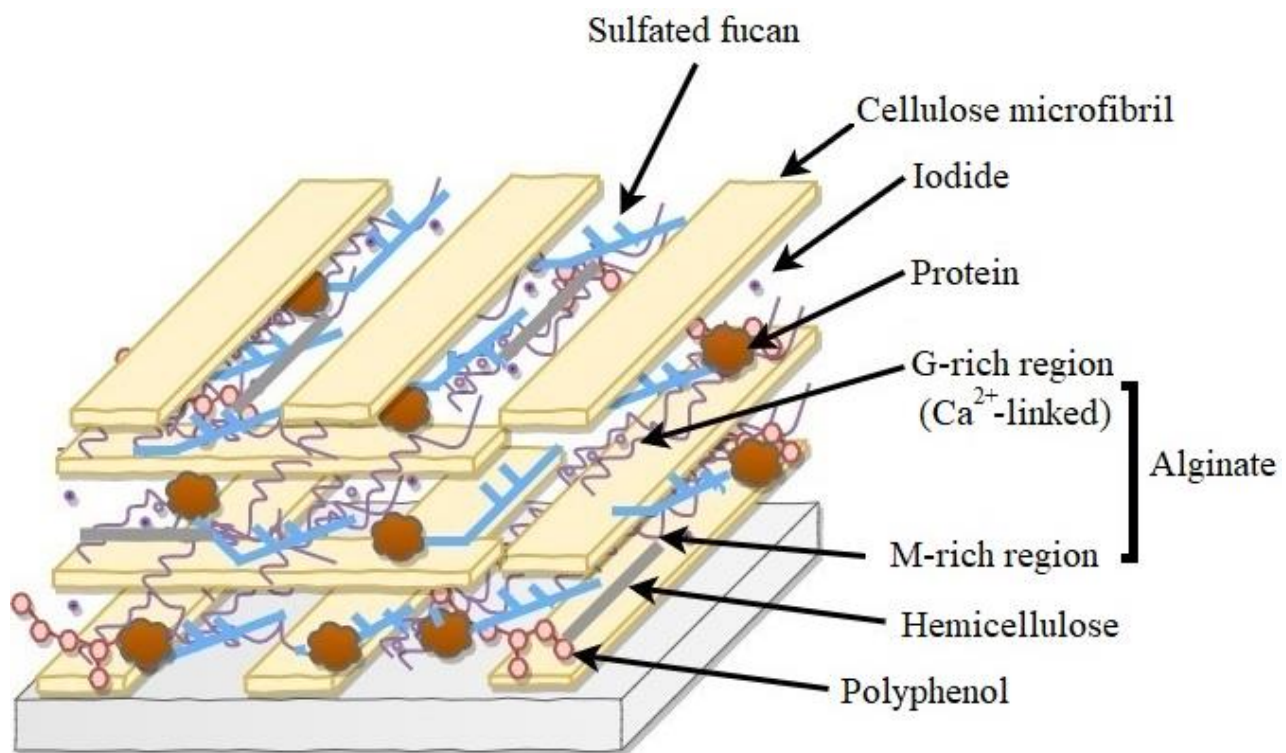


Figure 2-4 Simplified structural model of a Phaeophyte cell wall (redrawn from Charoensiddhi et al. (2017), and Deniaud-Bouët et al.(2014))

The complex and interconnected nature of the cell-wall and constituent polysaccharides provides difficulty during extraction. The cell-walls must be broken down to access entrapped polysaccharides. Laminarin is found in vacuoles in the cell and thus the degradation of the cell wall may not increase its yield (Kadam et al., 2015b). Alginate (a phycocolloid) with rheology altering properties has been reported as a possible cause of decreased extraction efficiencies of other water-soluble polysaccharides (mass transfer is hindered by increased viscosity) (Garcia-Vaquero et al., 2017; Holdt and Kraan, 2011).

### 2.4.1. Laminarin

First extracted and isolated by Schmiedeberg (1885), laminarin functions as part of the carbon metabolism of Phaeophyte species. It is reported as constituting the largest portion of dry mass in the *Laminaria* and *Saccharina* genera (Devillé et al., 2004; Holdt and Kraan, 2011). Total laminarin content in brown seaweeds (reported up to 35 % dry weight) is dependent on a number of factors, such as frond age, nutritive salts, season (via water temperature and light intensity), and water depth (Boney, 1965; Chizhov et al., 1998).

Rioux et al. (2009) and Chapman and Craigie (1978) found that the reduction in available nutritive salts, nitrites, and nitrates coincided with reduced growth and increased laminarin accumulation in *S. longicuris*. These trends outweighed season, with samples from May 2005 having twice as much laminarin as June 2006 (5.3 % as opposed to < 2.5 %), and half of the nitrite and nitrate levels (5 vs 11 mmol·m<sup>-3</sup>). Maximum laminarin content was found in the August-September period (summer / autumn crossover in Canada, where the samples were harvested), when the nitrite and nitrate levels were lowest at the sampling site. The dependence on nutritive salt concentrations shows that seasonal variability is not the only challenge to large-scale production of a consistent product (Kadam et al., 2015)

Laminarin is formed from 1,3-β-D-glucan with additional branching provided by 1,6-β-bonds. The ratio of these links varies with species, with a 1,3-β-D-glucopyranose backbone exhibiting 6-O branching along with the mentioned 1,6-β-links (Shin et al., 2009). This branching determines the solubility of the molecule (which generally consist of 20-25 glucose monomers) (Zhang & Row, 2015). The reducing-end of a laminarin polysaccharide can be either mannitol or glucose, referred to as M- and G- chain laminarin respectively (Figure 2-5, 1,6-β-D-branching omitted for simplicity). Harvest season, species, frond maturity, and the presence or absence of nutritive salts determine the ratio of M- to G- types. The different structures within the plant also contain differing concentrations of the polysaccharide. It has been found in the fronds and stipes of all brown seaweeds.

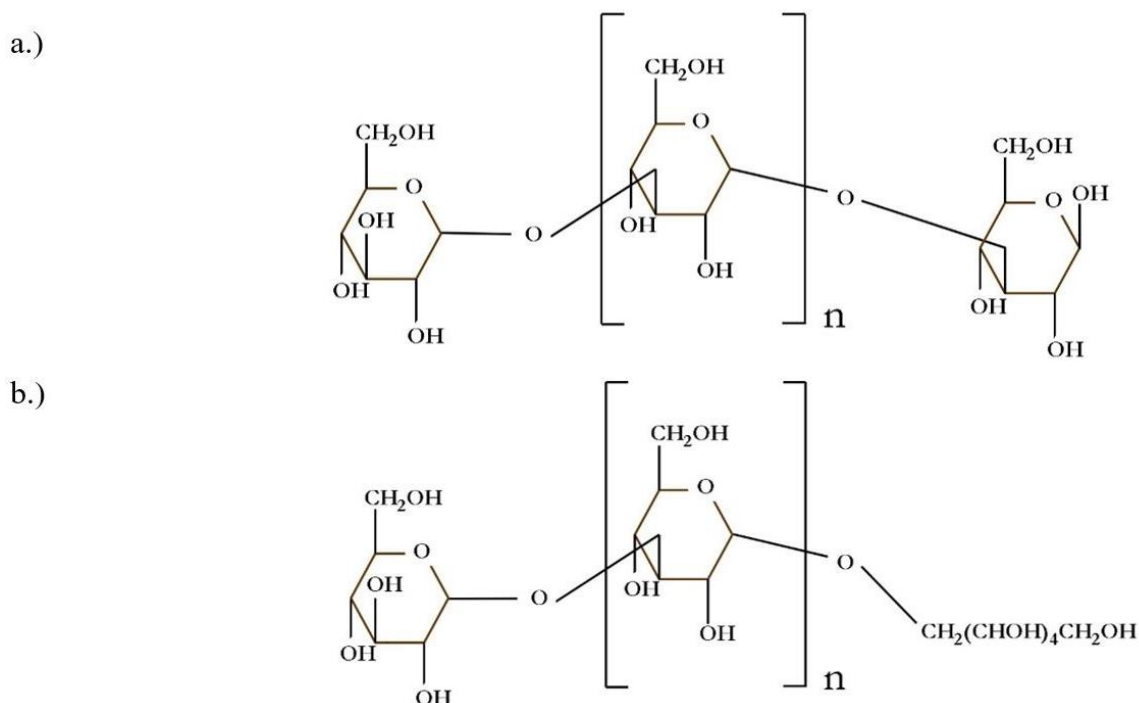


Figure 2-5 Structural formulae of unbranched a.) M-chain laminarin b.) G-chain laminarin (redrawn from Kadam et al. (2015b))

Within the metabolism of brown algae, laminarin acts as an interchangeable carbon storage compound with the polyol mannitol, much like starch with sucrose in higher plants. Mannitol can itself constitute a large portion of the dry weight of phaeophytes, as large as 20 – 30 %, and carbon-storage switches back and forth between mannitol and laminarin in the year, leading to the requirement of well-timed harvesting if laminarin is the desired compound to be extracted (Reed et al., 1985).

The value of laminarin stems from various biological activities it possesses when ingested. It exhibits immunostimulatory, anti-tumour, wound-healing, anti-thrombotic, anti-coagulant, anti-viral, antibacterial, and anti-inflammatory activities (Choi et al., 2012; Kadam et al., 2015b). Laminarin is resistant to digestion in the small intestine of mammals, classifying the polysaccharide as dietary fibre. It plays a prebiotic role through microbial fermentation in the colon (Devillé et al., 2004). Holdt and Kraan (2011) reported that laminarin protects against radiation, plays a role in reducing cholesterol levels in serum, mediates hypertension, and brings down unhealthy lipid levels in the body and blood. Molecular modification with sulfation, oxidation, reduction, and radiation can be used to enhance the antioxidant activity of laminarin (Choi et al., 2012). These physiological functions are the push behind the commercialisation of the polysaccharide.

## 2.5. Seaweed processing and laminarin extraction

The general order of extraction for laminarin relies on washing, size reduction, pretreatment, and precipitation with either mineral acids or bases (Garcia-Vaquero et al., 2017; Kadam et al., 2015b). The seaweed is washed with distilled water in order to displace external organisms that may grow on the seaweed (epiphytes), impurities, sand, and salt residues. The seaweed is then often dried, with milling employed as the next step (on dry or wet raw material) to homogenise the seaweed and provide a more favourable surface-to-volume ratio (Garcia-Vaquero et al., 2017; Hahn et al., 2012; Imbs et al., 2016).

Fucoidan and laminarin are most commonly co-precipitated as a polysaccharide fraction using ethanol. Co-extraction is targeted in studies or designs that aim to incorporate downstream separations and multiple product streams. Alginic acid co-extraction can be limited with the use of acidic conditions, which converts the molecule to an insoluble form (Hahn et al., 2012; Lorbeer et al., 2017).

### 2.5.1. Chemical extraction of seaweed polysaccharides

Chemical extractions of the laminarin molecule are the most prevalent in literature, with the most common methods applying temperatures above 60 °C and mild acidic conditions (Black et al., 1951; Devillé et al., 2004; Zha et al., 2012; Zhang and Row, 2015). It is undesirable to precipitate alginate with the polysaccharide fraction, as this results in lower purities of the targeted polysaccharides. Acidic conditions convert alginate ions to insoluble alginic acid and prevent the molecule from co-precipitating (Hahn et al., 2012; Lorbeer et al., 2017). The removal of alginic acid with the insoluble residue is likely the reason that acidic extraction conditions are preferable for laminarin extraction and therefore the most commonly reported.

Cong et al. (2016) and Dinesh et al. (2016) made use of CaCl<sub>2</sub> solutions to extract alginate from pre-extracted polysaccharide fractions. Aqueous calcium chloride (2 %) was also used by Mian and Percival (1973) as the first step in a sequential extraction scheme. This method (Figure 2-6) allowed both alginate and laminarin extraction and split the fucoidan fraction between streams. It was replicated by Rioux et al. (2007). Co-extraction makes this particular method a viable laminarin extraction scheme, although it makes use of many different chemicals, and could lead to increased costs in effluent treatment and specialised materials of construction.

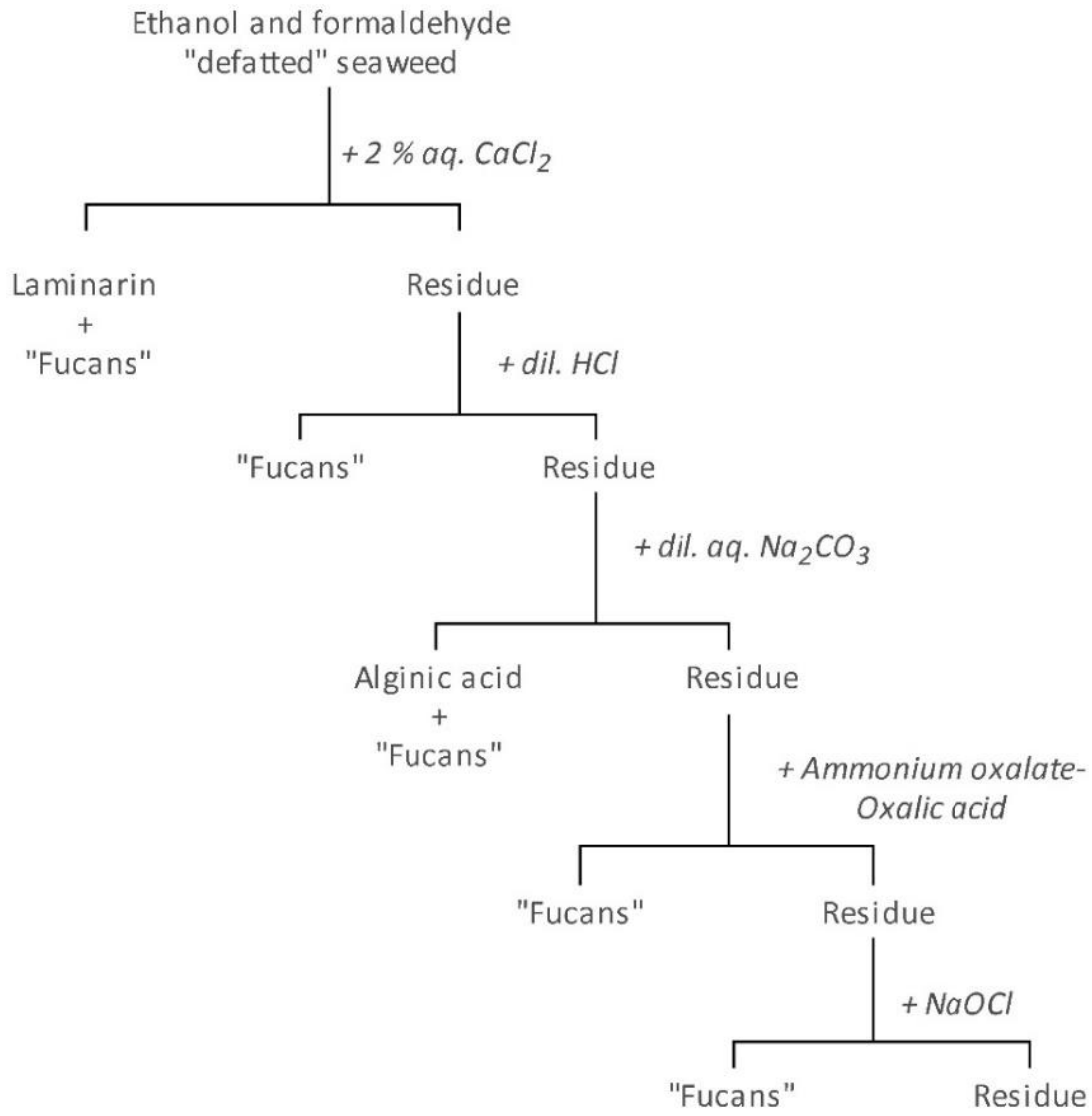


Figure 2-6 Flow-diagram illustrating stepwise polysaccharide extraction (Mian and Percival, 1973)

## Laminarin chemical extraction

The summary of chemical laminarin extractions (Figure 2-7) shows that the laminarin polysaccharide has been successfully extracted from many brown seaweed species. Black et al. (1951) extracted laminarin from *Laminaria saccharina* with both hydrochloric and sulfuric acid treatments at a reported optimal pH of 2.4. Isolation of the molecule involved either allowing the sample to stand and precipitate or adding ethanol or acetone to precipitate the polysaccharide fraction. Additional ethanol or acetone was not always necessary, leading to the conclusion that the material contained both water-soluble and water-insoluble laminarins. Difficulties in extraction and precipitation were attributed to increased viscosity from alginate, and the inhibition of mass-transfer. The same set of experiments also found that lengthy extraction times were unnecessary and opted for 2 hours at most. These experiments made use of a technique of enriching the supernatant by adding more algal sample to supernatants.

Hot acidic conditions enhance extraction efficiency and the updated method of Devillé et al. (2004) (0.09 M HCl at 70°C, 150 minute extraction) resulted in a more efficient laminarin extraction when compared to the methods of Yvin et al. (1999) and Black et al. (1951). These are some of the only comparable results performed on the same batch of seaweed (*Laminaria saccharina*). Zhang and Row (2015) reported similar optimum conditions for pH and temperature when processing *L. japonica*, with optimisation of size exclusion chromatography (SEC) separation arriving at 0.1 M HCl and 80 °C, with a longer extraction period of 240 minutes. Abdel-Fattah and Hussein (1973), Ermakova et al. (2013), Jin et al. (2014), and Voronova et al. (1991) all made use of either HCl (usually 0.1 M) or H<sub>2</sub>SO<sub>4</sub>. Zha et al. (2012) used only water while Rioux et al. (2010, 2007) made use of CaCl<sub>2</sub>. The summary made it evident that the most common extraction conditions that occurred in reviewed literature was 0.1 HCl (pH 1.0) between 60 and 80 °C.

Enzymatic extraction with selective enzymes could reduce the requirement of hot acidic conditions and result in the extraction of laminarin polysaccharides with more structural integrity (increased biological function, increased value). Involving less chemicals in extraction could also simplify the process design to less steps and lower capital costs. It is also likely to reduce the dependence on specialised materials of construction for reactors, negating the requirement for expensive acid-resistant alloys.



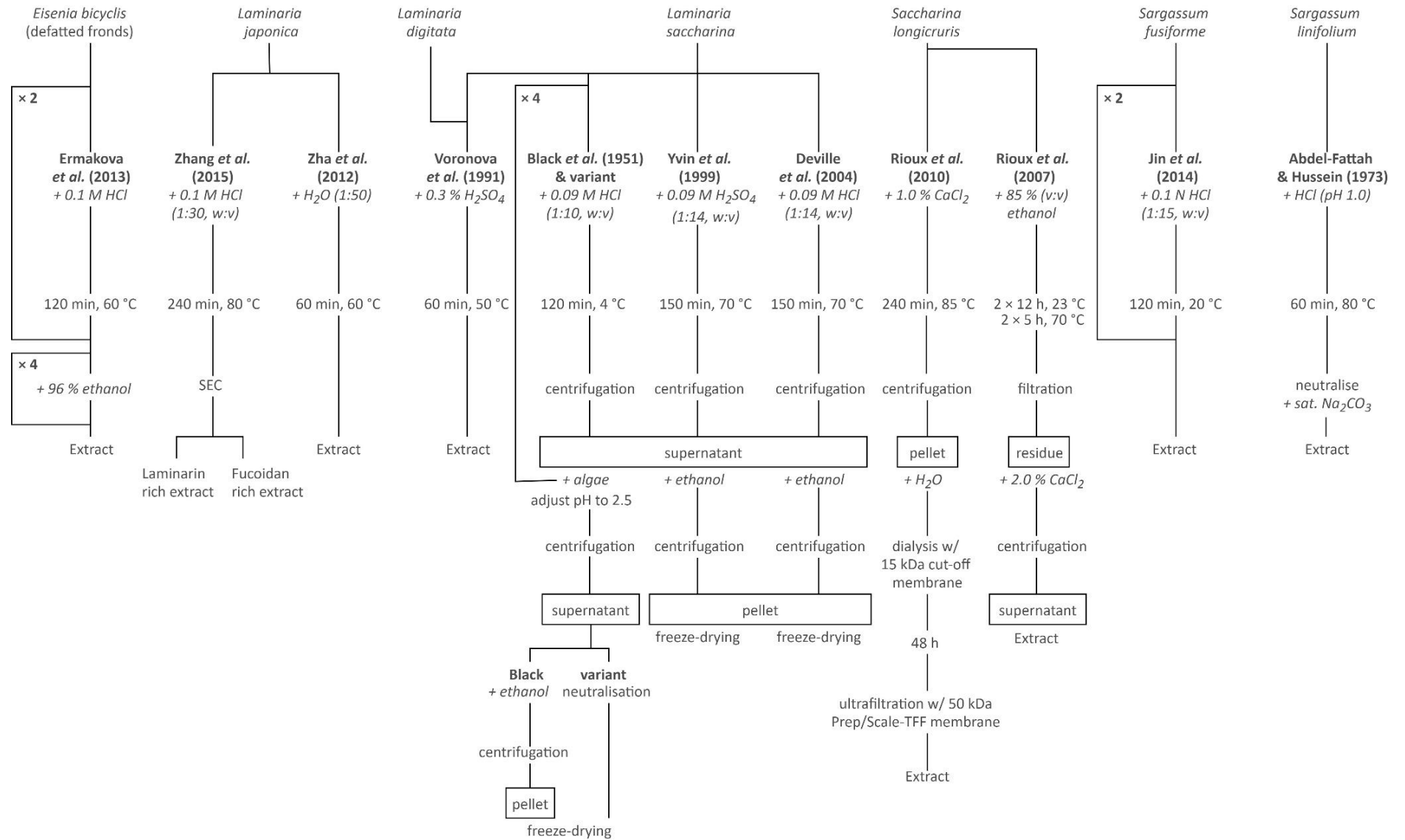


Figure 2-7 Flow-diagram of chemical extraction procedures for laminarin from various seaweeds



## 2.5.2. Enzyme-assistant extraction (EAE)

Enzyme-assistant extraction relies on the specificity provided by selective enzymes. It is an alternative extraction method gauged towards the breakdown of the cell wall. This method has resulted in increased bio-activities and yields from seaweed-derived extracts and is an accepted method for extraction (Wijesinghe and Jeon, 2012a). Despite suppliers providing optimum conditions for pH and temperature, complex substrate mixtures still require optimisation studies to improve extraction. This is particularly important when experimenting with feedstocks that the enzyme mixture was not designed for. The use of EAE in seaweed research has been towards releasing bioactive compounds entrapped in cell walls (Figure 2-8).

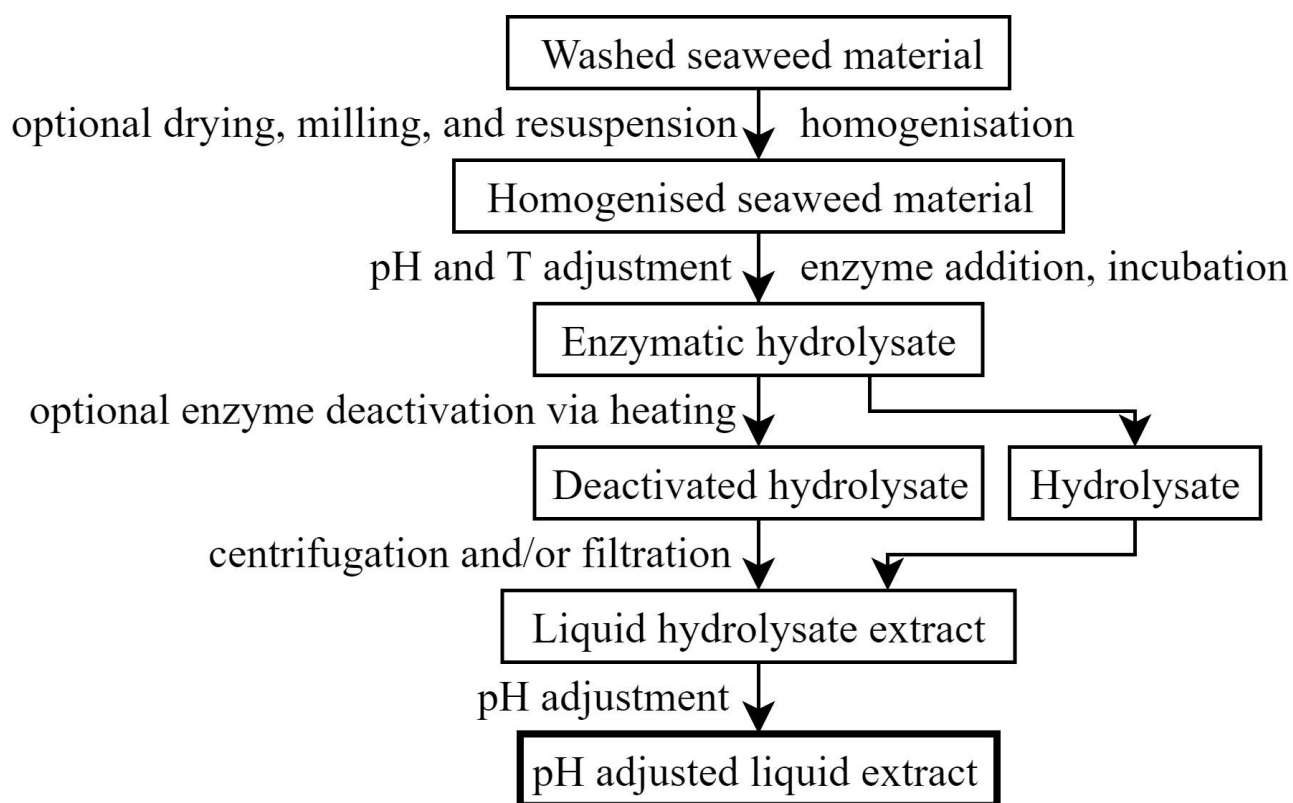


Figure 2-8 General EAE procedure for preparation of enzyme extracts from seaweed (redrawn from Wijesinghe and Jeon (2012a))

The optional drying and powdering of the seaweed material (Figure 2-8) is useful in controlling solids-loading for lab-scale testing. Drying at low temperatures (30 to 40 °C) should have no appreciable effect on the compounds present but is unnecessary when subsequent resuspension in water is intended. Drying is, however, an energy intensive process and the ability to analyse moisture content can bypass the step effectively. Deactivation of the enzyme is another energy intensive step that would be required for analysis (to prevent samples from changing), but is unnecessary for large scale processing.

## Enzyme effect on brown seaweed polysaccharides

Commercially available enzymes that have found reported use in brown seaweed extractions (Table 2-1) include both carbohydrases and proteases, with Heo et al. (2003) reporting the activity of the carbohydrases as superior to the proteases on brown seaweeds, *Ecklonia cava* in particular. This makes sense when considering that carbohydrates make up the majority of seaweed chemical makeup (>50 %) against the crude protein fraction (< 20 %) (Mišurcová, 2011). Top performing carbohydrases in this study were Celluclast, Viscozyme, and AMG – all achieving a 40 % solubilised yield on *E. cava* in 0.1 N acetate buffer. The extractions from Heo et al. (2003) and Heo et al. (2005) both incubated dry powdered brown seaweed samples (1 % w/v) in 0.1 N buffers (acetate and phosphate) with 10 % (v/dw) enzyme dosing for 12 hours. This was to ensure hydrolysis but is an analytical scale extraction technique and likely not reproducible at an industrial scale. Heo et al. (2003) also compared the radical scavenging of Celluclast liquid extracts to solvent extracts (ethanol, methanol, ether, chloroform, acetone, and hexane) and found the Celluclast to be superior.

The use of Viscozyme, Celluclast, Neutrase, Ultraflo, and Flavourzyme for extraction was investigated by Charoensiddhi et al.(2016). Only Viscozyme, Celluclast, and a mixture of the two enzymes resulted in increased glucose yield from the free sugar fraction. Enzymatic extraction was used for alginate and fucoidan extraction. It was reported that a reduction in the molecular weight of both compounds took place. This molecular-weight reduction may have been due to hydrolysis of similar bonds found in both compounds, and the study reported low hydrolytic efficiency. It was theorised that both laminarin and cellulose were also targeted by the enzymes to achieve an increased glucose yield. Buffers used in the study were found to severely hinder carbohydrate extraction (Charoensiddhi et al., 2016).

If a selective cellulase (1,4- $\beta$ -glucanase or other) were used that affects 1,3- $\beta$  and sulfated polysaccharide links minimally, then the yield of polysaccharides (alginate, fucoidan, and laminarin) could theoretically be improved. This extraction mechanism could also leave extracted compounds with more structural and chemical integrity as opposed to traditional methods. Further hydrolysis would be dependent on additional (perhaps even enzymatic) steps, allowing manufacturers and researchers choice over molecular weights (and bioactivities as a result).

Table 2-1 points to Celluclast (which includes cellulase / endoglucanase I (EG I) from *Trichoderma reesei* ATCC 26921) as a suitable choice of enzyme with high specificity to cellulose. Despite the theorised co-specificity on laminarin in Charoensiddhi et al. (2016), there are studies that have checked the specificity of the EG I against laminarin (1,3- $\beta$  and 1,6- $\beta$  links) and reported inactivity while still finding activity against 1,4- $\beta$  links in barley glucan (Bailey et al., 1993; Nakazawa et al., 2008).

Table 2-1 Optimum hydrolysis conditions for a variety of commercial enzymes

Enzyme	Optima		Activities	Organism	Source
	pH	T (°C)			
Viscozyme	4.5	50	Arabanase, cellulase, $\beta$ -glucanase, hemicellulase and xylanase	<i>Aspergillus aculeatus</i>	Heo et al. (2005), Charoensiddhi et al. (2016)
AMG	4.5	60	Exo-1,4- $\alpha$ -D-glucosidase	<i>Aspergillus niger</i>	Heo et al. (2005)
Celluclast	4.5	50	Cellulase	<i>Trichoderma reesei</i> ATCC 2692	Heo et al. (2005), Charoensiddhi et al. (2016)
Termamyl	6.0	60	Heat-stable $\alpha$ -amylase	<i>Bacillus licheniformis</i>	Heo et al. (2005)
Protamex	6.0	40	Protease complex	<i>Bacillus</i> sp.	Heo et al. (2005)
Kojizyme	6.0	40	Amino- and carboxy-peptidase	<i>Aspergillus oryzae</i>	Heo et al. (2005)
Neutrase	6.0	50	Metallo-endoprotease	<i>Bacillus amyloliquefaciens</i>	Heo et al. (2005), Charoensiddhi et al. (2016)
Ultraflo	7.0	60	Heat-stable multi-active $\beta$ -glucanase	<i>Humicola insolens</i>	Heo et al. (2005), Charoensiddhi et al. (2016)
Umamizyme	7.0	50	Endo- and exo-peptidase complex	<i>Aspergillus oryzae</i>	Heo et al. (2005)
Flavourzyme	7.0	50	Endo-protease and exo-peptidase	<i>Aspergillus oryzae</i>	Heo et al. (2005), Charoensiddhi et al. (2016)
Alcalase	8.0	50	Endo-peptidase	<i>Bacillus licheniformis</i>	Heo et al. (2005), Charoensiddhi et al. (2016)

## Hybridised extraction

Hybridised extraction (the combination of more than one extraction method) is possible, particularly when EAE is paired with ultrasound-assistant extraction (UAE) and microwave-assistant extraction (MAE). The combination of EAE and UAE is used as ultrasound-assisted enzymatic hydrolysis (UAEH) and has been used to extract R-phycoerythrin (R-PE) from the Rhodophyte species *Grateloupia turuturu*. That study showed higher temperatures to aid liquefaction of water-soluble compounds and that suitable temperatures should be chosen when attempting to extract heat-sensitive target compounds (and not damage their structural integrity and worth) (Le Guillard et al., 2015).

Charoensiddhi et al. (2015) made use of a microwave-assisted enzymatic extraction and found that it improved phlorotannin recovery. This same study also showed shorter enzymatic hydrolysis times to result in higher total phenolic content (TPC) and antioxidant activities. A threefold increase in ferric reducing ability of plasma (FRAP) was found in extraction with Viscozyme and Celluclast as opposed to extraction with water alone.

## 2.6. Polysaccharide purification techniques

Post-extraction purification increases the value of a crude extract. The foremost techniques that have found use in increasing the value of seaweed polysaccharides (the mixture of laminarin and fucoidan specifically) are size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), chromatography by affinity and ultrafiltration via molecular-weight cut-off membranes (Garcia-Vaquero et al., 2017). Chromatographic methods tend to be used for analytical purposes whereas ultrafiltration is more commonly used on an industrial scale. Only the methods where laminarin has been enriched are considered here.

The application of IEC has found extensive industrial use in the separation of charged molecules due to its relatively low associated costs, high sample handling capacity, and its ease of automation. Anion-exchange chromatography was used by Anastyuk et al. (2012) and Imbs et al. (2016) to separate laminarin and fucoidan. Ion-exchange resin with a positive charge was used to separate the net-negative charge molecules of sulfated polysaccharides (fucoidan) from the charge-free laminarin.

Zhang and Row (2015) made use of high-performance SEC to separate laminarin and fucoidan, proving the viability of the concept. SEC operates as a gradient-type exclusion method, separating molecules based on their elution times through a packed column of porous beads with specific pore sizes (Meyer, 2010). This separation technique creates a continuous distribution of molecular weights and could require less maintenance and encounter less clogging than the discrete size cut-offs provided by ultrafiltration

systems. No mention has been found in literature of industrial scale use of this technology for this particular separation.

The use of membranes has been implemented in other fields on an industrial scale. (Garcia-Vaquero et al., 2017). It has been used in dairy-related separations, as well as for bacterial exo-polysaccharide purifications. It should apply well to polysaccharide fractionation. It is likely to scale to an industrial level better than chromatographic methods. Excessive fouling is a reported problem to be overcome and diafiltration has been suggested along with sequential ultrafiltration and diafiltration with decreasing molecular-weight cut-offs to curb the problem (Marcati et al., 2014; Patel et al., 2013).

Ultrafiltration is the likely choice of separation technique for laminarin / fucoidan separation on a large scale. Anion-exchange chromatography is more specifically gauged to the task and likely to result in better separations and purities (charge differences are discrete while molecular weight of the polysaccharides are a distribution), but this purification may not be necessary for the certain target markets (food, nutraceuticals). Purification techniques chosen must correspond to the product and market requirements (Garcia-Vaquero et al., 2017).

## **2.7. Quantification techniques for laminarin**

Specificity is required in either the breakdown of the laminarin molecule or the quantification of the released glucose in order to quantify the polysaccharide. Analytical enzymes can provide this specificity in either step (Kadam et al., 2015b). Precipitation of the joint polysaccharide fraction (fucoidan and laminarin) via ethanol is a common step in extraction and purification protocols (Garcia-Vaquero et al., 2017), but quantification of the laminarin polysaccharide in crude extracts could simplify processes.

### **2.7.1. Selective enzymatic hydrolysis of laminarin**

Endo-1,3- $\beta$ -D-glucanase (laminarinase) (EC 3.2.1.39) selectively hydrolyses links in 1,3- $\beta$ -D-glucans, while showing low activity towards 1,4- $\beta$ -D-links (McCarthy et al., 2003). A laminarinase derived from *Trichoderma* spp. (endo-1,3(4)- $\beta$ -D-glucanase, EC 3.2.1.6) has been used to selectively hydrolyse laminarin in order to quantify the glucose released and thereby quantify the laminarin polysaccharide (Devillé et al., 2007, 2004). Glucose oxidase (EC 1.1.3.4) was added for a second incubation period following incubation of seaweed sample with laminarinase, quantifying glucose against a known standard (Kadam et al., 2015a). Devillé et al. (2004) quantified released reducing-sugars with the dinitrosalicylic acid (DNS) assay, and found the assay to be a suitable choice of sugar quantification when measuring enzyme-hydrolysed laminarin. The DNS assay is a simple, short, and cost effective method, all advantages when processing multiple samples (Miller, 1959).

Purportedly, the most exact method for quantification was presented by Becker et al. (2017). This method also involved enzymatic hydrolysis using specifically isolated enzymes. Enzymes isolated from *Formosa* spp. (FbGH17A (1,3- $\beta$ -D-glucanase), and FbGH30 (1,6- $\beta$ -D-glucanase)) were used to hydrolyse laminarin, after which glucose or reducing-sugars quantification was carried out.

An amperometric method was devised by Miyanishi et al. (2004) using  $\beta$ -(1,3)-glucanase (obtained from *Bacillus clausii*) and glucose oxidase (obtained from *Aspergillus niger*) and involved the use of a bioreactor with enzymes immobilised in glass beads. Correlation was made between a calibrated electrode response and laminarin concentration. Immobilised enzymes and increased sample requirement would make this method more viable in an industrial setting where continuous monitoring of the laminarin content is desirable.

### **2.7.2. Acid hydrolysis of laminarin in polysaccharide fraction**

Acid hydrolysis can be used to liberate monosaccharides of all polysaccharides in a mixture. This lack of specificity is only acceptable when it is assumed that laminarin being tested is the only glucose-containing polysaccharide present. This assumption has been made after polysaccharide precipitation with ethanol (Charoensiddhi et al., 2016). A method of laminarin quantification in crude liquid extracts could not utilise this step, and it is uncertain how much lower molecular weight laminarin would remain in solution after precipitation. High performance liquid chromatography (HPLC) determination of glucose or glucose oxidase enzyme would likely be the logical next step after acidic hydrolysis of samples. Ethanol precipitation and the subsequent drying (unless freeze drying is used) is not ideal with small sample volumes where mass loss could significantly influence results, it is also time consuming. The acid hydrolysis, subsequent neutralisation of individual samples, centrifugation, and filtration in preparation for HPLC are time consuming steps and impractical for large sample numbers.

## **2.8. Characterisation techniques for laminarin**

While quantification of laminarin refers to the measurement of the polysaccharide concentration, characterisation refers to molecular properties. Molecular weight is dependent on extraction conditions, as is molecular structure (Shin et al., 2009). Biological functions (and therefore value) of the extracted laminarin is dependent on these factors as well as modifying steps like irradiation and sulfation (Kadam et al., 2015b).

High-performance size exclusion (HPSEC) (Zha et al., 2012), its combination with multi-angle laser light scattering (HPSEC-MALLS) (Rioux et al., 2007), matrix-assisted laser desorption / ionisation time of flight mass spectrometry (MALDI-TOF-MS), fast atomic bombardment mass spectrometry (FAB-MS)

(Chizhov et al., 1998), and electrospray ionisation mass spectrometry (ESI-MS) (Rioux et al., 2010) have all been used in some form to determine laminarin molecular weight.

The  $\beta$ -glucan structure of laminarin was successfully characterised by Shin et al. (2009) with terahertz time-domain spectroscopy. This technique used refractive index and dielectric constants and the unique profiles of materials in the terahertz domain allowed their identification.

## **2.9. Conclusions, recommendations and research gaps**

The multiple bioactivities that laminarin exhibits label the polysaccharide as a high-value compound, well suited to the food and nutraceutical market (Kadam et al., 2015b). Reported abundance of laminarin in brown seaweeds and their relative ease of harvest point towards a possible commercial product in laminarin, although low extraction yields with current methods must be overcome. Despite high value and potential economic interest in the laminarin polysaccharide, there is no standardised protocol for quantification. EAE studies have been focussed on the formation of crude seaweed extracts, using carbohydrase enzyme mixtures that could result in the hydrolysis of valuable laminarin. A promising enzyme for the enzymatic extraction of laminarin is Celluclast (a commercial cellulase with reported low activity against laminarin), which has shown utility in other brown seaweed EAE studies (Heo et al., 2005, 2003). Conventional techniques for extraction of laminarin have been published (Kadam et al., 2015b) but the effect of EAE on laminarin extraction is only barely described in Charoensiddhi et al. (2016), and proper quantification and optimisation of laminarin was not carried out in that study.

Laminarin extraction via EAE should be investigated, optimised, and compared to conventional extraction techniques. Laminarin extraction with a selective cellulase enzyme should be compared to extraction with blended carbohydrase enzymes (including  $\beta$ -glucanase). The effect of harvest season and nitrate salt content in seawater on laminarin content should also be investigated using these methods as these are major factors in determining suitable feedstock.





# Chapter 3

## *Aims and objectives*

### **3.1. Aims & objectives**

The aim of the project was to optimise the batch-style extraction of laminarin using enzymatic hydrolysis.

Broad objectives (and sub-objectives) to be completed included:

1. Development of a spectrophotometric assay to quantify laminarin in solution
2. Optimisation of laminarin extraction
  - a. Choice of enzyme
  - b. Choice of independent variables
  - c. Choice of experimental range
  - d. Selection of appropriate assays and extra responses to measure
  - e. Experimentation and sample collection
  - f. Response measurement
  - g. Mathematical optimisation of responses
  - h. Validation of response surface models
3. Statistical comparison of results from validation experiments with alternative enzyme and conventional dilute-acid thermal hydrolysis.
4. Statistical comparison of two batches of *E. maxima* algal material harvested May 2018 and June 2019.
5. Comparison of spectrophotometric laminarin method results with HPLC testing method

### **3.2. Study scope**

The scope of the study included the lab-scale optimisation of laminarin extraction from *Ecklonia maxima* (harvested May 2018) with a selected enzyme (Celluclast® 1.5L). Subsequent comparisons were made to conventional extraction technique, alternative enzyme (Accellerase® 1500) extraction technique, and a second batch of *E. maxima* material (June 2019).

### **3.3. Deliverables**

The deliverables of this project include the following:

1. An enzymatic protocol for quantification of laminarin
2. Optimum conditions for laminarin extraction
3. Thesis document

# Chapter 4

## *Experimental procedures*

For responses to be analysed, the creation of samples was first required. A bioreactor setup was used to achieve this and spectrophotometric assays were used to quantify the responses. Work regarding the response surfaces is presented in Chapter 5, while sampling and quantification methods are presented in this chapter

Enzymatic hydrolysis was chosen for the study as the effect of EAE on the extraction of laminarin is not fully described in literature, nor has optimisation been carried out for the extraction of this polysaccharide using EAE methods. The majority of studies in available literature performed enzymatic hydrolysis on dried and milled samples of brown seaweed. The decision was made to avoid this time- and energy-intensive drying step and to use a colloid mill for size reduction of the seaweed samples.

Assays chosen for this investigation were kept to spectrophotometric methods, making it possible to process and quantify many samples without lengthy chromatographic preparation steps (acid hydrolysis, neutralisation, and filtration). The responses were measured in the supernatant of the enzymatically hydrolysed *E. maxima* and subsequently modelled with RSM. These responses included the concentrations of laminarin, reducing sugars, inorganic sulfates (as a proxy for fucoidan), and total phenolics alongside solubilised yield (including supernatant dissolved solids, supernatant mass fraction, and pellet-solids loading) and antioxidant capacity. HPLC was used to test glucose content in the polysaccharide fraction of selected ethanol precipitated supernatants, although this method was not used for the full CCD.

### **4.1. Harvesting, preparation, enzymatic extraction and sampling**

The experimental requirement of this study was to measure the amount of laminarin polysaccharide in enzymatic hydrolysate from *E. maxima*, alongside other responses. General enzymatic hydrolysis was carried out in order to collect samples for testing. Monitoring and maintenance of pH and temperature was performed, as well as the constant agitation of the mixture. A closed reactor was used to minimise evaporation and time series sampling was carried out at every 1.5 hours (0, 1.5, 3, 4.5, 6 hours). The maximum sampled amount during the hydrolysis run did not exceed 10 % of the total batch size (500 ml). The experimental apparatus (Figure 4-1) was purchased from Glass-Chem (Stellenbosch, ZA).

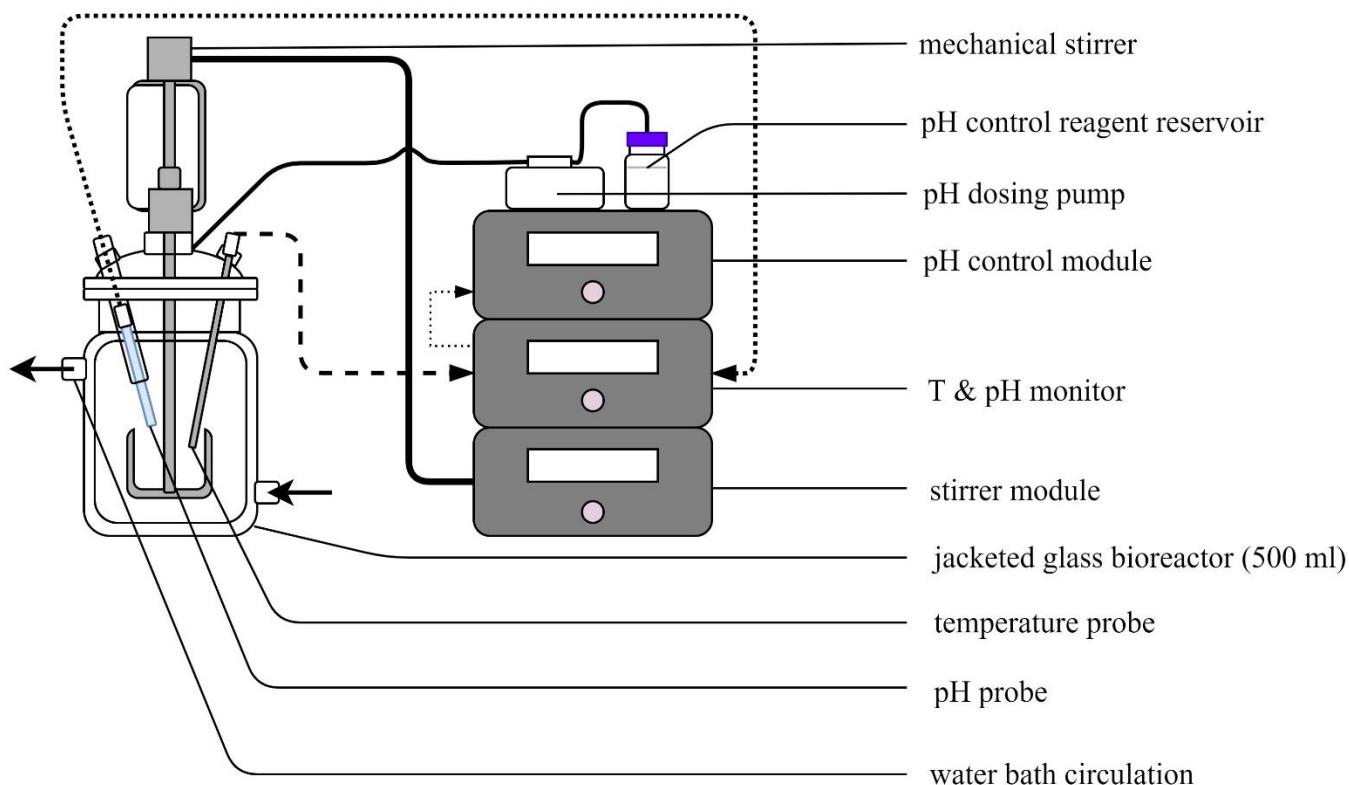


Figure 4-1 Jacketed glass bioreactor experimental setup for enzymatic extraction

*Ecklonia maxima* used in this study was harvested in May 2018 at a concession area in the Western Cape province of South Africa. The harvested kelp was washed and the fronds and stipes separated before a Comitrol® colloid mill was used on the wet material (washed seaweed mixed 50:50 by weight with water) for size reduction. Samples were frozen at  $-20\text{ }^{\circ}\text{C}$  and defrosted once to be mixed, repackaged, and refrozen in 500 ml bottles for ease of use. Only fronds were used in this investigation.

The experimental protocol for sample creation (Figure 4-2) required multiple steps. Prior to each hydrolysis experiment, a 500 ml sample was defrosted overnight at room temperature. The solids loading was tested and corrected to 5 % by weight to keep solids loading uniform between runs. 500 g of this sample was then loaded into the reactor shown in Figure 4-1, where the connected water bath was already at the required temperature. The sample was brought to temperature before adjustment to required pH was carried out with 1 M HCl or 1 M NaOH. Samples of  $\approx 12\text{ ml}$  from the reactor were moved to 15 ml conical bottom tubes (CBT) while the hydrolysate was agitated to prevent settling of insoluble solids. After centrifugation, the enzyme in the supernatant was deactivated at  $90\text{ }^{\circ}\text{C}$  for 5 minutes and the supernatant was partitioned for storage and freezing in 500  $\mu\text{l}$  aliquots. Deactivation occurred after centrifugation to ensure that no additional solubilisation would take place in the mixed hydrolysate at  $90\text{ }^{\circ}\text{C}$ .

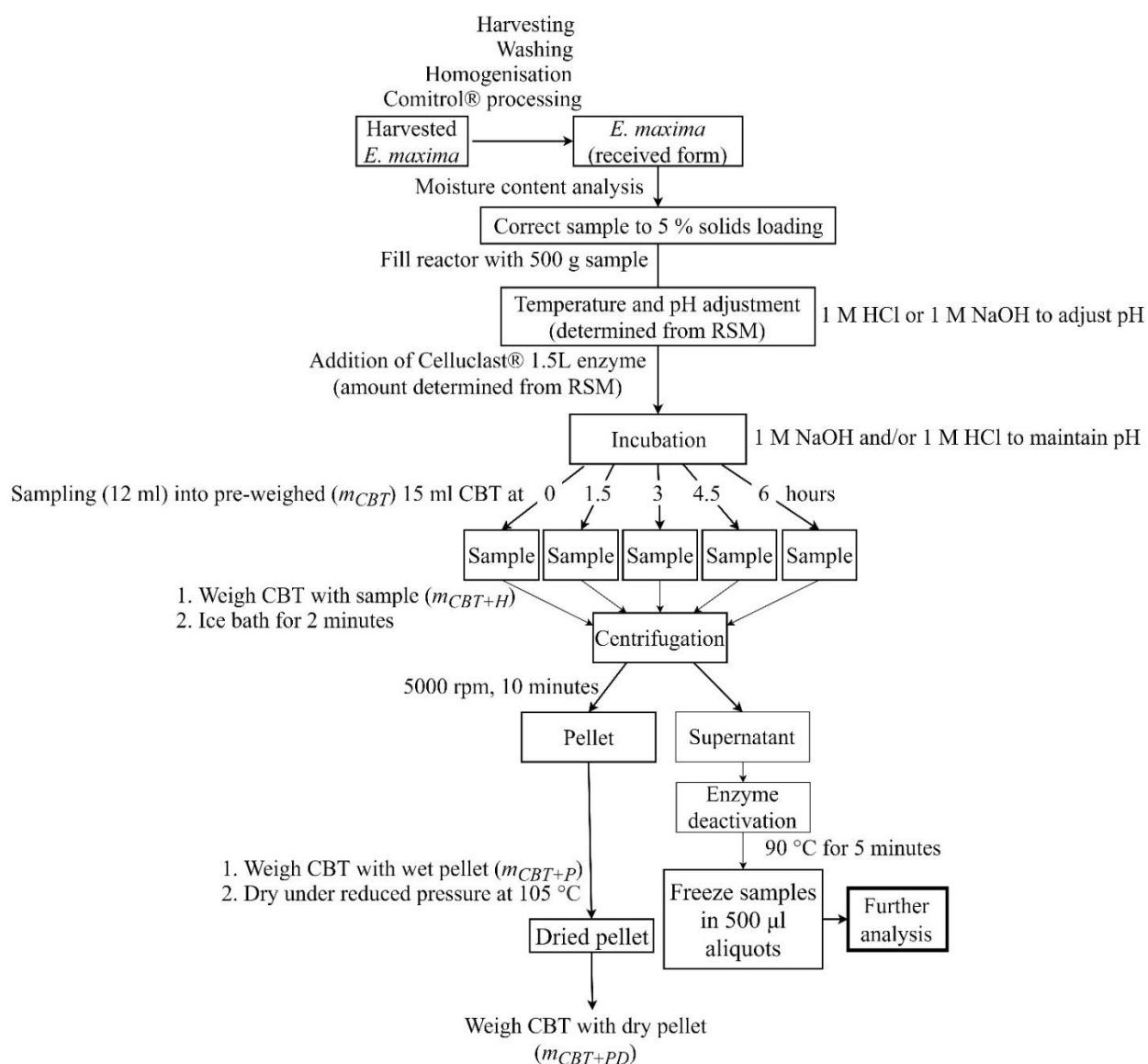


Figure 4-2 Flow diagram of the experimental protocol for enzymatic extraction

## 4.2. Response determinations and assays

All reagents were obtained from Merck ZA except endo-1,3-β-D-glucanase, which was purchased from Megazyme (Wicklow, Ireland). All spectrophotometric testing was carried out with a Biotek ELx800 microplate reader, running 96-well plates. Moisture analysis of supernatant samples was performed with a KERN DBS halogen lamp moisture analyser, with constant drying at a temperature of 180 °C.

Dissolved solids and solubilised yield determination coincided with enzymatic extractions and will be covered first. Laminarin testing required the use of a reducing sugars test method and thus the reducing sugars method is first presented in order to find correct dilution factors. The reducing sugar measurement was also used to track enzyme activity. Inorganic sulfate, total phenolic content, and antioxidant capacity testing are covered last, in that order.

#### 4.2.1. Supernatant solids loading and solubilised yield measurement

Dissolved solids in the supernatant ( $DS$ ) at each sampling point was determined via moisture analysis. Equation 4-1 below gives the variable definition.

$$DS = 1 - \mu_{M/W} \quad (4-1)$$

Where  $\mu_{M/W}$  is the average moisture content of the sample ( $r = 6$ , sample size = 500  $\mu\text{l}$ ). In order to define the solubilised yield ( $SY$ ), further definitions of supernatant mass fraction ( $F_{SN}$ ) and pellet solids loading fraction ( $SL_P$ ) were required. This is shown in Equation 4-2 and 4-3 following.

$$F_{SN} = \frac{m_{CBT+H} - m_{CBT+P}}{m_{CBT+H} - m_{CBT}} \quad (4-2)$$

$$SL_P = \frac{m_{CBT+PD} - m_{CBT}}{m_{CBT+P} - m_{CBT}} \quad (4-3)$$

Where  $m_{CBT}$  is the mass of an empty CBT [ $g$ ],  $m_{CBT+H}$  is the mass of the full CBT after sampling [ $g$ ],  $m_{CBT+P}$  is the mass of the CBT after decanting supernatant [ $g$ ], and  $m_{CBT+PD}$  is the mass of the CBT after the pellet has been dried [ $g$ ].

The decision was made to dry the entire pellet at reduced pressure and 105 °C instead of using a moisture analyser. This was for both practical time-wise consideration and to combat non-representative sampling from the CBT. Solubilised yield ( $SY$ ) is defined by Equation 4-4.

$$SY = \frac{F_{SN} \cdot DS}{F_{SN} \cdot DS + (1 - F_{SN}) \cdot SL_P} \quad (4-4)$$

#### 4.2.2. Reducing sugar measurement

Reducing sugars were quantified with an adapted version of the DNS (dinitrosalicylic acid) protocol, where phenol was not included in the reagent (Miller, 1959; Sumner, 1921). DNS reagent was prepared weekly when required and consisted of 0.75 % dinitrosalicylic acid, 0.75 % sodium hydroxide, 0.04 % sodium sulphite and 10 % sodium potassium tartrate tetrahydrate (Rochelle salt). It was stored in a foil-covered bottle and refrigerated at 4 °C. The decision to pre-mix sodium potassium tartrate tetrahydrate was made to allow more efficient processing of multiple samples, this was an adaptation taken from Devillé et al. (2007). Glucose was used as the standard. The full protocol and standard curve are included in Appendix A.1., and a short method summary is provided below.

1. 400  $\mu\text{l}$  of sample and 1600  $\mu\text{l}$  of demineralised water were mixed in a 2 ml microcentrifuge tube (MCT), resulting in a dilution factor of 5.

2. 500  $\mu\text{l}$  of diluted sample was pipetted to 3 other 1.5 ml MCTs. Standards ( $2.5 - 0 \text{ g}\cdot\text{l}^{-1}$  glucose solutions) were prepared in parallel.
3. 500  $\mu\text{l}$  of DNS was added to each MCT, and the mixtures were incubated at  $90 \text{ }^\circ\text{C}$  for 5 minutes, followed by 3 minutes in an ice bath to stop further colour development.
4. After samples had reached ambient temperature, they were pipetted into microplate wells (200  $\mu\text{l}$  per well) and absorbance was read at 590 nm with a microplate reader.
5. Reducing sugar concentration *RS* of a sample was calculated from the glucose standard curve and is reported in  $\text{gGlE}\cdot\text{l}^{-1}$ .

#### 4.2.3. Laminarin measurement

Laminarin determination was carried out using a combination of enzymatic incubation with buffer solution and the reducing sugars protocol above. The laminarinase enzyme releases reducing sugars via the selective hydrolysis of laminarin, and the difference between the selectively hydrolysed sample and non-hydrolysed “blank” sample is quantifiable by any reducing sugars assay. The protocol was adapted from the standard laminarinase assay (SIGMA, 1995), where DNS assay was used in place of the Nelson-Somogyi reducing sugars assay. Replacement of the Nelson-Somogyi assay with the DNS assay was a safety consideration, as the Nelson-Somogyi assay requires the use of molybdic acid and arsenic acid. The full protocol and mixing of reagents is included in Appendix A.2., with a short description of the assay given below.

1. 100  $\mu\text{l}$  demineralised water and 100  $\mu\text{l}$  enzyme solution ( $150 \text{ U}\cdot\text{ml}^{-1}$ ) were added to MCTs B (blank) and H (hydrolysis) respectively.
2. 450  $\mu\text{l}$  of sample and 1350  $\mu\text{l}$  of demineralised were mixed in a 2 ml MCT, resulting in a dilution factor of 4. 400  $\mu\text{l}$  of this diluted sample was added to both MCT B and H (resulting in a dilution factor of 5). Standard solutions of laminarin from *Laminaria digitata* (Merck, ZA) ( $10 - 0 \text{ g}\cdot\text{l}^{-1}$ ) were prepared alongside samples in this manner to obtain a laminarin vs reducing sugars released standard curve.
3. Samples (and laminarin standards) were incubated at  $37 \text{ }^\circ\text{C}$  for 22 hours in a temperature controlled water bath.
4. After incubation, a short centrifuge spin was used to remove condensed liquid from the cap. Glucose standard samples ( $2.5 - 0 \text{ g}\cdot\text{l}^{-1}$  glucose solution) were prepared in parallel and 500  $\mu\text{l}$  of DNS was added to each MCT.
5. Mixtures were heated at  $90 \text{ }^\circ\text{C}$  for 5 minutes, followed by 3 minutes in an ice bath to prevent further colour development.

6. After samples had reached ambient temperature, they were pipetted into microplate wells (200 µl per well) and the absorbance was read at 590 nm via microplate reader.
7. Reducing sugar content of samples (and laminarin standards) was determined via glucose standard curve. Laminarin reducing sugar equivalent was calculated via Equation 4-5.

$$RS_L = RS_H - RS_B \quad (4-5)$$

where

$RS_L$  is the laminarin reducing sugar equivalent concentration [gGIE·l<sup>-1</sup>]

$RS_H$  is the measured reducing sugar concentration of the 1,3-β-D-glucanase incubated supernatant sample [gGIE·l<sup>-1</sup>]

$RS_B$  is the measured reducing sugar concentration of the blank incubated sample [gGIE·l<sup>-1</sup>]

8. The  $RS_L$  values measured from laminarin standard solutions were paired with the known laminarin concentration of the laminarin standard solutions to construct a secondary standard curve. Laminarin concentrations ( $L$ ) of all samples were calculated from respective  $RS_L$  values using this secondary standard curve, and reported in gLE·l<sup>-1</sup> (grams laminarin equivalent per litre). This calculation is stated explicitly in Equation 4-6 following.

$$L = \frac{RS_L - c_L}{m_L} \quad (4-6)$$

Where  $c_L$  is the y-intercept of the  $L(x)$  vs  $RS_L(y)$  standard curve [gGIE·l<sup>-1</sup>], and  $m_L$  is the slope of the  $L(x)$  vs  $RS_L(y)$  standard curve [gGIE·gLE<sup>-1</sup>].

#### 4.2.4. Inorganic sulfate measurement

The concentration of the sulfated polysaccharide fucoidan in solution was measured via the proxy of inorganic sulfate concentration. Testing follows the method set forth by Dodgson (1961), adapted to a smaller assay size of 2 ml. 4 % TCA (trichloroacetic acid) and a gelatin-barium chloride reagent (0.5 % gelatin, 0.5 % barium chloride) are prepared as in Appendix A.3. Potassium sulfate was prepared as a standard and the resultant standard curve is also shown in Appendix A.3.. A summary of the method is given below.

1. Sample is diluted by a factor of 4 – after which 80 µl is pipetted into a 2 ml CBT. A standard (1 - 0 g·l<sup>-1</sup> SO<sub>4</sub><sup>2-</sup>) is prepared in parallel.
2. 1520 µl of 4 % TCA is added, followed by 400 µl of gelatin-barium chloride reagent.
3. After mixing, incubate at 25 °C for 20 minutes
4. Pipette 300 µl into microplate wells and read absorbance at 450 nm with microplate reader.
5. Report inorganic sulfate content as grams sulfate equivalents per litre (gSE·l<sup>-1</sup>).



#### 4.2.5. Total phenolics measurement

Testing of total phenolics was carried out to further characterise extracts, and the response was expected to increase as the cell-wall was hydrolysed. An adapted version of the method presented in Fogliano, Verde, Randazzo & Ritieni (1999) was used. 10.6 % Folin-Ciocalteu (FC) reagent was used in conjunction with a 1.54 % sodium carbonate solution and gallic acid standard ( $0.5 \text{ g}\cdot\text{l}^{-1}$ ). Reagent preparation and standard curves are shown in Appendix A.4., with the method summarised below. The protocol was carried out under low light conditions.

1. 20  $\mu\text{l}$  of sample was added to a 2 ml MCT.
2. 940  $\mu\text{l}$  10.6 % FC solution was added to the MCT and mixed via vortex for 1 minute.
3. 1040  $\mu\text{l}$  was added to the MCT in two amounts of 520  $\mu\text{l}$ , ensuring good mixing.
4. Samples were then incubated at 25 °C for 2 hours in darkness.
5. Samples were shaken to remove bubbles and 300  $\mu\text{l}$  developed solution pipetted into microplate wells
6. Absorbance was read at 750 nm via microplate reader.
7. *TP* was reported as grams gallic acid equivalents per litre ( $\text{gGAE}\cdot\text{l}^{-1}$ ).

#### 4.2.6. Antioxidant capacity measurement

The antioxidant capacity method used was also adapted from Fogliano *et al.* (1999) and made use of DMPD (dimethyl-4-phenylenediamine). 100 mM (2.09 %) solution of DMPD in demineralised water was prepared alongside 50 mM (0.8 %) ferric chloride solution and 100 mM sodium acetate buffer (pH 5.25). TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, vitamin E analogue) was used as a standard, dissolved in absolute ethanol. A dilution factor of 20 was chosen due to full reagent decolouring with all undiluted samples. The full protocol and standard curve are presented in Appendix A.5., with the method summarised as follows.

1. 500  $\mu\text{l}$  DMPD solution and 100  $\mu\text{l}$  ferric chloride solution were added to 50 ml of sodium acetate buffer solution. This formed the coloured radical cation solution.
2. 80  $\mu\text{l}$  of diluted sample was added to a 2 ml MCT. A three point TROLOX standard ( $0.2\text{-}0 \text{ g}\cdot\text{l}^{-1}$ ) was developed alongside the sample. 1.6 ml coloured radical cation solution was then added to the samples and standards. Uninhibited signal was measured by adding 1.6 ml of the coloured radical cation solution to an empty MCT. All sample MCTs were mixed for 10 minutes.
3. 300  $\mu\text{l}$  of the developed solutions were pipetted into microplate wells.
4. Absorbances of solutions were read at 490 nm via microplate reader.
5. Antioxidant capacity was reported as grams TROLOX equivalents per litre ( $\text{gTE}\cdot\text{l}^{-1}$ )

### 4.3. Unit conversion

To make data comparison with literature easier during discussion, all spectrophotometric data (originally reported as  $\text{g}\cdot\text{l}^{-1}$ ) was converted to units of  $\text{g}\cdot\text{gDW}^{-1}$  (gram per gram dry weight) and co-reported. This conversion is shown in Equation 4-7. Unit converted data is located in Appendix I.

$$y_c = \frac{y_s}{DS_c} \times SY \quad (4-7)$$

Where  $y_c$  is a generic sample response after unit conversion [ $\text{g}\cdot\text{gDW}^{-1}$ ],  $y_s$  is a generic spectrophotometric sample response before unit conversion [ $\text{g}\cdot\text{l}^{-1}$ ],  $DS_c$  is the dissolved solids in the supernatant of the sample [ $\text{g}\cdot\text{l}^{-1}$ ], and  $SY$  is the solubilised yield of the sample [ $\text{gDS}\cdot\text{gDW}^{-1}$ ].

While this conversion allowed the comparison of data to external literature, it was the spectrophotometric responses that were used for optimisation, where the liquid extract was viewed as the product and the concentrations of laminarin and the other compound groups in solution were the quantities of interest.

## Chapter 5

# *Response surface analysis and modelling*

In this chapter, the analysis and modelling of each response is presented. Models were assessed via three criteria, namely adjusted  $R^2$ , lack of fit p-value, and individual factor p-values. Statistical significance ( $p < 0.05$ ) was highlighted and outlined in all tables, and p-values that showed near-significance ( $0.05 < p < 0.1$ ) were highlighted only. All data is included in Appendix C, with ANOVA tables included in Appendix D, and response surface regression coefficients in Appendix E. Validation of models takes place later in the chapter (Section 5.3) and discussion regarding all modelling and validation takes place in Section 5.4.

### 5.1. Response surface methodology

A central-composite design (CCD) was proposed as an efficient tool for optimisation, as it allows the detection and modelling of curvature in the response through the regression of quadratic models (Bezerra et al., 2008). An  $\alpha$ -value of 1.682 was used to ensure rotatability (and therefore uniform prediction error), and blocking was deemed unnecessary as samples will originate from the same batch of harvested material. Laboratory conditions were expected to remain constant. Coded variables were used for pH, temperature (K) and enzyme-to-substrate-ratio ( $v/dw$ ) respectively. The CCD chosen consisted of a full factorial design augmented with star points and centre point (with replication). Equation 5-1 determines the number of experiments to be carried out in the initial CCD.

$$N = k^2 + 2k + c_p \quad (5-1)$$

Where  $N$  is the number of experimental runs,  $k$  is the number of factors to be optimised, and  $c_p$  is the number of centre point replicates in the CCD. In this case  $c_p$  was chosen as five to give an acceptable indication of experimental error, confirming a total experimental count of twenty when optimising three factors. A characteristic CCD studies each factor at five levels, namely the set of coded variables

$$[-\alpha, -1, 0, +1, +\alpha]$$

The relationship between the coded variables and actual experimental variables corresponds to the linear relationships made explicit in Equations 5-2 to 5-4.

Table 5-1 Independent variables in experimental and coded form

Experimental variable	Variable range	Coded variable	Variable range
pH	[3.0, 6.0]	pH	[-1.682, +1.682]
Temperature (°C)	[40.0, 60.0]	T	[-1.682, +1.682]
E/S Ratio (w/w)	[0.00, 0.04]	ES	[-1.682, +1.682]

$$pH_{actual} = 0.8918 \cdot pH_{coded} + 4.5 \quad (5-2)$$

$$T_{actual} = 5.9453 \cdot T_{coded} + 50 \quad (5-3)$$

$$ES_{actual} = 0.0119 \cdot ES_{coded} + 0.02 \quad (5-4)$$

These factors at five levels resulted in the design presented in Appendix B. Coded variables were used during response surface modelling, which allowed effect comparisons on a standardised basis. Optima were reported in actual values, and intermediate graphical representations used coded variable axes. Final results and figures were presented once converted back to actual values.

The main response variable optimised was the laminarin concentration in the supernatant fraction, but all responses were modelled using the same general regression equation form. A second order function with quadratic and interaction terms formed the basis of modelling. Equation 5-5 shows a generalised equation of the form required.

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{1 \leq i < j}^k \beta_{ij} x_i x_j + \varepsilon \quad (5-5)$$

Where  $y$  is the response,  $x_i$  represents the modelling variables,  $\beta_0$  is the constant regressed coefficient,  $\beta_i$  represents the linear regression coefficient,  $\beta_{ii}$  the quadratic regression coefficient, and  $\beta_{ij}$  the second-order interaction regression coefficient. The residual/error is represented by  $\varepsilon$ . Standard practice was followed in omitting the third-order interaction term (Bezerra et al., 2008). Equation 5-6 shows the specific form Equation 5-5 took in this study.

$$y = A + B \cdot pH_{coded} + C \cdot pH_{coded}^2 + D \cdot T_{coded} + E \cdot T_{coded}^2 + F \cdot ES_{coded} + G \cdot ES_{coded}^2 + H \cdot pH_{coded} \cdot T_{coded} + I \cdot pH_{coded} \cdot ES_{coded} + J \cdot T_{coded} \cdot ES_{coded} \quad (5-6)$$

Where  $y$  is any response modelled, and  $A$  to  $J$  are response-surface coefficients, to be determined by sum-of-squares regression during modelling. A summary of all response variables to be tested in the hydrolysate supernatant is given in Table 5-2 below, along with the rationale for measuring each particular variable. Analytical methods were described in Chapter 4.

Table 5-2 Response variables to be tested, along with relevant units

Response variable	Symbol	Units	Testing rationale
Solubilised yield	<i>SY</i>	%	Assessment of substrate solubilisation
Supernatant dissolved solids	<i>DS</i>	%	As above
Reducing sugar	<i>RS</i>	gGLE·l <sup>-1</sup>	Enzyme activity tracking, and preparation for laminarin testing
Laminarin	<i>L</i>	gLE·l <sup>-1</sup>	Focus of project, optimisation
Inorganic sulfate	<i>S</i>	gSE·l <sup>-1</sup>	Fucoidan proxy, assessment of coextraction with laminarin
Total phenolic content	<i>TP</i>	gGAE·l <sup>-1</sup>	Coextraction assessment
Antioxidant capacity	<i>AO</i>	gTE·l <sup>-1</sup>	Correlation study with other responses

Once data was collected for a particular response (20 experiments, 5 sampling points each), TIBCO® Statistica™ (13.5.0.17) was used to analyse the data. Pure error was chosen as the ANOVA (analysis of variance) error term. CCD response surface coefficients were regressed with a 95 % confidence interval, meaning that statistical significance was noted at p-values of < 0.05. Statistical “near-significance” in models was noted when  $0.05 < p < 0.10$ . After response surfaces were modelled and optima mathematically calculated, validation experiments were conducted at the optimum conditions. Validation error  $\varepsilon_{val}$  is defined via Equation 5-7.

$$\varepsilon_{val} = \frac{|y_{model} - y_{measured}|}{y_{measured}} \quad (5-7)$$

Where  $y_{model}$  is a chosen model predicted response variable, and  $y_{measured}$  is a chosen experimentally measured response variable.

## 5.2. Response surface results

### 5.2.1. Solubilised yield

Solubilised yield (*SY*, defined in Equation 4-4) data is presented in Appendix C.1, Table C-1. The solubilised yield response showed a range between 43 to 56 % (Table 5-3), with the maximum occurring at higher pH (5.4), higher temperature (44.1 °C), and higher enzyme dosage (3.2 % (*v/dw*)) than the minimum. These were preliminary indicators of the effect of each variable.

Table 5-3 Summary of key *SY* experimental data points

Response	Point nature	Value (%)	SO	pH	T (°C)	ES ( <i>v/dw</i> )	t (hrs)
<i>SY</i>	Maximum	56.0 ± 0.7	6	5.4	44.1	0.032	6
	Minimum	43.4 ± 1.1	11	4.5	40.0	0.020	3

Summarised ANOVAs (Table 5-4 and Appendix D, Table D-1) showed the significant effect of linear enzyme-to-substrate ratio from 1.5 to 6 hours (all p-values < 0.04). Near-significance ( $0.05 < p < 0.10$ ) was exhibited at 0 hours for the quadratic effect of pH ( $p \approx 0.07$ ) and the interaction between pH and temperature ( $p \approx 0.08$ ) factors. All  $R^2$  values were low (< 0.63) and the adjusted  $R^2$  values were all less than  $\approx 0.28$  (1.5 hours), meaning that less than 28 % of the experimental variance displayed was explained by the regressed *SY* response surface models. Higher enzyme loadings lead to increased responses.

Table 5-4 Solubilised yield (*SY*) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-1)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.757	0.429	0.799	0.981	0.592
pH (Q)	0.071	0.294	0.401	0.384	0.413
T (L)	0.859	0.442	0.198	0.574	0.360
T (Q)	0.862	0.337	0.424	0.369	0.545
ES (L)	0.257	0.013	0.017	0.007	0.040
ES (Q)	0.744	0.740	0.946	0.605	0.528
pH by T	0.085	0.331	0.485	0.105	0.988
pH by ES	0.851	0.381	0.922	0.597	0.734
T by ES	0.708	0.418	0.629	0.764	0.385
Lack of fit	0.240	0.321	0.166	0.131	0.339
Pure error	3.175	3.259	4.264	4.018	5.131
$R^2$	0.452	0.623	0.492	0.568	0.489
Adj- $R^2$	0	0.284	0.035	0.180	0.030

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Model regression coefficients (Table 5-5 and Appendix E, Table E-1) showed that the linear effect of enzyme-to-substrate ratio played the largest role in the response surfaces from 1.5 to 6 hours (coefficients between  $\approx 1.69$  and  $\approx 2.35$ ), although no definitive increasing or decreasing trend was seen.

The regressed optima (Table 5-6) showed a change from pH 3.0 as the optimum for 0 and 1.5 hours to pH 6 from 3 hours onwards. This pH switch was paired with the switch in temperature from high (60 °C) to low (< 46 °C), also occurring from 3 hours onwards. This indicated rate-based behaviour and a trade-off between optimum operating conditions and enzyme longevity. All optima indicated the requirement for higher enzyme, whereas optima for pH and temperature moved away from lower pH (3.0) and higher temperature (60 °C).

Table 5-5 Solubilised yield (*SY*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-1)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time <i>t</i> (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	47.253	48.908	49.926	50.871	51.212
pH (L)	B	-0.157	-0.420	0.150	0.013	0.350
pH (Q)	C	1.076	0.558	0.499	0.504	0.532
T (L)	D	-0.090	0.408	0.829	0.326	0.618
T (Q)	E	0.086	0.505	-0.474	-0.521	-0.387
ES (L)	F	0.617	1.841	1.953	2.352	1.695
ES (Q)	G	-0.162	-0.167	0.039	-0.292	-0.405
pH by T	H	-1.350	-0.687	-0.550	-1.400	0.012
pH by ES	I	-0.125	0.613	0.075	0.400	0.288
T by ES	J	-0.250	0.563	-0.375	0.225	-0.763

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-6 Optimum conditions for *SY* responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
Hydrolysis time <i>t</i> (hours)	0	1.5	3	4.5	6
pH	3.0	3.0	6.0	6.0	6.0
T (°C)	60.0	60.0	45.4	40.6	45.1
ES ( <i>v/dw</i> )	0.035	0.040	0.040	0.040	0.040
Model predicted <i>SY</i> (%)	54.7	57.7	55.5	57.9	56.1

The resultant response surfaces (Figure 5-1) agreed with the trends shown. A conspicuous shift from concave surfaces to convex surfaces occurred over time, confirming the effect of extended hydrolysis time on solubilised yield. Figure 5-1a, where enzyme dosage was kept constant at 4.0 % (*v/dw*) shows that a saddle-shape developed, confirming the favourable nature for mid-range temperatures ( $\approx 45$  °C) to the solubilised yield response. Figure 5-1b (constant temperature at 45 °C) showed the effect of enzyme dosage to be greater at higher pH, evidenced by the larger slope developing further up the coded pH axis. Figure 5-1c shows a predicted mid-range optimum temperature by 6 hours, displayed in the negative quadratic curvature along the temperature axis.



