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**M.Sc. in Bioeconomics**

**The Suitability of Brewers' Spent Grain (BSG)  
for 2<sup>nd</sup> Generation Bioethanol Production:  
A Combined Biochemical and Economic Assessment**

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## **ΔΙΠΜΣ ΣΤΗ ΒΙΟΟΙΚΟΝΟΜΙΑ**

**Η Αξιολόγηση του Στερεού Αποβλήτου Ζυθοποιίας  
(Brewers' Spent Grain - BSG)  
στην Παγωγή Βιοαιθανόλης 2<sup>ης</sup> Γενιάς:  
Μία Συνδυαστική Βιοχημική και Οικονομική Ανάλυση**

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*Στην οικογένειά μου και στα αγαπημένα μου πρόσωπα*

## Ευχαριστίες

Κατά την διάρκεια εκπόνησης της εν λόγω Διπλωματικής Εργασίας, από την υλοποίηση των πειραμάτων έως και τη συγγραφή του κειμένου, η υποστήριξη όλων των μελών του Εργαστηρίου Μικροβιολογίας αποτέλεσε παράγοντα μείζονος σημασίας σε ολόκληρη την προσπάθεια.

Θα ήθελα πρωτίστως να ευχαριστήσω τον επιβλέποντά μου, Αναπληρωτή Καθηγητή κ. Δημήτρη Γ. Χατζηνικολάου όπου, χάρη στην την αμέριστη συμβολή και αδιάκοπη ενασχόλησή του με τη διπλωματική εργασία, κατάφερα να φέρω εις πέρας την πλήρωσή της.

Η εμπιστοσύνη που επέδειξε στα αποτελέσματα καθώς επίσης και η πίστη στις ικανότητές μου, με ώθησαν στην επιτυχή ολοκλήρωση του παρόντος πονήματος. Η συνεργασία μαζί του ήταν μία συνεχής διαδραστική εμπειρία η οποία κατέστη αναγκαία για την αποτελεσματική διεκπεραίωση όλων των πειραματικών εργασιών καθώς επίσης και στην δημιουργία προστιθέμενης αξίας κατά την αξιολόγηση των αποτελεσμάτων.

Επιπροσθέτως, θα ήθελα να ευχαριστήσω ιδιαιτέρως τους δύο καθηγητές από την Τριμελή μου Επιτροπή και συγκεκριμένα τον κ. Κωνσταντίνο Βοργιά και την κ. Κλαίρη Οικονομίδου για τη διαρκή στήριξη και τις πολύτιμες συμβουλές τους μέσω των οποίων είχα τη δυνατότητα να βελτιωθώ και να πετύχω καλύτερα αποτελέσματα.

Σημαντική ήταν η υποστήριξη όλων των μελών του Εργαστηρίου Μικροβιολογίας καθώς συνέδραμαν στην διατήρηση ενός ευχάριστου κλίματος.

Τέλος, θα ήθελα να ευχαριστήσω όλους τους παράγοντες του ΔΠΜΣ Βιοοικονομίας που μου έδωσαν την ευκαιρία να συμμετάσχω στο συγκεκριμένο πρόγραμμα σπουδών, καθώς καθ' όλη την πορεία μου μέσα σε αυτό εφοδιάστηκα με απαραίτητες γνώσεις και εμπειρία ώστε να καταφέρω να στοχεύσω σε υψηλότερους επαγγελματικούς στόχους και να εξελιχθώ επιστημονικά.

# **The Suitability of Brewers' Spent Grain (BSG) for 2<sup>nd</sup> Generation Bioethanol Production: A Combined Biochemical and Economic Assessment**

**Keywords:** Brewers' Spent Grain (BSG), pretreatment, acid, alkali, optimization, costs, *Zymomonas mobilis* 8b, hydrolysis, fermentation, Single Vessel Approach (SVA), Separate Hydrolysis – Fermentation (SHF)

## **Abstract**

The present work represents a comprehensive evaluation of the suitability of Brewers' Spent Grain (BSG) derived from Greek breweries, as a substrate for the production of 2<sup>nd</sup> generation bioethanol. The study incorporates the first comparative optimization between mild alkaline and mild acid pretreatment of BSG with respect to its susceptibility against enzymatic hydrolysis, in addition to the evaluation of the performance of the ethanologenic bacterium *Zymomonas mobilis* 8b in the fermentation of the produced sugars. Additionally, a cost-perspective analysis is carried out for the determination of the optimal pretreatment parameters.

In contrast to other pretreatment optimization attempts, where only two independent parameters are used for the optimization, in our approach the optimization was performed with respect to three independent variables, namely, pretreatment temperature, time and alkali/acid load, with the sugar yield produced by the subsequent enzymatic hydrolysis as the dependent variable. In a second, innovative, approach, the obtained experimental results were used in order to access the pretreatment optimization process from an economical perspective, using the specific sugar yield - the amount of sugars produced per unit capital - as a dependent variable.

Depending on the optimization target (sugar yield or specific sugar yield), a different set of optimum pretreatment conditions was obtained. This was true for both alkaline and acid pretreatment although for the latter, the corresponding optima differ only with respect to the acid load. Acid pretreatment, proved relatively more cost effective compared to the alkaline, with slightly higher overall sugar yields. The differences that have risen between the two optimization strategies, proves that our approach may result in accountable reductions in the cost per kg of reducing sugar obtained.

The second part of our work concerned the evaluation of the genetically modified strain of *Z. mobilis* 8b for the production of ethanol from BSG hydrolysates. Two operational modes for the hydrolysis and fermentation process were evaluated; Separate Hydrolysis and Fermentation (SHF) and Single Vessel Approach (SVA).

The most important result obtained from our fermentation data, is the verification that *Z. mobilis* 8b is able to ferment the sugars in the BSG hydrolysates in SVA mode, especially after alkaline pretreatment where the microorganism ferments the BSG derived sugars at levels equal to the maximum theoretical. Oddly enough, the sugars obtained from BSG, hydrolyzed and fermented in SHF mode, proved to be the less suitable substrates for ethanol production by *Z. mobilis* 8b, despite the fact that the hydrolysate was fortified by the necessary salts.

In overall, our results prove that ethanol production from BSG, represents a potentially economically viable perspective, where its outcome is strongly dependent on the experimentally verified selection of pretreatment conditions, microorganism, and overall biorefinery approach (SVA or SHF).

# **Η Καταλληλότητα του Στερεού Αποβλήτου Ζυθοποιίας (Brewers' Spent Grain - BSG)**

**για την Παραγωγή Βιοαιθανόλης 2<sup>ης</sup> Γενιάς:**

## **Μία Συνδυαστική Βιοχημική και Οικονομική Αξιολόγηση**

**Σημαντικοί Όροι:** Στερεό Απόβλητο Ζυθοποιίας (Brewers' Spent Grain - BSG), προκατεργασία, όξινη, αλκαλική, αριστοποίηση, κόστη, *Zymomonas mobilis* 8b, υδρόλυση, αλκοολική ζύμωση, Προσέγγιση Ενιαίου Αντιδραστήρα (SVA), Διαχωρισμός Υδρόλυσης – Ζύμωσης (SHF)

### **Περίληψη**

Η παρούσα εργασία αποτελεί μian κατά το δυνατό πλήρη αξιολόγηση της καταλληλότητας του Στερεού Αποβλήτου Ζυθοποιίας (Brewers' Spent Grain - BSG) Ελληνικής ζυθοποιίας, ως υπόστρωμα για την παραγωγή βιοαιθανόλης δεύτερης γενιάς. Η μελέτη περιλαμβάνει την πρώτη συγκριτική αριστοποίηση μεταξύ της ήπιας αλκαλικής και ήπιας όξινης προκατεργασίας του BSG ως προς την επακόλουθη ενζυμική υδρόλυση και επιπλέον, της αξιολόγησης της απόδοσης του βακτηρίου *Zymomonas mobilis* 8b στη ζύμωση των παραγόμενων σακχάρων σε αιθανόλη. Επιπρόσθετα, επιχειρείται και η αριστοποίηση της προκατεργασίας ως προς την κεφαλαιουχική απόδοση σε αναγωγικά σάκχαρα.

Σε αντίθεση με άλλες προσπάθειες αριστοποίησης της προκατεργασίας, όπου χρησιμοποιούνται μόνο δύο ανεξάρτητες μεταβλητές στη βελτιστοποίηση, στην προσέγγισή μας η αριστοποίηση πραγματοποιήθηκε συναρτήσει τριών ανεξάρτητων μεταβλητών: της θερμοκρασίας προκατεργασίας, της διάρκειας προκατεργασίας και της αναλογίας μάζας βάσης/οξέος προς μάζα BSG. Ως εξαρτημένη μεταβλητή επιλέχθηκε η απόδοση σε σάκχαρα μετά την ενζυμική υδρόλυση. Σε μια δεύτερη, καινοτόμο προσέγγιση, τα αποτελέσματα που προέκυψαν χρησιμοποιήθηκαν για την αξιολόγηση της διαδικασίας αριστοποίησης της προκατεργασίας από οικονομική άποψη, χρησιμοποιώντας την ειδική απόδοση σακχάρων - την ποσότητα των σακχάρων που παράγονται ανά μονάδα κόστους προκατεργασίας - ως εξαρτημένη μεταβλητή.

Ανάλογα με την αντικειμενική συνάρτηση αριστοποίησης (απόδοση σακχάρων ή ειδική απόδοση σακχάρων), ελήφθησαν διαφορετικά σεντ βέλτιστων συνθηκών προκατεργασίας. Αυτό συνέβη τόσο στην αλκαλική όσο και στην όξινη προκατεργασία, αν και για την

τελευταία, τα αντίστοιχα άριστα διέφεραν μόνο σε σχέση με τη συγκέντρωση οξέος. Η όξινη προκατεργασία αποδείχθηκε σχετικά πιο αποδοτική σε σχέση με την αλκαλική, με ελαφρώς υψηλότερες συνολικές αποδόσεις σακχάρων. Οι διαφορές που έχουν προκύψει μεταξύ των δύο στρατηγικών βελτιστοποίησης αποδεικνύουν ότι η προσέγγισή μας μπορεί να οδηγήσει σε σημαντικές μειώσεις του κόστους ανά kg παραγόμενων αναγωγικών σακχάρων.

Το δεύτερο μέρος της εν λόγω εργασίας αφορά στην αξιολόγηση του γενετικά τροποποιημένου στελέχους *Z. mobilis* 8b ως προς την παραγωγή αιθανόλης από τα προϊόντα υδρόλυσης του BSG. Για τη συνολική διαδικασία υδρόλυσης και ζύμωσης εξετάστηκαν δύο τρόποι λειτουργίας: Διαχωρισμός Υδρόλυσης και Ζύμωσης (Separate Hydrolysis Fermentation - SHF) και Προσέγγιση Ενιαίου Αντιδραστήρα (Single Vessel Approach - SVA).

Το σημαντικότερο αποτέλεσμα που προκύπτει από τα δεδομένα της αλκοολικής ζύμωσης είναι η επαλήθευση ότι το *Z. mobilis* 8b είναι ικανό να ζυμώνει τα σάκχαρα στα προϊόντα υδρόλυσης του BSG στη λειτουργία SVA, ειδικά μετά από αλκαλική προκατεργασία, όπου ο μικροοργανισμός ζυμώνει τα σάκχαρα BSG σε επίπεδα ίσα με το θεωρητικό μέγιστο. Ομοίως, τα σάκχαρα που λαμβάνονται από το BSG, υδρολυμένα και ζυμωμένα στη λειτουργία SHF, αποδείχθηκαν τα λιγότερο κατάλληλα υποστρώματα για την παραγωγή αιθανόλης από το *Z. mobilis* 8b, παρά το γεγονός ότι το προϊόν υδρόλυσης ενισχύθηκε με τα απαραίτητα άλατα.

Συνολικά, τα αποτελέσματά μας αποδεικνύουν ότι η παραγωγή αιθανόλης από το BSG αντιπροσωπεύει μια δυνητικά οικονομικά βιώσιμη προοπτική, όπου η έκβασή της εξαρτάται σε μεγάλο βαθμό από την επιλογή των συνθηκών προκατεργασίας που έχουν επαληθευτεί πειραματικά, του μικροοργανισμού και της συνολικής προσέγγισης ως προς τη λειτουργία του βιοδιωλιστηρίου (SVA ή SHF).



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**Figure 3.17.** 2D-contour plot and 3D-mesh plot of the mild acid pretreatment cost optimization results. Combined effect of pretreatment time (min) - X<sub>2</sub> and H<sub>2</sub>SO<sub>4</sub> load (mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) - X<sub>3</sub> on sugar yield at the saddle point temperature of 59 °C (X<sub>1, opt</sub>'=-0.215) as determined by the regression model .....

**Figure 3.18.** 2D-contour plot and 3D-mesh plot of the mild acid pretreatment cost optimization results. Combined effect of pretreatment temperature (°C) - X<sub>1</sub> and H<sub>2</sub>SO<sub>4</sub> load (mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) - X<sub>3</sub> on sugar yield at the optimum pretreatment time of 44 min (X<sub>2, opt</sub>=0.13) as determined by the regression model .....

**Figure 3.19.** 2D-contour plot and 3D-mesh plot of the mild acid pretreatment cost optimization results. Combined effect of pretreatment temperature (°C) - X<sub>1</sub> and pretreatment time (min) - X<sub>2</sub> on sugar yield - X<sub>3</sub> on sugar yield at the optimum H<sub>2</sub>SO<sub>4</sub> load (139 mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) (X<sub>3, opt</sub>=0.238) as determined by the regression model .....

**Figure 3.20.** 2D-contour plot and 3D-mesh plot of the mild acid pretreatment cost optimization results. Combined effect of pretreatment time (min) - X<sub>2</sub> and H<sub>2</sub>SO<sub>4</sub> load (mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) - X<sub>3</sub> on sugar yield at temperature T = 95 °C (X<sub>1</sub>=1).....

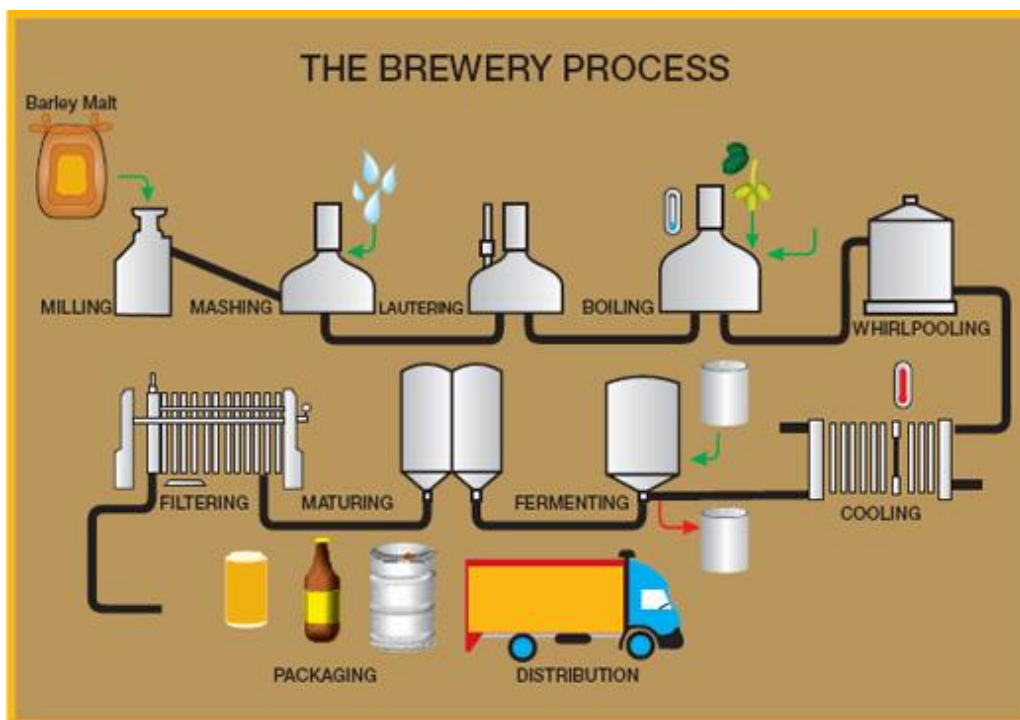
**Figure 3.21.** Reducing sugars and ethanol concentration during the course of SVA fermentation. Data points represent the mean of triplicate flasks. Left graph: SVA on NaOH pretreated material, Right graph: SVA on H<sub>2</sub>SO<sub>4</sub> pretreated material .....

**Figure 3.22.** Reducing sugars consumption and ethanol concentration, during the course of fermentation of enzymatically pretreated suspensions of pretreated BSG (SHF fermentation mode). Data points represent the mean of triplicate flasks. Left graph: SHF on NaOH pretreated material, Right graph: SHF on H<sub>2</sub>SO<sub>4</sub> pretreated material.....

# 1. Introduction

## 1.1. The Brewery Industry and its Wastes

Brewing Industry is one of the most profitable global businesses, with annual revenues of \$294.5 billion (2017), as a result of the increasing trend in worldwide beer consumption within the last decades. This fact has led to large volumes of global beer production, with China being the world leader, producing 48.9 billion L/year, followed by the United States with 22.5 billion L/year and Brazil with 12.4 billion L/year (dos Santos Mathias et al. 2014). Apart from the final product of the brewing process (beer), there is also a big percentage of residues being produced (Figure 1.1). There are four categories of wastes; brewer spent grain (BSG), residual brewing yeast and trub and diatomaceous earth. The first three wastes are responsible for the loss approximately 20 L per 100 L of water used in the brewing process, especially because of their high water content which fluctuates between 80% and 90% (dos Santos Mathias et al. 2014).



**Figure 1.1.** Schematic representation of the brewing process. Adopted from <https://www.eightdegrees.ie/brewing-process-2/>.

Brewers' Spent Grain (BSG) is the residue which occurs during the mashing step (Figure 1.1). In that process, there is an exhaustion of malted grains milled, as all the important soluble compounds which constitute the sweet wort are extracted. In this step, the bagasse formed has an important role as a filter element. This residue has a high nutritional value and it is the largest solid residue produced, resulting in a great volume of residue throughout the year, with low or no cost for its acquisition (Aliyu and Bala 2011).

BSG is a significant by-product in the overall brewing process, corresponding to about 85% of the total waste generated. It contributes to, on average, 30-60% of the biochemical oxygen demand (BOD) and suspended solids (Fillaudeau et al. 2006). Normally, for every 100 kg of processed grains, 125 to 130 kg of wet bagasse are generated, containing about 80% to 85% of moisture when obtained in filtration tanks, or 50% to 55%, when obtained in the press filter. This amount corresponds to around 14 to 20 kg of bagasse for each hectoliter of beer produced (Fillaudeau et al. 2006), which draws between 0.5% and 1% of the produced wort extract (dos Santos Mathias et al. 2014). The production of BSG in only Europe itself, is approximately over 3.4 million tons (Stojceska et al. 2008).

During the mashing process, about 80% of the malt mass is solubilized, leaving its the insoluble fractions in the bagasse. Although Brewers' Spent Grain composition varies with the species of barley used, the specifics of the malting process, as well as the type of milling, mashing and clarification applied on the malt (Santos et al. 2003; Celus et al. 2006), exhausted malt is predominantly a fibrous lignocellulosic material with significant lipid and protein content, containing nutritional value equivalent to about one fifth of the value of barley (Tang et al. 2009). BSG generated, represents on average 31% of the original malt weight, therefore, the recycling options of this residue are significant for the economical processing and bioconversion into added-value products (Nigam 2017).

## ***1.2. Current Disposal Methods of Brewers' Spent Grain (BSG)***

Modern day brewing technological advances have been developed to reduce the amount of waste produced and to generate useful materials from the by-products of brewing. There are economic advantages in reducing product waste and producing valuable by-products for the brewing industry, in addition to the fact that reusing the residues generated contributes to



circular economy and consequently, to the protection of the environment (Kerby and Vriesekoop 2017).

The most common use of BSG is commercialization to animal feed production, as it has been found to be an excellent protein source for ruminants (Nigam 2017). Regardless of the location or brewery size, the main method of BSG disposal is as animal feed. Large breweries typically have their BSG removed by animal feed producers who process BSG as a base material for animal feed, rather than having it delivered directly to farms (Kerby and Vriesekoop 2017).

BSG may also be added to other process residues to increase their market value, for example, trub, brewery residual yeast and diatomaceous earth (Briggs et al. 2004). Beyond its reuse in these applications, some of the BSG components could be useful as precursors for food grade chemicals or as carbon source for microbial fermentations (Gupta et al. 2010). Feed trade sources reported that availability of distillery by-products in feeds was at high level in year 2012 and its usage in animal feeds has increased by 51% since 2007. Industrially produced lysine and threonine are added in the animal feed compositions using barley grain to obtain a balanced nutritional diet. However, these essential amino acids are added at additional cost (Nigam 2017). Another nutritional application of BSG is its use in high-fibre cookies which have been tested to provide health benefits (Prentice et al. 1978). Furthermore, barley residue has been evaluated for its potential as a functional baking ingredient in a study that showed that addition of 25% and 35% BSG significantly increased the protein content of the snacks and the addition of 15% doubled the content of dietary fibre (Ktenioudaki et al. 2012).

