Production of high added value products from sweet sorghum using high efficiency bioprocesses

Doctoral Thesis

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Η έγκριση της διδακτορικής διατριβής από την Ανωτάτη Σχολή Χημικών Μηχανικών του Ε.Μ.Πολυτεχνείου δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα. (Ν. 5434/1932, Άρθρο 202).

To my parents Spyridon and Vasiliki

ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

ΠΑΥΛΟΣ ΧΡΙΣΤΑΚΟΠΟΥΛΟΣ Σ. Καθηγητής Ε.Μ.Π.	Επιβλέπων Κθηγητής
ΣΕΡΑΦΕΙΜ ΠΑΠΑΝΙΚΟΛΑΟΥ Επικ. Καθηγητής Γ.Π.Α.	Μέλος συμβουλευτικής και εξεταστικής επιτροπής
ΕΥΑΓΓΕΛΟΣ ΤΟΠΑΚΑΣ Επικ. Καθηγητής Ε.Μ.Π.	Μέλος συμβουλευτικής και εξεταστικής επιτροπής
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ABSTRACT

Aim of this thesis was the evaluation of the potential of sweet sorghum stalks to serve as raw material for the production of several forms of biofuels. Initially, ethanol production was evaluated in two different process configurations (either whole stalks or the lignocellulosic fraction (bagasse) alone). In order to improve enzymatic digestibility of bagasse, microwave-assisted hydrothermal pretreatment was evaluated and optimized. Under optimal conditions, cellulose content was very high (66.84% w/w) and the yield of cellulose conversion reached 30.42% after 8h of digestion. During ethanol production trials, 12h of saccharification was proven to be sufficient, reaching a cellulose conversion of 59.8%. Ethanol concentration at the subsequent fermentation reached 41.4g/L with a volumetric productivity equal to 1.88g/L·h. Addition of extra fresh enzymes at the start-up of the fermentation had a positive effect on ethanol production.

When the whole stalks were evaluated as raw material for ethanol production under high solids content, it was found during the initial experiments that incorporation of an enzymatic treatment step prior to fermentation results in increasing ethanol production and productivity. In order to optimize the hydrolysis step, the combined effect of hydrolysis duration and enzyme load on ethanol productivity was evaluated and optimized. Under the optimal conditions found for enzymatic hydrolysis (8.32 FPU/g enzyme load for 8.6h), ethanol production reached 62.53 g/L and productivity 2.98 g/L·h. Finally, when the effect of the solid content was evaluated, it was found that initial solids content of 35% w/w resulted in very high ethanol production with the highest productivity and very high relative yield comparing to the maximum theoretical.

Based on the obtained results for ethanol production, it can be concluded that utilization of the stalks is more beneficial, as less steps are included and the obtained ethanol concentration was higher comparing to the bagasse. For this reason, the same process was used for the evaluation of microbial lipids production which could serve as raw material for biodiesel. Three different oleaginous yeasts were evaluated as candidates, namely *L. starkeyi* CBS 1807, *T. fermentans* CBS 439.83 and *R. toruloides* CCT 0783. Initially the ability of

the stalks to support yeast growth both as nitrogen and carbon source was evaluated by supplementing stalks with external nitrogen source. In all the yeasts it was found that addition of external nitrogen source results in decrease in lipid production. The ability of the stalks to provide with the necessary nitrogen it is beneficial for the process economics, as nitrogen supplementation is considered to increase the production cost. Presence of the enzymatic saccharification prior to yeast cultivation had a positive effect in lipid production for all the yeasts and solid contents. The highest lipid production concentration was observed when *R. toruloides* was used and reached 13.77 g/L when the cultivation took place on juice that came from 20% w/w solids. The biodiesel parameters of the obtained oils were predicted and all of them have appropriate characteristics to be used as biodiesel.

Finally, the ability of stalks to serve as raw material for the production of methane by anaerobic digestion was examined. Two different treatments were evaluated in order to increase the yield of methane production, namely a mild thermal and an enzymatic treatment. Thermal treatment had a negative effect on methane yield. On the other hand, enzymatic treatment in one-step (similar to SSF process) increased methane yield from 238 mL CH_4/g VS to 274 mL CH_4/g VS. In a final step, the combined effect of enzyme load and I/S ratio was evaluated and resulted in a highest methane yield equal to 284.37 mL CH_4/g VS when the enzyme load was equal to 13FPU/g at a I/S ratio equal to 0.7.

ΠΕΡΙΛΗΨΗ

Σκοπός της παρούσας διδακτορικής διατριβής ήταν η αξιολόγηση της δυνατότητας χρησιμοποίησης του γλυκού σόργου ως πρώτη ύλη για την παραγωγή διαφόρων μορφών βιοκαυσίμων. Αρχικώς αξιολογήθηκε η δυνατότητα παραωγής βιοαιθανόλης με την χρήση δύο διαφορετικών στρατηγηκών (είτε την χρήση όλων των στελεχών του σόργου είτε με την χρήση μόνο του λιγνινοκυτταρινούχου κλάσματος, την βαγάσση). Σε μια προσπάθεια να βελτιωθεί η ενζυμική υδρόλυση της βαγάσσης, μελετήθηκε και βελτιστοποιήθηκε η εφαρμογή υδροθερμική προκατεργασίας με την χρήση μικρομυμάτων. Κάτω απο τις βέλτιστες συνθήκες προκατεργασίας, παρατηρήθηκε υψηλή συγκέντρωση κυτταρίνης (66.84% w/w) καθώς και υψηλή υδρόλυση της κυτταρίνης, η οποία ανήλθε σε 30.42% μετά από 8 ώρες υδρολύσεως. Κατά την διάρκεια των πειραμάτων παραγωγής αιθανόλης, παρατηρήθηκε οτι υδρόλυση 12 ωρών ήταν αρκετή για την αποτελεσματική υδρόλυση της κυτταρίνης, η οποία ανήλθε σε 59.8%. Η συγκέντρωση της αιθανόλης στην ακόλουθη ζύμωση ανήλθε σε 41.4g/L με την παραγωγικότητα να φτάνει σε 1.88g/L·h. Η προσθήκη επιπλέον φρέσκων ενζύμων κατά την έναρξη της ζύμωσης είχε θετική επίδραση στην παραγόμενη αιθανόλη.

Κατά την διάρκεια των πειραμάτων όπου χρησιμοποιήθηκαν τα στελέχη του σόργου σε συνθήκες υψηλής συγκέντρωσης στερεών, βρέθηκε οτι η ενσωμάτωση ενός ξεχωριστού σταδίου ενζυμικής υδρόλησης πριν την ζύμωση είχε ως αποτέλεσμα την αύξηση της παραγωγής αιθανόλης καθώς και της παραγωγικότητας. Προκειμένου να βελτιωθεί το στάδιο της υδρολύσεως, μελετήθηκε η συνδυασμένη επίδραση του χρόνου υδρόλυσης και του ενζυμικού φορτίου στην παραγωγικότητα της αιθανόλης. Κάτω από τις βέλτιστες συνθήκες που βρέθηκαν (ενζυμικό φορτίο ίσο με 8.32 FPU/g και χρονική διάρκεια 8.6 ωρών), η παραγωγή αιθανόλης έφτασε τα 62.53 g/L με την παραγωγικότητα να είναι 2.98 g/L·h. Τέλος, κατά την διάρκεια της μελέτης της επίδρασης της αρχικής συγκέντρωσης στερεών, βρέθηκε ότι συγκέντρωση στερεών ίση με 35% w/w οδήγησε στην υψηλή παραγωγή αιθανόλης, στην μέγιστη παραγωγικότητα καθώς και σε έναν από τους υψηλότερους συντελεστές απόδοσης, σε σύγκριση με τις άλλες συγκεντρώσεις που μελετήθηκαν.

Λαμβάνοντας υπόψην τα αποτελέσματα σχετικά με την παραγωγή αιθανόλης, καταλήξαμε οτι η χρήση των στελεχών του γλυκού σόργου είναι πιο αποτελεσματική, καθώς λιγότερα στάδια είναι απαραίτητα και η συγκέντρωση της παραγώμενης αιθανόλης ήταν υψηλότερη σε σχέση με την χρήση της βαγάσσης. Για

αυτόν τον λόγο αποφασίστηκε να χρησιμοποιηθεί η ίδια διεργασία κατά την διάρκεια των πειραμάτων μελέτης παραγωγής μικροβιακών λιπιδίων τα οποία μπορούν να χρησιμοποιηθούν για την παραγωγή βιοντίζελ. Τρείς ελαιογόνες ζύμες μελετήθηκαν για αυτόν τον σκοπό, πιο συγκεκριμένα οι ζύμες *L. starkeyi* CBS 1807, *T. fermentans* CBS 439.83 και *R. toruloides* CCT 0783. Αρχικώς εξετάστηκε η δυνατότητα των στελεχών του σόργου να υποστηρίξουν την ανάπτυξη των διαφόρων ζυμών παρέχοντας τόσο τον άνθρακα όσο και το απαραίτητο άζωτο. Αυτό πραγματοποιήθηκε μελετώντας την επίδραση που είχε η προσθήκη εξωτερικής πηγής αζώτου στο μέσον της καλλιέργειας. Παρατηρήθηκε ότι η προσθήκη του εξωγενούς αζώτου είχε αρνητική επίδραση και στις τρείς ζύμες, μειώνοντας την παραγωγή μικροβιακού λίπους. Η ικανότητα των στελεχών του σόργου να προσφέρουν τόσο τον απαραίτητο άνθρακα όσο και το απαραίτητο άζωτο είναι πολύ σημαντική για την μείωση του κόστους παραγωγής, καθώς η προσθήκη αζώτου θεωρείται ότι αυξάνει το συνολικό κόστος. Επιπλέον, η ύπαρξη ενός σταδίου ενζυμικής σακχαροποίηση πριν το στάδιο παραγωγής λίπους είχε θετική επίδραση στο παραγώμενο λίπος, ανεξαρτήτως της χρησιμοποιηθέντας ζύμης και της συγκέντρωσης στερεών. Η υψηλότερη συγκέντρωση λίπους παρατηρήθηκε όταν καλλιεργήθηκε η ζύμη *R. toruloides* σε υπόστρωμα που προέκυψε από συγκέντρωση στερεών ίση με 20% w/w, έχοντας αφαιρέσει τα στερεά, και ανήλθε σε 13.77 g/L. Στο τελικό στάδιο αυτής της μελέτης, έγινε πρόβλεψη των ιδιοτήτων του βιοντίζελ το οποίο θα προέκυπτε αν χρησιμοποιούνταν τα λίπη που προέυψαν απο τις τρείς ζύμες που μελετήθηκαν. Βρέθηκε ότι και οι τρεις παράγουν λιπίδια που θα έδιναν καλές ιδιότητες στο παραχθέν βιοντίζελ.

Στο τελικό στάδιο της παρούσας μελέτης, εξετάστηκε η δυνατότητα χρήσης των στελεχών του σόργου για την παραγωγή βιομεθανίου μέσω αναερόβιας χώνευσης. Αρχικώς, μελετήθηκε η επίδραση δύο διαφορετικών κατεργασιών πριν την αναερόβια χώνευση στην απόδοση σε μεθάνιο. Πιο συγκεκριμένα μελετήθηκαν μια ήπια θερμική κατεργασία και μια ενζυμική. Η θερμική κατεργασία βρέθηκε ότι είχε αρνητική επίδραση στην απόδοση σε μεθάνιο. Από την άλλη μεριά, η ενζυμική κατεργασία όταν εφαρμόστηκε σε ένα στάδιο με την αναερόβια χώνευση (προσομοιάζοντας την διεργασία SSF) βελτίωσε την απόδοση σε μεθάνιο από 238mL CH_4/g VS σε 274mL CH_4/g VS. Σε ένα τελικό στάδιο, εξετάστηκε η συνδυαστική δράση του ενζυμικού φορτίου και του λόγου I/S και βρέθηκε ότι η υψηλότερη απόδοση σε μεθάνιο επιτεύχθηκε για ενζυμικό φορτίο ίσο με 13FPU/g και λόγο I/S ίσο με 0.7 και ανήλθε σε 284.37mL CH_4/g VS.

LIST OF PAPERS

The thesis is based on the following papers:

1. Matsakas L, Bonturi N, Miranda EA, Rova U, Christakopoulos P: High concentrations of dried sorghum stalks as a biomass feedstock for single cell oil production by *Rhodosporidum toruloides*. *Biotechnol Biofuel*, *accepted for publication*.

2. Matsakas L, Sterioti AA, Rova U, Christakopoulos P: Use of dried sweet sorghum for the efficient production of lipids by the yeast *Lipomyces starkeyi* CBS 1807. *Ind Crop Prod* 2014, 62:367-372.

3. Matsakas L, Rova U, Christakopoulos P: Evaluation of dried sweet sorghum stalks as raw material for methane production. *Biomed Res Int* 2014, 2014:731731.

4. Matsakas L, Christakopoulos P: Optimization of ethanol production from high dry matter liquefied dry sweet sorghum stalks. *Biomass Bioenerg* 2013, 51:91-98.

5. Matsakas L, Christakopoulos P: Fermentation of liquefacted hydrothermally pretreated sweet sorghum bagasse to ethanol at high-solids content. *Bioresource Technol* 2013, 127:202-208.

Publications written within the duration of the thesis but not included in this thesis:

1. Matsakas L, Topakas E, Christakopoulos P: New trends in microbial production of 3-hydroxypropionic acid. *Curr Biochem Eng* 2014, 1:141-154.