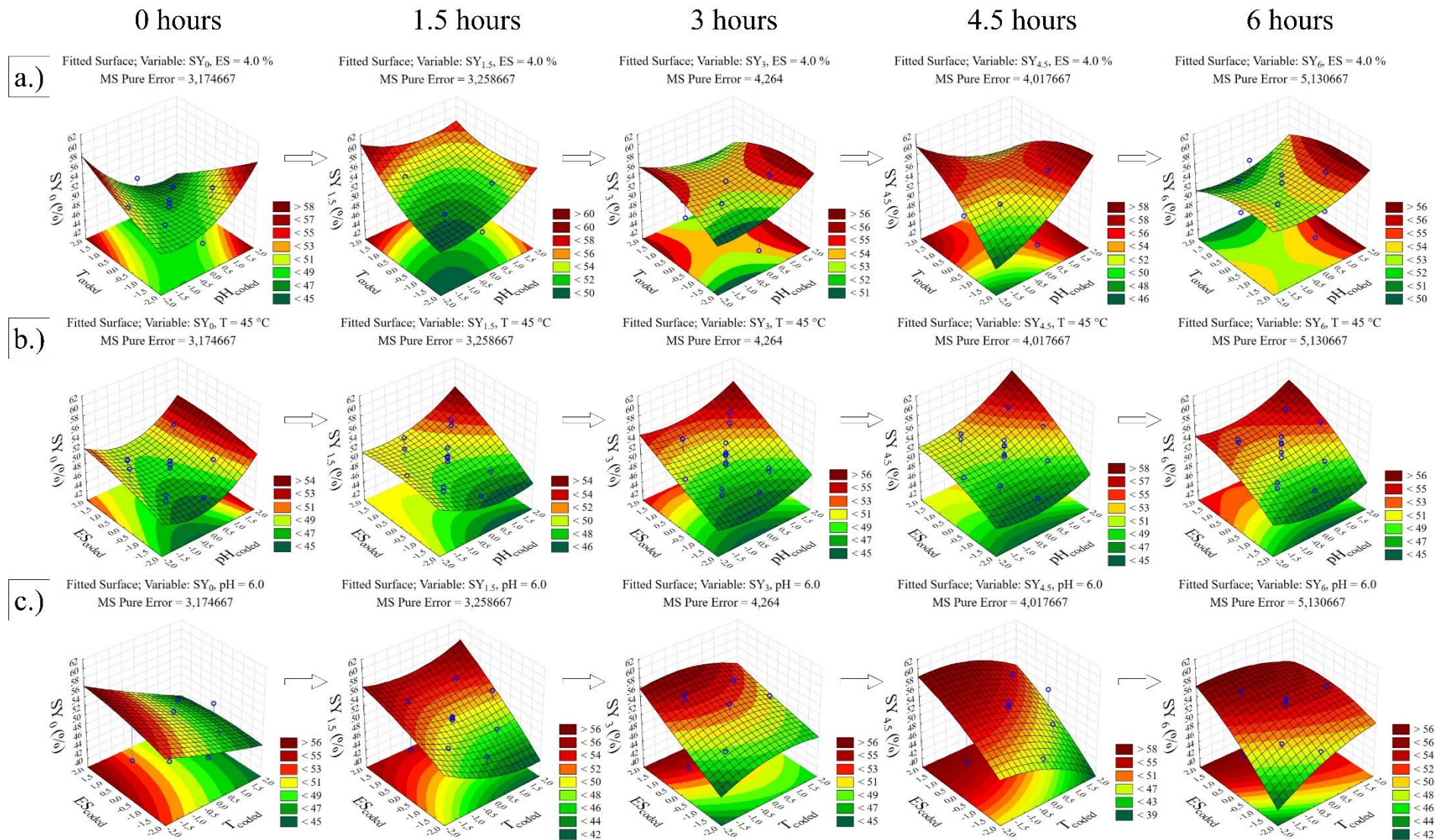


Figure 5-1 Response surface plots over time for solubilised yield, set at optimum conditions (pH 6.0, 45 °C, and 4.0 % (v/dw) enzyme dosage. a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant temperature (45 °C), c.) Constant pH (6.0)



## Supernatant dissolved solids

The supernatant dissolved solids (*DS*) was defined in Equation 4-1 and contributed to the definition of solubilised yield (*SY*). Dissolved solids data is presented in Appendix C, Table C-2 and the summary of minimum and maximum experimental data observations (Table 5-7) showed the response to remain approximately between 3 - 4 %.

Table 5-7 Summary of key *DS* experimental data points

Response	Point nature	Value	SO <sup>1</sup>	pH	T (°C)	ES (v/dw)	t (hrs)
<i>DS</i>	Maximum	3.93 ± 0.11 %	6	5.4	44.1	0.032	4.5
	Minimum	2.96 ± 0.09 %	20	4.5	50	0.020	0

<sup>1</sup>Standard order of experiments in RSM

ANOVAs over the five sampling points (Table 5-8 and Appendix D, Table D-2) indicated no consistent significant effects. A significant effect was found only in the linear enzyme-to-substrate term at 1.5 hours ( $p \approx 0.02$ ).  $R^2$  values of all 5 time points did not exceed 0.8, with 1.5 hours showing the highest  $R^2$  value of  $\approx 0.69$ , while the adjusted  $R^2$  at 1.5 hours was the only non-zero ( $\approx 0.41$ ). There was no significant lack-of-fit (all  $p > 0.4$ ), but the regressed response surfaces did not fit the data well. All time points except 1.5 hours did not show independent variable significance. Any pH and temperature within the experimental range could be used, with increased ES resulting in higher solids loading at early time points.

Table 5-8 Supernatant dissolved solids (*DS*) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-2)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.607	0.260	0.747	0.821	0.961
pH (Q)	0.751	0.574	0.477	0.664	0.929
T (L)	0.224	0.989	0.980	0.796	0.668
T (Q)	0.635	0.392	0.556	0.802	0.701
ES (L)	0.409	0.020	0.503	0.190	0.348
ES (Q)	0.713	0.363	0.795	0.598	0.402
pH by T	0.340	0.176	0.397	0.159	0.413
pH by ES	0.920	0.890	0.701	0.513	0.622
T by ES	0.389	0.806	0.683	0.698	0.977
Lack of fit	0.582	0.686	0.402	0.637	0.642
Pure error	0.036	0.027	0.058	0.051	0.023
$R^2$	0.381	0.689	0.211	0.430	0.287
Adj- $R^2$	0	0.409	0	0	0

Significant factors (CI = 95 %) are shaded and outlined. Values reported to 3 d.p.

Model regression coefficients for the *DS* responses (Table 5-9 and Appendix E, Table E-2) further indicated a lack of significant effects in the response. The linear coefficient of the enzyme-to-substrate term at 1.5 hours (0.149) was the largest and the early increase in the mean/intercept term over time (3.261% at 0 hours to 3.529 % at 1.5 hours) was consistent with the “fast-washing phase” as described by Ferreira et al (2019)

Table 5-9 Supernatant dissolved solids (*DS*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-2)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time <i>t</i> (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	3.261	3.529	3.520	3.561	3.595
pH (L)	B	0.028	-0.056	-0.022	-0.015	-0.002
pH (Q)	C	0.017	-0.026	0.049	0.027	-0.004
T (L)	D	-0.071	-0.001	-0.002	-0.017	0.019
T (Q)	E	0.025	0.040	-0.040	0.016	0.016
ES (L)	F	0.046	0.149	0.047	0.092	0.042
ES (Q)	G	-0.019	-0.043	-0.017	-0.033	-0.037
pH by T	H	-0.071	-0.091	-0.079	-0.132	-0.048
pH by ES	I	-0.007	-0.008	-0.035	-0.056	-0.028
T by ES	J	-0.063	-0.015	-0.037	-0.033	-0.002

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Common trends could still be observed in the optima obtained from each model (Table 5-10). Low pH (3.00) and high temperature (> 55 °C) combinations were common across all time points. Enzyme-to-substrate ratio optimum increased over time – an indication that the addition of enzyme had some effect – despite lacking significance in the ANOVA. *DS* was therefore considered at highest response with low pH, high temperature, and high enzyme-to-substrate ratio (pH 3.00, 60 °C, 4.0 % (*v/dw*) enzyme dosage).

Table 5-10 Optimum conditions for *DS* responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
Hydrolysis time <i>t</i> (hrs)	0	1.5	3	4.5	6
pH	3.0	3.0	3.0	3.0	3.0
T (°C)	60.0	60.0	55.1	60.0	60.0
ES ( <i>v/dw</i> )	0.005	0.039	0.040	0.040	0.034
Model predicted <i>DS</i> (%)	3.443	4.030	3.853	4.178	3.853

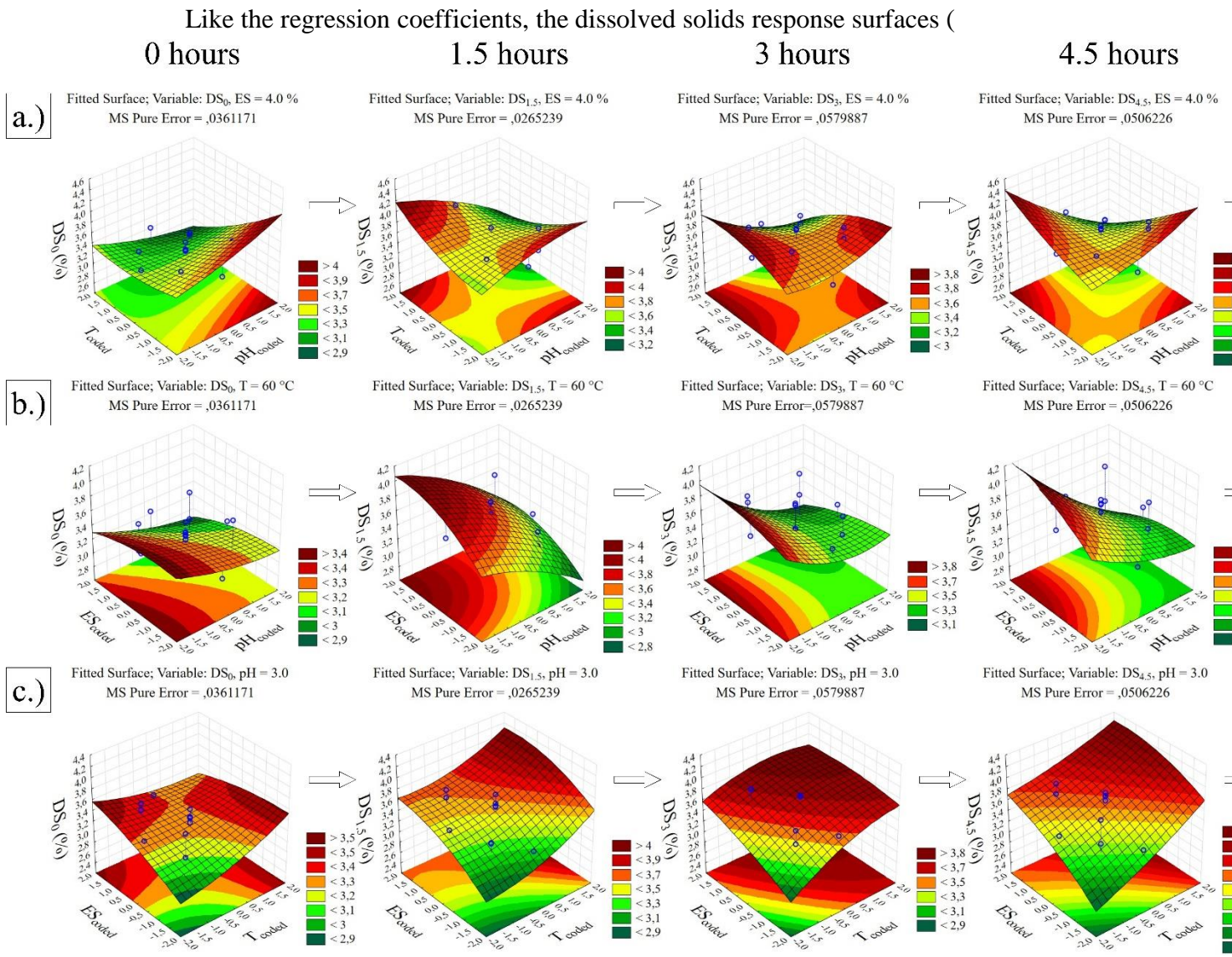


Figure 5-2) did not show definitive trends or changes over time. Figure 5-2b and c indicated an increased response with lower pH and higher temperature in earlier sampling points, with levelling by 6 hours. Despite lack of significance, this levelling indicates an enzymatic hydrolysis “catch-up” to conventional acid hydrolysis (low pH / high temperature).



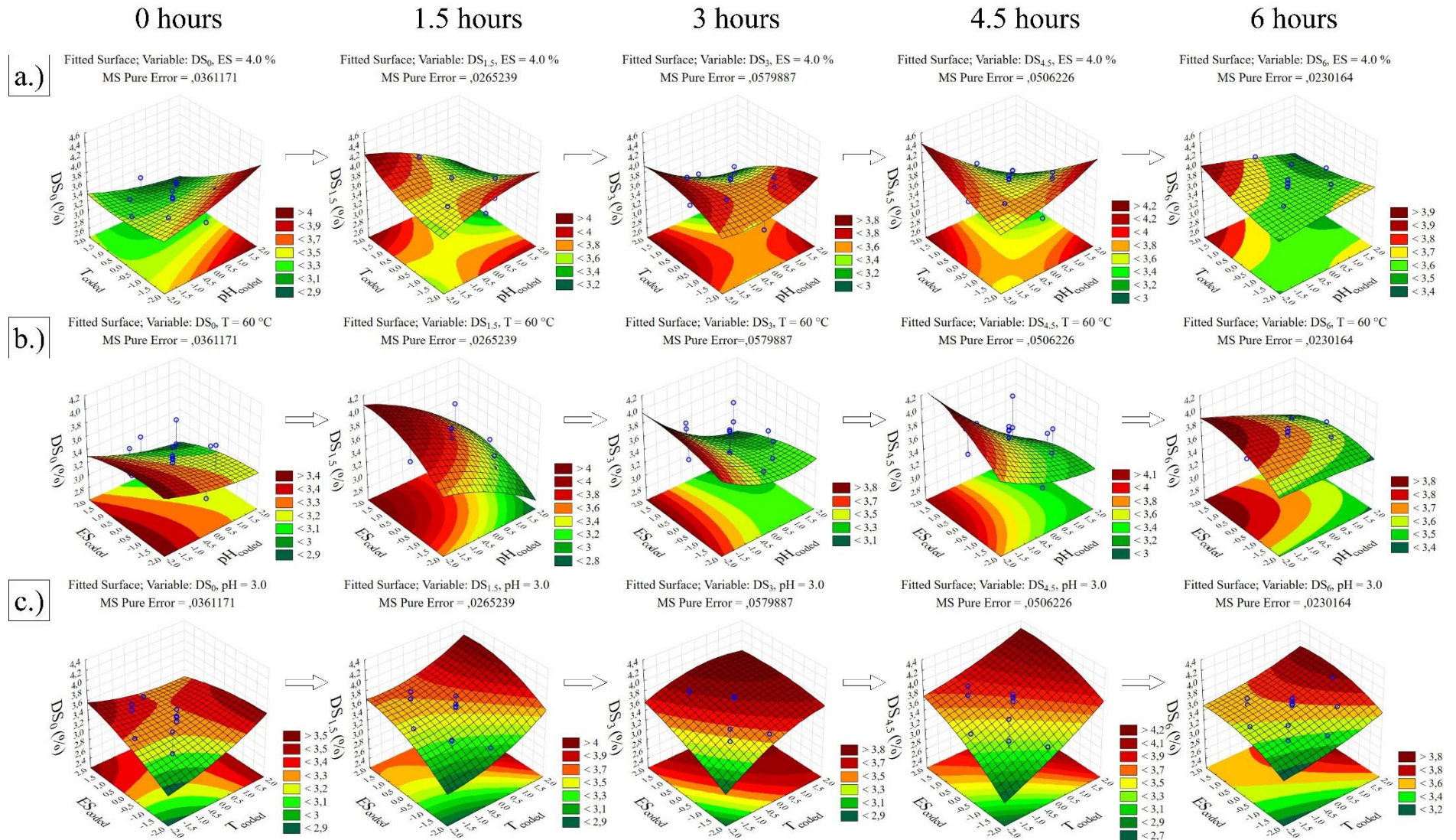


Figure 5-2 Response surface plots over time for supernatant dissolved solids, set at optimum conditions (pH 3.0, 60 °C, and 4.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant temperature (60 °C), c.) Constant pH (3.0)

## Supernatant mass fraction

Solubilised yield was heavily dependent on relative mass fractions of supernatant and pellet when sampling from the reactor (followed by centrifuging). The supernatant mass fraction  $F_{SN}$  (previously defined in Equation 4-2) was therefore analysed in the same way as other responses, constructing the five response surfaces. Data for  $F_{SN}$  are included alongside  $SY$  in Appendix C.1 (Figure C-3 and Table B2-2). Maximum and minimum values for experimental  $F_{SN}$  (Table 5-11) showed a ranged from 73.8 to 83.0 %. The maximum response in the CCD occurred in the same sample (therefore the same conditions) as the maximum  $SY$  response. Minimum  $F_{SN}$  occurred with lower enzyme loading (0.8 % (v/dw)) and higher temperature (55.9 °C). While both minimum and maximum data points both took place at pH 5.4, temperature and enzyme-to-substrate ratio differed. This gave a preliminary indication that pH was likely not a significant factor to supernatant mass fraction response, while temperature and enzyme-to-substrate ratio could be.

Table 5-11 Summary of key supernatant mass fraction ( $F_{SN}$ ) experimental data points

Response	Point nature	Value (%)	SO	pH	T (°C)	ES (v/dw)	t (hrs)
$F_{SN}$	Maximum	83.0 ± 0.1	6	5.4	44.1	0.032	6
	Minimum	73.8 ± 0.1	7	5.4	55.9	0.008	4.5

The summary of ANOVAs for  $F_{SN}$  (Table 5-12 and Appendix D, Table D-3) indicated enzyme to play a large role in the response. Statistical significance was shown in the linear effect of enzyme-to-substrate ratio from 1.5 to 6 hours (all  $p < 0.02$ ), as well as the quadratic effect of pH at 0 hours ( $p \approx 0.04$ ). The choice of pH and temperature (within experimental range) was not statistically significant, and only an increase in enzyme addition significantly increased  $F_{SN}$ . Higher  $R^2$  and adjusted  $R^2$  values were exhibited in these ANOVA compared to both  $DS$  and  $SY$  (all  $R^2 > 0.55$ , adjusted  $R^2 > 0.16$ ). The best fitting model was achieved at 3 hours (Adj- $R^2 \approx 0.47$ ).

The summary of surface coefficients for  $F_{SN}$  (Table 5-13 and Appendix E, Table E-3) shows that linear enzyme-to-substrate coefficients (1.5 to 6 hours, highlighted) were consistently higher than other factors (1.30 - 2.34 vs  $< 0.53$ ), confirming the absence of other significant effects.

Table 5-12 Supernatant mass fraction ( $F_{SN}$ ) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-3)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.622	0.563	0.362	0.650	0.715
pH (Q)	0.044	0.830	0.878	0.898	0.981
T (L)	0.677	0.610	0.947	0.761	0.764
T (Q)	0.836	0.973	0.785	0.376	0.2002
ES (L)	0.586	0.019	0.009	0.008	0.009
ES (Q)	0.659	0.727	0.712	0.799	0.751
pH by T	0.123	0.705	0.490	0.437	0.972
pH by ES	0.662	0.362	0.380	0.841	0.972
T by ES	0.950	0.924	0.796	0.973	0.585
Lack of fit	0.572	0.469	0.725	0.532	0.295
Pure error	1.139	1.991	3.784	4.035	3.763
R <sup>2</sup>	0.558	0.567	0.722	0.681	0.602
Adj-R <sup>2</sup>	0.160	0.178	0.471	0.395	0.243

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-13 Supernatant mass fraction ( $F_{SN}$ ) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-3)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time t (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	78.44	78.03	78.17	78.54	79.01
pH (L)	B	-0.15	0.24	0.53	0.26	0.20
pH (Q)	C	0.75	0.08	0.08	-0.07	-0.01
T (L)	D	-0.13	-0.21	0.04	-0.17	-0.17
T (Q)	E	0.06	0.01	-0.15	-0.51	-0.76
ES (L)	F	0.17	1.30	2.20	2.34	2.18
ES (Q)	G	0.13	0.14	-0.20	-0.14	-0.17
pH by T	H	-0.70	-0.20	-0.51	-0.60	0.03
pH by ES	I	0.18	-0.50	-0.66	-0.15	0.02
T by ES	J	-0.02	0.05	-0.19	0.02	-0.40

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optima regressed from models (Table 5-14) showed a change at 4.5 hours. Low pH (3.0) and high temperature (60 °C) changed to high pH (6.0) and lower temperatures (< 47 °C). This agreed with the shift in optima found at 3 hours in SY analysis. High enzyme substrate loading (4.0 % (v/dw)) was consistent in the predicted optima throughout all five models, agreeing with the significant effects shown.

Table 5-14 Optimum conditions for  $F_{SN}$  responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
<b>Hydrolysis time t (hours)</b>	0	1.5	3	4.5	6
<b>pH</b>	3.0	3.0	3.0	6.0	6.0
<b>T (°C)</b>	60.0	60.0	60.0	43.4	46.9
<b>ES (v/dw)</b>	0.040	0.040	0.040	0.040	0.040
<b>Model predicted <math>F_{SN}</math> (%)</b>	82.8	82.3	83.1	82.5	82.8

The response surfaces (Figure 5-3) showed the effect of enzyme dosage and hydrolysis time graphically, with conspicuous optimums developing in Figure 5-3a and c indicating mid-range temperatures to be beneficial to the response (as was found with the solubilised yield responses). Figure 5-3a and b both show a lack effect from pH, showcased by the near-horizontal contours along the pH axis and Figure 5-3b and c both show the positive effect of enzyme dosage on the response, showing the steepest slope of the three variables. These observations agree with the statistical analysis, where the largest and only significant effect was enzyme dosage.



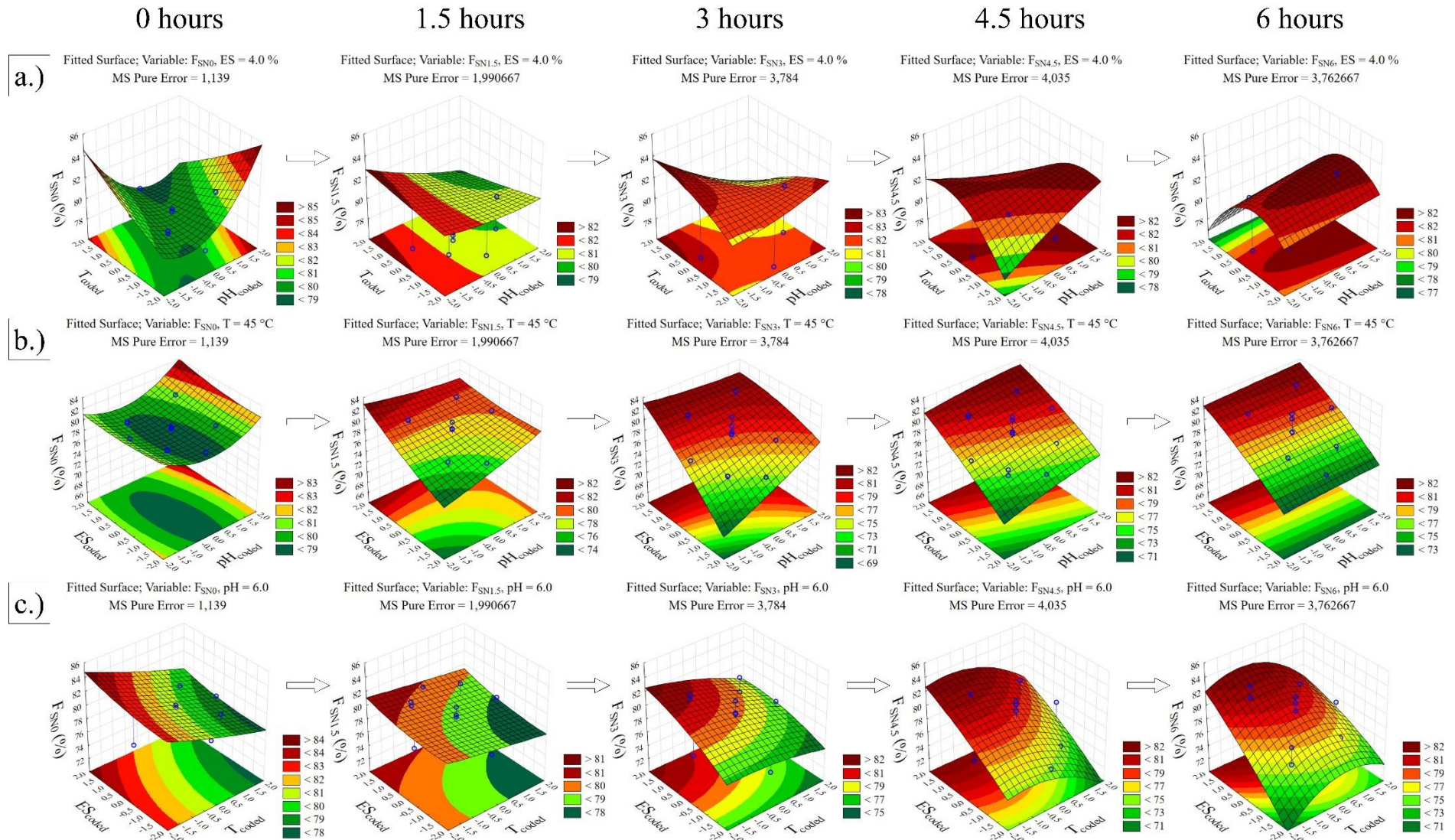


Figure 5-3 Response surface plots over time for supernatant mass fraction, set at optimum conditions (pH 6.0, 45 °C, and 4.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant temperature (45 °C), c.) Constant pH (6.0)



## Pellet-solids loading

Apart from the supernatant mass fraction and the dissolved solids responses, solubilised yield was also dependent on the solids-loading of the pellet after centrifugation ( $SL_P$ , Equation 4-3). Response surfaces were analysed for this parameter as well, with proxy errors derived from the centre-point experiments SO 15 to 20. The maximum and minimum pellet solids loading data points from the CCD (Table 5-15 and Appendix C.4) showed a range from 10.8 to 14.8 %. It was noted that for an increased solubilised yield response,  $SL_P$  must decrease. The table suggests that the effect of enzyme dosage may not aid in the decrease of the  $SL_P$  response, with SO 19 at 6 hours maintaining a higher value ( $14.2 \pm 0.8$  %) than SO 13 ( $10.8 \pm 0.8$  %).

Table 5-15 Summary of key  $SL_P$  experimental data points

Response	Point nature	Value (%)	SO	pH	T (°C)	ES (v/dw)	t (hrs)
$SL_P$	Maximum	$14.8 \pm 1.0$	19	4.5	50	0.020	0
	Minimum	$10.8 \pm 0.8$	13	4.5	50	0.000	6

ANOVAs of the  $SL_P$  response (Table 5-16 and Appendix D, Table D-4) showed the significant effect of linear enzyme-to-substrate parameter from 3 to 6 hours (p-values  $< 0.024$ ), as well as the linear temperature effect from 4.5 to 6 hours (p-values  $< 0.035$ ). The quadratic effect of pH was significant at 4.5 hours only. Response surfaces from 3 hours showed reasonable adjusted  $R^2$  values (all  $> 0.48$ ), with the best model (4.5 hours) showing the highest adjusted  $R^2$  value of  $\approx 0.85$ , explaining 85 % of the experimental variance at that time point. While the quadratic effect of pH was significant at 4.5 hours, pH did not show a significant effect elsewhere and should be maintained between the boundaries of the experiment.

Response surface coefficients for  $SL_P$  (Table 5-17 and Appendix E, Table E-4) showed that linear coefficients of the enzyme-to-substrate ratio were consistently positive, with 3 to 6 hours (0.759 – 0.909) showing the largest coefficients. This positive enzyme effect was a similar trend to that found in  $F_{SN}$ . The linear temperature effect (significant at 4.5 and 6 hours) showed a consistently negative and decreasing surface regression coefficient, indicating the role temperature plays in decreasing the pellet-solids loading. These effects worked against one another over the course of the experiment, where  $SY$  indicated a combination of higher temperature and enzyme dosage as favourable.

Table 5-16 Pellet-solids loading ( $SL_P$ ) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-4)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.951	0.676	0.566	0.440	0.284
pH (Q)	0.978	0.366	0.399	0.039	0.240
T (L)	0.427	0.352	0.208	0.027	0.034
T (Q)	0.871	0.790	0.970	0.087	0.269
ES (L)	0.627	0.164	0.023	0.001	0.014
ES (Q)	0.969	0.995	0.918	0.286	0.865
pH by T	0.752	0.848	0.570	0.558	0.094
pH by ES	0.602	0.173	0.081	0.083	0.278
T by ES	0.650	0.440	0.570	0.083	0.929
Lack of fit	0.911	0.860	0.994	0.910	0.356
Pure error	0.910	1.499	0.979	0.258	0.570
R <sup>2</sup>	0.211	0.548	0.783	0.922	0.729
Adj-R <sup>2</sup>	0	0.140	0.587	0.852	0.485

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-17 Pellet-solids loading ( $SL_P$ ) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-4)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time t (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	13.522	13.177	12.830	13.082	12.871
pH (L)	B	-0.017	0.147	0.164	0.115	0.245
pH (Q)	C	-0.007	-0.320	-0.240	-0.372	-0.265
T (L)	D	-0.223	-0.339	-0.387	-0.426	-0.589
T (Q)	E	-0.043	-0.090	-0.010	-0.284	-0.247
ES (L)	F	0.133	0.540	0.865	0.909	0.759
ES (Q)	G	0.010	-0.002	-0.028	-0.160	0.035
pH by T	H	0.113	-0.088	-0.213	-0.113	-0.550
pH by ES	I	0.188	-0.688	-0.763	-0.388	-0.325
T by ES	J	-0.163	-0.363	-0.213	-0.388	-0.025

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optimum (minimum) conditions for each response surface of  $SL_P$  (Table 5-18) showed low pH (3.0) and enzyme dosage (0 %) to be consistent from 1.5 hours onwards. The predicted response was lowest with high temperature (60 °C) from 4.5 to 6 hours, although the fluctuation between 40 and 60 °C for predicted optima does show uncertainty in the results.

Table 5-18 Optimum conditions for  $SL_P$  responses, from response surfaces (in experimental range)

<b>Independent variable</b>	<b>Optimum conditions observed in response surfaces</b>				
<b>Hydrolysis time t (hours)</b>	0	1.5	3	4.5	6
<b>pH</b>	3.0	3.0	3.0	3.0	3.0
<b>T (°C)</b>	60.0	40.0	40.0	60.0	60.0
<b>ES (v/dw)</b>	0.040	0.000	0.000	0.000	0.000
<b>Model predicted <math>SL_P</math> (%)</b>	11.978	8.207	7.602	8.655	9.551

The response surfaces of pellet-solids loading (Figure 5-4) confirmed the positive effect of middle-point temperature and pH on the response. These conditions, ideally suited to enzyme activity, predicted a higher pellet-solids loading even when enzyme was not present (Figure 5-4a). The combination of high enzyme dosage and low pH also showed as favourable in the plots (Figure 5-4b). The effect of temperature was not visually apparent in either Figure 5-4a or c.

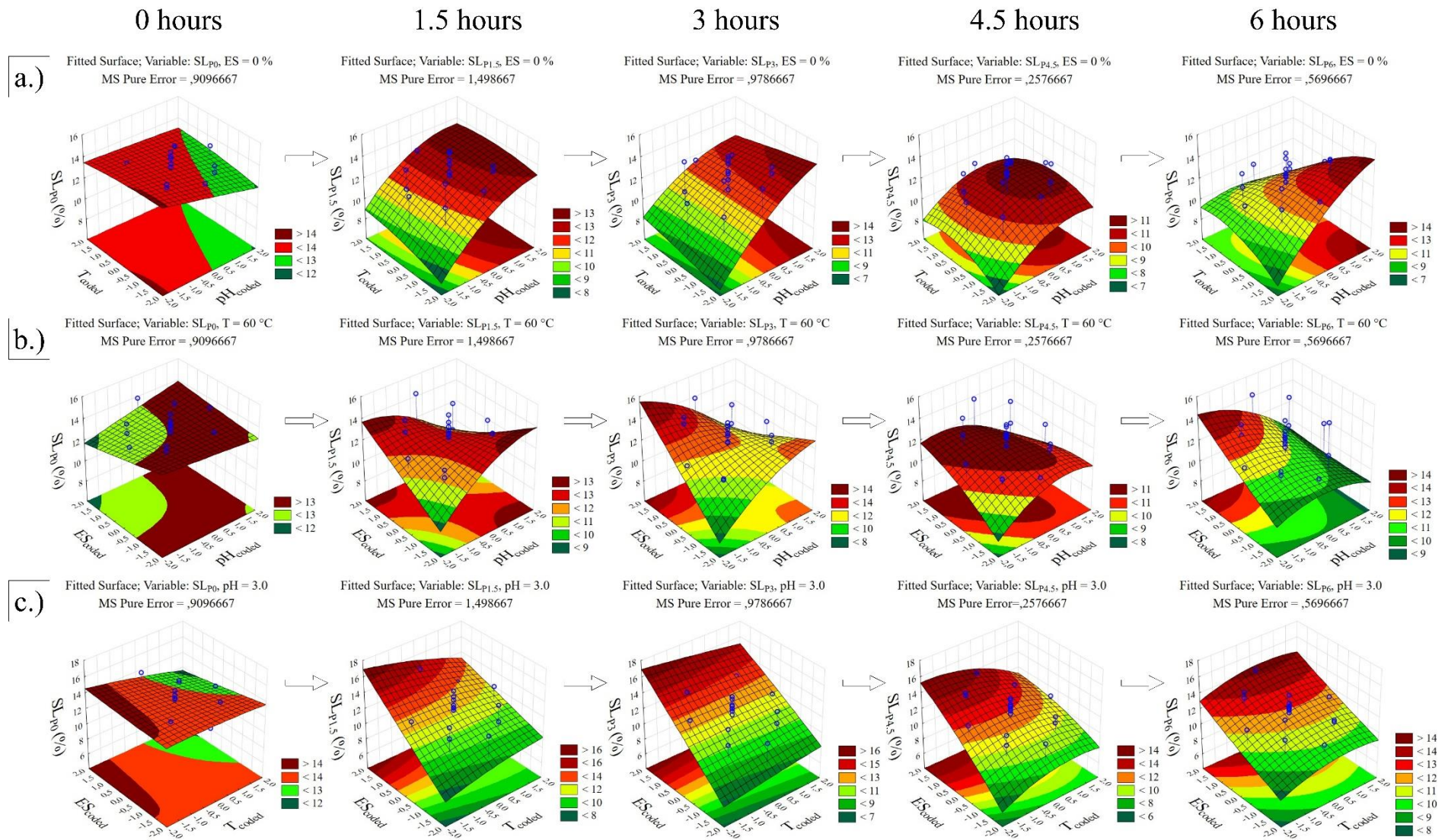


Figure 5-4 Response surface plots over time for pellet-solids loading, set at optimum conditions (pH 3.0, 60 °C, and 0 % (v/dw) enzyme dosage).  
 a.) Constant enzyme dosage (0 % (v/dw)), b.) Constant temperature (60 °C), c.) Constant pH (3.0)

## 5.2.2. Reducing sugars

The reducing sugar content of the supernatant (*RS*) was determined via spectrophotometric measurement as described in Chapter 4. Key data (Table 5-19 and Appendix C6) showed a range from  $\approx 4.4$  to  $\approx 7.2$   $\text{gGlE}\cdot\text{l}^{-1}$ . Maximum and minimum responses occurred at the same pH (3.6) and maximum *RS* occurred with both higher temperature (55.9 °C) and higher enzyme-to-substrate ratio (3.2 % (*v/dw*)), which gave a preliminary indication of the significance of these factors.

Table 5-19 Summary of key *RS* experimental data points

Response	Point nature	Value ( $\text{gGlE}\cdot\text{l}^{-1}$ )	SO	pH	T (°C)	ES ( <i>v/dw</i> )	t (hrs)
<i>RS</i>	Maximum	$7.24 \pm 0.14$	4	3.6	55.9	0.032	6
	Minimum	$4.44 \pm 0.11$	1	3.6	44.1	0.008	0

ANOVAs for *RS* (Table 5-20 and Appendix D, Table D-5) showed that the linear enzyme-to-substrate term showed the greatest significance to *RS* from 1.5 to 6 hours (all  $p < 0.007$ ). Near-significance ( $0.05 < p < 0.10$ ) was seen in the linear temperature effect at 1.5 and 4.5 hours ( $p$ -values of  $\approx 0.067$  and  $\approx 0.088$  respectively) and also in the linear pH effect at 6 hours ( $p \approx 0.055$ ). Adjusted  $R^2$  values exceeded 0.6 (except at 0 hours), with the fit highest at 4.5 and 6 hours ( $\approx 0.78$  and  $\approx 0.76$  respectively). Overall it was indicated from the ANOVAs that increased enzyme addition was the only statistically significant treatment for an increased *RS* response. Temperature and pH must be maintained within experimental space.

Table 5-20 Reducing sugars (*RS*) factor significance  $p$ -values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-5)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.946	0.470	0.361	0.198	0.055
pH (Q)	0.934	0.344	0.420	0.341	0.201
T (L)	0.176	0.067	0.193	0.088	0.126
T (Q)	0.638	0.967	0.804	0.189	0.263
ES (L)	0.150	0.003	0.007	0.001	0.000
ES (Q)	0.673	0.444	0.628	0.929	0.588
pH by T	0.281	0.249	0.405	0.389	0.227
pH by ES	0.431	0.428	0.464	0.440	0.358
T by ES	0.335	0.483	0.513	0.526	0.886
Lack of fit	0.555	0.665	0.897	0.745	0.374
Pure error	0.128	0.133	0.222	0.125	0.090
$R^2$	0.492	0.827	0.802	0.885	0.874
Adj- $R^2$	0.035	0.671	0.623	0.781	0.760

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.



The regression coefficients determined from the RSM models for *RS* (Table 5-21, Appendix E, Table E-5) confirmed the dominant effect of the linear enzyme-to-substrate term on the models from 1.5 to 6 hours (0.531 – 0.656), while the mean (a model predicted centre-point) increased from  $\approx 5.0$  to  $\approx 6.4$  gGIE $\cdot$ l $^{-1}$  over the six hours.

Table 5-21 Reducing sugars (*RS*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-5)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time <i>t</i> (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	4.995	5.731	6.021	6.306	6.440
pH (L)	B	-0.007	0.077	0.128	0.142	0.203
pH (Q)	C	0.008	-0.101	-0.109	-0.098	-0.116
T (L)	D	0.153	0.231	0.192	0.202	0.149
T (Q)	E	0.047	0.004	-0.033	-0.141	-0.100
ES (L)	F	0.164	0.531	0.567	0.656	0.656
ES (Q)	G	0.042	0.080	0.064	-0.009	-0.046
pH by T	H	-0.153	-0.168	-0.151	-0.118	-0.146
pH by ES	I	-0.108	-0.111	-0.132	-0.105	-0.107
T by ES	J	0.135	0.098	0.117	0.085	0.016

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optima of the five response surface models (Table 5-22) showed that, as time progressed, the optimum of the *RS* response moved from pH 3.0 to pH 3.7 at 6 hours. A slight reduction of temperature optimum was observed at the 6 hour mark as well ( $T = 58.9$  °C). The high addition of enzyme was consistent at 4.0 % (*v/dw*) for the duration of the experiment.

Table 5-22 Optimum conditions for *RS* responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
Hydrolysis time <i>t</i> (hours)	0	1.5	3	4.5	6
pH	3.0	3.0	3.1	3.4	3.7
T (°C)	60.0	60.0	60.0	60.0	58.9
ES ( <i>v/dw</i> )	0.040	0.040	0.040	0.040	0.040
Model predicted <i>RS</i> (gGIE $\cdot$ l $^{-1}$ )	6.94	7.90	8.00	7.70	7.54

The response surfaces for *RS* (Figure 5-5) showed the positive effect of hydrolysis time, with Figure 5-5a also showing optima formation at midrange temperature and pH as time progressed. Figure 5-5b and c both show the positive effect of elevated enzyme dosage to the response, visibly more of an effect than either pH or temperature.

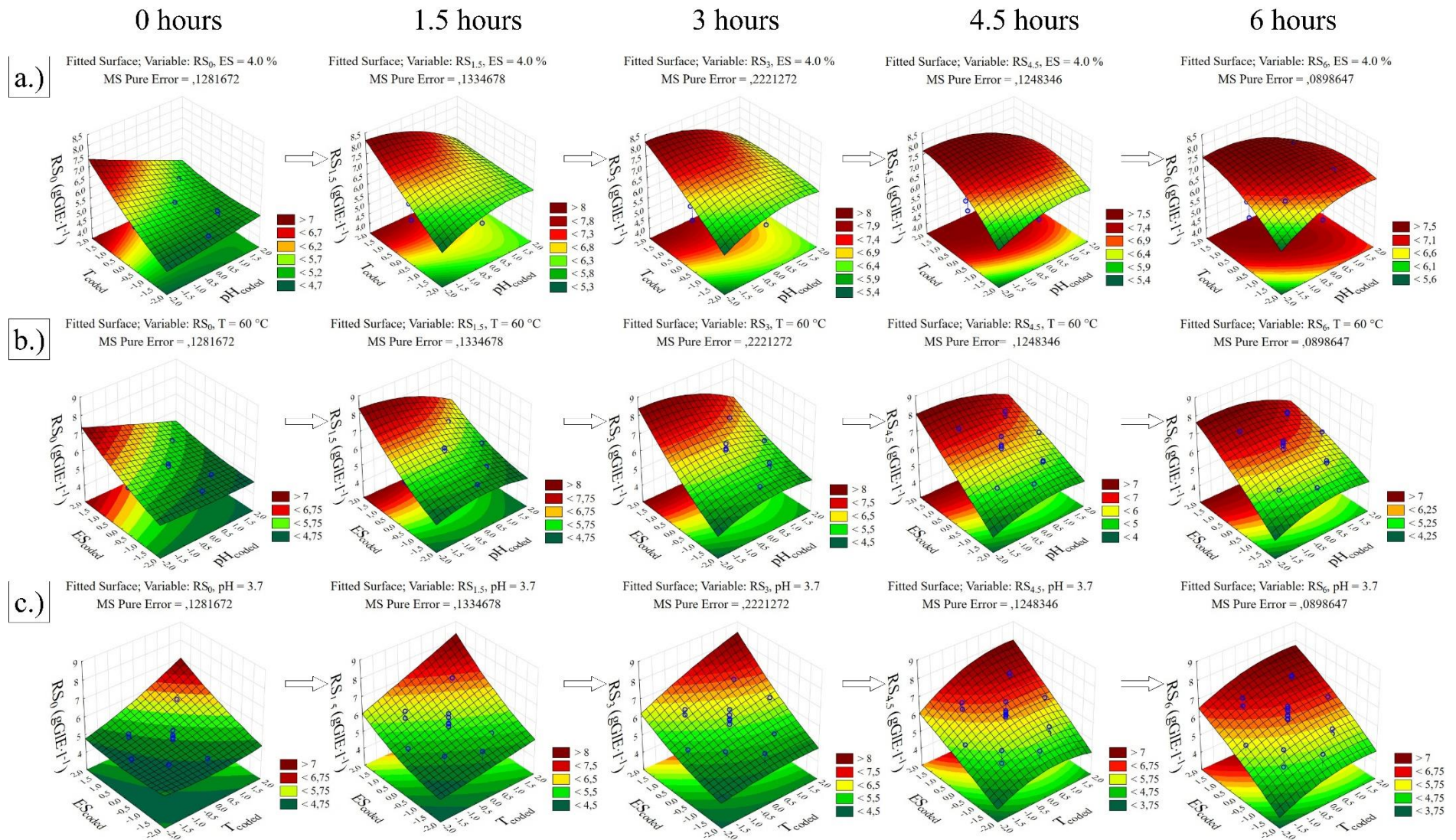


Figure 5-5 Response surface plots over time for reducing sugar concentration, set at optimum conditions (pH 3.7, 60 °C, and 4.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant temperature (60 °C), c.) Constant pH (3.7)

### 5.2.3. Laminarin

The laminarin response ( $L$ ) data is found in Appendix C.7., including measurements of the blank reducing sugars  $RS_B$  and enzymatically hydrolysed reducing sugars  $RS_H$ . The differences between these measurements ( $RS_L$ , defined in Equation 4-5) was converted to  $\text{gLE}\cdot\text{l}^{-1}$  (grams laminarin equivalent per litre) via a secondary standard (Appendix A.2.). This standard was obtained by hydrolysing laminarin from *Laminaria digitata* (Merck) using the methods stated in Chapter 4. Key data for the  $L$  response (Table 5-23 and Appendix C.7.) showed a range barely exceeding  $1.0 \text{ gLE}\cdot\text{l}^{-1}$  and dropping almost to zero. These data points indicated that a lower pH and higher temperature combination should be optimal, and higher enzyme dosage was also exhibited in the maximum response.

Table 5-23 Summary of key  $L$  experimental data points

Response	Point nature	Value ( $\text{gLE}\cdot\text{l}^{-1}$ )	SO	pH	T ( $^{\circ}\text{C}$ )	ES ( $v/dw$ )	t (hrs)
$L$	Maximum	$1.04 \pm 0.07$	4	3.6	56.0	0.032	1.5
	Minimum	$0.15 \pm 0.07$	5	5.4	44.1	0.008	0

ANOVAs for  $L$  (Table 5-24 and Appendix D, Table D-6) indicated temperature and pH to play significant roles in the laminarin response. No lack-of-fit was shown and all adjusted  $R^2$  were greater than 0.75, explaining the majority of experimental variance. Consistent significance was shown in the linear pH effects (all p-values  $< 0.002$ ) and also the quadratic pH effects (all p-values  $< 0.046$ , except at 4.5 hours). Linear and quadratic temperature effects also showed as consistently significant, other than the quadratic effect at 4.5 hours. The lowest p-values, and therefore highest statistical significance were shown by both the linear pH and linear temperature effects, while enzyme-to-substrate ratio did not have a statistically significant impact (only through the pH by enzyme-to-substrate ratio effect at 6 hours). The conditions pointed towards the conventional methods of laminarin extraction (pH 1.0,  $70^{\circ}\text{C}$ ) suggested by (Devillé et al., 2004), and indicated no enzyme requirement.

Regression coefficients of the laminarin response (Table 5-25 and Appendix E, Table E-6) showed trends in the obtained surface models over time. The table shows that there was no discernible trends in coefficients over time. The means (equivalent to the response surfaces centre points) did not increase over time. The linear coefficients of temperature remained at  $\approx 0.1$  while linear pH coefficients fluctuated between -0.237 and -0.142. The consistent positive nature of the linear temperature coefficient confirmed the positive effect of temperature on  $L$  while the negative linear pH coefficients indicated the requirement for lower pH. The linear enzyme-to-substrate coefficient remained near zero, which confirmed its lack of influence on laminarin release.



Table 5-24 Laminarin (*L*) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-6)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.000	0.000	0.000	0.002	0.001
pH (Q)	0.043	0.046	0.013	0.109	0.009
T (L)	0.000	0.002	0.001	0.026	0.002
T (Q)	0.043	0.032	0.002	0.051	0.220
ES (L)	0.150	0.962	0.983	0.259	0.067
ES (Q)	0.267	0.927	0.592	0.818	0.508
pH by T	0.160	0.819	0.249	0.701	0.699
pH by ES	0.237	0.114	0.357	0.805	0.013
T by ES	0.103	0.238	0.328	0.335	0.187
Lack of fit	0.061	0.094	0.470	0.726	0.178
Pure error	0.003	0.005	0.003	0.009	0.005
R <sup>2</sup>	0.889	0.901	0.959	0.878	0.889
Adj-R <sup>2</sup>	0.789	0.811	0.923	0.769	0.788

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-25 Laminarin (*L*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-6)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time t (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	0.417	0.424	0.401	0.405	0.396
pH (L)	B	-0.166	-0.237	-0.161	-0.153	-0.142
pH (Q)	C	0.039	0.049	0.051	0.049	0.074
T (L)	D	0.128	0.107	0.108	0.082	0.110
T (Q)	E	0.039	0.054	0.078	0.065	0.025
ES (L)	F	0.025	0.001	0.000	0.033	0.043
ES (Q)	G	0.018	0.002	-0.008	0.006	-0.013
pH by T	H	0.032	0.006	0.024	-0.014	0.010
pH by ES	I	-0.026	-0.048	-0.018	-0.009	-0.091
T by ES	J	0.039	0.033	0.020	0.036	0.037

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Time point optima (Table 5-26) further exhibited a lack of time-based behaviour. Low pH (3.0) and high temperature (60 °C) were maintained as optima for the duration of the 6 hours, with enzyme-to-substrate ratio also high at 4 % (*v/dw*) in all five measurements. Laminarin was the response of greatest interest

during this study, leading to these conditions being tested during model validation, and duplicated without enzyme.

Table 5-26 Optimum conditions for *L* responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
<b>Hydrolysis time <i>t</i> (hours)</b>	0	1.5	3	4.5	6
<b>pH</b>	3.0	3.0	3.0	3.0	3.0
<b>T (°C)</b>	60.0	60.0	60.0	60.0	60.0
<b>ES (v/dw)</b>	0.040	0.040	0.040	0.040	0.040
<b>Model predicted <i>L</i> (gLE·l<sup>-1</sup>)</b>	1.323	1.513	1.237	1.365	1.472

The response surface plots for laminarin (Figure 5-6) illustrate the overall lack of time-based behaviour in the response, as well as the effects of the significant variables. Figure 5-6a (4.0 % (v/dw) enzyme dosage) shows a lack of surface change over time when compared to other responses, and Figure 5-6b shows the same at 0.0 % (v/dw) enzyme. The surfaces between 0.0 % and 4.0 % (v/dw) enzyme do not differ much visually, showing diminished enzyme influence when compared to solubilised yield and reducing sugars responses. Figure 5-6c and d also show no surface-change over time, and show the effect of both high temperature and low pH, illustrating the mathematical responses observed.

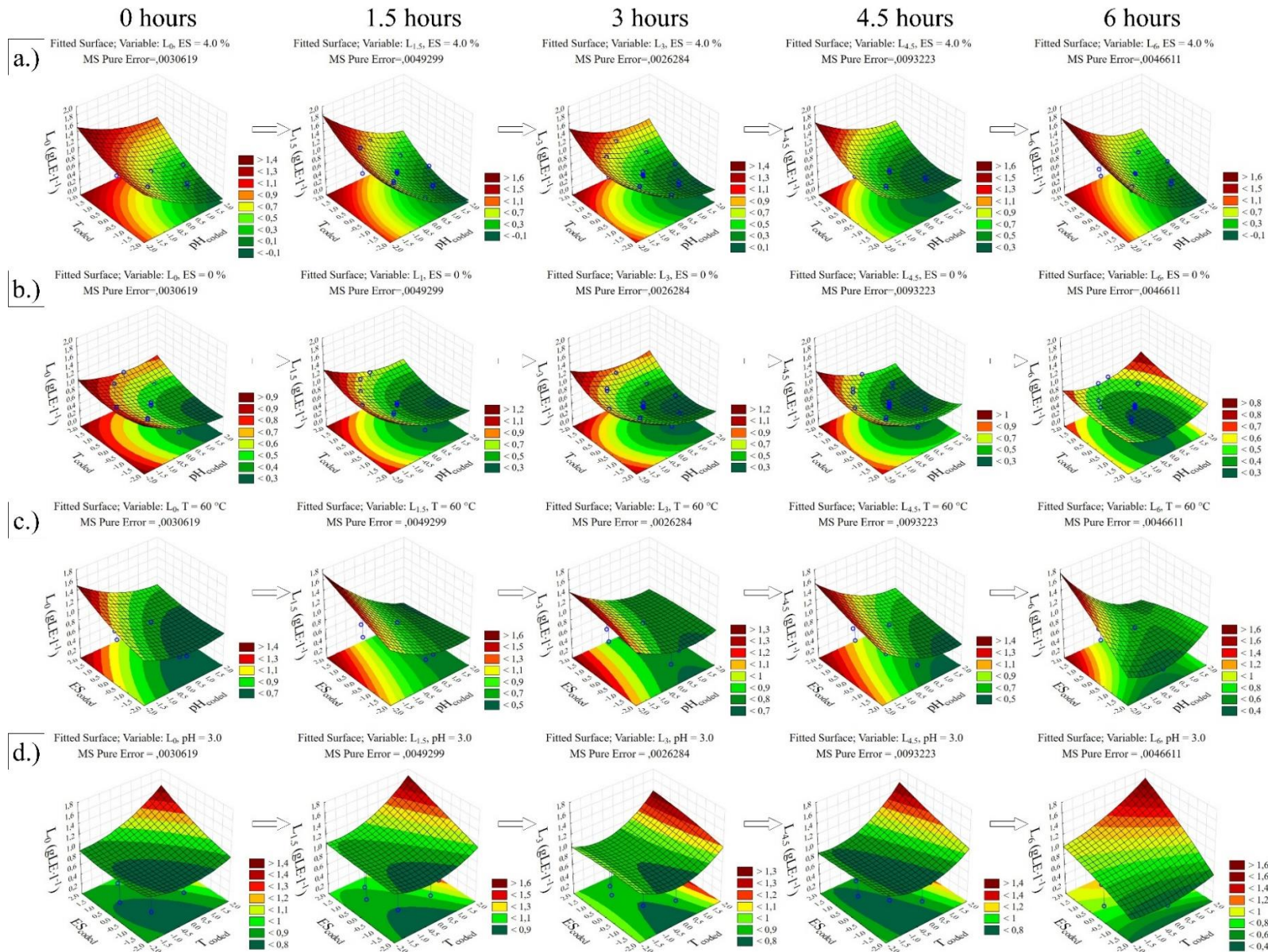


Figure 5-6 Response surface plots over time for laminarin concentration, set at optimum conditions (pH 3.0, 60 °C, and 4.0 % (v/dw) enzyme dosage).  
a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant enzyme dosage (0 % (v/dw)), c.) Constant temperature (60 °C), d.) Constant pH (3.0)

## 5.2.4. Inorganic sulfate

Quantification of inorganic sulfate served as a proxy measurement for the sulfated polysaccharide fucoidan. Data (summarised in Table 5-27, full dataset in Appendix C, Table C-6) was reported in units of  $\text{gSE}\cdot\text{l}^{-1}$  (sulfate equivalents per litre) and showed a spread between  $\approx 0.34$  and  $\approx 1.22 \text{ gSE}\cdot\text{l}^{-1}$ . Maximum and minimum data points occurred at opposite sides of the CCD in terms of all three independent variables (SO 1 vs SO 8), offset by 4.5 hours. This relationship continued at 4.5 hours, where SO 1 showed a lower experimental response than SO 8 by half ( $0.63 \pm 0.17 \text{ gSE}\cdot\text{l}^{-1}$ ), indicating that higher temperature and enzyme loading were beneficial to the response, while lower pH was not.

Table 5-27 Summary of key *S* experimental data points

Response	Point nature	Value ( $\text{gSE}\cdot\text{l}^{-1}$ )	SO	pH	T ( $^{\circ}\text{C}$ )	ES ( $v/dw$ )	t (hrs)
<i>S</i>	Maximum	$1.22 \pm 0.13$	8	5.4	56.0	0.032	4.5
	Minimum	$0.34 \pm 0.03$	1	3.6	44.1	0.008	0

ANOVAs (Table 5-28, Appendix D, Table D-7) show that adjusted  $R^2$  values of all models exceeded  $\approx 0.50$ , except at 3 hours ( $\approx 0.20$ ), meaning that variability was not accounted for at 3 hours. Statistical significance was exhibited for the linear pH effect across all five time points (all  $p < 0.032$ ) and the linear effect of temperature was significant from 0 to 4.5 hours (all  $p < 0.042$ ). The linear effect of enzyme-to-substrate ratio was significant for all times except 3 hours (all  $p < 0.037$ ). Additional significance was shown in the quadratic temperature and pH effects at 0 hours ( $p \approx 0.015$  and  $p \approx 0.004$  respectively). Near-significance was shown for the pH by enzyme-to-substrate interaction at 0 and 1.5 hours, and also by the quadratic pH effect at 4.5 and 6 hours. The significant effects highlighted showed that all three independent variables contributed to the concentration of inorganic sulfate in the supernatant.

The response surface regression coefficients for the inorganic sulfate (Table 5-29, Appendix E, Table E-7) showed that the mean of the models (equivalent to the response surface model centre point) increased over time ( $a \approx 77\%$  increase from 0.526 to 0.930). The linear pH coefficient was higher at 4.5 to 6 hours ( $> 0.11$ ) when compared to 0 to 3 hours ( $< 0.09$ ). The linear temperature coefficient was highest at 0 hours (0.096) and consistently lower until 6 hours, where the effect was no longer significant. The linear enzyme-to-substrate coefficient was highest at 4.5 and 6 hours. The 3 hour model did not fit these trends, but this was consistent with the decreased model performance at this sample point (lower adjusted  $R^2$  and fewer significant effects) shown from the ANOVAs in Table 5-28.

Table 5-28 Inorganic sulfates (*S*) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-7)

Factor	p-Values				
	Hydrolysis time <i>t</i> (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.003	0.007	0.032	0.004	0.006
pH (Q)	0.004	0.539	0.768	0.095	0.064
T (L)	0.002	0.025	0.033	0.042	0.139
T (Q)	0.015	0.314	0.974	0.533	0.278
ES (L)	0.019	0.037	0.528	0.024	0.021
ES (Q)	0.147	0.643	0.433	0.661	0.378
pH by T	0.747	0.209	0.530	0.499	0.688
pH by ES	0.022	0.026	0.840	0.918	0.258
T by ES	0.160	0.679	0.863	0.881	0.836
Lack of fit	0.295	0.163	0.280	0.500	0.789
Pure error	0.004	0.005	0.009	0.007	0.010
R <sup>2</sup>	0.902	0.742	0.583	0.831	0.861
Adj-R <sup>2</sup>	0.814	0.510	0.207	0.679	0.737

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-29 Inorganic sulfates (*S*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-7)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time <i>t</i> (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	0.526	0.777	0.813	0.894	0.930
pH (L)	B	0.088	0.082	0.074	0.117	0.127
pH (Q)	C	0.084	0.012	-0.008	-0.046	-0.064
T (L)	D	0.096	0.059	0.074	0.063	0.049
T (Q)	E	0.060	0.020	0.001	0.015	-0.033
ES (L)	F	0.058	0.052	0.017	0.074	0.092
ES (Q)	G	0.028	0.009	0.021	0.010	0.026
pH by T	H	0.007	-0.035	0.022	0.022	0.015
pH by ES	I	-0.072	-0.075	-0.007	-0.003	-0.046
T by ES	J	-0.036	-0.011	-0.006	0.005	0.008

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optimum conditions for the response surfaces (Table 5-30) showed a common trend of higher pH and temperature (6.0 and 60 °C) until the optimum pH lowered at 4.5 and 6 hours. The 6 hour optimum conditions also indicated a lowered temperature (56.4 °C) when compared to the first 4.5 hours. Enzyme-to-substrate loading was only indicated as optimal from 3 hours onwards. The optimal value of *S* predicted



at 0 hours ( $1.56 \text{ gSE}\cdot\text{l}^{-1}$ ) was the highest prediction made and indicated that prolonged extractions were not beneficial to inorganic sulfate release. Despite this, the extraction conditions indicated the combination of higher pH, higher temperature, and higher enzyme-substrate loading as favourable.

Table 5-30 Optimum conditions for  $S$  responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
Hydrolysis time $t$ (hours)	0	1.5	3	4.5	6
pH	6.0	6.0	6.0	5.9	5.0
T ( $^{\circ}\text{C}$ )	60.0	60.0	60.0	60.0	56.4
ES ( $v/dw$ )	0.000	0.000	0.040	0.040	0.040
Model predicted $S$ ( $\text{gSE}\cdot\text{l}^{-1}$ )	1.56	1.19	1.16	1.33	1.21

The response surfaces obtained during modelling (Figure 5-7) showed the positive effect of both higher temperature and pH when enzyme dosage was held constant (Figure 5-7a), the same figure that indicated a very clear change from concave surface to convex surface over the 6 hour period. Figure 5-7b (constant temperature of  $56.4^{\circ}\text{C}$ ) showed the positive effect of pH to outweigh enzyme dosage in surface contributions, with a steeper slope along that axis. Figure 5-7c showed that the temperature effect also outweighed enzyme dosage, with a similar observation made of the relative slopes. These graphical forms agreed with the statistical analysis and interpretation.

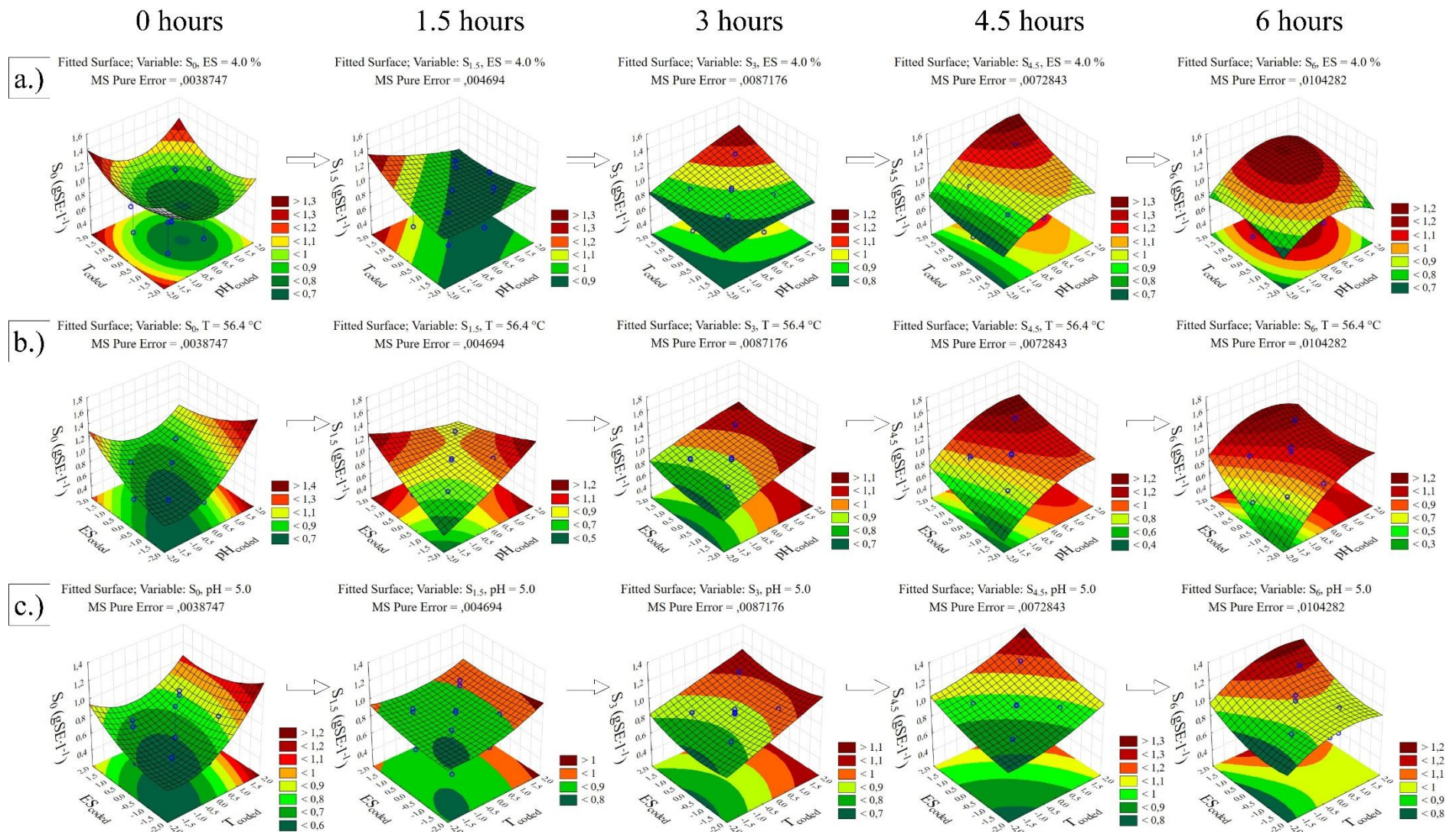


Figure 5-7 Response surface plots over time for inorganic sulfate concentration, set at optimum conditions (pH 5.0, 56.4 °C, and 4.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (4.0 % (v/dw)), b.) Constant temperature (56.4 °C), c.) Constant pH (5.0)



### 5.2.5. Total phenolics

Total phenolics data across the RSM is located in Appendix C.9. The maximum and minimum experimental data obtained (Table 5-31) showed a higher experimental response at SO 12, which indicated the positive effect that temperature had on the response. Lower pH and temperature resulted in the minimum response of SO 1 (which is consistently the lowest response over the data set).

Table 5-31 Summary of key *TP* experimental data points

Response	Point nature	Value (gGAE·l <sup>-1</sup> )	SO	pH	T (°C)	ES (v/dw)	t (hrs)
<i>TP</i>	Maximum	0.411 ± 0.002	12	4.5	60	0.02	3
	Minimum	0.286 ± 0.003	1	3.6	44.1	0.008	0

The ANOVA summary for the *TP* response (Table 5-32 and Appendix D, Table D-8), showed consistent significance in the linear pH and temperature effects. The linear pH p-values from ANOVA became more significant over the run (lowest p-values at 3 to 6 hours). The linear temperature effect remained most significant overall, with p-values for 0 to 4.5 all below 0.01. The highest adjusted R<sup>2</sup> was found at 3 hours (≈ 0.78), indicating the best model (which showed no significant effect of enzyme). Significant lack of fit was experienced at 4.5 hours, so the extra significance of the linear enzyme-to-substrate effect was not taken as meaningful.

Table 5-32 Total phenolics (*TP*) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-8)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.037	0.027	0.000	0.001	0.001
pH (Q)	0.504	0.200	0.149	0.004	0.563
T (L)	0.009	0.010	0.004	0.001	0.021
T (Q)	0.419	0.075	0.116	0.187	0.443
ES (L)	0.557	0.429	0.512	0.002	0.549
ES (Q)	0.199	0.700	0.732	0.092	0.795
pH by T	0.236	0.078	0.027	0.890	0.138
pH by ES	0.180	0.296	0.127	0.169	0.867
T by ES	0.841	0.796	0.300	0.747	0.658
Lack of fit	0.574	0.145	0.229	0.012	0.344
Pure error	0.000	0.000	0.000	0.000	0.000
R <sup>2</sup>	0.780	0.688	0.885	0.760	0.848
Adj-R <sup>2</sup>	0.582	0.407	0.781	0.544	0.711

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

The mean/intercept surface coefficients (Table 5-33 and Appendix E, Table E-8) suggested that the release of total phenolics took place mostly by 1.5 hours. The linear coefficients of temperature showed a consistent lowering trend over time, from 0.019 at 0 hours to 0.011 by 6 hours. Both linear pH and linear temperature coefficients remained the highest coefficients in each model. The linear enzyme-to-substrate coefficients were also consistently positive, which was taken to indicate a minor effect despite statistical insignificance.

Table 5-33 Total phenolics (*TP*) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-8)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time <i>t</i> (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	0.331	0.363	0.370	0.364	0.368
pH (L)	B	0.013	0.012	0.021	0.015	0.024
pH (Q)	C	0.003	-0.006	-0.004	-0.010	-0.002
T (L)	D	0.019	0.016	0.013	0.014	0.011
T (Q)	E	0.004	0.008	0.005	-0.003	0.003
ES (L)	F	0.003	0.003	0.002	0.011	0.002
ES (Q)	G	0.007	0.002	-0.001	-0.004	0.001
pH by T	H	-0.008	-0.011	-0.010	0.000	-0.007
pH by ES	I	-0.009	-0.006	-0.006	-0.004	-0.001
T by ES	J	0.001	0.001	0.004	-0.001	-0.002

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optima for each *TP* model (Table 5-34) showed an upward trend of optimum pH from 3.0 at 0 hours to 6.0 at 6 hours. Optimum temperature within the experimental range remained at 60 °C over the course of the experiment, and the enzyme loading optimum lowered from 4.0 % to 0 (v/dw) by 6 hours. The model predicted optimum *TP* dropped after 1.5 hours, indicating that prolonged extraction was likely not necessary.

Table 5-34 Optimum conditions for *TP* responses, from response surfaces (in experimental range)

Independent variable	Optimum conditions observed in response surfaces				
Hydrolysis time <i>t</i> (hours)	0	1.5	3	4.5	6
pH	3.0	3.2	3.8	5.0	6.0
T (°C)	60.0	60.0	60.0	60.0	60.0
ES (v/dw)	0.040	0.040	0.040	0.031	0.000
Model predicted <i>TP</i> (gGAE·l <sup>-1</sup> )	0.436	0.440	0.419	0.388	0.414

The surface plots for total phenolic concentration (Figure 5-8) confirmed the lack of enzyme effect (Figure 5-8b and c) and the positive effects of both pH and temperature (Figure 5-8a).

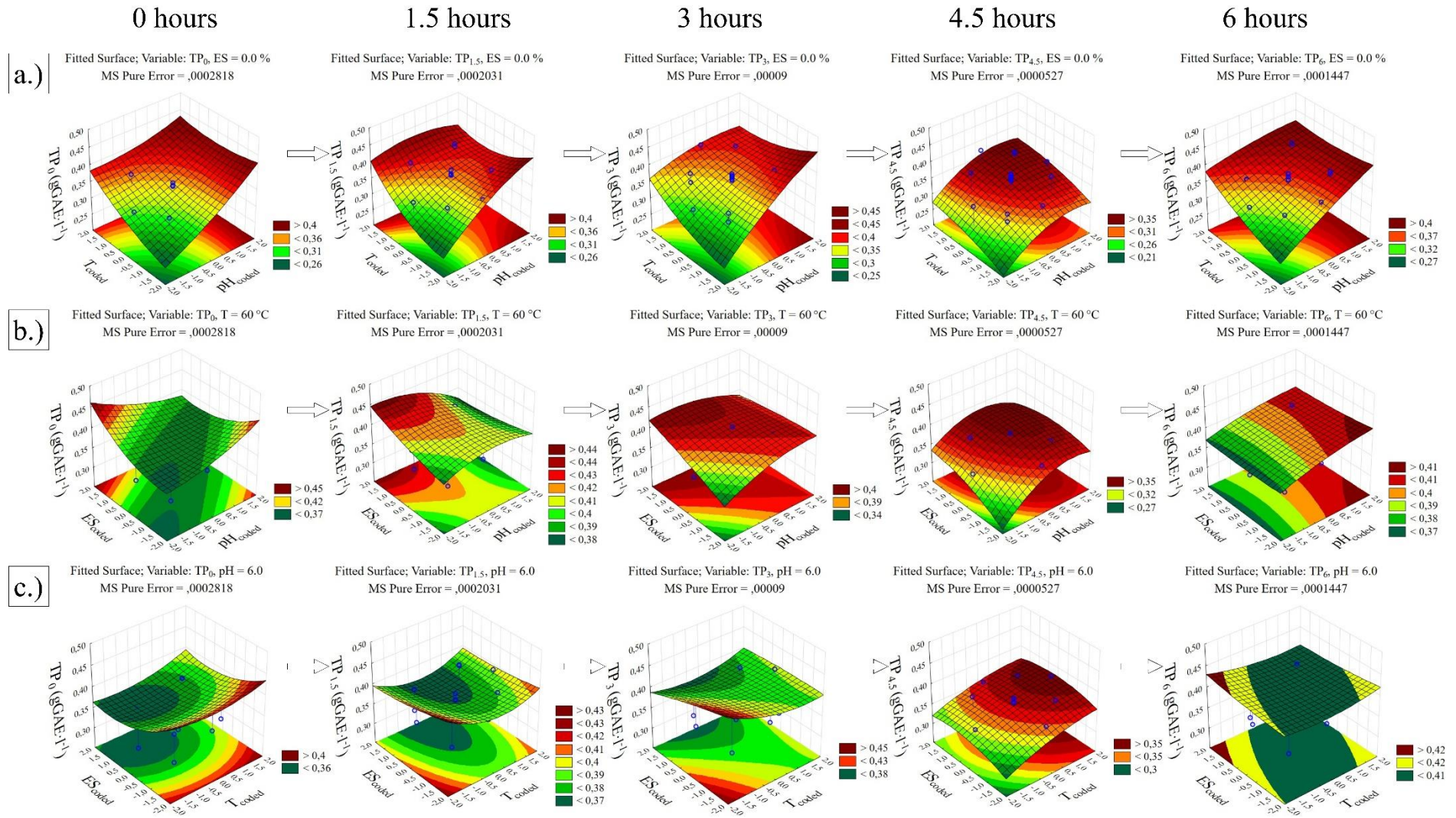


Figure 5-8 Response surface plots over time for total phenolic concentration, set at optimum conditions (pH 6.0, 60 °C, and 0.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (0.0 % (v/dw)), b.) Constant temperature (60 °C), c.) Constant pH (6.0)

### 5.2.6. Antioxidant capacity

When testing the antioxidant capacity of the collected samples, the undiluted samples consistently decoloured the DMPD solution to an  $\approx 80\%$  inhibition reading (equivalent to  $\approx 0.25 \text{ gTE}\cdot\text{l}^{-1}$ ). This was taken as the “saturated” response and so a factor of dilution of 20 was implemented for all samples, to ensure no saturation, and to keep data comparable within the CCD. Antioxidant capacity data is included in Appendix C.10 and the maximum and minimum points of the antioxidant capacity data set (Table 5-35) showed preliminary effect indications. Maximum *AO* ( $1.55 \pm 0.05 \text{ gTE}\cdot\text{l}^{-1}$ ) was found at 0 hours and centre point conditions, giving no indication of preliminary significance. The minimum value of  $1.10 \pm 0.03 \text{ gTE}\cdot\text{l}^{-1}$  was likely more telling and indicated both pH and temperature as important factors.

Table 5-35 Summary of key *AO* experimental data points

Response	Point nature	Value ( $\text{gTE}\cdot\text{l}^{-1}$ )	SO	pH	T ( $^{\circ}\text{C}$ )	ES ( $v/dw$ )	t (hrs)
<i>AO</i>	Maximum	$1.55 \pm 0.05$	16	4.5	50.0	0.020	0
	Minimum	$1.10 \pm 0.03$	1	3.6	44.1	0.008	1.5

The summarised ANOVAs (Table 5-36 and Appendix D, Table D-9) showed no significant effect at 0 hours, where the response surface showed the lowest adjusted  $R^2$  value ( $\approx 0.07$ ). Adjusted  $R^2$  values for 1.5 to 6 hours all exceeded 0.5, with the highest being 1.5 hours ( $\approx 0.65$ ). Significant lack of fit was experienced at 1.5 hours ( $p \approx 0.014$ ), forcing the response to be treated cautiously. Common and consistent trends in the significant factors of *AO* include both quadratic pH and linear temperature effects. 3 hours indicates the quadratic temperature and pH by enzyme-to-substrate interaction to be significant as well, but these points are isolated.

Changes over time were more easily identified in regression coefficients (Table 5-37 and Appendix E, Table E-9). The table showed that the mean did not experience a rise during the experiment, but the linear temperature coefficient was increased from 3 hours onwards (if the 0 hour linear temperature coefficient is discounted). The quadratic pH coefficient remained consistently negative, likely indicating an optimum pH close to the centre point of 4.5.

Table 5-36 Antioxidant capacity (AO) factor significance p-values from ANOVAs at different hydrolysis times (Summarised data from Appendix D, Table D-9)

Factor	p-Values				
	Hydrolysis time t (hrs)				
	0	1.5	3	4.5	6
pH (L)	0.897	0.976	0.126	0.198	0.119
pH (Q)	0.196	0.000	0.010	0.038	0.028
T (L)	0.172	0.031	0.001	0.023	0.045
T (Q)	0.375	0.105	0.043	0.798	0.306
ES (L)	0.586	0.707	0.594	0.656	0.821
ES (Q)	0.997	0.013	0.977	0.699	0.178
pH by T	0.368	0.001	0.058	0.084	0.219
pH by ES	0.469	0.050	0.024	0.185	0.564
T by ES	0.676	0.340	0.311	0.699	0.564
Lack of fit	0.796	0.014	0.060	0.858	0.670
Pure error	0.011	0.001	0.001	0.005	0.004
R <sup>2</sup>	0.512	0.818	0.761	0.807	0.750
Adj-R <sup>2</sup>	0.073	0.654	0.546	0.633	0.525

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Table 5-37 Antioxidant capacity (AO) regression coefficients for different factors at different hydrolysis times according to Equation 5-6 (Summarised data from Appendix E, Table E-9)

Factor	Coefficient in Eq. 5-6	Regression coefficients				
		Hydrolysis time t (hrs)				
		0	1.5	3	4.5	6
Mean/intercept	A	1.411	1.336	1.382	1.412	1.371
pH (L)	B	0.004	0.000	0.019	0.028	0.032
pH (Q)	C	-0.041	-0.073	-0.040	-0.051	-0.051
T (L)	D	0.045	0.019	0.066	0.061	0.045
T (Q)	E	-0.027	0.012	0.027	-0.005	-0.019
ES (L)	F	0.016	0.003	0.006	-0.009	0.004
ES (Q)	G	0.000	-0.023	0.000	0.007	-0.026
pH by T	H	-0.036	-0.064	-0.033	-0.053	-0.031
pH by ES	I	-0.029	-0.021	-0.043	-0.037	0.014
T by ES	J	-0.016	-0.009	-0.015	-0.010	-0.014

Significant factors (CI = 95 %) shaded and outlined. Values reported to 3 d.p.

Optima for each model over time (Table 5-38) showed that the optimal pH across the run remained between 3.6 and 4.9, at an average of  $\approx 4.2$ . Higher temperature ( $> 56.0$  °C) was a common optima across the design, while high enzyme loading was only indicated as optimal at 0 hours (and could therefore be disregarded). The optimal release was deemed to require a pH between 4 and 5 (from 3 hours onwards),



with shortened extraction times able to use lower pH. Temperature should be maintained at 60 °C, although this was due to confining the optimum conditions to the predefined ranges for pH, temperature, and enzyme dosage. No enzyme addition was necessary for the increased *AO* response, but a lack of significance on the linear and quadratic enzyme-to-substrate effects indicated that it should show no detrimental effect. Prediction accuracy was assessed in the validation section following.

Table 5-38 Optimum conditions for *AO* responses, from response surfaces (in experimental range)

<b>Independent variable</b>	<b>Optimum conditions observed in response surfaces</b>				
<b>Hydrolysis time t (hours)</b>	0	1.5	3	4.5	6
<b>pH</b>	3.6	3.8	4.9	4.5	4.3
<b>T (°C)</b>	56.0	60.0	60.0	60.0	58.9
<b>ES (v/dw)</b>	0.040	0.021	0.000	0.000	0.016
<b>Model predicted <i>AO</i> (gTE·l<sup>-1</sup>)</b>	1.47	1.44	1.61	1.56	1.40

The response surface plots of antioxidant capacity (Figure 5-9) confirm the regressed results and statistical analyses undertaken. Figure 5-9a shows both consistent significant effects, with curvature shown clearly along the pH axis and the linear effect of temperature made apparent. Figure 5-9b and c both show enzyme dosage to have little effect on the response when compared to either pH or temperature.