Finally, another method used for the utilization of BSG is composting. The composting of BSG by itself has been found to be difficult because of its high moisture content. Nevertheless, when combined with other waste streams, BSG can be successfully composted (Stocks et al. 2002).

### ***1.3. Potential Uses of BSG***

Apart from the typical methods of reusing BSG in dietary and nutritional applications, waste barley may also be used as a useful resource-material for microbial fermentations as well as for commercial/industrial use.

The carbohydrate content of BSG makes it a potential substrate for various applications; the sugars that are released after chemical or enzymatic processing can be microbiologically converted into various bioproducts, such as organic acids, ethanol, glycerol, butanol, etc. The use of BSG as a substrate in a growth medium for a variety of suitable microorganisms provides a cheaper carbon source compared to defined media for economical production of a desired product (Nigam 2017).

BSG has been proposed to be used as a substrate (carbon source) following its hydrolysis, for lactic acid production, in a bioprocess employing *Lactobacillus delbrueckii* (Mussatto et al. 2008a; Singh nee' Nigam 2009). Xylitol production is another potential field of application of BSG, since xylitol can be produced by fermentation from xylose using acid hydrolysates of waste BSG (Aliyu and Bala 2011). Xylitol is an important alternative to sucrose as a sweetener with many applications in the food industry (Nigam 2017), and hence, BSG use in xylitol production could reduce the cost of the whole process.

BSG can also be used in microbial enzyme production. It is well known that enzyme production on industrial scale using defined media, incorporates a high cost of production, thus the utilization of waste BSG as a cheap raw material could be the key to reduce the overall costs and making enzyme production more profitable (Vandamme 2009).

Another use of BSG could be in protein hydrolysate production, as the barley waste may consist of 10-24% protein content on a dry weight basis (Robertson et al. 2010). Finally, BSG can also be used as a cheaper supporting matrix for the immobilization of yeast cells instead of synthetic polymers, and such prepared biocatalysts were successfully used as starter cultures for wine making and beer production (Tsaousi et al. 2010).

#### ***1.4. BSG as a Substrate for 2<sup>nd</sup> Generation Bioethanol Production***

Due to the fact that cellulose and hemicellulose are the most abundant renewable natural resources of reduced carbon in the biosphere, BSG, containing appreciable amounts of cellulose and hemicellulose, is a potential feedstock for the production of 2<sup>nd</sup> generation bioethanol. The production of fuel bioethanol is predominantly from agricultural crops, rich in cellulose and starch (Aggarwal et al. 2001), and conventionally, alcohol biofuels have been produced on industrial scale by fermentation of sugars derived from wheat, corn, sugar beets, sugarcane and molasses (1<sup>st</sup> generation biofuels). Such commercially produced biofuels have

the drawback of being dependent on food crops (Gnansounou 2010; Nigam and Singh 2011). Hence, there is a need to promote a faster deployment of sustainable second generation bioethanol that will not compete with human food production (Singh et al. 2011). With the increasing demand for ethanol, there is not only search for cheaper, abundant and annually renewable substrates, but also for the development of an efficient and less expensive technology so that ethanol can be made available at a lower cost from these substrates (Shindo and Tachibana 2006; Vandamme 2009; Nigam and Singh 2011). Therefore, an alternative method has been proposed through the utilization of residual biomass in BSG by economical bioprocessing, namely, the Second Generation Ethanol Production from BSG (Liguori et al. 2015). Using BSG as a source of energy production will also contribute towards making brewery industry more sustainable by producing less waste and contributing to the value chain (Mussatto 2014).

In order to liberate the fermentable sugars from the recalcitrant lignocellulosic materials, a pretreatment stage is usually required to thermally or chemically modify the material and facilitate higher sugar yields during subsequent enzymatic conversion. Therefore, the development of economical yet effective pretreatments is required (Wilkinson et al. 2014). An important factor when evaluating potential lignocellulosic biomass pretreatments is their ability to operate efficiently at high solids loadings, which is a requirement for any biofuel production process to be economically viable on a commercial scale (Lu et al. 2009; Petersen et al. 2009; Galbe and Zacchi 2012). The technical difficulty and cost associated with recycling pretreatment chemical reagents - for example, ethanol in an Organosolv process (Alriols et al. 2010) or ionic liquids (Dennewald et al. 2011) - encourages the use of low-cost, abundant, and dilute reagents such as common acids and alkalis in processes where their regeneration is not required. Pretreatment of lignocellulosic biomass such as BSG requires careful optimization because it must be severe enough to deconstruct the matrix sufficiently, to facilitate enhancement of the subsequent enzymatic hydrolysis, yet not so energy intensive as to negate the net energy balance of the putative biofuel production process. Furthermore, excessive severity of pretreatment results in the formation of lignocellulosic degradation products (including furfural, 5-hydroxymethylfurfural (HMF), ferulic, and p-coumaric acid) that are known yeast-inhibitory compounds with the potential to impact on downstream ethanol yields (Palmqvist and Hahn-Hägerdal 2000; Luo et al. 2002; Carneiro et al. 2008). The primary mode of action of pretreatment strategies for lignocellulosic biomass initially

was the removal in part of either hemicellulose, lignin, or both. This enhances the accessibility of cellulose to any cellulase class enzymes present and improves its enzymatic digestion to glucose (Carvalho et al. 2008).

Enzymatic hydrolysis of cellulose is a reaction initially carried out by cellulase enzymes, which are highly specific (Sharma et al. 2016). Cellulases are usually a mixture of several enzymes, among which at least three major groups are involved in the hydrolysis of cellulose: (1)  $\alpha$ -1,4-endoglucanase, which attacks regions of low crystallinity in the cellulose fiber creating free chain ends; (2)  $\alpha$ -1,4-exoglucanase or cellobiohydrolase, which degrades the molecule further by removing cellobiose units from the free chain ends; (3)  $\beta$ -glucosidase or cellobiase, which hydrolyzes cellobiose to produce glucose (Bayer et al. 1998; van den Brink and de Vries 2011; Guerriero et al. 2015; Sharma et al. 2016). Because enzymatic hydrolysis is a heterogeneous reaction and requires direct physical contact between enzyme and substrate, the enzymes must diffuse from the bulk aqueous solution to the particle surface, diffuse through physical barriers such as lignin, adsorb on the substrate surface, and then catalyze the hydrolysis (Chang and Holtzaple 2000; Cao and Tan 2002). Consequently, these reactions are complex and can be affected by the physicochemical properties of the substrate such as crystallinity, degree of polymerization, surface area, and lignin and hemicellulose contents (Chang and Holtzaple 2000; Vuong and Wilson 2009).

In lignocellulosic materials, cellulose is physically associated with hemicellulose, and physically and chemically associated with lignin. The presence of these two fractions is reported to make the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis (Gilbert 2010; Wilkinson et al. 2014). The lignocellulosic ethanol industry drives forward the development of pretreatment and enzymatic hydrolysis technology to hydrolyse lignocellulosic carbohydrates to fermentable sugars (Sun and Cheng 2002; Kemppainen et al. 2016). Due to the high hemicellulose content of BSG, it is very important at the industrial level, that a mixture of both cellulases and hemicellulases class enzymes to be used in the hydrolysis process, in order to achieve a higher sugar yield production (Saha 2003; Carvalho et al. 2008).

After the production of fermentable sugars through the hydrolysis process, follows the microbial fermentation. The most important factor for microorganism selection for the fermentation process is the convertibility of released sugars to ethanol. A large number of facultative anaerobic fungi, yeasts and bacteria have been employed for this with the yeast

*Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* being the most prominent in terms of productivity and ethanol yield (Gnansounou 2010; Ho et al. 2014; Alfenore and Molina-Jouve 2016; Rastogi and Shrivastava 2018). The main drawback of these two strains is the fact that their wild-type strains do not have the ability to ferment xyloses into ethanol.

In order to overcome this problem, *Z. mobilis* wild-type strain has been genetically modified with the introduction of genes that allow xylose metabolism (Mohagheghi et al. 2004). Recently, the strain has been studied and found to be adapted/evolved for enhanced tolerance to the toxic compounds present in corn stover hydrolysates. The adapted strain SS3 has higher xylose utilization rate and produce more ethanol than the parent strain (Mohagheghi et al. 2015). However, there are no studies available concerning the microbial fermentation of produced sugars from BSG pretreatment/hydrolysis process.

## 2. Materials and Methods

### 2.1. Compositional Analysis of BSG

#### 2.1.1. Raw material and moisture determination

Raw BSG was obtained from Athenian Brewery S.A. (Leof. Kifisou 102, Egaleo 122 41, Attica). It was split into 3-4 kg batches and stored at -20 °C in plastic bags. Raw material was dried at 65 °C until constant weight (for approx. 3 days), subjected to size reduction (< 1 mm particle size) in a benchtop mill (IKA®-Labortechnik, Model MFC, Type DCFH 48, JANKE & KUNKEL GmbH) and finally stored in airtight vessels until use. Moisture content of the raw and pretreated BSG fractions was determined in an IR moisture determination balance (Kett, Model FD-600).

#### 2.1.2. Reducing sugars, glucose and protein concentration

Reducing sugars concentration in the various samples throughout this work, was determined by the DiNitro Salicylic acid (DNS) method (Miller 1959). 200 µL of properly diluted sample were mixed with 200 µL of DNS reagent and boiled for 5 min. Absorbance was determined at 540 nm in a microplate reader (ThermoScientific Inc.), against a blank prepared exactly the same way using ddH<sub>2</sub>O. Two different reference curves were prepared with glucose and xylose at the range of 0 to 2 g/L. Results are expressed as reducing sugars equivalents of glucose or xylose.

Glucose concentration was also determined through the glucose-specific Glucose Oxidase - Peroxidase (GOD-PAP) method. A commercial Kit (Biosis, Agios Dimitrios, Greece) was employed according to the manufacturer's recommendations.

Protein concentrations were determined in the microplate at 595 nm using the Bradford assay (Bradford 1976).

#### 2.1.3. Determination of soluble sugars and protein in raw BSG

Raw and dry BSG was suspended in ddH<sub>2</sub>O at 10% w/v and incubated at 90 °C for 30 min under occasional stirring. An aliquot was centrifuged (10000xg) and glucose, reducing sugars and protein content was determined in the supernatant.

#### 2.1.4. Determination of holocellulose and cellu-hemicellulose content of raw and pretreated BSG

The determination of BSG composition (cellulose and hemicellulose content) was based on available TAPPI (Technical Association of the Pulp and Paper Industry, [www.tappi.org](http://www.tappi.org)) protocols for plant biomass analysis, with some modifications. Compositional analysis was applied both to the raw and the pretreated materials. Prior to any manipulation, the humidity of every biomass sample was determined (Paragraph 2.1.1) in order all calculations to be based on the net dry biomass. All steps were performed at least in triplicates.

For holocellulose (cellulose + hemicellulose) determination, approximately 700 mg ( $\pm 0.1$  mg) of dried biomass were placed in a wide-neck 100 mL conical flask followed by the addition of 50 mL distilled water. The flask was covered and slowly heated up to boiling temperature under gentle stirring. 1 mL of acetic acid and 0.7 g NaClO<sub>2</sub> were successively and slowly added and boiling continued for 50 min. Additional 1 mL of acetic acid and 0.7 g NaClO<sub>2</sub> were added and the mixture was boiled for another 50 min. During this time occasional dH<sub>2</sub>O addition was taking place in order to keep the volume around 35-40 mL. The flask was cooled to room temperature and its content was quantitatively transferred into a 50 mL PP-tube. The tube was centrifuged (4000 rpm, 15 min) and the supernatant was carefully decanted. The precipitated holocellulose was washed 3-times with distilled water, each followed by centrifugation. 100 mg of sodium bicarbonate were added, and the holocellulose precipitate was washed 2 additional times with distilled water to neutrality, plus a final one with 96% ethanol. The tube content was quantitatively transferred in pre-weighed low-tare aluminum plates, dried at 60 °C overnight followed by 3 h at 100 °C, cooled in a dessicator and weighed ( $\pm 0.1$  mg). The dry material on the aluminum plate was considered as ***holocellulose*** and its weight was used to determine the holocellulose percentage in biomass.

The whole amount of dried holocellulose (from above) was weighed ( $\pm 0.1$  mg) and placed in an 100 mL flask. 50 mL of 2 % w/v HCl were added, the flask was covered and the mixture was boiled for 2h in order to solubilize the hemicelluloses. The flask was then cooled to room temperature, its content was quantitatively transferred into a 50 mL PP-tube, and the same washing-neutralization procedure used for holocellulose, was applied for the precipitated cellulose. **Cellulose** percentage in holocellulose was determined from the weight of the corresponding dry material, while the remaining percentage is regarded as **hemicelluloses**.

## ***2.2. Effect of enzyme load and time on the Enzymatic Hydrolysis of BSG***

### ***2.2.1. Preparation of enzyme mix***

The enzyme mix used throughout this work consisted of an equivolume preparation of Novozyme NS22192 and Novozyme HTec2, designated by the manufacturer as a mostly cellulase and mostly hemicellulase mix, respectively. The working solution was prepared by initially mixing 1 v from each preparation with 2 v of 150 mM citrate-phosphate buffer pH 5. This intermediate solution was desalted in a GE-Healthcare PD-10 column equilibrated with the same buffer. The final working enzyme stock prepared through this procedure, contained approx. 30 mg/mL protein.

### ***2.2.2. Evaluation of optimum hydrolysis conditions***

Three conditions with different protein loads were evaluated, namely, 50, 100, and 200 mg protein / g of raw BSG. For each condition, 300 mg of raw BSG were pre-weighted in two 15-mL PP-tubes. The volume of enzyme mix that corresponds to each protein load was added, along with pH 5 buffer up to a final volume of 4 mL. An extra tube was used as a blank, containing 300 mg of raw BSG and 4 mL of pH 5 buffer, without enzyme. Hydrolysis took place under mild shaking at 50 °C. 400 µL aliquots were obtained from each tube at different time intervals, centrifuged, and reducing sugars concentration was determined in the clarified supernatants.

## ***2.3. Optimization of BSG pretreatment***

### ***2.3.1. General procedure for the determination of optimum pretreatment conditions***

Alkali (dilute NaOH) and acid (dilute H<sub>2</sub>SO<sub>4</sub>) pretreatments were performed in 15 mL PP-tubes at a combination of temperature, pretreatment time and acid/alkali load conditions (see Results and Discussion). For each condition, 300 mg ( $\pm$  0.1 mg) of raw and dry BSG were placed in the tube followed by the addition of 1.8 mL of NaOH or H<sub>2</sub>SO<sub>4</sub> solution of the appropriate concentration in order to achieve the required alkali/acid load. Following mixing in a vortex, tubes were incubated in a water bath for a certain temperature and time. After the end of each pretreatment, the suspensions were neutralized by the addition of equivalent amounts of HCl or NaOH solution (0.45 mL) followed by the addition of 1 mL of enzyme



mix (Paragraph 2.2.1) and citrate-phosphate buffer pH 5 up to a final volume of 4 mL. The tubes were then incubated for  $20 \pm 2$  h at 50 °C with mild stirring and released sugars concentration was determined in the clarified (14000 x g) supernatants. Each pretreatment condition was conducted in triplicate tubes, while two additional tubes where the enzyme mix was replaced with buffer, was processed for every pretreatment condition.

#### 2.3.2. Re-evaluation of enzyme load at the optimal conditions of each pretreatment

Optimum enzyme load was additionally evaluated at the optimum conditions of the mild alkali and acid pretreatments determined in the previous paragraph. The general pretreatment procedure was followed at the corresponding optimum conditions for each pretreatment, with the application of four different enzyme loads, namely, 55, 95, 135, and 175 mg protein / g of raw BSG (4 mL final volume).

### **2.4. Ethanol production from BSG hydrolysates**

#### 2.4.1. Microorganism

The strain of *Zymomonas mobilis* 8b was used (Mohagheghi et al. 2004). This strain has been genetically modified in order to be able to ferment xylose, in addition to glucose (which is the natural substrate of the wild-type version of the microorganism). *Z. mobilis* 8b was maintained and propagated in agar plates containing DSMZ medium 1445 (without carbon source) supplemented with chloramphenicol and either glucose or xylose at an initial concentration of 10 g/L.

#### 2.4.2. Fermentation modes

Two fermentation modes were evaluated, namely, the Single Vessel Approach (SVA) and the Separate Hydrolysis and Fermentation (SHF). In the first, all process steps, pretreatment, enzymatic hydrolysis and fermentation are performed in the same vessel, following appropriate pH adjustments relevant to each step. In SHF, pretreatment proceeds independently and the pretreated material is subsequently hydrolyzed and fermented in a different vessel.

#### 2.4.3 Single Vessel Approach (SVA)

For the SVA, optimum pretreatment conditions (temperature, time, NaOH / H<sub>2</sub>SO<sub>4</sub> load) on raw BSG were applied in a 100 mL flask at the working concentration of 166.67 g solids/L. Following pretreatment, the pH of the suspension was adjusted to 5 and hydrolysis took place by the addition of enzyme mix (0.1 g protein / g BSG) at 83.3 g BSG solids/L.

Following completion of hydrolysis, the pH of the suspension was adjusted to 6. Fermentation took place in 13 mL anaerobic fermentation vials, at 30 °C and under mild shaking. The fermentation medium consisted of 10.8 mL pretreatment/hydrolysis mix, supplemented with 1.2 mL of 10x DSMZ 1445 medium (without carbon source) and 0.5 mL of *Z. mobilis* 8b suspension adjusted to an OD<sub>600nm</sub> of 40. For each experimental condition, *Z. mobilis* 8b inoculum was prepared from either glucose or xylose cultures.

400 µL aliquots were obtained from each vial at different time intervals, centrifuged, and reducing sugars concentration was determined in the clarified supernatants using the DNS method. Ethanol concentration was also determined in the clarified supernatants on an SRI 8610 gas chromatograph, using a Supelcowax-10 column.

#### 2.4.4. Separate Hydrolysis and Fermentation

For the separate hydrolysis/ fermentation approach, 150 gr of BSG were initially pretreated with NaOH and H<sub>2</sub>SO<sub>4</sub> at the optimum determined conditions for the mild alkali and mild acid pretreatment, respectively. Following each pretreatment, the treated material was washed with tap water on a 0.125 mm sieve, following neutralization at pH 5. The pretreatment supernatants were stored in PP-tubes at -20 °C for reducing sugars determination and further analysis. Both pretreated solids of each occasion were dried (<10% humidity), milled (0.7 mm) and stored.

A pilot hydrolysis was first carried out, in order to determine the initial reducing sugars content. 300 mg of NaOH / H<sub>2</sub>SO<sub>4</sub> pretreated BSG were placed in six 15-mL PP-tubes for each occasion, as well as 1 mL of enzyme and 3 mL of buffer pH 5. Hydrolysis took place under mild shaking at 50 °C. 400 µL aliquots were obtained from each tube, centrifuged, and reducing sugars concentration was determined in the clarified supernatants using the DNS method.