2. Sarris D, Matsakas L, Aggelis G, Koutinas AA, Papanikolaou S: Aerated vs non-aerated conversions of molasses and olive millwastewaters blends into bioethanol by *Saccharomyces cerevisiae* under non-aseptic conditions. *Ind Crop Prod* 2014, 56:83-93.

3. Matsakas L, Kekos D, Loizidou M, Christakopoulos P: Utilization of Household Food Waste for the production of ethanol at high dry material content. *Biotechnol Biofuel* 7:4.

Peer-reviewed abstracts in conference proceedings:

1. Christakopoulos P, Matsakas L, Rova U. Use of high dry matter liquefied dry sweet sorghum stalks for the production of second generation energy carriers. Joint Monash/LTU symposium on Biorefinery Applications, Melbourne, Australia, 10-12 December 2014. Oral presentation.

2. Matsakas L, Sjöblom M, Rova U, Christakopoulos P. Use of high dry matter liquefied dry sweet sorghum stalks for the production of second generation energy carriers. FP1306 COST Action, First WG1 Meeting, Brussels, Belgium, 1-2 October 2014. Oral presentation.

3. Christakopoulos P, Matsakas L. Production of Single Cell Oil from liquefied sweet sorghum stalks**.** 36th Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, Florida, USA, 28 April - 1 May 2014, Abstract book p. 19. Oral presentation.

4. Bonturi N, Matsakas L, Nilsson R, Christakopoulos P, Berglund K, Miranda EA, Rova U. Evaluation of methodologies for lipid extraction from *Lipomyces starkeyi* and *Rhodosporidium toruloides*. 36th Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, Florida, USA, 28 April - 1 May 2014, Abstract book p. 48. Poster presentation.

5. Matsakas L, Paschos T, Christakopoulos P. Ethanol production from the oleaginous yeast *Trichosporon fermentans***.** 36th Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, Florida, USA, 28 April- 1 May 2014, Abstract book p. 55. Poster presentation.

6. Antonopoulou I, Matsakas L, Christakopoulos P. Evaluation of the enzymatic system of the fungus *Sporotrichum thermophile* for the efficient hydrolysis of cellulose and bagasse. 9th Panhellenic Conference of Chemical Engineering. 23-25 May 2013, Athens, Greece. Poster presentation.

7. Konti K, Matsakas L, Mamma D, Kekos D. Biodegradation of 1,3-dichloro-2-propanol and 3-chloro-1,2-propanediol by the bacterium *Pseudomonas putida* DSM437. 9th Panhellenic Conference of Chemical Engineering. 23-25 May 2013, Athens, Greece. Poster presentation.

8. Matsakas L, Sterioti AA, Spanopoulos A, Christakopoulos P. Use of liquefied dry sweet sorghum stalks for the production of lipids by *Lipomyces starkeyi* CBS 1807 cells**.** 5th Panhellenic conference «Modern

trends in the field of lipids», Greek Lipid Forum, 29 March 2013, Athens Greece, Abstract book p.68. Poster presentation.

9. Sarris D, Matsakas L, Koutinas AA, Komaitis M, Papanikolaou S. Bio-ethanol production during growth of the yeast *Saccharomyces cerevisiae* MAK 1 on mixtures of molasses and olive mill wastewaters under non-sterile conditions. 5^{o} 5th Panhellenic conference «Modern trends in the field of lipids», Greek Lipid Forum, 29 March 2013, Athens Greece, Abstract book p.51. Poster presentation.

10. Matsakas L, Christakopoulos P. Utilization of liquefied sweet sorghum stalks for the production of ethanol and single cell oil. Bio4Energy Conference, Skellefteå Sweden, 19-20 March 2013. Oral presentation

11. Matsakas L, Antonopoulou I, Christakopoulos P. Optimization of ethanol production on liquefacted dry sweet sorghum stalks**.** 15th European Congress on Biotechnology, Istanbul Turkey, 23-26 September 2012, New Biotechnology 29, S41. Poster presentation

12. Antonopoulou I, Matsakas L, Christakopoulos P. Evaluation of *Sporotrichum thermophille*'s cellulolytic system for an efficient hydrolysis of cellulose. 15th European Congress on Biotechnology, Istanbul Turkey, 23-26 September 2012, New Biotechnology 29, S40. Poster presentation

13. Matsakas L, Mamma D, Christakopoulos P. Ethanol production using liquefacted dry sweet sorghum stalk particles at high-solid concentrations. 7th International Conference on Renewable Resources and Biorefineries, Bruges Belgium, 8-10 June 2011, Abstract book p. 127. Poster presentation

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Contents

1. INTRODUCTION

Renewable energy alternatives have been subjected to a huge research activity due to rising environmental problems combined with an imminent shortage of fossil fuels which creates energy insecurity (Li et al., 2010; Sarris et al., 2013; Papanikolaou and Aggelis, 2011a). Estimations have shown that the greenhouse gas (GHG) emissions have increased by 70% from 1970 to 2004 (Galbe and Zacchi, 2002). Another report has evaluated the GHG emissions for 2012 to have been increased by 2.6% and 58% compared to the emissions in 2011 and 1990 respectively (Le Quéré et al., 2012). It was mentioned that the total CO_2 emissions attributed to petroleum products reached 10.9 giga metric tons during 2007, where transport section contributed with 60% of the total emissions (Hervé et al., 2011). It is obvious that GHG emissions rapidly increased during the last decades, which contributes to several environmental problems such as global warming. Moreover, most of the countries worldwide are dependent on the importation of fossil fuels to meet their energy demands, which make them dependent on the exporter. This fact results in energy insecurity which could become crucial.

According to a report released by I.E.A. (IEA, 2013), the global energy supply is based mostly on fossil fuels (81.6% of the total energy) where oil accounts for 31.5%, natural gas 21.3% and coal 28.8%. Renewable energy like biofuels and wastes accounts for 10% and hydropower only 2.3%. Finally nuclear power represents 5.1% of the total energy supply. Recently E.I.A. (2014) projected that the global energy consumption will increase by 56% from 2010 to 2040 mostly due to the high increase in the energy demands of the non-OECD countries (Figure 1) which are presenting high rates of

economic growth (http://www.eia.gov). More specifically 85% of this increase is attributed to non-OECD Asia and Middle East (EIA, 2014). These increasing rates of fossil fuel consumption apart from the environmental problems that they cause will result in the depletion of the fossil resources. Indeed, according to some researchers oil reserves could be depleted in the next 41 years whereas natural gas in the next 64 years (Goldember, 2007). All these facts have alerted the society to take action in order to ensure energy supply for the future generation. For example according to the directive 2003/30/EC, countries member of EU should have incorporated incorporate 2% and 5.75% of renewable fuels in the transport section by 2005 and 2010 respectively (EC, 2003).

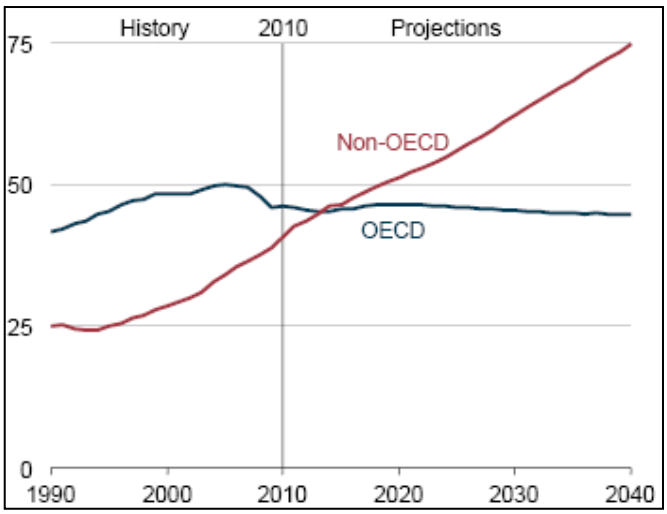

Figure 1: Daily consumption in million barrels of petroleum and other liquid fuels in OECD and non-OECD countries during the period 1990-2040.
Source: E.I.A. International Energy Outlook 2014 (EIA, 2014).

1.1 Renewable energy

Renewable energy is seen as an excellent solution to both reduce the negative impact of humanity on the environment and make the societies energy independent. Several forms of renewable energy are used, like hydropower, solar energy, geothermal, biofuels, etc. The importance in increasing the use of renewable energy is shown in Figure 2, where the average annual growth during the last years of different renewable energy is presented. There is a clear rising trend in the production of all the forms of renewable energy, except from the production of ethanol during 2012. This slight decrease could be attributed to the saturation of the sources that are used for ethanol production and the need to exploit new ones. The interest for new investments on renewable energy production units had increased during the last years from 40 billion US dollars in 2004 to 279 billion US dollars in 2011, with a minor decline to 244 billion in 2012 (REN 21, 2013). It is obvious that investors consider renewable energy as a profitable business that in the future will play an important role in the energy production worldwide.

Concerning the biofuels production, several options are available such as woodchips, pellets, methanol, ethanol, biodiesel, biogas, syngas, DME, etc (Nigam and Singh, 2011). Utilization of woodchips and pellets is considered to be a primary and more traditional forms of biofuel as they are natural and unprocessed materials, whereas the others are considered to be more advanced forms. Regarding the transportation sector, which accounts for 32.6% of the total energy consumption in EU (EU, 2010), liquid fossil fuels (i.e. gasoline or diesel) are mostly used which reduced the number of potential renewable alternatives compared to for example electricity production. Ethanol, biodiesel and biogas are some paradigms of renewable fuels that could replace the use of fossil fuels in the transport section. Ethanol and biodiesel are the most widely

used; as they can replace gasoline and petroleum respectively (Matsakas et al., 2014). Figure 3 shows a clear increase in their global production from 1975 to 2010. Finally, after having been upgraded by carbon dioxide removal and increase of the methane concentration, biogas can be used as vehicle fuel with slight modifications of the vehicle. Biofuels are generally considered to be the most promising option of renewable energy for short term use due to their market maturity (Nigam and Singh, 2011) and their compatibility with the existing infrastructure. Production of biofuels does not only have a positive impact by reducing the GHG emissions produced by oil burning, but also but also by improving the energy security with the use of domestic natural resources for their production and by creating new jobs especially in rural areas.

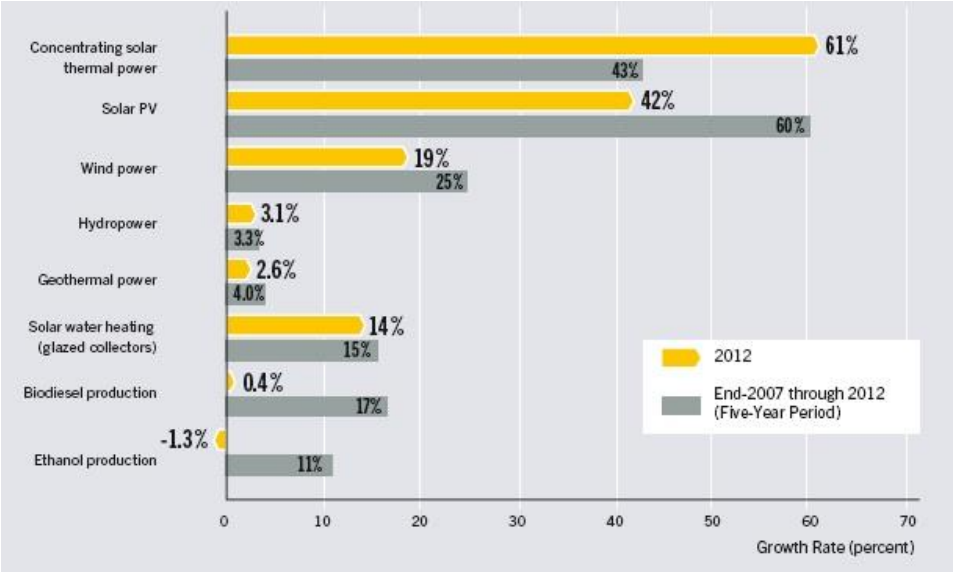

Figure 2: Average annual growth of different forms of renewable energy as an average during the five years period 2007-2012 and during 2012.
Source: REN 21, 2013.

Generally, biofuels can be distinguished in three generations. In the first generation the raw materials used are mainly sugars, grains and seeds which represent the storage organs of plants (Hervé et al., 2011). Some characteristic examples of raw materials used during first generation biofuels are sugarcane, corn, potatoe, wheat, palmoil, rapsoil, soybean,etc (Halvík et al., 2011; Naik et al., 2010). The use of these raw materials for biofuels production was blamed to contribute to the increase of the food prices globally which resulted in severe criticism against biofuels (Papanikolaou and Aggelis, 2011a; Pinzi et al., 2013). Moreover, first generation biofuels are also blamed not to efficiently contribute to the reduction of GHG emissions (Naik et al., 2010; Hervé et al., 2011) or to reduce the efficiency of land use (Nigam and Singh, 2010).

In order to deal with the *food vs fuel* dilemma, researchers have turned their interest to the utilization of non-food sources for biofuel production, such as lignocelluloses biomass, moving to the second generation biofuels. Lignocellulose, which will be discussed later, includes different kind of straws (like wheat, corn, rice, etc), leaves, branches, sawdust or even energy grassed or trees. Despite the obvious advantages of second generation biofuels, there are still some concerns about land-use change and competition in land use with crops that are cultivated for food (Brennan and Owende, 2010).