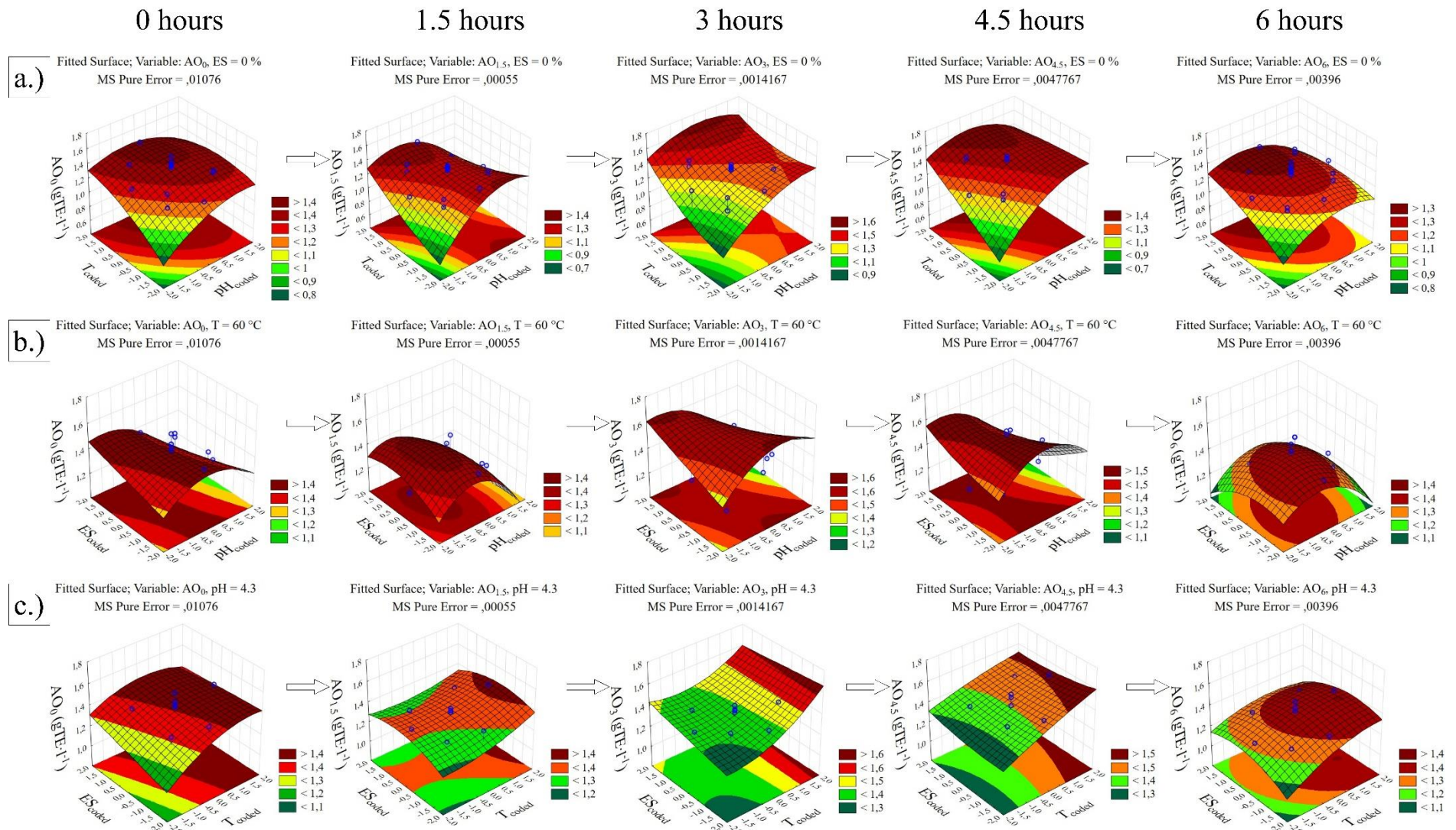


Figure 5-9 Response surface plots over time for antioxidant capacity, set at optimum conditions (pH 4.3, 60 °C, and 0.0 % (v/dw) enzyme dosage). a.) Constant enzyme dosage (0.0 % (v/dw)), b.) Constant temperature (60 °C), c.) Constant pH (4.3)



### 5.3. Response surface validation

A summary of the optimal conditions reported from all models is given below in Table 5-39, with the primary responses outlined, and non-fitting models highlighted in grey.

Table 5-39 Optimum conditions for modelled responses, from response surface models

Response	Optimum conditions from RSM														
	pH					T (°C)					ES (v/dw) (%)				
t (hours)	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6
<b>DS</b>	3.0	3.0	3.0	3.0	3.0	60.0	60.0	55.1	60.0	60.0	0.5	3.9	4.0	4.0	3.4
<b>SY</b>	3.0	3.0	6.0	6.0	6.0	60.0	60.0	45.4	40.6	45.1	3.5	4.0	4.0	4.0	4.0
<b>F<sub>SN</sub></b>	3.0	3.0	3.0	6.0	6.0	60.0	60.0	60.0	43.4	46.9	4.0	4.0	4.0	4.0	4.0
<b>SL<sub>P</sub></b>	3.0	3.0	3.0	3.0	3.0	60.0	40.0	40.0	60.0	60.0	4.0	0.0	0.0	0.0	0.0
<b>RS</b>	3.0	3.0	3.1	3.4	3.7	60.0	60.0	60.0	60.0	58.9	4.0	4.0	4.0	4.0	4.0
<b>L</b>	3.0	3.0	3.0	3.0	3.0	60.0	60.0	60.0	60.0	60.0	4.0	4.0	4.0	4.0	4.0
<b>S</b>	6.0	6.0	6.0	5.9	5.0	60.0	60.0	60.0	60.0	56.4	0.0	0.0	4.0	4.0	4.0
<b>TP</b>	3.0	3.2	3.8	5.0	6.0	60.0	60.0	60.0	60.0	60.0	4.0	4.0	4.0	3.1	0
<b>AO</b>	3.6	3.8	4.9	4.5	4.3	56.0	60.0	60.0	60.0	58.9	4.0	2.1	0	0	1.6

It was noted from Table 5-39 that the majority of responses (including laminarin) showed model maximum responses at low pH (3.0), high temperature (60°C), and high enzyme loading (4 % (v/dw)). The exception to this appeared to be the inorganic sulfate response (with a pH optimum of 6.0, consistently in the higher range of the experimental design). *DS*, *L*, and *SL<sub>P</sub>* maintained lower pH optima, while other responses moved to higher pH optima in the course of the experiment.

The focus of the optimisation was laminarin, so these optimum extraction conditions were tested during validation (pH 3.0, 60 °C, 4.0 % (v/dw) enzyme dosage). Optimum laminarin extraction conditions (determined in Section 5.2.3) did not statistically require the addition of enzyme, but the predicted optima in the models consistently indicated the upper boundary of enzyme addition (4.0 % (v/dw)) as optimal. For this reason, the experiment was repeated without enzyme as an extra validation run (autohydrolysis at the same pH and temperature). The consistent low pH and high temperature combination agreed with the reported literature conventional extraction optima of pH 1.0 and 70 °C (Devillé et al., 2004) and pH 1.0 and 80 °C (Zhang and Row, 2015).

Most of the models that showed the consistent significant effect of enzyme loading (*SY*, *F<sub>SN</sub>*, and *RS*) showed no significance with either pH or temperature effects. The exception to this was *SL<sub>P</sub>*, which showed the linear effect of temperature to be significant from 3 to 4.5 hours. An experiment at centre-

point temperature and pH conditions (50 °C, pH 4.5), with higher enzyme loading (4 % (v/dw)), was chosen to validate the action of the Celluclast enzyme on each response. These conditions replicated the conditions of the SO 14 experiment (an axial point in the CCD), and the moderate conditions were chosen in an attempt to minimise the influence of high temperature and low pH and assess enzyme action independently from these effects. It was noted that these conditions were chosen on a pre-existing experiment and that this may have prevented true validation of the models in the centre of the design, but this was secondary to the study of laminarin extraction. The conditions of the three chosen validation runs are summarised in Table 5-40.

Table 5-40 Conditions of model validation runs

Exp.	Exp. conditions			Description
	pH	T (°C)	ES (v/dw)	
V	3.0	60.0	0.04	Validation of maximum laminarin release (in CCD)
VB	3.0	60.0	-	Repeat of V without enzyme addition
VE	4.5	50.0	0.04	Enzyme run (Celluclast)/ central region validation of CCD

Predicted and experimental responses resulting from validation (Table 5-41) show good model predictions (prediction errors less than 10 %) highlighted in dark grey, and reasonable predictions (prediction error between 10 % and 20 %) highlighted in a lighter grey. The error in prediction for all eight response surface models was highest under condition set V (a total average prediction error across all nine response of  $\approx$  30 %). Although average prediction errors of *DS* (10.0 %), *SY* (6.3 %), and  $F_{SN}$  (3.7 %) were good (< 10 %), these errors were higher than the errors under VB or VE conditions. Large average over-predictions occurred for *RS* (31.0 %), *S* (89.8 %), *L* (67.2 %), and *AO* (22.8 %), while *TP* was mostly over predicted (except at 4.5 hours, previously established as a non-fitting model in section 5.2.5).

The blank enzyme run VB showed good average prediction error for *DS* (3.9 %), *SY* (4.3 %),  $F_{SN}$  (2.5 %), *TP* (8.4 %), and *AO* (5.8 %). The laminarin response *L* was again over predicted (33.2 % on average), while *RS* was under predicted by an average of 11.5 %.

Response surface models performed well under the conditions of VE, with average errors all sitting under 10 %, except for *L* (12.6 %) and *TP* (13.6 %). This pointed to decreased prediction performance away from the central region of the CCD (as shown by V and VB).

Table 5-41 Model validations (V, VB, and VE)

Response	Model validation at pH = 3.0, T = 60.0 °C, ES = 4.0 % (v/dw) (V)															
	Predicted					Measured					Prediction error $\varepsilon$ (%)					
t (hours)	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6	$\mu$
<b>DS (%)</b>	3.28	4.03	3.83	4.18	3.84	3.44	3.45	3.62	3.56	3.64	4.7	16.7	5.6	17.3	5.7	10.0
<b>SY (%)</b>	54.7	57.7	54.8	57.9	50.8	50.9	50.9	53.8	54.4	52.0	7.5	13.4	2.0	6.4	2.3	6.3
<b>F<sub>SN</sub> (%)</b>	82.8	82.3	83.1	81.9	78.1	80.3	78.3	78.3	79.1	77.7	3.2	5.0	6.2	3.4	0.5	3.7
<b>SL<sub>P</sub> (%)</b>	12.0	13.3	14.7	11.7	13.8	13.5	12.0	11.2	11.3	11.7	11.0	10.6	31.5	3.6	17.5	14.8
<b>RS (gGIE·l<sup>-1</sup>)</b>	6.94	7.90	8.00	7.68	7.48	5.19	5.83	5.94	6.00	6.07	33.7	35.5	34.5	28.0	23.2	31.0
<b>L (gLE·l<sup>-1</sup>)</b>	1.32	1.51	1.24	1.37	1.47	0.97	1.04	1.01	0.85	0.54	36.5	45.2	23.1	60.6	171	67.2
<b>S (gSE·l<sup>-1</sup>)</b>	1.21	1.22	0.82	0.83	0.86	0.38	0.49	0.60	0.67	0.71	215	152	37.3	23.5	21.0	89.8
<b>TP (gGAE·l<sup>-1</sup>)</b>	0.44	0.44	0.42	0.35	0.37	0.32	0.33	0.34	0.36	0.33	36.3	34.3	23.5	3.1	12.3	21.9
<b>AO (gTE·l<sup>-1</sup>)</b>	1.45	1.35	1.60	1.54	1.14	1.18	1.16	1.15	1.14	1.13	22.9	16.3	39.0	34.9	0.6	22.8
Response	Model validation at pH = 3.0, T = 60.0 °C, ES = 0.0 % (v/dw) (VB)															
<b>DS (%)</b>	3.44	3.57	3.68	3.74	3.55	3.36	3.47	3.53	3.49	3.44	2.5	2.7	4.2	7.1	3.1	3.9
<b>SY (%)</b>	53.3	51.8	50.8	51.0	51.0	48.3	48.6	50.8	49.0	51.1	10.3	6.6	0.0	4.1	0.2	4.3
<b>F<sub>SN</sub> (%)</b>	83.4	74.8	73.0	73.0	73.2	79.7	73.4	73.9	74.8	75.1	4.6	1.8	1.2	2.4	2.5	2.5
<b>SL<sub>P</sub> (%)</b>	13.5	9.6	8.7	8.7	9.6	12.9	9.9	9.7	10.7	9.9	4.3	2.7	10.0	19.5	3.6	8.0
<b>RS (gGIE·l<sup>-1</sup>)</b>	5.01	4.94	4.68	4.40	4.57	5.30	5.25	5.36	5.41	5.33	5.5	6.1	12.8	18.7	14.3	11.5
<b>L (gLE·l<sup>-1</sup>)</b>	0.87	1.05	1.02	1.00	0.60	0.70	0.86	0.80	0.57	0.73	24.5	22.3	27.3	74.6	17.2	33.2
<b>S (gSE·l<sup>-1</sup>)</b>	0.81	0.68	0.76	0.54	0.25	0.50	0.57	0.90	0.72	0.68	61.9	19.9	15.8	25.9	63.7	37.4
<b>TP (gGAE·l<sup>-1</sup>)</b>	0.37	0.39	0.35	0.29	0.37	0.35	0.35	0.35	0.36	0.36	5.5	11.5	0.0	20.2	4.8	8.4
<b>AO (gTE·l<sup>-1</sup>)</b>	1.33	1.27	1.43	1.42	1.28	1.43	1.39	1.39	1.44	1.41	7.1	8.3	3.0	1.7	9.0	5.8
Response	Model validation at pH = 4.5, T = 50.0 °C, ES = 4.0 % (v/dw) (VE)															
<b>DS (%)</b>	3.28	3.66	3.55	3.62	3.56	3.28	3.66	3.55	3.62	3.56	4.7	4.6	0.8	1.0	3.3	2.9
<b>SY (%)</b>	47.8	51.5	53.3	54.0	52.9	49.4	52.8	53.7	54.0	55.2	3.1	2.3	0.6	0.1	4.1	2.0
<b>F<sub>SN</sub> (%)</b>	79.1	80.6	81.3	82.1	82.2	79.7	81.3	81.8	82.1	82.5	0.8	0.8	0.6	0.0	0.3	0.5
<b>SL<sub>P</sub> (%)</b>	13.8	14.1	14.2	14.2	14.2	13.7	13.6	13.9	13.9	14.1	0.8	3.6	2.3	1.6	1.2	1.9
<b>RS (gGIE·l<sup>-1</sup>)</b>	5.39	6.85	7.16	7.39	7.41	4.95	6.32	6.86	7.22	7.25	8.9	8.4	4.4	2.3	2.2	5.2
<b>L (gLE·l<sup>-1</sup>)</b>	0.51	0.43	0.38	0.48	0.43	0.47	0.48	0.51	0.46	0.39	8.5	11.0	26.3	5.0	12.2	12.6
<b>S (gSE·l<sup>-1</sup>)</b>	0.70	0.89	0.90	1.05	1.16	0.65	0.83	0.97	0.99	1.14	7.8	7.2	7.3	5.5	1.4	5.9
<b>TP (gGAE·l<sup>-1</sup>)</b>	0.35	0.37	0.37	0.37	0.37	0.29	0.32	0.33	0.33	0.40	21.2	16.2	12.1	12.7	5.6	13.6

<b><i>AO</i></b> ( <b>gTE·l<sup>-1</sup></b> )	1.44	1.28	1.39	1.42	1.30	1.21	1.33	1.27	1.22	1.29	18.6	4.1	9.4	16.4	1.2	9.9
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All models were considered validated in the central region of the CCD, including laminarin (which showed good to reasonable prediction except at 3 hours), and total phenolics (good to reasonable prediction except at 0 hours). The prediction errors of non-spectrophotometric responses (dissolved solids, solubilised yield, supernatant mass fraction, and pellet solids loading) showed general better performance across the three condition sets. Spectrophotometric models could not predict responses with reasonable accuracy at condition set V, with only reducing sugar, total phenolics, and antioxidant capacity response surfaces able to do so at condition set VB.

## 5.4. Response surface discussion

The main focus of this study was to optimise the release of laminarin from the *E. maxima* kelp using Celluclast carbohydrase enzyme. Optimisation was carried out on a single batch (harvested May 2018) and the resultant response surfaces indicated optimal laminarin release conditions in the general direction of the conventional extractions used by Devillé et al (2004) (pH 1.0 and 70 °C), and Zhang and Row (2015) (pH 1.0 and 80 °C). Other responses were measured and analysed and all were discussed in this section, consolidating all response surface analyses. Comparison to measurements found in literature was reserved for Chapter 6 and 7, with response surface model significant effects taking precedence in this section. All responses involved in the definition of solubilised yield are discussed first and followed by spectrophotometric responses.

### 5.4.1. Solubilised yield

Response surface modelling of solubilised yield ( $SY$ ) showed the consistent significant effect of linear enzyme dosage. Analysis of the other contributing variables to the  $SY$  calculation, namely supernatant mass fraction ( $F_{SN}$ ) and pellet solids loading ( $SL_P$ ) also showed this variable to be consistently significant over all experiments. While temperature and pH did not contribute significantly to the response of  $SY$  at each sampling point, the response surface optima shifted from conditions of pH 3.0 and 60 °C for 0 to 1.5 hours to pH 6.0 and lower temperatures of 40 – 45 °C for 3 to 6 hours. This was likely due to the faster hydrolysis effects of dilute-acid thermal hydrolysis (low pH/high temperature) compared to the sustained enzymatic hydrolysis over time (when the enzyme was not denatured at low pH/high temperature) (Offei et al., 2018). There are no papers that directly replicate the conditions used in this study (solids loading, enzyme dosage, wet/dry processing, extraction time), but Celluclast has been reported by Charoensiddhi et al. (2016), Heo et al. (2005), and Wang et al. (2010) to increase solubilised yield in seaweed hydrolysis, confirming the significance shown by linear enzyme dosage.

## Supernatant dissolved solids

Response surface analysis of the supernatant dissolved solids (*DS*) did not identify pH or temperature as significant, with the linear effect of enzyme dosage at 1.5 hours being the only significant effect. Early significance of enzyme dosage suggested that appreciable enzymatic hydrolysis occurred without extended incubation. The mean (equivalent to a centre-point model prediction) of the response surface models consistently increased from 0 to 6 hours and the predicted optimum conditions obtained from the five sequential *DS* response surfaces all indicated the requirement of lower pH (3.0), higher temperature (60°C), and increased enzyme addition (4.0 % (v/dw) Celluclast) (confined to the predefined model range pH, temperature, and enzyme-dosage). The lack of significant effect from independent variables was likely due to large experimental errors present in the measurement of dissolved solids, as well as 3 hours being a long enough extraction time to obtain similar results from all dosages of enzyme (up to 4.0 % (v/dw)) and all pH and temperatures in the experimental range. The predicted optimum extraction conditions of the model were viewed as a combination of enzymatic and conventional hydrolysis.

It is possible that the dilute nature of the supernatant samples (< 4 % as mentioned) and the use of moisture analysis machinery intended for testing larger sample volumes (KERN DBS halogen lamp moisture analyser) contributed to the large experimental errors present in the supernatant dissolved solids data. Larger sample volumes were impractical, as samples were limited due to the time-sampling requirement of the study. Small changes in the dissolved solids measurements, paired with experimental errors derived from using small test volumes and a moisture analyser on an open laboratory benchtop likely prevented the response surface ANOVAs from showing the significant effects of pH, temperature, and enzyme-to-substrate loading in the analysed CCDs.

## Supernatant mass fraction

The response surface ANOVAs of  $F_{SN}$  indicated the significant positive effect of linear enzyme-to-substrate ratio from 1.5 to 6 hours. Despite pH and temperature not showing any significant effect in these ANOVAs, the predicted optima of the first three sampling points (0 to 3 hours) indicated optimum conditions of pH 3.0, 60 °C, and 4.0 % (v/dw) enzyme dosage (identical to validation treatment V). The predicted optima of 4.5 to 6 hours changed to pH 6.0 and  $\approx$  45 C, while still maintaining 4.0 % (v/dw) enzyme dosage. This change in optimum prediction was also likely caused by the initial fast release at low pH, high temperature, and high enzyme combinations followed by the subsequent denaturing of the enzyme around 4.5 hours. This would leave experimental treatments with more enzyme-inhabitable pH and temperature to continue increasing  $F_{SN}$  towards later sampling points.

The positive effect of enzyme addition on the  $F_{SN}$  response likely resulted from the hydrolysis and solubilisation of glucose polysaccharides in the cell wall matrix, as well as the possible hydrolysis of similar bonds in fucoidan and alginate, as was theorised by Charoensiddhi et al. (2016). Similar bonds to  $\beta$ -glucans (the natural substrate of the enzyme) may exist in the alginic acid polysaccharide. Degradation of these bonds could theoretically result in a reduction in hydrocolloid properties and the ability of the polysaccharide to absorb water (gel). The hypothesized hydrolysis of the alginic acid by Celluclast could have reduced the swelling/moisture absorbing ability of the polysaccharide, resulting in less moisture retention in the pellet and a larger supernatant mass fraction. This effect of decreased molecular weight on gel strength was investigated by Draget et al. (1994), and shown to result in lower swelling and decreased moisture absorption. Alginic acid / alginate concentration was not investigated in this study and should be quantified in further studies of this nature.

### **Pellet-solids loading**

Response surfaces obtained for pellet-solids loading ( $SL_P$ ) showed the consistent significant positive effect from the linear enzyme-to-substrate ratio (from 3 to 6 hours), as well as linear temperature from 4.5 to 6 hours. The  $SL_P$  response was initially investigated due to its effect on solubilised yield. The surfaces predicted optimum (minimum) conditions of pH 3.0 and 0 % enzyme loading with 40 °C at 1.5 to 3 hours and 60 °C at 4.5 and 6 hours. The change in predicted optimum temperature was likely also connected with the possible enzymatic hydrolysis of the alginic acid polysaccharide over time as theorised for  $F_{SN}$ .

The positive effect of enzyme on the pellet-solids loading response (undesirable) would result from the same mechanism as for supernatant mass fraction. While alginic acid bonds were theoretically broken, and the moisture absorbing ability of the pellet reduced, the solids loading of the pellet would increase with diminished hydrocolloid behaviour.

### **5.4.2. Reducing sugars**

The response surface modelling and analysis of reducing sugars showed the consistent significant effect of the linear enzyme-to-substrate term. Optimal conditions predicted from the resultant models increased from a pH of 3.0 at 0 and 1.5 hours towards 3.7 by 6 hours, while optimal temperature and enzyme-to-substrate loading were constantly predicted at 60 °C and 4.0 % ( $v/dw$ ) enzyme dosage. An increase in the optimal pH over time was shown in other responses as well, such as solubilised yield, supernatant mass fraction, total phenolics, and antioxidant capacity. While neither total phenolics nor antioxidant capacity ANOVAs indicated any significant effect of enzyme dosage, the increase in the optimal pH was



still likely due to the trade-off between fast initial dilute-acid thermal hydrolysis and the sustained enzymatic hydrolysis over time at milder temperature and pH. The optimal region of Celluclast activity, according to Herlet et al. (2017), lies between 45 and 60 °C and pH 4.0 and 5.5, and this could explain the lack of significance found from either variables, as the CCD mostly overlapped with the optimal activity region.

Validation of the *RS* model was possible at treatment VE, where prediction errors were all below 10 %. As with other spectrophotometric responses, this prediction error increased for validation experiment VB and V. The lack of prediction accuracy may have been caused by the choice of pH and temperature range for the CCD. In more detail, conditions of the SO 4 CCD experiment [-1,+1,+1] (pH 3.6, 56 °C, 3.2 % (*v/dw*) enzyme dosage) were likely located on the activity “plateau” shown in Herlet et al. (2017). The proposed experiment V (pH 3.0, 60 °C, 4.0 % (*v/dw*)) sat closer to the edge of the plateau, and the subsequent drop in activity associated with this pH and temperature combination may not have been accounted for in the 20 experiments of the CCD, leading to the consistent over-prediction error at treatment V. This prediction problem was likely consistent for all measured responses at treatment V.

#### **5.4.3. Laminarin**

Laminarin responses surfaces showed the quadratic and linear effects of both pH and temperature to be significant. Each surface predicted optimum conditions for laminarin extraction at pH 3.0, 60 °C, and 4.0 % (*v/dw*) Celluclast enzyme dosage. The prediction of enzyme requirement was consistent in the models, despite showing no statistical significance to the response. This was the purpose behind the validation testing at VB conditions. The predicted optimum conditions pointed towards conventional dilute-acid thermal hydrolysis reported by both Devillé et al. (2004) and Zhang and Row (2015) and suggested the lack of Celluclast enzyme utility in extracting laminarin. The low pH/high temperature combination predicted as optimal pointed towards the solubility of laminarin as the main effect in determining extraction efficiencies and would explain the near-consistent use of acid-based hydrolysis treatments summarised in Section 2.5.1. Comparisons of the validation runs obtained in this section to alternate treatments such as Accellerase enzyme and conventional dilute-acid thermal hydrolysis (Section 6.2.3.) shed more light on the extraction of laminarin.

#### **5.4.4. Inorganic sulfates**

Response surface modelling of the inorganic sulfates concentration consistently showed the significant effect of all three independent variables (pH, temperature, and enzyme dosage) at multiple sampling points. Predicted optimal conditions (confined within the predefined experimental range) regressed from

the five response surfaces showed a decrease in optimal predicted pH from 6.0 at 3 hours to 5.0 at 6 hours. Temperature and enzyme dosage optima remained at the upper bound of the design (60 °C and 4.0 % (v/dw) Celluclast).

The prediction of higher pH (6.0) as optimal for the extraction of inorganic sulfates agreed with Hahn et al. (2012), but disagreed with Hifney et al. (2016), which observed an opposite trend and made use of buffer solutions in their study. Temperature was shown to have a positive effect on fucoidan extraction up to 80 °C by Zhang and Row (2015), and this would likely apply to inorganic sulfates as well. The effect of enzyme (in particular Celluclast) was reported by Charoensiddhi et al. (2016) to increase fucoidan extraction (measured as fucose) in pH adjusted water, and this would also coincide with the increased measurement of inorganic sulfate. The same study reported a reduction in fucoidan molecular weight and hypothesized that Celluclast was responsible for this hydrolysis.

January et al. (2019) reported acid hydrolysis to be necessary for the analysis of the sulfate content in fucoidan, but also reported that hydrolysis of the fucoidan molecule could occur with other methods (enzymatic methods included). Enzymatic hydrolysis of fucoidan by Celluclast could explain the decrease in molecular weight when enzymatic extraction was used. Celluclast has also been linked with increased solubilisation of algal material via the hydrolysis of the cellulose fibres of the cell wall. This cell wall hydrolysis and subsequent release of entrapped components (fucoidans, proteins, polyphenols, alginates, and others) would increase the observed sulfate response in solution (from fucoidan). Further increase in the measurement of sulfates could then derive from a combination of released fucoidan from the cell wall and the hydrolysis of the fucoidan itself, liberating further sulfate groups for analysis. It is likely that further testing of both the fucose content of extracts and the use of chromatographic methods would result in more definitive answers regarding the fucoidan polysaccharide.

#### **5.4.5. Total phenolics**

Total phenolic concentration was quantified with the Folin-Ciocalteu method, which is commonly used but has been shown to be non-specific and subject to interference from sugars, proteins, and other molecules (Generalić Mekinić et al., 2019). Despite this interference, the assays widespread usage makes it possible to compare results from other studies.

The response surfaces of the total phenolics concentration in the extract supernatants consistently indicated that the linear effects of temperature and pH were significant to the *TP* response. The predicted response optimum conditions moved steadily from a pH of 3.0 at 0 hours to 6.0 at 6 hours, paired with consistent higher temperature (60 °C). Enzyme dosage of 4.0 % (v/dw) Celluclast was indicated as

optimal from 0 to 3 hours, with a 0 % (*v/dw*) enzyme dosage predicted at 6 hours. The effect of temperature is consistent with literature, with many studies reporting an increase in phenolic content to coincide with elevated temperatures (60 °C and greater) (Generalić Mekinić et al., 2019), while low pH is reported to negatively affect the extraction of phenolic compounds by Kadam et al. (2015a), and theorised to coincide with the degradation of polyphenolic compounds.

#### **5.4.6. Antioxidant capacity**

The response surfaces of the antioxidant capacity data indicated the significant effect of the linear temperature term and quadratic pH term. Optimum conditions for antioxidant capacity were therefore predicted at higher temperature (60 °C) and midrange pH ( $\approx 4.5$ ), with no enzyme requirement. A decrease in total phenolic content, antiradical power, and ORAC (oxygen radical absorbance capacity) was observed by Wang et al. (2010) when investigating the use of Celluclast for creating antioxidative extracts from the red seaweed *Palmaria palmate*. This confirmed the negative effect of the enzyme on the antioxidant capacity response, which showed consistently low enzyme requirement in the predicted optimum conditions of the response surface models, despite not showing a significant effect.

# Chapter 6

## *Treatment comparisons*

Alternate treatments were tested against the chosen Celluclast enzyme to confirm enzyme utility and choice. Accellerase® 1500 (DuPont) (“Accellerase”, “treatment A”) was chosen as an alternative carbohydrase blend because of its use in bioethanol production and advertised inclusion of  $\beta$ -glucanases (assumed to include a 1,3- $\beta$ -glucanase. Conventional dilute-acidic thermal hydrolysis (“treatment C”) was chosen as a previously established, effective method of laminarin extraction. Methodology for statistical comparison and error propagation are presented first, followed by results for each responses comparisons and finally by discussion.

### **6.1. Treatment comparison methodology**

Statistical analysis was carried out on the spectrophotometric responses only ( $\text{g}\cdot\text{l}^{-1}$ ), and only during discussion and conclusions were responses converted to literature comparable data ( $\text{g}\cdot\text{gDW}^{-1}$ ). The challenge that arose with this approach was the propagation of experimental error from supernatant dissolved solids analysis. Where replication was not practical or possible (mass based measurements), errors and variances provided by the repeated centre-point experiments were used.

#### **6.1.1. Error and error propagation**

The  $t$  distribution was chosen to calculate confidence intervals, as this took the effect of replication into account and gave a more conservative error than standard error alone. The limited number and volume of samples confined the number of replicates, preventing the use of the normal distribution. Testing of samples came with the assumptions of both unknown mean ( $\mu$ ) and unknown variance ( $\sigma^2$ ). The confidence interval  $\Delta$  was defined by Equation 6-1 following (Montgomery and Runger, 2014).

$$\Delta = \bar{X} - \mu = \left( S / \sqrt{n} \right) \cdot t(\alpha, n - 1) \quad (6-1)$$

Where  $\bar{X}$  is the sample mean,  $\mu$  is the population mean,  $S$  is the sample standard deviation,  $n$  is the sample size, and  $t(\alpha, n - 1)$  is the inverse of the two-tailed student’s  $t$  distribution. Quadrature error propagation was necessary with multiple responses. Take two variables  $x$  and  $y$  with uncertainty parameters  $\Delta_x$  and  $\Delta_y$  respectively. The equations 6-2 to 6-4 display the propagation of these errors.

$$\Delta_{x \cdot y} = |x \cdot y| \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta y}{y}\right)^2} \quad (6-2)$$

$$\Delta_{x/y} = |x/y| \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta y}{y}\right)^2} \quad (6-3)$$

$$\Delta_{x-y} = \Delta_{x+y} = \sqrt{\Delta_x^2 + \Delta_y^2} \quad (6-4)$$

All error propagations of measurements were handled in this manner. True replication of analysis was carried out in triplicate at least (three complete analyses, on three separate occasions). Microplate well absorbance readings were considered pseudo-replicates due to multiple measurements of the same sample (one sample split between multiple microplate wells) and all microplates were read once only.

### 6.1.2. Variance propagation for Tukey HSD test

In order for the standard Tukey HSD (honest significant difference) post hoc test to be used, responses that required propagation of error (laminarin concentration and solubilised yield) needed to have their sample variances propagated for the HSD test to calculate a mean critical difference. The methods used were derived from Lee and Forthofer (2006), and are shown in Equations 6-5 to 6-8. Only elementary operations were required and were carried out in a stepwise fashion. Sample variances were used following the same propagation rules as population variances.

$$s_{aA+bB}^2 = a^2 s_A^2 + b^2 s_B^2 + 2abs_{AB} \quad (6-5)$$

$$s_{aA-bB}^2 = a^2 s_A^2 + b^2 s_B^2 - 2abs_{AB} \quad (6-6)$$

$$s_{A \cdot B}^2 = (AB)^2 \cdot \left[ \left(\frac{s_A}{A}\right)^2 + \left(\frac{s_B}{B}\right)^2 + 2 \frac{s_{AB}}{AB} \right] \quad (6-7)$$

$$s_{A/B}^2 = (AB)^2 \cdot \left[ \left(\frac{s_A}{A}\right)^2 + \left(\frac{s_B}{B}\right)^2 - 2 \frac{s_{AB}}{AB} \right] \quad (6-8)$$

Where  $s_A^2$  and  $s_B^2$  are sample variances of quantities A and B respectively, and  $s_{AB}^2$  is the sample covariance between A and B.

## 6.2. Treatment comparison results

The main objective behind the response surface analysis was the optimisation of the laminarin response ( $L$ ). The response surface models over predicted the  $L$  response at the proposed conditions (V), but the experimental data obtained at these conditions were still highest. This result was taken as positive as it

pushed the experimental space towards predefined conventional extraction methods. To confirm or disprove the effect of enzyme and hydrolysis time on all responses, the Tukey HSD (honest significant difference) test was implemented on each response for the full grouping of V, VB, and VE experiments with simultaneous comparisons made to an Accellerase experiment (the same conditions as VE, including enzyme-substrate ratio), and the conventional laminarin extraction method reported by Devillé et al. (2004). Each response was also measured on the raw seaweed material (R) following the sample preparation methods described in Section 4.1. The five experiments chosen for comparison (Table 6-1) are summarised below.

Table 6-1 Summary of experimental conditions for Tukey HSD testing

Exp.	Exp. conditions			Description
	pH	T (°C)	ES (v/dw)	
<b>V</b>	3.0	60.0	0.04	Validation of maximum laminarin release (in CCD)
<b>VB</b>	3.0	60.0	-	Repeat of V without enzyme addition
<b>VE</b>	4.5	50.0	0.04	Enzyme run (Celluclast)/ central region validation of CCD
<b>A</b>	4.5	50.0	0.04	Enzyme run (Accellerase)
<b>C</b>	1.0	70.0	-	Conventional extraction conditions (Deville et al., 2004)

### 6.2.1. Solubilised yield

In order to implement the Tukey HSD on the *SY* response,  $F_{SN}$  (supernatant mass fraction) and  $SL_P$  (pellet solids loading) required the use of proxy errors. This was implemented as stated in the previous section, via the sample variance and error parameter calculated from the repeated centre points (SO 15 – 20). Data, calculations, and HSD results regarding solubilised yield (*SY*) are shown in Appendix F (Table F-1) and the results are shown graphically in Figure 6-1. The mean critical difference (MCD) was calculated as  $\approx 4.4\%$  solubilised yield, with a span of sample means from  $\approx 48.6$  to  $\approx 57.3\%$  solubilised yield (MCD  $\approx 50\%$  of the sample span). This gave an indication of large relative errors and sample variances in the *SY* response (a combination of  $DS$ ,  $F_{SN}$ , and  $SL_P$  via Equation 4-4, defined in Chapter 4).

It was clear from Figure 6-1 that treatment C had the highest mean solubilised yield. At 0 hours, C was significantly higher than other treatments ( $57.3 \pm 15.30\%$ ), despite the large propagated error parameter. At 1.5 hours, the *SY* response of experiment C ( $56.1 \pm 17.6\%$ ) was significantly higher than experiments V and VB only. At 3 hours experiment C was only significantly higher than experiment VB, with this observation repeated at 4.5 hours. At 6 hours there was no significance between treatments.

The pairing of V and VB showed a significant difference ( $V > VB$ ) at 4.5 hours only, with only experiment V producing a significant increase above the respective raw material *SY* response. This



showed a lack of statistically significant increase in the *SY* response due to the addition of Celluclast enzyme at pH 3.0 and 60 °C, and agreed with the observations made between V and VB for the *DS* response shown next. The means of experiment V were consistently larger than VB from 0 to 4.5, and it is likely that large experimental error prevented significant differences from being observed between treatments.

Experiments VE and A showed no significant difference when compared to one another at each time point. Neither of these enzyme experiments significantly increased above their raw material readings, although this could be attributed to the larger errors present in the *SY* response, stemming from the propagation of error from the *DS* (dissolved solids),  $F_{SN}$  (supernatant mass fraction), and  $SL_P$  (pellet solids loading) experimental results.

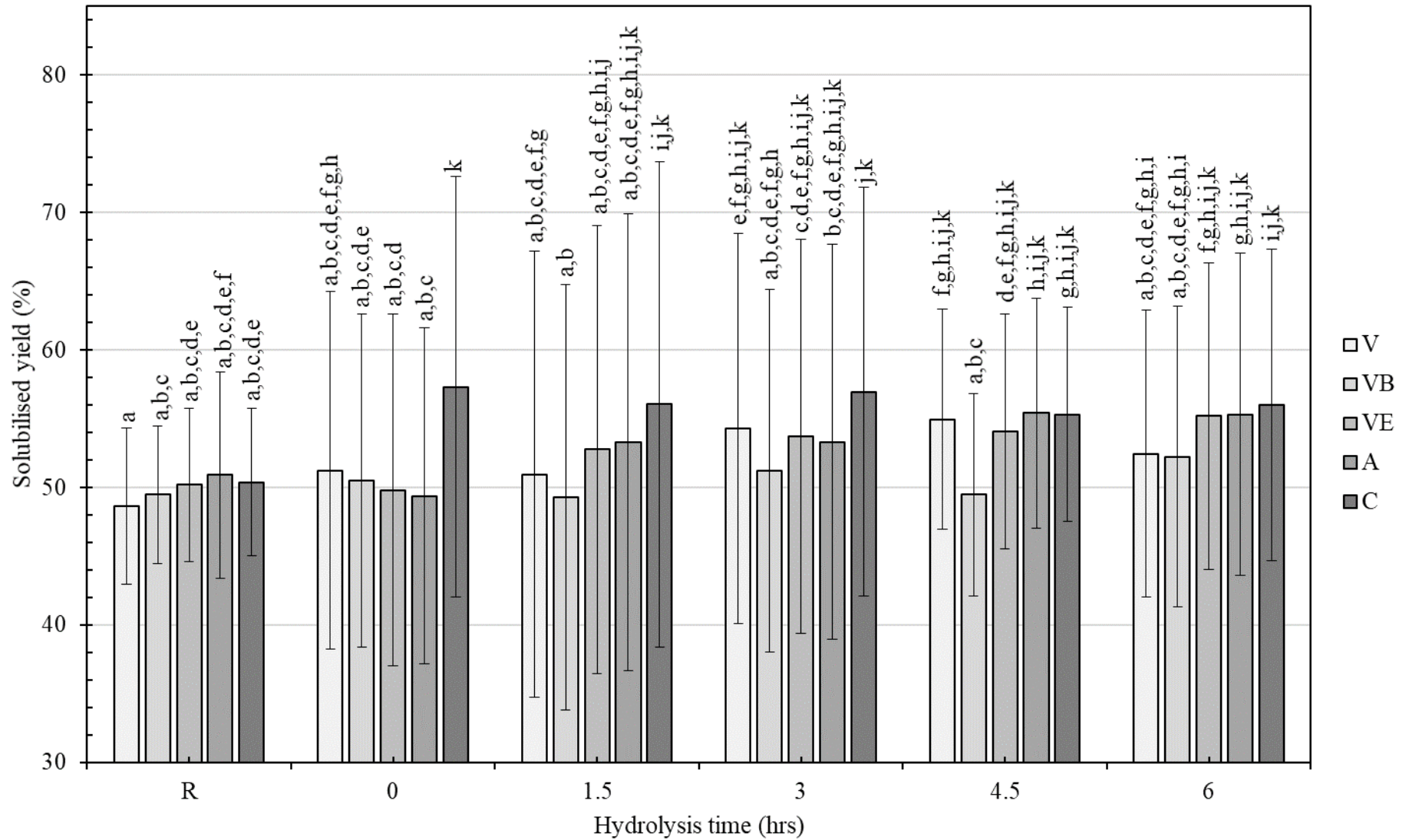


Figure 6-1 Solubilised yield (SY) validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 4.3816 %,  $p < 0.05$ )

## Supernatant dissolved solids

Treatment comparisons for supernatant dissolved solids (Figure 6-2) showed similarly large errors to the solubilised yield results, suggesting that the supernatant dissolved solids measurement was at least partially to blame for the error in solubilised yield calculations. ANOVAs and Tukey HSD post-hoc test results for the *DS* response are shown in Appendix F (Table F-2) and the MCD calculated from the HSD test was  $\approx 0.4\%$  ( $\approx 50\%$  of the *DS* response range), indicating a large error in the measurement of the dissolved solids response.

Figure 6-2 showed that the dissolved solids measured in the supernatant of the raw samples (R) were not significantly different from one another, indicating that pre-hydrolysis moisture correction, sampling, and centrifugation were all repeatable and that each sample was representative. The *DS* response obtained from experiment C (conventional acidic extraction) consistently exhibited the highest mean across the sampling times (highest at 6 hours –  $4.01 \pm 0.24\%$ ), although experimental error shown in measurement prevented this increase from being statistically significant. The 6 hour response of experiment VB ( $3.44 \pm 0.07\%$ ) and the 0 hour response of experiment A ( $3.18 \pm 0.08\%$ ) were the only *DS* responses significantly lower than treatment C at their respective sampling points.

The means of experiment V were higher than VB at all time points, this was not however statistically significant, and neither treatment significantly increased the *DS* response above that of the raw material. This means that the addition of 4% (*v/dw*) Celluclast enzyme to the pH and temperature combination of 3.0 and 60 °C did not significantly increase the *DS* response.

Dissolved solids responses of experiments VE and A (an enzyme comparison pairing) were not significantly different from one another at any sampling points. Experiment A was significantly higher than the raw material response of A at 4.5 hours, while experiment VE only achieved this significant increase at 6 hours.

The superior response *DS* occurred for experiment C, showing a significant increase at 0 hours (after heating to 70 °C and pH adjustment to 1.0) above the dissolved solids measurement of the raw material. While not significantly increased above other treatments at each time point, it is the only *DS* response to show a consistent significant difference from the raw material reading, where enzymatic methods (VE and A) took time to achieve this as mentioned.

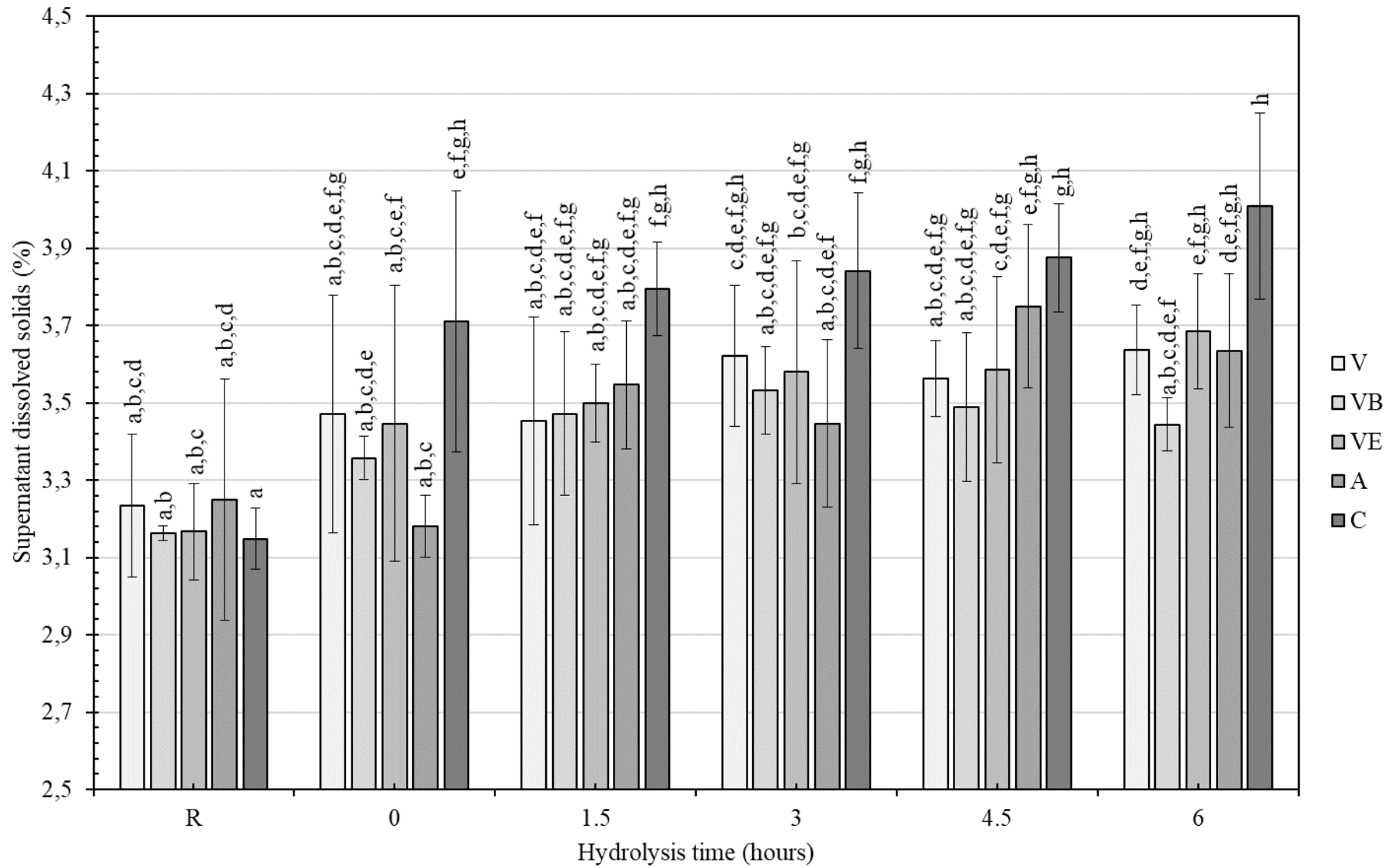


Figure 6-2 Dissolved solids (*DS*) validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.41931 %,  $p < 0.05$ ).

## Supernatant mass fraction

As stated during the presentation of the solubilised yield treatment comparison results, the  $F_{SN}$  parameter used a proxy error derived from the repeated centre points of the original CCD. ANOVAs and Tukey HSD results are shown in Appendix F (Table F-3) and results are displayed in Figure 6-3. The resultant MCD from the Tukey test was  $\approx 3.18$  % supernatant mass fraction ( $\approx 35$  % of the span between  $\approx 73.4$  and  $\approx 82.5$  % shown in the results). There was no significant increase shown for any of the  $F_{SN}$  responses over the  $F_{SN}$  observed on the raw material (R), although significant decreases below the raw material were observed in both acidic treatment experiments VB (1.5 - 6 hours), and C (1.5, 4.5, and 6 hours).

Experiment V showed a significantly higher response over VB from 1.5 to 4.5 hours, indicating the positive effect of the Celluclast treatment on the  $F_{SN}$  response at pH 3.0 and temperature 60 °C. Centre point conditions (pH 4.5 and 50 °C) with the same enzyme loading still outperformed at these conditions, with treatment VE significantly higher than V (and all treatments other than A) at 3 and 6 hours ( $81.3 \pm 1.48$  and  $82.5 \pm 1.7$  % respectively). The  $F_{SN}$  responses of VE, while highest by mean, were not significantly higher than the responses of experiment A (Accellerase) at any of the sampling points of the CCD.

Increased temperature and decreased pH treatment (experiment C) exhibited a significantly higher response over VB only at 3 hours. This showed that while increases in temperature and decreases in pH increased the  $F_{SN}$  response, they did not compete with the three tested enzymatic methods (V, VE, A).

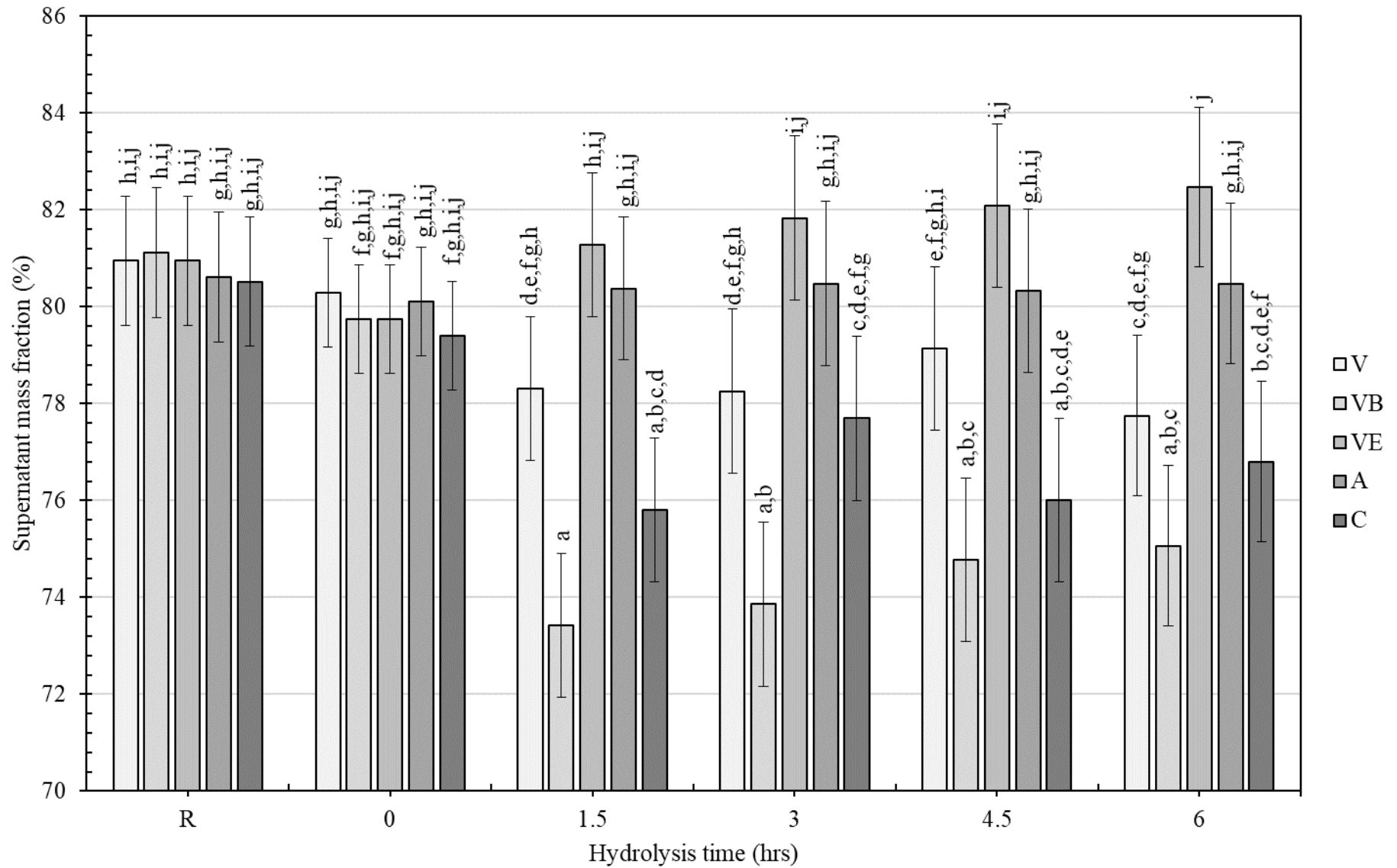


Figure 6-3 Supernatant mass fraction ( $F_{SN}$ ) validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 3.17807 %,  $p < 0.05$ ).



## Pellet-solids loading

The comparisons between  $SL_P$  responses to alternative treatments are shown in Figure 6-4. The Tukey HSD test and ANOVA are shown in Appendix F (Table F-4), and the MCD calculated was  $\approx 1.9\%$ . Unlike other responses investigated in this thesis, it was desirable for the  $SL_P$  response to be minimal, as increased solubilisation of the seaweed material was desired.

Figure 6-4 shows that the pellet-solids loading response of the raw material, while not significantly different from one another, showed more variability than previous responses presented. Non-enzyme treatments VB and C were consistently the lowest responses (desirable for this response) from 1.5 hours onwards, where both responses were significantly lower than their respectively raw material readings. These were the only two responses to drop below 10 % pellet-solids loading, and neither changed significantly in the 1.5 hours onwards.

Enzymatic treatments VE and A were not significantly different from one another at any sampling point, despite the mean of VE being consistently larger than A. VE was significantly larger than treatments V, VB, and C from 3 to 6 hours, whereas A was not. While Celluclast and Accellerase experiments were shown to be superior for the  $F_{SN}$  response, this was not the case with  $SL_P$ .

Enzymatic treatment and validation experiment V was only significantly larger than the autohydrolysis blank experiment VB at 1.5 hours. This showed that the errors calculated via the error proxy method did not allow a meaningful comparison between V and VB that would prove the effect of Celluclast enzyme at pH 3.0 and 60 °C on pellet-solids loading. The highest responses of  $SL_P$  required no enzyme addition, as well as decreased pH and elevated temperature.

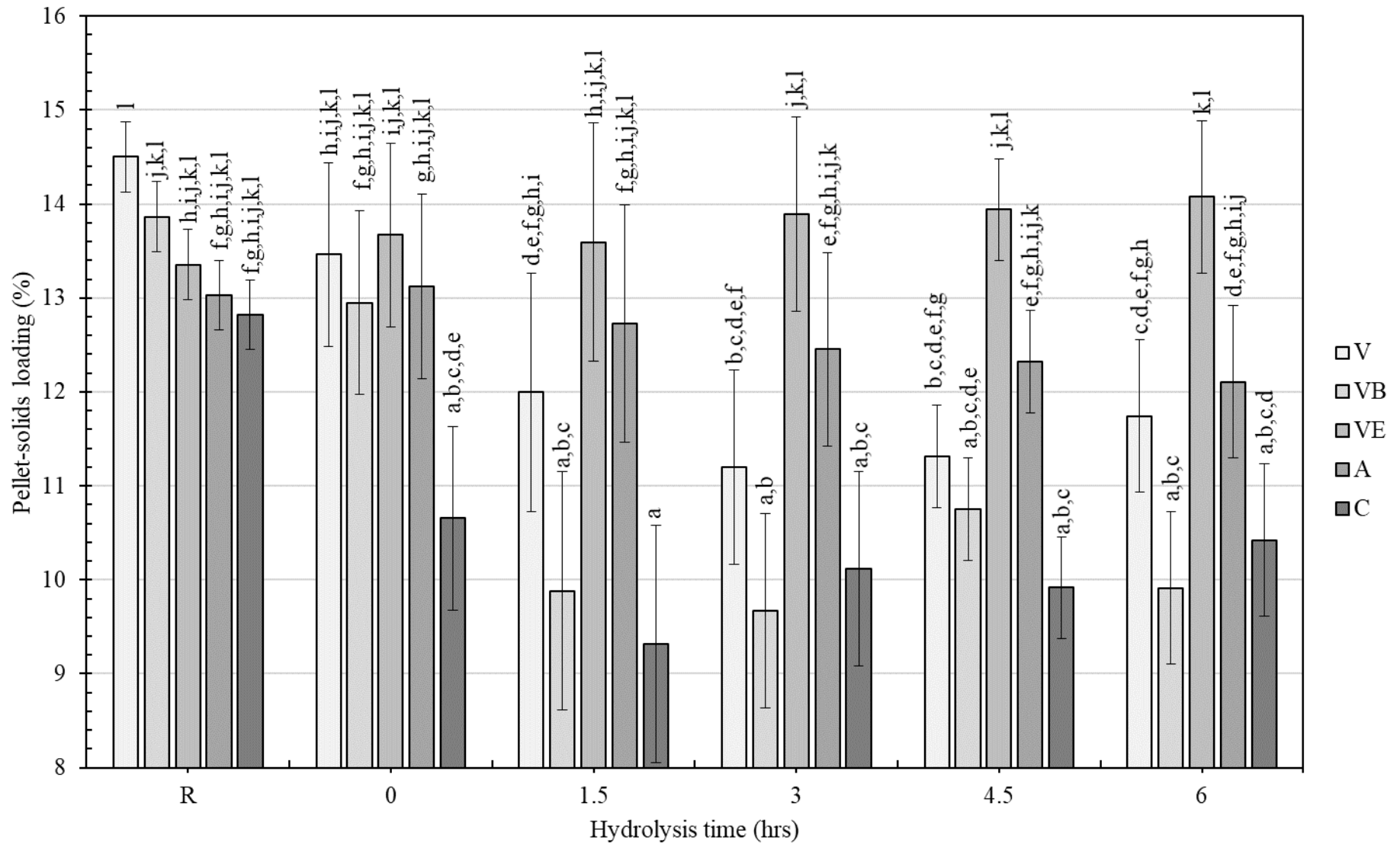


Figure 6-4 Pellet-solids loading ( $SL_P$ ) validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 1.867 %,  $p < 0.05$ ).

### 6.2.2. Reducing sugars

The *RS* response treatment comparison is shown in Figure 6-5, with ANOVAs and Tukey HSD calculations and tables included in Appendix F (Table F-5). The calculated MCD was  $\approx 0.34 \text{ gGIE}\cdot\text{l}^{-1}$ , while the mean *RS* responses spanned between  $\approx 4.2$  and  $\approx 7.6 \text{ gGIE}\cdot\text{l}^{-1}$ . The MCD was therefore approximately 10 % of the range, and the lowest relative MCD presented so far.

The highest responses for *RS* occurred with both experiment VE and A (4.0 % (*v/dw*) Celluclast and Accellerase enzyme runs at centre point conditions of pH 4.5 and 50 °C). This validated the milder pH and temperature choices made for the comparison of enzyme action only. Experiment VE and A were not significantly different from one another from 1.5 hours to 6 hours, showing neither to be superior for the release of reducing sugars from the batch studied (May 2018). The significant difference at 0 hours could likely be attributed to experimental error. VE and A both exhibited significant increase over time until 4.5 hours ( $7.22 \pm 0.15 \text{ gGIE}\cdot\text{l}^{-1}$  and  $7.32 \pm 0.11 \text{ gGIE}\cdot\text{l}^{-1}$  respectively), suggesting this amount of time to be sufficient at the dosage chosen. Extended hydrolysis would likely still result in significant increases, albeit over longer periods.

The next highest response other than VE and A was experiment C (conventional dilute-acid thermal hydrolysis at pH 1.0 and 70 °C), which showed a positive significant difference at 0 hours ( $5.72 \pm 0.05 \text{ gGIE}\cdot\text{l}^{-1}$ ) above the other treatments. Treatment C continued to show statistically significant increases in *RS* until 4.5 hours ( $6.63 \pm 0.04 \text{ gGIE}\cdot\text{l}^{-1}$ ) and exhibited a significantly higher response than V at 0, 1.5 and 4.5 hours and VB at for 0 to 6 hours.

VB maintained a significantly lower response than other treatments from 1.5 to 6 hours, showing that either enzyme (Celluclast or Accellerase ) or lowered pH and elevated temperatures were required to increase the reducing sugars response. VB did not significantly increase from 0 hours ( $5.30 \pm 0.16 \text{ gGIE}\cdot\text{l}^{-1}$ ) to 6 hours ( $5.33 \pm 0.12 \text{ gGIE}\cdot\text{l}^{-1}$ ), unlike the harsher conditions of experiment C. Comparison of VB and V showed that 4.0 % (*v/dw*) dosage of Celluclast significantly increased the released reducing sugars from 1.5 to 6 hours under pH 3.0 and 60 °C. The V experimental response however only significantly increased until 1.5 hours ( $5.83 \pm 0.12 \text{ gGIE}\cdot\text{l}^{-1}$ ), suggesting enzyme denaturation under prolonged periods of time at the conditions of pH 3.0 and 60 °C.

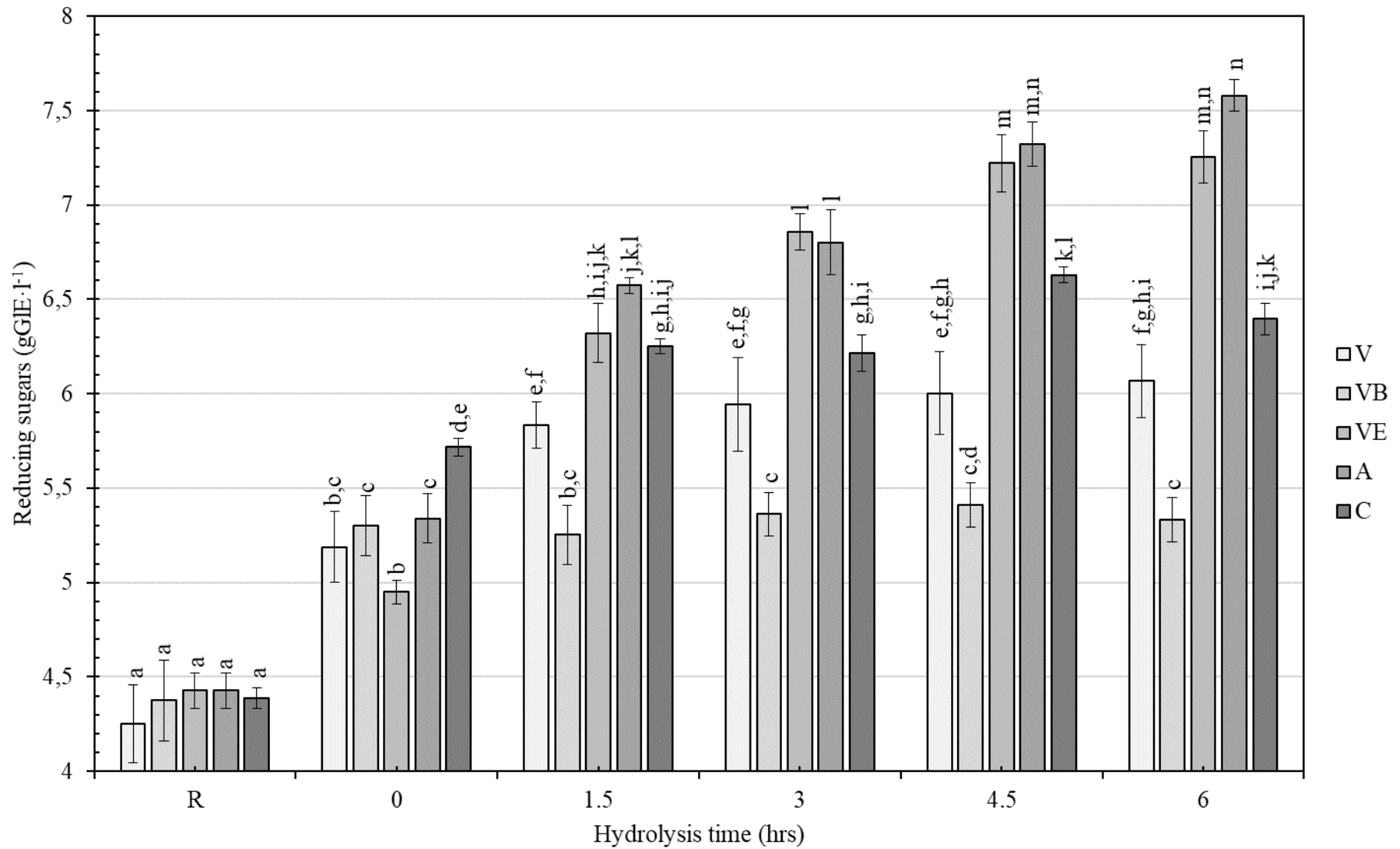


Figure 6-5 Reducing sugar (*RS*) model validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.34411 gGIE·l<sup>-1</sup>,  $p < 0.05$ )

### 6.2.3. Laminarin

The Tukey HSD test, ANOVAs, and calculations pertaining to the laminarin content of the supernatant are shown in Appendix F (Table F-6), with results shown in Figure 6-6. Calculated MCD was  $\approx 0.31 \text{ gLE}\cdot\text{l}^{-1}$ , while the data spanned from approximately 0.3 to  $2.6 \text{ gLE}\cdot\text{l}^{-1}$ , resulting in a  $\approx 13 \%$  error relative to the range of results.

The conventional dilute-acid thermal hydrolysis method (experiment C) resulted in a significantly higher response than other treatments, with a laminarin response fluctuating between  $\approx 2$  and  $\approx 2.5 \text{ gLE}\cdot\text{l}^{-1}$ . This was a  $\approx 100 \%$  improvement over the next highest response (experiment V,  $\approx 1 \text{ gLE}\cdot\text{l}^{-1}$ ) and comparisons to other treatments (VB, VE, and A) were even more favourable for the conventional hydrolysis. The significantly lower values in experiment C at 1.5 and 6 hours when compared to other time points could likely be attributed to experimental error.

The pairing of experiments V (significantly higher than VE and A from 0 to 3 hours) and VB (significantly higher than VE and A only at 1.5 hours) showed no significant difference at each time point. Experiment VE was significantly higher than the raw material readings (R) from 0 to 4.5 hours, while VB was only significantly improved over R at 1.5 and 3 hours. Neither of these responses changed significantly over time, with the exception of a significant drop in experiment V at 6 hours.

The comparison between VE (Celluclast) and A (Accellerase) showed no significant differences at any time point, while both significantly underperformed against C, and to a lesser significant extent V and VB. The means of experiment VE did not increase above  $0.5 \text{ gLE}\cdot\text{l}^{-1}$  except at 3 hours ( $0.51 \pm 0.16 \text{ gLE}\cdot\text{l}^{-1}$ ). The lack of utility in using enzymes for laminarin extraction was easily observable.

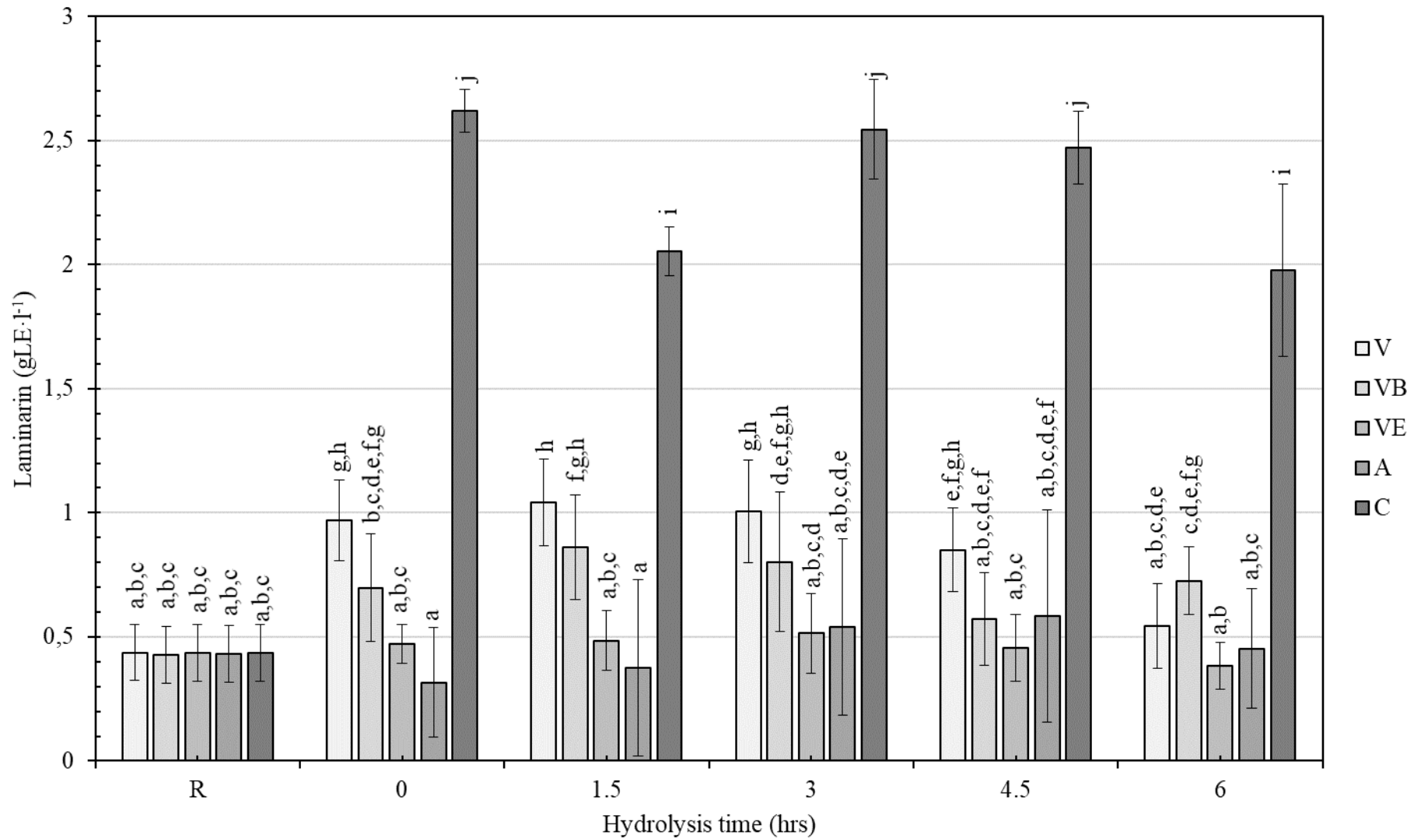


Figure 6-6 Laminarin (*L*) model validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.3149 gLE·l<sup>-1</sup>,  $p < 0.05$ )



#### 6.2.4. Inorganic sulfate

The measurement of inorganic sulfate ( $S$ ), as mentioned previously, served as a proxy measurement for the sulfated polysaccharide fucoidan (which is often coextracted with laminarin). Figure 6-7 shows the treatment comparison and all calculations, ANOVAs, and Tukey HSD testing regarding the  $S$  response is shown in Appendix F (Table F-7).

Figure 6-7 indicates the highest response for the release of inorganic sulfates as the 6 hour sampling point of experiment VE ( $1.14 \pm 0.10 \text{ gSE}\cdot\text{l}^{-1}$ , 4.0 % ( $v/dw$ ) Celluclast, pH 4.5, and 50 °C). This was significantly higher than the next largest response at 6 hours (A), and experiment VE was also significantly highest at 4.5 and 1.5 hours. The next largest responses were experiment A and VB, which were statistically equivalent from 1.5 to 4.5 hours.

The addition of enzyme at pH 3.0 and 60 °C (V vs VB) did not show a significant effect at any time but 3 hours (where VB was larger). This called the reading of VB at 3 hours into question. Further decreases in pH and increases in temperature from experiment VB (experiment C) resulted in the lowest attained inorganic sulfate responses. Experiment C significantly increases from a raw material reading of  $0.35 \pm 0.02$  to  $0.49 \pm 0.01 \text{ gSE}\cdot\text{l}^{-1}$  at 0 hours (coming up to temperature, followed by pH adjustment to 1.0). Over the course of hydrolysis the inorganic sulfate response of experiment C then significantly decreased from 0 hours to the lowest point of  $0.25 \pm 0.02 \text{ gSE}\cdot\text{l}^{-1}$  at 4.5 hours.

It was clear from the data that enzymatic extraction methods under milder pH and temperature outperformed both conventional acidic extraction (the worst  $S$  response) and both experiments at pH 3.0 and 60 °C (regardless of enzyme addition).

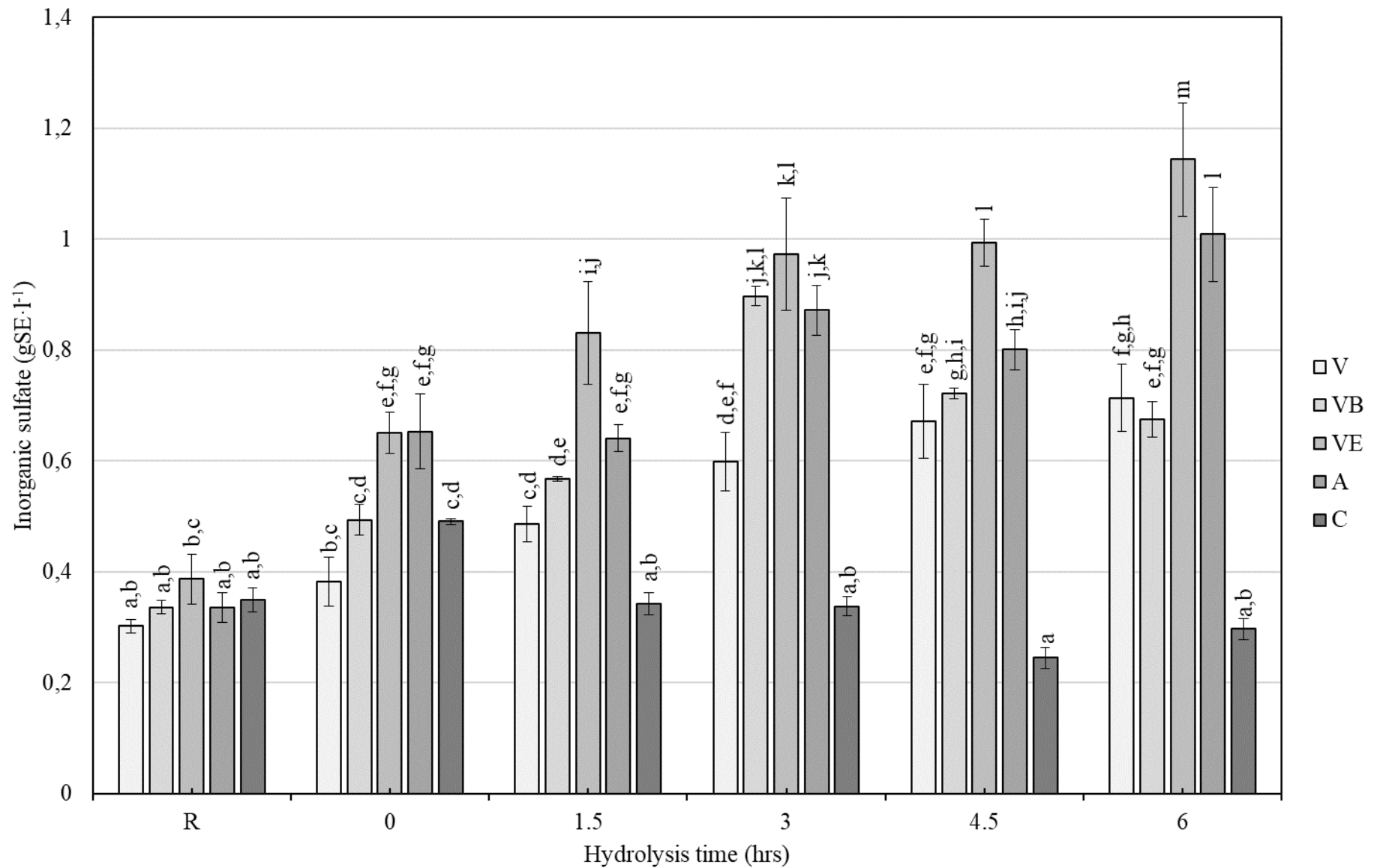


Figure 6-7 Inorganic sulfate (*S*) model validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.116572 gSE·l<sup>-1</sup>,  $p < 0.05$ )

### 6.2.5. Total phenolics

The Tukey results for the total phenolic content in the five experiments are shown in Appendix F (Table F-8), and shown graphically in Figure 6-8. The mean critical difference was calculated as  $\approx 0.018 \text{ gGAE}\cdot\text{l}^{-1}$ , against a span of results from  $\approx 0.24$  to  $\approx 0.40 \text{ gGAE}\cdot\text{l}^{-1}$  (MCD  $\approx 11$  % of experimental span).

Figure 6-8 indicates that the response of experiment VB was significantly higher than other treatments at 0, 1.5, and 6 hours, with experiment V statistically equivalent at 3 and 4.5 hours. V and VB were both significantly higher than all other treatments at 0 and 4.5 hours while at 1.5 and 3 hours there was no significant difference between V and VE. The lowest responses occurred in experiment C from 1.5 hours onwards, where the response did not significantly change over time.

Enzymatic treatments VE and A were not significantly different from 1.5 to 4.5 hours, where a conspicuous spike occurred in VE. This spike was not consistent with the gradual release curves seen for enzyme extractions and was likely an experimental anomaly or error to be treated with caution. Experiment VE increased significantly until 1.5 hours ( $0.32 \pm 0.01 \text{ gGAE}\cdot\text{l}^{-1}$ , an increase of  $\approx 23$  % over the raw value reading of  $0.26 \pm 0.00 \text{ gGAE}\cdot\text{l}^{-1}$ ) where a plateau was reached until 4.5 hours, this was further evidence that the value at 6 hours was anomalous and discountable. Experiment A increased significantly until 3 hours, but once again, the differences between treatment VE and A were not significantly different.

While experiment V and VB resulted in the highest responses, conventional treatment C showed significantly lower responses, indicating sensitivity of the total phenolics response to the further decrease in pH and increase in temperature used by conventional extraction techniques. The effects of pH and temperature were already found to be significant on the total phenolics response during the response surface modelling and analysis of Chapter 5.