For the actual SHF process, NaOH/H<sub>2</sub>SO<sub>4</sub> pretreated BSG was hydrolyzed at the standard conditions (pH 5, 0.1 g protein / g pretreated material, 83.33 g solids/L). Following

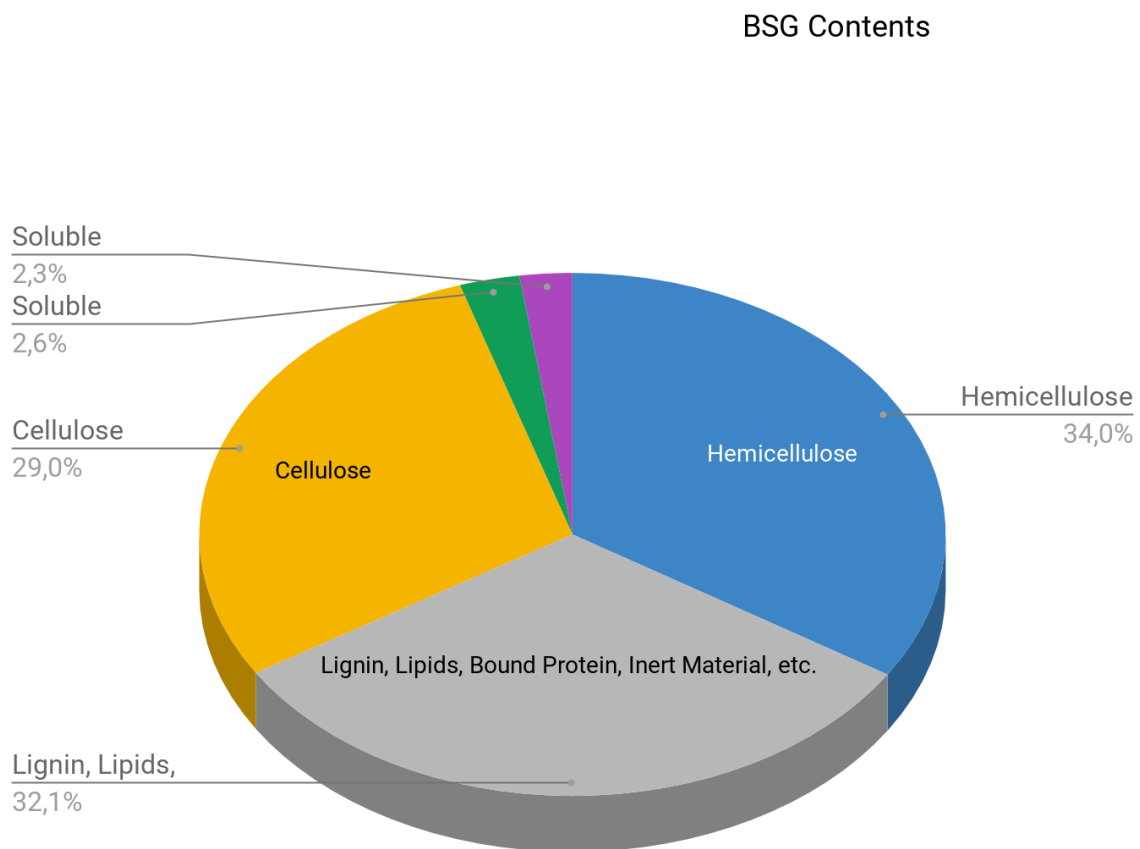
completion of hydrolysis, the pH of the suspension was adjusted to 6. Fermentation took place in 13 mL anaerobic fermentation vials, at 30 °C and under mild shaking. The fermentation medium consisted of 10.8 mL hydrolysis mix, supplemented with 1.2 mL of 10x DSMZ 1445 medium (without carbon source) and 0.5 mL of *Z. mobilis* 8b suspension adjusted to an OD<sub>600nm</sub> of 40. For each experimental condition, *Z. mobilis* 8b inoculum was prepared from either glucose or xylose cultures.

400 µL aliquots were obtained from each vial at different time intervals, centrifuged, and reducing sugars concentration was determined in the clarified supernatants using the DNS method. Ethanol concentration was also determined in the clarified supernatants on an SRI 8610 gas chromatograph, using a Supelcowax-10 column.

### 3. Results & Discussion

#### 3.1. Compositional Analysis of BSG

Raw BSG was analysed for its soluble sugars and protein, as well as for its cellulose and hemicelluloses content as described in Materials and Methods section. The obtained results are shown in Figure 3.11.



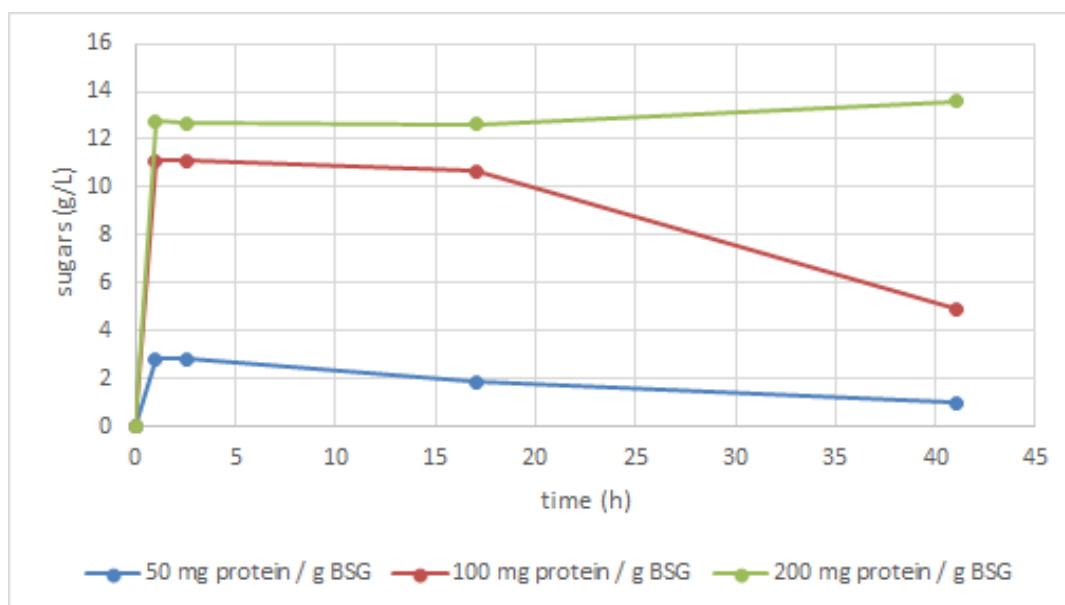
**Figure 3.1.** Compositional analysis of raw BSG. Percentages are calculated on a dry basis.

As shown in Figure 3.1 the BSG used in this work (originating mostly from Greek barley varieties) contains over 60% holocellulose (cellulose and hemicelluloses) with hemicelluloses representing 54% and cellulose 46% (dry basis). The determined holocellulose value is significantly higher than those reported in other studies, where the holocellulose content is usually found at levels below 50% w/w (Mussatto and Roberto 2006; Mussatto et al. 2008b;

Wilkinson et al. 2014; Kemppainen et al. 2016; Ravindran et al. 2018). This difference could probably reflect specific differences on the malt characteristics or the mashing process as well as difference in the applied methodology. Our results though, agree with all these studies on the fact that the hemicellulose content in raw BSG is higher than that of cellulose, a fact that suggests that any economically sustainable utilization of BSG for the production of ethanol should definitely involve the fermentation of pentoses obtained from its hemicellulosic fraction.

### 3.2. Effect of enzyme load and processing time on BSG hydrolysis

Following the procedures described in Materials and Methods, the obtained results are shown in the diagram below:



**Figure 3.2.** Effect of incubation time on total reducing sugars release from raw BSG at different enzyme loads. BSG was hydrolysed by the addition of the indicated protein loads (from the enzyme mix) per g raw and dry BSG at 50 °C and pH 5. Total reducing sugars were measured by the DNS method (Paragraph 2.1.2) and expressed as glucose equivalents.

As shown in the Figure above, hydrolysis of raw BSG by the commercial enzyme mix was completed already from the 2<sup>nd</sup> hour of incubation, regardless of the enzyme load applied. This result is very important from an economic point of view since it contributes to the overall process productivity. Reducing sugars' concentration remained practically constant till the 20th hour, and as a result, this hydrolysis incubation time was chosen for all subsequent experimental sets.

Enzyme load significantly affected the final reducing sugars concentration. The observed effect was much stronger between 50 and 100 mg protein per g raw BSG and practically insignificant between 100 and 200 mg per g BSG. Taking into account the fact that enzyme cost is the second most important cost factor in biorefinery applications (Lynd et al. 2008) an enzyme load of 100 mg/g was chosen for the rest of this work.

### ***3.3. Economic evaluation of the most suitable alkali and acid for the pretreatment***

In literature, for mild acid and mild alkali pretreatment of lignocellulosic materials, the most commonly used reagents are hydrochloric or sulfuric acid and potassium or sodium hydroxide, respectively (Galbe and Zacchi 2012).

For the alkaline pretreatment in the vast majority of studies NaOH is employed, due to its significantly lower price and high activity towards sugar yield (Chen et al. 2013). For the acid pretreatment the choice between H<sub>2</sub>SO<sub>4</sub> and HCl is more obscure, since both acids are highly active in the initial decomposition of the lignocellulosic matrix (Saha et al. 2005). Thus our choice had to be based on the economic efficiency of these acids based on available data on their relative prices. Tables 3.1 to 3.3 show the price per kilogram for HCl, H<sub>2</sub>SO<sub>4</sub> and NaOH as obtained from various vendors at the internet was eventually selected, due to its activity in relate to HCl and after the economic assessment carried out in the table shown below:

**Table 3.1.** Median internet prices for hydrochloric acid as obtained from various internet sources.

<b>HCl</b>				
<b>Source</b>	<b>Concentration (w/w)</b>	<b>Price (\$/ton)</b>	<b>Mass per ton (kg)</b>	<b>Cost (\$/kg HCl)</b>
Vendor 1	33.5%	265	335	\$0.791
Vendor 2	32.5%	107.5	325	\$0.331
Vendor 3	32%	125	320	\$0.391
Vendor 4	31%	185	310	\$0.597
Vendor 5	35%	185	350	\$0.529

Vendor 6	37%	300	370	\$0.811
Vendor 7	36%	195	360	\$0.542
Vendor 8	31%	325	310	\$1.048
Vendor 9	31.5%	85	315	\$0.270
<b>Average Cost of HCl (\$/kg HCl)</b>				<b>\$0.590</b>

**Table 3.2.** Median internet prices for sulfuric acid as obtained from various internet sources.

<b>H<sub>2</sub>SO<sub>4</sub></b>				
<b>Source</b>	<b>Concentration (w/w)</b>	<b>Price (\$/ton)</b>	<b>Mass (kg)</b>	<b>Cost (\$/kg H<sub>2</sub>SO<sub>4</sub>)</b>
Vendor 1	98%	230	980	\$0.235
Vendor 2	98%	220	980	\$0.224
Vendor 3	98%	225	980	\$0.230
Vendor 4	98%	275	980	\$0.281
Vendor 5	98%	325	980	\$0.332
Vendor 6	98%	250	980	\$0.255
<b>Average Cost of H<sub>2</sub>SO<sub>4</sub> (\$/kg H<sub>2</sub>SO<sub>4</sub>)</b>				<b>\$0.259</b>

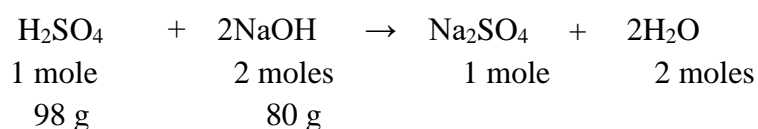
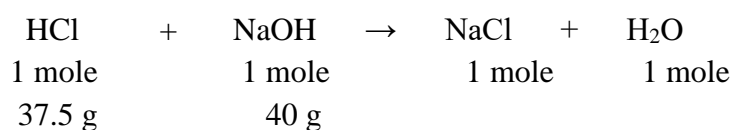
**Table 3.3.** Median internet prices for sodium hydroxide as obtained from various internet sources.

<b>NaOH</b>				
<b>Source</b>	<b>Concentration (w/w)</b>	<b>Price (\$/ton)</b>	<b>Mass (kg)</b>	<b>Cost (\$/kg NaOH)</b>
Vendor 1	100%	350	1000	\$0.350
Vendor 2	100%	407.5	1000	\$0.408
Vendor 3	100%	520	1000	\$0.520
Vendor 4	100%	490	1000	\$0.490
Vendor 5	100%	810	1000	\$0.810
Vendor 6	100%	515	1000	\$0.515
Vendor 7	100%	510	1000	\$0.510

Vendor 8	100%	550	1000	\$0.550
Vendor 9	100%	475	1000	\$0.475
Vendor 10	100%	300	1000	\$0.300
Vendor 11	100%	600	1000	\$0.600
<b>Average Cost of NaOH (\$/kg NaOH)</b>				<b>\$0.503</b>

All costs shown in Tables 3.1 to 3.3 were obtained from alibaba.com, from several sellers who offer HCl, H<sub>2</sub>SO<sub>4</sub> and NaOH for industrial use.

By comparing the average costs of HCl and H<sub>2</sub>SO<sub>4</sub>, the cost per kg of H<sub>2</sub>SO<sub>4</sub> is approximately two times lower than the relevant cost of HCl. However, when one of these two acids is used in the pretreatment of lignocellulosic material its neutralization has to be taken into account regarding the cost evaluation. The neutralization reaction equations of HCl by NaOH and H<sub>2</sub>SO<sub>4</sub> by NaOH are the following:



According to the stoichiometry of the above equations:

1 kg of HCl (0.590 \$) requires 1.067 kg (0.537 \$) of NaOH at a total cost of **1.127 €**, while

1 kg of H<sub>2</sub>SO<sub>4</sub> (0.259 \$) requires 0.816 kg (0.411 \$) of NaOH at a total cost of **0.67 €**

Based on the above economic analysis, the choice of **sulfuric acid** for the mild acid pretreatment of BSG is straightforward.



### ***3.3. Experimental optimization of the alkaline and acid pretreatment of BSG***

#### *3.3.1. Definition of the independent parameter space and experimental design settings*

As mentioned in the Materials and Methods section, pretreatment of BSG was optimized with respect to three independent process parameters, namely:

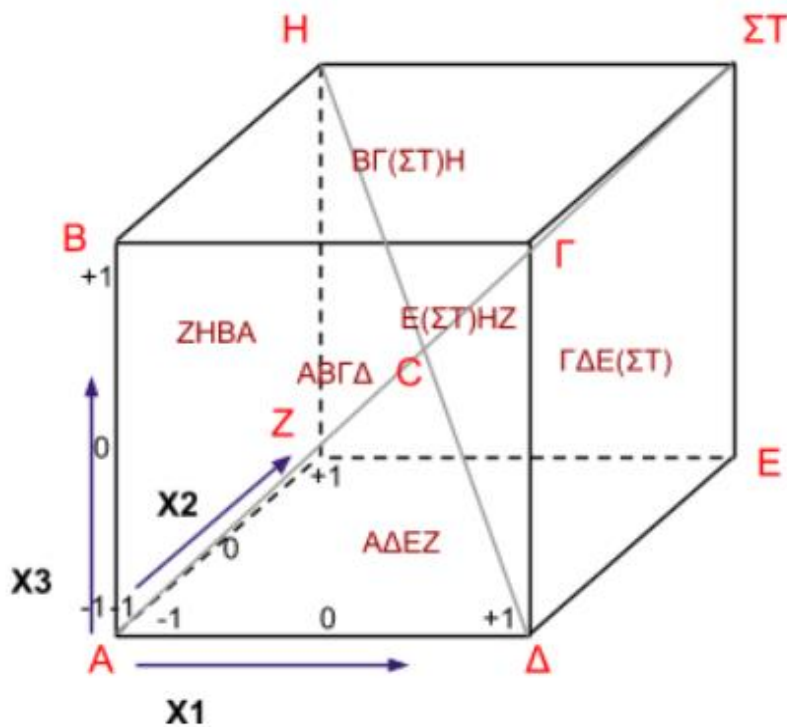
**X<sub>1</sub>**: Pretreatment temperature, in °C

**X<sub>2</sub>**: Pretreatment time, in min

**X<sub>3</sub>**: Alkali/Acid load, in mg per g dry and raw BSG

The dependent variable, **Y**, for the optimization, was the concentration of the total reducing sugars released following enzymatic hydrolysis of the pretreated material, performed at the conditions determined in Paragraph 3.2.

A Central Composite Design (CCD) approach was applied in order to experimentally materialize the complete optimization procedure. According to the CCD model, the three independent parameters of the experiment,  $X_1$ ,  $X_2$  and  $X_3$  (Temperature, Time, NaOH / H<sub>2</sub>SO<sub>4</sub> load, respectively) form a three-dimensional space, namely, a cube (Figure 3.3). Each edge of the cube represents the values of  $X_1$ ,  $X_2$  and  $X_3$ ; from minimum to maximum, while the median is the means of the values. The combinations of  $X_1$ ,  $X_2$  and  $X_3$  to be evaluated are all 8 vertices of the cube (i.e. A, B, Γ, Δ, E, ΣT, H, Z), as well as the centers of all six faces (i.e. ABΓΔ, ΓΔE(ΣT), E(ΣT)HZ, ZHBA, AΔEZ, BΓ(ΣT)H). Finally, the center of the cube, C, represents the condition where all parameters acquire their central values. It also has more weight than the rest conditions, consequently it is going to be evaluated in multiple tests.



**Figure 3.3.** Independent parameter space model used in the central composite design experiments.

The minimum, median and maximum physical values used for each independent parameter are given in the Table 3.4. For normalization of the response function, the range of each independent parameter was normalized between -1 and 1 (see also Table 1).

**Table 3.4.** Physical and normalized independent parameter values employed in the Central Composite Design experiments for pretreatment optimization.

Parameter	Minimum	Median	Maximum
Temperature ( $X_1$ ), °C	35	65	95
	-1	0	1
Time ( $X_2$ ), min	10	40	70
	-1	0	1
NaOH / H <sub>2</sub> SO <sub>4</sub> load ( $X_3$ ), mg / g raw BSG	40	120	200
	-1	0	1

Following the analysis presented above, the experimental sets performed for the alkali/acid pretreatment optimization are summarized in Tables 3.5 and 3.6, that follow:

**Table 3.5.** Actual experimental conditions used in the optimization of BSG pretreatment.

Experiment #	Experimental conditions (Figure 3.3)	Temperature (°C)	Time (min)	NaOH / H <sub>2</sub> SO <sub>4</sub> load (mg / g BSG)
1	A	35	10	40
2	B	35	10	200
3	Γ	95	10	200
4	Δ	95	10	40
5	E	95	70	40
6	ΣΤ	95	70	200
7	Z	35	70	40
8	H	35	70	200
9	ΑΒΓΔ	65	10	120
10	ΓΔΕ(ΣΤ)	95	40	120
11	Ε(ΣΤ)ΗΖ	65	70	120
12	ΖΗΒΑ	35	40	120
13	ΑΔΕΖ	65	40	40
14	ΒΓ(ΣΤ)Η	65	40	200
15	C	65	40	120
16	C	65	40	120
17	C	65	40	120

**Table 3.6.** Normalized experimental conditions used in the optimization of BSG pretreatment.

Pretreatment Conditions Normalization				
Experiment #	Experimental conditions (Figure 3.3)	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>
1	A	-1	-1	-1
2	B	-1	-1	1
3	Γ	1	-1	1
4	Δ	1	-1	-1
5	E	1	1	-1
6	ΣΤ	1	1	1
7	Z	-1	1	-1
8	H	-1	1	1
9	ΑΒΓΔ	0	-1	0
10	ΓΔΕ(ΣΤ)	1	0	0
11	Ε(ΣΤ)ΗΖ	0	1	0
12	ΖΗΒΑ	-1	0	0
13	ΑΔΕΖ	0	0	-1
14	ΒΓ(ΣΤ)Η	0	0	1
15	C	0	0	0
16	C	0	0	0
17	C	0	0	0

### 3.3.2. Pretreatment optimization results and regression analysis

The experimental data sets presented in Tables 3.2 and 3.3 were performed as described in Materials and Methods. The obtained results for the alkali and acid pretreatment are given in Tables 3.7 and 3.8, respectively.

**Table 3.7.** Experimental results obtained for the **mild alkali pretreatment** of BSG at all experimental conditions. Y<sub>1</sub>: Reducing sugars concentration (g/L) in the pretreatment/hydrolysis tube with no added enzyme (blank). Y<sub>2</sub>: Reducing sugars concentration (g/L) in the pretreatment/hydrolysis vessel with enzyme added (0.1 g protein / per g raw BSG). Y: Net reducing sugars concentration (g/L) in the pretreatment/hydrolysis vessel corresponding to enzyme action on the pretreated material. Values represent the average of triplicate experiments.

NaOH Pretreatment Results							
Experiment #	Experimental conditions (Figure 3.3)	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y <sub>1</sub>	Y <sub>2</sub>	Y
1	A	-1	-1	-1	0.18	4.10	<b>3.92</b>
2	B	-1	-1	1	1.26	9.91	<b>8.65</b>
3	Γ	1	-1	1	0.41	17.71	<b>17.30</b>
4	Δ	1	-1	-1	0.31	8.13	<b>7.82</b>
5	E	1	1	-1	0.73	17.39	<b>16.66</b>
6	ΣΤ	1	1	1	3.05	20.94	<b>17.89</b>
7	Z	-1	1	-1	0.90	6.62	<b>5.72</b>
8	H	-1	1	1	3.84	20.65	<b>16.81</b>
9	ΑΒΓΔ	0	-1	0	2.54	18.96	<b>16.42</b>
10	ΓΔΕ(ΣΤ)	1	0	0	0.85	19.12	<b>18.27</b>
11	Ε(ΣΤ)ΗΖ	0	1	0	1.60	21.44	<b>19.84</b>
12	ZHBA	-1	0	0	0.82	14.26	<b>13.44</b>
13	ΑΔΕΖ	0	0	-1	0.37	10.16	<b>9.79</b>
14	ΒΓ(ΣΤ)Η	0	0	1	4.90	26.52	<b>21.62</b>
15	C	0	0	0	2.52	18.19	<b>15.67</b>

**Table 3.8.** Experimental results obtained for the **mild acid pretreatment of BSG** at all experimental conditions. Y<sub>1</sub>: Reducing sugars concentration (g/L) in the pretreatment/hydrolysis tube with no added enzyme (blank). Y<sub>2</sub>: Reducing sugars concentration (g/L) in the pretreatment/hydrolysis vessel with enzyme added (0.1 g protein / per g raw BSG). Y: Net reducing sugars concentration (g/L) in the pretreatment/hydrolysis vessel corresponding to the enzyme action on the pretreated material. Values represent the average of triplicate experiments.