Finally, the third generation biofuels is based on photosynthetic algae (Lam and Lee, 2012). During the third generation biofuels production, carbon dioxide is captured and used for the storage of algae lipids which are involved in the production of biodiesel.

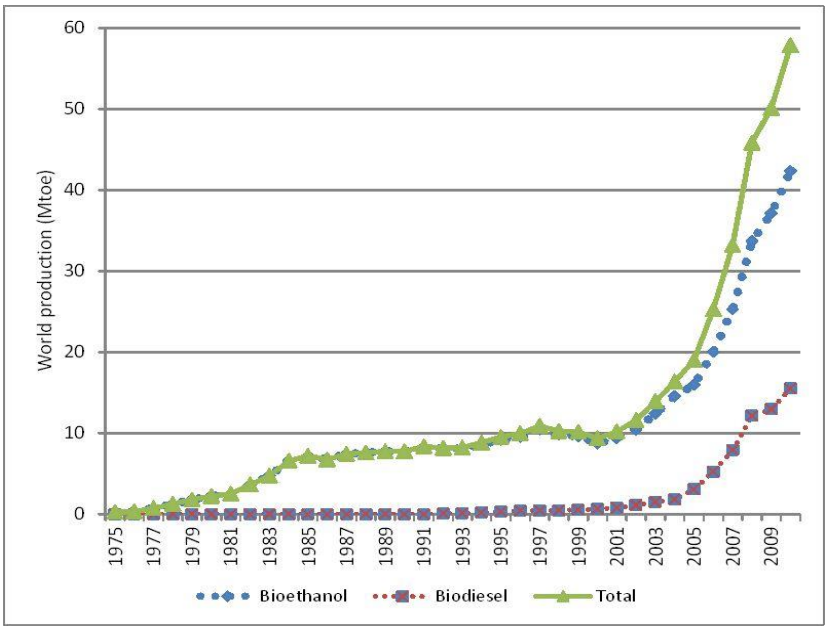

Figure 3: Global production of bioethanol and biodiesel in million tons between the years 1975 and 2009.
Source: Hervé et al., 2011

1.2 Ethanol production

The use of ethanol as a vehicle fuel is known since 1860s with Nicholas Otto and with Henry Ford in 1896 for his first car (Datta et al., 2010). It is considered as one of the most important biofuels as it presents several environmental benefits when used (Cardona Alzate and Sánchez Toro, 2006). It is estimated that globally about 820 million cars and light trucks are working with ethanol (Datta et al., 2010). Ethanol can be used in blends with gasoline in which the percentage variation in ethanol from 5% (E5) to 100% (E100). Conventional cars can use up to E10 without modifications but as the ethanol percentage increases some modification are necessary (such as in the fuel

injection, fuel pump, evaporative system, etc) and for ethanol blends higher than 25% engine modification are also required (The Royal Society, 2008).

Ethanol as a fuel presents a high octane number (Christakopoulos and Topakas, 2010) with even small amounts of ethanol in the blend causing a disproportionately large increase in octane number of the blend (Datta et al., 2010). Moreover, the higher oxygen content improves the efficiency of the combustion (Niven, 2005). On the other hand the energy content of ethanol represents 66% of the gasoline energy content (Nigam and Singh, 2011). Concerning the environmental effect of ethanol use, emissions of GHG are generally considered to be reduced. More specifically, ethanol burn results in lower emission in carbon monoxide (CO), fine particulate matter (PM), volatile organic compounds (VOCs), sulfur oxides, benzene and hydrocarbons comparing to fossil fuels (CFDC, 2010). On the other hand, some findings indicate that there is an increase in nitrogen oxides (NO_x), acetaldehyde, ethanol, formaldehyde and acrolein emissions (Niven, 2005). Moreover, blends of ethanol up to 10% w/w in gasoline results in increase of the Reid Vapor Pressure, which in turn results in increase of the evaporative emissions (Poulopoulos et al., 2001).

Common substrates for ethanol production are sugars and starch. However, there is an increasing effort to move towards the second generation ethanol where lignocellulose is used. The most commonly used microorganism is the yeast *Saccharomyces cerevisiae*, although some other yeasts (like *Kluyveromyces* sp.), bacteria (like *Zymomonas mobilis*) or fungi (like *Fusarium oxysporum*) could also be used. The two biggest producers of ethanol worldwide are USA and Brazil, using corn and sugars from sugarcane respectively (Oliveira et al., 2005). Until recently (2005) Brazil was leading in the production of ethanol worldwide, with a constant high production rate since the 80s. The last years

USA took the lead presenting an impressive increase in the annual ethanol production as can be seen in Figure 4 (Wang et al., 2012). According to Gnansounou (2010) ethanol production in Europe represented 5% of the global production in 2008, with Germany and France being the main producers (Balat, 2007). Despite the fact that there is an increasing interest in utilizing alternative sources for ethanol fermentation (such as lignocellulose), the main sources of ethanol production in Europe are cereals and sugar beet (Demirbas 2009).

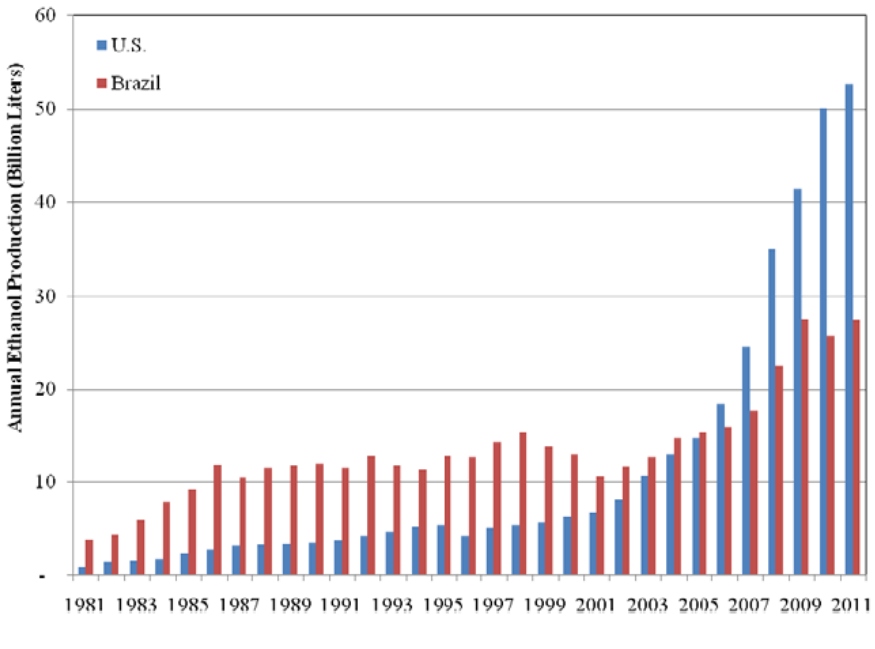

Figure 4: Annual ethanol production in USA and Brazil between 1981 and 2011.
Source: Wang et al., 2012

When it comes to the utilization of sugars or starch, the produced ethanol is called first generation. The use of sugars is simple, as the yeast can directly grow on them and convert them into ethanol. However the use of starch requires a step of hydrolysis, where starch is converted into glucose by specific amylolytic enzymes, namely glucoamylase and α-amylase, under 'dry grind' or 'wet mill' processes (Bothast and Schlicher, 2005; Shigechi et al., 2004).

There is a rising interest in exploiting lignocellulose for the production of second generation bioethanol, in order to minimize usage of food sources. However, the utilization of lignocelluloses is more complex compared to that of sugars and starch. The main challenge is to efficiently release the sugars from mainly cellulose using specific enzymes. For this reason, prior to enzymatic hydrolysis a pretreatment step is necessary in most cases (Matsakas et al., 2014). Pretreatment and hydrolysis of cellulose will be discussed later.

No matter which raw materials is used, the production and the use of bioethanol contribute to a decrease of CO_2 emissions. On the other hand, the source of raw materials contributes to a different degree of CO_2 reduction, where cellulosic ethanol presents a bigger reduction than corn. Wang et al. (2012) calculated that GHG emissions (in CO_2 equivalents) to be 94g/MJ for gasoline production, whereas for ethanol produced from corn and sugar cane are 76g/MJ and 45g/MJ respectively. When cellulosic ethanol is produced, the emissions are even lower, between 22 and 29 g/MJ depending on the cellulose source, according to the same study. Thus, the reduction of GHG emissions is dependent on the blend that is used and the source of ethanol. With a higher percentage of ethanol in the blend, higher reduction in GHG is observed. For corn ethanol, a reduction of 1% in E10 blend has been calculated, which is increased to a reduction of 14-19% for E85 blend (Wang et al., 1999). When E85 of cellulosic ethanol is used, GHG emission can be

reduced by 64% (Wang, 2005). As suggested by Wang et al. (2009) improvement of corn ethanol production could result in higher reduction in GHG emissions, but on the other hand it is obvious that in order to achieve the highest reduction, ethanol production should move towards cellulosic ethanol. Despite the clear environmental benefits of cellulosic ethanol production it is estimated even in 2020 44% of ethanol will be produced by coarse grains and 34% from sugarcane (Vivekanandhan et al., 2013). In order to improve the share of cellulosic ethanol in the total ethanol production, new technologies, aiming in reducing the production costs need to be applied. In the time being, some pilot/demo plants are operating for cellulosic ethanol production, such as Inbicon in Denmark, Sekab in Sweden, KL Energy and POET LLC in USA, Abengoa in Spain, Iogen in Canada and Chemtex in Italy, which will contribute to a better understanding of the process at industrial level (Cannella and Jørgensen, 2013; Menon and Rao, 2012).

1.3 Biodiesel production

Biodiesel is considered to be an important renewable fuel that could replace fossil diesel in compression ignition engines. Apart from being renewable, biodiesel has attracted the interest because it is biodegradable, nontoxic and improve the lubricity of the fuel as well as reducing GHG formation (Li et al., 2008; Zhu et al., 2008; Matsakas et al., 2014; Demirbas, 2008). Biodiesel consists of a mixture of fatty acid esters which corresponds to the product of the transesterification of triacylglycerols (TAGs) with an alcohol in the presence of a catalyst (Economou et al., 2010; Agarwal, 2007). The different sources of TAGs can be plant oils or animal fats.

Vegetable oils were positively tested directly as a fuel by Rudolf Diesel on his engine when this was not possible with animal fats due to their rich

composition in saturated fatty acids which makes them solids at room temperature (Ma and Hanna, 1999; Shay, 1993). Despite the environmental benefits of using vegetable oils as fuel, several problems have been mentioned such as clogging problems, high viscosity, deposit problems due to polymerization of the unsaturated components, lower volatility, thickening and gelling of the lubricating oil (Nigam and Singh, 2011; Shay, 1993; Ma and Hanna, 1999). In order to improve oils characteristics, different techniques were applied including pyrolysis, micro-emulsification, transesterification, catalytic craking etc (Sharma et al., 2008; Demirbas, 2008). Transesterification is considered to be the most promising one (Nigam and Singh, 2011). Bases, acids or enzymes can be used as catalysts during the transesterification process. (Ma and Hanna, 1999). The use of enzymes presents some advantages such as the mild reaction conditions, easy glycerol purification and the ability to convert free fatty acids present in the oil into biodiesel (Luković et al., 2011). On the other hand, the reactions proceeds at slow rates, the cost of the enzymes is still high and the enzymes present low stability in the presence of methanol (Luković et al., 2011). The main byproduct of transesterification process is glycerol, where 10 kg are produced for 100 kg of produced biodiesel (Chatzifragkou and Papanikolaou, 2012).

Only small amounts of biodiesel were produced until the beginning of this century, but then production rapidly increased (Figure 3). The main producer of biodiesel globally is EU which accounts for the 71% of the global biodiesel production during 2007 (Baier et al., 2009), with Germany and France having the lead in EU (Timilsina and Shrestha, 2011). The main oils that are used include soybean in USA, rapeseed in EU and palm (Demirbas, 2008). Coding of biodiesel blends is the same as for ethanol, where the letter 'E' is changed to the letter 'B'; and the specifications for B100 are covered by the standard

ASTM D6751 (http://www.astm.org/Standards/D6751.htm). Blends up to B20 can be directly used in common engines whereas higher blends might require some few engine modifications (Demirbas, 2008).

As it was previously mentioned, biodiesel is contributing to the reduction of gas emissions. An increase in biodiesel percentage in the blend leads to a higher reduction of different gases with the exception of NOx (Figure 5). The source of the oil used for biodiesel production also affects the emissions (Guarieiro and Guarieiro, 2013).

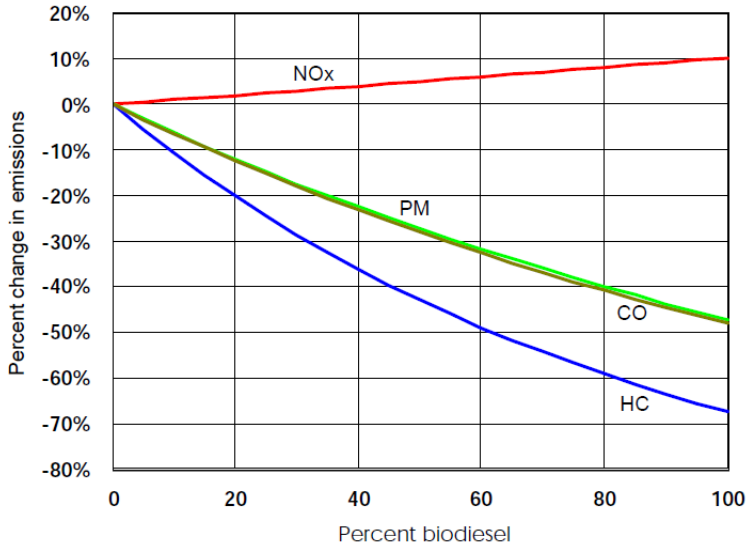

Figure 5: Changes in emissions of different blends of biodiesel. NOx: Mono-nitrogen oxide, PM: Particulate matter, CO: Carbon monoxide, HC: Hydrocarbons.
Source: EPA, 2002.