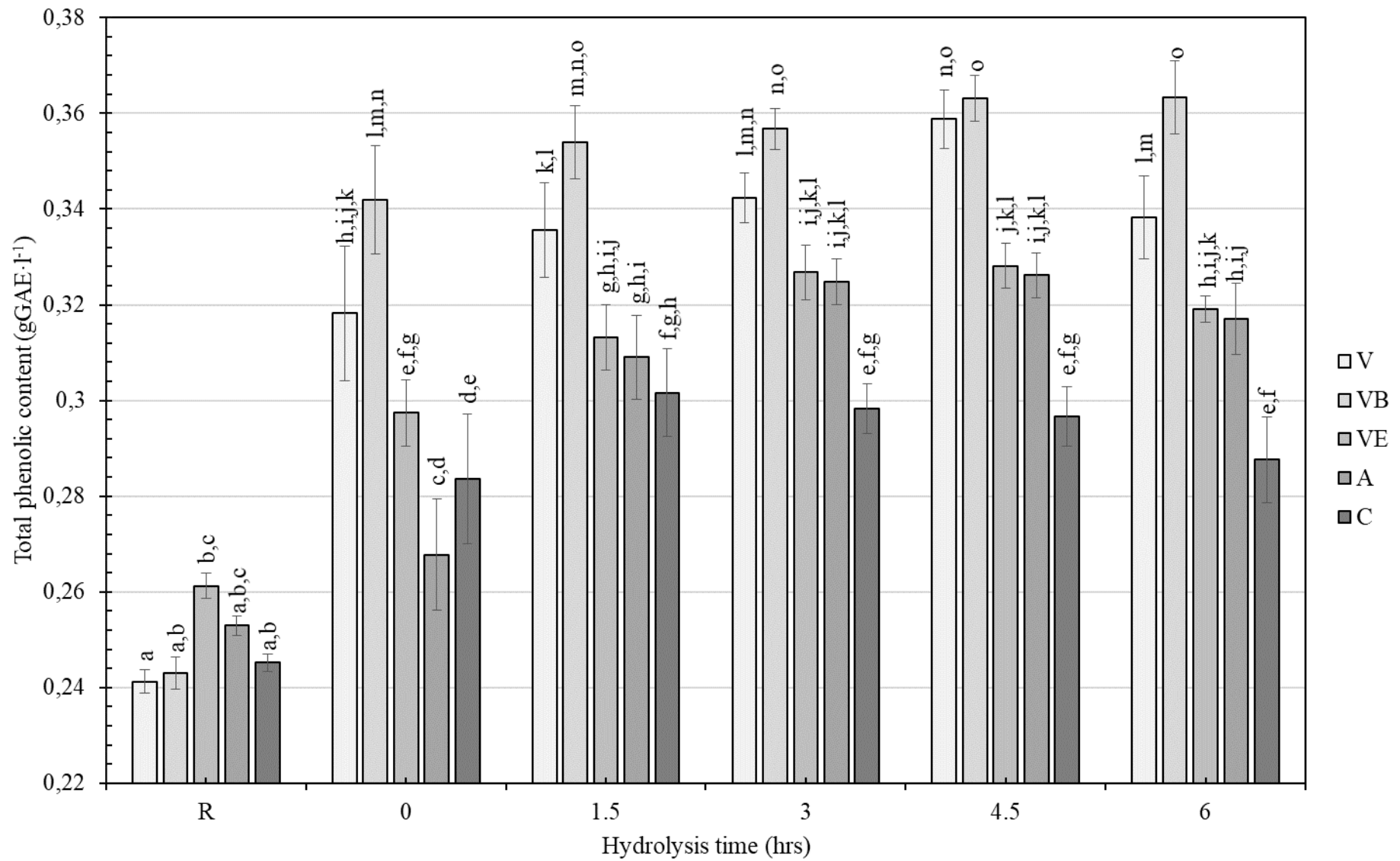


Figure 6-8 Total phenolics (*TP*) model validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.01796 gGAE·l<sup>-1</sup>,  $p < 0.05$ )

### 6.2.6. Antioxidant capacity

The antioxidant capacity Tukey HSD determinations and ANOVAs for the comparison of the five selected runs are located in Appendix F (Table F-9), while the results are shown graphically in Figure 6-9. The *AO* responses span from  $\approx 0.9$  to  $\approx 1.6$   $\text{gTE}\cdot\text{l}^{-1}$ , with a MCD of  $\approx 0.1$   $\text{gTE}\cdot\text{l}^{-1}$  ( $\approx 14\%$  of the experimental range).

The raw material antioxidant readings for all five treatments were significantly different, with the readings of VE and A higher than V, VB, and C. Despite this, the conventional extraction method C resulted in the highest *AO* response (significantly so at 0 and 1.5 hours). The experimental response of C increased significantly from 0 to 1.5 hours, followed by a drop and plateau from 3 hours onwards.

Experiment VB, statistically the second highest response at 0 hours, did not significantly change over the course of the 6 hour experiment. Experiment V, which included 4.0% (*v/dw*) Celluclast loading over VB, showed significantly lower antioxidant capacity responses than VB and also did not change significantly from 0 hours ( $0.94 \pm 0.02$   $\text{gTE}\cdot\text{l}^{-1}$ ), where the response of VB was  $1.43 \pm 0.04$   $\text{gTE}\cdot\text{l}^{-1}$ .

Enzyme responses VE and A did not significantly differ from one another over the course of the experiment, except at 4.5 hours, where VE was significantly lower than A. This VE response at 4.5 hours did not fit the VE responses over time (being significantly lower than the 6 hour response), and was likely an experimental error or anomaly. It is likely that the lack of significant difference between VE and A is consistent in reality. Discounting the VE response at 4.5 hours, neither VE nor A experienced significant increase past 1.5 hours. These two responses, along with treatment V consistently experienced the lowest antioxidant responses – making it likely that the low pH of conventional extraction was responsible for the increase in antioxidant capacity, and that enzymatic treatments may be detrimental to the antioxidant capacity response (significantly reduced antioxidant response of V compared to VB).

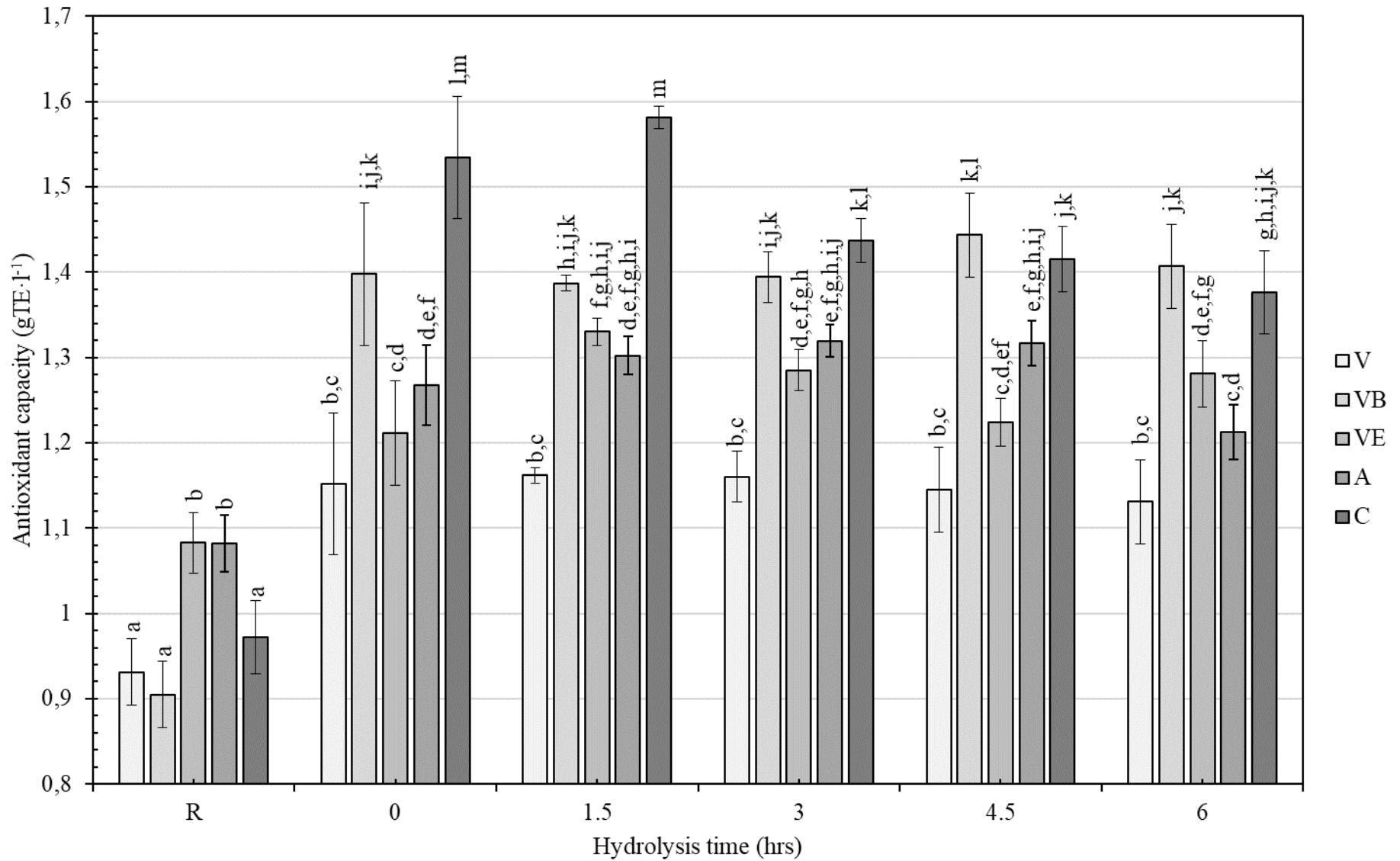


Figure 6-9 Antioxidant capacity ( $AO$ ) model validation experiments V, VB, and VE compared with experiments A and C. Different letters indicate statistically different groups (Tukey HSD procedure,  $MCD = 0.1035 \text{ gTE} \cdot \text{l}^{-1}$ ,  $p < 0.05$ )



### 6.3. Treatment comparison discussion

As implemented in the previous chapter, discussion was split between general discussion and response-specific discussions. All responses found in the definition of solubilised yield were grouped together. Of the different treatments compared (three validation runs, Accellerase, and conventional dilute-acid thermal hydrolysis), some responses benefitted from acidic / conventional hydrolysis and some benefitted more so from enzymatic hydrolysis. Duplicate observations made in this chapter were referred back to explanations presented in the discussion of response surface methodology in Chapter 5.

Conventional hydrolysis (experiment C) attained the highest responses for dissolved solids, solubilised yield, antioxidant capacity, and laminarin concentration. The laminarin response attained via treatment C remained at  $\approx 2.5 \text{ gLE}\cdot\text{I}^{-1}$ ,  $\approx 100 \%$  greater than any other treatment. While enzymatic methods performed in *DS* and *SY*, this required hydrolysis time when compared to treatment C. Treatment C attained maximum responses at 0 hours (first sampling point after pH and temperature adjustment) for laminarin, dissolved solids, and solubilised yield, indicating that prolonged hydrolysis was not required for these responses under dilute-acid thermal hydrolysis.

The validation runs V and VB obtained the largest total phenolic response over all, where enzymatic methods were lower, and the lowest response was from conventional extraction. Enzymatic methods proved superior with regards to the release of reducing sugars, inorganic sulfate, and supernatant mass fraction.

The performance of Accellerase and Celluclast did not significantly differ across all responses, implying similar makeups in enzyme mixture. Celluclast is not reported to contain 1,3- $\beta$ -D-glucanases or hemicellulases but it may be possible that the target compounds of these two specific enzyme classes (laminarin and hemicellulase) do not affect general solubilisation and compound release as much as cellulose microfibril hydrolysis. Detailed information on both mixture compositions are likely proprietary information and not readily available.

#### 6.3.1. Solubilised yield

The lack of significant difference between treatments showed the *SY* experimental response to be susceptible to errors in the components that make up the response (*DS*,  $F_{SN}$ , and  $SL_P$ ). While enzymatic methods were able to solubilise material over time when observing the mean solubilised yield alone, experimental errors prevented this change from being significant. Conventional dilute-acid thermal hydrolysis (pH 1.0 and 70 °C) was able to achieve a significant increase in solubilised yield above that

of the raw material as early as the 0 hour sampling point ( $57.3 \pm 15.30$  %, sampling occurring after temperature and pH adjustment). The faster action of dilute-acid thermal hydrolysis was shown again in the treatment comparison and was previously discussed in Section 5.4.1 regarding the response surface modelling of the solubilised yield response. If sufficient reactor volumes were available to provide adequate residence time for the chemicals ( $>1.5$  hours) then enzymatic hydrolysis would be a viable method for solubilisation of seaweed material. Values for solubilised yield presented in this study are consistent with the  $\approx 50$  to  $60$  % reported by Charoensiddhi et al. (2015), where 24-hour Celluclast extraction of *Ecklonia radiata* (1 % solids loading, 10 % (v/dw) enzyme dosage) resulted in a solubilised yield of approximately 56 % (compared to  $\approx 55$  % after 6 hours in this study). Different feedstocks, seasons, harvesting, and processing techniques make further comparisons to other studies impractical. The second batch tested in this study even showed appreciable difference (presented and discussed in Chapter 7).

### **Supernatant dissolved solids**

Comparison to conventional extraction (C) showed the highest means in the primary batch, although large errors also prevented this from being termed as significant. A large relative Tukey HSD MCD ( $\approx 50$  % of the *DS* response range) during the comparison of V, VB, VE, A, and C pointed to the supernatant dissolved solids measurement as being the major cause of the uncertainty found in solubilised yield.

The conclusion was made that the solubilisation performance of the different treatments could not be definitively compared using the *DS* response as defined, and while studies report solubilised yields, they do not report dissolved solids in the supernatant – preventing direct comparison to literature. Causes of error regarding dissolved solids measurement were discussed previously in Section 5.4.1.

### **Supernatant mass fraction**

Tukey HSD testing and comparison of the three validation runs (V, VB, and VE) to other treatments (A and C) showed that Celluclast treatment VE outperformed the proposed optimum of treatment V significantly at 3 and 6 hours. This confirmed that milder pH and temperature were conducive to an increased  $F_{SN}$  response with enzyme and agreed with the significant effect of enzyme dosage reported during response surface modelling of  $F_{SN}$  (5.2.1). The blank enzyme treatment VB showed that Celluclast enzyme significantly increased  $F_{SN}$  under these conditions (pH 3.0 and 60 °C), suggesting that the enzyme continued to function at this pH/temperature combination. Further comparisons to the  $F_{SN}$  response of Accellerase (treatment A) and conventional dilute-acid thermal hydrolysis (C) showed that

both enzymatic treatments VE and A (4.0 % (v/dw), pH 4.5, and 50 °C) were not significantly different. Treatment C (pH 1.0 and 70 °C autohydrolysis) underperformed against both enzymes runs (VE and A) and the difference in performance between enzymatic and conventional treatments on the supernatant mass fraction results was reasoned to be caused by the presence of alginic acid and the possible enzymatic hydrolysis of alginate by both carbohydrase mixtures (Celluclast and Accellerase).

The presence of the hydrocolloid alginic acid could explain the drop in the  $F_{SN}$  treatments seen at lower pH, where alginate would have become insoluble alginic acid and would therefore be centrifuged out with the pellet (Vauchel et al., 2008). Acting as a hydrocolloid, it would also likely absorb further water from the supernatant into the pellet, further decreasing the measured value of  $F_{SN}$ . *E. maxima* is reported to consist of between 22 and 40 % alginic acid by dry weight (Amosu et al., 2015), enough for the solubility and hydrocolloid characteristics of the polysaccharide to significantly alter the  $F_{SN}$  response between experiments. The effect of degradation of alginate and molecular weight reduction on gelling ability was discussed in Section 5.4.1.

### **Pellet-solids loading**

The lowest (favourable in this case) of the three validation responses for  $SL_P$  was pH 3.0 and 60 °C autohydrolysis (treatment VB), measuring  $\approx 10$  % pellet solids loading over 0 to 6 hours. Comparison of the model validation runs to conventional (C) and Accellerase (A) hydrolysis showed that the overall lowest  $SL_P$  responses came from conventional extraction C (pH 1.0 and 70 °C), which dropped from an  $SL_P$  response of  $\approx 13$  % in the raw material to  $\approx 10$  % from 0 hours onwards. Enzymatic treatments at 4.0 % (v/dw), pH 4.5, and 50 °C for both Celluclast and Accellerase underperformed against both conventional dilute-acid thermal hydrolysis and the blank validation run, and did not significantly change from the  $SL_P$  readings of the raw materials. Neither Celluclast nor Accellerase enzymes significantly changed the  $SL_P$  response, but enzyme loading was reported as significantly negative to  $SL_P$  during response surface analysis. These observations were also reasoned to be caused by the presence and behaviour of the alginic acid hydrocolloid.

Low pH would convert alginate to insoluble alginic acid and the hydrocolloid would then be confined to the pellet when centrifuging. The drop in  $SL_P$  (and coinciding drop in  $F_{SN}$ ) experienced at low pH and high temperature conditions of more acidic extractions could be explained by the hydrocolloid properties (gel-formation) of alginic acid likely absorbing water back from the supernatant, increasing both the mass of the pellet and simultaneously decreasing the pellet solids loading (Vauchel et al., 2008).

### 6.3.2. Reducing sugars

Comparison of the validation experiments to treatment A (Accellerase) and C (conventional dilute-acid thermal hydrolysis) indicated that the enzymatic performances of Celluclast and Accellerase did not differ significantly at pH 4.5 and 50 °C (consistent with other responses). Enzymatic treatments at these conditions significantly outperformed conventional extraction and the proposed validation runs to achieve maximum reducing sugar responses of  $7.25 \pm 0.14 \text{ gGIE}\cdot\text{l}^{-1}$  ( $105 \pm 22 \text{ mgGIE}\cdot\text{gDW}^{-1}$ ) and  $7.58 \pm 0.08 \text{ gGIE}\cdot\text{l}^{-1}$  ( $111 \pm 25 \text{ mgGIE}\cdot\text{gDW}^{-1}$ ) for Celluclast and Accellerase respectively at 6 hours. Conventional extraction resulted in a maximum reducing sugar response of  $6.63 \pm 0.04 \text{ gGIE}\cdot\text{l}^{-1}$  ( $91 \pm 14 \text{ mgGIE}\cdot\text{gDW}^{-1}$ ) at 4.5 hours.

The magnitude of the results were consistent with those reported in literature, particularly dilute acid treatments and enzymatic treatments, with reducing sugar results ranging from 50 to 540  $\text{mgGIE}\cdot\text{gDW}^{-1}$  in various macroalgal species in the review bioethanol review paper of Offei et al. (2018). It was impractical to compare the results of one batch of seaweed to another with regards to different extraction techniques, making it more important to compare general treatments. Abd-Rahim et al., (2014) showed that it was possible to obtain higher reducing sugar yields above that release from dilute-acid hydrolysis alone, via the addition of Celluclast enzyme ( $573 \text{ mgGIE}\cdot\text{gDW}^{-1}$  from  $396 \text{ mgGIE}\cdot\text{gDW}^{-1}$ ). The superior response of both carbohydrases in this study above that of conventional dilute acid thermal hydrolysis extraction is therefore supported in literature.

Enzymatic hydrolysis likely outperformed dilute-acid thermal hydrolysis because of the high catalytic efficiency of enzymatic methods in the temperature and pH range investigated. Higher temperatures (above the 70 °C used in the conventional hydrolysis method) are generally used in dilute acid thermal hydrolysis (> 100 °C) (Yanagisawa et al., 2013), and the combination of pH 1.0 and 70 °C was derived from a study optimising the extraction laminarin, and not reducing and simple sugars (Deville et al., 2004). It is therefore likely that if the temperature of the “conventional” extraction method were raised above 100 °C there would be both an increase in reducing and simple sugar extraction and a decrease in the yield of laminarin, as was shown at 90 °C by Zhang and Row (2015) in their study on laminarin and fucoidan extraction and separation via size-exclusion chromatography.

### 6.3.3. Laminarin

Comparison of the validation laminarin extractions to conventional extraction (pH 1.0 and 70 °C) and Accellerase extraction at 4.5 and 50 °C (Section 6.2.3) showed that all treatments were inferior to the conventional dilute-acid thermal hydrolysis method ( $\approx 2.3 \text{ gLE}\cdot\text{l}^{-1}$ ,  $\approx 33 \text{ mgLE}\cdot\text{gDW}^{-1}$ ). Celluclast and

Accellerase treatments at 4.0 % (v/dw), pH 4.5, and 50 °C (VE and A) were unable to release more than  $\approx 0.5 \text{ gLE}\cdot\text{l}^{-1}$  ( $\approx 8 \text{ mgLE}\cdot\text{gDW}^{-1}$ ) in comparison. The method referred to as “conventional”, as previously mentioned, was derived from Devillé et al. (2004). In that particular study ethanol precipitation was used to prepare extracts, and extraction reported as a percentage of the ethanol precipitated extract. Kadam et al. (2015a) reported laminarin concentrations between approximately 32 to 43  $\text{mgLE}\cdot\text{gDW}^{-1}$  when using 0.1 HCl (pH 1.0) extraction similar to Devillé et al. (2004) on *Ascophyllum nodosum* and *Laminaria hyperborea*. Rioux et al. (2009) observed similar amounts in two batches harvested roughly a year apart (May 2005 and June 2006), showing laminarin concentrations of  $\approx 53 \text{ mgLE}\cdot\text{gDW}^{-1}$  and  $< 25 \text{ mgLE}\cdot\text{gDW}^{-1}$  respectively. The conventional hydrolysis method of this study therefore produced results consistent with literature, and enzymatic methods were shown to underperform significantly. Low yields were attributed to seasonal and geographical factors and a full seasonality study would be required if laminarin extraction were pursued as a commercial endeavour. The requirement for ethanol precipitation in order to obtain higher purities of laminarin is likely not suitable for mass processing.

#### 6.3.4. Inorganic sulfates

Comparison to Accellerase extraction (pH 4.5, 50 °C, 4.0 % (v/dw) enzyme dosage), and the “conventional” hydrolysis conditions for laminarin (pH 1.0 and 70 °C) (Section 6.2.4) showed that Celluclast at pH 4.5 and 50 °C significantly outperformed all treatments except Accellerase, yielding a maximum sulfate concentration in solution of  $16.5 \pm 3.8 \text{ mgSE}\cdot\text{gDW}^{-1}$  at 6 hours from a raw material reading of  $5.9 \pm 1.0 \text{ mgSE}\cdot\text{gDW}^{-1}$ , and indicating a  $\approx 180$  % increase in release when compared to Accellerase (maximum of  $14.8 \pm 3.6 \text{ mgSE}\cdot\text{gDW}^{-1}$  at 6 hours) and conventional conditions (maximum of  $7.3 \pm 2.2 \text{ mgSE}\cdot\text{gDW}^{-1}$  at 0 hours). Enzymatic conditions both showed significant increases until 6 hours, indicating that further inorganic sulfate release was likely possible if either enzyme dosage (shown as a significant factor during response surface modelling of Chapter 5) or hydrolysis time were increased. The lowering of pH resulting in lowered inorganic sulfate response likely indicates that coextraction of laminarin and fucoidan may not occur under the same conditions. A step-wise process with mild enzymatic conditions up front, followed by dilute-acid thermal hydrolysis on the residue could result in the reasonable extraction of both valuable polysaccharides.

While low pH was shown by Hahn et al., (2012) to be detrimental to the sulfate response, it was also reported to increase fucoidan yield overall. In that particular study, it was advised to minimise the sulfate content extracted, while simultaneously optimising fucoidan recovery. This was reasoned because increased sulfate release is linked to the hydrolysis of the fucoidan polysaccharide itself. Despite this proposed method by Hahn et al. (2012), the method of fucoidan quantification in that paper is unclear,

and chromatographic methods may have been used. Enzymatic methods (both Celluclast and Accellerase) were shown to be superior in inorganic sulfate/fucoidan release, consistent with the reported findings of Charoensiddhi et al. (2016). That particular paper did not report fucoidan release in terms of inorganic sulfate, but rather in terms of fucose, making quantitative comparison impossible. It is likely necessary to quantify both inorganic sulfate and fucose to quantify fucoidan, with inorganic sulfate alone varying between  $\approx 3$  to 33 % of the polysaccharide weight (Zhang et al., 2020). The assessment of the coextraction of laminarin and fucoidan would benefit from these extra analyses.

### 6.3.5. Total phenolics

Comparison to alternate extractions showed that enzymatic (both Celluclast and Accellerase at 4.0 % (*v/dw*), pH 4.5, and 50 °C) and conventional dilute-acid thermal hydrolysis (pH 1.0 and 70 °C) underperformed against the blank validation run VB (pH 3.0 and 60 °C). VB obtained a maximum total phenolic response of  $5.3 \pm 1.1$  mgGAE·gDW<sup>-1</sup> at 6 hours, although this was not significantly higher than VB at 1.5 to 4.5 hours ( $4.8 - 4.9$  mgGAE·gDW<sup>-1</sup>) and so extraction past 1.5 hours did not significantly improve the release of phenolic compounds.

Dilute acidic extraction (pH  $\approx 1.5$ ) was found to be superior to water extraction by Kadam et al. (2015c), and the extraction time of phenolic compounds from brown seaweed in literature varies, although extended extractions usually net higher phenolic responses. It was likely that the increased acidic conditions of the conventional extraction method (pH 1.0, 70 °C, maximum of  $4.3 \pm 1.4$  mgGAE·gDW<sup>-1</sup> at 1.5 hours) degraded the polyphenolics present and therefore showed lower experimental responses (Kadam et al., 2015a). Results in literature show a large range from 0.09 mgGAE·gDW<sup>-1</sup> up to  $\approx 150$  mgGAE·gDW<sup>-1</sup> but this may be due to the non-specific nature of the Folin-Ciocalteu method, and interference by other bioactive compounds in the solutions tested (Generalić Mekinić et al., 2019).

### 6.3.6. Antioxidant capacity

Comparison to Accellerase at pH 4.5 and 50 °C and the conventional laminarin extraction method (pH 1.0 and 70 °C) (Section 6.2.6) showed that conventional dilute-acid thermal hydrolysis outperformed enzymatic treatments significantly to obtain a maximum antioxidant capacity of  $1.58 \pm 0.01$  gTE·l<sup>-1</sup> ( $22.5 \pm 7.2$  mgTE·gDW<sup>-1</sup>). The blank autohydrolysis at pH 3.0 and 60 °C also significantly outperformed enzymatic treatments (including its pH and temperature paired enzyme experiment V), confirming that enzymatic action lead to a decrease in the antioxidant capacity of the seaweed extracts (as stated from RSM analysis, and Wang et al. (2010)).



Kadam et al. (2015a) observed 0.1M acidic treatment (pH 1.0) to show superior DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition (a similar mechanism to the DMPD used), while increased temperature extractions were generally reported to increase antioxidant extraction into solution (Balboa et al., 2013). These studies confirm the observed effects of this investigation, and the increased temperature and decreased pH likely facilitated increased antioxidant capacity from the breakdown of the cell wall and subsequent release of bioactives into solution (Balboa et al., 2013). Comparison to literature was not possible due to the choice of consistent dilution factor to allow response surface modelling. Full antioxidant capacity characterisation at differing concentration of extract would be necessary on the final chosen laminarin / polysaccharide extract.

# Chapter 7

## *Batch comparison*

Further to the treatment comparisons, a second batch of kelp material was harvested (June 2019) and processed in the same fashion as the first batch (May 2018). Comparison of this second batch was made under a set of chosen treatment from the previous chapters and this formed the basis of a simple preliminary batch/seasonality comparison. Methodology is presented first as before, followed by results and discussions regarding each responses batch comparisons.

### **7.1. Batch comparison methodology**

A second batch of kelp material (referred to as B2) was tested under three of the experimental treatments defined in Chapter 6, namely that of conventional extraction (conditions described for experiment C), Celluclast extraction (experiment VE), and Accellerase extraction (experiment A). These conditions were chosen for their different extraction mechanisms (acid-based extraction, cellulase mixture extraction, and cellulase/hemicellulase/ $\beta$ -glucanase mixture extraction). Experimental conditions for V and VB were excluded as they were seen as combinations (V) and extensions (VB) of the three conditions. The Tukey HSD test was again used to test for significant differences between these three responses on each batch, resulting in a total of six responses to be compared over time. Error propagation was carried out in the same way as in Chapter 6, therefore negating the need to state new methodology.

### **7.2. Batch comparison results**

Results of the batch comparison (May 2018 (B1) vs June 2019 (B2)) are presented in this section following the same structure as Chapter 6. Solubilised yield was presented first with all of the sub-responses (supernatant dissolved solids, supernatant mass fraction, and pellet-solids loading), with other responses following. Discussion follows in Section 7.3.

#### **7.2.1. Solubilised yield**

Tukey HSD results and ANOVAs for the batch comparison study are included in Appendix G (Table G-1), while the results are shown graphically in Figure 7-1. The calculated MCD was  $\approx 4.5\%$ , and the solubilised yields of B1 (consistently significantly higher than B2) sat approximately 15% higher than

all B2 solubilised yields. The solubilised yield of raw material R was significantly lower in B2 ( $\approx 33\%$ ), approximately 66 % of the raw material reading of B1 ( $\approx 50\%$ ).

The solubilised yield response of treatment VE (4.0 % (v/dw) Celluclast, pH 4.5, and 50 °C) showed a significant increase in B2 until 1.5 hours (40.3 % solubilised, a 20 % relative increase above the raw material reading of 33.7 %), where it plateaued for the rest of the experiment. While not a significant increase between 0 and 1.5 hours on B1 (due to high propagated error), the same trend was noticed in the means of B1, where half of the increase in mean occurred between 0 and 1.5 hours ( $\approx 5\%$  increase over raw material reading as compared to the  $\approx 10\%$  increase at 6 hours). It was evident that a larger increase occurred for B2 relative to the raw material.

Accellerase (treatment A) mirrored the observed trends of Celluclast, but significantly increased until 3 hours in B2 ( $37.1 \pm 8.7\%$ ). Each time point was not significantly different from other treatments in B2, except VE B2 at 1.5 hours and C B2 at 0 hours (A B2 was lower in both cases).

Treatment C (conventional extraction) increased significantly over the raw material at 0 hours (after temperature and pH adjustment) in both B1 and B2. Enzymatic treatments did not achieve the same significant increase above raw material until 3 hours (VE B2), 4.5 hours (A B2), and 6 hours (VE B1 and VE B2). While C B1 achieved a steady plateau after 0 hours, C B2 showed significant fluctuation.

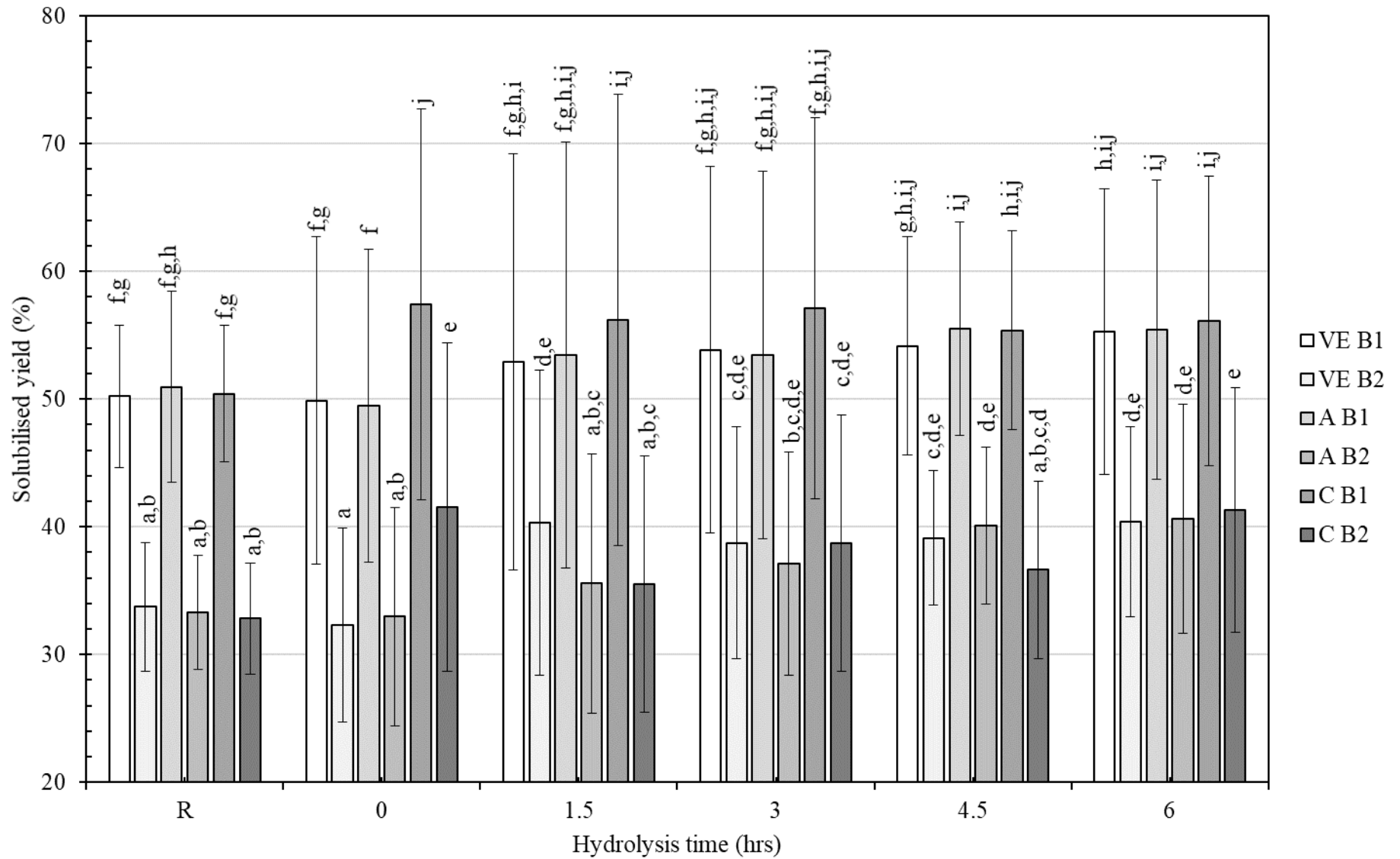


Figure 7-1 Solubilised yield (SY) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 4.454 %,  $p < 0.05$ )

### **Supernatant dissolved solids**

Tukey HSD results and ANOVAs for the supernatant dissolved solids batch comparison are included in Appendix G (Table G-2), with the comparisons shown graphically in Figure 7-2. The figure shows that while the B1 *DS* results are higher by mean, only the conventional extraction (C) *DS* response from 3 to 6 hours is significantly higher for B1 as opposed to B2. A large mean critical difference of  $\approx 0.6\%$  prevents further differences from being identified as significant. The response errors are consistent with those shown in both response surface analysis and treatment comparisons are the reason for these errors is likely consistent.

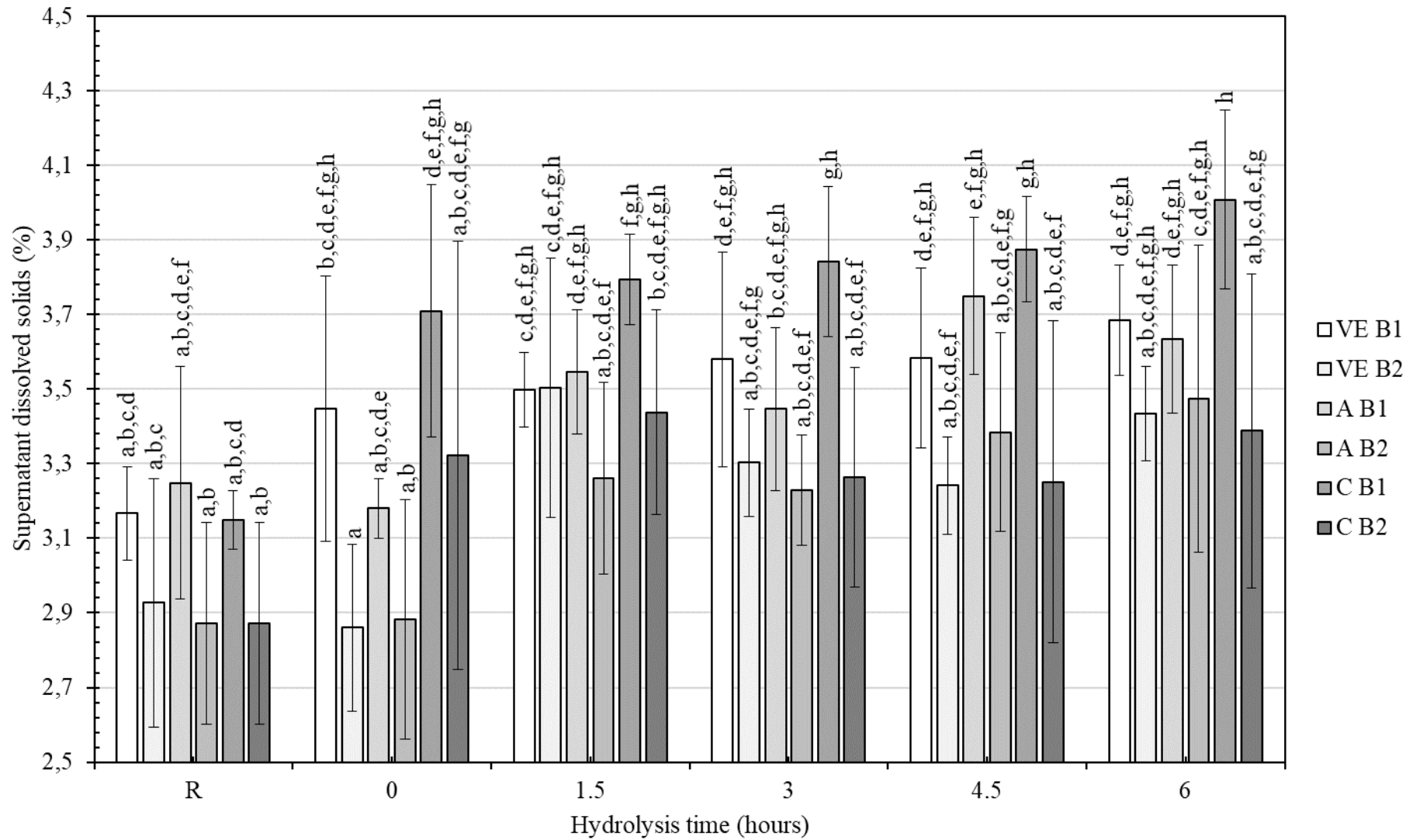


Figure 7-2 Supernatant dissolved solids (*DS*) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.57762 %,  $p < 0.05$ )



## Supernatant mass fraction

As the supernatant mass fraction  $F_{SN}$  contributed to the solubilised yield response, it was likely to find batch differences like those found for solubilised yield. Tukey HSD results and ANOVAs are included in Appendix G (Table G-3), while the results are shown graphically in Figure 7-3. The MCD was calculated as  $\approx 3.2\%$ . A vast difference could be seen between batches in the figure, with the  $F_{SN}$  response of B1 measuring between approximately 75 and 80% while B2 lay from 55 to 65%. A larger drop in  $F_{SN}$  was also observed from the preheating step (R to 0 hours) in B2 than in B1 and this drop was significant for both A B2 and C B2.

Treatment VE B2 showed a significant increase only until 1.5 hours following a minor drop at 0 hours. A significant difference from the raw material reading was only achieved at 4.5 hours. VE B2 was only significantly larger than A B2 at 1.5 hours, and was significantly larger than C B2 from 1.5 to 4.5 hours. B1 partially showed this trend, with VE B1 being consistently significantly larger than C B1 from 1.5 hours onward. Treatment A B2 significantly increased until 3 hours and was significantly larger than C B2 only at 4.5 hours, indicating that there was more consistently no significant difference between these treatments the second batch.

A non-smooth response was shown over time in C B2 where a significant drop in  $F_{SN}$  occurred between the raw material and 0 hours, followed by two successive periods (0 – 1.5 hours and 3 – 4.5 hours) of no significant change. An upward trend was however clear by 6 hours, although the reading at this sampling point was not significantly different from the raw material, having only recovered from the initial drop experienced in the  $F_{SN}$  response at 0 hours.

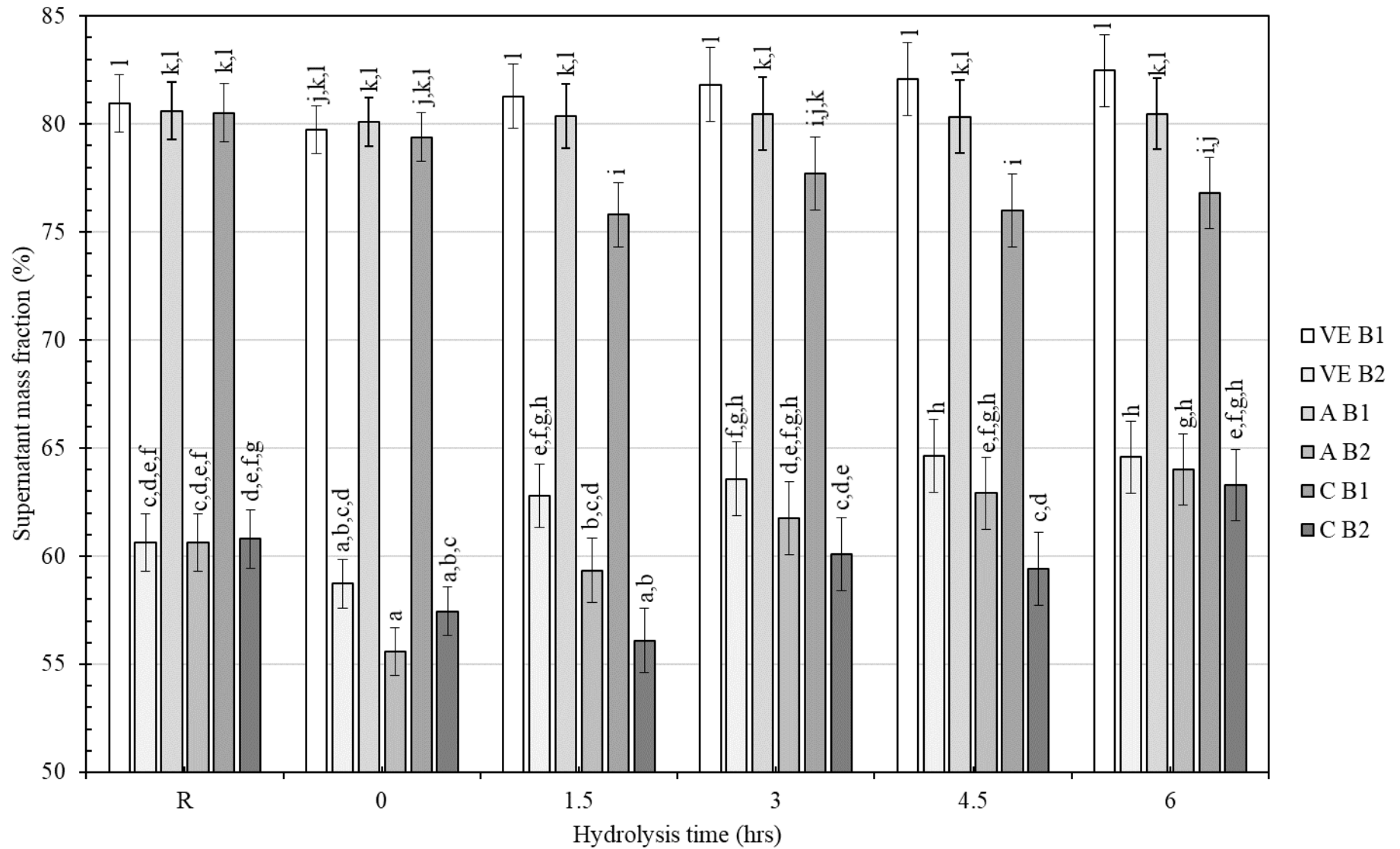


Figure 7-3 Supernatant mass fraction ( $F_{SN}$ ) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 3.2448 %,  $p < 0.05$ )

## Pellet-solids loading

The ANOVA and Tukey HSD post hoc test results for the  $SL_p$  batch comparison are located in Appendix G (Table G-4), with the results shown graphically in Figure 7-4. A MCD of  $\approx 1.9\%$  was calculated via Tukey HSD test and the error proxy method was described in Section 6.1.2.

The primary batch (B1) was significantly larger than B2 for all treatments except for C. While B2 consistently showed C B2 to be lower by mean, this was not a significant difference when compared to VE B2 and A B2. In both batches, the enzymatic methods were not shown to be significantly different other than at 6 hours (where VE showed a larger  $SL_p$  response). Neither enzymatic method differed significantly from the raw material reading in either batch. In contrast, experiment C B1 (also shown in Section 6.2.1) showed a significant decrease below the raw material ( $\approx 13$  to  $\approx 10\%$ ). C B2 also showed a significant decrease from the raw material, although this was only at 0 hours following temperature and pH adjustment ( $\approx 9$  to  $\approx 6.2\%$ ).

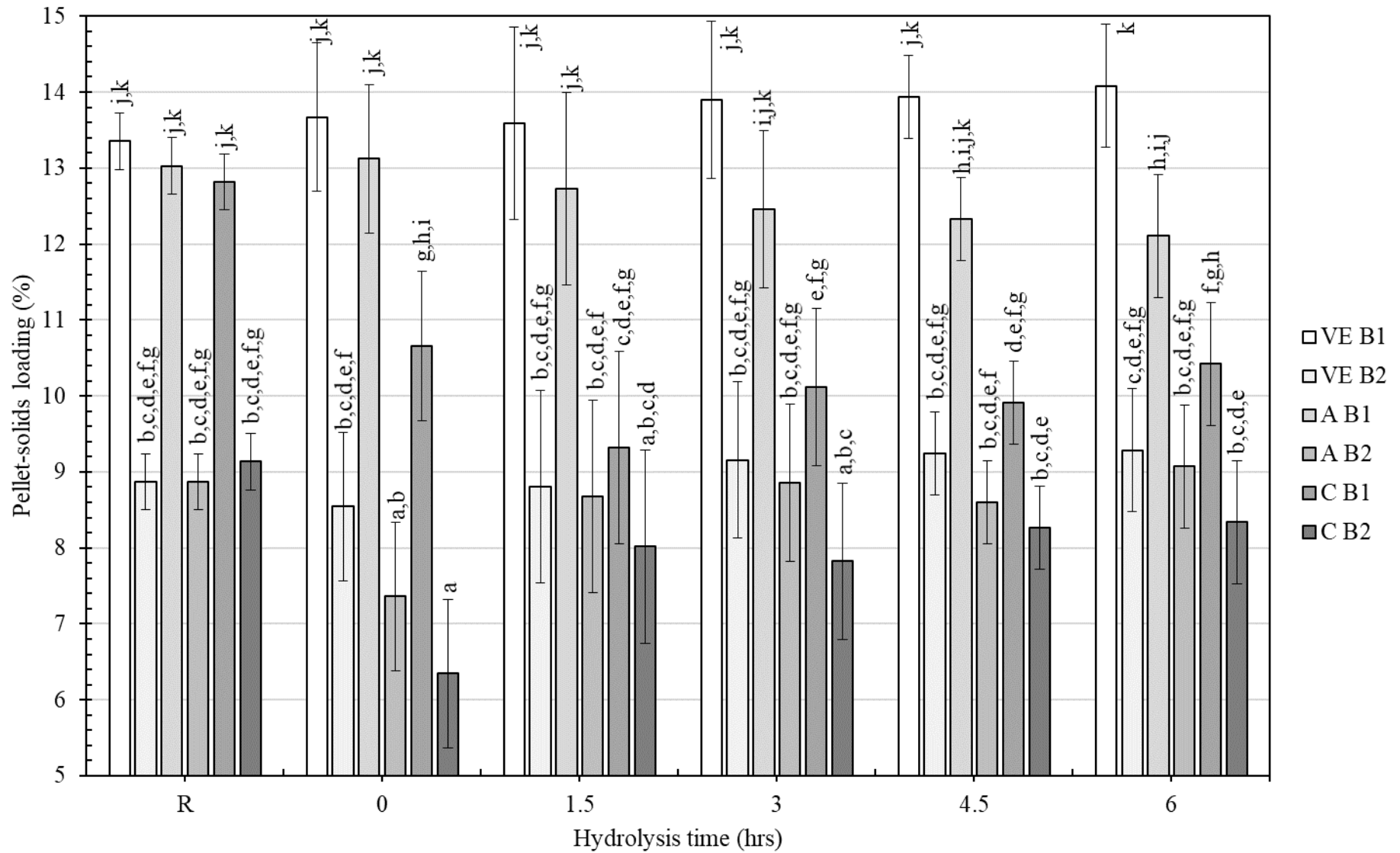


Figure 7-4 Pellet-solids loading ( $SL_p$ ) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 1.906 %,  $p < 0.05$ )

### 7.2.2. Reducing sugars

Tukey HSD results and ANOVAs for the reducing sugars response (*RS*) are located in Appendix G (Table G-5), with the results shown graphically along with the compared responses in Figure 7-5. The MCD calculated from the Tukey HSD test was  $\approx 0.2 \text{ gGlE}\cdot\text{l}^{-1}$ , a low relative error in comparison to the mass-based responses discussed. The lower relative error of spectrophotometric methods was consistent through both response surface modelling and treatment comparisons of the previous chapters.

Figure 7-5 makes it immediately apparent that there was vast batch differences in the raw material readings, with B1 raw material measured at  $\approx 4.4 \text{ gGlE}\cdot\text{l}^{-1}$ , and B2 raw material at  $\approx 1.1 \text{ gGlE}\cdot\text{l}^{-1}$  (a  $\approx 75\%$  decrease). The highest response attained in B2 was from treatment A (Accellerase), which showed a significant increase between each sample point until 6 hours ( $5.94 \pm 0.08 \text{ gGlE}\cdot\text{l}^{-1}$ ). This A B2 response was significantly larger than VE B2 (Celluclast) from 1.5 hours onwards to the mentioned maximum at 6 hours, whereas the two enzyme treatments were not significantly different from one another over the middle sampling points (3 and 4.5 hours). While VE B1 and VE B2 showed similar increases over their respective raw material readings ( $\approx 2.8$  and  $\approx 2.7 \text{ gGlE}\cdot\text{l}^{-1}$  respectively), A B1 increased by  $\approx 3.1 \text{ gGlE}\cdot\text{l}^{-1}$  and A B2 exhibited a much larger  $\approx 4.9 \text{ gGlE}\cdot\text{l}^{-1}$  increase.

Conventional extraction on the second batch (experiment C B2) remained significantly lower than treatments VE and A from 1.5 hours onwards. The same occurred for treatment C B1, albeit by a smaller margin than was shown in B2. Conventional extraction in B1 showed significant increase until 4.5 hours (a  $\approx 2.2 \text{ gGlE}\cdot\text{l}^{-1}$  increase over raw material), followed by a significant drop at 6 hours. The same treatment on the second batch increased significantly until 3 hours, where it attained only a  $\approx 1.2 \text{ gGlE}\cdot\text{l}^{-1}$  increase from a raw material reading of  $1.05 \pm 0.02 \text{ gGlE}\cdot\text{l}^{-1}$ .

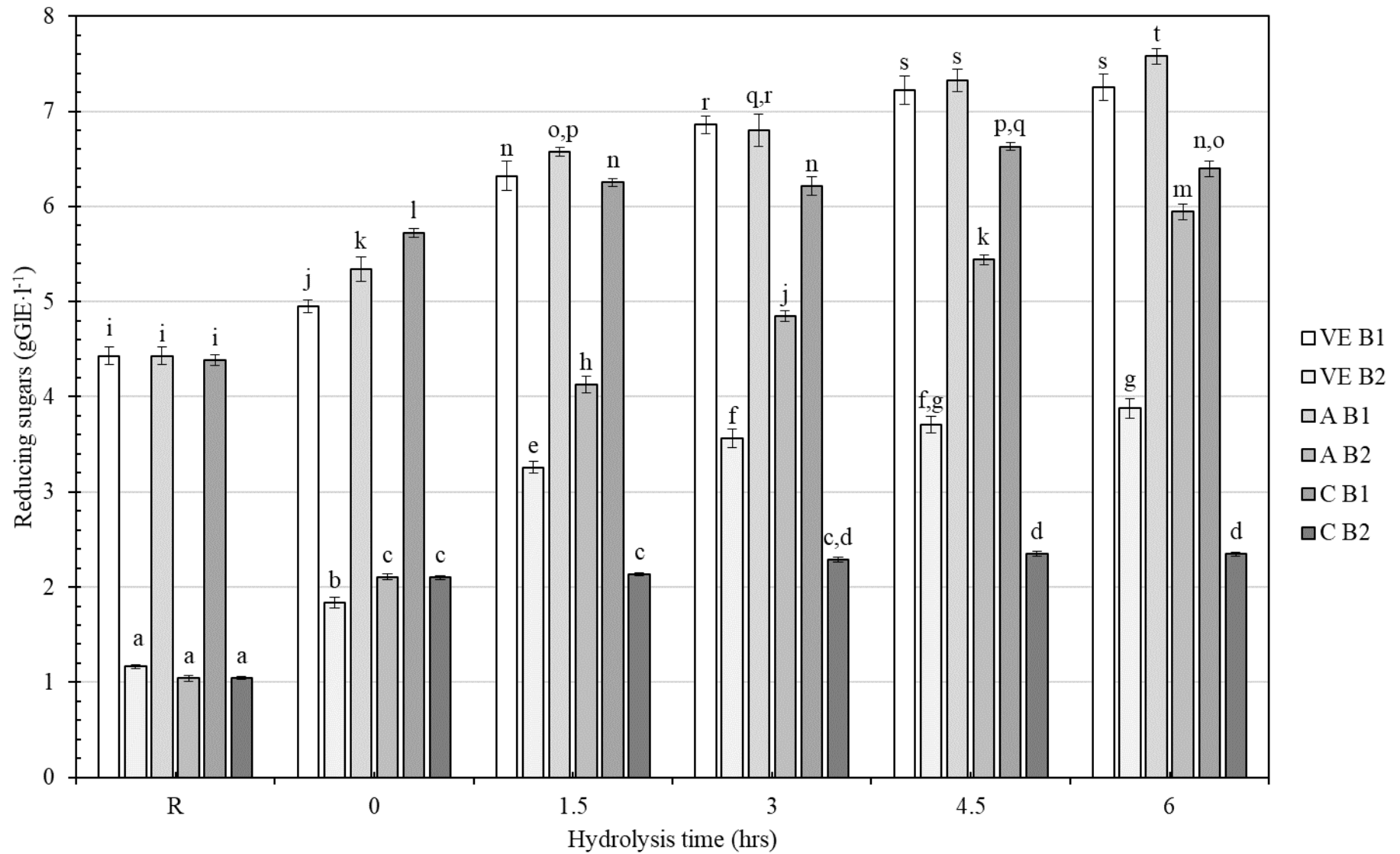


Figure 7-5 Reducing sugars (RS) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.211478 gGLE·l<sup>-1</sup>, p < 0.05)

### 7.2.3. Laminarin

The Tukey HSD results and ANOVAs for the laminarin batch comparison are included in Appendix G (Table G-6), and the responses and significant groupings shown in Figure 7-6. The MCD in the data set was determined as  $\approx 0.22 \text{ gLE}\cdot\text{l}^{-1}$ .

Figure 7-6 shows clearly that the conventional extraction treatment on B1 was superior to all other responses. The raw material reading between batches held a similar ratio to that of the reducing sugars, where B1 exhibited a response of  $\approx 0.44 \text{ gLE}\cdot\text{l}^{-1}$  as opposed to the B2 response of  $\approx 0.13 \text{ gLE}\cdot\text{l}^{-1}$  (a  $\approx 70\%$  decrease between batches). This decrease was shown throughout the responses when comparing B1 and B2, but was most evident when comparing conventional treatment (C). While C B1 was the superior response to other treatments on the first batch ( $\approx 2.5 \text{ gLE}\cdot\text{l}^{-1}$  for the duration of the hydrolysis), C B2 was not significantly different from the raw material response at any point. In fact, none of the measurements taken from the second batch exhibited any laminarin response that differed from the raw material. The extraction of so little laminarin in the second batch indicated that very little of the polysaccharide was present and available for extraction, pointing to seasonality playing a role. With laminarin content of  $\approx 0.2 \text{ gLE}\cdot\text{l}^{-1}$ , the laminarin concentration in solution of B2 was  $\approx 8\%$  of that found in B1.

Enzymatic responses were only comparable to conventional extraction technique in B2 because of the low amount of laminarin present in the batch. The differences observed between treatments in B1 (as discussed prior in Section 6.2.3), showed clearly that enzymatic methods of extraction could not compete for the release of laminarin into solution.



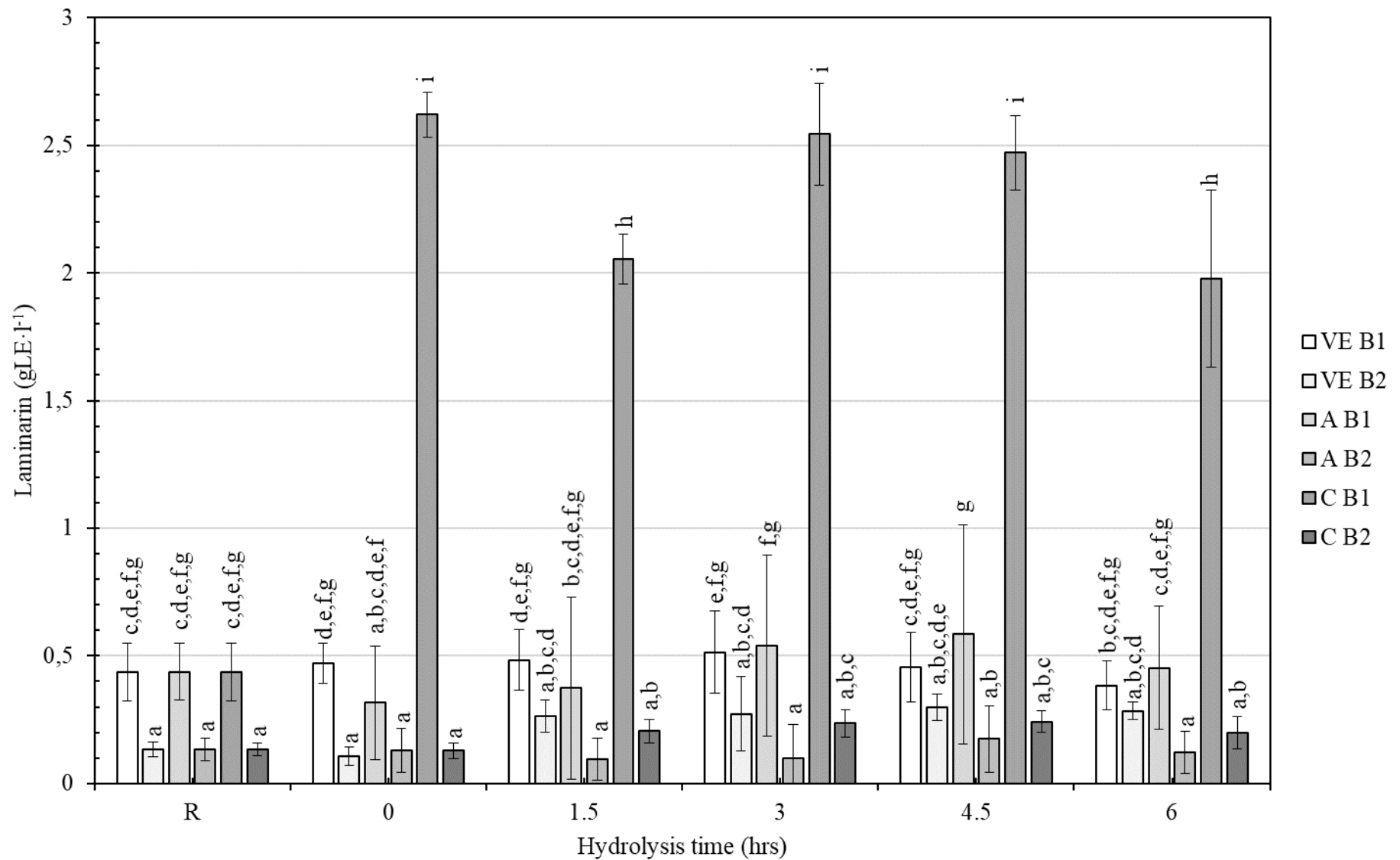


Figure 7-6 Laminarin (*L*) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.22476 gLE·l<sup>-1</sup>,  $p < 0.05$ )

#### 7.2.4. Inorganic sulfate

The Tukey HSD results and ANOVAs for the inorganic sulfate response batch comparison are found in Appendix G (Table G-7) and the results are shown in Figure 7-7. The MCD calculated from the data was  $\approx 0.28 \text{ gSE}\cdot\text{l}^{-1}$ .

Figure 7-7 shows that inorganic sulfate responses for B1 were significantly lower than that of B2. The raw material readings for B2 were approximately  $\approx 1.3 \text{ gSE}\cdot\text{l}^{-1}$  compared to B1 samples with  $\approx 0.35 \text{ gSE}\cdot\text{l}^{-1}$  (this equated to a  $\approx 270\%$  increase in B2). This raw material comparison between batches showed opposite trends to the reducing sugars, laminarin, and solubilised yield parameters discussed thus far.

The significantly highest response from 0 hours to 3 hours was the Accellerase treatment (A) when applied to B2. It was however not significantly higher than Celluclast at 4.5 and 6 hours. Experiment A B2 showed a significant increase over time until 1.5 hours ( $2.59 \pm 0.25 \text{ gSE}\cdot\text{l}^{-1}$ ), while VE B2 increases significantly until 4.5 hours.

The conventional extraction conditions applied to the second batch (C B2) showed a significant decrease over time from a raw material *S* response of  $1.21 \pm 0.09 \text{ gSE}\cdot\text{l}^{-1}$  to  $0.65 \pm 0.03 \text{ gSE}\cdot\text{l}^{-1}$  at 3 hours (just above half of the raw material response). The conventional conditions applied to the primary batch (C B1) did not show any significant trends in comparison, although the mean did drop until 4.5 hours.

Enzymatic treatments proved superior over the decreasing response shown with conventional extraction, with this trend being consistent between batches. B2 showed a superior inorganic sulfate response in the raw material, and enzymatic treatments resulted in higher increases in B2 (an increase of  $\approx 1.2 \text{ gSE}\cdot\text{l}^{-1}$  to  $\approx 2.5 \text{ gSE}\cdot\text{l}^{-1}$  at 6 hours) in comparison with B1 (increase by  $\approx 0.65 \text{ gSE}\cdot\text{l}^{-1}$  to  $\approx 1.0 \text{ gSE}\cdot\text{l}^{-1}$ ). Enzymatic methods therefore released approximately twice as much inorganic sulfate in the second batch.

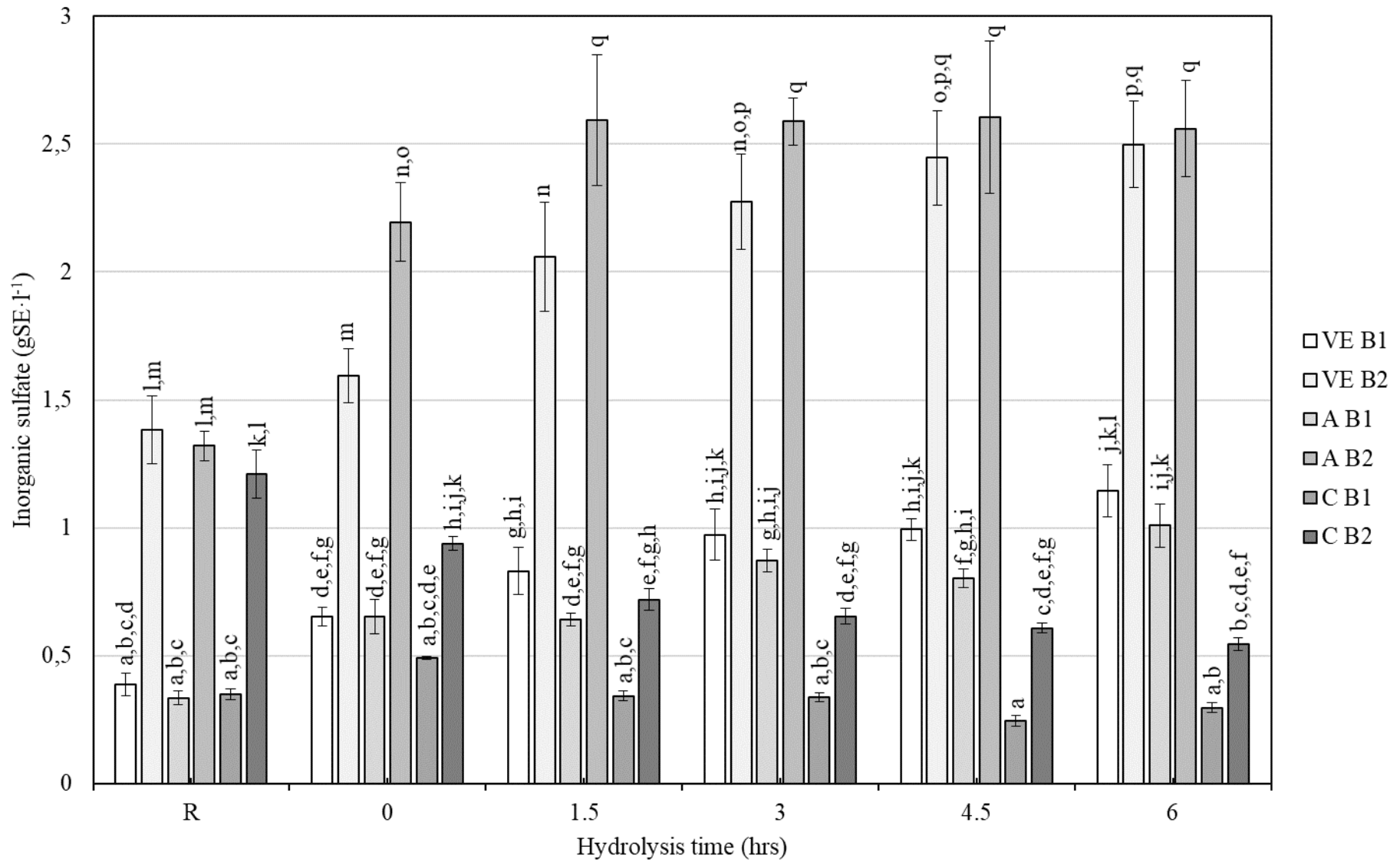


Figure 7-7 Inorganic sulfate (*S*) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.281469 gSE·l<sup>-1</sup>,  $p < 0.05$ )

### 7.2.5. Total phenolics

Tukey HSD results and ANOVAs pertaining to the total phenolic content batch comparison are shown in Appendix G (Table G-8), with the results shown graphically in Figure 7-8. The calculated MCD was  $\approx 0.017 \text{ gGAE}\cdot\text{l}^{-1}$ .

Figure 7-8 shows that the second batch exhibited a significantly higher *TP* response at all sampling points when compared to the primary batch. Comparing raw material readings, B1 showed a response of  $\approx 0.25 \text{ g}\cdot\text{l}^{-1}$  when compared to  $\approx 0.32 \text{ g}\cdot\text{l}^{-1}$  exhibited by B2. These differences became more pronounced over the course of hydrolysis time, with treatment A exhibiting the highest response from 1.5 hours onwards. The second highest response came from treatment VE and conventional extraction conditions C resulted in the lowest response. This response ranking changed slightly from the first batch, where both enzymes were statistically equivalent and outperformed the response of conventional extraction.

Treatment A increased significantly in B2 until 6 hours (a maximum response of  $\approx 0.6 \text{ gGAE}\cdot\text{l}^{-1}$ ), whereas VE did not experience significant increase past 1.5 hours ( $\approx 0.45 \text{ gGAE}\cdot\text{l}^{-1}$ ). The observation of Accellerase outperforming Celluclast in B2 but not in B1 was also observed in both the reducing sugars (*RS*) response and the inorganic sulfates (*S*) response. As mentioned in Section 6.2.5, where the treatment comparisons were made against model validation runs, this discounted the VE B1 response shown at 6 hours as an anomaly.

Pre-hydrolysis the total phenolics response of B1 equated to  $\approx 80 \%$  of B2, with this ratio dropping to  $\approx 50 \%$  with treatment A after 6 hours and  $\approx 70 \%$  with Celluclast after 6 hours (if the 6 hours VE B1 point is discounted and assumed equivalent to 4.5 hours).

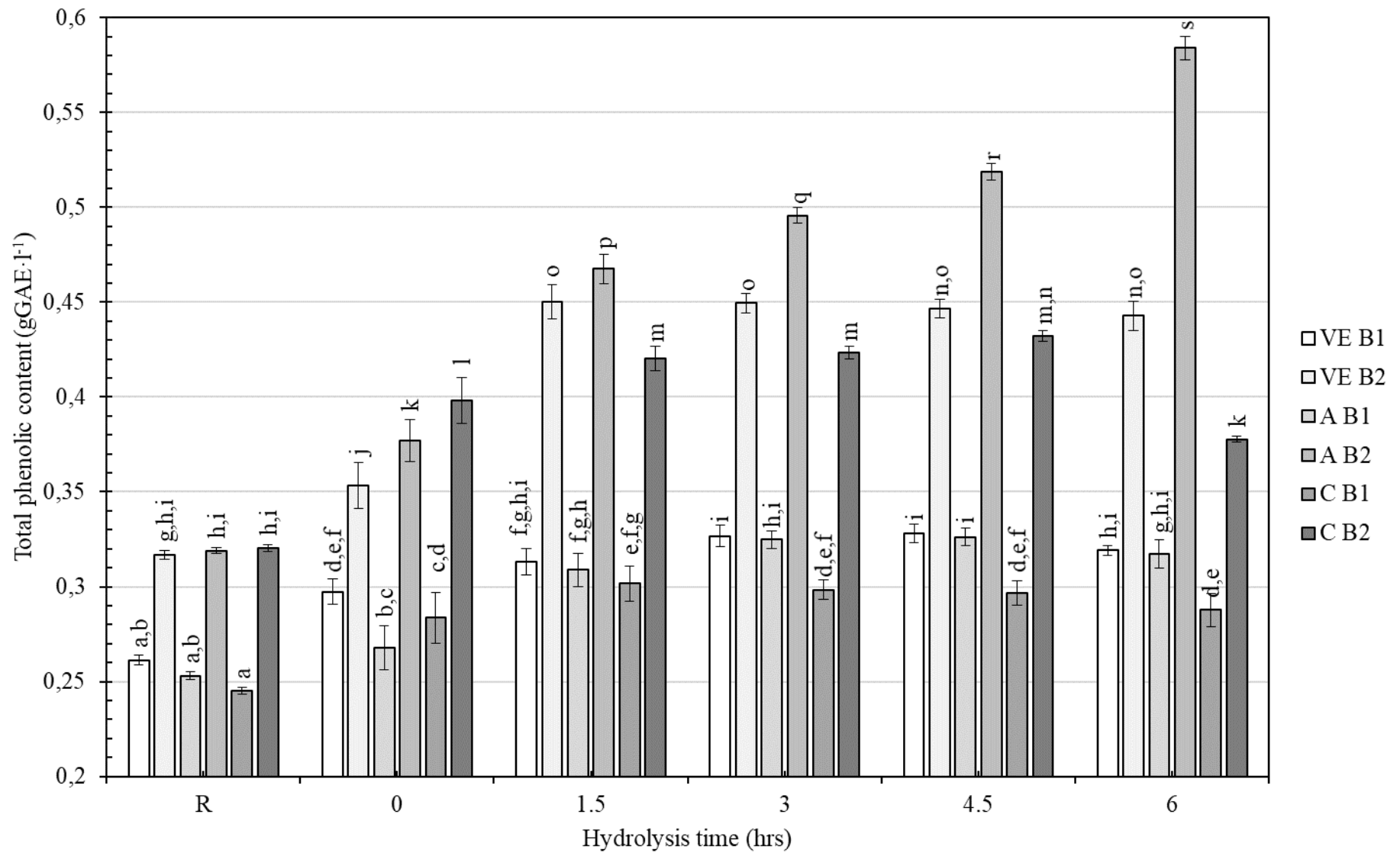


Figure 7-8 Total phenolic (TP) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey HSD procedure, MCD = 0.01711 gGAE·l<sup>-1</sup>, p < 0.05)

### 7.2.6. Antioxidant capacity

Tukey HSD testing and ANOVAs for antioxidant capacity are included in Appendix G (Table G-9), while the results are shown in Figure 7-9. The calculated MCD was  $\approx 0.09 \text{ gTE}\cdot\text{l}^{-1}$ .

Figure 7-9 shows that B2 exhibited a significantly higher antioxidant capacity in the raw material supernatant than B1 ( $\approx 1.50 \text{ gTE}\cdot\text{l}^{-1}$  compared to  $\approx 1.00 \text{ gTE}\cdot\text{l}^{-1}$ ), as well as larger antioxidant capacities with each experimental treatment. While the conventional acid treatment exhibited a higher response consistently when testing B1 (increasing significantly until 3 hours), the antioxidant capacity of Accellerase (treatment A) was significantly higher than treatment C at 1.5 hours. This 1.5 hour result was significantly larger than both the 0 and 3 hour of the same experiment (A B2), meaning that it was likely an anomalous experimental error and should have tested lower. This assumption allowed the observation that treatment C was significantly larger than enzymatic treatments for 0 to 4.5 hours. At 6 hours experiment A B2 was not significantly different from C B2, indicating that the enzyme response had increased more gradually over time and drew even with the conventional dilute-acid thermal hydrolysis conditions.

Experiment VE B2 was significantly lower than both A B2 and C B2 for the duration of the experiment (0 to 6 hours). In B1 this was not the case, as the enzymatic treatments were not significantly different from one another. This was also observed in the batch comparisons of responses *RS*, *S*, and *TP* making it likely that experiment A B2 occurred with some sort of systematic error.

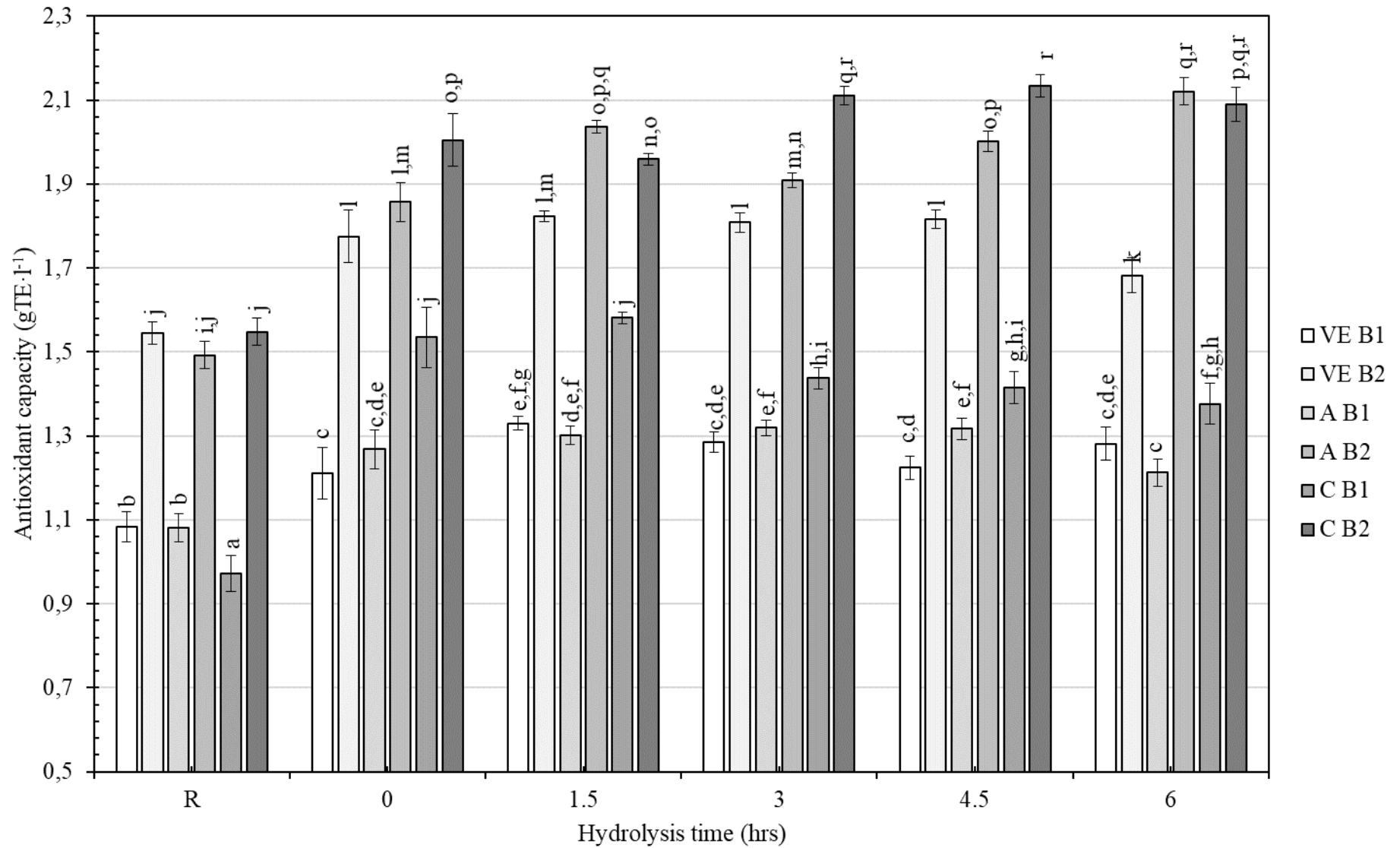


Figure 7-9 Antioxidant capacity (*AO*) batch comparison over time at experimental conditions VE, A, and C. Different letters indicate statistically different groups (Tukey-Kramer HSD procedure, MCD = 0.08938 gTE·l<sup>-1</sup>,  $p < 0.05$ )



### 7.3. Batch comparison discussion

Treatment effects were consistent between batches, although responses were vastly different. The primary batch resulted in significantly larger responses for solubilised yield ( $SY$ ), supernatant mass fraction ( $F_{SN}$ ), pellet solids loading ( $SL_P$ ), reducing sugars ( $RS$ ), and laminarin ( $L$ ). The secondary batch showed larger responses for inorganic sulfate ( $S$ ), total phenolics ( $TP$ ), and antioxidant capacity ( $AO$ ). Significant differences could not be seen between the dissolved solids ( $DS$ ) responses of the two batches, although B1 was consistently larger by mean.