<b>H<sub>2</sub>SO<sub>4</sub> Pretreatment Results</b>							
Experiment #	Experimental conditions (Figure 3.3)	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y <sub>1</sub>	Y <sub>2</sub>	Y
1	A	-1	-1	-1	0.48	1.85	<b>1.37</b>
2	B	-1	-1	1	0.52	7.47	<b>6.95</b>
3	Γ	1	-1	1	3.78	22.41	<b>18.63</b>
4	Δ	1	-1	-1	0.33	2.23	<b>1.90</b>
5	E	1	1	-1	0.36	5.77	<b>5.41</b>
6	ΣΤ	1	1	1	19.77	39.15	<b>19.38</b>
7	Z	-1	1	-1	0.36	3.22	<b>2.86</b>
8	H	-1	1	1	0.63	8.90	<b>8.27</b>
9	ΑΒΓΔ	0	-1	0	1.13	4.15	<b>3.02</b>
10	ΓΔΕ(ΣΤ)	1	0	0	20.46	43.54	<b>23.08</b>
11	Ε(ΣΤ)ΗΖ	0	1	0	0.74	8.07	<b>7.33</b>
12	ZHBA	-1	0	0	0.56	8.97	<b>8.41</b>
13	ΑΔΕΖ	0	0	-1	0.24	4.40	<b>4.16</b>
14	ΒΓ(ΣΤ)Η	0	0	1	0.70	10.27	<b>9.57</b>
15	C	0	0	0	1.29	8.10	<b>6.81</b>

### 3.3.3. Regression Analysis

Using the sugar yield data of all 15 experiment conditions, Sigmaplot software was used in order to determine the relation between the experiment parameters (Temperature, time, NaOH / H<sub>2</sub>SO<sub>4</sub> load) and sugar yield (g/L). The regression model used was a nonlinear, polynomial, 2<sup>nd</sup> order equation:

$$Y = a_0 + a_1 \cdot X_1 + a_2 \cdot X_2 + a_3 \cdot X_3 + a_{11} \cdot X_1^2 + a_{22} \cdot X_2^2 + a_{33} \cdot X_3^2 + a_{12} \cdot X_1 \cdot X_2 + a_{13} \cdot X_1 \cdot X_3 + a_{23} \cdot X_2 \cdot X_3 \quad (1)$$

where,

**Y**, is the final hydrolysis yield expressed as reducing sugars equivalent [the concentration of reducing sugars after pretreatment and enzymatic hydrolysis in the reaction vessel, Y in Tables 3.4 and 3.5],

**X<sub>1</sub>**, corresponds to the normalized temperature (°C),

**X<sub>2</sub>**, corresponds to the normalized duration of pretreatment (min) ,

**X<sub>3</sub>**, corresponds to the normalized NaOH or H<sub>2</sub>SO<sub>4</sub> load (g NaOH per g dry untreated material), and

**a<sub>ij</sub>**, model constants

The experimental results from Table 1 were fitted into equation (1) in order to obtain the values of the model constants for every substrate. For this reason we applied the non-linear regression routines of SigmaPlot™ software, and the obtained results are reported in Table 3.8. The optimum set of independent variables was determined by zeroing the three first derivatives of equation 1:

$$\frac{\partial y}{\partial x_1} = a_1 + 2 \cdot a_{11} \cdot x_{1,opt} + a_{12} \cdot x_{2,opt} + a_{13} \cdot x_{3,opt} = 0 \quad (2.1)$$

$$\frac{\partial y}{\partial x_2} = a_2 + a_{12} \cdot x_{1,opt} + 2 \cdot a_{22} \cdot x_{2,opt} + a_{23} \cdot x_{3,opt} = 0 \quad (2.2)$$

$$\frac{\partial y}{\partial x_3} = a_3 + a_{13} \cdot x_{1,opt} + a_{23} \cdot x_{2,opt} + 2 \cdot a_{33} \cdot x_{3,opt} = 0 \quad (2.3)$$

The above set of linear equations was solved for each substrate using the *lsolve* routine of MathCad™ software package (ver. 14).

#### 3.3.4. Regression analysis results for the mild alkaline pretreatment

Table 3.9 shows the results of the fitting the experimental results of the alkaline pretreatment (Table 3.7) in the regression equation (1).

**Table 3.9.** Regression coefficients determined from the regression analysis of the alkaline pretreatment.

<b>Coefficients</b>	<b>Regression coefficient values</b>	<b>P-value of the coefficient</b>
a <sub>0</sub>	17.9824	0.0001
a <sub>1</sub>	2.9400	0.0285
a <sub>2</sub>	2.2810	0.0643
a <sub>3</sub>	3.8360	0.0105
a <sub>11</sub>	-2.7056	0.2137
a <sub>22</sub>	-0.4306	0.8299
a <sub>33</sub>	-2.8556	0.1934
a <sub>12</sub>	-0.0662	0.9547
a <sub>13</sub>	-0.6387	0.5793
a <sub>23</sub>	-0.2362	0.8356
<b>R<sup>2</sup> of the model</b>	<b>0.8880</b>	

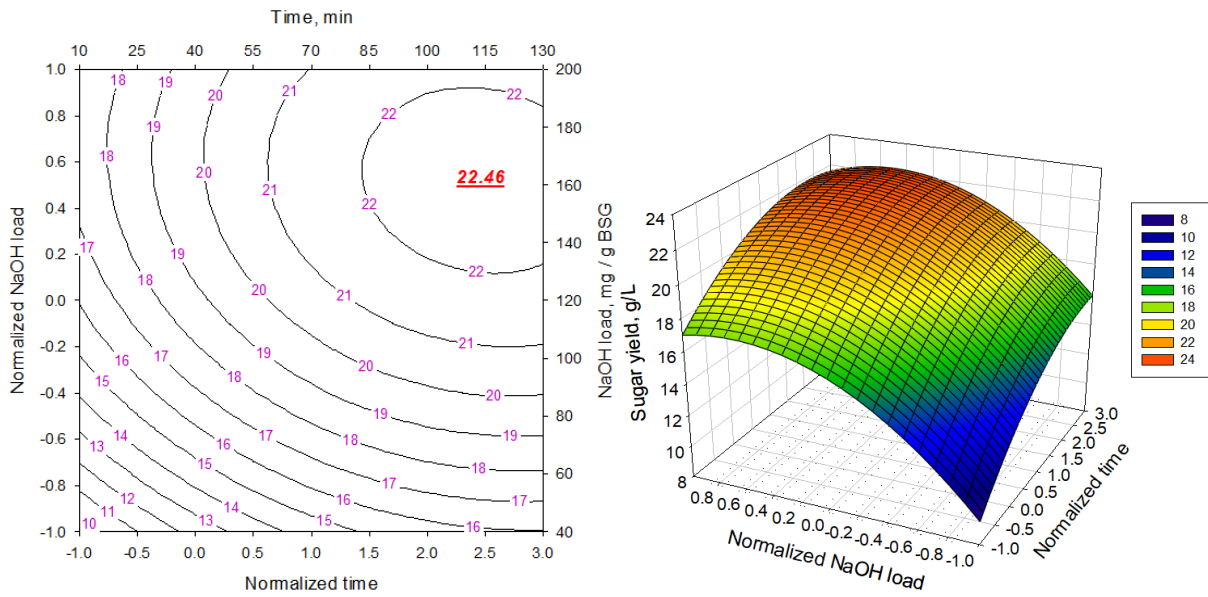
Based on the data from Table 3.9, solving the linear system resulting from zeroing the first derivatives of equation (1), the following optimum model values for the independent parameters were obtained.

**Table 3.10.** Optimal model conditions for the alkaline pretreatment of BSG.

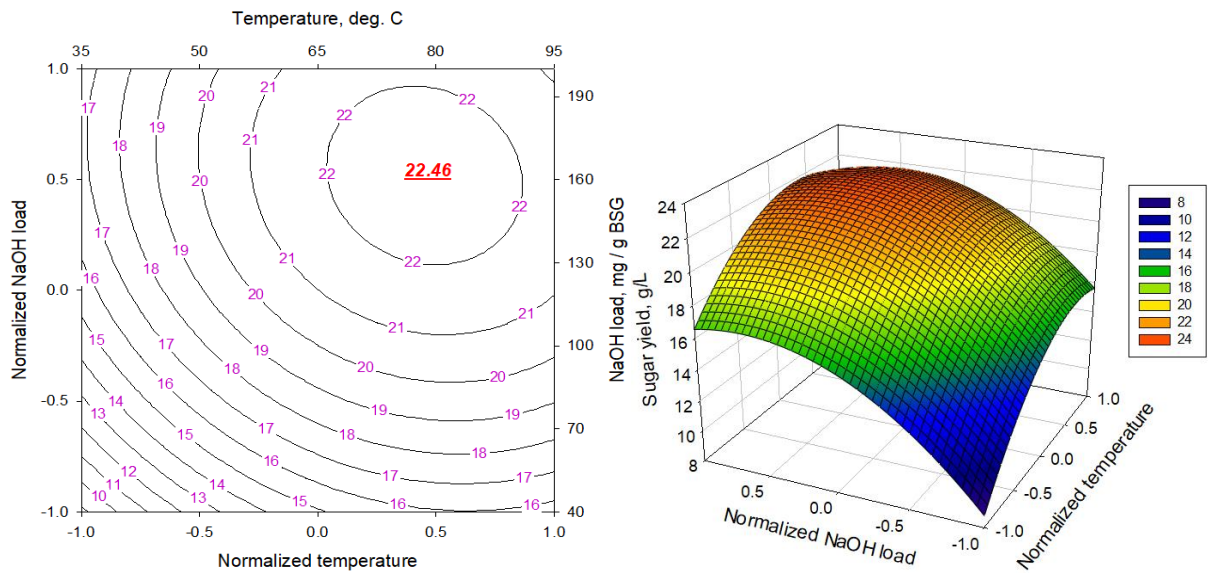
<b>Alkali Pretreatment - Optimal Conditions</b>		
<b>Temperature*</b>	<b>Time*</b>	<b>NaOH load*</b>
<i>Physical Values</i>		
79 °C	114 min	161 (mg / g BSG)
<i>Normalized Value</i>		
0.452	2.472	0.519

The 2D-binary contour and 3D-mesh diagrams at the optimum value of each independent parameter of the alkaline pretreatment, are shown in Figures 3.4 to 3.6.

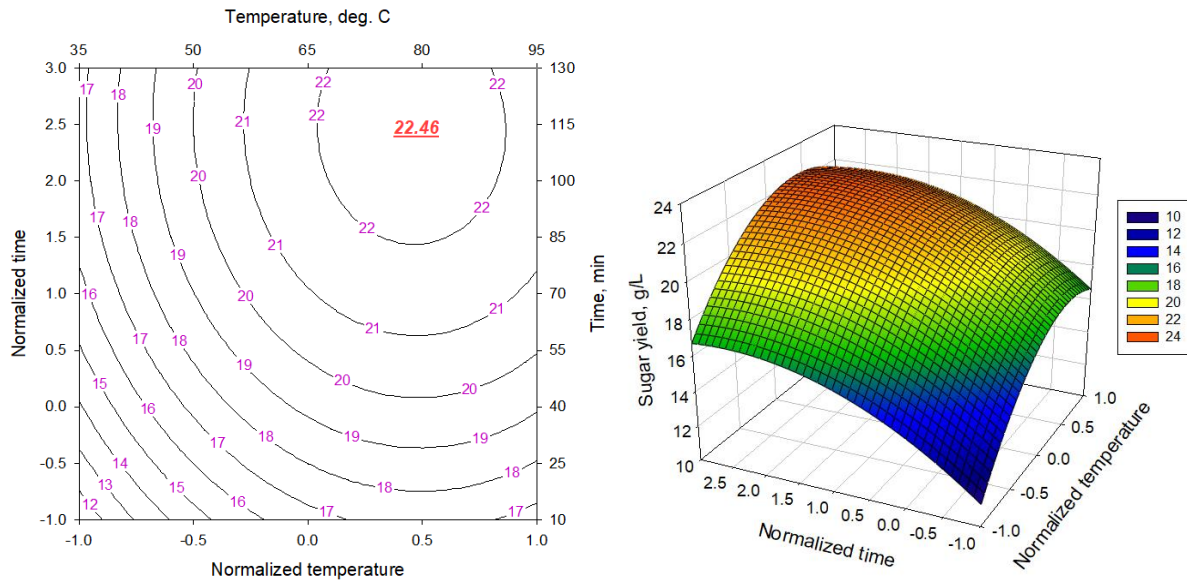




**Figure 3.4.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** optimization results. Combined effect of **pretreatment time (min) -  $X_2$**  and **NaOH load (mg NaOH per g raw BSG) -  $X_3$**  on sugar yield at the **optimum temperature of 79 °C ( $X_{1, opt}=0.452$ )** as determined by the regression model.



**Figure 3.5.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** optimization results. Combined effect of **pretreatment temperature (°C) -  $X_1$**  and **NaOH load (mg NaOH per g raw BSG) -  $X_3$**  on sugar yield at the **optimum pretreatment time of 114 min ( $X_{2, opt}=2.472$ )** as determined by the regression model.



**Figure 3.6.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** optimization results. Combined effect of **pretreatment temperature** ( $^{\circ}\text{C}$ ) -  $X_1$  and **pretreatment time** (min) -  $X_2$  on sugar yield -  $X_3$  on sugar yield at the **optimum NaOH load** (161 mg NaOH per g raw BSG) ( $X_{3, \text{opt}}=0.519$ ) as determined by the regression model.

As far as the results of the alkaline pretreatment optimization are concerned, the experimental results fitted relatively well to the model, yielding a regression equation at an  $R^2$  of 0.89 (Table 3.6). The first order terms showed the strongest effect on sugar yield since their coefficients ( $a_1$ ,  $a_2$  and  $a_3$ ) revealed P-values well below 0.1. Higher order terms were less significant (P-values  $> 0.1$ ), with the quadratic term of NaOH load (P-value for  $a_{33}=0.1934$ ) and pretreatment temperature (P-value for  $a_{11}=0.2137$ ) having a small but not negligible effect. The effect of interactions among the independent parameters of the model are totally not significant (P-values  $>0.5$ ).

From a practical point of view, our results on the alkaline pretreatment optimization of BSG reveal that the NaOH load is the independent parameter with the highest impact on the obtained sugar yield, followed by the pretreatment temperature ( $|a_3| > |a_1| > |a_2|$  and  $|a_{33}| > |a_{11}| > |a_{22}|$ ). Incubation time had the least (and mathematically most insensitive) effect on sugar yield. The latter is also reflected on the fact that the optimum value of incubation time obtained from the regression model lies well beyond the experimental independent parameter space employed ( $t_{\text{opt}} = 114$  min), that corresponds to  $X_{2, \text{opt}}=2.472 > 1$ . Although this result is posing questions concerning the validity of our model with respect to pretreatment time (and implies the expansion of our experimental data set to higher values of pretreatment times) we may very well rely on the insensitivity of pretreatment time and accept the obtained results. It

is characteristic from Figures 3.4 and 3.6 that over 95% of the maximum obtainable sugar yield of 22.46 g/L can be obtained even from approx. 70 min of pretreatment time (provided that the other two independent parameters are set on their optimum values).

### 3.3.5. Regression analysis results for the mild acid pretreatment

Table 3.11 shows the results of fitting the experimental results of the mild acid pretreatment (Table 3.8) in the regression equation (1).

**Table 3.11.** Regression coefficients determined from the regression analysis of the mild acid pretreatment.

<b>Coefficients</b>	<b>Regression coefficient values</b>	<b>P-value of the coefficient</b>
a <sub>0</sub>	8.9678	0.0028
a <sub>1</sub>	4.0540	0.0086
a <sub>2</sub>	1.1380	0.2929
a <sub>3</sub>	4.7100	0.0046
a <sub>11</sub>	6.2378	0.0223
a <sub>22</sub>	-4.3322	0.0727
a <sub>33</sub>	-2.6422	0.2250
a <sub>12</sub>	0.1813	0.8752
a <sub>13</sub>	2.4638	0.0721
a <sub>23</sub>	-0.3662	0.7498
<b>R<sup>2</sup> of the model</b>	<b>0.9250</b>	

Based on the data from Table 3.11, solving the linear system resulting from zeroing the first derivatives of equation (1), the following optimum model values for the independent parameters were obtained.

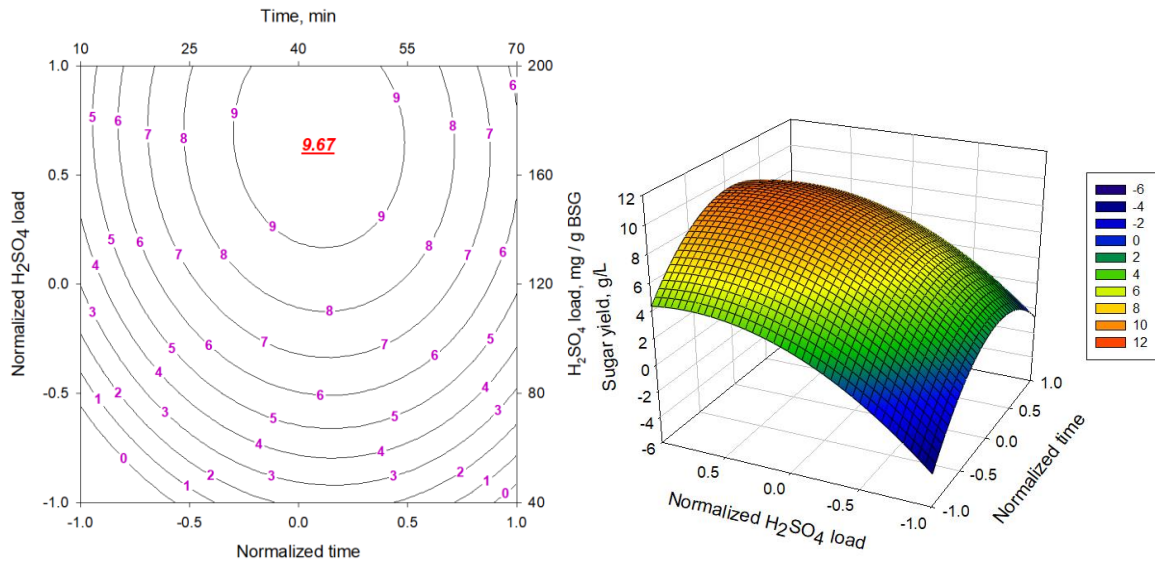
**Table 3.12.** Optimal model conditions for the acid pretreatment of BSG.

<b>Acid Pretreatment - Optimal Condition</b>		
<b>Temperature*</b>	<b>Time*</b>	<b>H<sub>2</sub>SO<sub>4</sub> load*</b>
<i>Physical Values</i>		
51 °C	43 min	174 (mg / g BSG)
<i>Normalized Values</i>		
-0.459	0.094	0.671

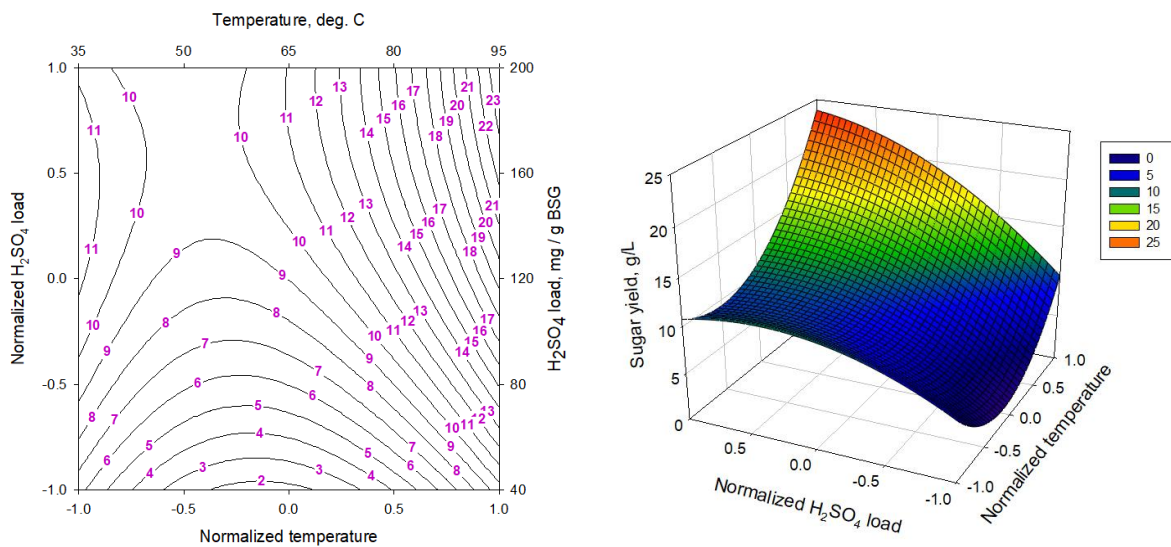
In Table 3.12 the values given for pretreatment temperature, do not correspond to a maximum but on saddle point (see analysis in the following paragraphs).