Like the case of ethanol production, the use of edible oils for biodiesel production is blamed to have increased their price. Unfortunately, FAO predicts that the use of vegetable oils will remain at high level even in 2020,

when it will contribute with 79% of the total biodiesel production (OECD-FAO, 2011). Alternative sources of oils should be exploited in order to decrease the use of edible oils for biodiesel production. Waste oils, which are recycled after used, can serve as raw materials, however the quality of them is low (Nigam and Singh, 2011) and the increased free fatty acid content can cause problems such as saponification during the transesterification process (Pinzi et al., 2013). Microbial oil could be another alternative source of non-edible oils which could serve as a raw material for biodiesel production (Papanikolaou and Aggelis, 2011a; Zhao et al., 2012).

Microorganisms capable of accumulating microbial oils can be found among different genera, like bacteria, yeasts and fungi (Leiva-Candia et al., 2014). Yeasts present some advantages over the other genera, such as high growth rates, high availability of candidates, cultivation that is not affected by climate conditions, ability to use low cost raw materials and more suitable morphology as well as being easier to handle in large-scale production (Ageitos et al., Zhao et al., 2012; Papanikolaou, 2011). Several yeast species have already been evaluated and have presented ability to accumulate high amounts of microbial oils, such as *Cryptococcus curvatus*, *Lipomyces starkeyi, Rhodotorula glutinis*, *Rhodosporidium toruloides*, *Yarrowia lipolytica, Trichosporon fermentans*, etc (Li et al., 2008; Ageitos et al., 2011; Papanikolaou, 2011). The main drawback of microbial oils is the high cost of the raw materials used for the cultivation. Koutinas et al. (2014) estimated that the production cost should be reduced by 50% in order to make microbial oils competitive with vegetable oils. The same authors calculated that the cost of glucose and yeast extract represent 79% and 16% respectively of the raw material costs during oil production from *R. toruloides* (Koutinas et al., 2014). In order to make microbial oil production economically feasible, low cost raw materials must be used, such as wastes (e.g. crude glycerol, used oils or fats, cheese whey etc) or

lignocellulosic biomass. Besides if the raw material used could provide the nitrogen necessary to support growth prior to lipid biosynthesis, the cost of lipid production would be further reduced. On the other hand nitrogen rich wastes should be avoided as they are not suitable for lipid biosynthesis, as lipid accumulation starts when nitrogen is depleted from the growth medium.

Synthesis of microbial oils (or lipids) starts when an essential element other than carbon is depleted from the culture broth; with nitrogen being the most common (Amaretti et al., 2010; Ratledge, 2004; Economou et al., 2010). More specifically, nitrogen limitation leads to the degradation of intra-cellular AMP (adenosine monophospahate) by the activity of AMP-desaminase yielding IMP (inosine monophosphate) and NH_4^+, which in turn results in deactivation of NAD(P)-dependent isocitrate dehydrogenase and citric acid accumulation. Citric acid accumulation inside the mitochondria above a critical value results in its transportation to the cytoplasm in exchange with malate and yields acetyl-CoA after cleavage by ATP-citrate lyase. Acetyl-CoA is then condensated to acyl-CoA which is the precursor of fatty acid biosynthesis through quasi-inverted β-oxidation reaction (Papanikolaou and Aggelis, 2011a; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2009). For non-oleaginous yeasts, after nitrogen depletion the growth stops and the accumulation of oils is less than 10% w/w while the excess of carbon is transformed into polysaccharides or remain unutilized (Ratledge, 2004; Ageitos et al., 2011; Amaretti et al., 2010).

During the cultivation of oleaginous microorganisms, it is important not only to know the concentration of nitrogen in the culture broth but also the ratio between carbon and nitrogen content, which is expressed by the C:N ratio (Carbon to Nitrogen ratio). There is a limit of C:N, characteristic for each microorganism, where accumulation of lipids takes place. Generally,

higher ratios facilitate the accumulation of lipids, but there is an upper limit where above this the growth of the microorganism can be inhibited. Other factors that affect the accumulation of single cell oil (SCO) are the form of nitrogen (organic or inorganic), the temperature, the pH, etc. At the end of the cultivation, cells should be separated from the broth followed by a lipid extraction step using organic solvents like methanol, chloroform, hexane etc.

In 2012 the Finnish oil company 'NESTE OIL' launched the first pilot plant in Europe capable of producing microbial oil from wastes and residues (such as straw) using yeasts (http://www.nesteoil.com). Few months later they declared that the first phase of the pilot plant was successfully completed and that they expected to achieve commercial-scale production by 2015 (http://www.nesteoil.com). Recently a partnership was announced between NESTE OIL and DONG Energy for the optimization of the pretreatment of these residues to enhance microbial oil production (http://www.nesteoil.com). This fact indicates the importance of further development of the processes for microbial oil production from renewable resources.

1.4 Biogas production

Biogas is the product of the anaerobic digestion of organic materials (Kafle et al., 2014; Liu et al., 2009). The two main components of biogas are carbon dioxide (CO_2) and methane (CH_4) and its total energy content is estimated between 16,630 and 26,081 kJ/m^3 depending on the ratio between these two components (Romano et al., 2009). Biogas is mainly used for electricity production after burning in CHP (Combined Heat and Power) systems and as a vehicle fuel (Jeihanipour et al., 2013). In order to use biogas as fuel the methane content should be increased by removing carbon dioxide and other

'contaminants' by upgrading processes such as water scrubbing, membranes etc, followed by liquefaction or compression of the gas (Swedish Gas Association, 2011).

As fuel, biogas can be used directly in vehicles with slight modifications such as installation of special fuel tank regulators to reduce the pressure and of a special fuel-air mixer (Clarke and DeBruyn, 2012). The benefits of using biogas as fuel are the negligible emissions of dust and particles, lower emissions of carbon monoxide, hydrocarbons, sulphur compounds and nitrogen oxides compared to fossil fuels and no release of GHG in the atmosphere (Swedish Gas Association, 2011). Despite the fact that biogas is more important to be used as vehicle fuel than for electricity production (due to the wide availability of other renewable solutions, such as wind and solar energy), relatively small volumes are directed to the transport section (Naik et al., 2010). Generally, biogas production is considered to have a better ratio between output and input of energy compared to ethanol, from a resource efficiency point of view (Jeihanipour et al., 2013) resulting in an output to input ratio as high as 28 (Zheng et al., 2014).

Biogas production in EU25 reached 4,898.9 ktoe during 2006 with Germany being the leading country (1,665.3 ktoe) with almost 4,000 biogas plants followed by UK (1,498.5 ktoe) and Italy (383.2 ktoe) (AEBIOM, 2009). According to the same report, until 2009 only Sweden (with a biogas production of 27.2 ktoe) had established a market for biomethane-driven cars and during 2008 17,000 vehicles were driving with biomethane (Figures 6 and 7). Biogas production in Greece was estimated to 29.8 ktoe of which the majority was produced as landfill gas (71.1 % of the total produced biogas).

Anaerobic digestion is a complex and multi-step biochemical process. Each step is catalyzed by a different microbial community, 'working' all together as

a unique system where the outputs of one step correspond to the inputs of the next step (Mshandete et al., 2005; Parawira, 2012). The main stages of this process include hydrolysis, acidogenesis, acetogenesis and methanogenesis. During the first step, complex compounds (such as lipids, polysaccharides and proteis) are hydrolyzed to more simple ones, which are further converted during the second step to volatile fatty acids (VFA) and alcohols by acidogen bacteria. Subsequently the VFAs which are longer than acetate are converted by acetogenic bacteria to acetate, CO_2 and H_2. During the last step these VFAs are converted to CH_4 and CO_2 by methanogens (Zhao et al., 2014; Adu-Gyamfi et al., 2012; Parawira, 2012). It is important to keep a balance between the different steps as the production of VFAs is a faster process than methanogenesis and their accumulation can result in pH decrease and methanogenesis inhibition (Griffin et al., 1998). The two first steps are catalyzed by bacteria, whereas methanogenesis is catalyzed by archaea. Two different operating temperatures of the processcan be distinguished; the mesophilic (25-35^{o}C) and the thermophilic (45-60 ^{o}C) temperatures (Liu et al., 2009). Thermophilic digestion presents some benefits, such as more favorable thermodynamically conditions leading to a higher methanogenic activity which leads to a faster digestion, and the prevention of contaminations (Xia et al., 2013; Lesteur et al.2010).

The main materials that are used for commercial biogas production are animal manures, municipal waste waters, food wastes, slaughterhouse wastes etc, where high yields have already been obtained resulting in the construction and operation of several plants. The need for further production of biogas resulted in the need to exploit alternative sources for anaerobic digestion such as lignocellulosic biomass. The bacteria present in the consortium have the ability to hydrolyze to some extent the cellulose and hemicellulose. On the other side, the addition of enzymes can be beneficial to

the process. The hydrolysis step is considered as the rate-limited step during exploitation of lignocellulosic biomass for biogas production (Parawira, 2012). Due to the recalcitrance of most lignocellulosic materials, materials undergo a pretreatment prior to the digestion process resulting in improvement of the biogas yields (Zheng et al., 2014).

Figure 6: Biogas filling unit in Grästorp, Sweden.
Source: http://biogasregionen.se/index.php?page=extensions

Anaerobic digestion not only facilitates to the production of biogas but also prevents from disposal of organic wastes in landfills. The latter result in uncontrolled gas emissions in the environment such as the methane, which is considered 20-23 times 'stronger' than carbon dioxide as a greenhouse gas (Zheng et al., 2014; Browne and Murphy, 2013). It also causes the production of leachates which contaminate underground waters. Besides, there is a huge concern about the shortage of landfill areas. Moreover, the digestate produced is rich in nitrogen, phosphorus, and potassium and presents peculiar rheology;

properties which make it an efficient biofertilizer (Kafle et al., 2014; Liu et al., 2009; Adu-Gyamfi et al., 2012).

Figure 7: Train moving with biogas in Sweden.
Source: http://commons.wikimedia.org/wiki/File:Biogast%C3%A5get_Amanda.jpg

1.5 Lignocellulosic biomass

Lignocellulosic biomass is considered one of the most ideal raw materials for biofuels production as it is an abundant low cost material (Xia et al., 2013). The total annual production reaches 100 billion tones organic matter of land biomass and 50 billion tons of aquatic biomass (Naik et al., 2010) with an estimated productivity in dry ash free biomass of 7.5 to 15 tons/ha/year (Datta et al., 2010). Lignocellulosic biomass can be derived from agricultural residues (such as wheat straw, corn cobs, rice straw), forest residues (hardwoods and softwoods), solids wastes from industry, paper wastes, etc (Tomás-Pejó et al., 2008; McKendry, 2002). Energy crops, such as sweet sorghum, *Miscanthus* and

switch grass are an interesting category of lignocellulosic biomass due to their high productivities resulting from the C4 pathway of CO_2 assimilation that they possess. As for CO_2 assimilation, there are two major pathways, either assimilating it with C3 compounds or with C4 compounds which is considered to be more productive and to have higher maximum efficiencies of light nitrogen and water than C3 assimilation (Xia et al. 2013).

Lignocellulose is a complex material of plants cell wall consisting of cellulose, hemicellulose, lignin extractives and several inorganic materials (Figure 8) (Taherzadeh and Karimi, 2008). The composition of lignocellulose in cellulose, hemicellulose and lignin depends on the source of the biomass. An average composition is considered to be 35-50% cellulose, 20-35% hemicellulose and 5-30% of lignin (Lynd et al., 2002).

Cellulose is a linear crystalline homopolymer of D-glucose units linked via β-1,4 glycosidic bonds presenting a degree of polymerization varying from 100 to 15,000 units depending on the origination of the substrate (Zheng et al., 2014; Taherzadeh and Karimi, 2008; Zhang and Lynd, 2004). Hydrogen bonds are formed by hydroxyl groups both in the same chain and between the cellulose chains and together with van der Waals forces are responsible for the stabilization of the cellulose chains and the high tensile strength. Cellulose chains form microfibrils which are packed into macrofibrils (Taherzadeh and Karimi, 2008). Depending on the degree of crystallinity there are two different cellulose regions, namely the amorphous (low crystallinity) and the crystalline (high crystallinity) regions (Sun et al., 2008). Crystalline regions are more difficult to be hydrolyzed compared to the amorphous.