While Celluclast and Accellerase extractions (treatments VE and A) performed similarly across all responses on the first batch of seaweed (May 2018). This was not true for the second batch (June 2019), and it is possibly attributable to either experimental error during sampling of experiment A B2, or the possible elevated presence of hemicellulases or other compound classes that Accellerase can hydrolyse (where Celluclast is unable to).

Laminarin was not present in comparable quantities in the second batch, according to the developed spectrophotometric assay. This is thought to have affected the batch variability of solubilised yield significantly (discussed further in Section 7.3.3). The untested alginic acid content is also likely to have played a role in the batch variabilities observed. Inorganic sulfate, total phenolics, and antioxidant capacity batch variabilities were attributed to geographical and seasonal factors. These are discussed in more detail in this section as well.

#### 7.3.1. Solubilised yield

Batch comparison of solubilised yield (Section 7.2.1) showed a significant difference between B1 ( $\approx 50 - 55\%$ ) and B2 ( $\approx 35 - 40\%$ ), evident in both raw and hydrolysed material. In both batches, the initial rise of the conventional treatment (C) was significantly larger than enzymatic treatments, as this was essentially a pre-heating and pH adjustment step before enzyme addition. No significant difference was seen in the three treatments (VE, A, and C) from 1.5 to 6 hours in the second batch (B2), as was the case in B1. This lack of significant difference was attributed to errors propagated from the supernatant dissolved solids measurement. High variability was expected between batches, and literature reports solubilised yields (via Celluclast, other enzymes, and water extraction) on different brown seaweeds varying from  $\approx 33$  to  $75\%$  (K. Habeebullah et al., 2019). All calculations of solubilised yield fell within this range and batch variability for the response was attributed to the varying presence of alginic acid (unquantified in this study) – in particular on the supernatant mass fraction and pellet-solids loading responses (discussed further below).

## Supernatant dissolved solids

Large errors stemming from the dissolved solids response were the likely cause of the lack of statistical significance observed in solubilised yield, and the reasons for this error were presented in the discussion section of Chapter 5 (Section 5.4.1) concerning the dissolved solids experimental responses. While enzymatic methods could compete with conventional acidic extraction, this required additional residence time in the reactor (4.5 - 6 hours minimum).

No significant differences were found between batches (mean critical difference of 0.6 % calculated via Tukey HSD test), with the exception of the treatment C exhibiting a significant difference between batches at 6 hours (May 2018 (B1) > June 2019 (B2)). Large errors in measurement prevented a significant difference from being stated, although B2 supernatant dissolved solids were consistently lower by mean. The consistent hierarchy of responses ( $DS\ B1 > DS\ B2$ ) was likely caused by either the presence of alginic acid (as previously mentioned) or the decreased laminarin/polysaccharide content suggested in the discussion regarding laminarin batch comparison (Section 7.3.3).

## Supernatant mass fraction

The batch variation results of the supernatant mass fraction (Section Chapter 0) showed a large difference between the two harvests, with May 2018 (B1) showing a  $F_{SN}$  response around  $\approx 80\%$  for both enzymatic treatment VE and A and  $\approx 76\%$  for conventional treatment C. The secondary batch B2 (June 2019) exhibited  $F_{SN}$  responses of  $\approx 65\%$  for enzymes and  $\approx 60\%$  for conventional. This section of the study corroborated the superior  $F_{SN}$  response of both Celluclast and Accellerase enzymes, as well as indicating batch variability likely due to increased presence of alginic acid in the secondary batch. Increased alginate would result in more gelling behaviour water absorption to the pellet/residue and the subsequent formation of larger pellets/residues with lower solids loadings. Gelling behaviour and moisture absorption was discussed in both Chapter 5 and 6 discussion sections on the supernatant mass fraction and pellet-solids loading responses.

## Pellet-solids loading

The batch variability study of pellet solids loading showed a significant difference in the response of the raw material, where the first batch exhibited a significantly larger  $SL_P$  response ( $\approx 13\%$  compared to  $\approx 9\%$ ). Enzymatic extractions on the second batch under pH 4.5 and 50 °C and 4.0 % ( $v/dw$ ) (VE and A) did not significantly change the response from that of the raw material, as was observed with B1.

The variation shown between the first and second batch, along with the lowering of  $SL_P$  response when using low pH treatments was consistent with the assumption that alginic acid played a role in the

solubilised yield response. The mechanism by which the varying alginic acid content would change the pellet-solids loading and supernatant mass fraction responses (and therefore solubilised yield) has already been discussed in the section.

### 7.3.2. Reducing sugars

The batch comparison study of reducing sugars showed that the raw, moisture corrected material of B1 exhibited a reducing sugar response of  $\approx 4.4 \text{ gGlE}\cdot\text{l}^{-1}$  ( $\approx 66 \text{ mgGlE}\cdot\text{gDW}^{-1}$ ) when compared to B2 ( $\approx 1.1 \text{ gGlE}\cdot\text{l}^{-1}$  ( $\approx 12 \text{ mgGlE}\cdot\text{gDW}^{-1}$ )). This variation was considerable, and the maximum responses for B2 treatments VE, A, and C were measured at  $3.87 \pm 0.10 \text{ gGlE}\cdot\text{l}^{-1}$  ( $44 \pm 8.5 \text{ mgGlE}\cdot\text{gDW}^{-1}$ ),  $5.94 \pm 0.08 \text{ gGlE}\cdot\text{l}^{-1}$  ( $67 \pm 19 \text{ mgGlE}\cdot\text{gDW}^{-1}$ ), and  $2.35 \pm 0.02 \text{ gGlE}\cdot\text{l}^{-1}$  ( $26 \pm 7 \text{ mgGlE}\cdot\text{gDW}^{-1}$ ) respectively. These results showed a high batch variation, which was likely not due to seasonal changes in composition (May 2018 vs June 2019), but to variation in the nitrates and nutritive salts in the ocean water where the kelps were harvested, as well as other varying geographically affected conditions (Rioux et al., 2009). Accellerase outperformed Celluclast significantly in the release of reducing sugars from the second batch, either by experimental error or by the action of extra enzymes present in the commercial enzyme mixture (hemicellulase, 1,3- $\beta$ -glucanase). While some results from the second batch were lower than the 50 to 540  $\text{mgGlE}\cdot\text{gDW}^{-1}$  range suggested by Offei et al. (2018), batch variability was expected, and the results were not regarded as unreasonable or unrealistic.

Increased reducing sugars release from the Accellerase enzyme above Celluclast on the second batch may have been caused by sampling error (as previously stated for all responses) or by the presence of elevated levels of compounds that the Accellerase enzyme mixture can degrade (with the additional enzymes found in that mixture and not in Celluclast). A comprehensive batch variability study with multiple batches and these two treatments would shed further light on the observed discrepancy.

### 7.3.3. Laminarin

Batch comparison of the laminarin response showed that the second batch (June 2019) did not exhibit a laminarin concentration in the supernatant above  $\approx 0.2 \text{ gLE}\cdot\text{l}^{-1}$  ( $\approx 3 \text{ mgLE}\cdot\text{gDW}^{-1}$ ), regardless of extraction method.

It was clear from the difference in laminarin responses between batches (May 2018 and June 2019) that season does not play the largest role in determining the quantity of the laminarin polysaccharide in *E. maxima*. Raw, moisture corrected supernatant material from May 2018 yielded laminarin at  $7 \text{ mgLE}\cdot\text{gDW}^{-1}$  when compared to the  $\approx 1.5 \text{ mgLE}\cdot\text{gDW}^{-1}$  yield of June 2019. Conventional extractions on these two harvested batches yielded  $\approx 27 - 40 \text{ mgLE}\cdot\text{gDW}^{-1}$  and  $\approx 1.5 - 3 \text{ mgLE}\cdot\text{l}^{-1}$  respectively,

despite being harvest in similar seasons and locations. Rioux et al. (2009) observed similar differences in two batches also harvested roughly a year apart (May 2005 and June 2006), showing laminarin concentrations of  $\approx 53 \text{ mgLE}\cdot\text{gDW}^{-1}$  and  $< 25 \text{ mgLE}\cdot\text{gDW}^{-1}$  respectively. Nutritive salts were tracked via a buoy in that study, and a decline in nutritive salts (nitrates in particular) coincided with the increase in the laminarin content of the seaweed harvested (*Saccharina longicuris*, Quebec, Canada). Frond age was also reported in this study as an important factor to consider, with older fronds containing higher laminarin amounts. These additional geographical factors should be monitored in further studies.

#### 7.3.4. Inorganic sulfates

The batch comparison of the inorganic sulfate results (Section 7.2.4) showed that the second batch of kelp (harvested June 2019) showed significantly higher amounts of inorganic sulfate in the raw material than the primary batch, which was harvested in May 2018 ( $14.6 \text{ mgSE}\cdot\text{gDW}^{-1}$  compared to  $5.5 \text{ mgSE}\cdot\text{gDW}^{-1}$ ). Enzymatic treatments released higher amounts of measurable sulfate than conventional treatments once more ( $28 - 30 \text{ mgSE}\cdot\text{gDW}^{-1}$  from both Celluclast and Accellerase extractions at 4.0 % ( $v/dw$ ) enzyme dosage, pH 4.5 and 50 °C. Once processed with enzymes, the sulfate response of the second batch was approximately twice as high as the primary batch.

The difference in batches were ascribed to factors that affect the general composition of the algal material, such as nutritive salt concentrations in seawater, harvest season, and geographical region (Garcia-Vaquero et al., 2017). It is likely that fucoidan was successfully extracted in the study, if the optimal conditions prescribed by Hahn et al. (2012) are correct (pH 2.7 and 41.8 °C), although different methods of analysis would be required to confirm this. Batch variability also showed laminarin to be present in higher quantities in B1 and inorganic sulfates/fucoidan to be higher in B2. This points to difficulties regarding coextraction from one batch of material, where only one of the polysaccharides is likely to be present in appreciable quantities per batch.

#### 7.3.5. Total phenolics

The batch comparison results for the total phenolic response (Section 7.2.5) showed significantly larger concentrations of total phenolics in the supernatant extracted from the secondary batch (June 2019), when compared to the primary batch harvested in May 2018. When the results were converted from concentrations to  $\text{mgGAE}\cdot\text{gDW}^{-1}$ , a closer pairing was observed with a maximum total phenolic response of  $6.6 \pm 1.8 \text{ mgGAE}\cdot\text{gDW}^{-1}$  found from Accellerase extraction after 6 hours at pH 4.5, 50 °C, and 4.0 % ( $v/dw$ ) enzyme dosage. This is approximately 40 % higher than the total phenolic response shown with

the same extraction conditions on the primary batch. The low pH conventional treatment (pH 1.0, 70 °C) on the secondary batch was once again the lowest response, below enzymatic treatments.

Measurement of the total phenolic content in the raw material showed spectrophotometric responses of  $\approx 0.25 \text{ gGAE}\cdot\text{l}^{-1}$  for B1 and  $\approx 0.32 \text{ gGAE}\cdot\text{l}^{-1}$  for B2. While the supernatant of B2 was enriched in comparison to B1 over all treatments, the total phenolics content of the two batches overall appears similar (raw material readings of  $\approx 3.8 \text{ mgGAE}\cdot\text{gDW}^{-1}$  and  $\approx 3.6 \text{ mgGAE}\cdot\text{gDW}^{-1}$  for B1 and B2 respectively). The difference in concentration in solution could be the result of the decreased solubilised yield experienced by B2 and caused by the difference in the relative mass fractions of supernatant between the batches ( $\approx 80\%$  and  $\approx 60\%$  in B1 and B2 respectively), which was likely caused by variability in the polysaccharide fraction and alginate. Alginic acids associate with polyphenols (Deniaud-Bouët et al., 2014) and the increased presence of alginate (theorised to cause much of the observed variability between treatments) may also have affected the ability of polyphenols to move into solution.

Polyphenol content of the brown seaweed *Ascophyllum nodosum* throughout the course of the year was reported by Tabassum et al. (2016) to vary between 2.1 and 49.8  $\text{mgGAE}\cdot\text{gDW}^{-1}$  and to reach a maximum in Summer (June, Ireland). The samples worked with in this study (*E. maxima*) were harvested in the same time period, but in the southern hemisphere, making it possible that the observed low concentrations of polyphenols under all extraction conditions was due to harvest season.

### 7.3.6. Antioxidant capacity

While the antioxidant capacities of the two batches (Section 7.2.6) were significantly different by concentration in the supernatant ( $\approx 1.0$  and  $\approx 1.5 \text{ gTE}\cdot\text{l}^{-1}$  for the raw material reading of B1 and B2 respectively), these readings were similar to one another on a dry weight basis ( $\approx 15$  and  $\approx 17 \text{ mgTE}\cdot\text{gDW}^{-1}$ ). The batches were harvested at similar periods in the year (May 2018 vs June 2019), and it was the variation in polysaccharide fraction (particularly alginate) that is suspected to be responsible for the difference of responses in solution (as stated in previous sections). Full characterisation of antioxidant capacities at differing extract concentration would be required to further investigate this parameter, and this was not within the scope of the study.

## Chapter 8

### *Laminarin testing comparison*

Other studies (Charoensiddhi et al., 2016) have made use of ethanol precipitation and glucose quantification via high performance liquid chromatography (HPLC) to estimate glucose-containing polysaccharides in extracts. This method was seen as non-specific to laminarin (not involving a laminarin specific enzyme). Nonetheless, a general comparison to the developed spectrophotometric method of laminarin measurement was carried out to ascertain the necessity of laminarinase when testing for the laminarin polysaccharide.

Methodology was presented first, showing the HPLC preparation steps followed. Results followed, and the discussion regarding the two measurement methods was presented last.

#### **8.1. HPLC testing methodology**

As a comparison to the developed laminarin spectrophotometric assay presented in Section 4.2.3, HPLC (high performance liquid chromatography) was also used to quantify the polysaccharide of selected samples. A TSP (Thermo Separations Product) HPLC with Shodex 101 RI detector (for glucose) and Biorad HPX-87H column (250 x 7.8mm with guard cartridge) was run at 65 °C with a 0.005M sulphuric acid mobile phase at 0.6 ml·min<sup>-1</sup>. The method involves the ethanol precipitation of the polysaccharide fraction, followed by acid hydrolysis of the resultant fraction to release glucose monomers and the subsequent neutralisation and filtration of the samples in preparation for HPLC testing. The adapted protocol for this testing is summarised as follows and is a combination of work derived from Charoensiddhi et al. (2016) and Sluiter et al. (2008).

1. Add 450 µl supernatant sample to a 2 ml MCT.
2. Add 1350 µl absolute ethanol to MCT (resulting in 75 % ethanol)
3. Incubate at 4 °C overnight
4. Centrifuge at 14,000 rpm for 10 minutes
5. Decant ethanol rich supernatant to second MCT
6. Wash pellet with further 450 µl ethanol, followed by 1 hour incubation at 4 °C
7. Centrifuge at 14,000 rpm again for 10 minutes. Combine supernatants.
8. Dry both supernatant and pellet at reduced pressure overnight.

9. Resuspend samples in 1.8 ml demineralised water (DF = 4), before pipetting 1.5 ml of this resuspension into McKenzie bottles and making the volume up to 4.5 ml (DF = 12) in order to check pH with probe.
10. Add 157  $\mu\text{l}$  of 72 % sulfuric acid to adjust sample to 4 % sulfuric acid. (DF = 12.4)
11. Prepare sugar recovery standards of 2.5 – 0.5  $\text{g}\cdot\text{l}^{-1}$  glucose solutions in identical fashion.
12. Autoclave all samples and sugar recovery standards at 121 °C for 1 hour.
13. Cool samples to room temperature, transfer to conical bottom tubes (15 ml) and neutralise to pH  $\approx$  6 carefully with calcium carbonate powder.
14. After settling, centrifuge the sample at 5000 rpm for 10 minutes.
15. Filter sample supernatants with 0.45  $\mu\text{m}$  syringe filters into 2 ml HPLC bottles.
16. HPLC test as normal against known glucose standards
17. Calculate average glucose recoveries  $\%R_{glucose}$  from known hydrolysed standards as shown in Equation 8-1.

$$\%R_{glucose} = \frac{C_{glucose,HPLC}}{C_{glucose,known}} \quad (8-1)$$

Where  $C_{glucose,HPLC}$  is the glucose response of the standard from HPLC testing [ $\text{g}\cdot\text{l}^{-1}$ ] and  $C_{glucose,known}$  is the glucose concentration of the standard [ $\text{g}\cdot\text{l}^{-1}$ ].

18. Correct the results of the tested samples using  $\%R_{glucose}$  as shown in Equation 8-2.

$$C_{sample} = \frac{C_{HPLC} \times DF}{\%R_{glucose}} \quad (8-2)$$

Where  $C_{sample}$  is the adjusted glucose reading of the sample [ $\text{g}\cdot\text{l}^{-1}$ ],  $C_{HPLC}$  is the HPLC glucose reading of the sample [ $\text{g}\cdot\text{l}^{-1}$ ], and  $DF$  is the dilution factor of the sample.

## 8.2. HPLC results

Laminarin concentration was tested in the polysaccharide rich fraction of the supernatant after ethanol precipitation. HPLC was used to quantify the concentration of glucose after dilute acid hydrolysis with sulphuric acid. The samples selected for testing with this method were the same samples as tested in the batch variability of Chapter 7, namely Celluclast and Accellerase at 4.0 % ( $v/dw$ ) enzyme dosage, pH 4.5, and 50 °C, along with conventionally extracted samples (pH 1.0 and 70 °C). All sampling points, including the raw material samples, were tested in both batches. An average glucose recovery ( $\%R_{glucose}$ , as defined in Equation 8-1) of 88.4 % was found from sugar recovery standards, and the relevant readings and calculations on all samples are shown in Appendix H. The results of the glucose



responses from the ethanol precipitated PRF (polysaccharide rich fraction) of each sample are shown graphically in Figure 8-1.

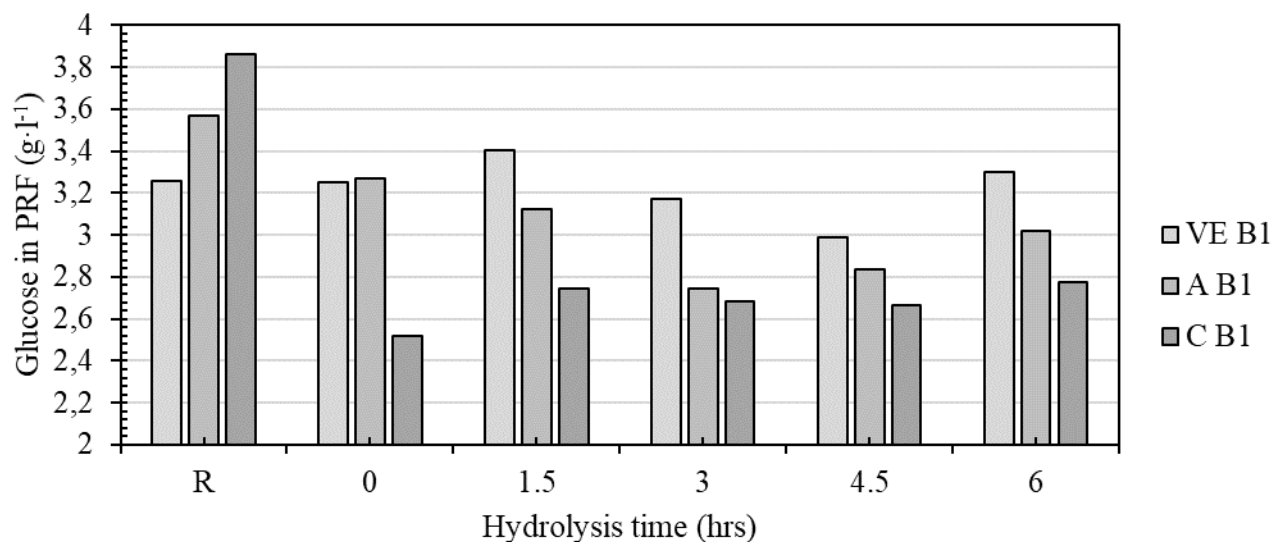


Figure 8-1 HPLC results for glucose concentration in ethanol precipitated and dilute acid hydrolysed polysaccharide rich fraction

The responses of the secondary batch were all below detectable limits, and so were not shown in Figure 8-1. Raw material showed an average laminarin concentration of  $3.56 \pm 0.75 \text{ g}\cdot\text{l}^{-1}$  ( $n = 3$ ), but the figure indicated high errors where raw material samples should have been more replicable. The figure shows that the three methods appear more similar in performance than was shown in Section 7.2.3, with C B1 agreeing closely with the spectrophotometric assay from 0 hours onwards ( $\approx 2.5 \text{ gLE}\cdot\text{l}^{-1}$ ), while the enzyme results (consistently larger than C B1) sat above the spectrophotometric assay response of  $\approx 0.5 \text{ gLE}\cdot\text{l}^{-1}$ . Changes from raw material readings showed that treatment C B1 decreased the laminarin concentration appreciably in solution from  $\approx 3.8 \text{ g}\cdot\text{l}^{-1}$  in the raw material to the  $\approx 2.5$  to  $2.7 \text{ g}\cdot\text{l}^{-1}$  reading shown until 6 hours. Despite the apparent error from the fluctuation of raw material readings, experimental responses of each treatment did not fluctuate by more than  $\approx 0.4 \text{ g}\cdot\text{l}^{-1}$  (A B1). Experiment VE B1 did not change appreciably from the raw material reading and moved between  $\approx 3$  and  $\approx 3.3 \text{ g}\cdot\text{l}^{-1}$ . Experiment A B1 lay between these two responses (VE B1 and C B1) other than at 0 hours, where it was within  $0.02 \text{ g}\cdot\text{l}^{-1}$  of the VE B1 glucose reading. While none of the responses increased the laminarin concentration above that of the raw material, Celluclast treatment consistently showed higher readings than conventional extraction for 0 to 6 hours.

The free sugars fraction (FSF, sugars lost in the ethanol rich supernatant) results for the primary batch are shown in Figure 8-2, with the data also located in Appendix H.

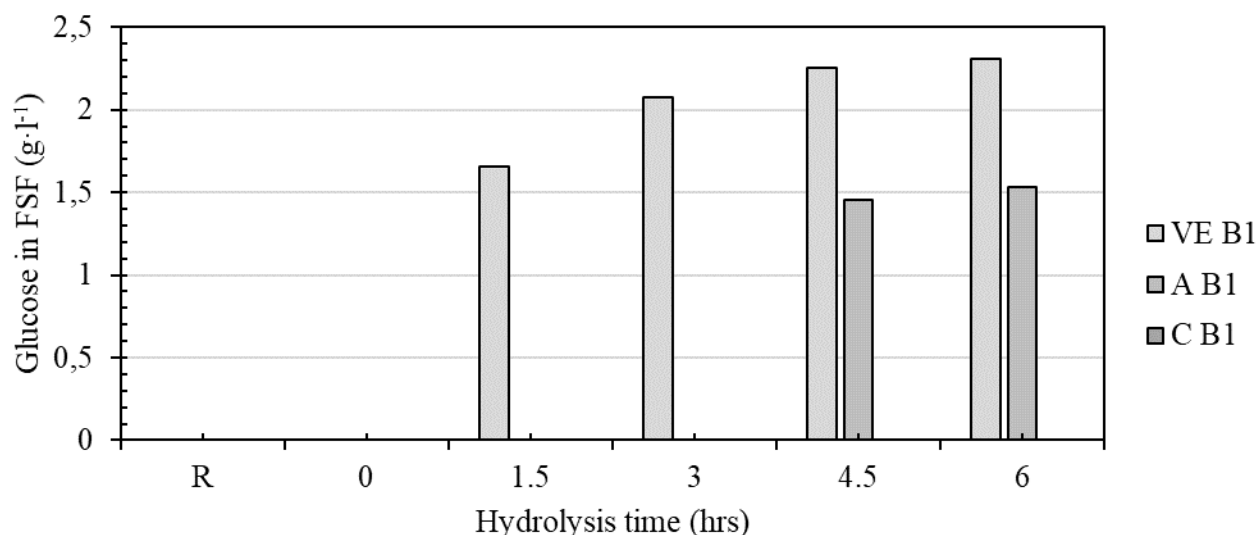


Figure 8-2 HPLC results for glucose concentration in ethanol precipitated and dilute acid hydrolysed free sugars fraction

It was shown that while glucose was present and increasing in the free sugars fraction of VE B1 from 1.5 to 6 hours, this occurred at a reduced rate in A B1 (only 4.5 and 6 hours were above detectable limits). C B1 did not increase above detectable limits in the free sugars fraction at any point in the experiment.

### 8.3. HPLC vs spectrophotometric assay discussion

HPLC testing for glucose after dilute acid hydrolysis of the ethanol precipitated polysaccharide rich fraction showed vastly different results in comparison to the spectrophotometric method of laminarin quantification. The results were however still shown to be independent of time, and the second batch showed no results above detectable limits (agreeing with the vast difference seen between batches with the spectrophotometric method). According to HPLC results, 4.0 % (*v/dw*) Celluclast pH 4.5 and 50 °C released between  $\approx 44$  and  $\approx 50$  mgGl·gDW<sup>-1</sup> (milligrams glucose per gram dry weight), 4.0 % (*v/dw*) Accellerase at the same pH and temperature released  $\approx 40$  to  $\approx 49$  mgGl·gDW<sup>-1</sup>, and the conventional experiment (pH 1.0, 70 °C) released  $\approx 37$  to  $\approx 39$  mgGl·gDW<sup>-1</sup>. Raw material registered between  $\approx 50$  and  $\approx 60$  mgGl·gDW<sup>-1</sup>, which agreed again with ranges found in Rioux et al. (2009).

The only responses that were similar between methods (spectrophotometric testing vs HPLC) were those that used the conventional extraction protocol of Devillé et al. (2004) (C B1 and C B2). For conventional extraction from the May 2018 batch (B1), both spectrophotometric and HPLC methods showed results between 30 and 40 mg·gDW<sup>-1</sup> (units simplified for comparison). The second batch (B2) showed low spectrophotometric measurements of  $< 3$  mg·gDW<sup>-1</sup> and HPLC testing showed the glucose concentration in these samples to be below detectable limits of the apparatus. Enzymatic methods of extraction, as well

as the raw material readings showed vastly different responses between the two testing methods. The spectrophotometric measurement of laminarin in the raw material was  $\approx 7 \text{ mg}\cdot\text{gDW}^{-1}$  compared to the  $\approx 50$  to  $\approx 60 \text{ mg}\cdot\text{gDW}^{-1}$  reported by the HPLC glucose testing method. Celluclast and Accellerase both showed spectrophotometric responses of  $\approx 5$  to  $\approx 8 \text{ mg}\cdot\text{gDW}^{-1}$  laminarin, compared to the HPLC testing range of  $\approx 40$  to  $\approx 48 \text{ mg}\cdot\text{gDW}^{-1}$ . It was evident that the effects of low pH and perhaps higher temperature play a role in the differences in analysis, and the likelihood that HPLC testing was not specific to laminarin.

The vast difference in the results of the HPLC and spectrophotometric methods for laminarin quantification with both enzyme treatments may have stemmed from additional molecules and cell-fragments with pH-dependent solubility (like alginate and phlorotannins) being present in the supernatants in the range between pH 3.0 and 6.0. These compounds may have interfered with the enzymatic action of the 1,3- $\beta$ -endoglucanase enzyme used in the developed spectrophotometric assay, likely by inhibition. In the HPLC method, all polysaccharides would have been ethanol precipitated, followed by dilute acid thermal hydrolysis and neutralisation. The HPLC method did not require further enzymatic hydrolysis, and would therefore not be inhibited, regardless of initial sample extraction conditions. This theory could explain the similar responses obtained from the two assays when dilute-acid thermal hydrolysis was used as the extraction technique, as these inhibitory molecules would be confined to the pellet during centrifugation. The effect of a prolonged endo-1,3- $\beta$ -D-glucanase hydrolysis time (22 hours) in the assay did not account for this interference, and the dinitrosalicylic acid reagent was not assumed to be at fault, having performed well enough to give consistent reducing sugars responses and blank reducing sugars ( $RS_B$ ) readings.

Enzyme inhibition on lipases has been reported from Hebridean brown seaweed bioactives, theorised to include alginates, fucoidans, and polyphenols (Chater et al., 2016), while phlorotannin oligomers have been reported to inhibit both  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes (Kellogg et al., 2014). Alginates and polyphenols were also reported to inhibit porcine  $\alpha$ -amylase (Zaharudin et al., 2018). While the Celluclast and Accellerase produced enzymatic extracts, these extracts were typically in pH regions where alginates, phlorotannins, and fucoidans would be reasonably co-extracted. Inhibition by these molecules may have played a role in the plateau found in the various responses of the enzyme experiments as well (inhibitory bioactives could have been increasingly released by enzymatic hydrolysis, and inhibited both Celluclast and Accellerase). These inhibitors could have remained in solution at less acidic extraction conditions and also inhibited the hydrolysis of laminarin by

endo-1,3- $\beta$ -glucanase, resulting in lower spectrophotometric readings for laminarin. This is one possible explanation for the observed results.

The lack of a laminarin-specific step in the HPLC protocol makes it more likely that the increased glucose readings in HPLC testing of the Celluclast and Accellerase were caused by water-soluble/ethanol-insoluble cellulose oligomers that hydrolysed to glucose during HPLC sample preparation. The developed spectrophotometric assay was therefore considered adequate for laminarin estimation in solution, and accuracy could increase with the addition of a 1,6- $\beta$ -glucanase to liberate more sugars from the laminarin molecule. The inclusion of laminarinase enzymes (both 1,3- and 1,6- $\beta$ -glucanases) into a hybrid HPLC method could result in more accurate readings, although the extensive preparation steps for HPLC testing may introduce considerable error into measurement (enough that spectrophotometric estimation would be equivalent for all intensive purposes).

## Chapter 9

### *Conclusions and recommendations*

Conclusions were paired with the objectives stated in Chapter 3. As such, each objective was stated first, and followed with the relevant conclusions from the study and any recommendations relevant to that objective. General recommendations were presented independently after conclusions.

#### **9.1. Conclusions and paired objectives**

The first objective to be achieved was the development of a spectrophotometric assay for the quantification of laminarin in solution. This was achieved via an enzymatic method (specific hydrolysis via endo-1,3- $\beta$ -glucanase) that could estimate laminarin content after various hydrolysis methods were carried out. Possible inhibition of the endo-1,3- $\beta$ -glucanase by polyphenols, phlorotannins, alginates, and other cell wall components was noted during comparison with HPLC-derived results when testing enzymatic extracts, but it was thought to be more likely that enzymatically released cellulose-oligomers contaminated the HPLC readings. The objective of laminarin assay development was therefore considered completed.

The second objective was the optimisation of laminarin extraction via enzymatic extraction. Laminarin extraction was not found to be dependent on enzyme dosage, but response surface ANOVAs showed the significant effect of both pH and temperature in linear and quadratic terms. Solubilised yield, supernatant mass fraction, pellet solids loading, and reducing sugars and inorganic sulfates concentration in the supernatant were found to be significantly influenced by Celluclast enzyme addition. Antioxidant activity and total phenolics were found to be significantly dependent on temperature and pH. Response surface optimisation of the laminarin response agreed with literature and moved the response towards low pH/high temperature treatments consistent with Deville et al. (2004). While optimisation was not completed for enzymatic extraction, it was proven that enzymatic methods could not compete with conventional dilute-acid thermal hydrolysis. The objective was considered successful in that it indicated previously reported optimal extraction conditions from literature.

Part of the optimisation process was model validation. All responses were measured and the models validated at three experimental conditions, including predicted laminarin optimum extraction conditions at the edge of the experimental design space (pH 3.0, 60 °C, 4.0 % (v/dw) Celluclast), an autohydrolysis repeat of this treatment at the same pH and temperature, and an enzyme treatment validation at

4.0 % (v/dw), pH 4.5 and 50 °C. All responses performed well in the midrange pH and temperatures, with low prediction errors, while giving larger errors towards the predicted laminarin optimum conditions. The performance of all models was validated within the spherical design of the original CCD, but not at the conditions prescribed as “laminarin optimal”. Nonetheless the prescribed “laminarin optimum” conditions yielded the highest response of laminarin in the CCD (but not significantly different from the blank enzyme run at the same pH and temperature). This lack of significant difference brought about by enzyme-addition meant that the Celluclast had no effect on the laminarin extraction, agreeing with the significant factors identified during RSM analysis. The conditions showed a move toward laminarin conventional extraction conditions proposed by Devillé et al. (2004) (pH 1.0 and 70 °C), taken as a positive sign that the regressed response surfaces for laminarin were predicting trends correctly, and giving the conclusion that enzymatic extraction was not suitable for laminarin extraction.

The third objective was the comparison of validation responses to alternate enzyme (Accellerase) and conventional extraction methods. Performance of Celluclast and Accellerase was not significantly different. While some responses showed increased performance with enzymatic methods (reducing sugars, inorganic sulfates, supernatant mass fraction, solubilised yield after 4.5 hours), others showed better responses with conventional conditions (pellet solids loading, antioxidants, and laminarin). The conventional extraction of laminarin (when measured with the spectrophotometric method) was found to be significantly better than enzyme methods.

The fourth objective was the statistical comparison between batches harvested in May 2018 and June 2019. Laminarin was found to be significantly increased in the May 2018 batch (by both spectrophotometric and HPLC quantification methods), along with reducing sugars, solubilised yield, and supernatant mass fraction. The batch from June 2019 showed elevated levels of inorganic sulfates, total phenolics, and antioxidant capacity.

The fifth objective was the comparison of the spectrophotometric assay laminarin results to the readings obtained by HPLC testing of the same samples. While both methods corroborated the contents of laminarin in the primary batch after acidic extraction, the results under enzymatic extractions were not in agreement. Cellulose-oligomers released during enzyme hydrolysis by both Celluclast and Accellerase were theorised as the cause of this difference, showing larger glucose amounts in HPLC testing. It was concluded that specificity was required to estimate the laminarin present in solution, a trait that was not inherent in the HPLC testing method implemented.

The final objective was to assess whether or not laminarin and fucoidan were co-extracted. Laminarin and inorganic sulfates (fucoidan proxy) indicated differing extraction requirements during response

surface modelling. Laminarin required low pH/high temperature combinations without the requirement for enzyme while inorganic sulfates required pH closer to neutral with indicated Celluclast enzyme requirement. These suggested that coextraction would not be possible in a one step process, but rather advocated the primary use of mild-conditioned enzymatic hydrolysis to extract fucoidan and the subsequent dilute-acid thermal hydrolysis of the residue to extract laminarin. Batch comparison showed, in the two batches tested, that laminarin and fucoidan were highest in B1 and B2 respectively (elevated levels for each polysaccharide occurred in different batches). This batch-related observation further showed coextraction of the polysaccharides to be unlikely. The objective was considered completed, although further, more detailed quantification of fucoidan (via fucose measurement) would provide more detail.

## **9.2. General conclusions and recommendations**

It is likely that the developed spectrophotometric assay for laminarin measurement could find use in an industrial setting, and the further addition of 1,6- $\beta$ -glucanase could increase the method accuracy. Ethanol precipitation of the polysaccharide rich fraction or the removal of small inhibitors via spin column should be investigated, along with inhibition of commercial and analytical enzymes by the cell-fragments present in kelp and seaweeds (alginic acid, polyphenols, etc.). It was concluded that enzymatic hydrolysis processes did not increase the extraction of laminarin, while conventional dilute-acid thermal hydrolysis showed high utility in that endeavour. Enzymatic hydrolysis was however shown to be beneficial to the release of fucoidan and reducing sugars, and in increasing solubilised yield.

The quantification of fucoidan should not be carried out solely by measuring inorganic sulfate content, as this results from two separate processes, namely the release and hydrolysis of fucoidan. Fucose should be quantified separately, and the effect of additional hydrolysis on samples other than by trichloroacetic acid should be confirmed.

Harvesting period, location, and other factors such as nitrate concentrations in seawater and frond age should be monitored and studied, with a monthly harvest taken of similar-sized kelp from the same colony. Alginate plays a large role in the physical behaviour of all brown seaweed extractions, meaning that all future samples in studies of this nature should be tested for alginate, as well as swelling/gelling ability and rheology.



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# *Appendix A*

## *Experimental protocols*

### **A.1. Reducing sugars assay (3,5-dinitrosalicylic acid)**

(Devillé et al., 2004; Miller, 1959; Sumner, 1921)

#### **A– Equipment:**

- 1.5 ml boil-proof microcentrifuge tubes
- Microcentrifuge tube floating rack
- Water bath capable of 90 °C
- 100 µl – 1000 µl micropipette
- Spectrometer capable of measuring absorbance at 590 nm

#### **B - Reagents:**

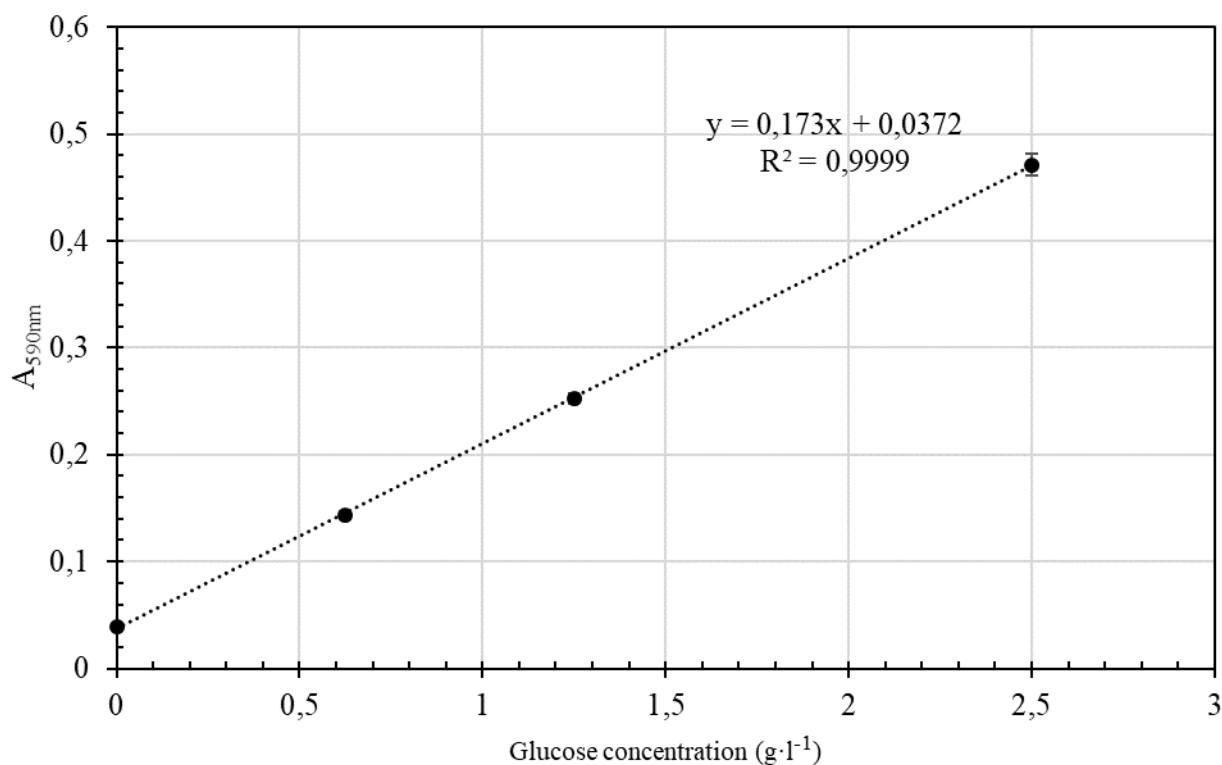
- 2.5 g·L<sup>-1</sup> Glucose standard solution
- DNS Acid Reagent (0.75 %)
  - 0.75 g 3,5-dinitrosalicylic acid
  - 0.75 g sodium hydroxide
  - 0.038 g sodium sulfite
  - 10 g potassium sodium tartrate tetrahydrate (Rochelle salt)
  - add demineralized water up to 100 ml

#### **C – Protocol:**

1. Add 500 µL of DNS acid reagent to 500 µL appropriately diluted sample in a microcentrifuge tube. Seal tube.
2. Heat at 90 °C for 5 minutes in hot water bath, followed by cooling to room temperature in a cool water bath.
3. Measure absorbance of sample at 590 nm.

**D – Notes:**

- Limit of standard linearity found at  $2.5 \text{ g}\cdot\text{L}^{-1}$ . Perform dilutions and include  $0 \text{ g}\cdot\text{L}^{-1}$  point in standard.
- Potassium sodium tartrate tetrahydrate (Rochelle salt) is used as a stabilizer for the brown-red colour developed by the DNS reagent's reaction with reducing sugars. It is pre-mixed with the DNS to avoid the delay of stabilization shown in other DNS methods.
- Other methods report 575 nm absorbance, 590 nm is chosen to accommodate a microplate reader with 590 nm filter.
- Use factor of dilution of 5 for seaweed supernatant samples.

**E - Standard curve:**

## A.2. Laminarin assay (endo-1,3- $\beta$ -D-glucanase)

(Devillé et al., 2004; Kadam et al., 2015a; SIGMA, 1995)

### A – Equipment:

- 1.5 ml boil-proof microcentrifuge tubes
- Microcentrifuge tube floating rack
- Water bath capable of 90 °C
- 100  $\mu$ l – 1000  $\mu$ l micropipette
- Spectrometer capable of measuring absorbance at 575nm/ 590 nm

### B - Reagents:

- 2.5 g·L<sup>-1</sup> Glucose standard solution
- Laminarinase (1,3-endo- $\beta$ -glucanase) enzyme solution (12 U/ml ), prepared immediately before use.
- 100 mM sodium acetate solution, pH adjusted to 5.0 with 32% HCl.
- DNS Acid Reagent (0.75 %)
  - 0.75 g 3,5-dinitrosalicylic acid
  - 0.75 g sodium hydroxide
  - 0.038 g sodium sulfite
  - 10 g potassium sodium tartrate tetrahydrate (Rochelle salt)
  - add demineralized water up to 100 ml

### C – Protocol:

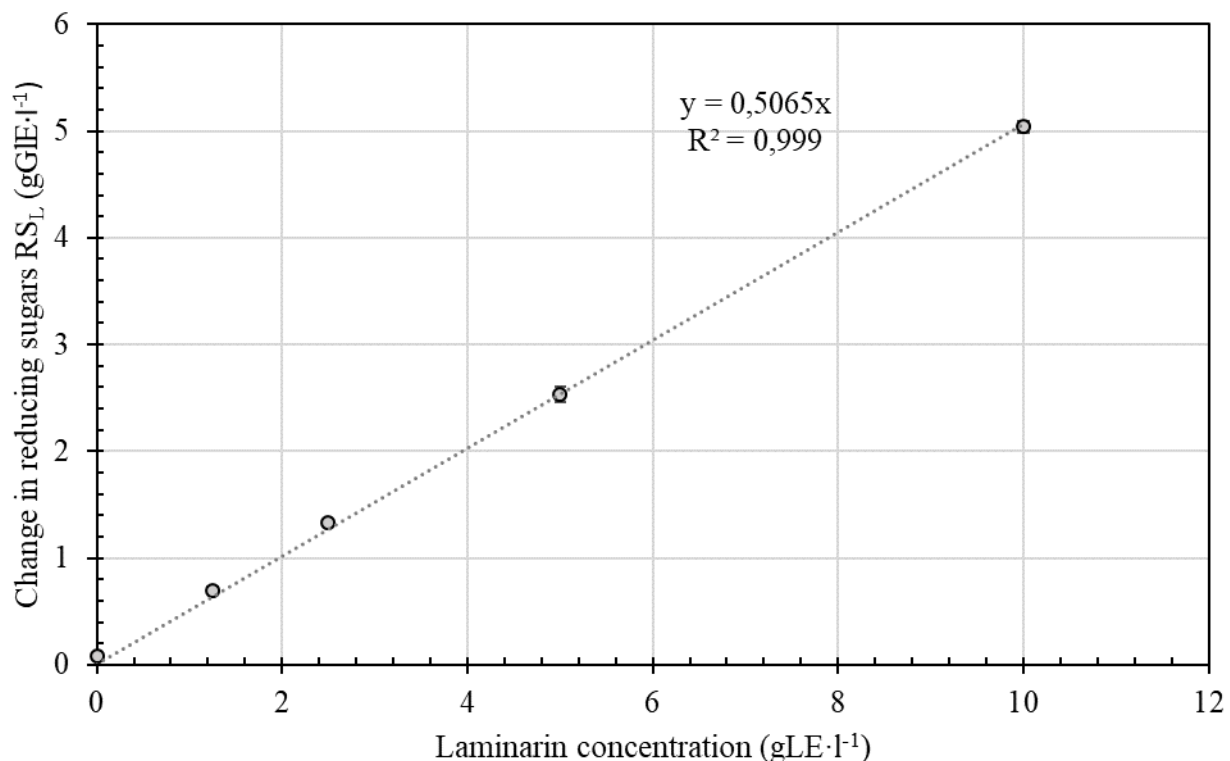
4. Add volume ratio of 1:3 (sample:buffer) in test tubes/ microcentrifuge tubes. Label one as blank.
5. Add 1 part demineralized to blank sample/buffer mixture (final volume ratio of 1:3:1, sample dilution factor of 5)
6. Add 1 part laminarinase enzyme solution to non-blank sample/ buffer mixture (final volume ratio of 1:3:1, sample dilution factor of 5)
7. Incubate for 1-22 hours at 37 °C.
8. Remove from water bath and add DNS reagent in 1:1 ratio with incubated solutions.
9. Heat at 90 °C for 5 minutes in hot water bath, followed by cooling to room temperature in a cool water bath.

10. Measure absorbance of samples at 575 nm / 590 nm.
11. The laminarin content is found via subtracting the blank glucose content from the non-blank glucose content.

### D – Notes:

- Limit of standard linearity found at  $2.5 \text{ g}\cdot\text{L}^{-1}$ . Perform dilutions and include  $0 \text{ g}\cdot\text{L}^{-1}$  point in standard.
- Potassium sodium tartrate tetrahydrate (Rochelle salt) is used as a stabilizer for the brown-red colour developed by the DNS reagent's reaction with reducing sugars. It is pre-mixed with the DNS to avoid the delay of stabilization shown in other DNS methods.
- Other methods report 575 nm absorbance, 590 nm is chosen to accommodate a microplate reader with 590 nm filter.
- Determine dilution factor required via a standard reducing sugars test on the sample being tested.
- Any reducing sugar or glucose determining method should work in place of DNS reagent.

### E - Standard curve:





### A.3. Inorganic sulfate test (barium chloride-gelatin)

(Dodgson, 1961)

#### A – Equipment:

- 2 ml boil-proof microcentrifuge tubes
- Microcentrifuge tube floating rack
- Water bath capable of 25 °C
- 100 µl – 1000 µl micropipette
- Spectrometer capable of measuring absorbance at 360 nm

#### B - Reagents:

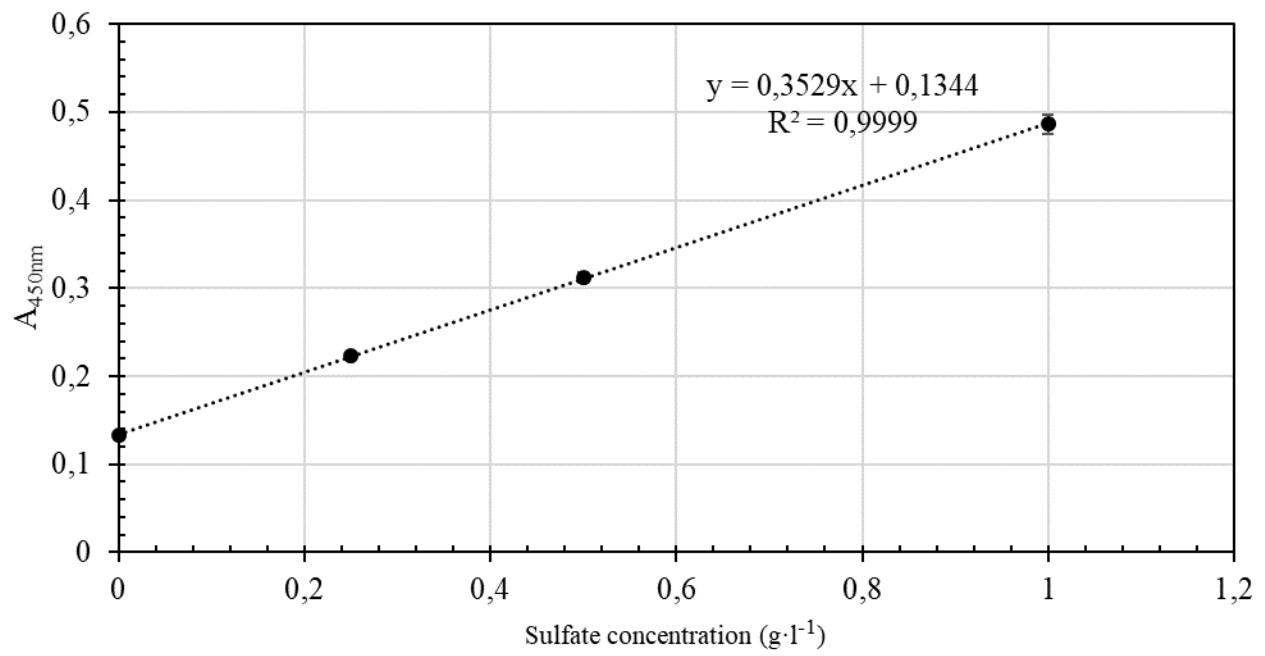
- 1 g·L<sup>-1</sup> Sulfate standard solution
- 4 % TCA (trichloroacetic acid) solution
- 0.5 % Gelatin solution (w/v), with 0.5 % BaCl<sub>2</sub> (dissolve 0.50 g gelatin in 100 ml demineralized water at 65 °C, refrigerate overnight and add / dissolve 0.50 g barium chloride)

#### C – Protocol:

12. Pipette 80 µL of appropriately diluted sample into 2 mL microcentrifuge tubes. Add 1520 µL of 4 % TCA, followed by 400 µL of BaCl<sub>2</sub>-gelatin.
13. After mixing, allow to stand at 25 °C for 20 minutes.
14. Pipette 300 µL into microplate wells and read at 360 nm.

#### D – Notes:

- Dilution factor of 4 used for kelp liquid hydrolysate samples

**E – Standard:**

## A.4. Total phenolics (Folin-Ciocalteu)

(Fogliano et al., 1999)

### A – Equipment:

- 2 ml microcentrifuge tubes
- Microcentrifuge tube floating rack
- Water bath capable of 25 °C
- 100 µl – 1000 µl micropipette
- 20 µl – 200 µl micropipette
- Spectrometer capable of measuring absorbance at 760 nm

### B - Reagents:

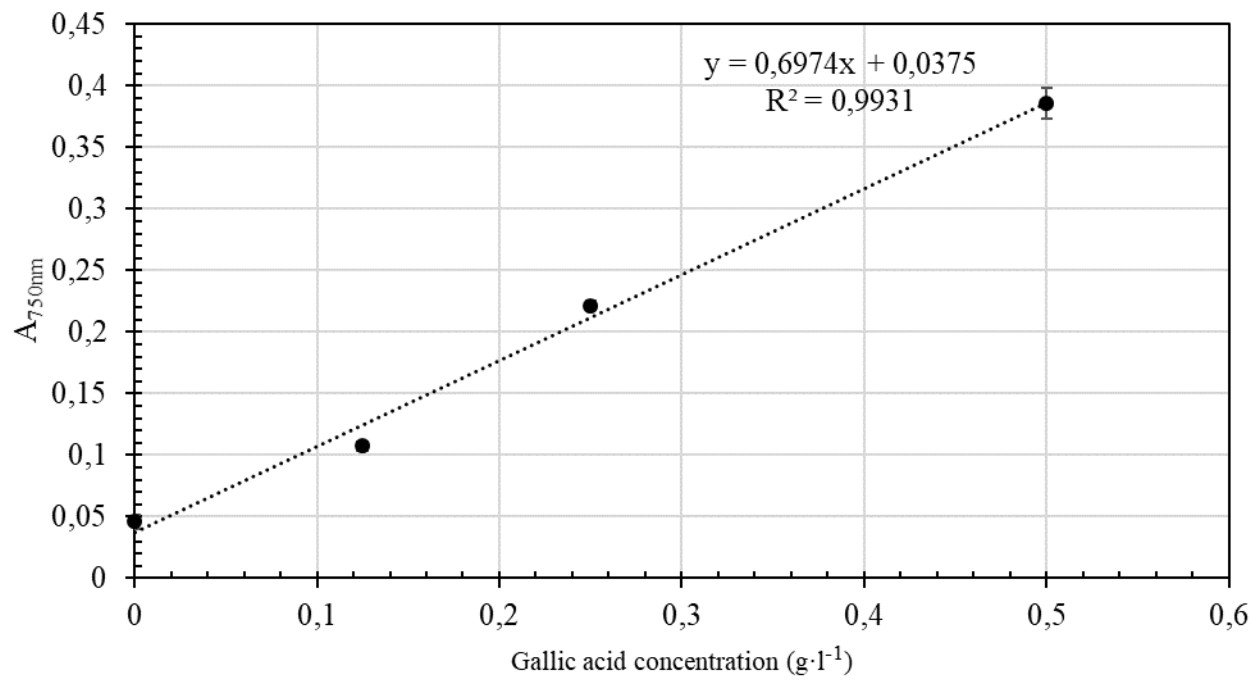
- 0.5 g·L<sup>-1</sup> gallic acid standard solution
- Deionized water
- Folin-Ciocalteu reagent (Sigma)
- 8 % sodium carbonate solution

### C – Protocol:

15. Add 20 µL of appropriately diluted sample to 840 µL of deionized water and 100 µl Folin-Ciocalteu reagent. Mix for 1 minute.
16. Add 200 µl of 8 % sodium carbonate and 840 µl of deionized water. Let stand at 25 °C for 2 hours in darkness.
17. Measure absorbance of sample at 750 nm.

### D – Notes:

8 % sodium carbonate, not 80 %.

**E – Standard**

## A.5. Antioxidants capacity assay (N-dimethyl-p-phenylenediamine)

(Fogliano et al., 1999)

### A – Equipment:

- 1.5 ml microcentrifuge tubes
- Microcentrifuge tube floating rack
- Water bath capable of 25 °C
- 100 µl – 1000 µl micropipette
- 20 µl – 200 µl micropipette
- Spectrometer capable of measuring absorbance at 490 nm

### B - Reagents:

- 0.2 g·L<sup>-1</sup> TROLOX solution in absolute ethanol
- Deionized water
- 100 mM DMPD solution (209 mg in 10 mL)
- 50 mM Ferric chloride solution
- 0.1 M acetate buffer solution, pH adjusted to 5.25

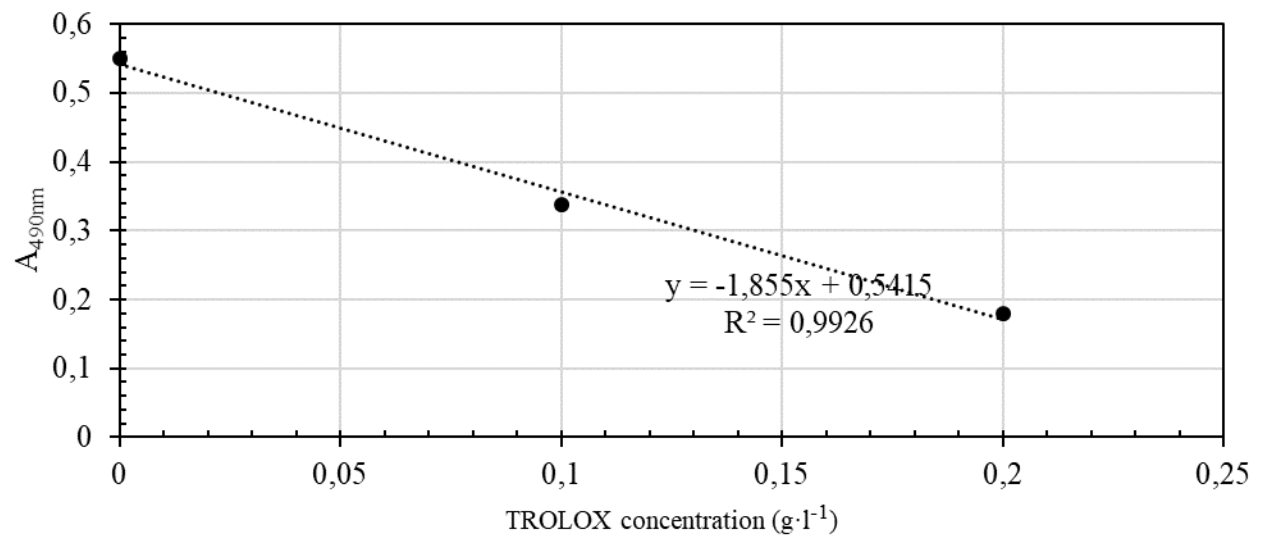
### C – Protocol:

18. Add 500 µL of DMPD solution and 100 µL ferric chloride solution to 50 mL of acetate buffer to obtain coloured radical cation.
19. Add 80 µL of sample to 1600 µL of prepared solution and stir/agitate continuously for 10 minutes. Measure absorbance at 490 nm.

### D – Notes:

Use a three point TROLOX standard curve (0.2, 0.1, and 0 g·L<sup>-1</sup>) along with an uninhibited signal reading (just the coloured radical cation solution) to calculate antioxidant capacity according to the equation below.

$$\text{inhibition of } A_{490\text{nm}} (\%) = \left(1 - \frac{A_f}{A_0}\right) \times 100$$

**E – Standard:**

Uninhibited signal  $A_{490nm} = 0.565 \pm 0.004$

# *Appendix B*

## *Experimental design*

Standard order	Random order	pH		T			ES ( $v/dw$ )	
		Actual	Coded	Actual (K)	Actual (°C)	Coded	Actual	Coded
1	13	3.61	-1.000	317.2	44.1	-1.000	0.008	-1.000
2	6	3.61	-1.000	317.2	44.1	-1.000	0.032	1.000
3	18	3.61	-1.000	329.1	55.9	1.000	0.008	-1.000
4	8	3.61	-1.000	329.1	55.9	1.000	0.032	1.000
5	3	5.39	1.000	317.2	44.1	-1.000	0.008	-1.000
6	12	5.39	1.000	317.2	44.1	-1.000	0.032	1.000
7	14	5.39	1.000	329.1	55.9	1.000	0.008	-1.000
8	1	5.39	1.000	329.1	55.9	1.000	0.032	1.000
9	11	3	-1.682	323.15	50.0	0.000	0.020	0.000
10	5	6	1.682	323.15	50.0	0.000	0.020	0.000
11	20	4.5	0.000	313.15	40.0	-1.682	0.020	0.000
12	10	4.5	0.000	333.15	60.0	1.682	0.020	0.000
13	4	4.5	0.000	323.15	50.0	0.000	0.000	-1.682
14	7	4.5	0.000	323.15	50.0	0.000	0.040	1.682
15	15	4.5	0.000	323.15	50.0	0.000	0.020	0.000
16	19	4.5	0.000	323.15	50.0	0.000	0.020	0.000
17	2	4.5	0.000	323.15	50.0	0.000	0.020	0.000
18	17	4.5	0.000	323.15	50.0	0.000	0.020	0.000
19	16	4.5	0.000	323.15	50.0	0.000	0.020	0.000
20	9	4.5	0.000	323.15	50.0	0.000	0.020	0.000



## Appendix C Experimental data

### C.1. Solubilised yield (SY) data

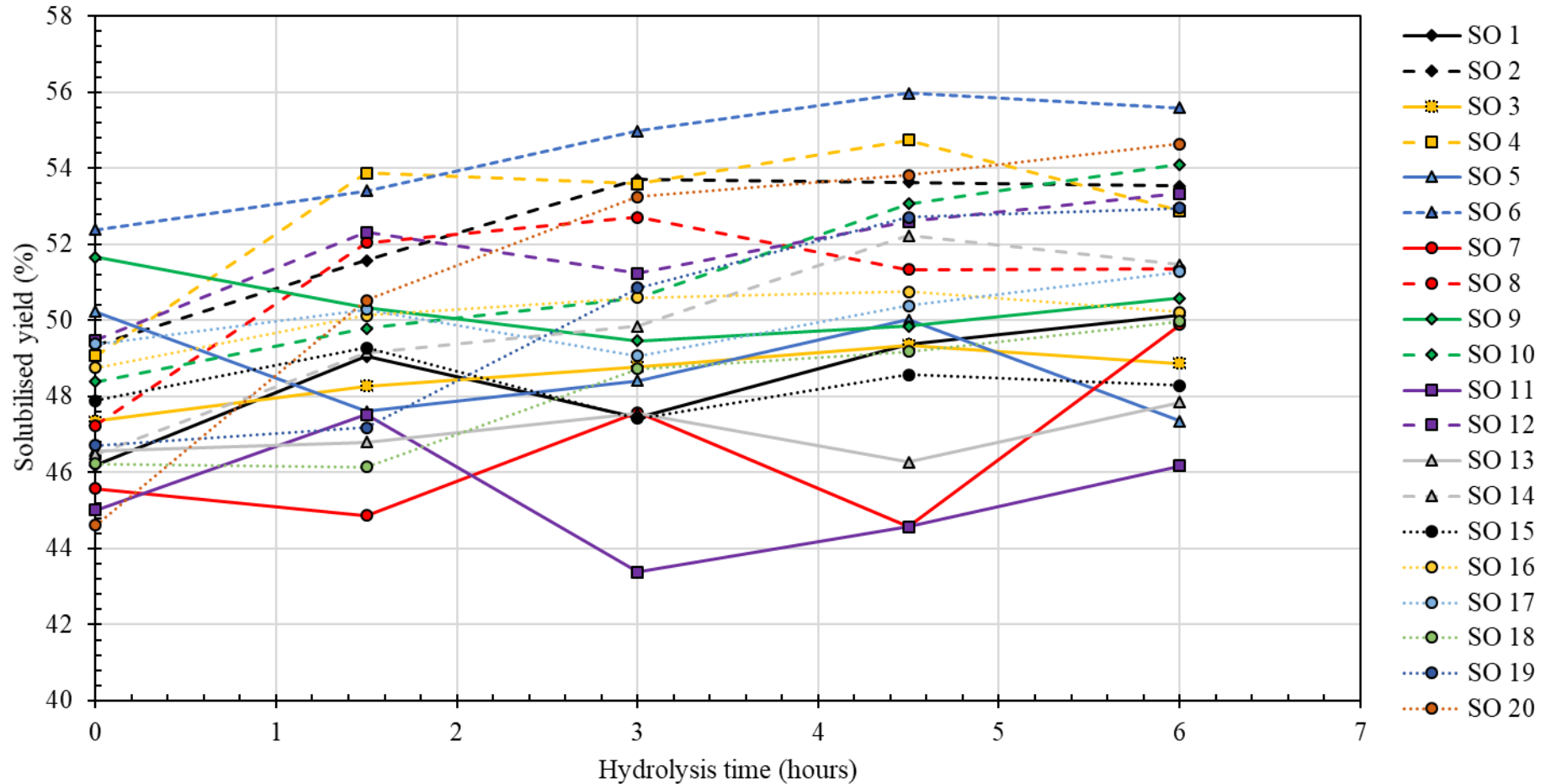


Figure C-1 Solubilised yield (SY) of experiments SO 1-20 over hydrolysis time

Table C-1 Solubilised yield data ( $SY$ ), with error parameter  $\Delta$  across experiments SO 1-20. (%)

SO	$SY_0$	$\Delta_{SY_0}$	$SY_{1.5}$	$\Delta_{SY_{1.5}}$	$SY_3$	$\Delta_{SY_3}$	$SY_{4.5}$	$\Delta_{SY_{4.5}}$	$SY_6$	$\Delta_{SY_6}$
1	46.2	0.7	49.1	0.3	47.4	0.3	49.4	0.7	50.1	0.3
2	49.3	0.6	51.6	0.7	53.7	0.7	53.6	0.7	53.5	0.9
3	47.4	1.2	48.3	0.6	48.8	0.9	49.4	0.9	48.9	0.6
4	49.1	1.5	53.9	1.2	53.6	0.5	54.7	0.6	52.9	1.0
5	50.2	0.9	47.6	1.3	48.4	0.7	50.0	1.1	47.4	0.6
6	52.4	1.1	53.4	0.8	55.0	0.5	56.0	0.7	55.6	0.8
7	45.6	1.5	44.9	1.3	47.6	1.4	44.6	0.3	49.9	0.6
8	47.2	1.5	52.1	1.3	52.7	0.6	51.3	1.1	51.4	0.4
9	51.7	1.1	50.3	0.3	49.5	0.5	49.9	0.7	50.6	0.7
10	48.4	0.7	49.8	0.8	50.6	0.8	53.1	0.4	54.1	0.7
11	45.0	0.7	47.5	0.8	43.4	1.1	44.6	0.8	46.2	1.0
12	49.5	1.3	52.3	0.7	51.2	1.1	52.6	0.6	53.3	0.5
13	46.6	1.3	46.8	1.3	47.6	0.6	46.3	1.2	47.9	0.5
14	46.5	0.8	49.2	1.0	49.9	1.1	52.2	0.8	51.5	1.1
15	47.9	0.7	49.3	0.5	47.4	0.8	48.6	1.1	48.3	1.0
16	48.8	0.9	50.1	1.2	50.6	0.2	50.8	0.6	50.2	0.9
17	49.4	1.1	50.3	1.0	49.1	1.2	50.4	1.0	51.3	0.6
18	46.2	0.8	46.2	1.2	48.7	0.8	49.2	0.7	50.0	0.7
19	46.7	1.0	47.2	0.5	50.9	0.8	52.7	0.8	53.0	0.5
20	44.6	0.9	50.5	0.9	53.3	0.9	53.8	0.5	54.6	0.9

### C.2. Supernatant dissolved solids (DS) data

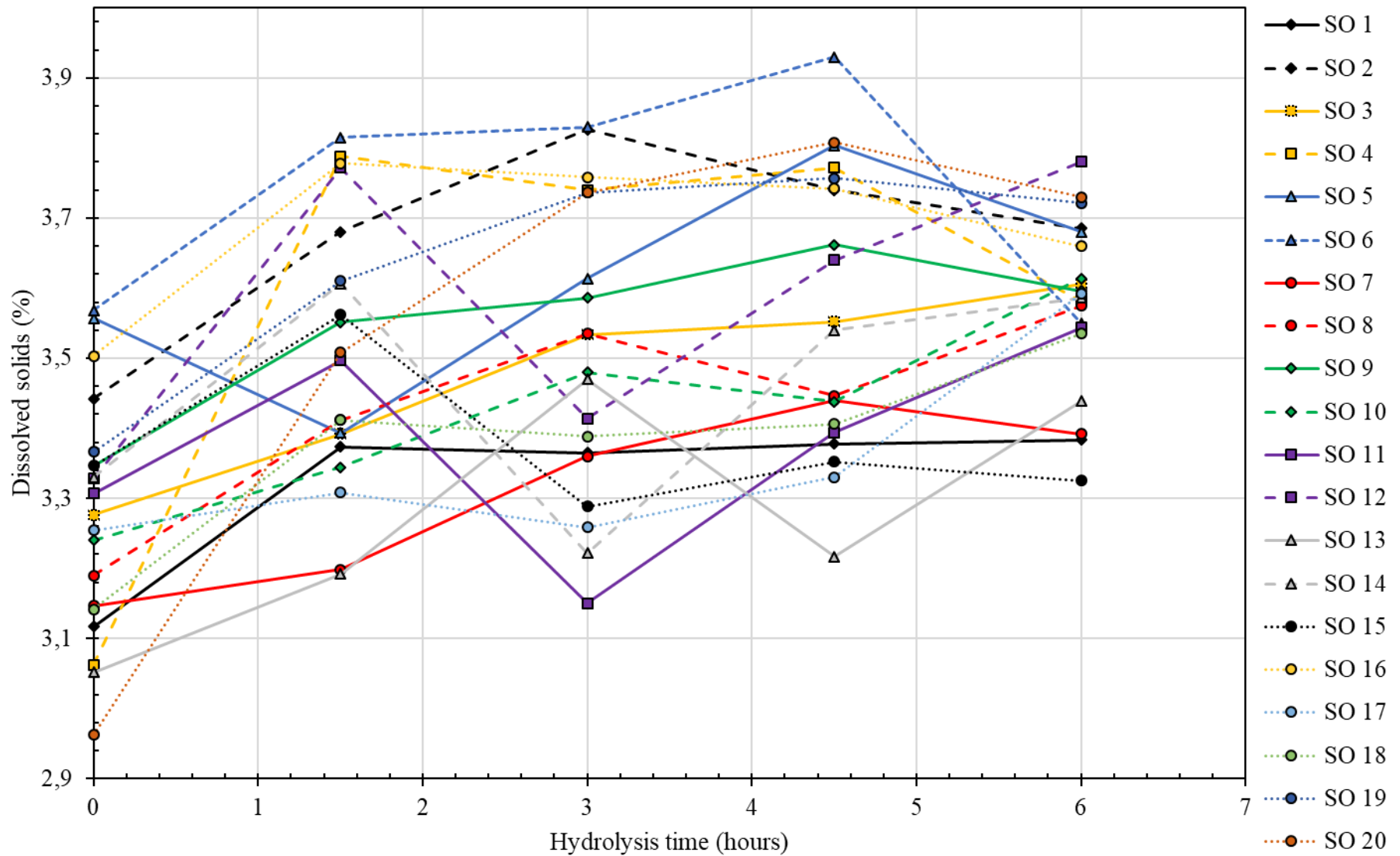


Figure C-2 Dissolved solids (DS) of experiments SO 1-20 over hydrolysis time

Table C-2 Dissolved solids data ( $DS$ ), with error parameter  $\Delta$  across experiments SO 1-20 (%)

SO	$DS_0$	$\Delta_{DS_0}$	$DS_{1.5}$	$\Delta_{DS_{1.5}}$	$DS_3$	$\Delta_{DS_3}$	$DS_{4.5}$	$\Delta_{DS_{4.5}}$	$DS_6$	$\Delta_{DS_6}$
1	3.117	0.085	3.373	0.037	3.365	0.041	3.378	0.038	3.383	0.036
2	3.442	0.057	3.680	0.104	3.826	0.038	3.740	0.111	3.685	0.135
3	3.276	0.127	3.392	0.032	3.533	0.127	3.552	0.159	3.606	0.055
4	3.062	0.096	3.788	0.127	3.740	0.075	3.772	0.062	3.582	0.150
5	3.557	0.127	3.393	0.179	3.614	0.085	3.803	0.165	3.680	0.058
6	3.568	0.153	3.815	0.121	3.830	0.056	3.930	0.108	3.550	0.097
7	3.147	0.185	3.198	0.174	3.360	0.120	3.440	0.039	3.392	0.044
8	3.190	0.196	3.412	0.183	3.535	0.087	3.447	0.155	3.575	0.059
9	3.347	0.149	3.552	0.042	3.586	0.041	3.662	0.108	3.596	0.072
10	3.240	0.089	3.344	0.065	3.480	0.107	3.438	0.060	3.614	0.056
11	3.307	0.094	3.497	0.111	3.150	0.143	3.394	0.052	3.543	0.146
12	3.328	0.179	3.772	0.100	3.414	0.121	3.640	0.065	3.780	0.076
13	3.052	0.164	3.192	0.166	3.470	0.084	3.217	0.158	3.438	0.077
14	3.330	0.107	3.607	0.151	3.222	0.128	3.540	0.112	3.586	0.170
15	3.347	0.095	3.562	0.072	3.288	0.106	3.352	0.098	3.325	0.137
16	3.503	0.119	3.778	0.183	3.758	0.032	3.742	0.076	3.660	0.101
17	3.254	0.116	3.308	0.137	3.258	0.158	3.330	0.118	3.592	0.041
18	3.142	0.108	3.412	0.156	3.388	0.084	3.406	0.051	3.535	0.088
19	3.366	0.103	3.610	0.029	3.737	0.121	3.757	0.117	3.722	0.080
20	2.962	0.091	3.508	0.127	3.736	0.113	3.808	0.055	3.730	0.146

### C.3. Supernatant mass fraction ( $F_{SN}$ ) data

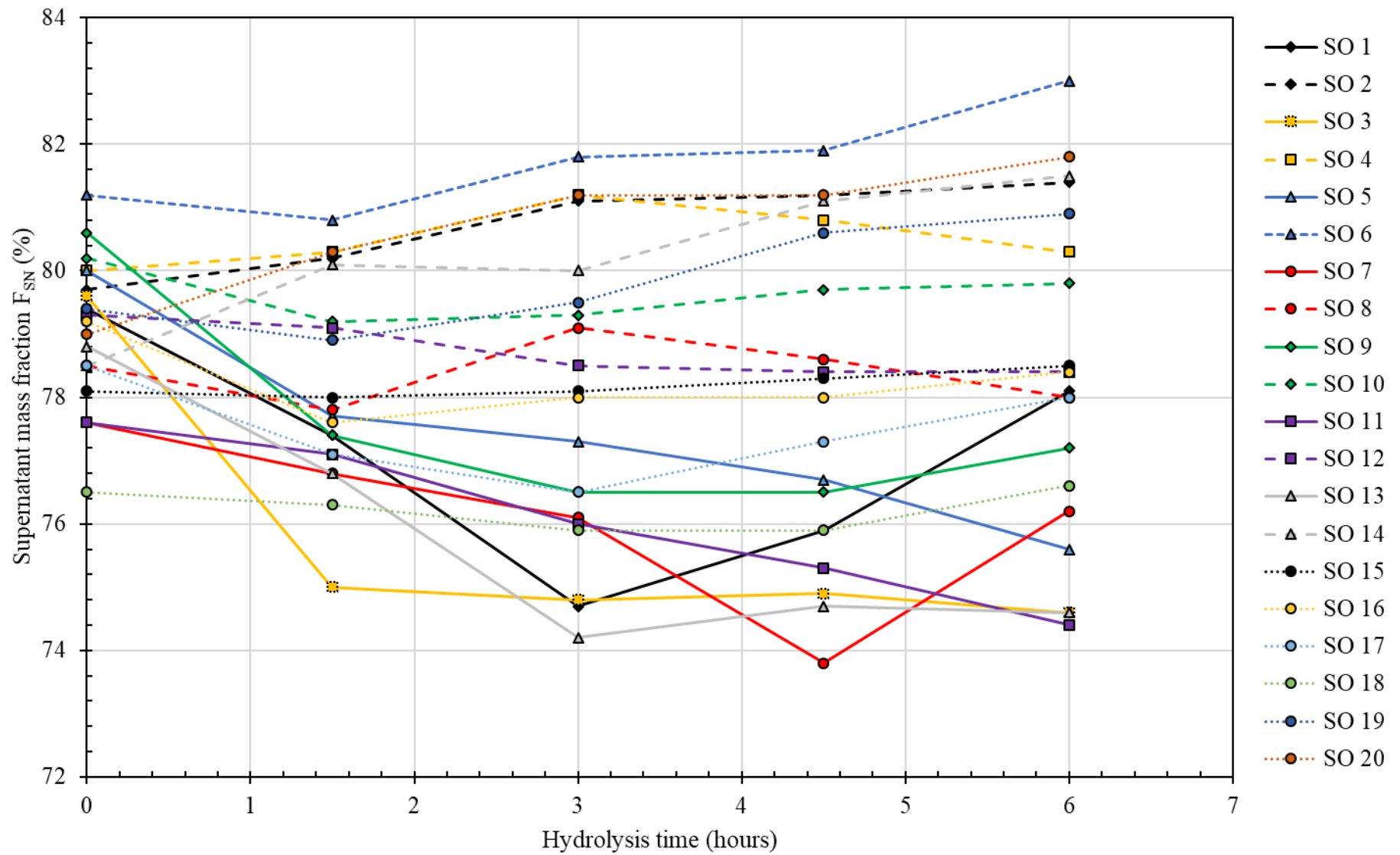


Figure C-3 Supernatant mass fraction ( $F_{SN}$ ) of experiments SO 1-20 over hydrolysis time

Table C-3 Supernatant mass fraction ( $F_{SN}$ ), with error parameter  $\Delta$  across experiments SO 1-20. (%)