Concerning the regression results of the acid pretreatment, the experimental results of Table 3.8 fitted well into the regression model ( $R^2=0.925$ ). The parameters with the highest linear effect and significance were the pretreatment temperature ( $a_1=4.05$ , P-value < 0.01) and H<sub>2</sub>SO<sub>4</sub> load ( $a_3=4.71$ , P-value < 0.01), while the effect of pretreatment time was again practically of no significance (P-value > 0.1). Oddly enough, among the quadratic terms of the independent parameters, the highest effect and significance were observed for pretreatment temperature and time with P-values 0.022 and 0.072, respectively, while the quadratic effect of H<sub>2</sub>SO<sub>4</sub> load was totally insignificant (Table 3.11). Finally the only interaction with some significance was that between pretreatment temperature and H<sub>2</sub>SO<sub>4</sub> load.

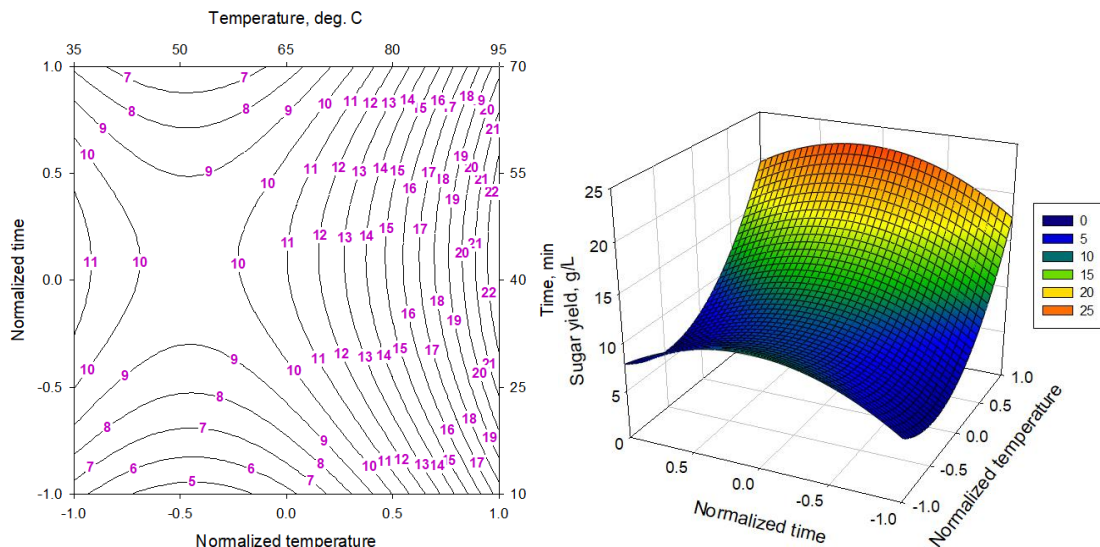
The 2D-binary contour and 3D-mesh diagrams at the optimum value of each independent parameter of the alkaline pretreatment, are shown in Figures 3.7 to 3.9.



**Figure 3.7.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** optimization results. Combined effect of **pretreatment time (min) - X<sub>2</sub>** and **H<sub>2</sub>SO<sub>4</sub> load (mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) - X<sub>3</sub>** on sugar yield at the **saddle point temperature of 51 °C (X<sub>1, opt.</sub> = -0.459)** as determined by the regression model.

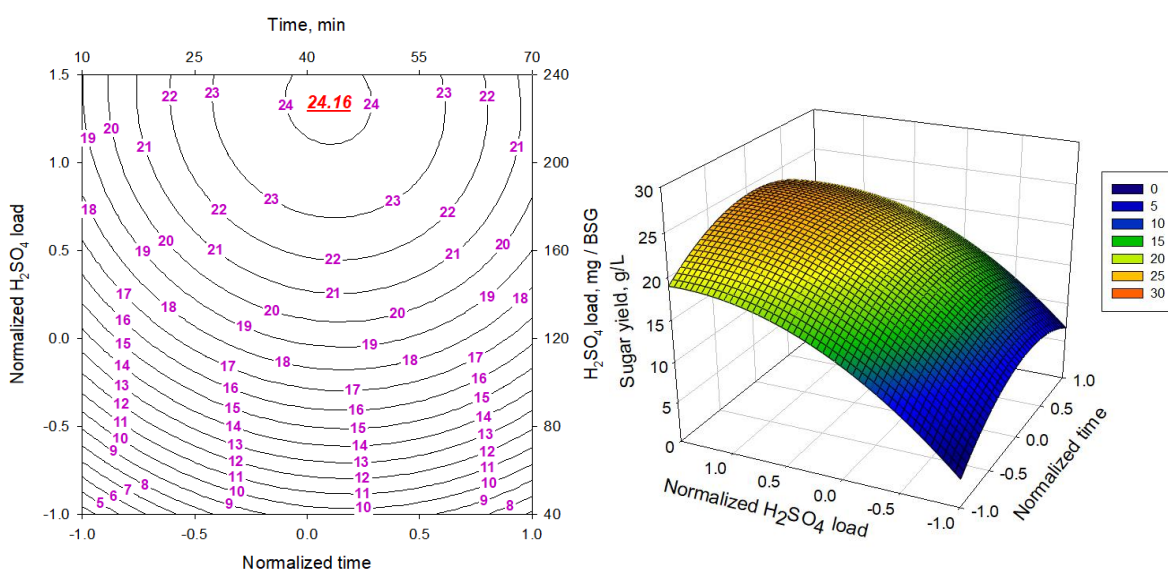


**Figure 3.8.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** optimization results. Combined effect of **pretreatment temperature (°C) - X<sub>1</sub>** and **H<sub>2</sub>SO<sub>4</sub> load (mg H<sub>2</sub>SO<sub>4</sub> per g raw BSG) - X<sub>3</sub>** on sugar yield at the **optimum pretreatment time of 43 min (X<sub>2, opt.</sub> = 0.094)** as determined by the regression model.



**Figure 3.9.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** optimization results. Combined effect of **pretreatment temperature** ( $^{\circ}\text{C}$ ) -  $X_1$  and **pretreatment time** (min) -  $X_2$  on sugar yield -  $X_3$  on sugar yield at the **optimum  $\text{H}_2\text{SO}_4$  load** (174 mg  $\text{H}_2\text{SO}_4$  per g raw BSG) ( $X_3$ ,  $\text{opt}=0.671$ ) as determined by the regression model.

Observing the results of our regression analysis as depicted in Figures 3.7 to 3.9 we realize that within the independent parameter space employed in our analysis, there is not a local optimum for the pretreatment temperature. As a result, a saddle point is observed in graphs 3.8 and 3.9 where the “real” optimum temperature always coincides with  $X_1 = 1$  ( $T = 95\text{ }^{\circ}\text{C}$ ). In Figure 3.10 we plot the graph of our regression equation for  $X_1 = 1$ .



**Figure 3.10.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** optimization results. Combined effect of **pretreatment time** (min) -  $X_2$  and  **$\text{H}_2\text{SO}_4$  load** (mg  $\text{H}_2\text{SO}_4$  per g raw BSG) -  $X_3$  on sugar yield at temperature  $T = 95\text{ }^{\circ}\text{C}$  ( $X_1=1$ ).

Comparing the graph in Figure 3.10 with the corresponding graph in Figure 3.7 (where  $X_1$  is set at the saddle point = -0.459) we observe that the theoretical optimum sugar yield increases from 9.7 to 24.2 g/L. This is of course expected since the saddle point ( $X_1 = -0.459$ ) does not correspond to a real maximum for the pretreatment temperature.

From a pragmatic point of view, and concerning the other two independent parameters, pretreatment time ( $X_2$  variable) has the least significant effect on the final sugar yield obtained. It is characteristic from the contour plots in Figures 3.7, 3.9 and 3.10 that sugar yield varies very little throughout the  $X_2$  (pretreatment time) range (at constant values for the other two independent parameters). The effect of  $H_2SO_4$  load is much more significant. The optimum  $H_2SO_4$  load at the saddle point of  $X_1$  is  $X_3 = 0.671$  (174 mg  $H_2SO_4$  per g raw BSG), where at  $X_1 = 1$  the optimum  $H_2SO_4$  load rises around  $X_3 = 1.3$  (224 mg  $H_2SO_4$  per g raw BSG). It is noteworthy to point out that at  $H_2SO_4$  load effect on sugar yield is much stronger at low  $H_2SO_4$  load values and relatively blunt for  $H_2SO_4$  load values over 0.

### 3.3.6. Expansion of acid pretreatment temperature range

Due to the fact that the regression model for the acid pretreatment of BSG did not yield an optimum for the pretreatment temperature, we considered necessary to perform additional experiments at temperatures higher than 95 °C, keeping pretreatment temperature and  $H_2SO_4$  load at their optimum conditions determined in the previous paragraph (Table 3.12).

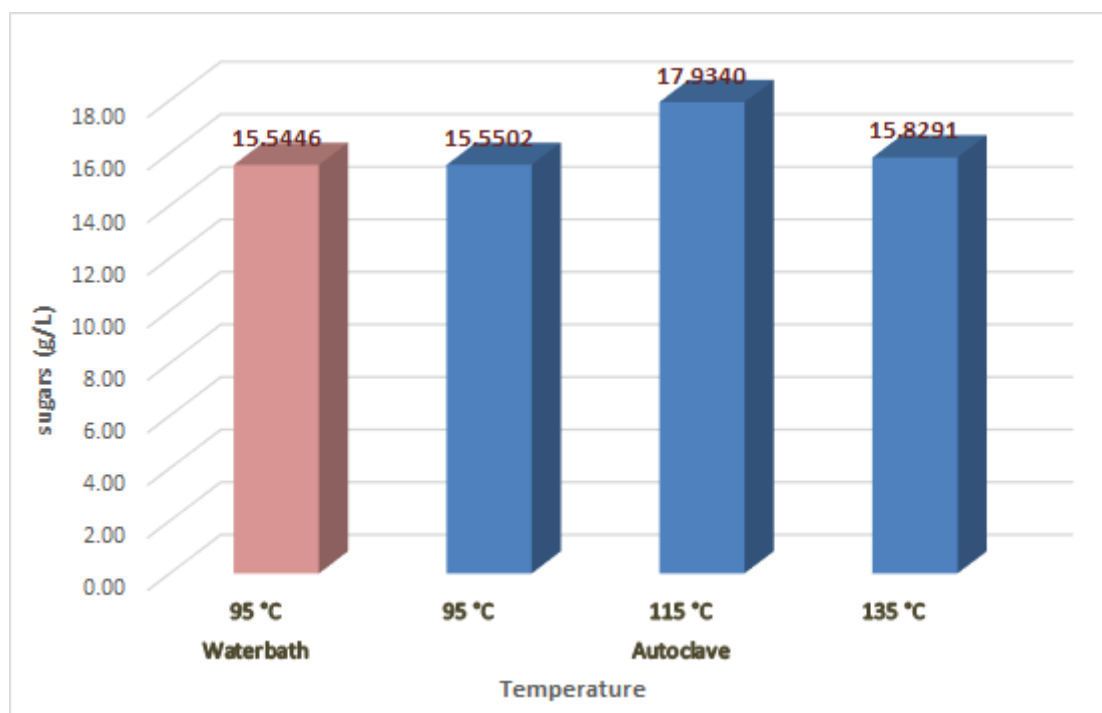
In order to achieve the higher than 100 °C temperatures required, we had to use the autoclave available to our laboratory. Since the heating regime is quite different between the two types of equipment the pretreatment condition at 95 °C was repeated both in the water bath as well as in the autoclave (Table 3.13). The corresponding results on sugar yield are given in the Table 3.14 and depicted in Figure 3.11.

**Table 3.13.** Additional H<sub>2</sub>SO<sub>4</sub> pretreatment conditions and hydrolysis

Condition	Heating apparatus	Temperature* (°C)	Time* (min)	H <sub>2</sub> SO <sub>4</sub> load* (mg / g BSG)	Enzyme load (mg protein / g BSG)
w	Water-bath	95	43	174	100
i	Autoclave	95	43	174	100
j	Autoclave	115	43	174	100
k	Autoclave	135	43	174	100

**Table 3.14.** Reducing Sugars from H<sub>2</sub>SO<sub>4</sub> pretreatment and Hydrolysis at Optimal Conditions in Water-bath and further temperatures evaluation in autoclave

	Water-bath	Autoclave		
Temperature	95 °C	95 °C	115 °C	135 °C
Sugars (g/L)	15.54	15.55	17.93	15.82

**Figure 3.11.** Effect of additional temperatures on the BSG acid pretreatment performance at optimum pretreatment time and H<sub>2</sub>SO<sub>4</sub> load values.



As shown in the results, the maximum sugar yield was obtained at  $T = 115\text{ }^{\circ}\text{C}$ , while at  $135\text{ }^{\circ}\text{C}$  the obtained sugar yield is similar to that at  $95\text{ }^{\circ}\text{C}$ . The use of temperatures above  $100\text{ }^{\circ}\text{C}$  implies the need of higher than atmospheric pressures, a fact that is expected to increase significantly the cost of the pretreatment vessel and equipment. This aspect combined with the fact that an increase from  $95$  to  $115\text{ }^{\circ}\text{C}$  results in only 15% increase in the sugar yield, fully justifies the selection of  $95\text{ }^{\circ}\text{C}$  as the optimum temperature for the acid pretreatment of BSG.

Furthermore, an important result is that we observed no bias between the two methods of heat provision for the pretreatment, between the autoclave and water-bath. This fact is supportive for robustness of our experimental approach.

### 3.3.7. Optimal pretreatment conditions for BSG with respect to reducing sugar yields.

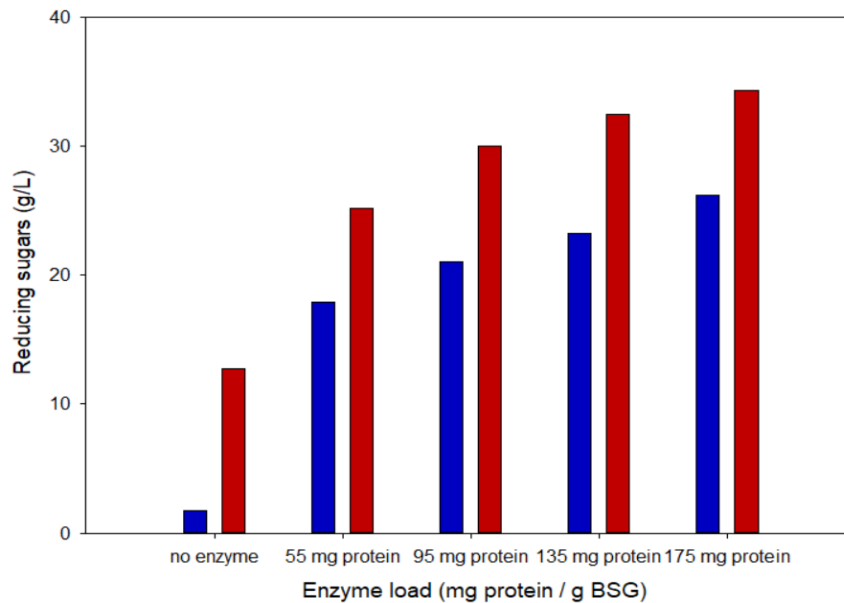
Table 3.15 summarizes our results for the optimization of BSG pretreatment with respect to the obtained sugar yield following enzymatic hydrolysis.

**Table 3.15.** Optimum values of the independent pretreatment parameters for BSG.

<b>Pretreatment</b>	<b>Temperature*</b>	<b>Time*</b>	<b>Load*</b>	<b>Sugar yield</b>
<b>Alkaline</b>	<b>79 °C</b>	<b>114 min</b>	<b>161 (mg / g BSG)</b>	<b>22.46 g/L</b>
<b>Acid<sub>saddle,p</sub></b>	<b>51 °C</b>	<b>43 min</b>	<b>174 (mg / g BSG)</b>	<b>9.67 g/L</b>
<b>Acid<sub>opt</sub></b>	<b>95 °C</b>	<b>46 min</b>	<b>224 (mg / g BSG)</b>	<b>24.16 g/L</b>

### 3.3.8. Reevaluation of enzyme load effect at optimal pretreatment conditions

As a final step in our BSG optimization experiments, we examined the effect of enzyme load (mg protein per g raw BSG) on the release of reducing sugars during the hydrolysis of optimally pretreated BSG. The corresponding results are given in Figure 3.12.



**Figure 3.12.** Effect of enzyme load on sugar yield at optimum pretreatment conditions. Raw BSG (at a concentration of 75 g/L = final concentration after enzyme addition) was subjected to either mild alkali or mild acid pretreatment at the optimal determined conditions (Table 3.15). Sugar yields were determined both after pretreatment (no enzyme) as well as after hydrolysis with the indicated enzyme load on the optimally pretreated materials.

As shown in Figure 3.12, alkaline pretreatment does not result in the release of significant amount of sugars. This is consistent with literature data regarding other lignocellulosic materials and is characteristic of the fact that the alkaline pretreatment mainly results in the solubilization of lignin and the loosening of the cellulose-hemicelluloses matrix (Wilkinson et al. 2014). As a result, almost all sugars released during the full alkaline pretreatment - hydrolysis process appear after the addition of the enzyme mix.

On the other hand, mild acid pretreatment has a significant hydrolysing effect on the hemicelluloses of BSG. Consequently, a significant amount of reducing sugars (mainly hemicellulosic pentoses and xylooligosaccharides) are released right after acid pretreatment and before enzyme addition (Ravindran et al. 2018). Subsequent enzyme addition results in an almost 2-fold increase in the total reducing sugars release.

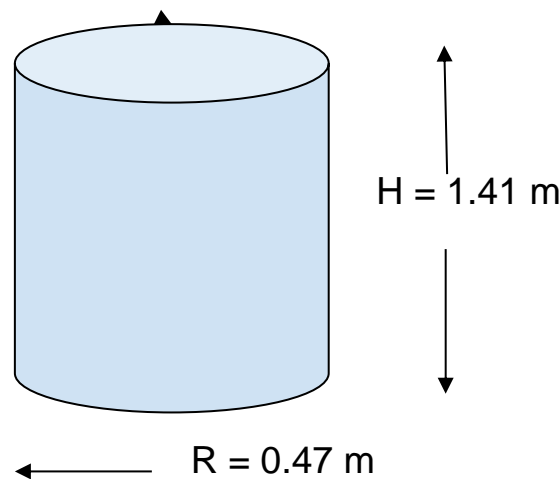
As far as the enzyme load is concerned, over 80% of the maximum obtainable yield of reducing sugars is released at an enzyme load of 100 mg protein per g of raw BSG. Taking into account that enzyme cost represents a significant part of the overall process cost in biorefinery applications (Aliyu and Bala 2011) justifies the use of this particular enzyme load value.

### 3.4. Economic optimization of BSG pretreatment parameters

The final part of this work deals with an initial financial optimization of both the mild alkaline and acid pretreatment, using the experimental data obtained from the physicochemical optimization. In order to perform this task, for each set of independent variables we assigned a total cost of the pretreatment process based on available information concerning the prices related to materials and energy.

#### 3.4.1. Definition of cost elements

As **the basis** of our calculations we used 1 m<sup>3</sup> volume of the pretreatment vessel. Assuming that this volume corresponds to a cylinder (the most usual shape employed for bioreactors) with a typical height to diameter ratio of 1.5 ( $H/D = 1.5$ ) we result to a cylinder of radius  $R=0.47$  m and height  $H = 1.41$  m.



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**Figure 3.13.** A typical pretreatment vessel of 1 m<sup>3</sup> unit volume.

For each pretreatment condition, the total cost is taken as the sum of the individual cost contributions of the corresponding independent parameters, namely; pretreatment temperature, pretreatment time and NaOH/H<sub>2</sub>SO<sub>4</sub> load.