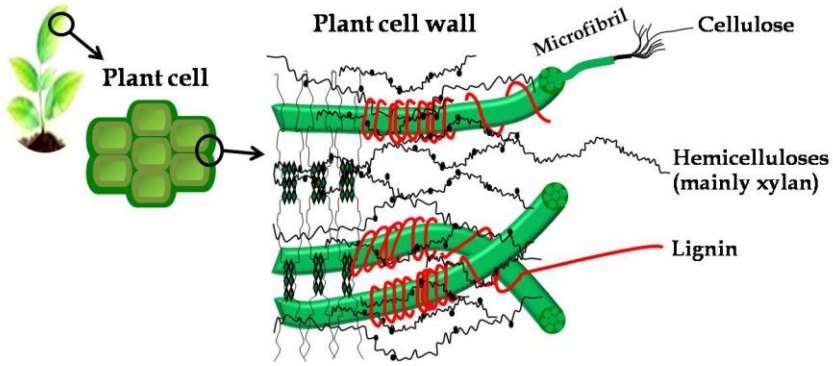

Figure 8: Typical structure of lignocellulose in cell plant wall.
Source: Ratanakhanokchai et al., 2013

Hemicellulose is an amorphous branched of heteropolymer consisting of pentoses (xylose and arabinose), hexoses (glucose, mannose, rhamnose and galactose) and uronic acids (glucuronic, methyl glucuronic and galacturonic), which is easier to be degraded than cellulose (Zheng et al., 2014). Their degree of polymerization is lower than that of cellulose and varies between 70 and 200 (Saha, 2003). Composition of hemicellulose in pentoses and hexoses as well as their structure depends on the source of biomass. Hemicellulose interacts with both lignin and cellulose to protect cellulose from enzymatic attack.

Lignin is a large and complex heteropolymeric poly-aromatic compound consisting mostly of phenypropane units such as sinapyl alcohol *p*-coumaryl alcohol, and coniferyl alcohol with hydroxyl, methoxyl and carbonyl functional units (Nigam and Singh, 2011; Stamatelou et al., 2012). It is also the most recalcitrant component of the cell wall (Zheng et al., 2014). Lignin is like the 'cement' surrounding cellulose and hemicellulose forming the three-dimensional structure of the cell wall, giving integrity, structural rigidity and preventing from swelling of lignocelluloses (Taherzadeh and Karimi, 2008;

Zheng et al., 2014). Up to now, applications of lignin are limited and it is mostly used to provide heat and electricity.

The structure of lignocellulose makes it resistant to degradation. More specifically, the main factors that affect the cellulose hydrolysis are the degree of cellulose crystallinity and polymerization, the accessible surface area, the presence of hemicellulose and lignin and the degree of acetylation of hemicellulose (Parawira, 2012; Kim and Holtzapple, 2006). In order to increase cellulose degradability, a pretreatment step needs to be incorporated in the process (Demirbas 2008). Indeed, an efficient pretreatment should lead to more easily hydrolysable cellulose, to a low degradation of the sugars present in cellulose and hemicellulose, to a low formation of inhibitors, and should reduce the operation cost as well as the energy consumption. It also implies a simple process configuration and a low environmental impact. There is a variety of pretreatment methods in the literature classified as physical, physicochemical, chemical and biological. Some examples of pretreatment techniques involve steam explosion, hydrothermal, dilute and concentrated acid hydrolysis, alkaline hydrolysis, supercritical CO_2, ozonolysis, organosolv, etc. Simple techniques such as chipping, grinding and milling can also reduce the cellulose crystallinity (Nigam and Singh, 2011).

Pretreatment efficiency strongly depends on the lignocellulosic material. For this reason there is no universal solution for all types of biomass. Hydrothermal pretreatment and steam explosion are two of the most effective pretreatment processes and have been widely evaluated in the literature. The main difference between these two is that in steam explosion the pressure is suddenly released to atmospheric at the end of the treatment and the material undergoes an explosive decompression. A catalyst such as SO_4 or a mild organic acid can also be included in the pretreatment, which results in

improvement of the subsequent enzymatic hydrolysis as it is more effective in hemicellulose removal (Ben-Ghedalia et al., 1981). On the other side, use of concentrated acids tends to be withdrawn due to the increasing of inhibitors formation, corrosive problems in the equipment, toxicity and environmental aspects.

Because of the strong interaction between cellulose and hemicellulose, in order to achieve efficient cellulose hydrolysis not only cellulolytic but also hemicellulolytic enzymes are required (Ratanakhanokchai et al., 2013). For this reason, a mixture of cellulose acting enzymes (endoglucanase, exoglucanase, β-glucosidase) and hemicellulose acting enzymes (such as xylanase, β-xylosidase, glucuronidase, acetylesterase, galactomannanase and glucomannanase) are employed (Nigam and Singh, 2011). Source of these enzymes can be either bacteria (e.g. *Clostridium, Cellulomonas, Acetovibrio*) or fungi (e.g. *Trichoderma, Mycelliophtora, Fusarium, Neurospora*) (Singh et al., 1992; Menon and Rao, 2012; Lynd et al., 2002). The most commonly used microorganism for the production of commercial enzyme solutions is *Trichoderma reesei* (Menon and Rao, 2012). For example, Novozymes and Genencor are two big companies which have recourse to *T. reesei*.

During hydrolysis of cellulose, endoglucanases (or endo-1,4-glucanase, EG, EC 3.2.1.4) act internally within cellulose chain on amorphous regions opening-up reducing and non-reducing ends where exoglucanase (or cellobiohydrolase, CBH, EC 3.2.1.91) starts hydrolyzing either from the reducing or non-reducing end releasing cellobiose units (Hayes, 2009). Moreover, CBH can also hydrolyze crystalline cellulose into cellobiose (Igarashi et al., 2009) and consist the major component of the fungal cellulose system reaching 40-70% of the total cellulose proteins (Sánchez, 2009). Finally β-glucosidase (BG, EC 3.2.1.21) hydrolyzes soluble cellodextrins and

cellobiose to form glucose (Lynd et al., 2002). A general overview of the enzyme action on cellulose is shown in Figure 9.

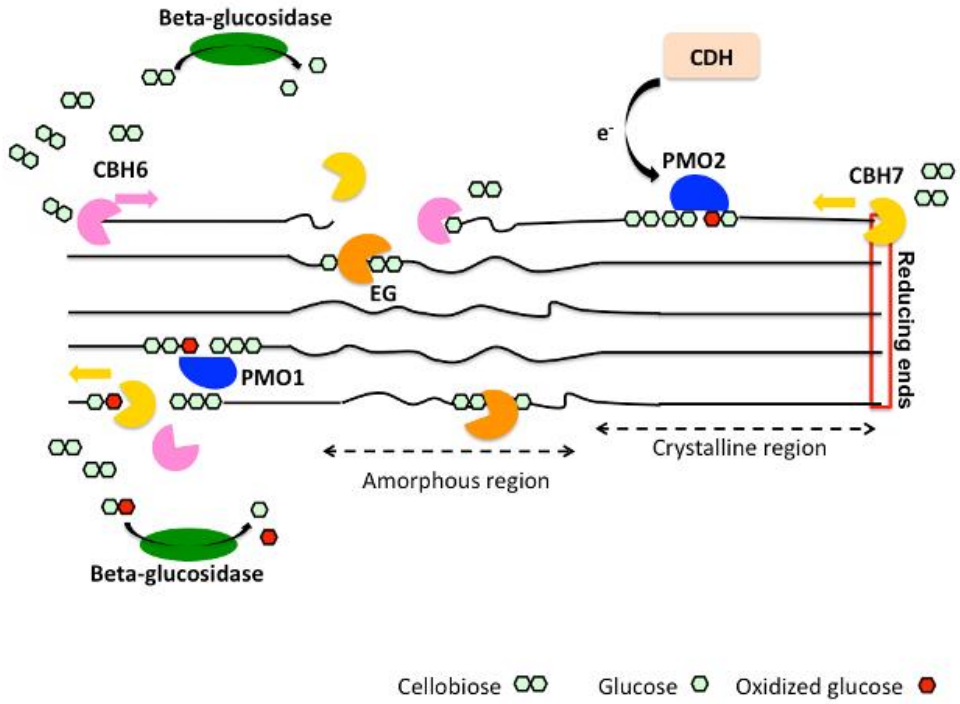

Figure 9: Synergistic action of different cellulolytic activities during cellulose degradation.
Source: Dimarogona et al., 2012

Cellulase activity presents end-product inhibition by both cellobiose and glucose, with the inhibition effect of cellobiose being much higher than that of glucose (Adrić et al., 2010). For this reason efficient β-glucosidase activity in the mixture is important to keep inhibition effects as low as possible. During the last years, a better understanding of some novel enzymatic activities enabled to find out that they could act on crystalline regions of cellulose. These enzymatic activities are the lytic polysaccharide

monoxygenases (LPMOs) and cellobiose dehydrogenases (CDHs). LPMOs act by oxidizing glucose units which makes cellulose more easily degradable whereas CDHs catalyze the reducing end of cellobiose, cellodextrins and other oligosaccharides to the corresponding lactones and act synergistically with LPMOs in cellulose hydrolysis (Dimarogona et al., 2012). The ability of these enzymes to act on the most recalcitrant part of cellulose is considered very important as it results in improved hydrolysis rates (Dimarogona et al., 2012; Dimarogona et al., 2013).

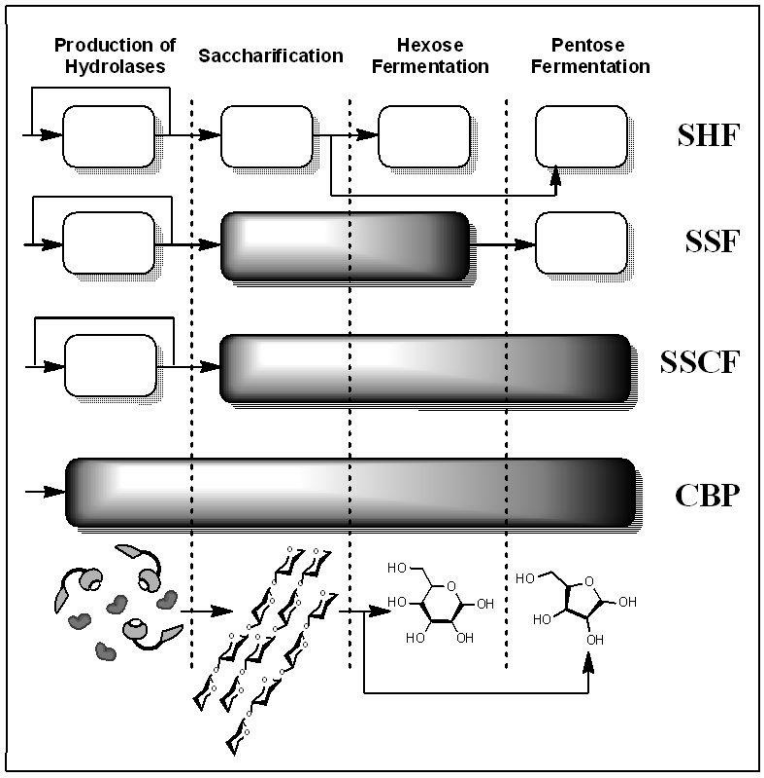

Figure 10: Different configurations of lignocellulose utilization as raw material for bioprocesses.
Source: Philbrook et al., 2013.

When it comes to the utilization of lignocellulose for biofuels or bio-based chemicals production, the main stages include pretreatment, production of enzymes, cellulose and/or hemicellulose hydrolysis, microbial conversion and product recovery. There are different process configurations which have been widely studied for ethanol production (Figure 10), namely SHF (Separate Hydrolysis and Fermentation), SSF (Simultaneous Saccharification and Fermentation) and CBP (Consolidated Bio-Process) (Menon and Rao, 2012). In all of them, pretreatment is taking place prior to enzymatic saccharification. A step may also be included where the enzymes are produced in order to reduce the cost of pursuing them. Product recovery follows these configurations and the recovery procedure depends on the product.

In SHF process enzymes are acting on cellulose and/or hemicellulose for a specific amount of time in a first stage followed by a second stage during which microbial conversion takes place. The main benefit of this configuration is the application of optimal temperature conditions of both enzymatic saccharification and microbial conversion, resulting in more efficient conversion at shorter duration. The two different carbohydrate fractions (six and five carbon sugars) could be either utilized together (if the microorganism used is capable of consuming pentoses) or in two different steps employing different microorganisms. The main drawback of SHF concerns the possible glucose inhibition on enzymes during the first step. Use of novel enzyme solutions with 'high tolerance' in glucose inhibition could overcome this problem (Cannella and Jørgensen, 2013).

In SSF configuration enzymatic hydrolysis and microbial conversation take place at the same stage where the temperature is as close as possible to the optimum for both enzymes and the microorganism. Due to the low tolerance of microorganisms to higher temperature, SSF usually works at lower

temperature than the optimum one for the enzymes. This results in lower saccharification rates but on the other hand the produced glucose is immediately consumed by the microorganism keeping its concentration low and in turn preventing from inhibition of cellulases. If the fermenting microorganism has the ability to utilize the pentoses fraction then SSF process is often characterised as SSCF (Simultaneous Saccharification and Co-Fermentation).

Finally, in CBP configuration only microorganisms which are capable of producing the hydrolytic enzymes and at the same time consuming the sugars and producing the desired product are used. During ethanol production, examples of microorganisms that are capable of both producing the enzymes and convert sugars to ethanol are *Fusarium oxysporoum, Neurospora crassa* and *Monilia* sp. (Taherzadeh and Karimi, 2007; Xiros and Christakopoulos, 2009). However ethanol production productivities of these microorganisms are low for commercial applications.