SO	$F_{SN,0}$	$\Delta_{F_{SN,0}}$	$F_{SN,1.5}$	$\Delta_{F_{SN,1.5}}$	$F_{SN,3}$	$\Delta_{F_{SN,3}}$	$F_{SN,4.5}$	$\Delta_{F_{SN,4.5}}$	$F_{SN,6}$	$\Delta_{F_{SN,6}}$
1	79.4	1.1	77.4	1.5	74.7	1.7	75.9	1.7	78.1	1.7
2	79.7	1.1	80.2	1.5	81.1	1.7	81.2	1.7	81.4	1.7
3	79.6	1.1	75.0	1.5	74.8	1.7	74.9	1.7	74.6	1.7
4	80.0	1.1	80.3	1.5	81.2	1.7	80.8	1.7	80.3	1.7
5	80.0	1.1	77.7	1.5	77.3	1.7	76.7	1.7	75.6	1.7
6	81.2	1.1	80.8	1.5	81.8	1.7	81.9	1.7	83.0	1.7
7	77.6	1.1	76.8	1.5	76.1	1.7	73.8	1.7	76.2	1.7
8	78.5	1.1	77.8	1.5	79.1	1.7	78.6	1.7	78.0	1.7
9	80.6	1.1	77.4	1.5	76.5	1.7	76.5	1.7	77.2	1.7
10	80.2	1.1	79.2	1.5	79.3	1.7	79.7	1.7	79.8	1.7
11	77.6	1.1	77.1	1.5	76.0	1.7	75.3	1.7	74.4	1.7
12	79.3	1.1	79.1	1.5	78.5	1.7	78.4	1.7	78.4	1.7
13	78.8	1.1	76.8	1.5	74.2	1.7	74.7	1.7	74.6	1.7
14	78.5	1.1	80.1	1.5	80.0	1.7	81.1	1.7	81.5	1.7
15	78.1	1.1	78.0	1.5	78.1	1.7	78.3	1.7	78.5	1.7
16	79.2	1.1	77.6	1.5	78.0	1.7	78.0	1.7	78.4	1.7
17	78.5	1.1	77.1	1.5	76.5	1.7	77.3	1.7	78.0	1.7
18	76.5	1.1	76.3	1.5	75.9	1.7	75.9	1.7	76.6	1.7
19	79.4	1.1	78.9	1.5	79.5	1.7	80.6	1.7	80.9	1.7
20	79.0	1.1	80.3	1.5	81.2	1.7	81.2	1.7	81.8	1.7

#### C.4. Pellet-solids loading ( $SL_P$ ) data

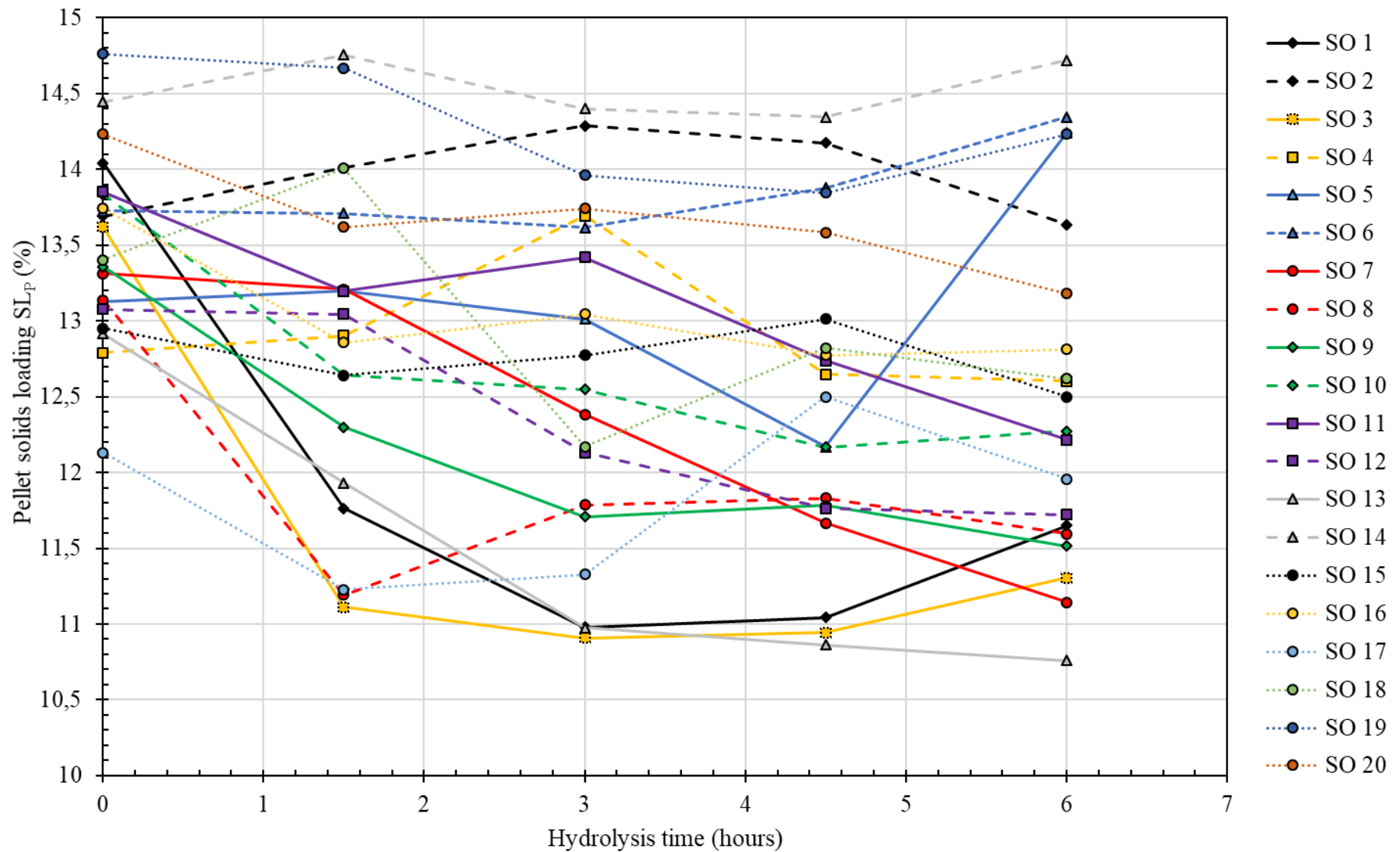


Figure C-4 Pellet solids loading ( $SL_P$ ) of experiments SO 1-20 over hydrolysis time



Table C-4 Pellet solids loading ( $SL_P$ ), with error parameter  $\Delta$  across experiments SO 1-20. (%)

SO	$SL_{P,0}$	$\Delta_{SL_{P,0}}$	$SL_{P,1.5}$	$\Delta_{SL_{P,1.5}}$	$SL_{P,3}$	$\Delta_{SL_{P,3}}$	$SL_{P,4.5}$	$\Delta_{SL_{P,4.5}}$	$SL_{P,6}$	$\Delta_{SL_{P,6}}$
1	14.0	1.0	11.8	1.3	11.0	1.0	11.0	0.5	11.7	0.8
2	13.7	1.0	14.0	1.3	14.3	1.0	14.2	0.5	13.6	0.8
3	13.6	1.0	11.1	1.3	10.9	1.0	10.9	0.5	11.3	0.8
4	12.8	1.0	12.9	1.3	13.7	1.0	12.6	0.5	12.6	0.8
5	13.1	1.0	13.2	1.3	13.0	1.0	12.2	0.5	14.2	0.8
6	13.7	1.0	13.7	1.3	13.6	1.0	13.9	0.5	14.3	0.8
7	13.3	1.0	13.2	1.3	12.4	1.0	11.7	0.5	11.1	0.8
8	13.1	1.0	11.2	1.3	11.8	1.0	11.8	0.5	11.6	0.8
9	13.4	1.0	12.3	1.3	11.7	1.0	11.8	0.5	11.5	0.8
10	13.8	1.0	12.6	1.3	12.5	1.0	12.2	0.5	12.3	0.8
11	13.9	1.0	13.2	1.3	13.4	1.0	12.7	0.5	12.2	0.8
12	13.1	1.0	13.0	1.3	12.1	1.0	11.8	0.5	11.7	0.8
13	12.9	1.0	11.9	1.3	11.0	1.0	10.9	0.5	10.8	0.8
14	14.4	1.0	14.8	1.3	14.4	1.0	14.3	0.5	14.7	0.8
15	12.9	1.0	12.6	1.3	12.8	1.0	13.0	0.5	12.5	0.8
16	13.7	1.0	12.9	1.3	13.0	1.0	12.8	0.5	12.8	0.8
17	12.1	1.0	11.2	1.3	11.3	1.0	12.5	0.5	12.0	0.8
18	13.4	1.0	14.0	1.3	12.2	1.0	12.8	0.5	12.6	0.8
19	14.8	1.0	14.7	1.3	14.0	1.0	13.8	0.5	14.2	0.8
20	14.2	1.0	13.6	1.3	13.7	1.0	13.6	0.5	13.2	0.8

**C.5. Mass measurement data**Table C-5 Supernatant (*SN*) and pellet (*P*) and dry pellet (*PD*) mass across experiments SO 1-20. (g)

SO	<i>SN</i> <sub>0</sub>	<i>P</i> <sub>0</sub>	<i>PD</i> <sub>0</sub>	<i>SN</i> <sub>1.5</sub>	<i>P</i> <sub>1.5</sub>	<i>PD</i> <sub>1.5</sub>	<i>SN</i> <sub>3</sub>	<i>P</i> <sub>3</sub>	<i>PD</i> <sub>3</sub>	<i>SN</i> <sub>4.5</sub>	<i>P</i> <sub>4.5</sub>	<i>PD</i> <sub>3</sub>	<i>SN</i> <sub>6</sub>	<i>P</i> <sub>6</sub>	<i>PD</i> <sub>3</sub>
1	11.25	2.92	0.41	9.89	2.89	0.34	9.96	3.37	0.37	10.24	3.26	0.36	11.04	3.09	0.36
2	10.35	2.63	0.36	10.41	2.57	0.36	10.20	2.38	0.34	11.00	2.54	0.36	10.60	2.42	0.33
3	10.92	2.79	0.38	9.72	3.24	0.36	9.80	3.30	0.36	9.83	3.29	0.36	10.12	3.45	0.39
4	10.29	2.58	0.33	10.12	2.48	0.32	10.43	2.41	0.33	10.62	2.53	0.32	10.00	2.46	0.31
5	10.33	2.59	0.34	10.01	2.88	0.38	9.97	2.92	0.38	9.99	3.04	0.37	9.79	3.16	0.45
6	11.01	2.55	0.35	10.41	2.48	0.34	10.58	2.35	0.32	11.09	2.45	0.34	11.93	2.44	0.35
7	10.67	3.08	0.41	10.50	3.18	0.42	10.26	3.23	0.40	10.14	3.60	0.42	10.65	3.32	0.37
8	10.00	2.74	0.36	9.70	2.77	0.31	9.94	2.63	0.31	9.62	2.62	0.31	9.77	2.76	0.32
9	10.89	2.62	0.35	10.58	3.09	0.38	10.29	3.16	0.37	10.23	3.14	0.37	10.27	3.04	0.35
10	10.26	2.53	0.35	10.27	2.69	0.34	10.39	2.71	0.34	10.34	2.63	0.32	10.93	2.77	0.34
11	10.23	2.96	0.41	9.68	2.88	0.38	9.93	3.13	0.42	9.55	3.14	0.40	10.23	3.52	0.43
12	9.94	2.60	0.34	10.47	2.76	0.36	9.96	2.72	0.33	9.87	2.72	0.32	9.90	2.73	0.32
13	10.05	2.71	0.35	9.46	2.85	0.34	9.43	3.28	0.36	9.22	3.13	0.34	9.28	3.16	0.34
14	9.83	2.70	0.39	9.80	2.44	0.36	10.02	2.50	0.36	10.48	2.44	0.35	10.21	2.31	0.34
15	9.90	2.78	0.36	9.55	2.69	0.34	9.77	2.74	0.35	9.71	2.69	0.35	9.95	2.72	0.34
16	9.96	2.62	0.36	9.70	2.80	0.36	9.76	2.76	0.36	9.74	2.74	0.35	10.18	2.81	0.36
17	9.96	2.72	0.33	9.59	2.85	0.32	10.05	3.09	0.35	9.80	2.88	0.36	9.81	2.76	0.33
18	9.48	2.91	0.39	9.90	3.07	0.43	9.58	3.04	0.37	9.84	3.12	0.40	10.36	3.17	0.40
19	10.45	2.71	0.40	11.19	3.00	0.44	10.28	2.65	0.37	10.80	2.60	0.36	11.03	2.60	0.37
20	10.31	2.74	0.39	10.49	2.57	0.35	11.28	2.62	0.36	11.43	2.65	0.36	11.60	2.58	0.34

### C.6. Reducing sugars (RS) data

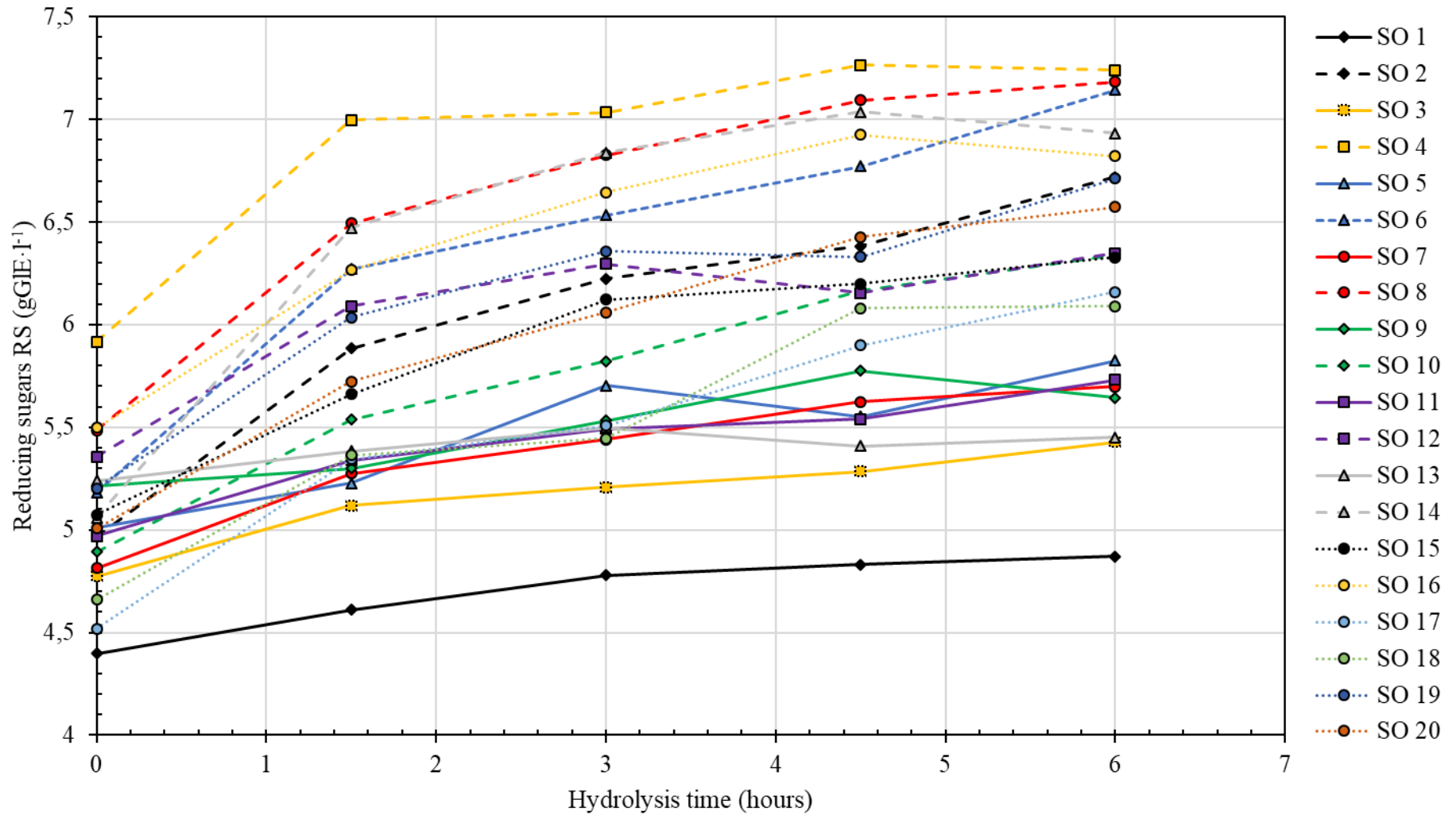


Figure C-5 Supernatant reducing sugars (RS) of experiments SO 1-20 over hydrolysis time

Table C-6 Supernatant reducing sugar ( $RS$ ), with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gGIE}\cdot\text{l}^{-1}$ )

SO	$RS_0$	$\Delta_{RS_0}$	$RS_{1.5}$	$\Delta_{RS_{1.5}}$	$RS_3$	$\Delta_{RS_3}$	$RS_{4.5}$	$\Delta_{RS_{4.5}}$	$RS_6$	$\Delta_{RS_6}$
1	4.398	0.111	4.611	0.101	4.778	0.093	4.831	0.105	4.870	0.078
2	4.965	0.088	5.885	0.129	6.222	0.114	6.381	0.107	6.719	0.116
3	4.773	0.082	5.117	0.055	5.207	0.104	5.285	0.074	5.429	0.098
4	5.916	0.112	6.996	0.121	7.034	0.214	7.264	0.114	7.241	0.140
5	5.012	0.131	5.227	0.170	5.704	0.140	5.552	0.225	5.825	0.156
6	5.182	0.090	6.270	0.105	6.533	0.180	6.772	0.125	7.144	0.107
7	4.812	0.088	5.274	0.119	5.440	0.127	5.623	0.120	5.699	0.103
8	5.486	0.203	6.494	0.143	6.826	0.137	7.095	0.137	7.183	0.146
9	5.212	0.139	5.299	0.181	5.532	0.148	5.776	0.224	5.643	0.184
10	4.894	0.104	5.536	0.122	5.823	0.212	6.165	0.291	6.341	0.249
11	4.969	0.124	5.338	0.092	5.492	0.090	5.540	0.107	5.731	0.217
12	5.358	0.112	6.090	0.180	6.296	0.144	6.154	0.113	6.347	0.114
13	5.241	0.130	5.384	0.140	5.497	0.113	5.408	0.156	5.451	0.177
14	5.058	0.127	6.472	0.102	6.838	0.111	7.037	0.172	6.932	0.294
15	5.073	0.315	5.661	0.231	6.121	0.190	6.199	0.188	6.328	0.212
16	5.497	0.155	6.266	0.147	6.646	0.102	6.924	0.131	6.820	0.158
17	4.518	0.102	5.344	0.129	5.508	0.100	5.900	0.109	6.159	0.186
18	4.660	0.044	5.365	0.068	5.445	0.128	6.080	0.124	6.091	0.109
19	5.202	0.104	6.035	0.168	6.357	0.114	6.330	0.249	6.712	0.127
20	5.008	0.118	5.722	0.136	6.060	0.123	6.426	0.141	6.574	0.143

### C.7. Laminarin ( $L$ ) (including blanks $RS_B$ and hydrolysed $RS_H$ ) data

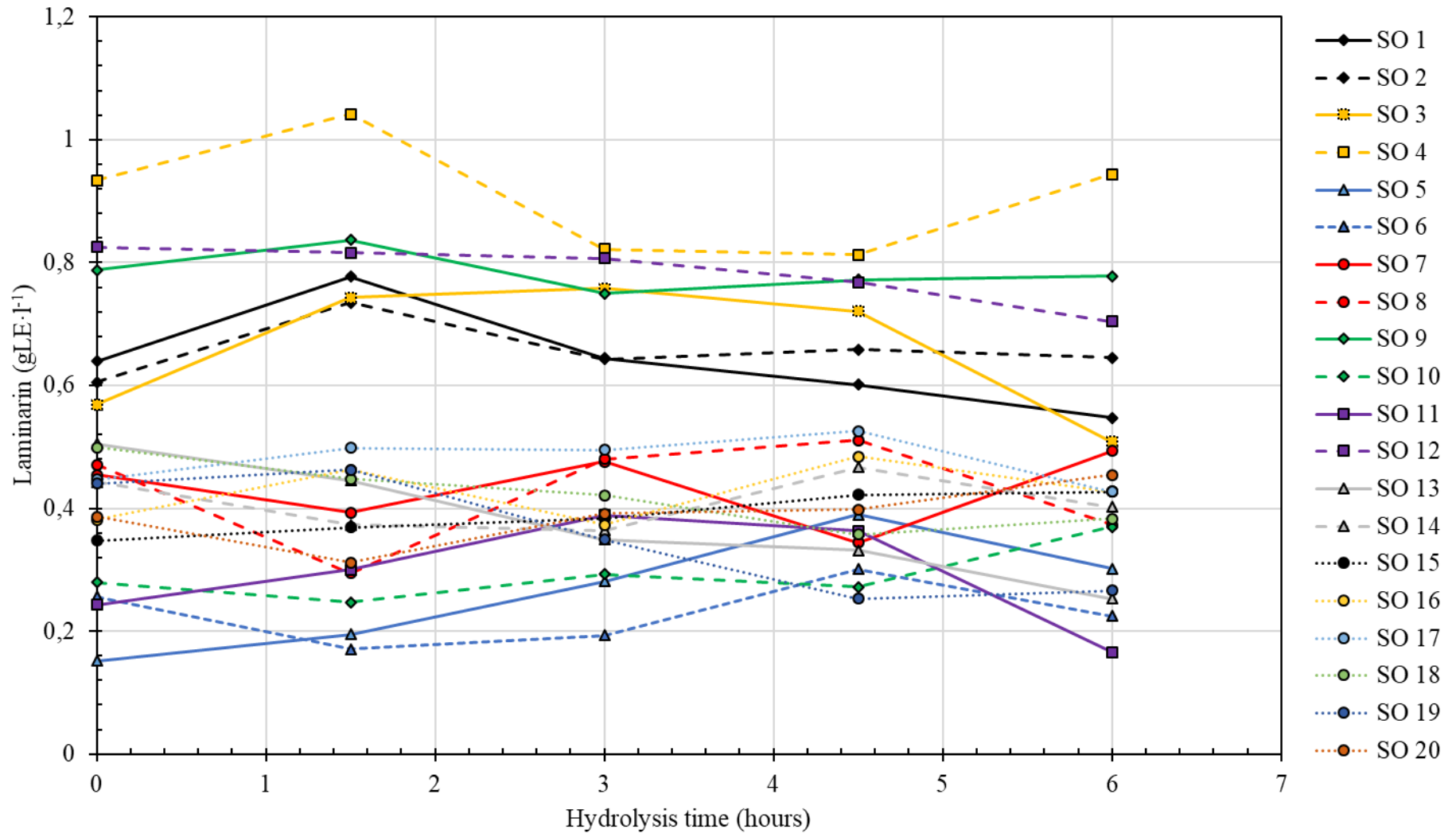


Figure C-6 Laminarin response ( $L$ ) of experiments SO 1-20 over hydrolysis time

Table C-7 Laminarin ( $L$ ), with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gLE}\cdot\text{l}^{-1}$ )

SO	$L_0$	$\Delta_{L_0}$	$L_{1.5}$	$\Delta_{L_{1.5}}$	$L_3$	$\Delta_{L_3}$	$L_{4.5}$	$\Delta_{L_{4.5}}$	$L_6$	$\Delta_{L_6}$
1	0.639	0.037	0.777	0.070	0.644	0.067	0.601	0.148	0.547	0.135
2	0.605	0.177	0.735	0.248	0.643	0.092	0.658	0.104	0.645	0.043
3	0.569	0.275	0.743	0.198	0.758	0.128	0.721	0.166	0.508	0.134
4	0.934	0.024	1.042	0.066	0.822	0.138	0.813	0.247	0.944	0.137
5	0.152	0.074	0.195	0.039	0.281	0.037	0.390	0.091	0.302	0.019
6	0.256	0.080	0.171	0.056	0.193	0.028	0.301	0.071	0.225	0.115
7	0.454	0.111	0.393	0.252	0.476	0.180	0.344	0.183	0.493	0.052
8	0.471	0.156	0.294	0.181	0.480	0.079	0.511	0.104	0.373	0.093
9	0.788	0.085	0.837	0.070	0.750	0.151	0.772	0.189	0.778	0.108
10	0.279	0.055	0.247	0.090	0.293	0.095	0.272	0.129	0.370	0.104
11	0.243	0.049	0.300	0.149	0.389	0.088	0.363	0.065	0.166	0.081
12	0.825	0.222	0.816	0.060	0.807	0.228	0.768	0.042	0.704	0.141
13	0.505	0.173	0.445	0.029	0.349	0.067	0.332	0.066	0.253	0.087
14	0.443	0.065	0.373	0.109	0.364	0.162	0.467	0.155	0.402	0.183
15	0.347	0.091	0.369	0.185	0.383	0.065	0.422	0.206	0.427	0.079
16	0.381	0.096	0.463	0.034	0.373	0.067	0.484	0.221	0.427	0.142
17	0.447	0.232	0.498	0.260	0.495	0.084	0.526	0.062	0.427	0.095
18	0.499	0.047	0.448	0.066	0.421	0.126	0.357	0.061	0.383	0.079
19	0.440	0.039	0.463	0.097	0.349	0.081	0.253	0.070	0.266	0.097
20	0.386	0.185	0.312	0.171	0.391	0.245	0.398	0.108	0.454	0.164

Table C-8 Blank sample reducing sugar ( $RS_B$ ) from laminarin assay, with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gGIE}\cdot\text{l}^{-1}$ )

SO	$RS_{B,0}$	$\Delta_{RS_{B,0}}$	$RS_{B,1.5}$	$\Delta_{RS_{B,1.5}}$	$RS_{B,3}$	$\Delta_{RS_{B,3}}$	$RS_{B,4.5}$	$\Delta_{RS_{B,4.5}}$	$RS_{B,6}$	$\Delta_{RS_{B,6}}$
1	4.585	0.037	4.735	0.096	4.838	0.055	4.763	0.043	4.976	0.077
2	5.048	0.171	5.800	0.126	6.126	0.080	6.168	0.040	6.215	0.124
3	4.663	0.055	5.186	0.153	4.954	0.099	5.234	0.099	4.934	0.135
4	5.283	0.062	6.331	0.068	6.203	0.113	6.358	0.096	6.400	0.158
5	5.491	0.066	5.834	0.135	5.959	0.127	6.064	0.074	6.197	0.204
6	5.395	0.085	6.393	0.171	6.552	0.041	6.918	0.033	7.327	0.112
7	4.546	0.111	4.931	0.206	4.990	0.157	5.129	0.126	5.319	0.038
8	5.022	0.100	6.057	0.133	6.283	0.111	6.492	0.038	6.562	0.028
9	4.822	0.047	5.236	0.060	5.284	0.109	5.458	0.186	5.490	0.015
10	4.948	0.072	5.463	0.111	5.734	0.060	5.898	0.088	6.060	0.063
11	5.001	0.048	5.456	0.055	5.447	0.104	5.553	0.060	5.635	0.030
12	5.089	0.199	5.911	0.120	6.128	0.177	5.955	0.096	5.988	0.051
13	4.640	0.162	4.746	0.105	5.020	0.085	5.086	0.055	5.015	0.061
14	5.256	0.087	6.229	0.167	6.571	0.133	6.789	0.085	6.917	0.141
15	4.686	0.072	5.393	0.123	5.771	0.082	5.880	0.069	6.105	0.059
16	5.363	0.084	6.202	0.166	6.246	0.108	6.739	0.217	6.519	0.142
17	4.499	0.131	5.183	0.141	5.707	0.088	5.992	0.073	5.818	0.099
18	4.561	0.114	5.272	0.188	5.516	0.139	5.693	0.050	5.975	0.054
19	4.695	0.052	5.546	0.100	5.806	0.166	5.874	0.097	6.101	0.046
20	4.733	0.130	5.501	0.151	5.735	0.179	5.925	0.021	6.123	0.087



Table C-9 Enzyme hydrolysed reducing sugar ( $RS_H$ ) from laminarin assay, with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gGIE}\cdot\text{l}^{-1}$ )

SO	$RS_{H,0}$	$\Delta_{RS_{H,0}}$	$RS_{H,1.5}$	$\Delta_{RS_{H,1.5}}$	$RS_{H,3}$	$\Delta_{RS_{H,3}}$	$RS_{H,4.5}$	$\Delta_{RS_{H,4.5}}$	$RS_{H,6}$	$\Delta_{RS_{H,6}}$
1	4.899	0.051	5.121	0.077	5.210	0.022	5.027	0.026	5.227	0.022
2	5.354	0.138	6.172	0.140	6.451	0.121	6.502	0.052	6.542	0.124
3	5.018	0.090	5.562	0.071	5.338	0.037	5.599	0.031	5.192	0.178
4	5.756	0.066	6.845	0.089	6.620	0.045	6.696	0.097	6.878	0.112
5	5.568	0.031	5.933	0.130	6.101	0.113	6.261	0.089	6.350	0.204
6	5.525	0.122	6.540	0.173	6.670	0.046	7.088	0.077	7.440	0.131
7	4.776	0.062	5.129	0.107	5.231	0.075	5.291	0.036	5.556	0.024
8	5.260	0.025	6.206	0.047	6.526	0.115	6.732	0.138	6.737	0.087
9	5.222	0.084	5.649	0.038	5.677	0.029	5.876	0.098	5.884	0.065
10	5.101	0.026	5.601	0.093	5.906	0.102	6.060	0.145	6.200	0.078
11	5.140	0.078	5.617	0.095	5.599	0.143	5.748	0.087	5.752	0.087
12	5.507	0.123	6.324	0.094	6.537	0.082	6.344	0.113	6.344	0.112
13	4.896	0.091	4.971	0.115	5.197	0.063	5.253	0.025	5.143	0.089
14	5.468	0.061	6.433	0.189	6.768	0.172	7.038	0.132	7.123	0.139
15	4.847	0.038	5.553	0.055	5.941	0.041	6.100	0.117	6.327	0.071
16	5.591	0.144	6.429	0.160	6.419	0.085	6.953	0.142	6.730	0.149
17	4.734	0.097	5.404	0.120	5.974	0.100	6.234	0.031	6.085	0.115
18	4.813	0.120	5.499	0.210	5.730	0.200	5.874	0.035	6.169	0.086
19	4.918	0.047	5.781	0.064	5.983	0.132	6.002	0.108	6.236	0.091
20	4.929	0.042	5.659	0.068	5.957	0.056	6.151	0.048	6.353	0.155

## C.8. Inorganic sulfate (S) data

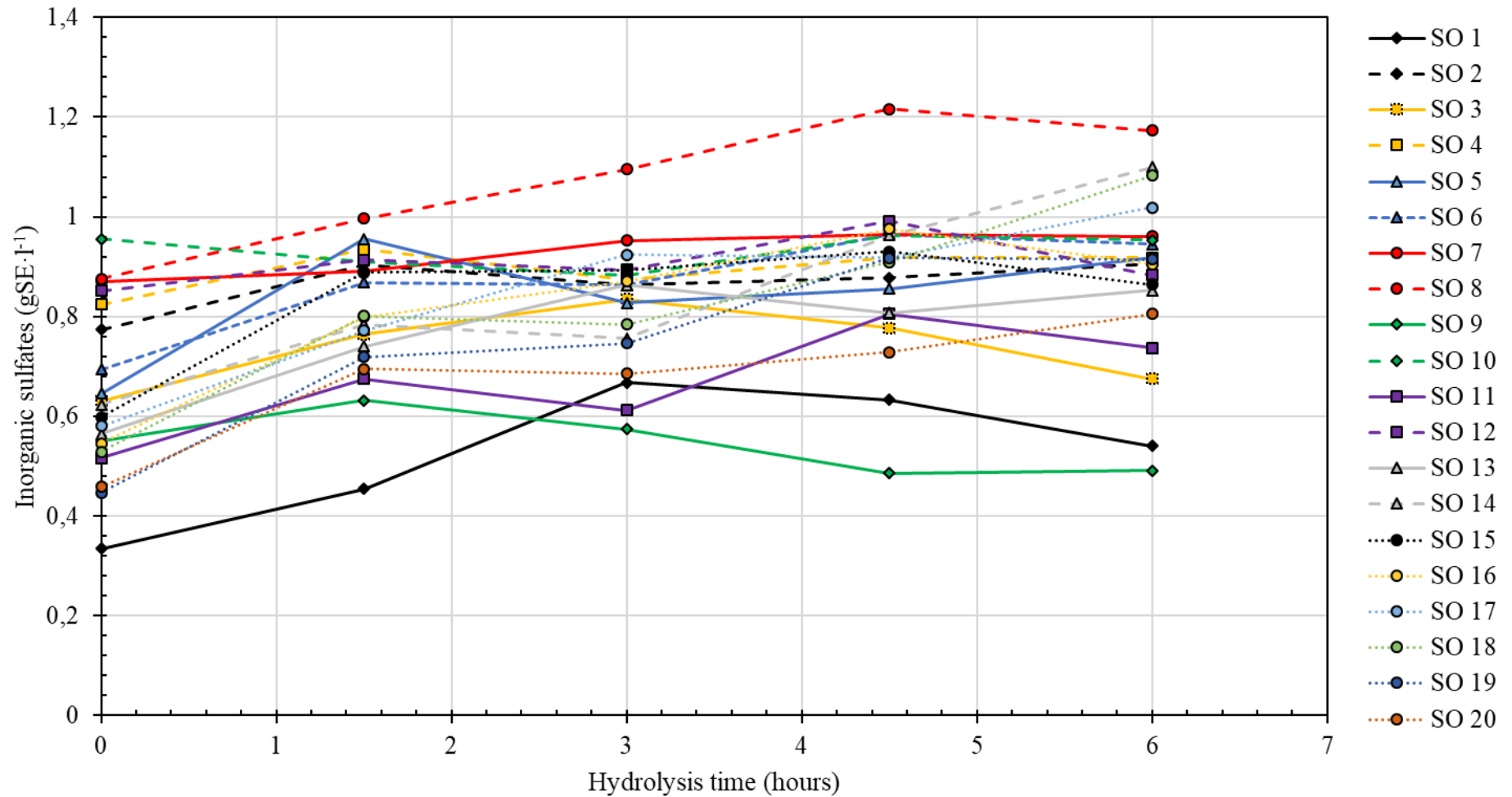


Figure C-7 Inorganic sulfate (S) of experiments SO 1-20 over hydrolysis time

Table C-10 Inorganic sulfate ( $S$ ), with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gSE}\cdot\text{l}^{-1}$ )

SO	$S_0$	$\Delta_{S_0}$	$S_{1.5}$	$\Delta_{S_{1.5}}$	$S_3$	$\Delta_{S_3}$	$S_{4.5}$	$\Delta_{S_{4.5}}$	$S_6$	$\Delta_{S_6}$
1	0.334	0.010	0.454	0.058	0.668	0.044	0.633	0.083	0.540	0.027
2	0.774	0.071	0.904	0.130	0.863	0.026	0.878	0.107	0.905	0.016
3	0.631	0.029	0.764	0.088	0.835	0.033	0.777	0.068	0.675	0.046
4	0.824	0.089	0.935	0.017	0.875	0.059	0.918	0.066	0.918	0.046
5	0.645	0.027	0.956	0.112	0.827	0.089	0.856	0.094	0.918	0.090
6	0.694	0.046	0.868	0.161	0.863	0.089	0.965	0.112	0.945	0.060
7	0.870	0.044	0.890	0.178	0.952	0.096	0.965	0.097	0.961	0.102
8	0.876	0.066	0.996	0.204	1.095	0.065	1.216	0.061	1.173	0.097
9	0.551	0.066	0.632	0.159	0.574	0.043	0.486	0.061	0.491	0.101
10	0.955	0.095	0.909	0.220	0.883	0.041	0.963	0.133	0.953	0.066
11	0.516	0.041	0.675	0.177	0.612	0.051	0.805	0.067	0.737	0.097
12	0.851	0.042	0.913	0.221	0.893	0.032	0.991	0.065	0.883	0.034
13	0.565	0.058	0.740	0.193	0.863	0.075	0.807	0.087	0.853	0.036
14	0.623	0.070	0.784	0.218	0.756	0.030	0.963	0.049	1.099	0.057
15	0.598	0.042	0.888	0.114	0.893	0.045	0.930	0.112	0.864	0.073
16	0.546	0.053	0.799	0.129	0.870	0.061	0.975	0.047	0.905	0.074
17	0.580	0.041	0.772	0.176	0.924	0.052	0.918	0.089	1.019	0.028
18	0.529	0.048	0.801	0.060	0.784	0.108	0.909	0.072	1.083	0.062
19	0.447	0.075	0.719	0.151	0.746	0.093	0.918	0.062	0.915	0.072
20	0.458	0.015	0.695	0.089	0.685	0.104	0.729	0.084	0.805	0.119

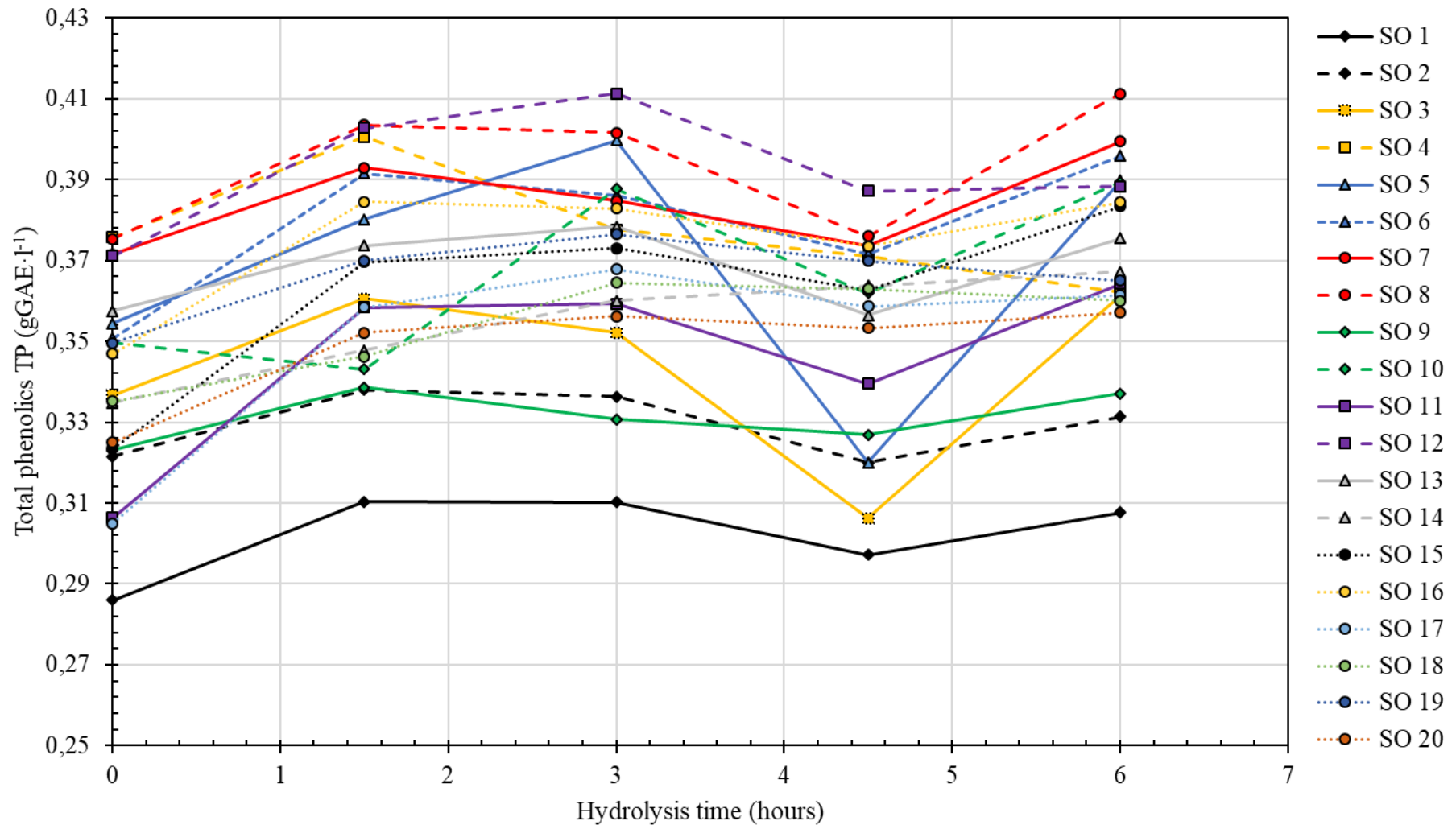
**C.9. Total phenolics (TP) data**

Figure C-8 Total phenolic (TP) of experiments SO 1-20 over hydrolysis time

Table C-11 Total phenolics ( $TP$ ), with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gGAE}\cdot\text{l}^{-1}$ )

SO	$TP_0$	$\Delta_{TP_0}$	$TP_{1.5}$	$\Delta_{TP_{1.5}}$	$TP_3$	$\Delta_{TP_3}$	$TP_{4.5}$	$\Delta_{TP_{4.5}}$	$TP_6$	$\Delta_{TP_6}$
1	0.286	0.003	0.310	0.013	0.310	0.018	0.297	0.008	0.308	0.002
2	0.322	0.002	0.338	0.017	0.336	0.003	0.320	0.009	0.331	0.002
3	0.337	0.001	0.361	0.001	0.352	0.002	0.306	0.003	0.361	0.002
4	0.376	0.004	0.401	0.015	0.378	0.005	0.371	0.003	0.362	0.003
5	0.354	0.004	0.380	0.006	0.400	0.001	0.320	0.003	0.390	0.007
6	0.351	0.002	0.391	0.011	0.386	0.016	0.372	0.002	0.396	0.006
7	0.371	0.004	0.393	0.026	0.385	0.001	0.373	0.002	0.399	0.001
8	0.375	0.002	0.403	0.009	0.402	0.004	0.376	0.004	0.411	0.007
9	0.323	0.004	0.339	0.020	0.331	0.001	0.327	0.003	0.337	0.002
10	0.350	0.004	0.343	0.008	0.388	0.004	0.362	0.009	0.389	0.004
11	0.306	0.007	0.358	0.011	0.359	0.001	0.340	0.006	0.364	0.002
12	0.371	0.005	0.403	0.004	0.411	0.002	0.387	0.007	0.388	0.005
13	0.357	0.004	0.374	0.004	0.378	0.005	0.357	0.005	0.375	0.011
14	0.335	0.002	0.348	0.002	0.360	0.002	0.364	0.007	0.367	0.005
15	0.323	0.006	0.370	0.006	0.373	0.002	0.363	0.004	0.383	0.008
16	0.347	0.004	0.385	0.003	0.383	0.002	0.373	0.009	0.384	0.013
17	0.305	0.004	0.358	0.011	0.368	0.007	0.359	0.006	0.361	0.003
18	0.335	0.007	0.346	0.005	0.364	0.006	0.363	0.003	0.360	0.003
19	0.350	0.004	0.370	0.005	0.376	0.005	0.370	0.002	0.365	0.003
20	0.325	0.006	0.352	0.001	0.356	0.004	0.353	0.004	0.357	0.010

### C.10. Antioxidant capacity (AO) data

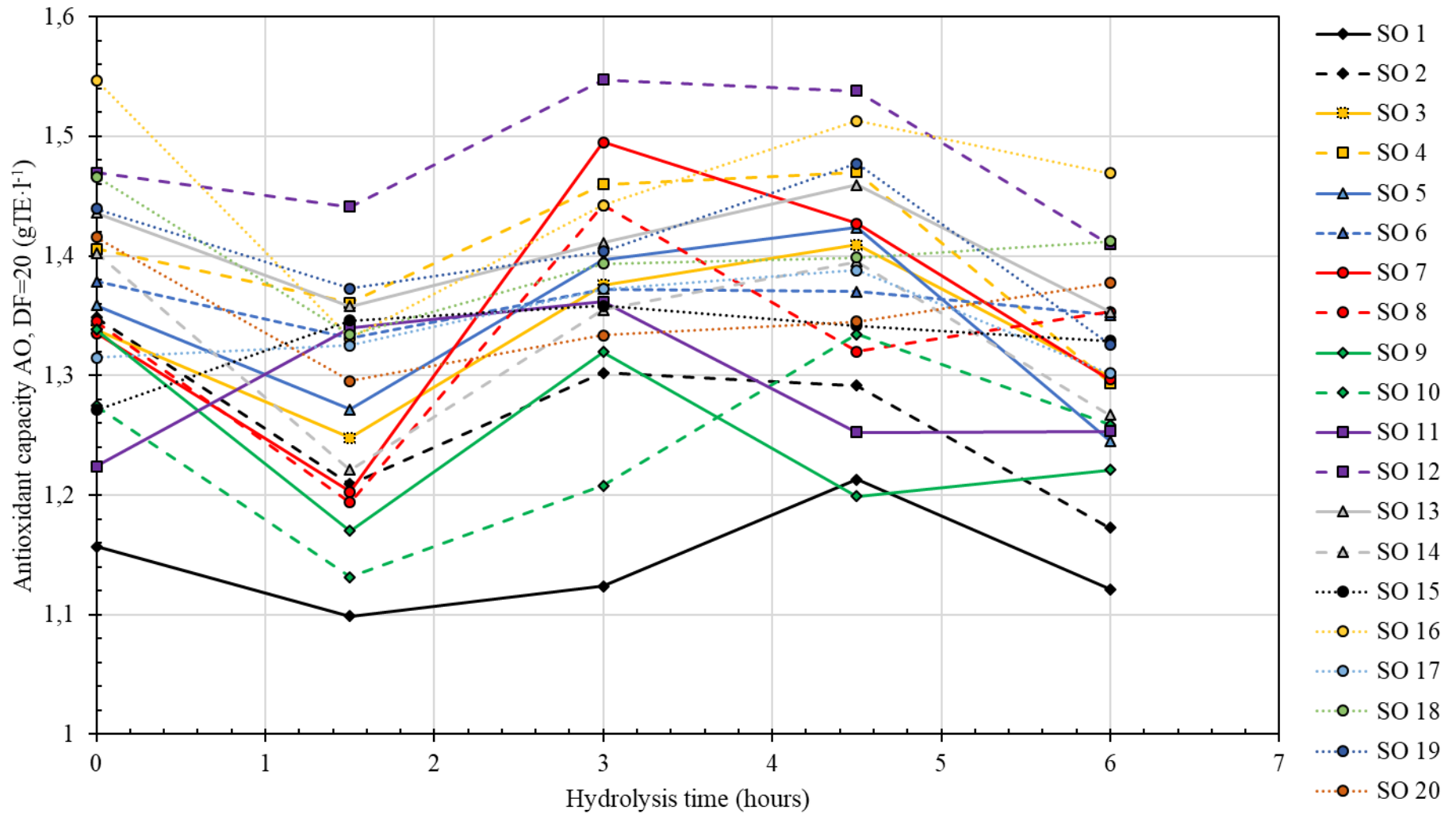


Figure C-9 Antioxidant capacity (AO) of experiments SO 1-20 over hydrolysis time

Table C-12 Antioxidant capacity ( $AO$ ), with error parameter  $\Delta$  across experiments SO 1-20. ( $\text{gTE}\cdot\text{l}^{-1}$ )

SO	$AO_0$	$\Delta_{AO_0}$	$AO_{1.5}$	$\Delta_{AO_{1.5}}$	$AO_3$	$\Delta_{AO_3}$	$AO_{4.5}$	$\Delta_{AO_{4.5}}$	$AO_6$	$\Delta_{AO_6}$
1	1.16	0.01	1.10	0.03	1.12	0.07	1.21	0.04	1.12	0.02
2	1.35	0.05	1.21	0.02	1.30	0.03	1.29	0.02	1.17	0.04
3	1.34	0.04	1.25	0.04	1.38	0.04	1.41	0.06	1.30	0.05
4	1.41	0.05	1.36	0.05	1.46	0.04	1.47	0.05	1.29	0.03
5	1.36	0.03	1.27	0.02	1.40	0.03	1.42	0.03	1.25	0.04
6	1.38	0.04	1.33	0.07	1.37	0.05	1.37	0.03	1.35	0.04
7	1.34	0.15	1.20	0.04	1.49	0.04	1.43	0.05	1.30	0.02
8	1.35	0.05	1.19	0.02	1.44	0.04	1.32	0.03	1.35	0.00
9	1.34	0.06	1.17	0.13	1.32	0.02	1.20	0.10	1.22	0.05
10	1.27	0.04	1.13	0.02	1.21	0.03	1.33	0.03	1.26	0.05
11	1.22	0.04	1.34	0.05	1.36	0.05	1.25	0.08	1.25	0.04
12	1.47	0.02	1.44	0.05	1.55	0.04	1.54	0.05	1.41	0.05
13	1.44	0.03	1.36	0.03	1.41	0.03	1.46	0.02	1.35	0.03
14	1.40	0.03	1.22	0.01	1.35	0.01	1.40	0.05	1.27	0.01
15	1.27	0.07	1.35	0.02	1.36	0.02	1.34	0.04	1.33	0.02
16	1.55	0.05	1.33	0.02	1.44	0.06	1.51	0.04	1.47	0.02
17	1.31	0.07	1.33	0.02	1.37	0.05	1.39	0.01	1.30	0.03
18	1.47	0.07	1.33	0.06	1.39	0.04	1.40	0.03	1.41	0.04
19	1.44	0.05	1.37	0.05	1.40	0.08	1.48	0.00	1.33	0.04
20	1.42	0.04	1.30	0.05	1.33	0.05	1.35	0.05	1.38	0.02



# Appendix D RSM ANOVA tables

Table D-1 ANOVA tables for Solubilised yield (*SY*) responses

ANOVA  $SY_{t=0hrs}$

$R^2=0.45165$ , Adj- $R^2=0$ , MS Pure error=3.174667

Factor	SS	df	MS	F	p
1. pH (L)	0.33845	1	0.33845	0.106609	0.757262
pH (Q)	16.67098	1	16.67098	5.251252	0.070512
2. T (L)	0.11113	1	0.11113	0.035005	0.858941
T (Q)	0.10559	1	0.10559	0.033260	0.862454
3. ES (L)	5.20585	1	5.20585	1.639811	0.256535
ES (Q)	0.37770	1	0.37770	0.118973	0.744187
1L by 2L	14.58000	1	14.58000	4.592608	0.084990
1L by 3L	0.12500	1	0.12500	0.039374	0.850525
2L by 3L	0.50000	1	0.50000	0.157497	0.707845
Lack of fit	30.96988	5	6.19398	1.951063	0.240424
Pure error	15.87333	5	3.17467		
Total SS	85.42550	19			

ANOVA  $SY_{t=1.5hrs}$

$R^2=0.62305$ , Adj- $R^2=0.28379$ , MS Pure error=3.258667

Factor	SS	df	MS	F	p
1. pH (L)	2.4133	1	2.41329	0.74057	0.428803
pH (Q)	4.4845	1	4.48449	1.37617	0.293592
2. T (L)	2.2739	1	2.27387	0.69779	0.441602
T (Q)	3.6723	1	3.67235	1.12695	0.336989
3. ES (L)	46.2650	1	46.26495	14.19751	0.013050
ES (Q)	0.4017	1	0.40168	0.12326	0.739830
1L by 2L	3.7812	1	3.78125	1.16037	0.330594
1L by 3L	3.0013	1	3.00125	0.92101	0.381286
2L by 3L	2.5313	1	2.53125	0.77677	0.418475
Lack of fit	25.2742	5	5.05483	1.55120	0.320858
Pure error	16.2933	5	3.25867		
Total SS	110.2720	19			

ANOVA  $SY_{t=3hrs}$

$R^2=0.49236$ , Adj- $R^2=0.03549$ , MS Pure error=4.264

Factor	SS	df	MS	F	p
1. pH (L)	0.3077	1	0.30771	0.07217	0.798942
pH (Q)	3.5823	1	3.58227	0.84012	0.401410
2. T (L)	9.3797	1	9.37967	2.19973	0.198144
T (Q)	3.2338	1	3.23378	0.75839	0.423665
3. ES (L)	52.0756	1	52.07560	12.21285	0.017383
ES (Q)	0.0219	1	0.02187	0.00513	0.945688
1L by 2L	2.4200	1	2.42000	0.56754	0.485177
1L by 3L	0.0450	1	0.04500	0.01055	0.922170
2L by 3L	1.1250	1	1.12500	0.26384	0.629382
Lack of fit	53.8827	5	10.77654	2.52733	0.165922
Pure error	21.3200	5	4.26400		
Total SS	148.1420	19			

ANOVA  $SY_{t=4.5hrs}$

$R^2=0.5681$ , Adj- $R^2=0.17938$ , MS Pure error=4.017667

Factor	SS	df	MS	F	p
1. pH (L)	0.0024	1	0.00242	0.00060	0.981375
pH (Q)	3.6588	1	3.65882	0.91068	0.383758
2. T (L)	1.4528	1	1.45284	0.36161	0.573834
T (Q)	3.9183	1	3.91833	0.97528	0.368717
3. ES (L)	75.5562	1	75.55620	18.80599	0.007453
ES (Q)	1.2256	1	1.22560	0.30505	0.604511
1L by 2L	15.6800	1	15.68000	3.90276	0.105176
1L by 3L	1.2800	1	1.28000	0.31859	0.596834
2L by 3L	0.4050	1	0.40500	0.10080	0.763696
Lack of fit	58.9967	5	11.79935	2.93687	0.131041
Pure error	20.0883	5	4.01767		
Total SS	183.1080	19			

ANOVA  $SY_{t=6hrs}$

$R^2=0.48942$ , Adj- $R^2=0.02989$ , MS Pure error=5.130667

Factor	SS	df	MS	F	p
1. pH (L)	1.6774	1	1.67743	0.326942	0.592210
pH (Q)	4.0812	1	4.08121	0.795453	0.413314
2. T (L)	5.2169	1	5.21686	1.016800	0.359558
T (Q)	2.1592	1	2.15924	0.420849	0.545127
3. ES (L)	39.2571	1	39.25712	7.651465	0.039546
ES (Q)	2.3610	1	2.36096	0.460167	0.527665
1L by 2L	0.0012	1	0.00125	0.000244	0.988150
1L by 3L	0.6613	1	0.66125	0.128882	0.734256
2L by 3L	4.6513	1	4.65125	0.906559	0.384754
Lack of fit	37.9068	5	7.58135	1.477654	0.339383
Pure error	25.6533	5	5.13067		
Total SS	124.4855	19			

Table D-2 ANOVA tables for Supernatant dissolved solids (*DS*) responses

ANOVA $DS_{t=0hrs}$						ANOVA $DS_{t=1.5hrs}$					
$R^2=.38066$ , Adj- $R^2=0$ , MS Pure error=.036117						$R^2=.68886$ , Adj- $R^2=.40883$ , MS Pure error=.026524					
Factor	SS	df	MS	F	p	Factor	SS	df	MS	F	p
1. pH (L)	0.010856	1	0.010856	0.300585	0.607095	1. pH (L)	0.042831	1	0.042831	1.614815	0.259730
pH (Q)	0.004051	1	0.004051	0.112177	0.751274	pH (Q)	0.009597	1	0.009597	0.361820	0.573727
2. T (L)	0.069420	1	0.069420	1.922080	0.224266	2. T (L)	0.000005	1	0.000005	0.000200	0.989269
T (Q)	0.009190	1	0.009190	0.254444	0.635401	T (Q)	0.023211	1	0.023211	0.875087	0.392494
3. ES (L)	0.029297	1	0.029297	0.811168	0.409057	3. ES (L)	0.303814	1	0.303814	11.454356	0.019580
ES (Q)	0.005464	1	0.005464	0.151293	0.713315	ES (Q)	0.026588	1	0.026588	1.002422	0.362686
1L by 2L	0.040186	1	0.040186	1.112663	0.339784	1L by 2L	0.065703	1	0.065703	2.477132	0.176326
1L by 3L	0.000406	1	0.000406	0.011245	0.919673	1L by 3L	0.000561	1	0.000561	0.021155	0.890038
2L by 3L	0.032131	1	0.032131	0.889638	0.388883	2L by 3L	0.001770	1	0.001770	0.066737	0.806445
Lack of fit	0.148705	5	0.029741	0.823461	0.581795	Lack of fit	0.083981	5	0.016796	0.633251	0.685836
Pure error	0.180585	5	0.036117			Pure error	0.132619	5	0.026524		
Total SS	0.531681	19				Total SS	0.696143	19			

ANOVA $DS_{t=3hrs}$						ANOVA $DS_{t=4.5hrs}$					
$R^2=.21095$ , Adj- $R^2=0$ , MS Pure error=.057989						$R^2=.43012$ , Adj- $R^2=0$ , MS Pure error=.050623					
Factor	SS	df	MS	F	p	Factor	SS	df	MS	F	p
1. pH (L)	0.006735	1	0.006735	0.116135	0.747117	1. pH (L)	0.002892	1	0.002892	0.057121	0.820593
pH (Q)	0.034278	1	0.034278	0.591113	0.476716	pH (Q)	0.010760	1	0.010760	0.212549	0.664136
2. T (L)	0.000039	1	0.000039	0.000668	0.980375	2. T (L)	0.003749	1	0.003749	0.074062	0.796393
T (Q)	0.023025	1	0.023025	0.397066	0.556280	T (Q)	0.003533	1	0.003533	0.069788	0.802188
3. ES (L)	0.030172	1	0.030172	0.520309	0.503019	3. ES (L)	0.116105	1	0.116105	2.293548	0.190338
ES (Q)	0.004335	1	0.004335	0.074760	0.795463	ES (Q)	0.015990	1	0.015990	0.315870	0.598360
1L by 2L	0.049770	1	0.049770	0.858273	0.396739	1L by 2L	0.138338	1	0.138338	2.732734	0.159219
1L by 3L	0.009591	1	0.009591	0.165396	0.701059	1L by 3L	0.025088	1	0.025088	0.495589	0.512869
2L by 3L	0.010878	1	0.010878	0.187590	0.682977	2L by 3L	0.008580	1	0.008580	0.169500	0.697611
Lack of fit	0.366598	5	0.073320	1.264378	0.401569	Lack of fit	0.181802	5	0.036360	0.718265	0.637314
Pure error	0.289944	5	0.057989			Pure error	0.253113	5	0.050623		
Total SS	0.832063	19				Total SS	0.763165	19			

ANOVA $DS_{t=6hrs}$					
$R^2=.28725$ , Adj- $R^2=0$ , MS Pure error=.023016					
Factor	SS	df	MS	F	p
1. pH (L)	0.000060	1	0.000060	0.002626	0.961118
pH (Q)	0.000201	1	0.000201	0.008743	0.929134
2. T (L)	0.004783	1	0.004783	0.207818	0.667599
T (Q)	0.003800	1	0.003800	0.165113	0.701299
3. ES (L)	0.024624	1	0.024624	1.069858	0.348388
ES (Q)	0.019323	1	0.019323	0.839532	0.401563
1L by 2L	0.018336	1	0.018336	0.796655	0.412986
1L by 3L	0.006328	1	0.006328	0.274940	0.622443
2L by 3L	0.000021	1	0.000021	0.000918	0.977003
Lack of fit	0.081628	5	0.016326	0.709302	0.642280
Pure error	0.115082	5	0.023016		
Total SS	0.275988	19			

Table D-3 ANOVA tables for Supernatant mass fraction ( $F_{SN}$ ) responsesANOVA  $F_{SN,t=0hrs}$  $R^2=.55814$ , Adj- $R^2=.16047$ , MS Pure error=1.139

Factor	SS	df	MS	F	p
1. pH (L)	0.31458	1	0.314579	0.276189	0.621675
pH (Q)	8.11925	1	8.119253	7.128404	0.044353
2. T (L)	0.22193	1	0.221934	0.194849	0.677346
T (Q)	0.05392	1	0.053919	0.047339	0.836360
3. ES (L)	0.38582	1	0.385824	0.338740	0.585812
ES (Q)	0.25064	1	0.250639	0.220052	0.658743
1L by 2L	3.92000	1	3.920000	3.441615	0.122732
1L by 3L	0.24500	1	0.245000	0.215101	0.662289
2L by 3L	0.00500	1	0.005000	0.004390	0.949742
Lack of fit	4.80600	5	0.961199	0.843898	0.571603
Pure error	5.69500	5	1.139000		
Total SS	23.76550	19			

ANOVA  $F_{SN,t=1.5hrs}$  $R^2=.56741$ , Adj- $R^2=.17809$ , MS Pure error=1.990667

Factor	SS	df	MS	F	p
1. pH (L)	0.76262	1	0.76262	0.38310	0.563057
pH (Q)	0.10180	1	0.10180	0.05114	0.830048
2. T (L)	0.58910	1	0.58910	0.29593	0.609814
T (Q)	0.00256	1	0.00256	0.00129	0.972765
3. ES (L)	23.06970	1	23.06970	11.58893	0.019162
ES (Q)	0.27080	1	0.27080	0.13603	0.727358
1L by 2L	0.32000	1	0.32000	0.16075	0.705026
1L by 3L	2.00000	1	2.00000	1.00469	0.362190
2L by 3L	0.02000	1	0.02000	0.01005	0.924053
Lack of fit	10.71109	5	2.14222	1.07613	0.468895
Pure error	9.95333	5	1.99067		
Total SS	47.76950	19			

ANOVA  $F_{SN,t=3hrs}$  $R^2=.7216$ , Adj- $R^2=.47104$ , MS Pure error=3.784

Factor	SS	df	MS	F	p
1. pH (L)	3.8054	1	3.80541	1.00566	0.361978
pH (Q)	0.0980	1	0.09797	0.02589	0.878465
2. T (L)	0.0186	1	0.01864	0.00492	0.946773
T (Q)	0.3129	1	0.31293	0.08270	0.785212
3. ES (L)	66.1402	1	66.14018	17.47891	0.008648
ES (Q)	0.5787	1	0.57870	0.15293	0.711856
1L by 2L	2.1012	1	2.10125	0.55530	0.489685
1L by 3L	3.5113	1	3.51125	0.92792	0.379645
2L by 3L	0.2812	1	0.28125	0.07433	0.796040
Lack of fit	10.7346	5	2.14692	0.56737	0.725474
Pure error	18.9200	5	3.78400		
Total SS	106.5180	19			

ANOVA  $F_{SN,t=4.5hrs}$  $R^2=.68132$ , Adj- $R^2=.39451$ , MS Pure error=4.035

Factor	SS	df	MS	F	p
1. pH (L)	0.9394	1	0.93937	0.23281	0.649834
pH (Q)	0.0737	1	0.07375	0.01828	0.897735
2. T (L)	0.4170	1	0.41701	0.10335	0.760851
T (Q)	3.7997	1	3.79965	0.94167	0.376416
3. ES (L)	74.8096	1	74.80959	18.54017	0.007673
ES (Q)	0.2916	1	0.29159	0.07227	0.798805
1L by 2L	2.8800	1	2.88000	0.71375	0.436748
1L by 3L	0.1800	1	0.18000	0.04461	0.841062
2L by 3L	0.0050	1	0.00500	0.00124	0.973281
Lack of fit	18.7189	5	3.74379	0.92783	0.531755
Pure error	20.1750	5	4.03500		
Total SS	122.0480	19			

ANOVA  $F_{SN,t=6hrs}$  $R^2=.60183$ , Adj- $R^2=.24348$ , MS Pure error=3.762667

Factor	SS	df	MS	F	p
1. pH (L)	0.5629	1	0.56292	0.14961	0.714827
pH (Q)	0.0023	1	0.00228	0.00061	0.981302
2. T (L)	0.3783	1	0.37825	0.10053	0.764008
T (Q)	8.2158	1	8.21584	2.18352	0.199542
3. ES (L)	65.0443	1	65.04430	17.28676	0.008843
ES (Q)	0.4248	1	0.42478	0.11289	0.750514
1L by 2L	0.0050	1	0.00500	0.00133	0.972332
1L by 3L	0.0050	1	0.00500	0.00133	0.972332
2L by 3L	1.2800	1	1.28000	0.34018	0.585039
Lack of fit	31.2705	5	6.25410	1.66215	0.295344
Pure error	18.8133	5	3.76267		
Total SS	125.7855	19			

Table D-4 ANOVA tables for Pellet-solids loading ( $SL_P$ ) responsesANOVA  $SL_{P,t=0hrs}$  $R^2=.21143$ , Adj- $R^2=0$ , MS Pure error=0.9096667

Factor	SS	df	MS	F	p
1. pH (L)	0.003783	1	0.003783	0.004158	0.951084
pH (Q)	0.000798	1	0.000798	0.000877	0.977520
2. T (L)	0.679122	1	0.679122	0.746561	0.427064
T (Q)	0.026394	1	0.026394	0.029015	0.871422
3. ES (L)	0.243262	1	0.243262	0.267419	0.627123
ES (Q)	0.001510	1	0.001510	0.001660	0.969075
1L by 2L	0.101250	1	0.101250	0.111305	0.752200
1L by 3L	0.281250	1	0.281250	0.309179	0.602148
2L by 3L	0.211250	1	0.211250	0.232228	0.650230
Lack of fit	1.231504	5	0.246301	0.270759	0.911037
Pure error	4.548333	5	0.909667		
Total SS	7.329500	19			

ANOVA  $SL_{P,t=3hrs}$  $R^2=.78259$ , Adj- $R^2=.58693$ , MS Pure error=0.9786667

Factor	SS	df	MS	F	p
1. pH (L)	0.36919	1	0.36919	0.37724	0.565953
pH (Q)	0.83197	1	0.83197	0.85010	0.398829
2. T (L)	2.04625	1	2.04625	2.09085	0.207816
T (Q)	0.00158	1	0.00158	0.00161	0.969531
3. ES (L)	10.22691	1	10.22691	10.44984	0.023138
ES (Q)	0.01141	1	0.01141	0.01166	0.918210
1L by 2L	0.36125	1	0.36125	0.36912	0.570015
1L by 3L	4.65125	1	4.65125	4.75264	0.081107
2L by 3L	0.36125	1	0.36125	0.36912	0.570015
Lack of fit	0.34355	5	0.06871	0.07021	0.994427
Pure error	4.89333	5	0.97867		
Total SS	24.08800	19			

ANOVA  $SL_{P,t=6hrs}$  $R^2=.72877$ , Adj- $R^2=.48466$ , MS Pure error=0.5696667

Factor	SS	df	MS	F	p
1. pH (L)	0.81951	1	0.819510	1.43858	0.284102
pH (Q)	1.01245	1	1.012455	1.77728	0.239996
2. T (L)	4.73433	1	4.734327	8.31070	0.034474
T (Q)	0.88191	1	0.881908	1.54811	0.268559
3. ES (L)	7.85750	1	7.857499	13.79315	0.013799
ES (Q)	0.01813	1	0.018126	0.03182	0.865428
1L by 2L	2.42000	1	2.420000	4.24810	0.094302
1L by 3L	0.84500	1	0.845000	1.48332	0.277588
2L by 3L	0.00500	1	0.005000	0.00878	0.928997
Lack of fit	4.03805	5	0.807610	1.41769	0.355532
Pure error	2.84833	5	0.569667		
Total SS	25.38950	19			

ANOVA  $SL_{P,t=1.5hrs}$  $R^2=.5476$ , Adj- $R^2=.14045$ , MS Pure error=1.498667

Factor	SS	df	MS	F	p
1. pH (L)	0.29422	1	0.294224	0.196324	0.676218
pH (Q)	1.47832	1	1.478317	0.986421	0.366221
2. T (L)	1.57400	1	1.573995	1.050264	0.352446
T (Q)	0.11796	1	0.117959	0.078710	0.790292
3. ES (L)	3.98504	1	3.985037	2.659055	0.163891
ES (Q)	0.00006	1	0.000063	0.000042	0.995094
1L by 2L	0.06125	1	0.061250	0.040870	0.847758
1L by 3L	3.78125	1	3.781250	2.523076	0.173058
2L by 3L	1.05125	1	1.051250	0.701457	0.440479
Lack of fit	2.66275	5	0.532550	0.355349	0.859658
Pure error	7.49333	5	1.498667		
Total SS	22.44950	19			

ANOVA  $SL_{P,t=4.5hrs}$  $R^2=.92236$ , Adj- $R^2=.85248$ , MS Pure error=0.2576667

Factor	SS	df	MS	F	p
1. pH (L)	0.18111	1	0.18111	0.70290	0.440039
pH (Q)	1.99318	1	1.99318	7.73550	0.038843
2. T (L)	2.47481	1	2.47481	9.60469	0.026879
T (Q)	1.15833	1	1.15833	4.49547	0.087478
3. ES (L)	11.29170	1	11.29170	43.82290	0.001184
ES (Q)	0.36784	1	0.36784	1.42759	0.285737
1L by 2L	0.10125	1	0.10125	0.39295	0.558259
1L by 3L	1.20125	1	1.20125	4.66203	0.083274
2L by 3L	1.20125	1	1.20125	4.66203	0.083274
Lack of fit	0.35125	5	0.07025	0.27264	0.909952
Pure error	1.28833	5	0.25767		
Total SS	21.11750	19			

Table D-5 ANOVA tables for Reducing sugars (RS) responses

ANOVA  $RS_{t=0hrs}$

$R^2=.4923$ , Adj- $R^2=.03536$ , MS Pure error=.1281672

Factor	SS	df	MS	F	p
1. pH (L)	0.000658	1	0.000658	0.005135	0.945649
pH (Q)	0.000961	1	0.000961	0.007499	0.934353
2. T (L)	0.318079	1	0.318079	2.481752	0.175993
T (Q)	0.032153	1	0.032153	0.250865	0.637732
3. ES (L)	0.369452	1	0.369452	2.882582	0.150299
ES (Q)	0.025767	1	0.025767	0.201043	0.672643
1L by 2L	0.186661	1	0.186661	1.456383	0.281482
1L by 3L	0.093745	1	0.093745	0.731423	0.431485
2L by 3L	0.145800	1	0.145800	1.137577	0.334934
Lack of fit	0.562810	5	0.112562	0.878243	0.554910
Pure error	0.640836	5	0.128167		
Total SS	2.370764	19			

ANOVA  $RS_{t=1.5hrs}$

$R^2=.82691$ , Adj- $R^2=.67112$ , MS Pure error=.1334678

Factor	SS	df	MS	F	p
1. pH (L)	0.081435	1	0.081435	0.61015	0.470081
pH (Q)	0.145820	1	0.145820	1.09255	0.343784
2. T (L)	0.727808	1	0.727808	5.45306	0.066779
T (Q)	0.000259	1	0.000259	0.00194	0.966578
3. ES (L)	3.844332	1	3.844332	28.80345	0.003022
ES (Q)	0.091998	1	0.091998	0.68929	0.444226
1L by 2L	0.226465	1	0.226465	1.69677	0.249477
1L by 3L	0.099013	1	0.099013	0.74185	0.428433
2L by 3L	0.076441	1	0.076441	0.57273	0.483292
Lack of fit	0.446175	5	0.089235	0.66859	0.665287
Pure error	0.667339	5	0.133468		
Total SS	6.432970	19			

ANOVA  $RS_{t=3hrs}$

$R^2=.80172$ , Adj- $R^2=.62327$ , MS Pure error=.2221272

Factor	SS	df	MS	F	p
1. pH (L)	0.224349	1	0.224349	1.01000	0.361031
pH (Q)	0.171572	1	0.171572	0.77241	0.419698
2. T (L)	0.503080	1	0.503080	2.26483	0.192679
T (Q)	0.015285	1	0.015285	0.06881	0.803537
3. ES (L)	4.389222	1	4.389222	19.75995	0.006732
ES (Q)	0.059267	1	0.059267	0.26682	0.627501
1L by 2L	0.183315	1	0.183315	0.82527	0.405301
1L by 3L	0.139656	1	0.139656	0.62872	0.463771
2L by 3L	0.110215	1	0.110215	0.49618	0.512629
Lack of fit	0.327519	5	0.065504	0.29489	0.896851
Pure error	1.110636	5	0.222127		
Total SS	7.253279	19			

ANOVA  $RS_{t=4.5hrs}$

$R^2=.88478$ , Adj- $R^2=.78108$ , MS Pure error=.1248346

Factor	SS	df	MS	F	p
1. pH (L)	0.274226	1	0.274226	2.19672	0.198403
pH (Q)	0.137867	1	0.137867	1.10440	0.341418
2. T (L)	0.559250	1	0.559250	4.47993	0.087885
T (Q)	0.288436	1	0.288436	2.31055	0.188973
3. ES (L)	5.879325	1	5.879325	47.09693	0.001004
ES (Q)	0.001094	1	0.001094	0.00877	0.929044
1L by 2L	0.111156	1	0.111156	0.89043	0.388689
1L by 3L	0.087571	1	0.087571	0.70150	0.440466
2L by 3L	0.057970	1	0.057970	0.46438	0.525863
Lack of fit	0.334594	5	0.066919	0.53606	0.744842
Pure error	0.624173	5	0.124835		
Total SS	8.321260	19			

ANOVA  $RS_{t=6hrs}$

$R^2=.87365$ , Adj- $R^2=.75993$ , MS Pure error=.0898647

Factor	SS	df	MS	F	p
1. pH (L)	0.560170	1	0.560170	6.23348	0.054711
pH (Q)	0.194592	1	0.194592	2.16538	0.201122
2. T (L)	0.301741	1	0.301741	3.35773	0.126375
T (Q)	0.142917	1	0.142917	1.59036	0.262915
3. ES (L)	5.871578	1	5.871578	65.33800	0.000470
ES (Q)	0.030055	1	0.030055	0.33444	0.588123
1L by 2L	0.170528	1	0.170528	1.89761	0.226813
1L by 3L	0.092020	1	0.092020	1.02399	0.358010
2L by 3L	0.002048	1	0.002048	0.02279	0.885906
Lack of fit	0.608684	5	0.121737	1.35467	0.373593
Pure error	0.449323	5	0.089865		
Total SS	8.373569	19			

Table D-6 ANOVA tables for Laminarin (*L*) responsesANOVA  $L_{t=0hrs}$  $R^2=.88885$ , Adj- $R^2=.78882$ , MS Pure error=.00306019

Factor	SS	df	MS	F	p
1. pH (L)	0.377323	1	0.377323	123.2331	0.000103
pH (Q)	0.022206	1	0.022206	7.2525	0.043141
2. T (L)	0.225479	1	0.225479	73.6411	0.000354
T (Q)	0.022407	1	0.022407	7.3179	0.042521
3. ES (L)	0.008854	1	0.008854	2.8916	0.149784
ES (Q)	0.004783	1	0.004783	1.5621	0.266673
1L by 2L	0.008321	1	0.008321	2.7175	0.160171
1L by 3L	0.005512	1	0.005512	1.8004	0.237377
2L by 3L	0.012168	1	0.012168	3.9740	0.102789
Lack of fit	0.069730	5	0.013946	4.5547	0.060812
Pure error	0.015309	5	0.003062		
Total SS	0.765091	19			

ANOVA  $L_{t=1.5hrs}$  $R^2=.9007$ , Adj- $R^2=.81134$ , MS Pure error=.0049299

Factor	SS	df	MS	F	p
1. pH (L)	0.766894	1	0.766894	155.5598	0.000059
pH (Q)	0.034314	1	0.034314	6.9604	0.046072
2. T (L)	0.156469	1	0.156469	31.7388	0.002443
T (Q)	0.042731	1	0.042731	8.6678	0.032103
3. ES (L)	0.000012	1	0.000012	0.0025	0.962242
ES (Q)	0.000045	1	0.000045	0.0092	0.927333
1L by 2L	0.000288	1	0.000288	0.0584	0.818612
1L by 3L	0.018050	1	0.018050	3.6613	0.113880
2L by 3L	0.008844	1	0.008844	1.7941	0.238090
Lack of fit	0.087968	5	0.017594	3.5687	0.094460
Pure error	0.024650	5	0.004930		
Total SS	1.134145	19			

ANOVA  $L_{t=3hrs}$  $R^2=.95949$ , Adj- $R^2=.92303$ , MS Pure error=.0026284

Factor	SS	df	MS	F	p
1. pH (L)	0.356201	1	0.356201	135.5199	0.000082
pH (Q)	0.036927	1	0.036927	14.0493	0.013318
2. T (L)	0.159953	1	0.159953	60.8556	0.000555
T (Q)	0.086931	1	0.086931	33.0737	0.002230
3. ES (L)	0.000001	1	0.000001	0.0005	0.983064
ES (Q)	0.000858	1	0.000858	0.3265	0.592476
1L by 2L	0.004465	1	0.004465	1.6988	0.249231
1L by 3L	0.002701	1	0.002701	1.0277	0.357222
2L by 3L	0.003081	1	0.003081	1.1722	0.328369
Lack of fit	0.014115	5	0.002823	1.0740	0.469731
Pure error	0.013142	5	0.002628		
Total SS	0.672855	19			

ANOVA  $L_{t=4.5hrs}$  $R^2=.87825$ , Adj- $R^2=.76868$ , MS Pure error=.0093223

Factor	SS	df	MS	F	p
1. pH (L)	0.319203	1	0.319203	34.24094	0.002065
pH (Q)	0.035302	1	0.035302	3.78689	0.109229
2. T (L)	0.091872	1	0.091872	9.85511	0.025687
T (Q)	0.060651	1	0.060651	6.50600	0.051223
3. ES (L)	0.015095	1	0.015095	1.61927	0.259156
ES (Q)	0.000551	1	0.000551	0.05911	0.817569
1L by 2L	0.001540	1	0.001540	0.16521	0.701218
1L by 3L	0.000630	1	0.000630	0.06759	0.805240
2L by 3L	0.010585	1	0.010585	1.13547	0.335340
Lack of fit	0.026417	5	0.005283	0.56675	0.725852
Pure error	0.046611	5	0.009322		
Total SS	0.599835	19			

ANOVA  $L_{t=6hrs}$  $R^2=.88864$ , Adj- $R^2=.78841$ , MS Pure error=.0046611

Factor	SS	df	MS	F	p
1. pH (L)	0.274780	1	0.274780	58.95222	0.000597
pH (Q)	0.079603	1	0.079603	17.07829	0.009062
2. T (L)	0.165589	1	0.165589	35.52604	0.001902
T (Q)	0.009135	1	0.009135	1.95993	0.220411
3. ES (L)	0.025281	1	0.025281	5.42386	0.067302
ES (Q)	0.002372	1	0.002372	0.50891	0.507514
1L by 2L	0.000780	1	0.000780	0.16737	0.699394
1L by 3L	0.066795	1	0.066795	14.33044	0.012817
2L by 3L	0.010878	1	0.010878	2.33383	0.187126
Lack of fit	0.056309	5	0.011262	2.41614	0.177562
Pure error	0.023305	5	0.004661		
Total SS	0.714916	19			