#### **A. Pretreatment temperature cost:**

The cost related to the pretreatment temperature ( $cX_1$ ) was considered equal to the cost of energy required to heat 1 m<sup>3</sup> of water (= 1000 kg) from ambient environmental temperature

(e.g. 15 °C) to the target pretreatment temperature that corresponds to each experimental pretreatment condition ( $T_i$ ).

$$(cX_1)_i = C_p * (T_i - 15) * 1000 * E_c \quad (3)$$

where,

$(cX_1)_i$ , is the pretreatment temperature cost at experimental condition,  $i$

$C_p$ , the specific heat capacity of water at constant pressure that equals to 4.186 (kJoule/kg/°K)

$E_c$ , the unit energy cost in ¢ per kJ

For our analysis,  $E_c$  was calculated from the available internet (<https://www.rockymountainpower.net/about/rar/ipc.html>) data on the cost of an industrial kWh which was taken equal to **0.0692 \$**.

Based on the fact that 1 kWh = 3600 kJ, the value of  $E_c$  in the pretreatment temperature cost equation (3) is equal to (6.92 ¢/kWh) \* (1/3600 kWh/kJ) = **0.000192 ¢/kJ**.

### **B. Pretreatment time cost:**

The cost related to the pretreatment temperature ( $cX_2$ ) was considered equal to the cost of energy consumed in order to “maintain” our unit pretreatment vessel in the temperature that corresponds to each particular experimental condition. This energy is equal to the heat losses from the walls of the vessel to the environment that take place during the entire corresponding pretreatment time.

The rate of energy flow,  $q$  (in Watts), between the two faces of an area  $A$  ( $m^2$ ) which are kept into a constant temperature difference  $\Delta T$ , is given by the equation:

$$q = A * U * \Delta T \quad (4)$$

where,

$U$ , is the overall heat transfer coefficient through  $A$  [ W/ $m^2$ /°K ]

$A$ , is the total surface of our 1  $m^3$  unit cylindrical treatment vessel = **5.57  $m^2$**  (Figure 3.13).

$\Delta T$ , is the temperature difference between the pretreatment temperature (inside the vessel) at a particular experimental condition,  $T_i$ , and the ambient environmental temperature of 15 °C, ( $T_i - 15$ ).

The value of the overall heat transfer coefficient that was used in our approach, was calculated using standard available heat transfer analysis tools ([https://www.engineeringtoolbox.com/overall-heat-transfer-coefficient-d\\_434.html](https://www.engineeringtoolbox.com/overall-heat-transfer-coefficient-d_434.html)). Our calculation was based on a 10 mm thick stainless steel pretreatment vessel wall, heat transfer through forced flow of water on the one side and convective heat transfer on air on the other side. Based on these assumptions the overall heat transfer coefficient values used was  $U = 47.6 \text{ (W/(m}^2 \text{ °K))}$ .

Based on the above, the pretreatment time cost,  $(cX_2)_i$ , at experimental condition  $i$ , can be obtained from the following equation (assuming constant heat flow throughout the entire pretreatment time,  $t_i$ ):

$$(cX_2)_i = q \text{ (J/s)} \times (kJ / 1000 J) \times (60 \text{ s} / \text{min}) \times (kWh / 3600 \text{ kJ}) \times (0.0692 \text{ \$} / \text{kWh}) \times t_i \quad (5)$$

Where,  $t_i$ , is the pretreatment time (min) at the pretreatment condition  $i$ .

### **C. NaOH/H<sub>2</sub>SO<sub>4</sub> load cost calculation for alkali and acid pretreatments:**

The reagent load cost for each pretreatment process,  $c(X_3)_i$ , is the sum of two cost elements:

- i*) the cost of pretreatment reagent (NaOH or H<sub>2</sub>SO<sub>4</sub>) needed to achieve the target NaOH/H<sub>2</sub>SO<sub>4</sub> load for experimental condition  $i$ , plus
- ii*) the cost of the equivalent amount of neutralizer reagent (H<sub>2</sub>SO<sub>4</sub> for alkaline and NaOH for acid pretreatment) needed to bring the whole amount of the pretreatment suspension in the 1 m<sup>3</sup> volume, back to neutrality.

Since the pretreatment of lignocellulosic material is a bulk and high-volume process, it does not require high quality and purity of NaOH and H<sub>2</sub>SO<sub>4</sub>. The cost per kg of these materials that was used for our calculations was obtained from the average minimum prices available from various internet vendors.

**Table 3.16.** Minimum internet prices for sulfuric acid as obtained from various internet sources.

<b>H<sub>2</sub>SO<sub>4</sub></b>				
<b>Source</b>	<b>Concentration (w/w)</b>	<b>Price (\$/ton)</b>	<b>Mass (kg)</b>	<b>Cost (\$/kg H<sub>2</sub>SO<sub>4</sub>)</b>
Vendor 1	98%	100	980	\$0.102
Vendor 2	98%	220	980	\$0.224
Vendor 3	98%	225	980	\$0.230
Vendor 4	98%	95	980	\$0.097
Vendor 5	98%	150	980	\$0.153
Vendor 6	98%	100	980	\$0.102
Vendor 7	98%	95	980	\$0.097
Vendor 8	98%	80	980	\$0.082
<b>Average minimum price for industrial H<sub>2</sub>SO<sub>4</sub> (\$/kg H<sub>2</sub>SO<sub>4</sub>)</b>				<b>\$0.136</b>

**Table 3.17.** Minimum internet prices for sodium hydroxide as obtained from various internet sources.

<b>NaOH</b>				
<b>Source</b>	<b>Concentration (w/w)</b>	<b>Price (\$/ton)</b>	<b>Mass (kg)</b>	<b>Cost (\$/kg NaOH)</b>
Vendor 1	99%	80	990	\$0.081
Vendor 2	99%	60	990	\$0.061
Vendor 3	99%	100	990	\$0.101
Vendor 4	99%	60	990	\$0.061
Vendor 5	99%	100	990	\$0.101
Vendor 6	99%	100	990	\$0.101
Vendor 7	99%	100	990	\$0.101
Vendor 8	99%	75	990	\$0.076
<b>Average minimum price for industrial (\$/kg NaOH)</b>				<b>\$0.085</b>

The concentration of BSG suspension during all of our pretreatment experiments was **167 g BSG per L** and this concentration was used for our cost calculations. Based on the above, the equation that yields the reagents load cost for the alkaline or acid pretreatment is the following:

$$c(\mathbf{X}_3)\mathbf{i} = \text{Pretreatment reagent cost (PRC)} + \text{Neutralization Reagent Cost (NRC)} \quad (6)$$

Based on the above analysis, and on the fact that 1 mole of  $\text{H}_2\text{SO}_4$  (98 g) requires 2 moles of NaOH (80 g) for its neutralization (and vice-versa),  $c(\mathbf{X}_3)\mathbf{i}$  takes the following form:

a) Alkaline pretreatment

$$\text{PRC}_{\text{Alkaline}} = (167 \text{ kg BSG}) * \mathbf{L}_i (\text{g NaOH/ kg BSG}) * (\text{kg NaOH/ } 1000 \text{ g NaOH}) * (0.085 \text{ \$/kg NaOH})$$

$$\text{NRC}_{\text{Alkaline}} = \text{PRC}_{\text{Alkaline}} * 98/80 (\text{kg H}_2\text{SO}_4/\text{kg NaOH}) * 0.136 (\text{\$/kg H}_2\text{SO}_4)$$

b) Acid pretreatment

$$\text{PRC}_{\text{Acid}} = (167 \text{ kg BSG}) * \mathbf{L}_i (\text{g H}_2\text{SO}_4/\text{kg BSG}) * (\text{kg H}_2\text{SO}_4/ 1000 \text{ g H}_2\text{SO}_4) * (0.136 \text{ \$/kg H}_2\text{SO}_4)$$

$$\text{NRC}_{\text{Acid}} = \text{PRC}_{\text{Acid}} * 80/98 (\text{kg NaOH/kg H}_2\text{SO}_4) * 0.085 (\text{\$/kg NaOH})$$

where,

$\mathbf{L}_i$ , is the NaOH/ $\text{H}_2\text{SO}_4$  load at the pretreatment condition,  $\mathbf{i}$ .

Table 3.18 summarizes the various cost elements used in our analysis above.

**Table 3.18.** NaOH / H<sub>2</sub>SO<sub>4</sub> pretreatment cost elements.

Condition	T (°C)	t (min)	NaOH / H <sub>2</sub> SO <sub>4</sub> load (mg / g BSG)	NaOH cost (€/kg)	H <sub>2</sub> SO <sub>4</sub> cost (€/kg)	Industrial KWh av. Cost (€)	Area m <sup>2</sup>	U[air-ss] (W/m <sup>2</sup> * K)	q (W)
A	35	10	40	8.5	13.6	6.92	5.57	47.6	5303
B	35	10	200	8.5	13.6	6.92	5.57	47.6	5303
Γ	95	10	200	8.5	13.6	6.92	5.57	47.6	21211
Δ	95	10	40	8.5	13.6	6.92	5.57	47.6	21211
E	95	70	40	8.5	13.6	6.92	5.57	47.6	21211
ΣΤ	95	70	200	8.5	13.6	6.92	5.57	47.6	21211
Z	35	70	40	8.5	13.6	6.92	5.57	47.6	5303
H	35	70	200	8.5	13.6	6.92	5.57	47.6	5303
ΑΒΓΔ	65	10	120	8.5	13.6	6.92	5.57	47.6	13257
ΓΔΕ(ΣΤ)	95	40	120	8.5	13.6	6.92	5.57	47.6	21211
Ε(ΣΤ)ΗΖ	65	70	120	8.5	13.6	6.92	5.57	47.6	13257
ZHBA	35	40	120	8.5	13.6	6.92	5.57	47.6	5303
ΑΔΕΖ	65	40	40	8.5	13.6	6.92	5.57	47.6	13257
ΒΓ(ΣΤ)Η	65	40	200	8.5	13.6	6.92	5.57	47.6	13257
C	65	40	120	8.5	13.6	6.92	5.57	47.6	13257

### 3.4.2. Regression analysis

For the cost optimization analysis, the dependent variable,  $Y_{sp}$ , used in the regression analysis for equation (1) is the specific sugar yield, expressed as the mass of sugars produced in the unit pretreatment volume of 1 m<sup>3</sup>, per \$ of pretreatment cost, that is given by the equation:

$$Y_{sp} = \frac{Y_2}{cX_1 + cX_2 + cX_3} \times 1000 \quad (6)$$



where,  $Y_2$ , is the total sugar yield after each pretreatment condition, presented in Tables 3.7 and 3.8 for alkaline and acid pretreatment, respectively. The corresponding values at each pretreatment condition are given in Tables 3.19 and 3.20.

**Table 3.19.** Parameter values used in the cost optimization regression analysis for the alkaline pretreatment.

Condition	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	cX <sub>1</sub>	cX <sub>2</sub>	cX <sub>3</sub>	Total Cost	Y <sub>sp</sub> (g/\$)
A	-1	-1	-1	\$1.61	\$0.07	\$1.68	\$3.35	1,223.69
B	-1	-1	1	\$1.61	\$0.07	\$8.39	\$10.06	985.51
Γ	1	-1	1	\$6.44	\$0.26	\$8.39	\$15.07	1,175.01
Δ	1	-1	-1	\$6.44	\$0.26	\$1.68	\$8.36	972.73
E	1	1	-1	\$6.44	\$1.84	\$1.68	\$9.83	1,769.44
ΣΤ	1	1	1	\$6.44	\$1.84	\$8.39	\$16.54	1,266.09
Z	-1	1	-1	\$1.61	\$0.46	\$1.68	\$3.71	1,782.55
H	-1	1	1	\$1.61	\$0.46	\$8.39	\$10.42	1,981.01
ΑΒΓΔ	0	-1	0	\$4.02	\$0.16	\$5.03	\$9.21	2,058.98
ΓΔΕ(ΣΤ)	1	0	0	\$6.44	\$1.05	\$5.03	\$12.45	1,535.97
Ε(ΣΤ)ΗΖ	0	1	0	\$4.02	\$1.15	\$5.03	\$10.13	2,117.35
ZHBA	-1	0	0	\$1.61	\$0.26	\$5.03	\$6.89	2,071.06
ΑΔΕΖ	0	0	-1	\$4.02	\$0.66	\$1.68	\$6.31	1,609.81
ΒΓ(ΣΤ)Η	0	0	1	\$4.02	\$0.66	\$8.39	\$13.02	2,036.32
C	0	0	0	\$4.02	\$0.66	\$5.03	\$9.67	1,881.36

**Table 3.20.** Parameter values used in the cost optimization regression analysis for the acid pretreatment.

Condition	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	cX <sub>1</sub>	cX <sub>2</sub>	cX <sub>3</sub>	Total Cost	Total Sugars/ Total Cost (g/\$)
A	-1	-1	-1	\$1.61	\$0.07	\$1.37	\$3.04	607.07
B	-1	-1	1	\$1.61	\$0.07	\$6.85	\$8.52	876.68
Γ	1	-1	1	\$6.44	\$0.26	\$6.85	\$13.53	1,656.51
Δ	1	-1	-1	\$6.44	\$0.26	\$1.37	\$8.05	277.56
E	1	1	-1	\$6.44	\$1.84	\$1.37	\$9.52	605.75
ΣT	1	1	1	\$6.44	\$1.84	\$6.85	\$15.00	2,611.03
Z	-1	1	-1	\$1.61	\$0.46	\$1.37	\$3.41	945.54
H	-1	1	1	\$1.61	\$0.46	\$6.85	\$8.88	1,002.16
ABΓΔ	0	-1	0	\$4.02	\$0.16	\$4.11	\$8.28	500.88
ΓΔE(ΣT)	1	0	0	\$6.44	\$1.05	\$4.11	\$11.52	3,778.55
E(ΣT)HZ	0	1	0	\$4.02	\$1.15	\$4.11	\$9.20	876.52
ZHBA	-1	0	0	\$1.61	\$0.26	\$4.11	\$5.96	1,504.79
ΑΔEZ	0	0	-1	\$4.02	\$0.66	\$1.37	\$6.00	732.38
BΓ(ΣT)H	0	0	1	\$4.02	\$0.66	\$6.85	\$11.48	894.88
C	0	0	0	\$4.02	\$0.66	\$4.11	\$8.74	926.40

### 3.4.3. Regression analysis results for the mild alkaline pretreatment cost optimization

Table 3.21 shows the results of the fitting the experimental results of the alkaline pretreatment (Table 3.19) in the regression equation (1).

**Table 3.21.** Regression coefficients determined from the regression analysis of the cost optimization of alkaline pretreatment.

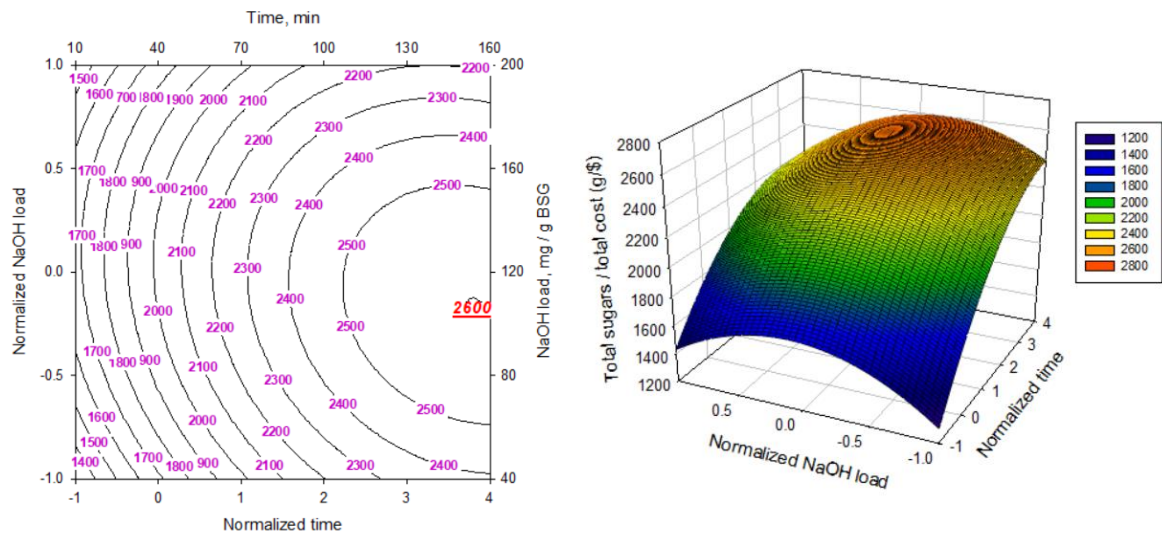
Coefficients	Regression coefficient values	P-value of the coefficient
a <sub>0</sub>	2079.5918	<0.0001
a <sub>1</sub>	-132.4572	0.2388
a <sub>2</sub>	250.0520	0.0529
a <sub>3</sub>	8.5723	0.9344
a <sub>11</sub>	-325.6327	0.9344
a <sub>22</sub>	-40.9823	0.8421
a <sub>33</sub>	-306.0847	0.1779
a <sub>12</sub>	-83.3215	0.4858
a <sub>13</sub>	-32.6670	0.7799
a <sub>23</sub>	-33.6240	0.7737
<b>R<sup>2</sup> of the model</b>	<b>0.7931</b>	

Based on the data from Table 3.21, solving the linear system resulting from zeroing the first derivatives of equation (1), the following optimum model values for the independent parameters were obtained.

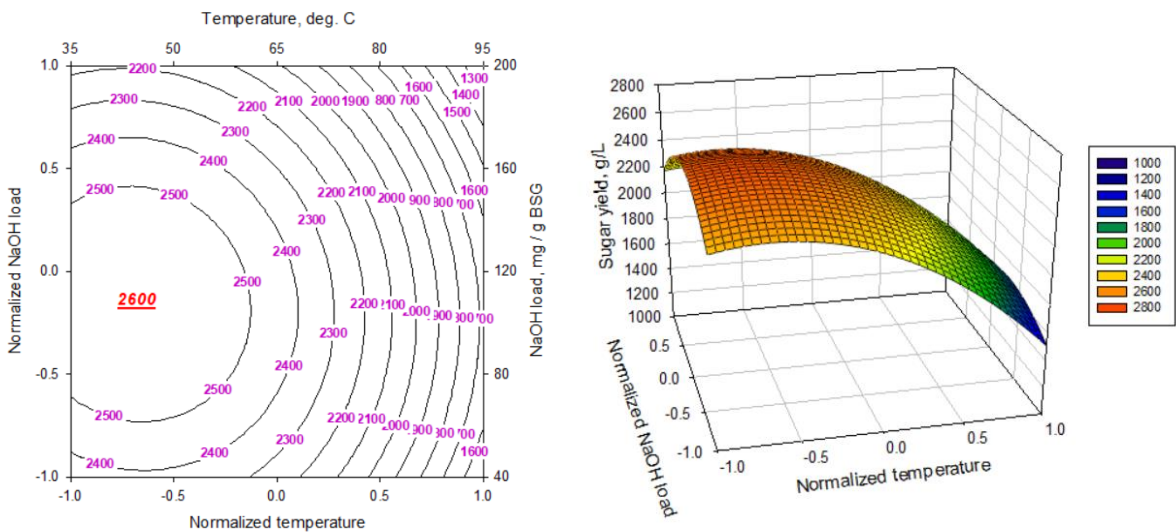
**Table 3.22.** Optimal model conditions for the cost optimization of alkaline pretreatment of BSG.

Alkali Pretreatment - Optimal Cost Condition		
Temperature*	Time*	NaOH load*
<i>Physical Values</i>		
44.5 °C	154 min	107 mg / g raw BSG
<i>Normalized Values</i>		
-0.683	3.81	-0.159

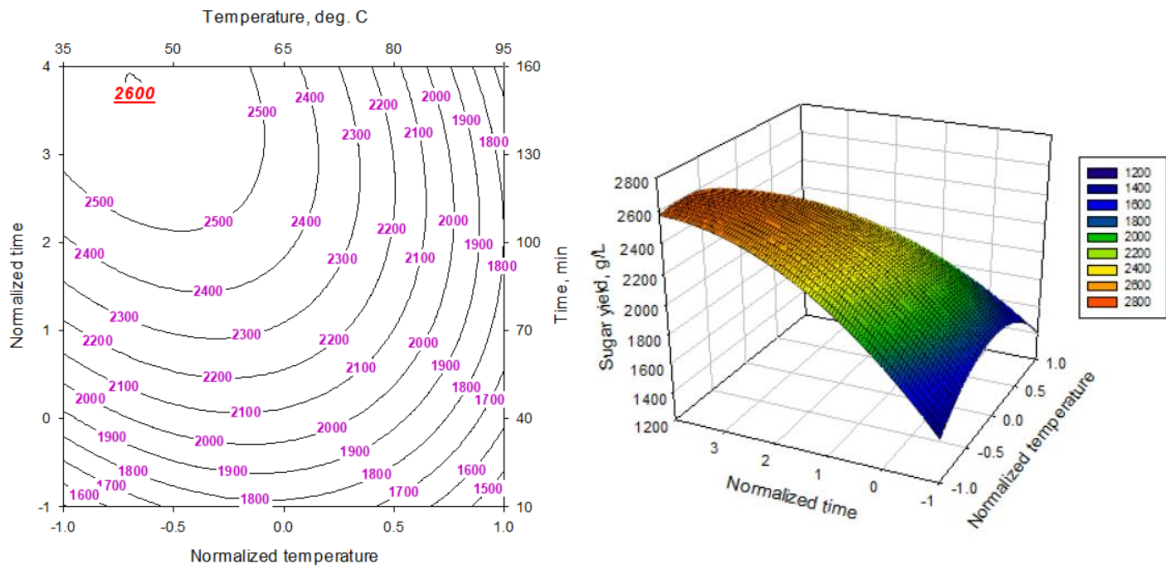
The 2D-binary contour and 3D-mesh diagrams at the optimum value of each independent parameter of the alkaline pretreatment, are shown in Figures 3.14 to 3.16.