1.6 Sweet sorghum

Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a very promising C4 energy crop presenting high photosynthetic activity and that can grow in temperate climates (Matsakas and Christakopoulos, 2013). It contains equal amounts of soluble (glucose, fructose and sucrose) and insoluble (cellulose and hemicellulose) carbohydrates (Mamma et al., 1995). Sugar concentrations up to 15-22°Bx in sweet sorghum juice have been reported (Kundiyana et al., 2010). Compared to other sugar crops (such as sugar beet and sugar cane) cultivation of sweet sorghum for sugar production is more beneficial. Because sugar beet is a root, its harvesting requires more energy and sugarcane cultivation is limited to only tropical regions (Whitfield et al., 2012).

Some characteristics of sweet sorghum such as shorter cultivation time (3-5 months), higher tolerance to harsh environmental conditions (such as drought and cold) and lower requirements in fertilization and irrigation than other crop plants, make it as an ideal solution for the exploitation of marginal lands (Gnansounou et al., 2005; Wu et al., 2010). More specifically, the requirements for irrigation of sweet sorghum are 1/3 and 1/2 of the needs of sugarcane and corn respectively (Wu et al., 2010). The fast growth rates enable to incorporate sweet sorghum cultivation into rotation with other crops or even to achievement of continuous production during the year in areas with appropriate climate (Whitfield et al., 2012). Furthermore, Han et al. (2011) found that the leaves of sweet sorghum contain high concentrations of nitrogen, phosphorus and potassium which could be used in the field in order to reduce the addition of fertilizers for the cultivation of other crops.

Sweet sorghum is cultivated in 99 countries around the world occupying around 44 million ha of mostly poor and semi-arid areas (Sakellariou-Makrantonaki et al., 2007). Due to its remarkable resistance to drought and saline solids together with the tolerance to waterlogging, FAO named it as the 'camel among crops' and concluded that cultivation of sweet sorghum could be the solution for the agricultural development in areas that have saline soils and that are affected by aridity (FAO, 2002). Finally, another advantage of sweet sorghum cultivation concerns the production of grains which can reach 5 to 25% of the total dry weight in maturity and could be used as animal feed or even incorporated in the production of biofuels (Whitfield et al., 2012).

Despite the excellent characteristics of the ethanol produced from sweet sorghum, its commercialization presents some serious technical challenges. The presence of soluble sugars together with the rich composition of the stalks and the high water content can lead to an easy contamination of the stalks by

microorganisms which would decrease dramatically their storage stability. The high water content also causes problems during transportation as the volume of stalks is high and also cooling units are required. Moreover, the seasonal characteristics of sorghum cultivation together combined with the low storage stability result in problems concerning the availability of stalks all over the year. It also impacts on the capital cost of the processing unit. In fact, high amounts of sweet sorghum are produced in a short period of time which requires a fast utilization of sweet sorghum leading to big facilities which will only operate seasonally. Bennett and Anex (2009) mentioned that if sweet sorghum could be stored for at least 6–8 months, the capital cost of the processing units would be rapidly reduced, as the same amount of sorghum would be processed by smaller units.

Two main strategies are considered when it comes to sweet sorghum utilization. In the first case, sweet sorghum stalks are used as it is resulting in the need of performing solid-state fermentation. Compared to submerged fermentation, solid-state fermentation is more difficult to achieve and makes the product recovery more complex. Moreover, in order to achieve higher sugar extractability (which is important in order to achieve high levels of biofuels production) sweet sorghum should be milled in small particles which are beyond the capacity of the harvesting equipment (Whitfield et al., 2012). The second strategy of sweet sorghum usage consists in first removing the juice and separate use of the juice and the remaining lignocellulosic fraction (the so called bagasse). Juice recovery is mostly done by pressing the stalks through a roller mill, which results often in less than 50% recovery (Whitfield et al., 2012; Prasad et al., 2006; Sun et al., 2010) and the ease of juice contamination requires the application of different preservation processes. Wu et al. (2010) found that that as much as 20% of the soluble sugars can be lost in 3 days if the juice is stored at room temperature. These technical challenges

makes important to either improve the current techniques in order to preserve sweet sorghum or explore alternative ones.

Until now the main research interest has been focused on the production of ethanol from sweet sorghum, by using either juice, bagasse or the whole stalks (Kundiyana et al., 2010; Mamma et al., 1995; Prasad et al., 2006; Wu et al., 2010; Shen et al., 2012). Apart from ethanol, sweet sorghum has also been used for lipid production by fungi (Economou et al., 2010), yeasts (Liang et al., 2012) and heterotrophic algae (Gao et al., 2010; Liang et al., 2010). Other products that have been produced from sweet sorghum are butanol and acetone (Yifeng et al., 2008), lactic acid (Yadav et al., 2011), Hydrogen (Ntaikou et al., 2010) and methane (Antonopoulou et al., 2010).

AIM OF THIS WORK

Aim of this work was to obtain a flexible process of sweet sorghum utilization which could allow the production of several biofuels or bio-based chemicals. During this work the production of several forms of biofuels was evaluated. This flexible process is able to switch between the production of liquid biofuels (ethanol and biodiesel) and gaseous (methane) depending on the needs. Moreover, the same process configuration could be applied in the future for the production of other bio-based chemicals. This process also provides the ability of switching from more traditional practices (juice separation) to an alternative one, where the stalks are used as they are (Figure 11). This way, when the production of juice is desired, production of biofuels could occur from the lignocellulosic fraction. Some of the major technical challenges that hinder commercialization of the use of sweet sorghum, such as low storage stability and efficient hydrolysis in the presence of soluble sugars, were solved. The application of high solids content was pre-requisite for this work, as it is considered very important for the commercialization of the process.

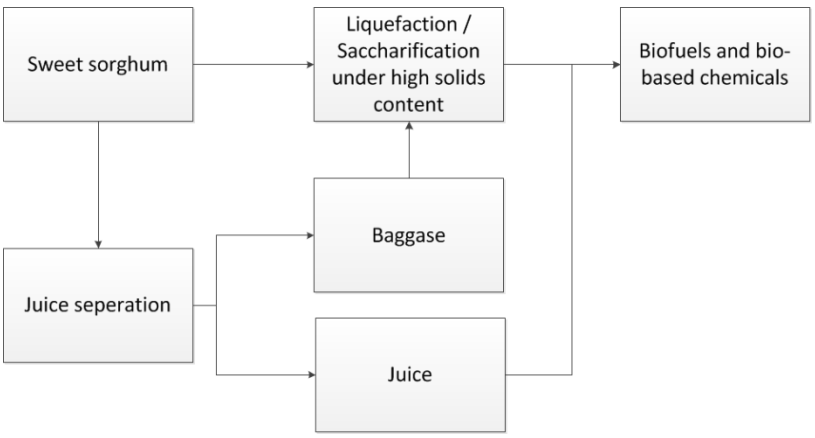

Figure 11: The flexible process proposed in this work for the use of sweet sorghum

2. MATERIALS & METHODS

2.1 Raw material, microorganisms and enzyme solutions

The sweet sorghum used during this work was of Keller variety and was kindly offered by Prof. George Skarakis, Department of Crop Science, Agricultural University of Athens. The leaves and the seeds were immediately removed by hand from the fresh stalks and the latter were stored in a freezer at -20°C in order to preserve them until usage. The composition of sweet sorghum in dry basis was (% w/w): Sucrose, 34.4; glucose, 8.2; fructose, 8.1; cellulose, 19.6; hemicellulose, 15.2; and insoluble lignin, 3.2. The concentration of volatile solid (VS) and total solids (TS) were 93.44% w/w and 95.69% w/w respectively.

The microorganisms involved in this work for the ethanol production were dry baker's yeast (Jotis, Athens, Greece) for the fermentation of bagasse and *Saccharomyces cerevisiae* MAK2 for the fermentation of dried stalks. The latter was kindly provided by Prof. Seraphim Papanikolaou, Department of Food Science & Technology, Agricultural University of Athens. For the experiments of single cell oil (or lipid) production three different yeasts were evaluated, namely *Lipomyces starkeyi* CBS 1807 and *Trichosporon* (or *Geotrichum*) *fermentans* CBS 439.83 which were obtained from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) and *Rhodosporidium toruloides* CCT 0783 which was pursued from Coleção de Culturas Tropicais (Campinas, Brazil). The anaerobic sludge used as an inoculum during the biogas production experiments was collected from the biogas plant in Boden, Sweden (Figure 12), where thermophilic co-digestion of sewage sludge and

food wastes at 55°C is taking place. The VS and TS content of the inoculum were found to be1.17% w/w and 2.04% w/w, respectively.

The enzyme solutions used during this work were obtained from Novozymes A/S (Bagsværd, Denmark). More specifically, either the enzyme solution Cellic® CTec2 or a mixture of Celluclast® 1.5L and Novozym® 188 at a ratio of 5:1 volumes were employed. The cellulose activity expressed as filter paper activity for Cellic® CTec2 was found to be 127 FPU/mL, whereas that of the mixture was 83 FPU/mL.

Figure 12: The biogas plant in Boden, Sweden, source of the thermophilic sludge.

2.2 Ethanol production from bagasse

2.2.1 Bagasse preparation

The sweet sorghum bagasse was prepared by removing the soluble sugars present in the stalks with a double aqueous extraction in a 30% w/v solution for 1h at 50°C. Subsequently, the solids were filtrated and washed thoroughly with distilled water in order to remove remaining sugars. Bagasse was finally dried at 80°C until constant weight. The dried sweet sorghum bagasse was milled in 0.50 mm particles using a laboratory mill. The cellulose and hemicellulose concentration were found to be 39.8% w/w and 34.5% w/w respectively.

2.2.2 Pretreatment of bagasse

During this work hydrothermal pretreatment was evaluated in order to make sweet sorghum bagasse more digestible from the enzymes. Hydrothermal pretreatment took place in a microwave digestion equipment (speedwave™ MWS-2, Berghof Instruments GmBH, Germany) (Figure 13). The sample was placed in special designed digestion vessels which are completely made of isostatically-pressed PTFE/TFM (Figure 14). Vessels were shielded with a small plastic cap made of the same material and a metal rupture and finally a bigger plastic cap placed on the top of the vessels keeping the shields in place (Figure 14). All the vessels were connected through a central vessel to the exhaust system. If high pressures were generated inside the digestion vessel, the plastic and metal shields were disrupted and the pressure was released through the exhaust outside of the room to protect the equipment and the operators. In order to be able to remotely determine the temperature of the vessels the system was equipped with an IR sensor. The power of the microwave was set up at 700 W during pretreatment.

Figure 13: The microwave digestion equipment

Figure 14: The digestion vessels with the pressure protection caps.

Pretreatment of bagasse took place at 10% w/v loading in 0.3% v/v acetic acid solution. Prior to hydrothermal pretreatment bagasse soaked at the solution of 0.3% acetic acid for 1 h at 80°C to fully hydrate bagasse fibers. In order to optimize the hydrothermal pretreatment of bagasse response surface methodology (RSM) was used. More specifically, the combined effect of treatment temperature and duration was evaluated. The temperature varied between 170 and 230°C and the duration between 9 and 51 minutes, resulting in 11 treatment combinations (see Results & Discussion). As a result (response) of the pretreatment, the efficiency of enzymatic hydrolysis after 8 h of incubation was taken into consideration. The results obtained were fitted to the equation described in the 'Response surface methodology' section (2.7) to describe the combined effect of temperature and duration on enzymatic hydrolysis efficiency and to optimize this combination.

At the end of the pretreatment the liquid fraction was separated from the solids by vacuum filtration. The solid residues were dried at 80°C overnight for structural carbohydrate analysis and saccharification experiments. All the pretreatments were performed in duplicates.

In each pretreatment the severity factor (SF) was determined according to Garrote et al. (1999) by using the following equation:

$$SF = \log(R) = \log\cdot\left[t \cdot e^{\frac{T-100}{14.75}} \right]$$

in which t and T are the pretreatment duration (min) and temperature (°C), respectively.

2.2.3 Enzymatic saccharification of pretreated bagasse

Enzymatic saccharification of pretreated bagasse was performed at either low solids concentration, in order to evaluate and to optimize the pretreatment, or at high solids concentration in order to use it as a substrate for ethanol production.

During the low solids concentration experiments, enzymatic saccharification was carried out in 2mL epperdorf tubes in an epperdorf thermomixer at 50°C for 8 hours with a mixing rate of 700 rounds per minute (rpm). The solids loading was adjusted to 3% w/v in 100mM citrate-phosphate buffer at pH=5.0 and Cellic® CTec2 was used at an enzyme load of 10FPU/g solids. Microbial contaminations were prevented by the addition of 0.02% (w/v) sodium azide. At different time intervals, samples were taken and the glucose concentration was determined in order to calculate the percentage of cellulose hydrolyzed by applying the following equation (Vasquez et al., 2006):

$$Eh = \frac{C_s}{F \cdot \left(CPn_o / WSR \right) \cdot \rho}$$

in which *Eh* represents the % hydrolysis of the initial cellulose presented in the raw material, C_s is the concentration of glucose released during the hydrolysis (g/L), *F* is the stoichiometric factor due to the hydration of molecules during the hydrolysis ($F_{hexoses}$= 1.111), CPn_o is the composition of the raw material in cellulose (g of cellulose per g of raw material, on dry basis), *WSR* is the water to solid ratio (g water/g of raw material) and ρ is the density of hydrolysates which is equal to 1025g/L.