Table D-7 ANOVA tables for Inorganic sulfates (S) responses

ANOVA  $S_{t=0hrs}$  $R^2=.90205$ , Adj- $R^2=.81389$ , MS Pure error=.0038747

Factor	SS	df	MS	F	p
1. pH (L)	0.105696	1	0.105696	27.27861	0.003402
pH (Q)	0.102577	1	0.102577	26.47372	0.003629
2. T (L)	0.127082	1	0.127082	32.79825	0.002272
T (Q)	0.051527	1	0.051527	13.29844	0.014799
3. ES (L)	0.045185	1	0.045185	11.66154	0.018943
ES (Q)	0.011422	1	0.011422	2.94775	0.146646
1L by 2L	0.000450	1	0.000450	0.11614	0.747113
1L by 3L	0.041761	1	0.041761	10.77783	0.021883
2L by 3L	0.010513	1	0.010513	2.71314	0.160442
Lack of fit	0.032217	5	0.006443	1.66297	0.295164
Pure error	0.019373	5	0.003875		
Total SS	0.526693	19			

ANOVA  $S_{t=1.5hrs}$  $R^2=.7419$ , Adj- $R^2=.5096$ , MS Pure error=.004694

Factor	SS	df	MS	F	p
1. pH (L)	0.091664	1	0.091664	19.52788	0.006898
pH (Q)	0.002037	1	0.002037	0.43406	0.539128
2. T (L)	0.047246	1	0.047246	10.06528	0.024743
T (Q)	0.005880	1	0.005880	1.25261	0.313919
3. ES (L)	0.037224	1	0.037224	7.93020	0.037281
ES (Q)	0.001138	1	0.001138	0.24238	0.643347
1L by 2L	0.009730	1	0.009730	2.07289	0.209480
1L by 3L	0.045451	1	0.045451	9.68281	0.026499
2L by 3L	0.000903	1	0.000903	0.19240	0.679232
Lack of fit	0.060041	5	0.012008	2.55822	0.162874
Pure error	0.023470	5	0.004694		
Total SS	0.323558	19			

ANOVA  $S_{t=3hrs}$  $R^2=.55292$ , Adj- $R^2=.15054$ , MS Pure error=.0141543

Factor	SS	df	MS	F	p
1. pH (L)	0.075537	1	0.075537	8.664850	0.032122
pH (Q)	0.000845	1	0.000845	0.096891	0.768153
2. T (L)	0.074486	1	0.074486	8.544297	0.032897
T (Q)	0.000010	1	0.000010	0.001138	0.974400
3. ES (L)	0.004011	1	0.004011	0.460111	0.527689
ES (Q)	0.006345	1	0.006345	0.727783	0.432560
1L by 2L	0.003961	1	0.003961	0.454311	0.530193
1L by 3L	0.000392	1	0.000392	0.044967	0.840439
2L by 3L	0.000288	1	0.000288	0.033037	0.862910
Lack of fit	0.075640	5	0.015128	1.735335	0.279967
Pure error	0.043588	5	0.008718		
Total SS	0.285629	19			

ANOVA  $S_{t=4.5hrs}$  $R^2=.83106$ , Adj- $R^2=.67901$ , MS Pure error=.0072843

Factor	SS	df	MS	F	p
1. pH (L)	0.187034	1	0.187034	25.67627	0.003876
pH (Q)	0.030870	1	0.030870	4.23795	0.094598
2. T (L)	0.053755	1	0.053755	7.37962	0.041947
T (Q)	0.003268	1	0.003268	0.44863	0.532670
3. ES (L)	0.074453	1	0.074453	10.22098	0.024075
ES (Q)	0.001578	1	0.001578	0.21656	0.661237
1L by 2L	0.003872	1	0.003872	0.53155	0.498658
1L by 3L	0.000085	1	0.000085	0.01160	0.918418
2L by 3L	0.000181	1	0.000181	0.02478	0.881078
Lack of fit	0.036450	5	0.007290	1.00077	0.499672
Pure error	0.036422	5	0.007284		
Total SS	0.431332	19			

ANOVA  $S_{t=6hrs}$  $R^2=.86139$ , Adj- $R^2=.73663$ , MS Pure error=.0104282

Factor	SS	df	MS	F	p
1. pH (L)	0.220670	1	0.220670	21.16094	0.005840
pH (Q)	0.058674	1	0.058674	5.62648	0.063793
2. T (L)	0.032337	1	0.032337	3.10089	0.138557
T (Q)	0.015405	1	0.015405	1.47723	0.278461
3. ES (L)	0.116382	1	0.116382	11.16039	0.020536
ES (Q)	0.009738	1	0.009738	0.93386	0.378246
1L by 2L	0.001891	1	0.001891	0.18135	0.687926
1L by 3L	0.017020	1	0.017020	1.63213	0.257510
2L by 3L	0.000496	1	0.000496	0.04758	0.835960
Lack of fit	0.024339	5	0.004868	0.46679	0.788588
Pure error	0.052141	5	0.010428		
Total SS	0.551744	19			



Table D-8 ANOVA tables for Total phenolics (*TP*) responsesANOVA  $TP_{t=0hrs}$  $R^2=0.78006$ , Adj- $R^2=0.58211$ , MS Pure error= $0.0002818$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.002253	1	0.002253	7.99577	0.036775
pH (Q)	0.000146	1	0.000146	0.51824	0.503827
2. T (L)	0.004773	1	0.004773	16.94016	0.009211
T (Q)	0.000218	1	0.000218	0.77406	0.419234
3. ES (L)	0.000111	1	0.000111	0.39528	0.557138
ES (Q)	0.000617	1	0.000617	2.18890	0.199076
1L by 2L	0.000512	1	0.000512	1.81711	0.235507
1L by 3L	0.000684	1	0.000684	2.42932	0.179825
2L by 3L	0.000012	1	0.000012	0.04436	0.841494
Lack of fit	0.001182	5	0.000236	0.83922	0.573919
Pure error	0.001409	5	0.000282		
Total SS	0.011781	19			

ANOVA  $TP_{t=1.5hrs}$  $R^2=0.688$ , Adj- $R^2=0.4072$ , MS Pure error= $0.0002031$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.001963	1	0.001963	9.66453	0.026587
pH (Q)	0.000442	1	0.000442	2.17620	0.200177
2. T (L)	0.003375	1	0.003375	16.61596	0.009575
T (Q)	0.001023	1	0.001023	5.03936	0.074758
3. ES (L)	0.000150	1	0.000150	0.73897	0.429272
ES (Q)	0.000034	1	0.000034	0.16677	0.699901
1L by 2L	0.000990	1	0.000990	4.87506	0.078306
1L by 3L	0.000276	1	0.000276	1.35955	0.296204
2L by 3L	0.000015	1	0.000015	0.07447	0.795847
Lack of fit	0.002797	5	0.000559	2.75461	0.145182
Pure error	0.001016	5	0.000203		
Total SS	0.012221	19			

ANOVA  $TP_{t=3hrs}$  $R^2=0.88483$ , Adj- $R^2=0.78117$ , MS Pure error= $0.00009$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.006280	1	0.006280	69.78040	0.000402
pH (Q)	0.000262	1	0.000262	2.91080	0.148701
2. T (L)	0.002178	1	0.002178	24.19633	0.004401
T (Q)	0.000325	1	0.000325	3.61589	0.115633
3. ES (L)	0.000045	1	0.000045	0.49748	0.512103
ES (Q)	0.000012	1	0.000012	0.13110	0.732093
1L by 2L	0.000861	1	0.000861	9.56806	0.027060
1L by 3L	0.000300	1	0.000300	3.33472	0.127402
2L by 3L	0.000120	1	0.000120	1.33472	0.300174
Lack of fit	0.000910	5	0.000182	2.02249	0.228993
Pure error	0.000450	5	0.000090		
Total SS	0.011809	19			

ANOVA  $TP_{t=4.5hrs}$  $R^2=0.75979$ , Adj- $R^2=0.5436$ , MS Pure error= $0.0000527$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.003103	1	0.003103	58.88359	0.000599
pH (Q)	0.001339	1	0.001339	25.40995	0.003964
2. T (L)	0.002814	1	0.002814	53.40070	0.000752
T (Q)	0.000123	1	0.000123	2.33484	0.187047
3. ES (L)	0.001754	1	0.001754	33.28332	0.002199
ES (Q)	0.000229	1	0.000229	4.33752	0.091750
1L by 2L	0.000001	1	0.000001	0.02135	0.889545
1L by 3L	0.000136	1	0.000136	2.58302	0.168926
2L by 3L	0.000006	1	0.000006	0.11622	0.747025
Lack of fit	0.002686	5	0.000537	10.19331	0.011741
Pure error	0.000264	5	0.000053		
Total SS	0.012279	19			

ANOVA  $TP_{t=6hrs}$  $R^2=0.84775$ , Adj- $R^2=0.71073$ , MS Pure error= $0.0001447$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.007566	1	0.007566	52.30178	0.000789
pH (Q)	0.000055	1	0.000055	0.38258	0.563314
2. T (L)	0.001612	1	0.001612	11.14121	0.020601
T (Q)	0.000100	1	0.000100	0.69244	0.443249
3. ES (L)	0.000060	1	0.000060	0.41244	0.549017
ES (Q)	0.000011	1	0.000011	0.07518	0.794908
1L by 2L	0.000450	1	0.000450	3.11060	0.138066
1L by 3L	0.000005	1	0.000005	0.03111	0.866925
2L by 3L	0.000032	1	0.000032	0.22120	0.657929
Lack of fit	0.001056	5	0.000211	1.45941	0.344193
Pure error	0.000723	5	0.000145		
Total SS	0.011685	19			

Table D-9 ANOVA tables for Antioxidant capacity (AO) responses

ANOVA  $AO_{t=0hrs}$  $R^2=.5123$ , Adj- $R^2=.07337$ , MS Pure error= $0.01076$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.000200	1	0.000200	0.018596	0.896852
pH (Q)	0.023929	1	0.023929	2.223926	0.196086
2. T (L)	0.027286	1	0.027286	2.535915	0.172160
T (Q)	0.010202	1	0.010202	0.948138	0.374914
3. ES (L)	0.003632	1	0.003632	0.337589	0.586429
ES (Q)	0.000000	1	0.000000	0.000011	0.997495
1L by 2L	0.010513	1	0.010513	0.976998	0.368329
1L by 3L	0.006613	1	0.006613	0.614545	0.468573
2L by 3L	0.002113	1	0.002113	0.196329	0.676214
Lack of fit	0.024474	5	0.004895	0.454903	0.796180
Pure error	0.053800	5	0.010760		
Total SS	0.160495	19			

ANOVA  $AO_{t=1.5hrs}$  $R^2=.81771$ , Adj- $R^2=.65366$ , MS Pure error= $0.00055$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.000001	1	0.000001	0.0010	0.976105
pH (Q)	0.076088	1	0.076088	138.3422	0.000078
2. T (L)	0.004881	1	0.004881	8.8742	0.030837
T (Q)	0.002142	1	0.002142	3.8941	0.105472
3. ES (L)	0.000087	1	0.000087	0.1589	0.706614
ES (Q)	0.007733	1	0.007733	14.0600	0.013298
1L by 2L	0.032513	1	0.032513	59.1136	0.000594
1L by 3L	0.003612	1	0.003612	6.5682	0.050472
2L by 3L	0.000613	1	0.000613	1.1136	0.339593
Lack of fit	0.025756	5	0.005151	9.3657	0.014111
Pure error	0.002750	5	0.000550		
Total SS	0.156380	19			

ANOVA  $AO_{t=3hrs}$  $R^2=.76127$ , Adj- $R^2=.54641$ , MS Pure error= $6.784$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.004761	1	0.004761	3.36102	0.126229
pH (Q)	0.023466	1	0.023466	16.56439	0.009635
2. T (L)	0.059250	1	0.059250	41.82377	0.001317
T (Q)	0.010368	1	0.010368	7.31879	0.042513
3. ES (L)	0.000458	1	0.000458	0.32333	0.594198
ES (Q)	0.000001	1	0.000001	0.00095	0.976560
1L by 2L	0.008450	1	0.008450	5.96471	0.058490
1L by 3L	0.014450	1	0.014450	10.20000	0.024164
2L by 3L	0.001800	1	0.001800	1.27059	0.310826
Lack of fit	0.032588	5	0.006518	4.60061	0.059681
Pure error	0.007083	5	0.001417		
Total SS	0.166175	19			

ANOVA  $AO_{t=4.5hrs}$  $R^2=.80688$ , Adj- $R^2=.63307$ , MS Pure error= $0.0047767$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.010498	1	0.010498	2.19766	0.198322
pH (Q)	0.037314	1	0.037314	7.81163	0.038222
2. T (L)	0.050167	1	0.050167	10.50246	0.022930
T (Q)	0.000349	1	0.000349	0.07309	0.797688
3. ES (L)	0.001070	1	0.001070	0.22409	0.655884
ES (Q)	0.000800	1	0.000800	0.16755	0.699242
1L by 2L	0.022050	1	0.022050	4.61619	0.084402
1L by 3L	0.011250	1	0.011250	2.35520	0.185454
2L by 3L	0.000800	1	0.000800	0.16748	0.699301
Lack of fit	0.008533	5	0.001707	0.35727	0.858448
Pure error	0.023883	5	0.004777		
Total SS	0.167855	19			

ANOVA  $AO_{t=6hrs}$  $R^2=.75005$ , Adj- $R^2=.5251$ , MS Pure error= $0.00396$ 

Factor	SS	df	MS	F	p
1. pH (L)	0.014001	1	0.014001	3.535549	0.118830
pH (Q)	0.037038	1	0.037038	9.353010	0.028158
2. T (L)	0.028064	1	0.028064	7.086916	0.044769
T (Q)	0.005135	1	0.005135	1.296662	0.306424
3. ES (L)	0.000225	1	0.000225	0.056867	0.820983
ES (Q)	0.009702	1	0.009702	2.450099	0.178291
1L by 2L	0.007813	1	0.007813	1.972854	0.219117
1L by 3L	0.001513	1	0.001513	0.381944	0.563625
2L by 3L	0.001513	1	0.001513	0.381944	0.563625
Lack of fit	0.013067	5	0.002613	0.659950	0.670261
Pure error	0.019800	5	0.003960		
Total SS	0.131495	19			

# Appendix E Response surface regression coefficients

Table E-1 Solubilised yield (SY) response surface regression coefficients (CI = 95 %)

Regression coefficients of $SY_{t=0hrs}$						
	Regr. C <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI
Mean	47.25267	0.726688	65.02474	0.000000	45.38466	49.12068
pH (L)	-0.15742	0.482141	-0.32651	0.757262	-1.39681	1.08196
pH (Q)	1.07555	0.469351	2.29156	0.070512	-0.13096	2.28205
T (L)	-0.09021	0.482141	-0.18710	0.858941	-1.32959	1.14918
T (Q)	0.08560	0.469351	0.18237	0.862454	-1.12091	1.29210
ES (L)	0.61741	0.482141	1.28055	0.256535	-0.62198	1.85679
ES (Q)	-0.16189	0.469351	-0.34492	0.744187	-1.36840	1.04461
1Lby2L	-1.35000	0.629947	-2.14304	0.084990	-2.96933	0.26933
1Lby3L	-0.12500	0.629947	-0.19843	0.850525	-1.74433	1.49433
2Lby3L	-0.25000	0.629947	-0.39686	0.707845	-1.86933	1.36933

Regression coefficients of $SY_{t=1.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	48.90839	0.736239	66.43007	0.000000	47.01583	50.80095
pH (L)	-0.42037	0.488478	-0.86057	0.428803	-1.67604	0.83530
pH (Q)	0.55783	0.475520	1.17310	0.293592	-0.66453	1.78020
T (L)	0.40804	0.488478	0.83534	0.441602	-0.84763	1.66372
T (Q)	0.50480	0.475520	1.06158	0.336989	-0.71756	1.72716
ES (L)	1.84056	0.488478	3.76796	0.013050	0.58489	3.09623
ES (Q)	-0.16695	0.475520	-0.35109	0.739830	-1.38931	1.05541
1Lby2L	-0.68750	0.638227	-1.07720	0.330594	-2.32811	0.95311
1Lby3L	0.61250	0.638227	0.95969	0.381286	-1.02811	2.25311
2Lby3L	0.56250	0.638227	0.88135	0.418475	-1.07811	2.20311

Regression coefficients of $SY_{t=3hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	49.92642	0.842184	59.28204	0.000000	47.76151	52.09132
pH (L)	0.15011	0.558770	0.26864	0.798942	-1.28626	1.58647
pH (Q)	0.49857	0.543948	0.91658	0.401410	-0.89969	1.89683
T (L)	0.82874	0.558770	1.48315	0.198144	-0.60762	2.26510
T (Q)	-0.47370	0.543948	-0.87086	0.423665	-1.87196	0.92456
ES (L)	1.95273	0.558770	3.49469	0.017383	0.51636	3.38909
ES (Q)	0.03895	0.543948	0.07161	0.945688	-1.35931	1.43721
1Lby2L	-0.55000	0.730068	-0.75335	0.485177	-2.42670	1.32670
1Lby3L	0.07500	0.730068	0.10273	0.922170	-1.80170	1.95170
2Lby3L	-0.37500	0.730068	-0.51365	0.629382	-2.25170	1.50170

Regression coefficients of $SY_{t=4.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	50.87113	0.817496	62.22799	0.000000	48.76969	52.97257
pH (L)	0.01331	0.542390	0.02453	0.981375	-1.38095	1.40757
pH (Q)	0.50387	0.528002	0.95430	0.383758	-0.85340	1.86114
T (L)	0.32616	0.542390	0.60134	0.573834	-1.06810	1.72042
T (Q)	-0.52143	0.528002	-0.98756	0.368717	-1.87871	0.83584
ES (L)	2.35212	0.542390	4.33659	0.007453	0.95786	3.74638
ES (Q)	-0.29162	0.528002	-0.55232	0.604511	-1.64890	1.06565
1Lby2L	-1.40000	0.708667	-1.97554	0.105176	-3.22169	0.42169
1Lby3L	0.40000	0.708667	0.56444	0.596834	-1.42169	2.22169
2Lby3L	0.22500	0.708667	0.31750	0.763696	-1.59669	2.04669

Regression coefficients of $SY_{t=6hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	51.21232	0.923816	55.43562	0.000000	48.83757	53.58706
pH (L)	0.35047	0.612931	0.57179	0.592210	-1.22512	1.92606
pH (Q)	0.53216	0.596672	0.89188	0.413314	-1.00163	2.06595
T (L)	0.61806	0.612931	1.00836	0.359558	-0.95753	2.19365
T (Q)	-0.38708	0.596672	-0.64873	0.545127	-1.92087	1.14672
ES (L)	1.69545	0.612931	2.76613	0.039546	0.11986	3.27103
ES (Q)	-0.40476	0.596672	-0.67836	0.527665	-1.93855	1.12904
1Lby2L	0.01250	0.800833	0.01561	0.988150	-2.04611	2.07111
1Lby3L	0.28750	0.800833	0.35900	0.734256	-1.77111	2.34611
2Lby3L	-0.76250	0.800833	-0.95213	0.384754	-2.82111	1.29611

<sup>1</sup>Regression coefficient

<sup>2</sup>Standard error – pure error

<sup>3</sup>Confidence interval

Table E-2 Supernatant dissolved solids ( $DS$ ) response surface regression coefficients (CI = 95 %)

Regression coefficients of $DS_{t=0hrs}$						
	Regr. C <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI
Mean	3.261454	0.077509	42.07812	0.000000	3.062209	3.460698
pH (L)	0.028194	0.051426	0.54826	0.607095	-0.104000	0.160389
pH (Q)	0.016767	0.050062	0.33493	0.751274	-0.111920	0.145454
T (L)	-0.071296	0.051426	-1.38639	0.224266	-0.203490	0.060898
T (Q)	0.025252	0.050062	0.50442	0.635401	-0.103435	0.153940
ES (L)	0.046317	0.051426	0.90065	0.409057	-0.085878	0.178511
ES (Q)	-0.019472	0.050062	-0.38896	0.713315	-0.148160	0.109215
1Lby2L	-0.070875	0.067191	-1.05483	0.339784	-0.243595	0.101845
1Lby3L	-0.007125	0.067191	-0.10604	0.919673	-0.179845	0.165595
2Lby3L	-0.063375	0.067191	-0.94321	0.388883	-0.236095	0.109345

Regression coefficients of $DS_{t=1.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	3.529197	0.066423	53.13233	0.000000	3.358452	3.699942
pH (L)	-0.056002	0.044070	-1.27075	0.259730	-0.169288	0.057283
pH (Q)	-0.025806	0.042901	-0.60151	0.573727	-0.136086	0.084475
T (L)	-0.000623	0.044070	-0.01413	0.989269	-0.113908	0.112663
T (Q)	0.040132	0.042901	0.93546	0.392494	-0.070148	0.150413
ES (L)	0.149152	0.044070	3.38443	0.019580	0.035866	0.262437
ES (Q)	-0.042953	0.042901	-1.00121	0.362686	-0.153233	0.067328
1Lby2L	-0.090625	0.057580	-1.57389	0.176326	-0.238640	0.057390
1Lby3L	-0.008375	0.057580	-0.14545	0.890038	-0.156390	0.139640
2Lby3L	-0.014875	0.057580	-0.25834	0.806445	-0.162890	0.133140

Regression coefficients of $DS_{t=3hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	3.520335	0.098213	35.84377	0.000000	3.267870	3.772801
pH (L)	-0.022206	0.065162	-0.34079	0.747117	-0.189711	0.145299
pH (Q)	0.048770	0.063434	0.76884	0.476716	-0.114291	0.211832
T (L)	-0.001685	0.065162	-0.02585	0.980375	-0.169190	0.165820
T (Q)	-0.039972	0.063434	-0.63013	0.556280	-0.203033	0.123090
ES (L)	0.047003	0.065162	0.72132	0.503019	-0.120502	0.214508
ES (Q)	-0.017344	0.063434	-0.27342	0.795463	-0.180406	0.145717
1Lby2L	-0.078875	0.085139	-0.92643	0.396739	-0.297731	0.139981
1Lby3L	-0.034625	0.085139	-0.40669	0.701059	-0.253481	0.184231
2Lby3L	-0.036875	0.085139	-0.43312	0.682977	-0.255731	0.181981

Regression coefficients of $DS_{t=4.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	3.560796	0.091764	38.80399	0.000000	3.324910	3.796682
pH (L)	-0.014551	0.060883	-0.23900	0.820593	-0.171056	0.141954
pH (Q)	0.027324	0.059268	0.46103	0.664136	-0.125029	0.179678
T (L)	-0.016569	0.060883	-0.27214	0.796393	-0.173074	0.139936
T (Q)	0.015657	0.059268	0.26417	0.802188	-0.136696	0.168010
ES (L)	0.092204	0.060883	1.51445	0.190338	-0.064301	0.248709
ES (Q)	-0.033310	0.059268	-0.56202	0.598360	-0.185663	0.119043
1Lby2L	-0.131500	0.079548	-1.65310	0.159219	-0.335984	0.072984
1Lby3L	-0.056000	0.079548	-0.70398	0.512869	-0.260484	0.148484
2Lby3L	-0.032750	0.079548	-0.41170	0.697611	-0.237234	0.171734

Regression coefficients of $DS_{t=6hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	3.595167	0.061875	58.10342	0.000000	3.436111	3.754222
pH (L)	-0.002104	0.041053	-0.05124	0.961118	-0.107633	0.103426
pH (Q)	-0.003737	0.039964	-0.09350	0.929134	-0.106467	0.098994
T (L)	0.018715	0.041053	0.45587	0.667599	-0.086815	0.124244
T (Q)	0.016239	0.039964	0.40634	0.701299	-0.086491	0.118969
ES (L)	0.042463	0.041053	1.03434	0.348388	-0.063067	0.147992
ES (Q)	-0.036617	0.039964	-0.91626	0.401563	-0.139348	0.066113
1Lby2L	-0.047875	0.053638	-0.89256	0.412986	-0.185756	0.090006
1Lby3L	-0.028125	0.053638	-0.52435	0.622443	-0.166006	0.109756
2Lby3L	-0.001625	0.053638	-0.03030	0.977003	-0.139506	0.136256

<sup>1</sup>Regression coefficient<sup>2</sup>Standard error – pure error<sup>3</sup>Confidence interval

Table E-3 Supernatant mass fraction ( $F_{SN}$ ) response surface regression coefficients (CI = 95 %)

Regression coefficients of  $F_{SN,t=0hrs}$

	Regr. C. <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI
Mean	78.44064	0.435272	180.2107	0.000000	77.32174	79.55954
pH (L)	-0.15177	0.288793	-0.5255	0.621675	-0.89414	0.59059
pH (Q)	0.75060	0.281132	2.6699	0.044353	0.02792	1.47327
T (L)	-0.12748	0.288793	-0.4414	0.677346	-0.86984	0.61489
T (Q)	0.06117	0.281132	0.2176	0.836360	-0.66151	0.78384
ES (L)	0.16808	0.288793	0.5820	0.585812	-0.57428	0.91045
ES (Q)	0.13188	0.281132	0.4691	0.658743	-0.59079	0.85455
1Lby2L	-0.70000	0.377326	-1.8552	0.122732	-1.66995	0.26995
1Lby3L	0.17500	0.377326	0.4638	0.662289	-0.79495	1.14495
2Lby3L	-0.02500	0.377326	-0.0663	0.949742	-0.99495	0.94495

Regression coefficients of  $F_{SN,t=3hrs}$

	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	78.17115	0.793367	98.53087	0.000000	76.13174	80.21057
pH (L)	0.52787	0.526381	1.00283	0.361978	-0.82524	1.88097
pH (Q)	0.08245	0.512418	0.16091	0.878465	-1.23476	1.39966
T (L)	0.03694	0.526381	0.07018	0.946773	-1.31617	1.39005
T (Q)	-0.14736	0.512418	-0.28757	0.785212	-1.46457	1.16985
ES (L)	2.20068	0.526381	4.18078	0.008648	0.84758	3.55379
ES (Q)	-0.20039	0.512418	-0.39107	0.711856	-1.51760	1.11682
1Lby2L	-0.51250	0.687750	-0.74518	0.489685	-2.28042	1.25542
1Lby3L	-0.66250	0.687750	-0.96329	0.379645	-2.43042	1.10542
2Lby3L	-0.18750	0.687750	-0.27263	0.796040	-1.95542	1.58042

Regression coefficients of  $F_{SN,t=6hrs}$

	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	79.00641	0.791128	99.86557	0.000000	76.97275	81.04007
pH (L)	0.20302	0.524895	0.38679	0.714827	-1.14626	1.55231
pH (Q)	-0.01259	0.510971	-0.02463	0.981302	-1.32608	1.30091
T (L)	-0.16642	0.524895	-0.31706	0.764008	-1.51571	1.18286
T (Q)	-0.75505	0.510971	-1.47767	0.199542	-2.06854	0.55845
ES (L)	2.18237	0.524895	4.15773	0.008843	0.83309	3.53166
ES (Q)	-0.17169	0.510971	-0.33600	0.750514	-1.48518	1.14181
1Lby2L	0.02500	0.685809	0.03645	0.972332	-1.73793	1.78793
1Lby3L	0.02500	0.685809	0.03645	0.972332	-1.73793	1.78793
2Lby3L	-0.40000	0.685809	-0.58325	0.585039	-2.16293	1.36293

Regression coefficients of  $F_{SN,t=1.5hrs}$

	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	78.03490	0.575437	135.6098	0.000000	76.55569	79.51411
pH (L)	0.23631	0.381789	0.6189	0.563057	-0.74511	1.21773
pH (Q)	0.08405	0.371662	0.2261	0.830048	-0.87134	1.03943
T (L)	-0.20769	0.381789	-0.5440	0.609814	-1.18911	0.77373
T (Q)	0.01334	0.371662	0.0359	0.972765	-0.94205	0.96872
ES (L)	1.29971	0.381789	3.4043	0.019162	0.31829	2.28113
ES (Q)	0.13708	0.371662	0.3688	0.727358	-0.81831	1.09247
1Lby2L	-0.20000	0.498832	-0.4009	0.705026	-1.48229	1.08229
1Lby3L	-0.50000	0.498832	-1.0023	0.362190	-1.78229	0.78229
2Lby3L	0.05000	0.498832	0.1002	0.924053	-1.23229	1.33229

Regression coefficients of  $F_{SN,t=4.5hrs}$

	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	78.53660	0.819257	95.86315	0.000000	76.43063	80.64257
pH (L)	0.26227	0.543559	0.48250	0.649834	-1.13500	1.65953
pH (Q)	-0.07153	0.529140	-0.13519	0.897735	-1.43173	1.28866
T (L)	-0.17474	0.543559	-0.32148	0.760851	-1.57201	1.22252
T (Q)	-0.51348	0.529140	-0.97040	0.376416	-1.87367	0.84672
ES (L)	2.34047	0.543559	4.30583	0.007673	0.94321	3.73773
ES (Q)	-0.14225	0.529140	-0.26882	0.798805	-1.50244	1.21795
1Lby2L	-0.60000	0.710194	-0.84484	0.436748	-2.42561	1.22561
1Lby3L	-0.15000	0.710194	-0.21121	0.841062	-1.97561	1.67561
2Lby3L	0.02500	0.710194	0.03520	0.973281	-1.80061	1.85061

<sup>1</sup>Regression coefficient  
<sup>2</sup>Standard error – pure error  
<sup>3</sup>Confidence interval

Table E-4 Pellet-solids loading ( $SL_P$ ) response surface regression coefficients (CI = 95 %)

Regression coefficients of $SL_{P,t=0hrs}$							Regression coefficients of $SL_{P,t=1.5hrs}$						
	Regr. C. <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	13.52231	0.388991	34.76252	0.000000	12.52238	14.52225	Mean	13.17690	0.499288	26.39139	0.000001	11.89344	14.46036
pH (L)	-0.01664	0.258087	-0.06448	0.951084	-0.68008	0.64679	pH (L)	0.14678	0.331266	0.44308	0.676218	-0.70477	0.99833
pH (Q)	-0.00744	0.251241	-0.02962	0.977520	-0.65327	0.63839	pH (Q)	-0.32028	0.322479	-0.99319	0.366221	-1.14924	0.50868
T (L)	-0.22300	0.258087	-0.86404	0.427064	-0.88643	0.44044	T (L)	-0.33949	0.331266	-1.02482	0.352446	-1.19104	0.51206
T (Q)	-0.04280	0.251241	-0.17034	0.871422	-0.68863	0.60304	T (Q)	-0.09047	0.322479	-0.28055	0.790292	-0.91943	0.73849
ES (L)	0.13346	0.258087	0.51713	0.627123	-0.52997	0.79690	ES (L)	0.54018	0.331266	1.63066	0.163891	-0.31136	1.39173
ES (Q)	0.01024	0.251241	0.04075	0.969075	-0.63560	0.65607	ES (Q)	-0.00208	0.322479	-0.00646	0.995094	-0.83104	0.82687
1Lby2L	0.11250	0.337207	0.33362	0.752200	-0.75432	0.97932	1Lby2L	-0.08750	0.432820	-0.20216	0.847758	-1.20010	1.02510
1Lby3L	0.18750	0.337207	0.55604	0.602148	-0.67932	1.05432	1Lby3L	-0.68750	0.432820	-1.58842	0.173058	-1.80010	0.42510
2Lby3L	-0.16250	0.337207	-0.48190	0.650230	-1.02932	0.70432	2Lby3L	-0.36250	0.432820	-0.83753	0.440479	-1.47510	0.75010
Regression coefficients of $SL_{P,t=3hrs}$							Regression coefficients of $SL_{P,t=4.5hrs}$						
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	12.83043	0.403474	31.79985	0.000001	11.79326	13.86759	Mean	13.08163	0.207027	63.18793	0.000000	12.54945	13.61381
pH (L)	0.16442	0.267696	0.61420	0.565953	-0.52372	0.85255	pH (L)	0.11516	0.137358	0.83839	0.440039	-0.23793	0.46825
pH (Q)	-0.24027	0.260595	-0.92201	0.398829	-0.91015	0.42961	pH (Q)	-0.37190	0.133714	-2.78128	0.038843	-0.71562	-0.02817
T (L)	-0.38708	0.267696	-1.44598	0.207816	-1.07522	0.30105	T (L)	-0.42569	0.137358	-3.09914	0.026879	-0.77878	-0.07260
T (Q)	-0.01046	0.260595	-0.04015	0.969531	-0.68034	0.65942	T (Q)	-0.28351	0.133714	-2.12025	0.087478	-0.62723	0.06022
ES (L)	0.86536	0.267696	3.23262	0.023138	0.17723	1.55349	ES (L)	0.90929	0.137358	6.61989	0.001184	0.55620	1.26238
ES (Q)	-0.02814	0.260595	-0.10798	0.918210	-0.69802	0.64174	ES (Q)	-0.15976	0.133714	-1.19482	0.285737	-0.50349	0.18396
1Lby2L	-0.21250	0.349762	-0.60756	0.570015	-1.11159	0.68659	1Lby2L	-0.11250	0.179467	-0.62686	0.558259	-0.57383	0.34883
1Lby3L	-0.76250	0.349762	-2.18005	0.081107	-1.66159	0.13659	1Lby3L	-0.38750	0.179467	-2.15917	0.083274	-0.84883	0.07383
2Lby3L	-0.21250	0.349762	-0.60756	0.570015	-1.11159	0.68659	2Lby3L	-0.38750	0.179467	-2.15917	0.083274	-0.84883	0.07383
Regression coefficients of $SL_{P,t=6hrs}$													
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	12.87069	0.307829	41.81123	0.000000	12.07940	13.66199							
pH (L)	0.24496	0.204237	1.19941	0.284102	-0.28004	0.76997							
pH (Q)	-0.26506	0.198819	-1.33315	0.239996	-0.77614	0.24603							
T (L)	-0.58878	0.204237	-2.88283	0.034474	-1.11379	-0.06377							
T (Q)	-0.24738	0.198819	-1.24423	0.268559	-0.75846	0.26370							
ES (L)	0.75852	0.204237	3.71391	0.013799	0.23351	1.28353							
ES (Q)	0.03547	0.198819	0.17838	0.865428	-0.47562	0.54655							
1Lby2L	-0.55000	0.266849	-2.06109	0.094302	-1.23596	0.13596							
1Lby3L	-0.32500	0.266849	-1.21792	0.277588	-1.01096	0.36096							
2Lby3L	-0.02500	0.266849	-0.09369	0.928997	-0.71096	0.66096							

<sup>1</sup>Regression coefficient  
<sup>2</sup>Standard error – pure error  
<sup>3</sup>Confidence interval

Table E-5 Reducing sugars (*RS*) response surface regression coefficients (CI = 95 %)

Regression coefficients of $RS_{t=0hrs}$							Regression coefficients of $RS_{t=1.5hrs}$						
	Regr. C. <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	4.994996	0.146012	34.20961	0.000000	4.619662	5.370331	Mean	5.730535	0.149000	38.45992	0.000000	5.347518	6.113553
pH (L)	-0.006942	0.096875	-0.07166	0.945649	-0.255968	0.242084	pH (L)	0.077220	0.098858	0.78112	0.470081	-0.176903	0.331343
pH (Q)	0.008167	0.094305	0.08660	0.934353	-0.234253	0.250587	pH (Q)	-0.100591	0.096236	-1.04525	0.343784	-0.347973	0.146792
T (L)	0.152613	0.096875	1.57536	0.175993	-0.096413	0.401639	T (L)	0.230852	0.098858	2.33518	0.066779	-0.023271	0.484975
T (Q)	0.047234	0.094305	0.50086	0.637732	-0.195186	0.289654	T (Q)	0.004238	0.096236	0.04404	0.966578	-0.243144	0.251620
ES (L)	0.164477	0.096875	1.69782	0.150299	-0.084549	0.413502	ES (L)	0.530561	0.098858	5.36688	0.003022	0.276438	0.784684
ES (Q)	0.042285	0.094305	0.44838	0.672643	-0.200135	0.284705	ES (Q)	0.079899	0.096236	0.83024	0.444226	-0.167484	0.327281
1Lby2L	-0.152750	0.126574	-1.20681	0.281482	-0.478118	0.172618	1Lby2L	-0.168250	0.129165	-1.30260	0.249477	-0.500278	0.163778
1Lby3L	-0.108250	0.126574	-0.85523	0.431485	-0.433618	0.217118	1Lby3L	-0.111250	0.129165	-0.86130	0.428433	-0.443278	0.220778
2Lby3L	0.135000	0.126574	1.06657	0.334934	-0.190368	0.460368	2Lby3L	0.097750	0.129165	0.75679	0.483292	-0.234278	0.429778
Regression coefficients of $RS_{t=3hrs}$							Regression coefficients of $RS_{t=4.5hrs}$						
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	6.021005	0.192220	31.32344	0.000001	5.526886	6.515123	Mean	6.306442	0.144101	43.76413	0.000000	5.936020	6.676865
pH (L)	0.128170	0.127534	1.00499	0.361031	-0.199666	0.456006	pH (L)	0.141703	0.095608	1.48213	0.198403	-0.104064	0.387470
pH (Q)	-0.109112	0.124151	-0.87887	0.419698	-0.428252	0.210028	pH (Q)	-0.097809	0.093071	-1.05090	0.341418	-0.337057	0.141438
T (L)	0.191930	0.127534	1.50493	0.192679	-0.135906	0.519766	T (L)	0.202361	0.095608	2.11658	0.087885	-0.043406	0.448128
T (Q)	-0.032568	0.124151	-0.26232	0.803537	-0.351707	0.286572	T (Q)	-0.141473	0.093071	-1.52005	0.188973	-0.380720	0.097775
ES (L)	0.566916	0.127534	4.44522	0.006732	0.239079	0.894752	ES (L)	0.656128	0.095608	6.86272	0.001004	0.410361	0.901895
ES (Q)	0.064129	0.124151	0.51654	0.627501	-0.255010	0.383269	ES (Q)	-0.008714	0.093071	-0.09362	0.929044	-0.247961	0.230534
1Lby2L	-0.151375	0.166631	-0.90844	0.405301	-0.579714	0.276964	1Lby2L	-0.117875	0.124917	-0.94362	0.388689	-0.438985	0.203235
1Lby3L	-0.132125	0.166631	-0.79292	0.463771	-0.560464	0.296214	1Lby3L	-0.104625	0.124917	-0.83755	0.440466	-0.425735	0.216485
2Lby3L	0.117375	0.166631	0.70440	0.512629	-0.310964	0.545714	2Lby3L	0.085125	0.124917	0.68145	0.525863	-0.235985	0.406235
Regression coefficients of $RS_{t=6hrs}$													
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI							
Mean	6.440481	0.122262	52.67750	0.000000	6.126195	6.754767							
pH (L)	0.202528	0.081118	2.49669	0.054711	-0.005994	0.411049							
pH (Q)	-0.116201	0.078967	-1.47152	0.201122	-0.319191	0.086789							
T (L)	0.148642	0.081118	1.83241	0.126375	-0.059879	0.357164							
T (Q)	-0.099584	0.078967	-1.26109	0.262915	-0.302574	0.103406							
ES (L)	0.655695	0.081118	8.08319	0.000470	0.447174	0.864217							
ES (Q)	-0.045667	0.078967	-0.57831	0.588123	-0.248657	0.157323							
1Lby2L	-0.146000	0.105986	-1.37754	0.226813	-0.418446	0.126446							
1Lby3L	-0.107250	0.105986	-1.01192	0.358010	-0.379696	0.165196							
2Lby3L	0.016000	0.105986	0.15096	0.885906	-0.256446	0.288446							

<sup>1</sup>Regression coefficient<sup>2</sup>Standard error – pure error<sup>3</sup>Confidence interval



Table E-6 Laminarin (L) response surface regression coefficients (CI = 95 %)

Regression coefficients of $L_{t=0hrs}$							Regression coefficients of $L_{t=1.5hrs}$						
	Regr. C <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.416981	0.022568	18.4767	0.000009	0.358968	0.474993	Mean	0.424336	0.028636	14.8181	0.000025	0.350724	0.497948
pH (L)	-0.166219	0.014973	-11.1010	0.000103	-0.204709	-0.127729	pH (L)	-0.236969	0.019000	-12.4724	0.000059	-0.285809	-0.188130
pH (Q)	0.039254	0.014576	2.6930	0.043141	0.001785	0.076723	pH (Q)	0.048796	0.018496	2.6383	0.046072	0.001252	0.096341
T (L)	0.128493	0.014973	8.5814	0.000354	0.090002	0.166983	T (L)	0.107038	0.019000	5.6337	0.002443	0.058198	0.155878
T (Q)	0.039431	0.014576	2.7052	0.042521	0.001962	0.076900	T (Q)	0.054453	0.018496	2.9441	0.032103	0.006909	0.101997
ES (L)	0.025462	0.014973	1.7005	0.149784	-0.013028	0.063952	ES (L)	0.000945	0.019000	0.0498	0.962242	-0.047895	0.049785
ES (Q)	0.018218	0.014576	1.2498	0.266673	-0.019251	0.055687	ES (Q)	0.001774	0.018496	0.0959	0.927333	-0.045771	0.049318
1Lby2L	0.032250	0.019564	1.6485	0.160171	-0.018040	0.082540	1Lby2L	0.006000	0.024824	0.2417	0.818612	-0.057812	0.069812
1Lby3L	-0.026250	0.019564	-1.3418	0.237377	-0.076540	0.024040	1Lby3L	-0.047500	0.024824	-1.9135	0.113880	-0.111312	0.016312
2Lby3L	0.039000	0.019564	1.9935	0.102789	-0.011290	0.089290	2Lby3L	0.033250	0.024824	1.3394	0.238090	-0.030562	0.097062
Regression coefficients of $L_{t=3hrs}$							Regression coefficients of $L_{t=4.5hrs}$						
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.400719	0.020910	19.1644	0.000007	0.346970	0.454469	Mean	0.405333	0.039379	10.29325	0.000149	0.304107	0.506559
pH (L)	-0.161500	0.013873	-11.6413	0.000082	-0.197161	-0.125838	pH (L)	-0.152883	0.026127	-5.85158	0.002065	-0.220044	-0.085722
pH (Q)	0.050620	0.013505	3.7482	0.013318	0.015904	0.085336	pH (Q)	0.049494	0.025434	1.94599	0.109229	-0.015886	0.114873
T (L)	0.108223	0.013873	7.8010	0.000555	0.072562	0.143885	T (L)	0.082019	0.026127	3.13929	0.025687	0.014858	0.149180
T (Q)	0.077667	0.013505	5.7510	0.002230	0.042951	0.112383	T (Q)	0.064873	0.025434	2.55069	0.051223	-0.000506	0.130253
ES (L)	0.000310	0.013873	0.0223	0.983064	-0.035352	0.035971	ES (L)	0.033246	0.026127	1.27251	0.259156	-0.033914	0.100407
ES (Q)	-0.007716	0.013505	-0.5714	0.592476	-0.042432	0.026999	ES (Q)	0.006184	0.025434	0.24312	0.817569	-0.059196	0.071563
1Lby2L	0.023625	0.018126	1.3034	0.249231	-0.022969	0.070219	1Lby2L	-0.013875	0.034136	-0.40646	0.701218	-0.101625	0.073875
1Lby3L	-0.018375	0.018126	-1.0137	0.357222	-0.064969	0.028219	1Lby3L	-0.008875	0.034136	-0.25999	0.805240	-0.096625	0.078875
2Lby3L	0.019625	0.018126	1.0827	0.328369	-0.026969	0.066219	2Lby3L	0.036375	0.034136	1.06558	0.335340	-0.051375	0.124125
Regression coefficients of $L_{t=6hrs}$													
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI							
Mean	0.395519	0.027845	14.20449	0.000031	0.323942	0.467095							
pH (L)	-0.141846	0.018474	-7.67803	0.000597	-0.189336	-0.094356							
pH (Q)	0.074321	0.017984	4.13259	0.009062	0.028091	0.120551							
T (L)	0.110114	0.018474	5.96037	0.001902	0.062624	0.157603							
T (Q)	0.025177	0.017984	1.39997	0.220411	-0.021052	0.071407							
ES (L)	0.043025	0.018474	2.32892	0.067302	-0.004465	0.090515							
ES (Q)	-0.012830	0.017984	-0.71338	0.507514	-0.059059	0.033400							
1Lby2L	0.009875	0.024138	0.40911	0.699394	-0.052173	0.071923							
1Lby3L	-0.091375	0.024138	-3.78556	0.012817	-0.153423	-0.029327							
2Lby3L	0.036875	0.024138	1.52769	0.187126	-0.025173	0.098923							

<sup>1</sup>Regression coefficient  
<sup>2</sup>Standard error – pure error  
<sup>3</sup>Confidence interval

Table E-7 Inorganic sulfates (S) response surface regression coefficients (CI = 95 %)

Regression coefficients of $S_{t=0hrs}$							Regression coefficients of $S_{t=1.5hrs}$						
	Regr. C. <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.525686	0.025387	20.70670	0.000005	0.460426	0.590946	Mean	0.776721	0.027943	27.79680	0.000001	0.704892	0.848550
pH (L)	0.087974	0.016844	5.22289	0.003402	0.044675	0.131272	pH (L)	0.081926	0.018539	4.41904	0.006898	0.034269	0.129583
pH (Q)	0.084367	0.016397	5.14526	0.003629	0.042217	0.126517	pH (Q)	0.011890	0.018048	0.65884	0.539128	-0.034502	0.058283
T (L)	0.096464	0.016844	5.72698	0.002272	0.053166	0.139763	T (L)	0.058818	0.018539	3.17258	0.024743	0.011161	0.106475
T (Q)	0.059795	0.016397	3.64670	0.014799	0.017645	0.101945	T (Q)	0.020199	0.018048	1.11920	0.313919	-0.026194	0.066592
ES (L)	0.057520	0.016844	3.41490	0.018943	0.014222	0.100819	ES (L)	0.052208	0.018539	2.81606	0.037281	0.004551	0.099865
ES (Q)	0.028152	0.016397	1.71690	0.146646	-0.013998	0.070302	ES (Q)	0.008885	0.018048	0.49232	0.643347	-0.037508	0.055278
1Lby2L	0.007500	0.022008	0.34079	0.747113	-0.049072	0.064072	1Lby2L	-0.034875	0.024223	-1.43975	0.209480	-0.097142	0.027392
1Lby3L	-0.072250	0.022008	-3.28296	0.021883	-0.128822	-0.015678	1Lby3L	-0.075375	0.024223	-3.11172	0.026499	-0.137642	-0.013108
2Lby3L	-0.036250	0.022008	-1.64716	0.160442	-0.092822	0.020322	2Lby3L	-0.010625	0.024223	-0.43863	0.679232	-0.072892	0.051642
Regression coefficients of $S_{t=3hrs}$							Regression coefficients of $S_{t=4.5hrs}$						
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.813384	0.038080	21.35986	0.000004	0.715496	0.911272	Mean	0.894277	0.034809	25.69091	0.000002	0.804797	0.983757
pH (L)	0.074371	0.025265	2.94361	0.032122	0.009425	0.139317	pH (L)	0.117027	0.023095	5.06718	0.003876	0.057659	0.176394
pH (Q)	-0.007656	0.024595	-0.31127	0.768153	-0.070879	0.055568	pH (Q)	-0.046283	0.022482	-2.05863	0.094598	-0.104076	0.011510
T (L)	0.073852	0.025265	2.92306	0.032897	0.008906	0.138798	T (L)	0.062739	0.023095	2.71655	0.041947	0.003371	0.122106
T (Q)	0.000830	0.024595	0.03373	0.974400	-0.062394	0.064053	T (Q)	0.015059	0.022482	0.66980	0.532670	-0.042734	0.072851
ES (L)	0.017138	0.025265	0.67832	0.527689	-0.047809	0.082084	ES (L)	0.073835	0.023095	3.19703	0.024075	0.014468	0.133203
ES (Q)	0.020982	0.024595	0.85310	0.432560	-0.042241	0.084206	ES (Q)	0.010462	0.022482	0.46536	0.661237	-0.047330	0.068255
1Lby2L	0.022250	0.033011	0.67403	0.530193	-0.062606	0.107106	1Lby2L	0.022000	0.030175	0.72908	0.498658	-0.055568	0.099568
1Lby3L	-0.007000	0.033011	-0.21205	0.840439	-0.091856	0.077856	1Lby3L	-0.003250	0.030175	-0.10770	0.918418	-0.080818	0.074318
2Lby3L	-0.006000	0.033011	-0.18176	0.862910	-0.090856	0.078856	2Lby3L	0.004750	0.030175	0.15741	0.881078	-0.072818	0.082318
Regression coefficients of $S_{t=6hrs}$													
	Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI		Regr. C.	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.930245	0.041649	22.33545	0.000003	0.823183	1.037307							
pH (L)	0.127115	0.027633	4.60010	0.005840	0.056082	0.198148							
pH (Q)	-0.063807	0.026900	-2.37202	0.063793	-0.132956	0.005341							
T (L)	0.048660	0.027633	1.76093	0.138557	-0.022373	0.119693							
T (Q)	-0.032695	0.026900	-1.21541	0.278461	-0.101843	0.036454							
ES (L)	0.092314	0.027633	3.34072	0.020536	0.021281	0.163347							
ES (Q)	0.025995	0.026900	0.96636	0.378246	-0.043154	0.095144							
1Lby2L	0.015375	0.036104	0.42585	0.687926	-0.077434	0.108184							
1Lby3L	-0.046125	0.036104	-1.27755	0.257510	-0.138934	0.046684							
2Lby3L	0.007875	0.036104	0.21812	0.835960	-0.084934	0.100684							

<sup>1</sup>Regression coefficient<sup>2</sup>Standard error – pure error<sup>3</sup>Confidence interval

Table E-8 Total phenolics (*TP*) response surface regression coefficients (CI = 95 %)

Regression coefficients of $TP_{t=0hrs}$						
	Regr. C <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI
Mean	0.330653	0.006846	48.29796	0.000000	0.313054	0.348251
pH (L)	0.012844	0.004542	2.82768	0.036775	0.001168	0.024520
pH (Q)	0.003183	0.004422	0.71989	0.503827	-0.008183	0.014550
T (L)	0.018695	0.004542	4.11584	0.009211	0.007019	0.030371
T (Q)	0.003890	0.004422	0.87981	0.419234	-0.007476	0.015257
ES (L)	0.002856	0.004542	0.62871	0.557138	-0.008820	0.014532
ES (Q)	0.006542	0.004422	1.47949	0.199076	-0.004825	0.017908
1Lby2L	-0.008000	0.005935	-1.34800	0.235507	-0.023256	0.007256
1Lby3L	-0.009250	0.005935	-1.55863	0.179825	-0.024506	0.006006
2Lby3L	0.001250	0.005935	0.21063	0.841494	-0.014006	0.016506

Regression coefficients of $TP_{t=1.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.363130	0.005812	62.47536	0.000000	0.348189	0.378071
pH (L)	0.011989	0.003856	3.10878	0.026587	0.002076	0.021902
pH (Q)	-0.005538	0.003754	-1.47520	0.200177	-0.015188	0.004112
T (L)	0.015720	0.003856	4.07627	0.009575	0.005806	0.025633
T (Q)	0.008427	0.003754	2.24485	0.074758	-0.001223	0.018078
ES (L)	0.003315	0.003856	0.85963	0.429272	-0.006598	0.013228
ES (Q)	0.001533	0.003754	0.40837	0.699901	-0.008117	0.011183
1Lby2L	-0.011125	0.005039	-2.20795	0.078306	-0.024077	0.001827
1Lby3L	-0.005875	0.005039	-1.16600	0.296204	-0.018827	0.007077
2Lby3L	0.001375	0.005039	0.27289	0.795847	-0.011577	0.014327

Regression coefficients of $TP_{t=3hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.370084	0.003869	95.64909	0.000000	0.360138	0.380030
pH (L)	0.021444	0.002567	8.35347	0.000402	0.014845	0.028043
pH (Q)	-0.004264	0.002499	-1.70611	0.148701	-0.010688	0.002160
T (L)	0.012628	0.002567	4.91898	0.004401	0.006029	0.019227
T (Q)	0.004752	0.002499	1.90155	0.115633	-0.001672	0.011176
ES (L)	0.001811	0.002567	0.70532	0.512103	-0.004788	0.008410
ES (Q)	-0.000905	0.002499	-0.36208	0.732093	-0.007329	0.005519
1Lby2L	-0.010375	0.003354	-3.09323	0.027060	-0.018997	-0.001753
1Lby3L	-0.006125	0.003354	-1.82612	0.127402	-0.014747	0.002497
2Lby3L	0.003875	0.003354	1.15530	0.300174	-0.004747	0.012497

Regression coefficients of $TP_{t=4.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.363947	0.002961	122.9233	0.000000	0.356336	0.371558
pH (L)	0.015074	0.001964	7.6736	0.000599	0.010024	0.020124
pH (Q)	-0.009640	0.001912	-5.0408	0.003964	-0.014555	-0.004724
T (L)	0.014355	0.001964	7.3076	0.000752	0.009305	0.019405
T (Q)	-0.002922	0.001912	-1.5280	0.187047	-0.007838	0.001994
ES (L)	0.011333	0.001964	5.7692	0.002199	0.006283	0.016383
ES (Q)	-0.003983	0.001912	-2.0827	0.091750	-0.008898	0.000933
1Lby2L	-0.000375	0.002567	-0.1461	0.889545	-0.006973	0.006223
1Lby3L	-0.004125	0.002567	-1.6072	0.168926	-0.010723	0.002473
2Lby3L	-0.000875	0.002567	-0.3409	0.747025	-0.007473	0.005723

Regression coefficients of $TP_{t=6hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	0.368345	0.004905	75.08817	0.000000	0.355735	0.380955
pH (L)	0.023538	0.003255	7.23200	0.000789	0.015171	0.031904
pH (Q)	-0.001960	0.003168	-0.61853	0.563314	-0.010104	0.006185
T (L)	0.010864	0.003255	3.33784	0.020601	0.002497	0.019230
T (Q)	0.002636	0.003168	0.83213	0.443249	-0.005508	0.010781
ES (L)	0.002090	0.003255	0.64222	0.549017	-0.006276	0.010457
ES (Q)	0.000869	0.003168	0.27419	0.794908	-0.007276	0.009013
1Lby2L	-0.007500	0.004252	-1.76369	0.138066	-0.018431	0.003431
1Lby3L	-0.000750	0.004252	-0.17637	0.866925	-0.011681	0.010181
2Lby3L	-0.002000	0.004252	-0.47032	0.657929	-0.012931	0.008931

<sup>1</sup>Regression coefficient<sup>2</sup>Standard error – pure error<sup>3</sup>Confidence interval

Table E-9 Antioxidant capacity (AO) response surface regression coefficients (CI = 95 %)

Regression coefficients of $AO_{t=0hrs}$						
	Regr. C <sup>1</sup>	SE.PE. <sup>2</sup>	t(5)	p	-95% CI <sup>3</sup>	+95% CI
Mean	1.410555	0.042306	33.34150	0.000000	1.301803	1.519307
pH (L)	0.003828	0.028069	0.13637	0.896852	-0.068327	0.075982
pH (Q)	-0.040749	0.027325	-1.49128	0.196086	-0.110989	0.029491
T (L)	0.044699	0.028069	1.59246	0.172160	-0.027455	0.116853
T (Q)	-0.026607	0.027325	-0.97372	0.374914	-0.096847	0.043634
ES (L)	0.016309	0.028069	0.58102	0.586429	-0.055845	0.088463
ES (Q)	-0.000090	0.027325	-0.00330	0.997495	-0.070330	0.070150
1Lby2L	-0.036250	0.036674	-0.98843	0.368329	-0.130524	0.058024
1Lby3L	-0.028750	0.036674	-0.78393	0.468573	-0.123024	0.065524
2Lby3L	-0.016250	0.036674	-0.44309	0.676214	-0.110524	0.078024

Regression coefficients of $AO_{t=3hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	1.381530	0.015351	89.99694	0.000000	1.342069	1.420990
pH (L)	0.018672	0.010185	1.83331	0.126229	-0.007509	0.044853
pH (Q)	-0.040352	0.009915	-4.06994	0.009635	-0.065839	-0.014866
T (L)	0.065867	0.010185	6.46713	0.001317	0.039686	0.092049
T (Q)	0.026823	0.009915	2.70533	0.042513	0.001336	0.052309
ES (L)	0.005791	0.010185	0.56862	0.594198	-0.020390	0.031973
ES (Q)	0.000306	0.009915	0.03088	0.976560	-0.025181	0.025793
1Lby2L	-0.032500	0.013307	-2.44227	0.058490	-0.066707	0.001707
1Lby3L	-0.042500	0.013307	-3.19374	0.024164	-0.076707	-0.008293
2Lby3L	-0.015000	0.013307	-1.12720	0.310826	-0.049207	0.019207

Regression coefficients of $AO_{t=6hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	1.370724	0.025665	53.40770	0.000000	1.304750	1.436699
pH (L)	0.032018	0.017028	1.88031	0.118830	-0.011754	0.075791
pH (Q)	-0.050696	0.016577	-3.05827	0.028158	-0.093307	-0.008084
T (L)	0.045332	0.017028	2.66213	0.044769	0.001559	0.089104
T (Q)	-0.018876	0.016577	-1.13871	0.306424	-0.061488	0.023736
ES (L)	0.004061	0.017028	0.23847	0.820983	-0.039712	0.047833
ES (Q)	-0.025947	0.016577	-1.56528	0.178291	-0.068559	0.016665
1Lby2L	-0.031250	0.022249	-1.40458	0.219117	-0.088442	0.025942
1Lby3L	0.013750	0.022249	0.61802	0.563625	-0.043442	0.070942
2Lby3L	-0.013750	0.022249	-0.61802	0.563625	-0.070942	0.043442

Regression coefficients of $AO_{t=1.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	1.336110	0.009565	139.6890	0.000000	1.311523	1.360697
pH (L)	0.000200	0.006346	0.0315	0.976105	-0.016113	0.016513
pH (Q)	-0.072662	0.006178	-11.7619	0.000078	-0.088542	-0.056782
T (L)	0.018905	0.006346	2.9790	0.030837	0.002592	0.035218
T (Q)	0.012191	0.006178	1.9733	0.105472	-0.003690	0.028071
ES (L)	0.002530	0.006346	0.3986	0.706614	-0.013783	0.018843
ES (Q)	-0.023165	0.006178	-3.7497	0.013298	-0.039045	-0.007284
1Lby2L	-0.063750	0.008292	-7.6885	0.000594	-0.085064	-0.042436
1Lby3L	-0.021250	0.008292	-2.5628	0.050472	-0.042564	0.000064
2Lby3L	-0.008750	0.008292	-1.0553	0.339593	-0.030064	0.012564

Regression coefficients of $AO_{t=4.5hrs}$						
	Regr. C	Pure Err.	t(5)	p	-95% CI	+95% CI
Mean	1.411518	0.028188	50.07551	0.000000	1.339059	1.483977
pH (L)	0.027725	0.018702	1.48245	0.198322	-0.020350	0.075800
pH (Q)	-0.050884	0.018206	-2.79493	0.038222	-0.097684	-0.004084
T (L)	0.060608	0.018702	3.24075	0.022930	0.012533	0.108683
T (Q)	-0.004922	0.018206	-0.27036	0.797688	-0.051722	0.041877
ES (L)	-0.008853	0.018702	-0.47339	0.655884	-0.056928	0.039222
ES (Q)	0.007452	0.018206	0.40933	0.699242	-0.039347	0.054252
1Lby2L	-0.052500	0.024435	-2.14853	0.084402	-0.115313	0.010313
1Lby3L	-0.037500	0.024435	-1.53467	0.185454	-0.100313	0.025313
2Lby3L	-0.010000	0.024435	-0.40924	0.699301	-0.072813	0.052813

<sup>1</sup>Regression coefficient  
<sup>2</sup>Standard error – pure error  
<sup>3</sup>Confidence interval

# Appendix F Treatment comparison ANOVAs and Tukey HSD results

Table F-1 ANOVA: Single Factor for Solubilised yield (SY) treatment comparison test

DESCRIPTION					Alpha	0.05		
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	6	2.92	0.49	0.000807	0.004037	0.008103	0.470477	0.502499
V0	6	3.07	0.51	0.000439	0.002196	0.008103	0.496436	0.528458
V1.5	6	3.06	0.51	0.000253	0.001266	0.008103	0.493625	0.525646
V3	6	3.26	0.54	0.000290	0.001448	0.008103	0.526719	0.558740
V4.5	6	3.30	0.55	0.000296	0.001479	0.008103	0.533533	0.565554
V6	6	3.15	0.52	0.000114	0.000569	0.008103	0.508562	0.540584
VBR	6	2.97	0.49	0.000258	0.001288	0.008103	0.478903	0.510925
VB0	6	3.03	0.51	0.000134	0.000672	0.008103	0.489085	0.521107
VB1.5	6	2.96	0.49	0.000228	0.001139	0.008103	0.476568	0.508589
VB3	6	3.07	0.51	0.000111	0.000553	0.008103	0.496181	0.528202
VB4.5	6	2.97	0.49	0.000299	0.001496	0.008103	0.478669	0.510691
VB6	6	3.13	0.52	-0.000022	-0.000109	0.008103	0.506455	0.538477
VER	6	3.01	0.50	0.000564	0.002822	0.008103	0.485837	0.517858
VE0	6	2.99	0.50	0.000557	0.002787	0.008103	0.482057	0.514078
VE1.5	6	3.17	0.53	0.000178	0.000890	0.008103	0.511704	0.543726
VE3	6	3.22	0.54	0.000792	0.003958	0.008103	0.521142	0.553164
VE4.5	6	3.25	0.54	0.000979	0.004895	0.008103	0.524862	0.556883
VE6	6	3.31	0.55	0.000458	0.002288	0.008103	0.535836	0.567858
AR	6	3.05	0.51	0.001301	0.006505	0.008103	0.493016	0.525038
A0	6	2.96	0.49	0.000158	0.000790	0.008103	0.477773	0.509795
A1.5	6	3.20	0.53	0.000245	0.001225	0.008103	0.517060	0.549082
A3	6	3.20	0.53	0.000471	0.002357	0.008103	0.516961	0.548983
A4.5	6	3.32	0.55	0.000731	0.003656	0.008103	0.538074	0.570096
A6	6	3.32	0.55	0.000471	0.002357	0.008103	0.537185	0.569207
CR	6	3.02	0.50	0.000369	0.001846	0.008103	0.487758	0.519780
C0	6	3.44	0.57	0.000415	0.002076	0.008103	0.557006	0.589028
C1.5	6	3.36	0.56	0.000196	0.000982	0.008103	0.544571	0.576592
C3	6	3.42	0.57	0.000311	0.001556	0.008103	0.553596	0.585618
C4.5	6	3.32	0.55	0.000262	0.001309	0.008103	0.537097	0.569119
C6	6	3.36	0.56	0.000151	0.000757	0.008103	0.544046	0.576067

n = 180, df = 150,  $\sum SS = 0.05909$ ,  $Q_{crit} = 5.4074$ , MCD = 0.04382

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	4.949441	29	0.170670	433.2236	6.35E-130	1.543210	1.308752	0.985843
Within Groups	0.059093	150	0.000394					
Total	5.008534	179	0.027981					

Table F-2 ANOVA: Single Factor for Dissolved solids (DS) treatment comparison test

DESCRIPTION					Alpha		0.05	
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	6	0.19	0.03	3.11E-06	1.56E-05	0.000775	0.030801	0.033866
V0	6	0.21	0.03	8.57E-06	4.29E-05	0.000775	0.033184	0.036249
V1.5	6	0.21	0.03	6.59E-06	3.3E-05	0.000775	0.033001	0.036066
V3	6	0.22	0.04	3.01E-06	1.51E-05	0.000775	0.034684	0.037749
V4.5	6	0.21	0.04	8.83E-07	4.41E-06	0.000775	0.034101	0.037166
V6	6	0.22	0.04	1.23E-06	6.15E-06	0.000775	0.034834	0.037899
VBR	6	0.19	0.03	3.47E-08	1.73E-07	0.000775	0.030101	0.033166
VB0	6	0.20	0.03	2.83E-07	1.41E-06	0.000775	0.032034	0.035099
VB1.5	6	0.21	0.03	4.07E-06	2.03E-05	0.000775	0.033184	0.036249
VB3	6	0.21	0.04	1.17E-06	5.83E-06	0.000775	0.033784	0.036849
VB4.5	6	0.21	0.03	3.36E-06	1.68E-05	0.000775	0.033351	0.036416
VB6	6	0.21	0.03	4.31E-07	2.15E-06	0.000775	0.032901	0.035966
VER	6	0.19	0.03	1.4E-06	6.99E-06	0.000775	0.030134	0.033199
VE0	6	0.21	0.03	1.15E-05	5.75E-05	0.000775	0.032934	0.035999
VE1.5	6	0.21	0.03	9.26E-07	4.63E-06	0.000775	0.033451	0.036516
VE3	6	0.21	0.04	7.53E-06	3.77E-05	0.000775	0.034268	0.037332
VE4.5	6	0.22	0.04	5.28E-06	2.64E-05	0.000775	0.034318	0.037382
VE6	6	0.22	0.04	2E-06	1E-05	0.000775	0.035318	0.038382
AR	6	0.19	0.03	8.85E-06	4.42E-05	0.000775	0.030951	0.034016
A0	6	0.19	0.03	5.88E-07	2.94E-06	0.000775	0.030268	0.033332
A1.5	6	0.21	0.04	2.51E-06	1.25E-05	0.000775	0.033934	0.036999
A3	6	0.21	0.03	4.3E-06	2.15E-05	0.000775	0.032934	0.035999
A4.5	6	0.23	0.04	4.04E-06	2.02E-05	0.000775	0.035968	0.039032
A6	6	0.22	0.04	3.58E-06	1.79E-05	0.000775	0.034818	0.037882
CR	6	0.19	0.03	5.58E-07	2.79E-06	0.000775	0.029951	0.033016
C0	6	0.22	0.04	1.04E-05	5.19E-05	0.000775	0.035568	0.038632
C1.5	6	0.23	0.04	1.34E-06	6.71E-06	0.000775	0.036418	0.039482
C3	6	0.23	0.04	3.66E-06	1.83E-05	0.000775	0.036884	0.039949
C4.5	6	0.23	0.04	1.81E-06	9.05E-06	0.000775	0.037218	0.040282
C6	6	0.24	0.04	5.25E-06	2.62E-05	0.000775	0.038551	0.041616

n = 180, df = 150,  $\sum SS = 0.00054$ ,  $Q_{crit} = 5.4074$ , MCD = 0.0041931

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	0.000854	29	2.94E-05	8.161686	6.49E-19	1.54321	1.166311	0.53571
Within Groups	0.000541	150	3.61E-06					
Total	0.001395	179	7.79E-06					

Table F-3 ANOVA: Single Factor for Supernatant mass fraction ( $F_{SN}$ ) treatment comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	6	4.86	0.81	0.000163	0.00081525	0.005877	0.797863	0.821088
V0	6	4.82	0.80	0.000114	0.0005695	0.005877	0.791363	0.814589
V1.5	6	4.70	0.78	0.000199	0.00099533	0.005877	0.771455	0.794681
V3	6	4.70	0.78	0.000261	0.00130533	0.005877	0.770938	0.794163
V4.5	6	4.75	0.79	0.000258	0.00129083	0.005877	0.779818	0.803044
V6	6	4.66	0.78	0.000248	0.00124133	0.005877	0.765881	0.789107
VBR	6	4.87	0.81	0.000163	0.00081525	0.005877	0.799515	0.82274
VB0	6	4.78	0.80	0.000114	0.0005695	0.005877	0.785838	0.809064
VB1.5	6	4.41	0.73	0.000199	0.00099533	0.005877	0.72264	0.745866
VB3	6	4.43	0.74	0.000261	0.00130533	0.005877	0.727038	0.750264
VB4.5	6	4.49	0.75	0.000258	0.00129083	0.005877	0.736086	0.759312
VB6	6	4.50	0.75	0.000248	0.00124133	0.005877	0.739058	0.762284
VER	6	4.86	0.81	0.000163	0.00081525	0.005877	0.797863	0.821088
VE0	6	4.78	0.80	0.000114	0.0005695	0.005877	0.785787	0.809013
VE1.5	6	4.88	0.81	0.000199	0.00099533	0.005877	0.801187	0.824413
VE3	6	4.91	0.82	0.000261	0.00130533	0.005877	0.806687	0.829913
VE4.5	6	4.92	0.82	0.000258	0.00129083	0.005877	0.809187	0.832413
VE6	6	4.95	0.82	0.000248	0.00124133	0.005877	0.813087	0.836313
AR	6	4.84	0.81	0.000163	0.00081525	0.005877	0.794521	0.817747
A0	6	4.81	0.80	0.000114	0.0005695	0.005877	0.789387	0.812613
A1.5	6	4.82	0.80	0.000199	0.00099533	0.005877	0.792187	0.815413
A3	6	4.83	0.80	0.000261	0.00130533	0.005877	0.793187	0.816413
A4.5	6	4.82	0.80	0.000258	0.00129083	0.005877	0.791687	0.814913
A6	6	4.83	0.80	0.000248	0.00124133	0.005877	0.793187	0.816413
CR	6	4.83	0.81	0.000163	0.00081525	0.005877	0.793583	0.816809
C0	6	4.76	0.79	0.000114	0.0005695	0.005877	0.782387	0.805613
C1.5	6	4.55	0.76	0.000199	0.00099533	0.005877	0.746387	0.769613
C3	6	4.66	0.78	0.000261	0.00130533	0.005877	0.765387	0.788613
C4.5	6	4.56	0.76	0.000258	0.00129083	0.005877	0.748387	0.771613
C6	6	4.61	0.77	0.000248	0.00124133	0.005877	0.756387	0.779613

$n = 180$ ,  $df = 150$ ,  $\sum SS = 0.03109$ ,  $Q_{crit} = 5.4074$ ,  $MCD = 0.03178$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	0.109859	29	0.003788	18.27838	6.4686E-36	1.54321	1.745393	0.735711
Within Groups	0.031088	150	0.000207					
Total	0.140947	179	0.000787					



Table F-4 ANOVA: Single Factor for Pellet solids loading ( $SL_p$ ) treatment comparison test

DESCRIPTION					Alpha		0.05	
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	6	0.870	0.145	1.3E-05	6.26E-05	0.00345	0.13818	0.15183
V0	6	0.808	0.135	8.7E-05	4.36E-04	0.00345	0.12779	0.14144
V1.5	6	0.720	0.120	1.5E-04	7.29E-04	0.00345	0.11312	0.12676
V3	6	0.672	0.112	9.7E-05	4.85E-04	0.00345	0.10517	0.11882
V4.5	6	0.679	0.113	2.7E-05	1.34E-04	0.00345	0.10629	0.11993
V6	6	0.705	0.117	6.0E-05	2.98E-04	0.00345	0.11060	0.12424
VBR	6	0.832	0.139	1.3E-05	6.26E-05	0.00345	0.13182	0.14547
VB0	6	0.777	0.129	8.7E-05	4.36E-04	0.00345	0.12267	0.13631
VB1.5	6	0.593	0.099	1.5E-04	7.29E-04	0.00345	0.09199	0.10563
VB3	6	0.580	0.097	9.7E-05	4.85E-04	0.00345	0.08990	0.10355
VB4.5	6	0.645	0.107	2.7E-05	1.34E-04	0.00345	0.10067	0.11431
VB6	6	0.595	0.099	6.0E-05	2.98E-04	0.00345	0.09230	0.10594
VER	6	0.801	0.134	1.3E-05	6.26E-05	0.00345	0.12673	0.14037
VE0	6	0.820	0.137	8.7E-05	4.36E-04	0.00345	0.12989	0.14353
VE1.5	6	0.816	0.136	1.5E-04	7.29E-04	0.00345	0.12912	0.14276
VE3	6	0.834	0.139	9.7E-05	4.85E-04	0.00345	0.13210	0.14575
VE4.5	6	0.836	0.139	2.7E-05	1.34E-04	0.00345	0.13257	0.14621
VE6	6	0.845	0.141	6.0E-05	2.98E-04	0.00345	0.13396	0.14761
AR	6	0.782	0.130	1.3E-05	6.26E-05	0.00345	0.12346	0.13710
A0	6	0.787	0.131	8.7E-05	4.36E-04	0.00345	0.12440	0.13804
A1.5	6	0.764	0.127	1.5E-04	7.29E-04	0.00345	0.12045	0.13409
A3	6	0.747	0.125	9.7E-05	4.85E-04	0.00345	0.11770	0.13134
A4.5	6	0.739	0.123	2.7E-05	1.34E-04	0.00345	0.11643	0.13007
A6	6	0.726	0.121	6.0E-05	2.98E-04	0.00345	0.11422	0.12787
CR	6	0.769	0.128	1.3E-05	6.26E-05	0.00345	0.12136	0.13501
C0	6	0.639	0.107	8.7E-05	4.36E-04	0.00345	0.09973	0.11338
C1.5	6	0.559	0.093	1.5E-04	7.29E-04	0.00345	0.08635	0.10000
C3	6	0.607	0.101	9.7E-05	4.85E-04	0.00345	0.09432	0.10796
C4.5	6	0.595	0.099	2.7E-05	1.34E-04	0.00345	0.09232	0.10597
C6	6	0.625	0.104	6.0E-05	2.98E-04	0.00345	0.09741	0.11105

$n = 180$ ,  $df = 150$ ,  $\sum SS = 0.01073$ ,  $Q_{crit} = 5.4074$ ,  $MCD = 0.01867$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	0.0429	29	0.0015	20.7063	0.0000	1.5432	1.8577	0.7605
Within Groups	0.0107	150	0.0001					
Total	0.0537	179	0.0003					

Table F-5 ANOVA: Single Factor for Reducing sugars (RS) treatment comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	16	68.02	4.25	0.146961	2.204412	0.064484	4.124567	4.378019
V0	16	83.00	5.19	0.123373	1.85059	0.064484	5.060649	5.314101
V1.5	16	93.33	5.83	0.052817	0.79226	0.064484	5.706461	5.959914
V3	16	95.10	5.94	0.216962	3.254433	0.064484	5.817211	6.070664
V4.5	16	96.04	6.00	0.17098	2.564698	0.064484	5.875649	6.129101
V6	16	97.07	6.07	0.133045	1.99568	0.064484	5.940399	6.193851
VBR	16	69.99	4.37	0.162975	2.444625	0.064484	4.247728	4.501181
VB0	16	84.79	5.30	0.090007	1.3501	0.064484	5.172711	5.426164
VB1.5	16	84.06	5.25	0.086635	1.29953	0.064484	5.126836	5.380289
VB3	16	85.79	5.36	0.04543	0.681446	0.064484	5.235399	5.488851
VB4.5	16	86.56	5.41	0.048478	0.727167	0.064484	5.283211	5.536664
VB6	16	85.32	5.33	0.048403	0.726047	0.064484	5.205961	5.459414
VER	16	70.84	4.43	0.030074	0.451109	0.064484	4.300586	4.554039
VE0	16	79.19	4.95	0.014599	0.218981	0.064484	4.822524	5.075976
VE1.5	16	101.13	6.32	0.086022	1.290334	0.064484	6.194086	6.447539
VE3	16	109.71	6.86	0.033116	0.496744	0.064484	6.730086	6.983539
VE4.5	16	115.55	7.22	0.081012	1.215178	0.064484	7.095399	7.348851
VE6	16	116.06	7.25	0.065846	0.987685	0.064484	7.126961	7.380414
AR	16	70.84	4.43	0.030074	0.451109	0.064484	4.300586	4.554039
A0	16	85.43	5.34	0.060877	0.913148	0.064484	5.212461	5.465914
A1.5	16	105.18	6.57	0.006093	0.091397	0.064484	6.446961	6.700414
A3	16	108.84	6.80	0.103673	1.555099	0.064484	6.675961	6.929414
A4.5	16	117.18	7.32	0.04901	0.735143	0.064484	7.196961	7.450414
A6	16	121.27	7.58	0.023994	0.359914	0.064484	7.452711	7.706164
CR	16	70.19	4.39	0.01005	0.150746	0.064484	4.260149	4.513601
C0	16	91.49	5.72	0.007548	0.113226	0.064484	5.591399	5.844851
C1.5	16	100.02	6.25	0.005002	0.075024	0.064484	6.124774	6.378226
C3	16	99.47	6.22	0.03252	0.487802	0.064484	6.090399	6.343851
C4.5	16	106.08	6.63	0.006226	0.093394	0.064484	6.503149	6.756601
C6	16	102.35	6.40	0.024098	0.361466	0.064484	6.270149	6.523601

$n = 480$ ,  $df = 450$ ,  $\sum SS = 29.9385$ ,  $Q_{crit} = 5.33647$ ,  $MCD = 0.34411$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	428.247	29	14.76714	221.9622	4E-246	1.492797	3.724599	0.930313
Within Groups	29.93849	450	0.06653					
Total	458.1855	479	0.956546					