**Figure 3.14.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** cost optimization results. Combined effect of **pretreatment time (min) -  $X_2$**  and **NaOH load (mg NaOH per g raw BSG) -  $X_3$**  on specific sugar yield ( $Y_{sp}$ ) at the **optimum temperature of 44.5 °C ( $X_{1, opt}=-0.683$ )** as determined by the regression model.



**Figure 3.15.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** cost optimization results. Combined effect of **pretreatment temperature (°C) -  $X_1$**  and **NaOH load (mg NaOH per g raw BSG) -  $X_3$**  on sugar yield at the **optimum pretreatment time of 154 min ( $X_{2, opt}=3.81$ )** as determined by the regression model.



**Figure 3.16.** 2D-contour plot and 3D-mesh plot of the **alkaline pretreatment** cost optimization results. Combined effect of **pretreatment temperature** ( $^{\circ}\text{C}$ ) -  $\mathbf{X}_1$  and **pretreatment time** (min) -  $\mathbf{X}_2$  on sugar yield -  $\mathbf{X}_3$  on sugar yield at the **optimum NaOH load** (107 mg NaOH per g raw BSG) ( $\mathbf{X}_3$ ,  $\text{opt}=-0.159$ ) as determined by the regression model.

Fitting the experimental results for the cost optimization analysis of the alkaline pretreatment into regression equation (1) yielded an  $R^2$  of 0.8 (Table 3.21). The most significant overall effect on the specific sugar yield was observed for the pretreatment time. This parameter, showed the strongest first order effect and also had the coefficient with the highest significance ( $P$ -value  $< 0.1$ ). Second order effects were less significant with those of pretreatment temperature and NaOH load having the strongest impact. The effect of interactions among the independent parameters of the model are totally not significant ( $P$ -values  $> 0.5$ ).

The fact that the cost of maintaining the temperature of pretreatment at a constant level, is the parameter with the lowest cost impact (Tables 3.19) results in the strong effect revealed for this parameter on the specific sugar yield. In contrast to the optimization with respect to sugar yield, NaOH load had a much less effect, similar to that of the pretreatment temperature.

3.4.4. Regression analysis results for the mild acid pretreatment cost optimization

Table 3.23 shows the results of fitting the experimental results of the mild acid pretreatment cost optimization (Table 3.20) in the regression equation (1).

**Table 3.23.** Regression coefficients determined from the regression analysis of the mild acid pretreatment cost optimization.

<b>Coefficients</b>	<b>Regression coefficient values</b>	<b>P-value of the coefficient</b>
a <sub>0</sub>	1359.6123	0.0098
a <sub>1</sub>	399.3158	0.0990
a <sub>2</sub>	212.2296	0.3314
a <sub>3</sub>	387.2946	0.1070
a <sub>11</sub>	1173.7522	0.0296
a <sub>22</sub>	-779.2159	0.1017
a <sub>33</sub>	-654.2869	0.1536
a <sub>12</sub>	102.345	0.6623
a <sub>13</sub>	382.2479	0.1438
a <sub>23</sub>	51.6677	0.8242
<b>R<sup>2</sup> of the model</b>	<b>0.8333</b>	

Based on the data from Table 3.23, solving the linear system resulting from zeroing the first derivatives of equation (1), the following optimum model values for the independent parameters were obtained.

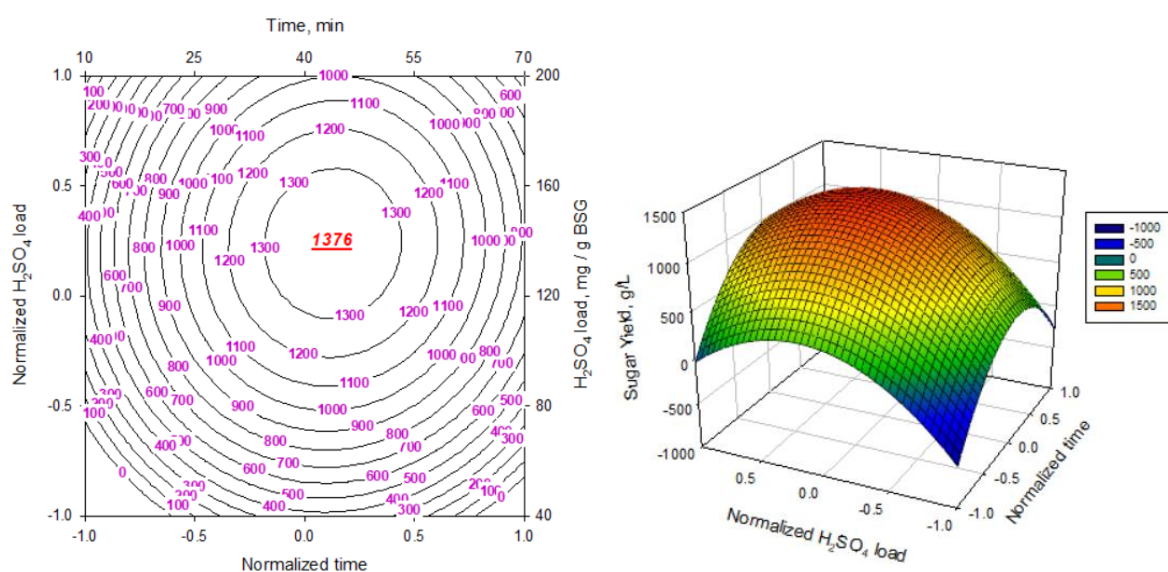
**Table 3.24.** Optimal model conditions for the acid pretreatment cost optimization of BSG.

<b>Acid Pretreatment - Optimal Cost Condition</b>		
<b>Temperature*</b>	<b>Time*</b>	<b>H<sub>2</sub>SO<sub>4</sub> load*</b>
<i>Physical Values</i>		
<b>59 °C</b>	44 min	139 mg / g raw BSG
<i>Normalized Values</i>		
<b>-0.215</b>	0.13	0.238

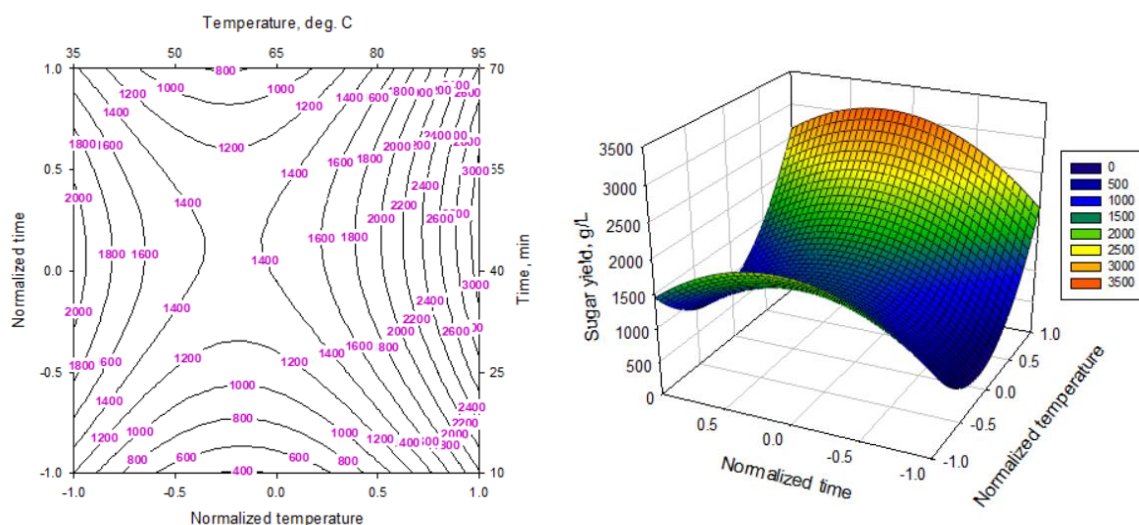
In Table 3.24 the values given for pretreatment temperature, do not correspond to a maximum but on saddle point (see analysis in the following paragraphs).

Concerning the regression results of the acid pretreatment cost optimization, the experimental results of Table 3.20 fitted relatively well into the regression model ( $R^2=0.833$ ). The parameters with the highest linear effect and significance were the pretreatment temperature ( $a_1=399.3$ , P-value = 0.09) and  $H_2SO_4$  load ( $a_3=387$ , P-value = 0.1), while the effect of pretreatment time was practically of no significance (P-value > 0.1). Among the quadratic terms of the independent parameters, the highest effect and significance were observed for pretreatment temperature and time with P-values 0.029 and 0.102, respectively, while the quadratic effect of  $H_2SO_4$  load was totally insignificant (Table 3.23). Finally the only interaction with some significance was that between pretreatment temperature and  $H_2SO_4$  load.

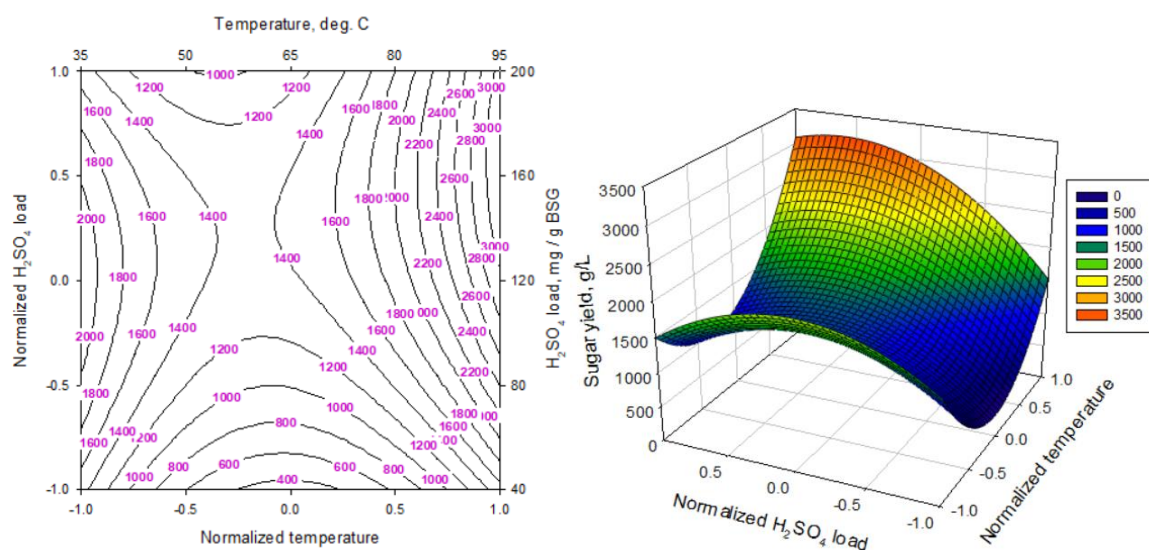
The 2D-binary contour and 3D-mesh diagrams at the optimum value of each independent parameter of the alkaline pretreatment, are shown in Figures 3.17 to 3.19.



**Figure 3.17.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** cost optimization results. Combined effect of **pretreatment time (min) -  $X_2$**  and  **$H_2SO_4$  load (mg  $H_2SO_4$  per g raw BSG) -  $X_3$**  on sugar yield at the **saddle point temperature of 59 °C ( $X_1, \text{opt.}=-0.215$ )** as determined by the regression model.



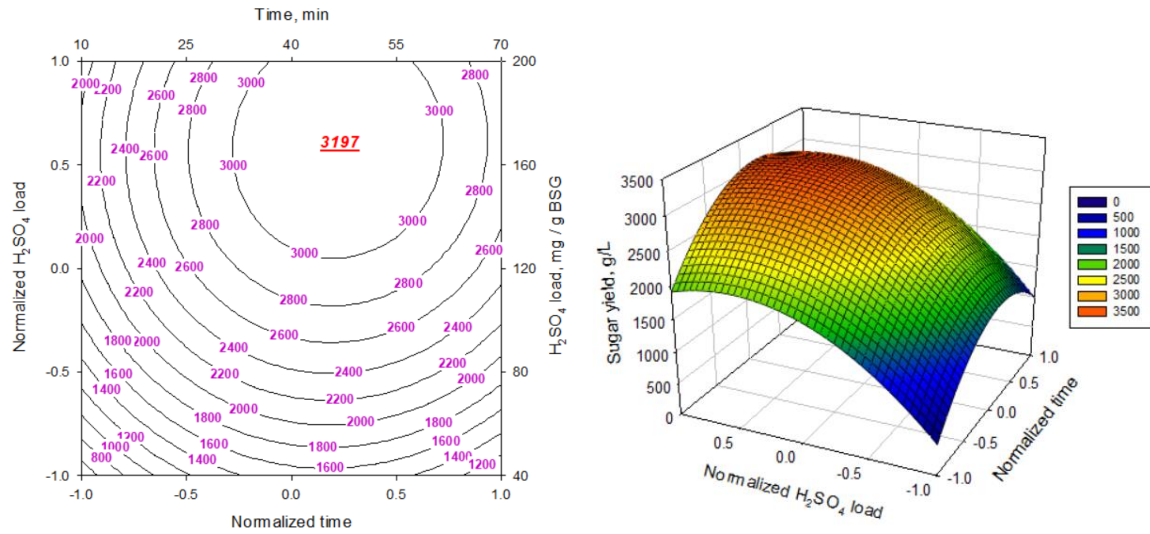
**Figure 3.18.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** cost optimization results. Combined effect of **pretreatment temperature (°C) -  $X_1$**  and  **$H_2SO_4$  load (mg  $H_2SO_4$  per g raw BSG) -  $X_3$**  on sugar yield at the **optimum pretreatment time of 44 min ( $X_{2, opt}=0.13$ )** as determined by the regression model.



**Figure 3.19.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** cost optimization results. Combined effect of **pretreatment temperature (°C) -  $X_1$**  and **pretreatment time (min) -  $X_2$**  on sugar yield -  $X_3$  on sugar yield at the **optimum  $H_2SO_4$  load (139 mg  $H_2SO_4$  per g raw BSG) ( $X_{3, opt}=0.238$ )** as determined by the regression model.

According to the results of our regression analysis as depicted in Figures 3.17 to 3.19, within the independent parameter space employed in our analysis, there is not a local optimum for the pretreatment temperature. As a result, a saddle point is observed in graphs 3.18 and 3.19 where the “real” optimum temperature always coincides with  $X_1 = 1$  ( $T = 95$  °C). In Figure 3.20 we plot the graph of our regression equation for  $X_1 = 1$ .





**Figure 3.20.** 2D-contour plot and 3D-mesh plot of the **mild acid pretreatment** cost optimization results. Combined effect of **pretreatment time (min) -  $X_2$**  and  **$H_2SO_4$  load (mg  $H_2SO_4$  per g raw BSG) -  $X_3$**  on sugar yield at temperature  $T = 95\text{ }^\circ\text{C}$  ( $X_1=1$ ).

Comparing the graph in Figure 3.20 with the corresponding graph in Figure 3.17 (where  $X_1$  is set at the saddle point = -0.215) we observe that the theoretical optimum specific sugar yield increases from 1376 to 3197 g reducing sugars / \$. This is of course expected since the saddle point ( $X_1 = -0.215$ ) does not correspond to a real maximum for the pretreatment temperature. It is noteworthy, that the optimum pretreatment time does not practically change when between  $X_1 = -0.215$  and  $X_1 = 1$ , while the optimum  $H_2SO_4$  load shows a significant increase from its saddle point value of 139 mg / g raw BSG to approximately 170 mg  $H_2SO_4$  per g raw BSG.

#### 3.4.5. Optimal pretreatment conditions for BSG with respect to the specific sugar yields.

Table 3.25 summarizes our results for the optimization of BSG pretreatment with respect to the specific sugar yields after cost optimization.

**Table 3.25.** Optimum values of the independent pretreatment parameters for BSG.

Pretreatment	Temperature*	Time*	Load*	Specific sugar yield
Alkaline	44.5 °C	154 min	107 (mg / g BSG)	2600 g/\$
Acid <sub>saddle.p</sub>	59 °C	44 min	139 mg / g BSG	1376 g/\$
Acid <sub>opt</sub>	95 °C	46 min	168 (mg / g BSG)	3196 g/\$

### ***3.5. Fermentative performance of Z. mobilis 8b on BSG hydrolysates***

The hydrolysates from the optimally pretreated BSG were used as substrates for the fermentative production of ethanol by the genetically modified bacterium *Z. mobilis* 8b. Two fermentations mode were examined, namely, a Single Vessel Approach (SVA) and Separate Hydrolysis and Fermentation (SHF) as described in Materials and Methods.

Wild-type *Z. mobilis* strains do not have the ability to ferment pentoses (such as xylose) but only hexoses (glucose). Strain 8b has been genetically modified by the incorporation of two genes that introduce exogenous xylose into the central Pentose Phosphate Pathway. Since the expression of two genes is controlled by the presence of xylose in the fermentation medium, in each fermentation mode, the inoculum used was prepared with either glucose or xylose as a carbon source.

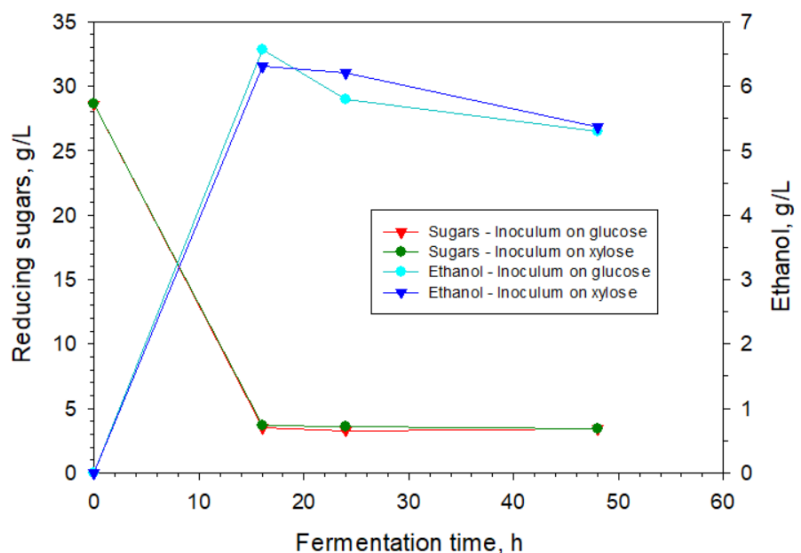
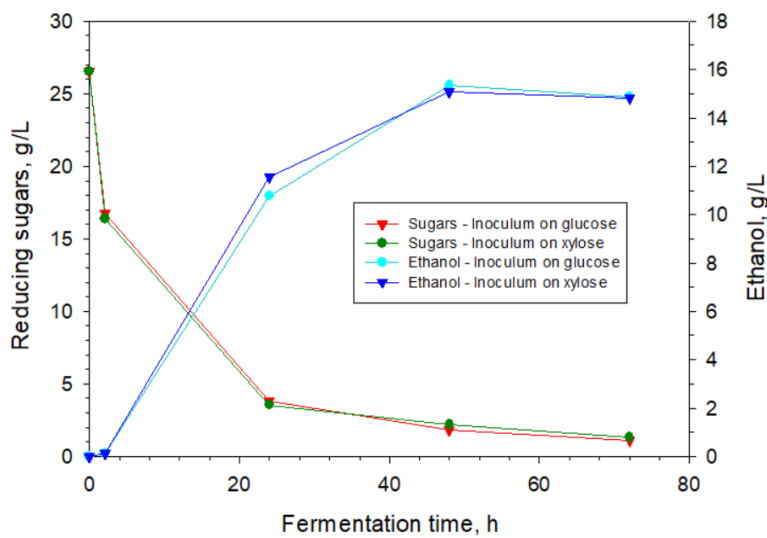
#### ***3.5.1. Fermentation in Single Vessel Mode***

BSG was alkali/acid pretreated and hydrolysed at optimal conditions determined in Paragraph 3.3 resulting in a final solid load of 83.33 g BSG solids/L. The concentrations of reducing sugars and the corresponding yields with respect to the dry BSG, and BSG holocellulose are given in Table 3.26.