Once the hydrothermal pretreatment was optimized according to the highest cellulose hydrolysis efficiency and sugar concentration, the optimum conditions were used to prepare a hydrolysate at high solids concentration which was used for ethanol production. A high concentration of solids was chosen in order to achieve a high concentration of glucose and in turn a high ethanol production rate. During this work the solid concentration was adjusted to 18% w/v and saccharification took place in a liquefaction/saccharification reactor which was designed and manufactured by Biotechnology Lab of Chemical Engineering School of National Technical University of Athens (Figure 15). This reactor consists of two vertical drum chambers and a rotating shaft for mixing the material. The shafts were controlled at a speed of 7rpm and the direction of the rotation programmed to shift between clock and anti-clock wise every minute. The drums were surrounded by an oil-filled heating jacket which enabled to control the temperature at desired levels. The basic idea of this design was that the material will be mixed and falling down by gravity, resulting in a better contact of the enzymes and the cellulose of the high solids mash. This better contact led to higher saccharification efficiency at shorter time duration. The temperature was adjusted to 50°C and the enzyme load (Cellic® CTec2) was set to 10FPU/g solids in 50mM citrate-phosphate buffer at pH=5.0 without

the addition of sodium azide. Saccharification took place for either 12 or 24 hours and the saccharified material was immediately used for the fermentation experiments after being cooled down to room temperature. At the end of the saccharification, the concentration of glucose and total reducing sugars in the slurries were determined.

Figure 15: The saccharification/liquefaction reactor of the Biotechnology Lab.

2.2.4 Fermentation of saccharified bagasse

Fermentation experiments were run in 100mL Erlenmeyer flasks containing 25g of non-sterilized slurry. The incubation process was performed anaerobically in an orbital shaker at 30°C with an agitation rate of 80rpm. Fermentation was carried out by adding dry baker's yeast at a concentration corresponding to 15mg yeast per gram of solids without any addition of other nutrients. At certain time intervals samples were removed from the cultures and centrifuged in order to remove the solids. Ethanol was then analyzed in the clear supernatant. All the trials were performed in duplicate.

2.3 Ethanol production from dried sweet sorghum stalks

2.3.1 Enzymatic liquefaction of sweet sorghum

Enzymatic liquefaction of dried sweet sorghum stalks were conducted in 100mL Erlenmeyer flasks containing 25g of different concentrations of dried sweet sorghum (as will be discussed later). The flasks were Incubated took place at 50°C and 180rpm in an orbital shaker. Sweet sorghum solutions were prepared in distilled water and the enzyme solution used was the mixture of Celluclast® 1.5L and Novozym® 188. Novozym® 188 was added at the start-up of inoculation and not during the liquefaction because it contained invertase activity (0.83U/mg) which would result in sucrose hydrolysis, thus increasing glucose concentration leading to cellulase inhibition. The enzyme load varied according to the experimental configuration (as will be discussed in Results & Discussion, section 3.2). All the trials were performed in duplicate and were cooled down to room temperature after the liquefaction. Finally, they were immediately used for the fermentation trials.

2.3.2 Media and growth conditions of the yeast

During this work the yeast strain *S. cerevisiae* MAK2 was used as a fermenting microorganism. Fermentations were performed in 100 mL Erlenmeyer flasks containing 25g of the non-sterilized liquefied sweet sorghum. Incubation took place anaerobically in an orbital shaker at 30°C and 80 rpm. The inoculum was 10% v/v of exponential growing pre-culture which was carried out in 250 mL Erlenmeyer flasks, containing 100 mL of pre-culture growth medium of the following composition (g/L): $(NH_4)_2HPO_4$, 5; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 5; sucrose, 10 (Mamma et al., 1995). Pre-culture media were inoculated with one loop of the yeast and incubated for 18h (in order the yeast growth to be at the end of

the exponential phase- Figure 16) at 30°C and 180 rpm before used. Prior to inoculation pre-culture media were autoclaved at 120°C for 20 minutes.

During fermentation experiments, samples were taken at certain time intervals, centrifuged in order to remove the solids and analyzed for ethanol and sugars. Each experiment was done in duplicate.

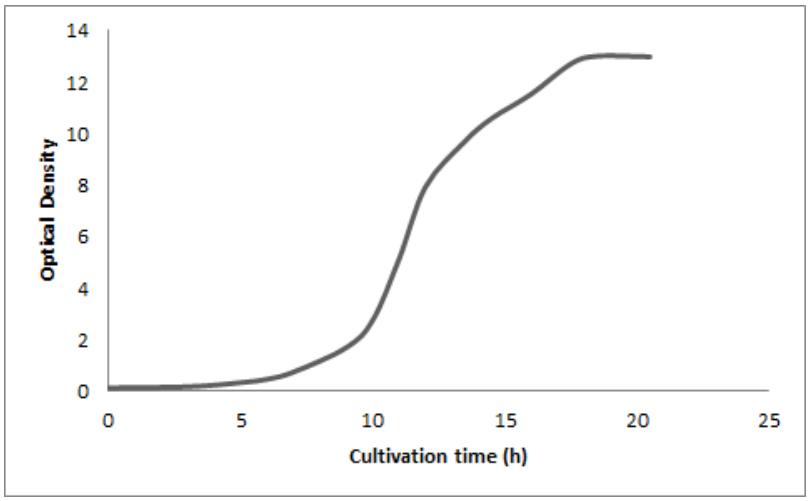

Figure 16: Growth curve of *S. cerevisiae* MAK2 in the pre-culture medium.

2.4 Lipids production from sweet sorghum stalks

2.4.1 Maintenance and pre-culture media for the growth of *Lipomyces starkeyi* and *Trichosporon fermentans*

The yeast was maintained at 4°C in slants of the following composition (g/L): Glucose, 20; Meat Peptone, 10; Yeast extract, 10; KH_2PO_4, 6; Na_2HPO_4, 2; agar, 20. Prior to fermentation, the yeast was cultivated in a pre-culture medium with the same composition without adding agar. The pH of the medium was adjusted to 6 for *L. starkeyi* and to 6.5 for *T. fermentans*.

Besides, prior to the inoculation, the pre-culture medium was sterilized at 121°C for 15min. Pre-cultures were inoculated with one loop of the yeast and incubated in an orbital shaker at 30°C and 200rpm for 48 and 24 hours for *L.starkeyi* and *T. fermentans* respectively. Media for lipid production trials were inoculated with 5% v/v of the pre-culture broth.

2.4.2 Lipids production from *Lipomyces starkeyi* and *Trichosporon fermentans* when cultivated on synthetic media

The yeasts were cultivated in 1L Erlenmeyer flasks containing 200mL cultivation broth of the following composition (g/L): KH_2PO_4, 7; Na_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2$, 0.15; $FeCl_3 \cdot 6H_2O$, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.06 (Papanikolaou and Aggelis, 2002). The pH of the broth was adjusted in the same way as the broth for the two yeasts. The initial sugar concentration in all the experiments was set to 40 g/L, where as carbon source glucose, fructose and sucrose were used separately or as a mixture. During the experiments where the effect of the different sugars was determined, a mixture of yeast extract and ammonium sulphate was used as nitrogen source at a concentration corresponding to a C:N ratio equal to 100. When the effect of the nitrogen source or the C:N ratio was evaluated, the mixture of the three sugars was used and the concentration of the nitrogen source was adjusted in order to achieved the desired C:N ratio.

2.4.3 Lipid production from *Lipomyces starkeyi* and *Trichosporon fermentans* when cultivated on liquefied sweet sorghum stalks

Enzymatic saccharification of sweet sorghum was performed at 50°C, at the combination of enzyme load and hydrolysis duration which was found to be optimum during ethanol production (Section 3.2). The same enzyme solutions were used and Novozym® 188 was added at the start-up of the

cultivation process. The media with sweet sorghum were sterilized (121°C, 15 min) prior to enzymatic saccharification. After the end of saccharification, the broths were left to cool down to room temperature and inoculated.

During the trials where solids were removed, sweet sorghum was squeezed through a coating sheet at the end of the saccharification and was centrifuged thereafter. The obtained liquids were then sterilized and utilized for the yeast cultivation. In order to evaluate the effect of enzymatic saccharification on lipids production, the same experiments (with and without the presence of solids) were performed without the addition of enzymes. When the solids were removed, sweet sorghum was pre-soaked at 50°C for 2 hours in order to facilitate the sugars extraction.

To prepare the sweet sorghum solutions, the stalks were dissolved in the same mineral solution as in the synthetic medium without adding either carbon or nitrogen source (unless otherwise stated). The incubation of the yeasts was done in an orbital shaker at 30°C and 200rpm. At different time intervals samples were taken and analyzed for the concentration of total sugars, biomass and lipids. All experiments were done in duplicates.

2.4.4 Maintenance and pre-culture media for the growth of *Rhodosporidium toruloides*

R. toruloides was maintained in agar plates containing a medium of the following composition (g/L): glucose, 20; meat peptone, 10; yeast extract, 10; KH_2PO_4, 6; Na_2HPO_4, 2; agar, 20. Prior to any experiment one loop of the yeast was inoculated into 250mL Erlenmeyer flasks containing 50mL of the pre-culture medium (with the same composition as in agar plates without including agar) and incubated at 30°C and 200rpm for 24h. The pH was adjusted to 5.5 and all the media were sterilized prior to inoculation at 121°C

for 15 min. Media for the trials of lipids production were inoculated with 5% v/v of the pre-culture broth.

2.4.5 Lipid production from *Rhodosporidium toruloides* when cultivated on liquefied sweet sorghum stalks

Saccharification of sweet sorghum stalks was performed at the same conditions as described in section 2.4.3 using a different nutrient solution. The cultivation broth, used for growth and lipids accumulation of *R. toruloides*, was supplemented with 1.5g/L of $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 each and 1% v/v of the trace element solution. The composition of the trace element solution was (g/L): $CaCl_2 \cdot 2H_2O$, 4.0; $FeSO_4 \cdot 7H_2O$, 0.55; citric acid$\cdot H_2O$, 0.52; $ZnSO_4 \cdot 7H_2O$, 0.10; $MnSO_4 \cdot H_2O$, 0.076; and 100μL/L of 18M H_2SO_4 (Wu et al., 2010). Incubation and sampling were also done as previously described in Section 2.4.3. All experiments were done in duplicates.

2.5 Biogas production from sweet sorghum stalks

2.5.1 Thermal and enzymatic treatment of sweet sorghum

During this work, the effect of three different treatments of dried sweet sorghum stalks on methane yield was studied including a mild thermal treatment, an enzymatic one and a combination of both. The thermal treatment was performed at 105°C for 1 h in an autoclave apparatus at a solid concentration of 20% w/w.

The enzymatic treatment, in both cases (combined and not combined with the thermal treatment) was performed at the same optimal conditions as for ethanol production and using the same enzyme solutions. Two different process configurations were applied during the enzymatic treatment, namely

the one-step and the two-steps process. In the one-step process, which is similar to the SSF process for the ethanol production, the enzymes were directly added in the sludge. However, for the two-step process, which is similar to SHF process, sweet sorghum was enzymatically pre-saccharified prior to the addition to sludge. During the two-step process, the saccharification was performed at 50°C at the optimal conditions for ethanol production and at 20% w/w solids content.

2.5.2 Biochemical Methane Potential (BMP) test

BMP tests were performed at the Automatic Methane Potential Test System (AMPTS II) equipment which was pursued from Bioprocess Control AB (Lund, Sweden) (Figure 17). Incubation took place in 500 mL glass bottles containing 400 g of total sample (inoculum and substrate). Flasks were sealed properly in order to prevent gas losses and the material was mixed gently. Mixing consisted in series of 10min mixing followed by 1min resting. Each bottle was connected with a CO_2-fixing unit (solubilizing all the produced gases and water vapors, except from methane), which consisted of 100mL glass flasks containing approximately 80mL of 3M NaOH and thymolphthalein as pH indicator. Finally, the volume of methane was measured at the gas flow meter unit. It is worth to mention that the room pressure and temperature were recorded every time the flow meter cells were opened in order to correlate the measured volumes to the normalized ones.

In every experimental batch, two different controls were also included. The first one contained only the inoculum in order to measure the methane production by the organic load still present in the sludge. This methane production was then removed from the methane produced during digestion of the sweet sorghum and calculate the yield of methane per gram of VS. The

second control contained the inoculum and the enzymes used. This way the methane production from the digestion of the enzymes was determined and subsequently removed this from the flasks that enzymes were also included. Additionally, a positive control was also included to evaluate the quality of the sludge containing avicel cellulose, where the methane yields are known from the literature.

Two different experimental batches were performed during this work. In the first one, the effect of the different treatments on methane yield was evaluated. The optimal treatment found was further optimized in the second batch by applying RSM (see Results & Discussion, section 3.7.2). In the first batch of experiments the Inoculum to Substrate ratio (I/S ratio) in terms of VS was equal to 2, whereas in the second batch it varied according to the experimental design (see Results & Discussion, section 3.7.2). Each flask was supplemented with 10mL/ L each of three solutions containing mineral and trace elements. Solution A composed of 7.21g/L of $(NH_4)_2HPO_4$ and solution B of 0.7g/L of $FeSO_4.7H_2O$. The composition of solution C was as follow (g/L): $CaCl_2 \cdot 2H_2O$, 22.5; NH_4CL, 35.9; $MgCl_2 \cdot 6H_2O$, 16.2; KCl, 117; $MnCl_2 \cdot 4H_2O$, 1.8; $CoCl_2 \cdot 6H_2O$, 2.7; H_3BO_3, 0.51; $CuCl_2 \cdot 6H_2O$, 0.24; $Na_2MoO_4 \cdot 2H_2O$, 0.23; $ZnCl_2$, 0.19; $NiCl_2 \cdot 6H_2O$, 0.2; H_2WO_4, 0.01 (Antonopoulou and Lyberatos, 2013; Vlassis et al., 2012). In order to remove oxygen from the sludge before the start-up of the digestion each flask was sparged with nitrogen for 90 sec. Incubation of the flasks took place in a water bath at 55°C and was ended when no significant amounts of methane production were observed. All the experiments lasted for a maximum of 21 days.