Table F-6 ANOVA: Single Factor for Laminarin (L) treatment comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	8	3.49	0.44	0.008472	0.067774	0.058564	0.320737	0.551634
V0	8	7.75	0.97	0.088905	0.711243	0.058564	0.853302	1.084198
V1.5	8	8.34	1.04	0.02264	0.181116	0.058564	0.926927	1.157823
V3	8	8.04	1.00	0.02196	0.175679	0.058564	0.889177	1.120073
V4.5	8	6.80	0.85	0.041675	0.333401	0.058564	0.734677	0.965573
V6	8	4.35	0.54	0.025123	0.200985	0.058564	0.428677	0.659573
VBR	8	3.42	0.43	0.008472	0.067774	0.058564	0.311587	0.542483
VB0	8	5.58	0.70	0.019878	0.159024	0.058564	0.581802	0.812698
VB1.5	8	6.89	0.86	0.00123	0.00984	0.058564	0.745802	0.976698
VB3	8	6.41	0.80	0.002965	0.023718	0.058564	0.686052	0.916948
VB4.5	8	4.57	0.57	0.015754	0.126034	0.058564	0.455677	0.686573
VB6	8	5.81	0.73	0.024466	0.195726	0.058564	0.610552	0.841448
VER	8	3.49	0.44	0.008472	0.067774	0.058564	0.320302	0.551198
VE0	8	3.77	0.47	0.002488	0.019904	0.058564	0.355677	0.586573
VE1.5	8	3.87	0.48	0.018817	0.150536	0.058564	0.368802	0.599698
VE3	8	4.11	0.51	0.00351	0.028083	0.058564	0.398677	0.629573
VE4.5	8	3.65	0.46	0.034659	0.27727	0.058564	0.340552	0.571448
VE6	8	3.08	0.38	0.004108	0.032866	0.058564	0.269302	0.500198
AR	8	3.45	0.43	0.008472	0.067774	0.058564	0.315944	0.546841
A0	8	2.53	0.32	0.020288	0.162308	0.058564	0.201302	0.432198
A1.5	8	2.99	0.37	0.008213	0.065704	0.058564	0.257927	0.488823
A3	8	4.32	0.54	0.065466	0.523728	0.058564	0.423927	0.654823
A4.5	8	4.68	0.58	0.010221	0.081764	0.058564	0.468927	0.699823
A6	8	3.63	0.45	0.00669	0.053521	0.058564	0.337802	0.568698
CR	8	3.49	0.44	0.008472	0.067774	0.058564	0.320302	0.551198
C0	8	20.96	2.62	0.009029	0.072231	0.058564	2.504677	2.735573
C1.5	8	16.44	2.05	0.002496	0.019967	0.058564	1.939052	2.169948
C3	8	20.36	2.54	0.012925	0.103404	0.058564	2.429052	2.659948
C4.5	8	19.77	2.47	0.021483	0.171868	0.058564	2.355552	2.586448
C6	8	15.81	1.98	0.192895	1.543158	0.058564	1.860302	2.091198

$n = 240$ ,  $df = 210$ ,  $\sum SS = 5.79195$ ,  $Q_{crit} = 5.377$ ,  $MCD = 0.3149$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	111.7617	29	3.85385	140.4575	3.3E-121	1.521631	4.205456	0.943981
Within Groups	5.761945	210	0.027438					
Total	117.5236	239	0.491731					

Table F-7 ANOVA: Single Factor for Inorganic sulfate (S) treatment comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	12	3.62	0.30	0.000379	0.004167	0.021792	0.258798	0.344535
V0	12	4.59	0.38	0.004682	0.051506	0.021792	0.339382	0.425118
V1.5	12	5.84	0.49	0.002527	0.027794	0.021792	0.443382	0.529118
V3	12	7.18	0.60	0.006813	0.074942	0.021792	0.555382	0.641118
V4.5	12	8.06	0.67	0.010977	0.120752	0.021792	0.628382	0.714118
V6	12	8.56	0.71	0.009151	0.100664	0.021792	0.670382	0.756118
VBR	12	4.03	0.34	0.000372	0.004092	0.021792	0.292965	0.378702
VB0	12	5.93	0.49	0.001892	0.020813	0.021792	0.450961	0.536698
VB1.5	12	6.81	0.57	5.21E-05	0.000573	0.021792	0.524818	0.610555
VB3	12	10.76	0.90	0.000749	0.008236	0.021792	0.853718	0.939455
VB4.5	12	8.66	0.72	0.000241	0.002653	0.021792	0.679095	0.764832
VB6	12	8.10	0.68	0.002566	0.028227	0.021792	0.632455	0.718192
VER	12	4.64	0.39	0.005024	0.055267	0.021792	0.343798	0.429535
VE0	12	7.81	0.65	0.003336	0.036692	0.021792	0.607965	0.693702
VE1.5	12	9.96	0.83	0.021255	0.2338	0.021792	0.787132	0.872868
VE3	12	11.68	0.97	0.02537	0.279067	0.021792	0.930465	1.016202
VE4.5	12	11.92	0.99	0.004515	0.049667	0.021792	0.950465	1.036202
VE6	12	13.72	1.14	0.025733	0.283067	0.021792	1.100465	1.186202
AR	12	4.02	0.34	0.001773	0.0195	0.021792	0.292132	0.377868
A0	12	7.83	0.65	0.011239	0.123625	0.021792	0.609632	0.695368
A1.5	12	7.69	0.64	0.00139	0.015292	0.021792	0.597965	0.683702
A3	12	10.46	0.87	0.005015	0.055167	0.021792	0.828798	0.914535
A4.5	12	9.61	0.80	0.003245	0.035692	0.021792	0.757965	0.843702
A6	12	12.10	1.01	0.017852	0.196367	0.021792	0.965465	1.051202
CR	12	4.19	0.35	0.001208	0.013292	0.021792	0.306298	0.392035
C0	12	5.88	0.49	7.29E-05	0.000802	0.021792	0.447522	0.533259
C1.5	12	4.11	0.34	0.001011	0.011123	0.021792	0.299804	0.385541
C3	12	4.06	0.34	0.000735	0.008081	0.021792	0.295112	0.380849
C4.5	12	2.94	0.25	0.000918	0.010096	0.021792	0.202168	0.287905
C6	12	3.56	0.30	0.000866	0.009527	0.021792	0.253802	0.339539

$n = 360$ ,  $df = 330$ ,  $\sum SS = 1.88504$ ,  $Q_{crit} = 5.34946$ ,  $MCD = 0.11657$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	21.96318	29	0.757351	132.9011	2.2E-163	1.501981	3.327926	0.913981
Within Groups	1.88054	330	0.005699					
Total	23.84372	359	0.066417					

Table F-8 ANOVA: Single Factor for Total phenolics (TP) treatment comparison test

DESCRIPTION					Alpha	0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>	
VR	12	2.90	0.24	1.42045E-05	0.000156	0.003357	0.234646	0.247854	
V0	12	3.82	0.32	0.00048572	0.005343	0.003357	0.311563	0.324771	
V1.5	12	4.03	0.34	0.000242152	0.002664	0.003357	0.329063	0.342271	
V3	12	4.11	0.34	6.91742E-05	0.000761	0.003357	0.335729	0.348937	
V4.5	12	4.31	0.36	9.22045E-05	0.001014	0.003357	0.352146	0.365354	
V6	12	4.06	0.34	0.000189659	0.002086	0.003357	0.331646	0.344854	
VBR	12	2.92	0.24	2.76061E-05	0.000304	0.003357	0.236479	0.249687	
VB0	12	4.10	0.34	0.000322333	0.003546	0.003357	0.335313	0.348521	
VB1.5	12	4.25	0.35	0.000144992	0.001595	0.003357	0.347313	0.360521	
VB3	12	4.28	0.36	4.54773E-05	0.0005	0.003357	0.350146	0.363354	
VB4.5	12	4.36	0.36	5.75379E-05	0.000633	0.003357	0.356563	0.369771	
VB6	12	4.36	0.36	0.000147152	0.001619	0.003357	0.356729	0.369937	
VER	12	3.14	0.26	1.78409E-05	0.000196	0.003357	0.254646	0.267854	
VE0	12	3.57	0.30	0.000118811	0.001307	0.003357	0.290813	0.304021	
VE1.5	12	3.76	0.31	0.000115659	0.001272	0.003357	0.306646	0.319854	
VE3	12	3.92	0.33	8.22045E-05	0.000904	0.003357	0.320146	0.333354	
VE4.5	12	3.94	0.33	5.40833E-05	0.000595	0.003357	0.321479	0.334687	
VE6	12	3.83	0.32	1.79015E-05	0.000197	0.003357	0.312479	0.325687	
AR	12	3.04	0.25	1.00833E-05	0.000111	0.003357	0.246313	0.259521	
A0	12	3.21	0.27	0.000332932	0.003662	0.003357	0.261146	0.274354	
A1.5	12	3.71	0.31	0.000189273	0.002082	0.003357	0.302396	0.315604	
A3	12	3.90	0.32	5.69318E-05	0.000626	0.003357	0.318146	0.331354	
A4.5	12	3.91	0.33	5.4697E-05	0.000602	0.003357	0.319563	0.332771	
A6	12	3.81	0.32	0.000136811	0.001505	0.003357	0.310479	0.323687	
CR	12	2.94	0.25	8.51515E-06	9.37E-05	0.003357	0.238563	0.251771	
C0	12	3.40	0.28	0.000453174	0.004985	0.003357	0.276979	0.290187	
C1.5	12	3.62	0.30	0.000207902	0.002287	0.003357	0.294979	0.308187	
C3	12	3.58	0.30	6.64242E-05	0.000731	0.003357	0.291729	0.304937	
C4.5	12	3.56	0.30	9.60606E-05	0.001057	0.003357	0.290063	0.303271	
C6	12	3.45	0.29	0.000199697	0.002197	0.003357	0.281063	0.294271	

n = 360, df = 330,  $\sum SS = 0.04463$ ,  $Q_{crit} = 5.34936$ , MCD = 0.01796

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	0.474506	29	0.016362	120.98671	2.9E-157	1.501981	3.175252	0.906241
Within Groups	0.044629	330	0.000135					
Total	0.519136	359	0.001446					

Table F-9 ANOVA: Single Factor for Antioxidant capacity (AO) treatment comparison test

DESCRIPTION					Alpha	0.05		
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VR	12	11.17	0.93	0.003762	0.041386	0.019349	0.892937	0.969063
V0	12	13.82	1.15	0.017264	0.189904	0.019349	1.11377	1.189896
V1.5	12	13.94	1.16	0.000213	0.00234	0.019349	1.123937	1.200063
V3	12	13.92	1.16	0.002155	0.0237	0.019349	1.122104	1.19823
V4.5	12	13.74	1.15	0.006106	0.067168	0.019349	1.107187	1.183313
V6	12	13.57	1.13	0.006014	0.066155	0.019349	1.092604	1.16873
VBR	12	10.86	0.91	0.003762	0.041386	0.019349	0.866937	0.943063
VB0	12	16.77	1.40	0.017264	0.189904	0.019349	1.35977	1.435896
VB1.5	12	16.64	1.39	0.000213	0.00234	0.019349	1.348937	1.425063
VB3	12	16.73	1.39	0.002155	0.0237	0.019349	1.356104	1.43223
VB4.5	12	17.32	1.44	0.006106	0.067168	0.019349	1.405187	1.481313
VB6	12	16.88	1.41	0.006014	0.066155	0.019349	1.368604	1.44473
VER	12	12.99	1.08	0.003177	0.034952	0.019349	1.044687	1.120813
VE0	12	14.53	1.21	0.009271	0.101983	0.019349	1.172854	1.24898
VE1.5	12	15.96	1.33	0.000624	0.006864	0.019349	1.291937	1.368063
VE3	12	15.42	1.29	0.001443	0.015876	0.019349	1.246937	1.323063
VE4.5	12	14.68	1.22	0.001961	0.021575	0.019349	1.185604	1.26173
VE6	12	15.37	1.28	0.003754	0.041291	0.019349	1.24302	1.319146
AR	12	12.98	1.08	0.002772	0.030493	0.019349	1.043854	1.11998
A0	12	15.22	1.27	0.005485	0.060335	0.019349	1.229854	1.30598
A1.5	12	15.62	1.30	0.001228	0.013511	0.019349	1.263854	1.33998
A3	12	15.83	1.32	0.000872	0.009588	0.019349	1.281104	1.35723
A4.5	12	15.80	1.32	0.001716	0.018881	0.019349	1.27852	1.354646
A6	12	14.55	1.21	0.002561	0.028173	0.019349	1.174354	1.25048
CR	12	11.67	0.97	0.004574	0.050309	0.019349	0.934437	1.010563
C0	12	18.41	1.53	0.012613	0.138739	0.019349	1.496354	1.57248
C1.5	12	18.97	1.58	0.000441	0.004848	0.019349	1.542937	1.619063
C3	12	17.24	1.44	0.001711	0.018816	0.019349	1.398937	1.475063
C4.5	12	16.99	1.42	0.003626	0.039887	0.019349	1.37752	1.453646
C6	12	16.52	1.38	0.005921	0.065136	0.019349	1.338187	1.414313

$n = 360$ ,  $df = 330$ ,  $\sum SS = 1.48256$ ,  $Q_{crit} = 5.34936$ ,  $MCD = 0.1035$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	9.807009	29	0.338173	75.27315	3.4E-127	1.501981	2.504548	0.856797
Within Groups	1.482561	330	0.004493					
Total	11.28957	359	0.031447					

## Appendix G

### Batch comparison ANOVAs and Tukey HSD results

Table G-1 ANOVA: Single Factor for Solubilised yield (SY) batch comparison test

DESCRIPTION					Alpha	0.05		
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VE B1 R	6	3.01391	0.50232	0.00057	0.00283	0.00807	0.48640	0.51824
VE B1 0	6	2.99390	0.49898	0.00056	0.00280	0.00807	0.48306	0.51490
VE B1 1.5	6	3.17478	0.52913	0.00018	0.00089	0.00807	0.51321	0.54505
VE B1 3	6	3.23157	0.53860	0.00080	0.00398	0.00807	0.52268	0.55452
VE B1 4.5	6	3.25054	0.54176	0.00098	0.00491	0.00807	0.52584	0.55768
VE B1 6	6	3.31764	0.55294	0.00046	0.00230	0.00807	0.53702	0.56886
VE B2 R	6	2.02489	0.33748	0.00078	0.00389	0.00807	0.32156	0.35340
VE B2 0	6	1.94050	0.32342	0.00004	0.00019	0.00807	0.30750	0.33934
VE B2 1.5	6	2.41882	0.40314	0.00023	0.00113	0.00807	0.38722	0.41906
VE B2 3	6	2.32477	0.38746	0.00014	0.00070	0.00807	0.37154	0.40338
VE B2 4.5	6	2.34813	0.39135	0.00012	0.00061	0.00807	0.37543	0.40727
VE B2 6	6	2.42247	0.40374	0.00003	0.00014	0.00807	0.38783	0.41966
A B1 R	6	3.05788	0.50965	0.00130	0.00652	0.00807	0.49373	0.52557
A B1 0	6	2.96763	0.49461	0.00016	0.00079	0.00807	0.47869	0.51053
A B1 1.5	6	3.20747	0.53458	0.00025	0.00123	0.00807	0.51866	0.55050
A B1 3	6	3.20647	0.53441	0.00047	0.00237	0.00807	0.51849	0.55033
A B1 4.5	6	3.33001	0.55500	0.00073	0.00367	0.00807	0.53908	0.57092
A B1 6	6	3.32631	0.55438	0.00041	0.00203	0.00807	0.53846	0.57030
A B2 R	6	1.99880	0.33313	0.00051	0.00255	0.00807	0.31721	0.34905
A B2 0	6	1.97801	0.32967	0.00008	0.00038	0.00807	0.31375	0.34559
A B2 1.5	6	2.13360	0.35560	0.00017	0.00086	0.00807	0.33968	0.37152
A B2 3	6	2.22823	0.37137	0.00011	0.00055	0.00807	0.35545	0.38729
A B2 4.5	6	2.40733	0.40122	0.00036	0.00179	0.00807	0.38530	0.41714
A B2 6	6	2.43849	0.40641	0.00041	0.00206	0.00807	0.39049	0.42233
C B1 R	6	3.02518	0.50420	0.00037	0.00185	0.00807	0.48828	0.52012
C B1 0	6	3.44461	0.57410	0.00042	0.00208	0.00807	0.55818	0.59002
C B1 1.5	6	3.37300	0.56217	0.00020	0.00099	0.00807	0.54625	0.57809
C B1 3	6	3.42687	0.57115	0.00031	0.00156	0.00807	0.55523	0.58707
C B1 4.5	6	3.32382	0.55397	0.00026	0.00131	0.00807	0.53805	0.56989
C B1 6	6	3.36734	0.56122	0.00015	0.00076	0.00807	0.54530	0.57714
C B2 R	6	1.96893	0.32816	0.00051	0.00256	0.00807	0.31224	0.34408
C B2 0	6	2.49111	0.41519	0.00048	0.00240	0.00807	0.39927	0.43110
C B2 1.5	6	2.13189	0.35532	0.00015	0.00076	0.00807	0.33940	0.37124
C B2 3	6	2.32340	0.38723	0.00016	0.00081	0.00807	0.37131	0.40315
C B2 4.5	6	2.19836	0.36639	0.00082	0.00408	0.00807	0.35047	0.38231
C B2 6	6	2.48028	0.41338	0.00039	0.00193	0.00807	0.39746	0.42930

n = 216, df = 180,  $\sum SS = 0.0703$ ,  $Q_{crit} = 5.521$ , MCD = 0.04454

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	2.131067	35	0.060888	155.90934	1.3E-116	1.488708	4.469358	0.961687
Within Groups	0.070296	180	0.000391					
Total	2.201363	215	0.010239					



Table G-2 ANOVA: Single Factor for Supernatant dissolved solids (DS) batch comparison test

DESCRIPTION	Alpha					0.05		
	Group	Count	Sum	Mean	Variance	SS	Std Err	Lower
VE B1 R	6	0.19	0.03	1.4E-06	6.99E-06	0.001046	0.029602	0.033731
VE B1 0	6	0.21	0.03	1.15E-05	5.75E-05	0.001046	0.032402	0.036531
VE B1 1.5	6	0.21	0.03	9.26E-07	4.63E-06	0.001046	0.032919	0.037048
VE B1 3	6	0.21	0.04	7.53E-06	3.77E-05	0.001046	0.033736	0.037864
VE B1 4.5	6	0.22	0.04	5.28E-06	2.64E-05	0.001046	0.033786	0.037914
VE B1 6	6	0.22	0.04	2E-06	1E-05	0.001046	0.034786	0.038914
VE B2 R	6	0.18	0.03	1.01E-05	5.03E-05	0.001046	0.027202	0.031331
VE B2 0	6	0.17	0.03	4.53E-06	2.27E-05	0.001046	0.026536	0.030664
VE B2 1.5	6	0.21	0.04	1.1E-05	5.52E-05	0.001046	0.032969	0.037098
VE B2 3	6	0.20	0.03	1.88E-06	9.41E-06	0.001046	0.030969	0.035098
VE B2 4.5	6	0.19	0.03	1.53E-06	7.67E-06	0.001046	0.030352	0.034481
VE B2 6	6	0.21	0.03	1.47E-06	7.34E-06	0.001046	0.032286	0.036414
A B1 R	6	0.19	0.03	8.85E-06	4.42E-05	0.001046	0.030419	0.034548
A B1 0	6	0.19	0.03	5.88E-07	2.94E-06	0.001046	0.029736	0.033864
A B1 1.5	6	0.21	0.04	2.51E-06	1.25E-05	0.001046	0.033402	0.037531
A B1 3	6	0.21	0.03	4.3E-06	2.15E-05	0.001046	0.032402	0.036531
A B1 4.5	6	0.23	0.04	4.04E-06	2.02E-05	0.001046	0.035436	0.039564
A B1 6	6	0.22	0.04	3.58E-06	1.79E-05	0.001046	0.034286	0.038414
A B2 R	6	0.17	0.03	6.69E-06	3.34E-05	0.001046	0.026652	0.030781
A B2 0	6	0.17	0.03	9.37E-06	4.69E-05	0.001046	0.026752	0.030881
A B2 1.5	6	0.20	0.03	6E-06	3E-05	0.001046	0.030552	0.034681
A B2 3	6	0.19	0.03	1.99E-06	9.95E-06	0.001046	0.030219	0.034348
A B2 4.5	6	0.20	0.03	6.49E-06	3.25E-05	0.001046	0.031786	0.035914
A B2 6	6	0.21	0.03	1.54E-05	7.7E-05	0.001046	0.032669	0.036798
C B1 R	6	0.19	0.03	5.58E-07	2.79E-06	0.001046	0.029419	0.033548
C B1 0	6	0.22	0.04	1.04E-05	5.19E-05	0.001046	0.035036	0.039164
C B1 1.5	6	0.23	0.04	1.34E-06	6.71E-06	0.001046	0.035886	0.040014
C B1 3	6	0.23	0.04	3.66E-06	1.83E-05	0.001046	0.036352	0.040481
C B1 4.5	6	0.23	0.04	1.81E-06	9.05E-06	0.001046	0.036686	0.040814
C B1 6	6	0.24	0.04	5.2E-06	2.6E-05	0.00105	0.03802	0.04215
C B2 R	6	0.17230	0.03	6.69E-06	3.34E-05	0.001046	0.026652	0.030781
C B2 0	6	0.19930	0.03	3E-05	0.00015	0.001046	0.031152	0.035281
C B2 1.5	6	0.20630	0.03	6.89E-06	3.44E-05	0.001046	0.032319	0.036448
C B2 3	6	0.19580	0.03	7.88E-06	3.94E-05	0.001046	0.030569	0.034698
C B2 4.5	6	0.19510	0.03	1.7E-05	8.49E-05	0.001046	0.030452	0.034581
C B2 6	6	0.20330	0.03	1.61E-05	8.03E-05	0.001046	0.031819	0.035948

n = 216, df = 180,  $\sum SS = 0.00118$ ,  $Q_{crit} = 5.521$ , MCD = 0.00578

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	0.001849	35	5.28E-05	8.043051	4.76E-22	1.488708	1.157803	0.53298
Within Groups	0.001182	180	6.57E-06					
Total	0.003031	215	1.41E-05					

Table G-3 ANOVA: Single Factor for Supernatant mass fraction ( $F_{SN}$ ) batch comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VE B1 R	6	4.85685	0.80948	0.00016	0.00082	0.00588	0.79788	0.82107
VE B1 0	6	4.78440	0.79740	0.00011	0.00057	0.00588	0.78580	0.80900
VE B1 1.5	6	4.87680	0.81280	0.00020	0.00100	0.00588	0.80120	0.82440
VE B1 3	6	4.90980	0.81830	0.00026	0.00131	0.00588	0.80670	0.82990
VE B1 4.5	6	4.92480	0.82080	0.00026	0.00129	0.00588	0.80920	0.83240
VE B1 6	6	4.94820	0.82470	0.00025	0.00124	0.00588	0.81310	0.83630
VE B2 R	6	3.63717	0.60620	0.00016	0.00082	0.00588	0.59460	0.61779
VE B2 0	6	3.52380	0.58730	0.00011	0.00057	0.00588	0.57570	0.59890
VE B2 1.5	6	3.76740	0.62790	0.00020	0.00100	0.00588	0.61630	0.63950
VE B2 3	6	3.81480	0.63580	0.00026	0.00131	0.00588	0.62420	0.64740
VE B2 4.5	6	3.87840	0.64640	0.00026	0.00129	0.00588	0.63480	0.65800
VE B2 6	6	3.87480	0.64580	0.00025	0.00124	0.00588	0.63420	0.65740
A B1 R	6	4.83681	0.80613	0.00016	0.00082	0.00588	0.79454	0.81773
A B1 0	6	4.80600	0.80100	0.00011	0.00057	0.00588	0.78940	0.81260
A B1 1.5	6	4.82280	0.80380	0.00020	0.00100	0.00588	0.79220	0.81540
A B1 3	6	4.82880	0.80480	0.00026	0.00131	0.00588	0.79320	0.81640
A B1 4.5	6	4.81980	0.80330	0.00026	0.00129	0.00588	0.79170	0.81490
A B1 6	6	4.82880	0.80480	0.00025	0.00124	0.00588	0.79320	0.81640
A B2 R	6	3.63720	0.60620	0.00016	0.00082	0.00588	0.59460	0.61780
A B2 0	6	3.33480	0.55580	0.00011	0.00057	0.00588	0.54420	0.56740
A B2 1.5	6	3.56040	0.59340	0.00020	0.00100	0.00588	0.58180	0.60500
A B2 3	6	3.70500	0.61750	0.00026	0.00131	0.00588	0.60590	0.62910
A B2 4.5	6	3.77460	0.62910	0.00026	0.00129	0.00588	0.61750	0.64070
A B2 6	6	3.84120	0.64020	0.00025	0.00124	0.00588	0.62860	0.65180
C B1 R	6	4.83117	0.80520	0.00016	0.00082	0.00588	0.79360	0.81679
C B1 0	6	4.76400	0.79400	0.00011	0.00057	0.00588	0.78240	0.80560
C B1 1.5	6	4.54800	0.75800	0.00020	0.00100	0.00588	0.74640	0.76960
C B1 3	6	4.66200	0.77700	0.00026	0.00131	0.00588	0.76540	0.78860
C B1 4.5	6	4.56000	0.76000	0.00026	0.00129	0.00588	0.74840	0.77160
C B1 6	6	4.60800	0.76800	0.00025	0.00124	0.00588	0.75640	0.77960
C B2 R	6	3.64748	0.60791	0.00016	0.00082	0.00588	0.59632	0.61951
C B2 0	6	3.44685	0.57447	0.00011	0.00057	0.00588	0.56288	0.58607
C B2 1.5	6	3.36600	0.56100	0.00020	0.00100	0.00588	0.54940	0.57260
C B2 3	6	3.60600	0.60100	0.00026	0.00131	0.00588	0.58940	0.61260
C B2 4.5	6	3.56400	0.59400	0.00026	0.00129	0.00588	0.58240	0.60560
C B2 6	6	3.79800	0.63300	0.00025	0.00124	0.00588	0.62140	0.64460

$n = 216$ ,  $df = 180$ ,  $\sum SS = 0.03731$ ,  $Q_{crit} = 5.521$ ,  $MCD = 0.03245$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	2.053327	35	0.058666	283.0671	3E-139	1.488708	6.868613	0.978589
Within Groups	0.037306	180	0.000207					
Total	2.090632	215	0.009724					

Table G-4 ANOVA: Single Factor for Pellet-solids loading ( $SL_P$ ) batch comparison test

DESCRIPTION		Alpha					0.05	
Group	Count	Sum	Mean	Variance	SS	Std Err	Lower	Upper
VE B1 R	6	0.80	0.13	1.3E-05	0.00006	0.003453	0.126738	0.140363
VE B1 0	6	0.82	0.14	8.7E-05	0.00044	0.003453	0.129895	0.143521
VE B1 1.5	6	0.82	0.14	1.5E-04	0.00073	0.003453	0.129126	0.142752
VE B1 3	6	0.83	0.14	9.7E-05	0.00049	0.003453	0.132112	0.145738
VE B1 4.5	6	0.84	0.14	2.7E-05	0.00013	0.003453	0.132576	0.146201
VE B1 6	6	0.84	0.14	6.0E-05	0.00030	0.003453	0.133973	0.147599
VE B2 R	6	0.53	0.09	1.3E-05	0.00006	0.003453	0.081880	0.095506
VE B2 0	6	0.51	0.09	8.7E-05	0.00044	0.003453	0.078605	0.092231
VE B2 1.5	6	0.53	0.09	1.5E-04	0.00073	0.003453	0.081229	0.094855
VE B2 3	6	0.55	0.09	9.7E-05	0.00049	0.003453	0.084768	0.098394
VE B2 4.5	6	0.55	0.09	2.7E-05	0.00013	0.003453	0.085605	0.099231
VE B2 6	6	0.56	0.09	6.0E-05	0.00030	0.003453	0.086007	0.099632
A B1 R	6	0.78	0.13	1.3E-05	0.00006	0.003453	0.123469	0.137095
A B1 0	6	0.79	0.13	8.7E-05	0.00044	0.003453	0.124409	0.138035
A B1 1.5	6	0.76	0.13	1.5E-04	0.00073	0.003453	0.120460	0.134086
A B1 3	6	0.75	0.12	9.7E-05	0.00049	0.003453	0.117709	0.131335
A B1 4.5	6	0.74	0.12	2.7E-05	0.00013	0.003453	0.116435	0.130061
A B1 6	6	0.73	0.12	6.0E-05	0.00030	0.003453	0.114233	0.127859
A B2 R	6	0.53	0.09	1.3E-05	0.00006	0.003453	0.081880	0.095506
A B2 0	6	0.44	0.07	8.7E-05	0.00044	0.003453	0.066792	0.080418
A B2 1.5	6	0.52	0.09	1.5E-04	0.00073	0.003453	0.079905	0.093531
A B2 3	6	0.53	0.09	9.7E-05	0.00049	0.003453	0.081800	0.095426
A B2 4.5	6	0.52	0.09	2.7E-05	0.00013	0.003453	0.079165	0.092791
A B2 6	6	0.54	0.09	6.0E-05	0.00030	0.003453	0.083898	0.097524
C B1 R	6	0.77	0.13	1.3E-05	0.00006	0.003453	0.121372	0.134998
C B1 0	6	0.64	0.11	8.7E-05	0.00044	0.003453	0.099741	0.113367
C B1 1.5	6	0.56	0.09	1.5E-04	0.00073	0.003453	0.086363	0.099989
C B1 3	6	0.61	0.10	9.7E-05	0.00049	0.003453	0.094328	0.107954
C B1 4.5	6	0.59	0.10	2.7E-05	0.00013	0.003453	0.092331	0.105957
C B1 6	6	0.63	0.10	6.0E-05	0.00030	0.003453	0.097419	0.111045
C B2 R	6	0.55	0.09	1.3E-05	0.00006	0.003453	0.084549	0.098175
C B2 0	6	0.38	0.06	8.7E-05	0.00044	0.003453	0.056640	0.070266
C B2 1.5	6	0.48	0.08	1.5E-04	0.00073	0.003453	0.073336	0.086962
C B2 3	6	0.47	0.08	9.7E-05	0.00049	0.003453	0.071414	0.085040
C B2 4.5	6	0.50	0.08	2.7E-05	0.00013	0.003453	0.075819	0.089445
C B2 6	6	0.50	0.08	6.0E-05	0.00030	0.003453	0.076557	0.090182

$n = 215$ ,  $df = 180$ ,  $\sum SS = 0.01287$ ,  $Q_{crit} = 5.521$ ,  $MCD = 0.01906$

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	0.10767	35	0.00308	43.0115	5.5E-70	1.4887081	2.6774218	0.8719168
Within Groups	0.01287	180	0.00007					
Total	0.12055	215	0.00056					

Table G-5 ANOVA: Single Factor for Reducing sugars (RS) batch comparison test

DESCRIPTION		Alpha			0.05			
Group	Count	Sum	Mean	Variance	SS	Std Err	Lower	Upper
VE B1 R	16	70.83700	4.42731	0.03007	0.45111	0.03897	4.35077	4.50386
VE B1 0	16	79.18800	4.94925	0.01460	0.21898	0.03897	4.87270	5.02580
VE B1 1.5	16	101.13300	6.32081	0.08602	1.29033	0.03897	6.24427	6.39736
VE B1 3	16	109.70900	6.85681	0.03312	0.49674	0.03897	6.78027	6.93336
VE B1 4.5	16	115.55400	7.22213	0.08101	1.21518	0.03897	7.14558	7.29867
VE B1 6	16	116.05900	7.25369	0.06585	0.98769	0.03897	7.17714	7.33023
VE B2 R	16	18.65885	1.16618	0.00151	0.02258	0.03897	1.08963	1.24273
VE B2 0	16	29.38000	1.83625	0.00952	0.14286	0.03897	1.75970	1.91280
VE B2 1.5	16	52.11800	3.25738	0.01451	0.21763	0.03897	3.18083	3.33392
VE B2 3	16	57.04100	3.56506	0.03211	0.48158	0.03897	3.48852	3.64161
VE B2 4.5	16	59.29100	3.70569	0.02623	0.39351	0.03897	3.62914	3.78223
VE B2 6	16	62.06500	3.87906	0.03642	0.54625	0.03897	3.80252	3.95561
A B1 R	16	70.83700	4.42731	0.03007	0.45111	0.03897	4.35077	4.50386
A B1 0	16	85.42700	5.33919	0.06088	0.91315	0.03897	5.26264	5.41573
A B1 1.5	16	105.17900	6.57369	0.00609	0.09140	0.03897	6.49714	6.65023
A B1 3	16	108.84300	6.80269	0.10367	1.55510	0.03897	6.72614	6.87923
A B1 4.5	16	117.17900	7.32369	0.04901	0.73514	0.03897	7.24714	7.40023
A B1 6	16	121.27100	7.57944	0.02399	0.35991	0.03897	7.50289	7.65598
A B2 R	16	16.70573	1.04411	0.00340	0.05107	0.03897	0.96756	1.12066
A B2 0	16	33.68400	2.10525	0.00372	0.05579	0.03897	2.02870	2.18180
A B2 1.5	16	66.00300	4.12519	0.02628	0.39420	0.03897	4.04864	4.20173
A B2 3	16	77.59800	4.84988	0.01036	0.15534	0.03897	4.77333	4.92642
A B2 4.5	16	87.03000	5.43938	0.00918	0.13764	0.03897	5.36283	5.51592
A B2 6	16	95.08800	5.94300	0.02249	0.33734	0.03897	5.86645	6.01955
C B1 R	16	70.19000	4.38688	0.01005	0.15075	0.03897	4.31033	4.46342
C B1 0	16	91.49000	5.71813	0.00755	0.11323	0.03897	5.64158	5.79467
C B1 1.5	16	100.02400	6.25150	0.00500	0.07502	0.03897	6.17495	6.32805
C B1 3	16	99.47400	6.21713	0.03252	0.48780	0.03897	6.14058	6.29367
C B1 4.5	16	106.07800	6.62988	0.00623	0.09339	0.03897	6.55333	6.70642
C B1 6	16	102.35000	6.39688	0.02410	0.36147	0.03897	6.32033	6.47342
C B2 R	16	16.72743	1.04546	0.00093	0.01388	0.03897	0.96892	1.12201
C B2 0	16	33.63800	2.10238	0.00153	0.02296	0.03897	2.02583	2.17892
C B2 1.5	16	34.10400	2.13150	0.00098	0.01471	0.03897	2.05495	2.20805
C B2 3	16	36.58800	2.28675	0.00210	0.03156	0.03897	2.21020	2.36330
C B2 4.5	16	37.62400	2.35150	0.00181	0.02710	0.03897	2.27495	2.42805
C B2 6	16	37.57200	2.34825	0.00175	0.02623	0.03897	2.27170	2.42480

n = 576, df = 540,  $\sum SS = 13.11974$ ,  $Q_{crit} = 5.427$ , MCD = 0.211478

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	2358.023	35	67.37209	2772.992	0	1.44497	13.1648	0.994098
Within Groups	13.11974	540	0.024296					
Total	2371.143	575	4.123727					

Table G-6 ANOVA: Single Factor for Laminarin (L) batch comparison test

DESCRIPTION		Alpha			0.05			
Group	Count	Sum	Mean	Variance	SS	Std Err	Lower	Upper
VE B1 R	8	3.4867	0.4358	0.0085	0.0593	0.0409	0.3553	0.5164
VE B1 0	8	3.7690	0.4711	0.0025	0.0174	0.0409	0.3906	0.5517
VE B1 1.5	8	3.8736	0.4842	0.0188	0.1317	0.0409	0.4036	0.5648
VE B1 3	8	4.1125	0.5141	0.0035	0.0246	0.0409	0.4335	0.5946
VE B1 4.5	8	3.6486	0.4561	0.0347	0.2426	0.0409	0.3755	0.5366
VE B1 6	8	3.0760	0.3845	0.0041	0.0288	0.0409	0.3039	0.4651
VE B2 R	8	1.0661	0.1333	0.0010	0.0068	0.0409	0.0527	0.2138
VE B2 0	8	0.8470	0.1059	0.0025	0.0173	0.0409	0.0253	0.1864
VE B2 1.5	8	2.1145	0.2643	0.0020	0.0139	0.0409	0.1837	0.3449
VE B2 3	8	2.1797	0.2725	0.0098	0.0684	0.0409	0.1919	0.3530
VE B2 4.5	8	2.3929	0.2991	0.0047	0.0329	0.0409	0.2185	0.3797
VE B2 6	8	2.2685	0.2836	0.0007	0.0046	0.0409	0.2030	0.3641
A B1 R	8	3.5025	0.4378	0.0076	0.0531	0.0409	0.3572	0.5184
A B1 0	8	2.5350	0.3169	0.0203	0.1420	0.0409	0.2363	0.3974
A B1 1.5	8	2.9872	0.3734	0.0082	0.0575	0.0409	0.2928	0.4540
A B1 3	8	4.3159	0.5395	0.0655	0.4583	0.0409	0.4589	0.6201
A B1 4.5	8	4.6752	0.5844	0.0102	0.0715	0.0409	0.5038	0.6650
A B1 6	8	3.6249	0.4531	0.0067	0.0468	0.0409	0.3725	0.5337
A B2 R	8	1.0661	0.1333	0.0010	0.0068	0.0409	0.0527	0.2138
A B2 0	8	1.0405	0.1301	0.0028	0.0193	0.0409	0.0495	0.2106
A B2 1.5	8	0.7680	0.0960	0.0022	0.0152	0.0409	0.0154	0.1766
A B2 3	8	0.7799	0.0975	0.0020	0.0140	0.0409	0.0169	0.1781
A B2 4.5	8	1.4038	0.1755	0.0012	0.0082	0.0409	0.0949	0.2560
A B2 6	8	0.9694	0.1212	0.0025	0.0174	0.0409	0.0406	0.2017
C B1 R	8	3.4827	0.4353	0.0080	0.0559	0.0409	0.3548	0.5159
C B1 0	8	20.9595	2.6199	0.0090	0.0632	0.0409	2.5394	2.7005
C B1 1.5	8	16.4344	2.0543	0.0025	0.0175	0.0409	1.9737	2.1349
C B1 3	8	20.3554	2.5444	0.0129	0.0905	0.0409	2.4639	2.6250
C B1 4.5	8	19.7690	2.4711	0.0215	0.1504	0.0409	2.3906	2.5517
C B1 6	8	15.8065	1.9758	0.1929	1.3503	0.0409	1.8952	2.0564
C B2 R	8	1.0661	0.1333	0.0010	0.0068	0.0409	0.0527	0.2138
C B2 0	8	1.0247	0.1281	0.0008	0.0054	0.0409	0.0475	0.2087
C B2 1.5	8	1.6367	0.2046	0.0036	0.0255	0.0409	0.1240	0.2852
C B2 3	8	1.8835	0.2354	0.0009	0.0063	0.0409	0.1549	0.3160
C B2 4.5	8	1.9368	0.2421	0.0006	0.0040	0.0409	0.1615	0.3227
C B2 6	8	1.6032	0.2004	0.0057	0.0398	0.0409	0.1198	0.2810

$n = 288$ ,  $df = 552$ ,  $\sum SS = 3.37394$ ,  $Q_{crit} = 5.49414$ ,  $MCD = 0.222476$

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	151.5143	35	4.328981	323.3318	4.4E-189	1.470017	6.357395	0.975107
Within Groups	3.373944	252.00	0.013389					
Total	154.8883	287.00	0.53968					

Table G-7 ANOVA: Single Factor for Inorganic sulfate (S) batch comparison test

DESCRIPTION					Alpha	0.05		
Group	Count	Sum	Mean	Variance	SS	Std Err	Lower	Upper
VE B1 R	12	4.64000	0.38667	0.00502	0.05527	0.05146	0.28550	0.48783
VE B1 0	12	7.81000	0.65083	0.00334	0.03669	0.05146	0.54967	0.75200
VE B1 1.5	12	9.96000	0.83000	0.02125	0.23380	0.05146	0.72883	0.93117
VE B1 3	12	11.68000	0.97333	0.02537	0.27907	0.05146	0.87217	1.07450
VE B1 4.5	12	11.92000	0.99333	0.00452	0.04967	0.05146	0.89217	1.09450
VE B1 6	12	13.72000	1.14333	0.02573	0.28307	0.05146	1.04217	1.24450
VE B2 R	12	16.58800	1.38233	0.04296	0.47252	0.05146	1.28117	1.48350
VE B2 0	12	19.13000	1.59417	0.02848	0.31329	0.05146	1.49300	1.69533
VE B2 1.5	12	24.73000	2.06083	0.11306	1.24369	0.05146	1.95967	2.16200
VE B2 3	12	27.28000	2.27333	0.08573	0.94307	0.05146	2.17217	2.37450
VE B2 4.5	12	29.35000	2.44583	0.08379	0.92169	0.05146	2.34467	2.54700
VE B2 6	12	29.99000	2.49917	0.07037	0.77409	0.05146	2.39800	2.60033
A B1 R	12	4.02000	0.33500	0.00177	0.01950	0.05146	0.23383	0.43617
A B1 0	12	7.83000	0.65250	0.01124	0.12363	0.05146	0.55133	0.75367
A B1 1.5	12	7.69000	0.64083	0.00139	0.01529	0.05146	0.53967	0.74200
A B1 3	12	10.46000	0.87167	0.00502	0.05517	0.05146	0.77050	0.97283
A B1 4.5	12	9.61000	0.80083	0.00324	0.03569	0.05146	0.69967	0.90200
A B1 6	12	12.10000	1.00833	0.01785	0.19637	0.05146	0.90717	1.10950
A B2 R	12	15.83900	1.31992	0.00859	0.09444	0.05146	1.21875	1.42108
A B2 0	12	26.33800	2.19483	0.05870	0.64565	0.05146	2.09367	2.29600
A B2 1.5	12	31.10900	2.59242	0.16093	1.77021	0.05146	2.49125	2.69358
A B2 3	12	31.06700	2.58892	0.02087	0.22954	0.05146	2.48775	2.69008
A B2 4.5	12	31.26500	2.60542	0.22063	2.42696	0.05146	2.50425	2.70658
A B2 6	12	30.72600	2.56050	0.08656	0.95211	0.05146	2.45933	2.66167
C B1 R	12	4.19000	0.34917	0.00121	0.01329	0.05146	0.24800	0.45033
C B1 0	12	5.88469	0.49039	0.00007	0.00080	0.05146	0.38922	0.59156
C B1 1.5	12	4.11207	0.34267	0.00101	0.01112	0.05146	0.24150	0.44384
C B1 3	12	4.05577	0.33798	0.00073	0.00808	0.05146	0.23681	0.43915
C B1 4.5	12	2.94044	0.24504	0.00092	0.01010	0.05146	0.14387	0.34620
C B1 6	12	3.56004	0.29667	0.00087	0.00953	0.05146	0.19550	0.39784
C B2 R	12	14.52700	1.21058	0.02155	0.23707	0.05146	1.10942	1.31175
C B2 0	12	11.26000	0.93833	0.00194	0.02137	0.05146	0.83717	1.03950
C B2 1.5	12	8.62000	0.71833	0.00442	0.04857	0.05146	0.61717	0.81950
C B2 3	12	7.85000	0.65417	0.00234	0.02569	0.05146	0.55300	0.75533
C B2 4.5	12	7.29000	0.60750	0.00093	0.01023	0.05146	0.50633	0.70867
C B2 6	12	6.54000	0.54500	0.00157	0.01730	0.05146	0.44383	0.64617

n = 432, df = 396,  $\sum SS = 12.58361$ ,  $Q_{crit} = 5.469727$ , MCD = 0.281469

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	271.9499	35	7.769998	244.518	6.2E-245	1.452957	4.514034	0.951759
Within Groups	12.58	396	0.031777					
Total	284.5335	431	0.660171					



Table G-8 ANOVA: Single Factor for Total phenolics (*TP*) batch comparison test

DESCRIPTION		Alpha			0.05			
<i>Group</i>	<i>Count</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>	<i>SS</i>	<i>Std Err</i>	<i>Lower</i>	<i>Upper</i>
VE B1 R	12	3.14	0.26	1.78E-05	0.000196	0.003129	0.255099	0.267401
VE B1 0	12	3.57	0.30	0.000119	0.001307	0.003129	0.291265	0.303568
VE B1 1.5	12	3.76	0.31	0.000116	0.001272	0.003129	0.307099	0.319401
VE B1 3	12	3.92	0.33	8.22E-05	0.000904	0.003129	0.320599	0.332901
VE B1 4.5	12	3.94	0.33	5.41E-05	0.000595	0.003129	0.321932	0.334235
VE B1 6	12	3.83	0.32	1.79E-05	0.000197	0.003129	0.312932	0.325235
VE B2 R	12	3.80	0.32	1.22E-05	0.000134	0.003129	0.310682	0.322985
VE B2 0	12	4.24	0.35	0.000366	0.004029	0.003129	0.347349	0.359651
VE B2 1.5	12	5.41	0.45	0.000203	0.002229	0.003129	0.444265	0.456568
VE B2 3	12	5.39	0.45	6.41E-05	0.000705	0.003129	0.443349	0.455651
VE B2 4.5	12	5.36	0.45	5.86E-05	0.000645	0.003129	0.440515	0.452818
VE B2 6	12	5.31	0.44	0.000148	0.001628	0.003129	0.436599	0.448901
A B1 R	12	3.04	0.25	1.01E-05	0.000111	0.003129	0.246765	0.259068
A B1 0	12	3.21	0.27	0.000333	0.003662	0.003129	0.261599	0.273901
A B1 1.5	12	3.71	0.31	0.000189	0.002082	0.003129	0.302849	0.315151
A B1 3	12	3.90	0.32	5.69E-05	0.000626	0.003129	0.318599	0.330901
A B1 4.5	12	3.91	0.33	5.47E-05	0.000602	0.003129	0.320015	0.332318
A B1 6	12	3.81	0.32	0.000137	0.001505	0.003129	0.310932	0.323235
A B2 R	12	3.83	0.32	5.45E-06	6E-05	0.003129	0.312849	0.325151
A B2 0	12	4.53	0.38	0.000301	0.003312	0.003129	0.371015	0.383318
A B2 1.5	12	5.61	0.47	0.000143	0.001573	0.003129	0.461432	0.473735
A B2 3	12	5.95	0.50	3.97E-05	0.000437	0.003129	0.489515	0.501818
A B2 4.5	12	6.22	0.52	4.72E-05	0.000519	0.003129	0.512349	0.524651
A B2 6	12	7.01	0.58	9.9E-05	0.001089	0.003129	0.577765	0.590068
C B1 R	12	2.94	0.25	8.52E-06	9.37E-05	0.003129	0.239015	0.251318
C B1 0	12	3.40	0.28	0.000453	0.004985	0.003129	0.277432	0.289735
C B1 1.5	12	3.62	0.30	0.000208	0.002287	0.003129	0.295432	0.307735
C B1 3	12	3.58	0.30	6.64E-05	0.000731	0.003129	0.292182	0.304485
C B1 4.5	12	3.56	0.30	9.61E-05	0.001057	0.003129	0.290515	0.302818
C B1 6	12	3.45	0.29	0.0002	0.0022	0.00313	0.28152	0.29382
C B2 R	12	3.85	0.32	7.36E-06	8.1E-05	0.003129	0.314349	0.326651
C B2 0	12	4.78	0.40	0.000355	0.003907	0.003129	0.391932	0.404235
C B2 1.5	12	5.05	0.42	0.000103	0.001135	0.003129	0.414265	0.426568
C B2 3	12	5.08	0.42	2.93E-05	0.000322	0.003129	0.417099	0.429401
C B2 4.5	12	5.19	0.43	2.23E-05	0.000245	0.003129	0.425932	0.438235
C B2 6	12	4.53	0.38	5.66E-06	6.23E-05	0.003129	0.371599	0.383901

$n = 432$ ,  $df = 396$ ,  $\sum SS = 0.04652$ ,  $Q_{crit} = 5.46973$ ,  $MCD = 0.01711$

## ANOVA

<i>Sources</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P value</i>	<i>F crit</i>	<i>RMSSE</i>	<i>Omega Sq</i>
Between Groups	2.853354	35	0.081524	693.9751	0	1.452957	7.604687	0.9825
Within Groups	0.04652	396	0.000117					
Total	2.899874	431	0.006728					



Table G-9 ANOVA: Single Factor for Antioxidant capacity (AO) batch comparison test

DESCRIPTION					Alpha	0.05		
Group	Count	Sum	Mean	Variance	SS	Std Err	Lower	Upper
VE B1 R	12	12.99	1.08	0.00318	0.034952	0.01634	1.050626	1.114874
VE B1 0	12	14.53	1.21	0.00927	0.101983	0.01634	1.178792	1.243041
VE B1 1.5	12	15.96	1.33	0.00062	0.006864	0.01634	1.297876	1.362124
VE B1 3	12	15.42	1.29	0.00144	0.015876	0.01634	1.252876	1.317124
VE B1 4.5	12	14.68	1.22	0.00196	0.021575	0.01634	1.191542	1.255791
VE B1 6	12	15.37	1.28	0.00375	0.041291	0.01634	1.248959	1.313208
VE B2 R	12	18.54	1.54	0.00166	0.01826	0.01634	1.512626	1.576874
VE B2 0	12	21.30	1.78	0.00964	0.106053	0.01634	1.743209	1.807458
VE B2 1.5	12	21.88	1.82	0.00046	0.005028	0.01634	1.790876	1.855124
VE B2 3	12	21.70	1.81	0.00126	0.0139	0.01634	1.776042	1.840291
VE B2 4.5	12	21.80	1.82	0.00119	0.013103	0.01634	1.784376	1.848624
VE B2 6	12	20.18	1.68	0.00426	0.046881	0.01634	1.649792	1.714041
A B1 R	12	12.98	1.08	0.00277	0.030493	0.01634	1.049792	1.114041
A B1 0	12	15.22	1.27	0.00548	0.060335	0.01634	1.235792	1.300041
A B1 1.5	12	15.62	1.30	0.00123	0.013511	0.01634	1.269792	1.334041
A B1 3	12	15.83	1.32	0.00087	0.009588	0.01634	1.287042	1.351291
A B1 4.5	12	15.80	1.32	0.00172	0.018881	0.01634	1.284459	1.348708
A B1 6	12	14.55	1.21	0.00256	0.028173	0.01634	1.180292	1.244541
A B2 R	12	17.91	1.49	0.00272	0.029957	0.01634	1.460209	1.524458
A B2 0	12	22.29	1.86	0.00526	0.057891	0.01634	1.824959	1.889208
A B2 1.5	12	24.43	2.04	0.00062	0.006864	0.01634	2.003876	2.068124
A B2 3	12	22.91	1.91	0.00073	0.00802	0.01634	1.877042	1.941291
A B2 4.5	12	24.02	2.00	0.00148	0.016253	0.01634	1.969376	2.033624
A B2 6	12	25.45	2.12	0.00256	0.028173	0.01634	2.088292	2.152541
C B1 R	12	11.67	0.97	0.00457	0.050309	0.01634	0.940376	1.004624
C B1 0	12	18.41	1.53	0.01261	0.138739	0.01634	1.502292	1.566541
C B1 1.5	12	18.97	1.58	0.00044	0.004848	0.01634	1.548876	1.613124
C B1 3	12	17.24	1.44	0.00171	0.018816	0.01634	1.404876	1.469124
C B1 4.5	12	16.99	1.42	0.00363	0.039887	0.01634	1.383459	1.447708
C B1 6	12	16.52	1.38	0.00592	0.06514	0.01634	1.34413	1.40837
C B2 R	12	18.58	1.55	0.00277	0.030442	0.01634	1.516042	1.580291
C B2 0	12	24.06	2.00	0.00959	0.105461	0.01634	1.972459	2.036708
C B2 1.5	12	23.51	1.96	0.00048	0.005328	0.01634	1.926876	1.991124
C B2 3	12	25.32	2.11	0.00121	0.013263	0.01634	2.078209	2.142458
C B2 4.5	12	25.60	2.13	0.00163	0.017941	0.01634	2.101459	2.165708
C B2 6	12	25.07	2.09	0.00406	0.044713	0.01634	2.057376	2.121624

n = 432, df = 396,  $\sum SS = 1.26878$ ,  $Q_{crit} = 5.46973$ , MCD = 0.08938

## ANOVA

Sources	SS	df	MS	F	P value	F crit	RMSSE	Omega Sq
Between Groups	49.97062	35	1.427732	445.6089	1.2E-294	1.452957	6.093774	0.972989
Within Groups	1.268785	396	0.003204					
Total	51.23941	431	0.118885					

# Appendix H

## HPLC results from laminarin testing

Table H-1 HPLC results from alternate laminarin test

SAMPLE CODE	V <sub>72%</sub> acid (ml)	TV (ml)	DF	Glucose <sub>HPLC</sub> (g·l <sup>-1</sup> )	Glucose <sub>sample</sub> (g·l <sup>-1</sup> )	%R <sub>glucose</sub>
SRS2.5	0.157	4.657	12.4	2.187888	2.5	0.875155
SRS1	0.157	4.657	12.4	0.88444	1	0.88444
SRS0.5	0.157	4.657	12.4	0.446279	0.5	0.892557
A B2 R_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 0_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 1.5_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 3_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 4.5_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 6_P	0.157	4.657	12.4	n.a.	n.a.	-
A B1 R_P	0.157	4.657	12.4	0.2538	3.5652448	-
A B1 0_P	0.157	4.657	12.4	0.2329	3.2716529	-
A B1 1.5_P	0.157	4.657	12.4	0.2222	3.1213451	-
A B1 3_P	0.157	4.657	12.4	0.1952	2.7420637	-
A B1 4.5_P	0.157	4.657	12.4	0.2019	2.8361817	-
A B1 6_P	0.157	4.657	12.4	0.2148	3.0173939	-
VE B1 R_P	0.157	4.657	12.4	0.2317	3.2547959	-
VE B1 0_P	0.157	4.657	12.4	0.2316	3.2533912	-
VE B1 1.5_P	0.157	4.657	12.4	0.2423	3.403699	-
VE B1 3_P	0.157	4.657	12.4	0.2257	3.1705112	-
VE B1 4.5_P	0.157	4.657	12.4	0.2126	2.9864895	-
VE B1 6_P	0.157	4.657	12.4	0.2348	3.2983431	-
VE B2 R_P	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 0_P	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 1.5_P	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 3_P	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 4.5_P	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 6_P	0.157	4.657	12.4	n.a.	n.a.	-
C B1 R_P	0.157	4.657	12.4	0.2747	3.8588366	-
C B1 0_P	0.157	4.657	12.4	0.1792	2.5173044	-
C B1 1.5_P	0.157	4.657	12.4	0.1955	2.746278	-
C B1 3_P	0.157	4.657	12.4	0.1911	2.6844692	-
C B1 4.5_P	0.157	4.657	12.4	0.1896	2.663398	-
C B1 6_P	0.157	4.657	12.4	0.1974	2.7729681	-
C B2 R_P	0.157	4.657	12.4	n.a.	n.a.	-
C B2 0_P	0.157	4.657	12.4	n.a.	n.a.	-
C B2 1.5_P	0.157	4.657	12.4	n.a.	n.a.	-
C B2 3_P	0.157	4.657	12.4	n.a.	n.a.	-

C B2 4.5_P	0.157	4.657	12.4	n.a.	n.a.	-
C B2 6_P	0.157	4.657	12.4	n.a.	n.a.	-
A B2 R_S	0.157	4.657	12.4	n.a.	n.a.	-
A B2 0_S	0.157	4.657	12.4	n.a.	n.a.	-
A B2 1.5_S	0.157	4.657	12.4	n.a.	n.a.	-
A B2 3_S	0.157	4.657	12.4	n.a.	n.a.	-
A B2 4.5_S	0.157	4.657	12.4	n.a.	n.a.	-
A B2 6_S	0.157	4.657	12.4	n.a.	n.a.	-
A B1 R_S	0.157	4.657	12.4	n.a.	n.a.	-
A B1 0_S	0.157	4.657	12.4	n.a.	n.a.	-
A B1 1.5_S	0.157	4.657	12.4	n.a.	n.a.	-
A B1 3_S	0.157	4.657	12.4	n.a.	n.a.	-
A B1 4.5_S	0.157	4.657	12.4	0.1036	1.4553166	-
A B2 6_S	0.157	4.657	12.4	0.1092	1.5339824	-
VE B2 R_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 0_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 1.5_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 3_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 4.5_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B2 6_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B1 R_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B1 0_S	0.157	4.657	12.4	n.a.	n.a.	-
VE B1 1.5_S	0.157	4.657	12.4	0.118	1.6576	-
VE B1 3_S	0.157	4.657	12.4	0.1476	2.0734048	-
VE B1 4.5_S	0.157	4.657	12.4	0.1603	2.2518075	-
VE B1 6_S	0.157	4.657	12.4	0.1644	2.309402	-
C B1 R_S	0.157	4.657	12.4	n.a.	n.a.	-
C B1 0_S	0.157	4.657	12.4	n.a.	n.a.	-
C B1 1.5_S	0.157	4.657	12.4	n.a.	n.a.	-
C B1 3_S	0.157	4.657	12.4	n.a.	n.a.	-
C B1 4.5_S	0.157	4.657	12.4	n.a.	n.a.	-
C B1 6_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 R_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 0_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 1.5_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 3_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 4.5_S	0.157	4.657	12.4	n.a.	n.a.	-
C B2 6_S	0.157	4.657	12.4	n.a.	n.a.	-

# Appendix I

## Converted data for discussion and comparison

Table I-1 Converted data and error parameter  $\Delta$  for treatments V and VB.

	V						VB					
	VR	V0	V 1.5	V3	V4.5	V6	VB R	VB 0	VB 1.5	VB 3	VB 4.5	VB 6
DS (%)	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.04	0.03	0.03
$\Delta_{DS}$ (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DS (g·l <sup>-1</sup> )	33.41	35.97	35.77	37.58	36.95	37.74	32.67	34.73	35.97	36.61	36.14	35.66
$\Delta_{DS}$ (g·l <sup>-1</sup> )	2.71	4.50	3.95	2.67	1.45	1.71	0.29	0.82	3.10	1.66	2.82	1.01
SY (%)	0.49	0.51	0.51	0.54	0.55	0.52	0.49	0.51	0.49	0.51	0.49	0.52
$\Delta_{SY}$ (%)	0.06	0.13	0.16	0.14	0.08	0.10	0.05	0.12	0.15	0.13	0.07	0.11
RS (gGIE·l <sup>-1</sup> )	4.25	5.19	5.83	5.94	6.00	6.07	4.37	5.30	5.25	5.36	5.41	5.33
$\Delta_{RS}$ (gGIE·l <sup>-1</sup> )	0.20	0.19	0.12	0.25	0.22	0.19	0.22	0.16	0.16	0.11	0.12	0.12
L (gLE·l <sup>-1</sup> )	0.44	0.97	1.04	1.00	0.85	0.54	0.43	0.70	0.86	0.80	0.57	0.73
$\Delta_L$ (gLE·l <sup>-1</sup> )	0.11	0.16	0.17	0.21	0.17	0.17	0.11	0.22	0.21	0.28	0.19	0.14
S (gSE·l <sup>-1</sup> )	0.30	0.38	0.49	0.60	0.67	0.71	0.34	0.49	0.57	0.90	0.72	0.68
$\Delta_S$ (gSE·l <sup>-1</sup> )	0.01	0.04	0.03	0.05	0.07	0.06	0.01	0.03	0.00	0.02	0.01	0.03
TP (gGAE·l <sup>-1</sup> )	0.24	0.32	0.34	0.34	0.36	0.34	0.24	0.34	0.35	0.36	0.36	0.36
$\Delta_{TP}$ (gGAE·l <sup>-1</sup> )	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.01
AO (gTE·l <sup>-1</sup> )	0.93	1.15	1.16	1.16	1.15	1.13	0.91	1.40	1.39	1.39	1.44	1.41
$\Delta_{AO}$ (gTE·l <sup>-1</sup> )	0.04	0.08	0.01	0.03	0.05	0.05	0.04	0.08	0.01	0.03	0.05	0.05
RS (mgGIE·gDS <sup>-1</sup> )	127	144	163	158	162	161	134	153	146	146	150	150
$\Delta_{RS}$ (mgGIE·gDS <sup>-1</sup> )	12	19	18	13	9	9	7	6	13	7	12	5
L (mgLE·gDS <sup>-1</sup> )	13	27	29	27	23	14	13	20	24	22	16	20
$\Delta_L$ (mgLE·gDS <sup>-1</sup> )	4	6	6	6	5	5	3	6	6	8	5	4
S (mgSE·gDS <sup>-1</sup> )	9	11	14	16	18	19	10	14	16	24	20	19
$\Delta_S$ (mgSE·gDS <sup>-1</sup> )	1	2	2	2	2	2	0	1	1	1	2	1
TP (mgGAE·gDS <sup>-1</sup> )	7	9	9	9	10	9	7	10	10	10	10	10
$\Delta_{TP}$ (mgGAE·gDS <sup>-1</sup> )	1	1	1	1	0	0	0	0	1	0	1	0
AO (mgTE·gDS <sup>-1</sup> )	28	32	32	31	31	30	28	40	39	38	40	39
$\Delta_{AO}$ (mgTE·gDS <sup>-1</sup> )	3	5	4	2	2	2	1	3	3	2	3	2
RS (mgGIE·gDW <sup>-1</sup> )	62	74	83	86	89	84	66	77	72	75	74	78
$\Delta_{RS}$ (mgGIE·gDW <sup>-1</sup> )	9	21	28	24	14	17	7	19	24	20	13	17
L (mgLE·gDW <sup>-1</sup> )	6	14	15	15	13	8	6	10	12	11	8	11
$\Delta_L$ (mgLE·gDW <sup>-1</sup> )	2	5	6	5	3	3	2	4	5	5	3	3
S (mgSE·gDW <sup>-1</sup> )	4	5	7	9	10	10	5	7	8	13	10	10
$\Delta_S$ (mgSE·gDW <sup>-1</sup> )	1	2	2	2	2	2	1	2	3	3	2	2
TP (mgGAE·gDW <sup>-1</sup> )	4	5	5	5	5	5	4	5	5	5	5	5
$\Delta_{TP}$ (mgGAE·gDW <sup>-1</sup> )	0	1	2	1	1	1	0	1	2	1	1	1
AO (mgTE·gDW <sup>-1</sup> )	14	16	17	17	17	16	14	20	19	20	20	21
$\Delta_{AO}$ (mgTE·gDW <sup>-1</sup> )	2	5	6	5	3	3	2	5	6	5	3	4

Table I-2 Converted data and error parameter  $\Delta$  for treatments VE B1 and VE B2.

	VE B1						VE B2					
	VE B1 R	VE B1 0	VE B1 1.5	VE B1 3	VE B1 4.5	VE B1 6	VE B2 R	VE B2 0	VE B2 1.5	VE B2 3	VE B2 4.5	VE B2 6
DS (%)	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.04	0.03	0.03	0.03
$\Delta_{DS}$ (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DS (g·r <sup>-1</sup> )	32.70	35.70	36.25	37.13	37.18	38.26	30.15	29.44	36.31	34.16	33.50	35.57
$\Delta_{DS}$ (g·r <sup>-1</sup> )	1.81	5.21	1.48	4.22	3.54	2.18	4.85	3.25	5.11	2.11	1.90	1.86
SY (%)	0.50	0.50	0.53	0.54	0.54	0.55	0.34	0.32	0.40	0.39	0.39	0.40
$\Delta_{SY}$ (%)	0.06	0.13	0.16	0.14	0.09	0.11	0.05	0.08	0.12	0.09	0.05	0.07
RS (gGIE·l <sup>-1</sup> )	4.43	4.95	6.32	6.86	7.22	7.25	1.17	1.84	3.26	3.57	3.71	3.88
$\Delta_{RS}$ (gGIE·l <sup>-1</sup> )	0.09	0.06	0.16	0.10	0.15	0.14	0.02	0.05	0.06	0.10	0.09	0.10
L (gLE·l <sup>-1</sup> )	0.44	0.47	0.48	0.51	0.46	0.38	0.13	0.11	0.26	0.27	0.30	0.28
$\Delta_L$ (gLE·l <sup>-1</sup> )	0.11	0.08	0.12	0.16	0.13	0.09	0.03	0.04	0.06	0.15	0.05	0.03
S (gSE·l <sup>-1</sup> )	0.39	0.65	0.83	0.97	0.99	1.14	1.38	1.59	2.06	2.27	2.45	2.50
$\Delta_S$ (gSE·l <sup>-1</sup> )	0.05	0.04	0.09	0.10	0.04	0.10	0.13	0.11	0.21	0.19	0.18	0.17
TP (gGAE·l <sup>-1</sup> )	0.26	0.30	0.31	0.33	0.33	0.32	0.32	0.35	0.45	0.45	0.45	0.44
$\Delta_{TP}$ (gGAE·l <sup>-1</sup> )	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01
AO (gTE·l <sup>-1</sup> )	1.08	1.21	1.33	1.29	1.22	1.28	1.54	1.78	1.82	1.81	1.82	1.68
$\Delta_{AO}$ (gTE·l <sup>-1</sup> )	0.04	0.06	0.02	0.02	0.03	0.04	0.03	0.06	0.01	0.02	0.02	0.04
RS (mgGIE·gDS <sup>-1</sup> )	135	139	174	185	194	190	39	62	90	104	111	109
$\Delta_{RS}$ (mgGIE·gDS <sup>-1</sup> )	8	20	8	21	19	11	6	7	13	7	7	6
L (mgLE·gDS <sup>-1</sup> )	13	13	13	14	12	10	4	4	7	8	9	8
$\Delta_L$ (mgLE·gDS <sup>-1</sup> )	4	3	3	5	4	3	1	1	2	4	2	1
S (mgSE·gDS <sup>-1</sup> )	12	18	23	26	27	30	46	54	57	67	73	70
$\Delta_S$ (mgSE·gDS <sup>-1</sup> )	2	3	3	4	3	3	9	7	10	7	7	6
TP (mgGAE·gDS <sup>-1</sup> )	8	8	9	9	9	8	11	12	12	13	13	12
$\Delta_{TP}$ (mgGAE·gDS <sup>-1</sup> )	0	1	0	1	1	0	2	1	2	1	1	1
AO (mgTE·gDS <sup>-1</sup> )	33	34	37	35	33	33	51	60	50	53	54	47
$\Delta_{AO}$ (mgTE·gDS <sup>-1</sup> )	2	5	2	4	3	2	8	7	7	3	3	3
RS (mgGIE·gDW <sup>-1</sup> )	68	69	92	99	105	105	13	20	36	40	43	44
$\Delta_{RS}$ (mgGIE·gDW <sup>-1</sup> )	9	20	29	29	20	22	3	5	12	10	6	9
L (mgLE·gDW <sup>-1</sup> )	7	7	7	7	7	6	1	1	3	3	3	3
$\Delta_L$ (mgLE·gDW <sup>-1</sup> )	2	2	3	3	2	2	0	0	1	2	1	1
S (mgSE·gDW <sup>-1</sup> )	6	9	12	14	14	17	15	18	23	26	29	28
$\Delta_S$ (mgSE·gDW <sup>-1</sup> )	1	3	4	4	3	4	4	5	8	7	5	6
TP (mgGAE·gDW <sup>-1</sup> )	4	4	5	5	5	5	4	4	5	5	5	5
$\Delta_{TP}$ (mgGAE·gDW <sup>-1</sup> )	0	1	1	1	1	1	1	1	2	1	1	1
AO (mgTE·gDW <sup>-1</sup> )	17	17	19	19	18	19	17	20	20	21	21	19
$\Delta_{AO}$ (mgTE·gDW <sup>-1</sup> )	2	5	6	5	3	4	4	5	7	5	3	4

Table I-3 Converted data and error parameter  $\Delta$  for treatments A B1 and A B2

	AB1						AB2					
	AB1 R	AB1 0	AB1 1.5	AB1 3	AB1 4.5	AB1 6	AB2 R	AB2 0	AB2 1.5	AB2 3	AB2 4.5	AB2 6
DS (%)	0.03	0.03	0.04	0.03	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03
$\Delta_{DS}$ (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DS (g·r <sup>-1</sup> )	33.57	32.84	36.77	35.70	38.96	37.72	29.57	29.67	33.72	33.36	35.04	35.98
$\Delta_{DS}$ (g·r <sup>-1</sup> )	4.56	1.18	2.44	3.19	3.10	2.91	3.95	4.68	3.76	2.16	3.91	6.03
SY (%)	0.51	0.49	0.53	0.53	0.56	0.55	0.33	0.33	0.36	0.37	0.40	0.41
$\Delta_{SY}$ (%)	0.08	0.12	0.17	0.14	0.08	0.12	0.04	0.09	0.10	0.09	0.06	0.09
RS (gGIE·l <sup>-1</sup> )	4.43	5.34	6.57	6.80	7.32	7.58	1.04	2.11	4.13	4.85	5.44	5.94
$\Delta_{RS}$ (gGIE·l <sup>-1</sup> )	0.09	0.13	0.04	0.17	0.12	0.08	0.03	0.03	0.09	0.05	0.05	0.08
L (gLE·l <sup>-1</sup> )	0.44	0.32	0.37	0.54	0.58	0.45	0.13	0.13	0.10	0.10	0.18	0.12
$\Delta_L$ (gLE·l <sup>-1</sup> )	0.11	0.22	0.36	0.36	0.43	0.24	0.04	0.09	0.08	0.13	0.13	0.08
S (gSE·l <sup>-1</sup> )	0.34	0.65	0.64	0.87	0.80	1.01	1.32	2.19	2.59	2.59	2.61	2.56
$\Delta_S$ (gSE·l <sup>-1</sup> )	0.03	0.07	0.02	0.04	0.04	0.08	0.06	0.15	0.25	0.09	0.30	0.19
TP (gGAE·l <sup>-1</sup> )	0.25	0.27	0.31	0.32	0.33	0.32	0.32	0.38	0.47	0.50	0.52	0.58
$\Delta_{TP}$ (gGAE·l <sup>-1</sup> )	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.01
AO (gTE·l <sup>-1</sup> )	1.08	1.27	1.30	1.32	1.32	1.21	1.49	1.86	2.04	1.91	2.00	2.12
$\Delta_{AO}$ (gTE·l <sup>-1</sup> )	0.03	0.05	0.02	0.02	0.03	0.03	0.03	0.05	0.02	0.02	0.02	0.03
RS (mgGIE·gDS <sup>-1</sup> )	132	163	179	191	188	201	35	71	122	145	155	165
$\Delta_{RS}$ (mgGIE·gDS <sup>-1</sup> )	18	7	12	18	15	16	5	11	14	10	17	28
L (mgLE·gDS <sup>-1</sup> )	13	10	10	15	15	12	5	4	3	3	5	3
$\Delta_L$ (mgLE·gDS <sup>-1</sup> )	4	7	10	10	11	6	2	3	2	4	4	2
S (mgSE·gDS <sup>-1</sup> )	10	20	17	24	21	27	45	74	77	78	74	71
$\Delta_S$ (mgSE·gDS <sup>-1</sup> )	2	2	1	3	2	3	6	13	11	6	12	13
TP (mgGAE·gDS <sup>-1</sup> )	8	8	8	9	8	8	11	13	14	15	15	16
$\Delta_{TP}$ (mgGAE·gDS <sup>-1</sup> )	1	0	1	1	1	1	1	2	2	1	2	3
AO (mgTE·gDS <sup>-1</sup> )	32	39	35	37	34	32	50	63	60	57	57	59
$\Delta_{AO}$ (mgTE·gDS <sup>-1</sup> )	4	2	2	3	3	3	7	10	7	4	6	10
RS (mgGIE·gDW <sup>-1</sup> )	67	80	96	102	104	111	12	23	44	54	62	67
$\Delta_{RS}$ (mgGIE·gDW <sup>-1</sup> )	14	20	30	29	18	25	2	7	13	13	12	19
L (mgLE·gDW <sup>-1</sup> )	7	5	5	8	8	7	2	1	1	1	2	1
$\Delta_L$ (mgLE·gDW <sup>-1</sup> )	2	4	5	6	6	4	1	1	1	2	2	1
S (mgSE·gDW <sup>-1</sup> )	5	10	9	13	11	15	15	24	27	29	30	29
$\Delta_S$ (mgSE·gDW <sup>-1</sup> )	1	3	3	4	2	4	3	8	9	7	7	8
TP (mgGAE·gDW <sup>-1</sup> )	4	4	4	5	5	5	4	4	5	6	6	7
$\Delta_{TP}$ (mgGAE·gDW <sup>-1</sup> )	1	1	1	1	1	1	1	1	2	1	1	2
AO (mgTE·gDW <sup>-1</sup> )	16	19	19	20	19	18	17	21	21	21	23	24
$\Delta_{AO}$ (mgTE·gDW <sup>-1</sup> )	3	5	6	6	3	4	3	6	7	5	4	7

Table I-4 Converted data and error parameter  $\Delta$  for treatments C B1 and C B2

	C B1						C B2					
	C B1 R	C B1 0	C B1 1.5	C B1 3	C B1 4.5	C B1 6	C B2 R	C B2 0	C B2 1.5	C B2 3	C B2 4.5	C B2 6
DS (%)	0.03	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03
$\Delta_{DS}$ (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
DS (g·r <sup>-1</sup> )	32.51	38.53	39.45	39.95	40.31	41.76	29.57	34.36	35.61	33.73	33.61	35.07
$\Delta_{DS}$ (g·r <sup>-1</sup> )	1.14	4.97	1.79	2.95	2.08	3.54	3.95	8.41	4.03	4.31	6.32	6.16
SY (%)	0.50	0.57	0.56	0.57	0.55	0.56	0.33	0.42	0.36	0.39	0.37	0.41
$\Delta_{SY}$ (%)	0.05	0.15	0.18	0.15	0.08	0.11	0.04	0.13	0.10	0.10	0.07	0.10
RS (gGIE·r <sup>-1</sup> )	4.39	5.72	6.25	6.22	6.63	6.40	1.05	2.10	2.13	2.29	2.35	2.35
$\Delta_{RS}$ (gGIE·r <sup>-1</sup> )	0.05	0.05	0.04	0.10	0.04	0.08	0.02	0.02	0.02	0.02	0.02	0.02
L (gLE·r <sup>-1</sup> )	0.44	2.62	2.05	2.54	2.47	1.98	0.13	0.13	0.20	0.24	0.24	0.20
$\Delta_L$ (gLE·r <sup>-1</sup> )	0.11	0.09	0.10	0.20	0.15	0.35	0.02	0.03	0.05	0.06	0.04	0.06
S (gSE·r <sup>-1</sup> )	0.35	0.49	0.34	0.34	0.25	0.30	1.21	0.94	0.72	0.65	0.61	0.55
$\Delta_S$ (gSE·r <sup>-1</sup> )	0.02	0.01	0.02	0.02	0.02	0.02	0.09	0.03	0.04	0.03	0.02	0.03
TP (gGAE·r <sup>-1</sup> )	0.25	0.28	0.30	0.30	0.30	0.29	0.32	0.40	0.42	0.42	0.43	0.38
$\Delta_{TP}$ (gGAE·r <sup>-1</sup> )	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.00
AO (gTE·r <sup>-1</sup> )	0.97	1.53	1.58	1.44	1.42	1.38	1.55	2.00	1.96	2.11	2.13	2.09
$\Delta_{AO}$ (gTE·r <sup>-1</sup> )	0.04	0.07	0.01	0.03	0.04	0.05	0.03	0.06	0.01	0.02	0.03	0.04
RS (mgGIE·gDS <sup>-1</sup> )	135	148	158	156	164	153	35	61	60	68	70	67
$\Delta_{RS}$ (mgGIE·gDS <sup>-1</sup> )	5	19	7	12	9	13	5	15	7	9	13	12
L (mgLE·gDS <sup>-1</sup> )	13	68	52	64	61	47	5	4	6	7	7	6
$\Delta_L$ (mgLE·gDS <sup>-1</sup> )	3	9	3	7	5	9	1	1	1	2	2	2
S (mgSE·gDS <sup>-1</sup> )	11	13	9	8	6	7	41	27	20	19	18	16
$\Delta_S$ (mgSE·gDS <sup>-1</sup> )	1	2	1	1	1	1	6	7	3	3	3	3
TP (mgGAE·gDS <sup>-1</sup> )	8	7	8	7	7	7	11	12	12	13	13	11
$\Delta_{TP}$ (mgGAE·gDS <sup>-1</sup> )	0	1	0	1	0	1	1	3	1	2	2	2
AO (mgTE·gDS <sup>-1</sup> )	30	40	40	36	35	33	52	58	55	63	63	60
$\Delta_{AO}$ (mgTE·gDS <sup>-1</sup> )	2	5	2	3	2	3	7	14	6	8	12	11
RS (mgGIE·gDW <sup>-1</sup> )	68	85	89	89	91	86	12	25	21	26	26	28
$\Delta_{RS}$ (mgGIE·gDW <sup>-1</sup> )	8	25	28	24	14	19	2	10	6	8	7	8
L (mgLE·gDW <sup>-1</sup> )	7	39	29	36	34	27	1	2	2	3	3	2
$\Delta_L$ (mgLE·gDW <sup>-1</sup> )	2	12	9	10	5	7	0	1	1	1	1	1
S (mgSE·gDW <sup>-1</sup> )	5	7	5	5	3	4	13	11	7	8	7	6
$\Delta_S$ (mgSE·gDW <sup>-1</sup> )	1	2	2	1	1	1	3	4	2	2	2	2
TP (mgGAE·gDW <sup>-1</sup> )	4	4	4	4	4	4	4	5	4	5	5	4
$\Delta_{TP}$ (mgGAE·gDW <sup>-1</sup> )	0	1	1	1	1	1	1	2	1	1	1	1
AO (mgTE·gDW <sup>-1</sup> )	15	23	23	21	19	18	17	24	20	24	23	25
$\Delta_{AO}$ (mgTE·gDW <sup>-1</sup> )	2	7	7	6	3	4	3	10	6	7	6	7