**Table 3.26.** Fermentable sugars yield after optimal alkali/acid pretreatment hydrolysis of raw BSG.

	Reducing sugars concentration	Reducing sugars yield (mg / g dry BSG)	Fermentable sugars (mg / gr BSG holocellulose)
BSG at optimal NaOH pretreatment	30.70 g/L	357	567
BSG at optimal H <sub>2</sub> SO <sub>4</sub> pretreatment	33.10 g/L	372	591

SVA was materialized by using the pretreated/hydrolyzed suspension, after fixing the pH at 6, directly as a fermentation medium for ethanol production by *Z. mobilis* 8b (see Materials and Methods). The results with respect to reducing sugars consumption and ethanol production in the fermentation medium are given in Figure 3.21.



**Figure 3.21.** Reducing sugars and ethanol concentration during the course of SVA fermentation. Data points represent the mean of triplicate flasks. Left graph: SVA on NaOH pretreated material, Right graph: SVA on H<sub>2</sub>SO<sub>4</sub> pretreated material.

The most important result drawn from *Z. mobilis* 8b SVA fermentation, is the fact that the particular microbial strain is able to grow anaerobically and produce significant amounts of ethanol directly on the pretreatment/hydrolysis suspension. In addition, there is no difference in the fermentation kinetics when the inoculum was prepared with either glucose or xylose as carbon source.

SVA on NaOH pretreated material proved to be the most successful with respect to ethanol production. Almost 95% of reducing sugars present in the fermentation vessel (26.5 g/L), were converted within 48 h to ethanol (15 g/L) at a conversion factor equal to the maximum

theoretical value (0.511 g ethanol/ g reducing sugars). On the contrary, SVA on H<sub>2</sub>SO<sub>4</sub> pretreated material produced significantly less ethanol (~6.5 g/L), despite the fact that maximum ethanol levels were obtained already from the 16<sup>th</sup> our of fermentation. These ethanol levels correspond to an ethanol conversion factor of 0.26 g ethanol / g reducing sugars that is only half of the maximum theoretical.

### 3.5.2. Fermentation in Separate Hydrolysis Fermentation mode

For the Separate Hydrolysis and Fermentation mode (SHF), the optimally pretreated BSG (alkali and acid) was washed, neutralized, and dried. The pretreated materials were subjected to compositional analysis and the results are shown in Table 3.27.

**Table 3.27.** Compositional analysis of raw and optimally pretreated BSG. All values are in g and data are reported per 100 g of raw material.

Contents (% w/w)	Raw BSG	NaOH pretreated BSG	H <sub>2</sub> SO <sub>4</sub> pretreated BSG
Moisture	7.1	4.0	9.7
Cellulose	29.0	43.0	38.8
Cellulose loss during pretreatment	-	0	17.9
Hemicellulose	34.0	24.4	18.0
Hemicellulose loss during pretreatment	-	47	67.5
Mass recovered after pretreatment	-	73.6	61.3
Reducing sugars lost in pretreatment supernatant	-	0.1	11.4

Pretreatment type had a profound effect on the holocellulose content and cellulose to hemicellulose ratio of the pretreated materials. In addition, it resulted in significant mass losses due to the solubilization of BSG constituents in the pretreatment supernatants. Both pretreatments increased the cellulose content in the pretreated material, an effect that was more intense for alkaline pretreatment. Hemicellulose content was decreased in both cases and especially for the acid pretreatment. This is explained, since it is known that acidic conditions cause a partial hemicellulose hydrolysis, a fact that is also verified by the relatively

high concentration of reducing sugars detected in the pretreatment supernatant of the acid hydrolysis.

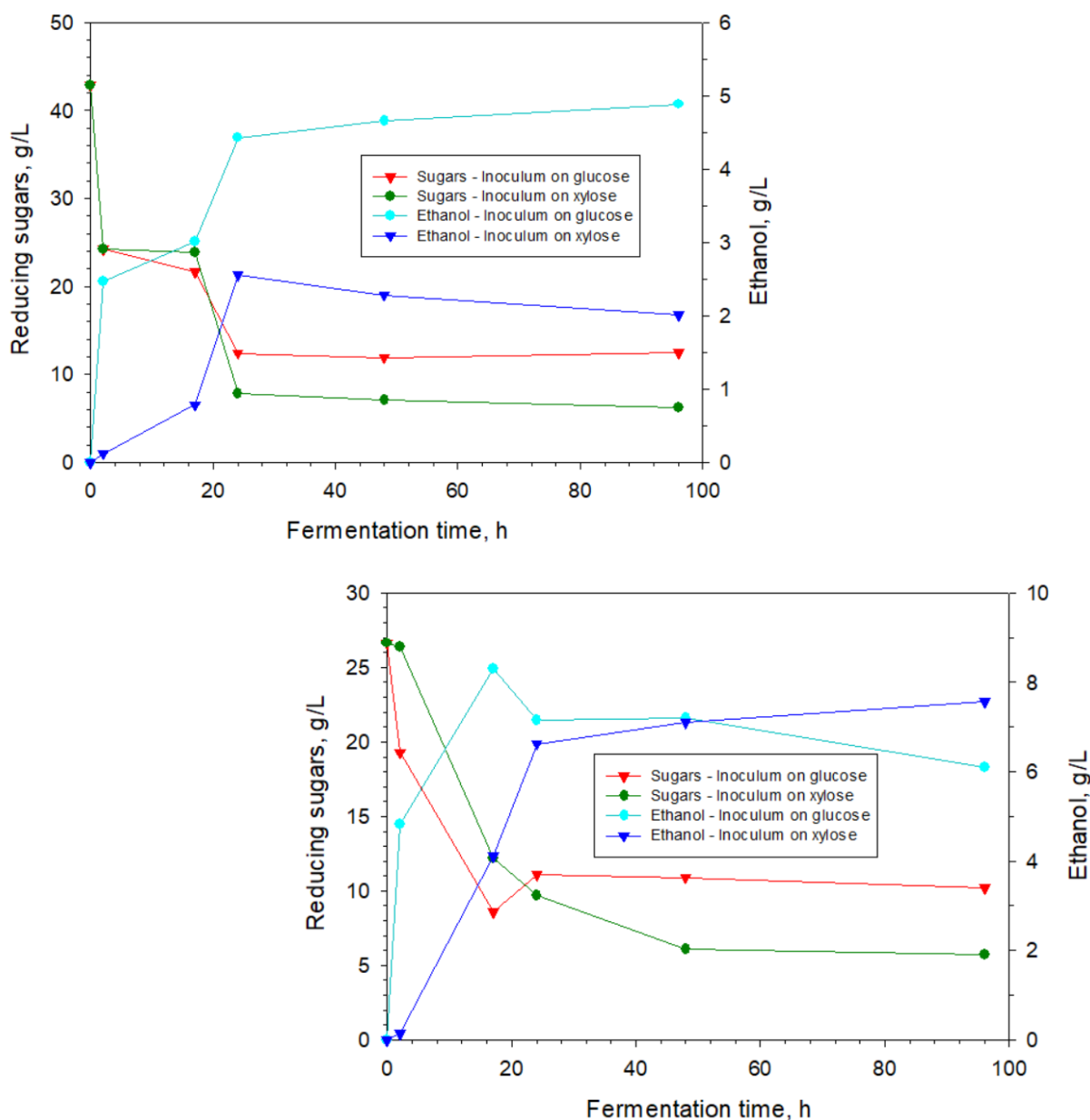
Raw and optimally pretreated BSG was subjected to enzymatic hydrolysis at a concentration of 83.33 g/L of solids load and the results are given in Table 3.28.

**Table 3.28.** Hydrolysis of raw and optimally NaOH and H<sub>2</sub>SO<sub>4</sub> pretreated BSG.

	Reducing sugars concentration (g/L)	Reducing sugars (g/kg dry raw / pretreated BSG)	Reducing sugars (g/kg holocellulose in raw / pretreated BSG)	Reducing sugars (g/kg dry raw BSG)
Raw BSG	12.64	163	241	163
NaOH pretreated BSG	49.65	620	884	456
H <sub>2</sub> SO <sub>4</sub> pretreated BSG	30.83	410	651	252

It is clear that the alkaline pretreatment is the most efficient for the production of reducing sugars from BSG, yielding 620 g sugars per kg of dry NaOH pretreated BSG, a value that represents almost 90% hydrolysis of the holocellulose in the pretreated material. When the above results are reduced per kg of raw material, almost half of the dry BSG biomass can potentially be converted to fermentable sugars.

The enzymatically hydrolyzed pretreated BSG (acid or alkali) suspensions were subsequently subjected to fermentation with *Z. mobilis* 8b as described in Materials and Methods, and the results are summarized in Figure 3.22.



**Figure 3.22.** Reducing sugars consumption and ethanol concentration, during the course of fermentation of enzymatically pretreated suspensions of pretreated BSG (SHF fermentation mode). Data points represent the mean of triplicate flasks. Left graph: SHF on NaOH pretreated material, Right graph: SHF on H<sub>2</sub>SO<sub>4</sub> pretreated material.

NaOH pretreated BSG hydrolysate proved to be a rather poor fermentation medium with respect to ethanol production, despite the fact that it has been fortified with the basal salts of *Zymomonas* medium DSMZ 1445. In the case of SHF of NaOH pretreated material, *Z. mobilis* 8b carbon source acclimatization had also a strong effect on ethanol yield. Specifically, ethanol yields after 48 h of fermentation were 0.161 and 0.073 g ethanol per g of reducing

sugars consumed, for glucose and fructose acclimatized cells, respectively. Maximum ethanol concentrations obtained were proportionally low, not exceeding 5 and 3 g/L, respectively.

H<sub>2</sub>SO<sub>4</sub> pretreated BSG hydrolysate, performed relatively better with respect to ethanol production from *Z. mobilis* 8b. Carbon source acclimatization did not significantly affected the maximum ethanol concentration achieved which, for both cases, it was around 8 g/L. *Z. mobilis* 8b acclimatization significantly affected sugar consumption and as a result, the corresponding ethanol yields were 0.461 and 0.362 g ethanol per g of reducing sugars consumed, for glucose and fructose acclimatized cells, respectively (90.2 and 70.8 % of the maximum theoretical yield).

## 4. Conclusions and prospects

The present study represents the first comparative optimization between mild alkaline and mild acid pretreatment of Brewers' Spent Grain (BSG) with respect to its susceptibility against enzymatic hydrolysis for second generation biorefinery applications. The available literature concerning the use of BSG as a lignocellulosic substrate for the production of fermentable sugars is rather limited, since until today, this substrate has been exploited as a low-cost animal feed, due to its high protein and lipid content. This has been verified by the results of our study, where the holocellulose content of the BSG obtained from Athenian Brewery S.A. was only about 63% (on a dry basis) with the rest being lipids and proteins.

**Table 4.1.** Polysaccharide composition of BSG from literature sources.

Reference	Cellulose	Hemicelluloses	Other
(Wilkinson et al. 2014)	20.7	19.9	59.4
(Ravindran et al. 2018)	19.2	26.9	53.9
(Mussatto et al. 2008b)	18.7	31.6	49.7
(Mussatto and Roberto 2006)	16.8	28.4	54.8
(Kemppainen et al. 2016)	18.1	25.3	56.6
This work	29.0	34.0	37.0

In contrast to other pretreatment optimization attempts, where only two independent parameters are usually used for the optimization, in our approach the optimization was performed with respect to three independent variables, namely,

Pretreatment temperature, (°C) -  $X_1$

Pretreatment time, (min) -  $X_2$

NaOH or H<sub>2</sub>SO<sub>4</sub> load (g reagent per kg raw BSG) -  $X_3$

A partial 3x3 experimental design was applied for the above three parameters, initially using the sugar yield after a subsequent enzymatic hydrolysis under uniform conditions,  $Y$ , as the optimization parameter (dependent variable).

In a second, innovative, approach the obtained experimental results were used in order to access the pretreatment optimization process from an economical perspective. For this reason,



the independent variables in each experimental condition were assigned to a specific cost. These individual costs were subsequently added-up to yield the total cost for the corresponding experimental condition. Following this analysis, the regression routine was reapplied with respect to the specific sugar yield,  $Y_{sp}$ , as the dependent variable, defined as the mass of sugars produced per \$. Table 4.2 summarizes the final results from these two optimization approaches.

**Table 4.2.** Optimization summary with respect to the total,  $Y$ , and specific sugar yield,  $Y_{sp}$ . Results are expressed on the basis of 167 kg/m<sup>3</sup> solid load during pretreatment, and a unit pretreatment volume of 1 m<sup>3</sup>.

Pretreatment	Temperature* (°C)	Time* (min)	Load* (mg / g BSG)	Y (kg/m <sup>3</sup> )	Y <sub>sp</sub> (kg/\$)	Actual Cost (\$/m <sup>3</sup> )	Model Cost (\$/m <sup>3</sup> )
<b>Sugar yield optimization</b>							
Alkaline	79	114	161	50.01	4.68	14.13	10.69
Acid	95	46	224	53.80	6.40	15.23	8.41
<b>Specific sugar yield optimization</b>							
Alkaline	44.5	154	107	37.23	5.79	8.25	6.43
Acid	95	46	168	50.46	7.13	13.31	7.10

Depending on the optimization target (sugar yield or specific sugar yield), a different set of optimum pretreatment conditions is obtained. This is true for both alkaline and acid pretreatment although for the latter, the corresponding optima differ only with respect to the H<sub>2</sub>SO<sub>4</sub> load.

For alkaline pretreatment, optimization with respect to sugar yield resulted in a maximum reducing sugars concentration of 50.0 g/L (or 300 kg/ ton raw BSG) at a cost of 21 ¢ per kg. At the optimum specific sugar yield conditions, there is a significant reduction in the final reducing sugars yield 37.2 g/L (223 kg / ton raw BSG) but this is compensated by the reduction of the cost per kg to 17 ¢ as a result of the reduced pretreatment temperature and NaOH load at optimum  $Y_{sp}$  conditions.

Acid pretreatment, proved relatively more cost effective compared to the alkaline, with slightly higher overall sugar yields. During yield optimization, it produced the highest sugar concentrations (53.8 g/L - 322 kg/ton raw BSG) with a cost of 16 ¢ per kg reducing sugars. Optimization with respect to specific sugar yield resulted in a slight decrease in the total

reducing sugars obtained (50.46 g/L - 302 kg/ton raw BSG) with a decrease in cost to 14 c per kg.

The analysis described in this work, is the first literature attempt to introduce a cost-oriented approach in the optimization of lignocellulosic materials' pretreatment studies. Although we followed a basic cost analysis, taking into account only cost factors affecting the materials acquisition and energy losses, we reached to the very important conclusion that when the pretreatment process is optimized with respect to the amount of sugars produced per unit capital, the optimum set of pretreatment conditions can differ significantly when compared to the optimum set when the optimization is performed only with respect to the sugar yield. This approach may result in accountable reductions in the cost per kg of reducing sugar obtained, as proved for the case of BSG, where optimization with respect to the sugar yield resulted in a 19% cost reduction for the alkaline pretreatment and 13% for the acid pretreatment of the material.

The second part of our work concerned the evaluation of the genetically modified strain of *Z. mobilis* 8b for the production of ethanol from BSG hydrolysates. It is well known that during pretreatment and subsequent enzymatic hydrolysis of a lignocellulosic material, a number of compounds may be released in the hydrolysate that could eventually interfere with the subsequent fermentation process. As a result, a common practice adopted in various bioethanol plants, is the separation and washing of the pretreated material right after pretreatment. In that way hydrolysis and fermentation take place in separate reactor (Separate Hydrolysis and Fermentation - SHF) a fact that substantially increases the installation costs of the facility.

In the case where pretreatment byproducts do not interfere with either hydrolysis or fermentation, there is a chance all related processes (pretreatment, hydrolysis and fermentation) to take place in a single vessel (Single Vessel Approach - SVA) with obvious positive results for the process economy. In this work we examined these two possibilities for both alkali and acid pretreated BSG, using *Z. mobilis* 8b inocula acclimatized both with either glucose or xylose as carbon source. The cumulative results of these fermentation experiments are summarized in Tables 4.3 and 4.4 that follow.

**Table 4.3.** Summary of fermentation results for the Single Vessel Approach. Results are expressed on the basis of 167 kg/m<sup>3</sup> solid raw BSG load fed in a unit pretreatment/hydrolysis/fermentation volume of 1 m<sup>3</sup>.

	Reducing sugars (kg/m <sup>3</sup> )	Final ethanol (kg/m <sup>3</sup> )	Ethanol yield on sugars (g ethanol /g sugars)	Ethanol yield on BSG (kg ethanol /ton raw BSG)
Alkaline pretreatment (Glucose/Xylose acclimatized cells)	61.53	31.44	0.511	188
Acid pretreatment (Glucose/Xylose acclimatized cells)	66.34	17.25	0.260	103

**Table 4.4.** Summary of fermentation results for the Separate Hydrolysis and Fermentation. Results are expressed on the basis of 167 kg/m<sup>3</sup> solid pretreated BSG load fed in a unit hydrolysis/fermentation volume of 1 m<sup>3</sup>.

	Reducing sugars (kg/m <sup>3</sup> )	Final ethanol (kg/m <sup>3</sup> )	Ethanol yield on sugars (g ethanol /g sugars)	Ethanol yield on BSG (kg ethanol /ton raw BSG)
Alkaline pretreatment (Glucose acclimatized cells)	99.50	16.04	0.161	70.6
Alkaline pretreatment (Xylose acclimatized cells)	99.50	7.26	0.073	31.9
Acid pretreatment (Glucose acclimatized cells)	61.78	28.48	0.461	105
Acid pretreatment (Xylose acclimatized cells)	61.78	22.36	0.362	82.5

The most important observation from the above data is the verification that *Z. mobilis* 8b is able to ferment the sugars in the BSG hydrolysates in an SVA mode. Especially for the alkaline pretreatment, the microorganism is capable to transform them almost quantitatively into ethanol. For the standard process parameters used throughout this work this corresponds to a final ethanol concentration in the pretreatment/hydrolysis/fermentation vessel over 30 g/L and an overall ethanol yield of 188 kg ethanol per ton raw BSG.

The chemical environment resulting from the acid pretreatment of BSG is less favorable with respect to ethanol production, meaning that this kind of pretreatment releases compounds that have an inhibitory effect in *Z. mobilis* 8b performance. The ethanol yield thus obtained is only 50% to the maximum theoretical, while the overall yield is around 100 kg of ethanol per ton raw BSG.

Oddly enough, the sugars obtained from the alkali pretreated BSG, hydrolyzed and fermented in SHF mode, proved to be the least suitable substrate for ethanol production by *Z. mobilis* 8b, despite the fact that the hydrolysate was fortified by the necessary salts. Even though in this mode we were able to obtain the highest reducing sugars concentration after hydrolysis, only a small percentage was subsequently fermented into ethanol resulting in overall ethanol yields well below 100 kg per g raw BSG. The fermentation of the sugars released during SHF of the acid pretreated BSG was notably more successful, especially when using *Z. mobilis* 8b cells acclimatized in glucose. This is probably due to the fact that acid pretreatment removes a significant amount of hemicellulose from BSG transforming the pretreated material to a cellulose-rich substrate, that yields mostly glucose after hydrolysis. An ethanol yield close to the maximum theoretical one was thus recorded accompanied by an overall yield of 105 kg per ton raw BSG.

In conclusion, our work proved that the fermentation of pretreated BSG hydrolysates by *Z. mobilis* 8b for the production of bioethanol, has a great potential for economic efficiency, despite the fact that its outcome is strongly dependent on the pretreatment conditions as well as on the overall biorefinery approach (SVA or SHF). The fermenting capacity of the *Z. mobilis* 8b strain used does not seem to be adversely affected by the potential inhibitors resulting from the alkali pretreatment of raw BSG (mainly phenolic compounds from the partial hydrolysis of lignin), which is of profound importance for overall process economics. More importantly, this allows the entire succession of all process steps (pretreatment - hydrolysis - fermentation) to be conducted in the same reaction vessel with the only external intervention being the pH adjustment of the suspension between steps. On the other hand, alkaline pretreatment is known that it mainly affects the lignin part of the substrate leaving intact most of the cellulose and hemicellulose for the subsequent hydrolytic attack. This results in a process, where the full carbohydrate potential of the substrate is being exploited towards ethanol by a strain such as *Z. mobilis* 8b which proved to ferment equally well both hexoses and pentoses under SVA conditions. Finally, the process appears to yield ground for

further improvement since the initial solid load of 167 g of raw BSG / L applied throughout the present work was selected for its suitability in laboratory studies. It is well known that industrial systems can operate at significantly higher loads that usually reach 300 g of dry solids per L. Under these conditions, it is anticipated that the scale-up of the proposed laboratory evaluated process will lead to final ethanol concentration higher than 40 g/L, which is the limit for an economically viable distillation.

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