Figure 17: The AMPTS II system of Bioprocess Control AB.

2.6 Analytical techniques

2.6.1 Sugar analysis

Total reducing sugars were determined according to dinitro-3,5-salicilic acid (DNS) method (Miller, 1959). In order to enable sucrose determination with the DNS method samples were treated with hydrochloric acid for 15 min at 70°C to enable sucrose hydrolysis and were subsequently neutralized with sodium hydroxide. Glucose was determined photometrically in a microplate apparatus by using the commercial enzyme kit of GOD/PAP (glucose oxidase / peroxidase).

2.6.2HPLC analysis

HPLC was used in order to determine ethanol and in some cases sugars (during structural carbohydrate analysis or during ethanol fermentation). The HPLC apparatus (Shimadzu LC-20AD) was equipped with a refractive index

detector (Shimadzu RID 10A). For ethanol analysis, an Aminex HPX-87H (Bio-Rad, 300 x 7.8mm, particle size 9μm) chromatography column was employed. The mobile phase used was 5mM H_2SO_4 in degassed HPLC-water at a flow rate of 0.6mL/min and the column temperature was set at 40°C. Analysis took place for 30 min. As for sugar analysis, an Aminex HPX-87P (Bio-Rad, 300 x 7.8mm, particle size 9μm) chromatography column was utilized. The mobile phase used was degassed HPLC-water at a flow rate of 0.6 mL/min and the column was operating at 70°C. Analysis took place for 35 min.

Prior to any analysis with HPLC, samples were filtrated through syringe filters with pore size of either 0.4 or 0.2μm in order to remove any remaining solids.

2.6.3 Biomass and lipid determination

During the experiments of lipid production, yeast biomass was determined in order to calculate the % w/w lipid content of cells. When solids were present, the biomass concentration was determined by plating samples on agar plates and incubating them at 30°C. The number of viable cells was expressed as colony forming units per mL (cfu/mL) and was correlated to cell dry mass (g/L) using a calibration curve. However, when solids were removed during the experiments, samples were centrifuged in order to separate the yeast biomass from the broth. The biomass was washed with distilled water to remove residual sugars and salts and was centrifuged again. Finally the yeast biomass was transferred to pre-weighted glass vials and the biomass concentration was estimated by the weight difference after being dried at 80-90°C until constant weight.

Subsequently, lipids were extracted from the dried biomass with a mixture of chloroform and methanol at a ratio of 2:1 volumes (Folch et al., 1957). Lipid quantification was performed gravimetrically after solvent evaporation under vacuum using a rotary evaporator and was expressed in g/L. Compositional analysis of the obtained lipids was done in a gas chromatography apparatus (Varian CP-3800, Agilent Technologies, USA) coupled with a capillary column (WCOT fused silica 100 m×0.25 mm coating CPSIL 88 for FAME). Prior to analysis fatty acid methyl-esters were formed from the yeast oil according to Appelqvist (1968). The temperature of the column oven was initially kept at 175°C for 26 min and thereafter increased up to 205°C at a rate of 2°C/min. Finally, the temperature remained constant at 205°C for 24 min. The respective temperatures of the detector and the injector were 270°C and 300°C. Helium was used as a carrier gas at a flow rate of 30ml/min.

2.6.4 Total solids (TS) and volatile solids (VS) analysis

TS content was analyzed as the weight difference before and after drying the samples at 105°C for 24 hours. The VS content was determined by subtracting the weight difference after drying the sample at 550°C for 2 hours to the TS content. Temperature during VS analysis was ramped up gradually to 550°C with about 1°C/min rate.

2.6.5 Structural carbohydrates analysis

Structural carbohydrate analysis was performed according to the official protocol of NREL (Sluiter et al., 2008).

2.6.6 Enzymatic activities determination

Total cellulase activity was measured on Whatman N°1 paper (Filter Paper Activity - FPA) by the standard IUPAC method (Ghose, 1987) and invertase (β-fructofuranosidase EC 3.2.1.26) activity was determined on sucrose according to Hoffman-Thoma et al. (1996). One unit (U) of enzyme activity in filter paper assay was defined as the amount of enzyme required to produce 1 μmol of glucose per minute and in invertase assay as the amount of enzyme required to hydrolyze 1μmol of sucrose per minute.

All assays were performed in an eppendorf themomixer at 50°C in 100 mM citrate - phosphate buffer of pH 5.0.

2.7 Response surface methodology

In order to improve some of the processes of this work, an experimental design (Response Surface Methodology-RSM) was employed. RSM estimates the interactions and the effects of chosen factors in one or more responses. It is then possible to build a model that can efficiently describe these interactions. Processes can also be optimized by this method. Different experimental designs can be used resulting in the generation of different combinations of the chosen factors which vary at certain levels. The responses of these combinations can be graphically represented and a quadratic or cubic model can describe the behavior of the responses. During this work, a Box-Wilson Circumscribed Central Composite (CCC) design was utilized generating 11 experimental combinations which included 3 replicates of the central points (Table 1).

The quadratic model applied was the following:

$$R = a_0 + a_1 \cdot x_1 + a_2 \cdot x_2 + a_3 \cdot x_1^2 + a_4 \cdot x_2^2 + a_5 \cdot x_1 \cdot x_2$$

Table 1: Treatment combinations generating from the experimental design

Treatment	Factors	
	X_1	X_2
1	-1	-1
2	1	-1
3	-1	1
4	1	1
5	0	0
6	0	0
7	-1.414	0
8	1.414	0
9	0	-1.414
10	0	1.414
11	0	0

where a_i is representing the different coefficients, x_1, and x_2 are the chosen factors and R the response. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The variance explained by the model is given by the multiple coefficient of determination, R^2. Fitting of the model, regression analysis and graphical representation were done by using either Sigma plot 11.0 (Systat software, Richmond, USA) or MODDE v.10 of Umetrics.

3. RESULTS & DISCUSSION

3.1 Fermentation of liquefacted hydrothermally pretreated sweet sorghum bagasse to ethanol at high-solids content

During this part of the thesis, the ability of utilizing sweet sorghum bagasse for ethanol production was evaluated. When it comes to usage of lignocelluloses for biofuels production, substrate consistency is one of the most important factors from energy balance and process economics point of view. High solids loadings result in higher sugar concentrations and in turn in higher ethanol concentrations, which allows a significant cut of both capital and production costs due to reduced equipment size and energy consumption during heating, cooling, and distillation. Concentrations of ethanol above 4% w/w are generally considered as the minimum prerequisite for a feasible large-scale distillation technology and require the solids loading to be more than 15% w/w (Fan et al., 2003; Wingren et al., 2003). At these consistencies there is practically no free water and the handling of the slurry becomes a challenge. Incorporation of a liquefaction/saccharification step prior to inoculation can result in the deconstruction of cellulose which in turn reduces the water binding capacity enabling submerged fermentation (Szijártó et al., 2011). Mixing of these high solids materials in conventional systems is not always efficient and results in poor contact of cellulases to cellulose. During the last few years, new advanced stirring systems have been evaluated by gravimetric mixing which enables the use of up to 40% w/w solids concentrations (Jørgensen et al., 2007; Larsen et al., 2008).

Use of untreated lignocellulosic materials usually results in very low enzymatic hydrolysis. In order to improve these yields, a pretreatment should

be included in the process. During this work, a hydrothermal pretreatment in the presence of acetic was done. Heating of the materials took place in a microwave oven to rapidly reach the desired temperature.

3.1.1 Evaluation and optimization of the hydrothermal pretreatment of bagasse

As previously discussed, in order to achieve high ethanol production, high hydrolysis rates are necessary. An appropriate pretreatment should result in a material with high cellulose content, which would be easily digestible by the enzymes. The increase in cellulose content is a result of hemicellulose solubilization. For this reason during this work a hydrothermal pretreatment was studied, where low concentration of acetic acid was used as a catalyst for hemicellulose solubilization. The pretreatment was carried out at a specially equipped microwave oven (as described in section 2.2.2) resulting in a rapid increase of the temperature to the desired levels. In traditional reactors, a certain time is needed for heating up the material. This rise in temperature can affect the leading to problems calculating the correct treatment duration.

In order to optimize the pretreatment, RSM was applied. The independent variables were pretreatment temperature and duration, while the response was the % hydrolysis of cellulose after 8 h of incubation. The tested temperatures for pretreatment were varying between 170 and 230°C with a duration changing between 9 and 51 minutes. The different combinations of pretreatment can be seen in Table 2. A second order regression model was applied to estimate the interactive effects of these two important factors and to calculate the pretreatment conditions which will result on the highest enzymatic hydrolysis of bagasse.

The composition of the pretreated bagasse is shown on Table 3 together with the calculated severity factor (SF) of each pretreatment combination. As can also been seen on Figure 18 there is a good correlation between severity factor and cellulose concentration in the pretreated material, which can be described by the following logarithmic equation:

$$C_c = 44.9838 \cdot \ln(SF) - 9.5886$$

where C_c is the concentration of cellulose (% w/w on dry basis) and SF the severity factor. The probability p-value is very low ($p<0.0001$) indicating the significance of the equation and the high coefficient of determination ($R^2=0.857$) indicates a very good correlation between the experimental and predicted values. At more severe treatment conditions cellulose concentration is increased, reaching even 66.84% w/w with pretreatment conditions of 230ºC and 30 minutes, presenting high potentials as it could result in high glucose concentration. Similar results of high cellulose concentration was also achieved with other raw materials, such as *Eucalyptus globulus* (Romaní,et al., 2012), wheat straw (Moreno et al., 2013; Alfani et al., 2000), aspenwood (Mes-Hartree et al., 1988) and sugarcane bagasse (Rocha et al., 2001).

The glucose released (Table 3) after 8 h of enzymatic hydrolysis can be described by the following exponential equation (Figure 19):

$$C_{glc} = 0.2289 \cdot e^{0.6238 \cdot SF}$$

where C_{glc} represents the glucose concentration released from raw material (g/L) and SF the severity factor. The probability p-value is very low ($p<0.0001$) and the coefficient of determination is high ($R^2=0.853$) indicating once again a very good correlation between predicted and experimental values. It can be concluded that high glucose levels can be achieved when the

pretreatment takes place at high severity factor. This is not only the result of the higher cellulose content of the pretreated materials but also the fact that cellulose becomes more susceptible to hydrolysis.

Table 2: Process variables used during this work, showing the pretreatment combinations and the enzymatic hydrolysis.

Treatment	Coding setting level (X_1= Temperature, X_2=duration)		Actual level (X_1= Temperature, X_2=duration)		Enzymatic hydrolysis (% of cellulose)
	X_1	X_2	X_1	X_2	Eh
1	-1	-1	180	15	14.06
2	1	-1	220	15	17.85
3	-1	1	180	45	12.66
4	1	1	220	45	25.46
5	0	0	200	30	21.70
6	0	0	200	30	21.75
7	-1.414	0	170	30	14.82
8	1.414	0	230	30	30.42
9	0	-1.414	200	9	12.86
10	0	1.414	200	51	15.44
11	0	0	200	30	21.52
12	2	1	240	45	19.01

Table 3: Composition of pretreated bagasse under different combinations and severity factor of the pretreatments.

Run	Severity factor	Cellulose (% w/w)	Hemicellulose (%w/w)	Glucose release (g/L)
1	3.53	44.60	20.91	2.14
2	4.71	63.17	3.54	3.85
3	4.01	57.90	15.24	2.50
4	5.19	60.93	2.00	5.30
5	4.42	55.78	15.57	4.14
6	4.42	57.33	13.32	4.26
7	3.54	46.46	18.27	2.35
8	5.30	66.84	n.d.	6.95
9	3.90	52.63	12.43	2.31
10	4.65	59.32	9.83	3.13
11	4.42	55.40	14.54	4.07

n.d. = not detected

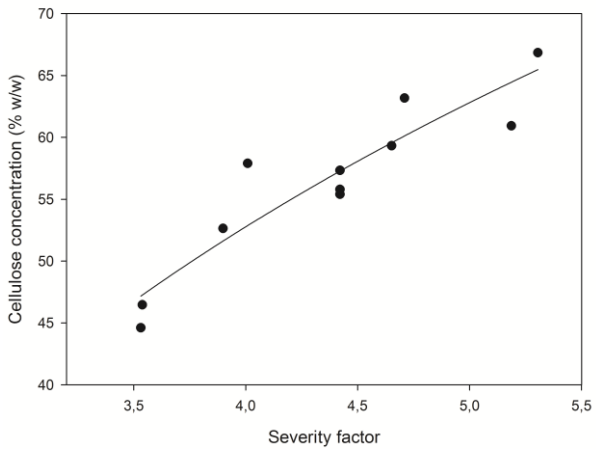

Figure 18: Relationship between severity factor and cellulose concentration of pretreated bagasse

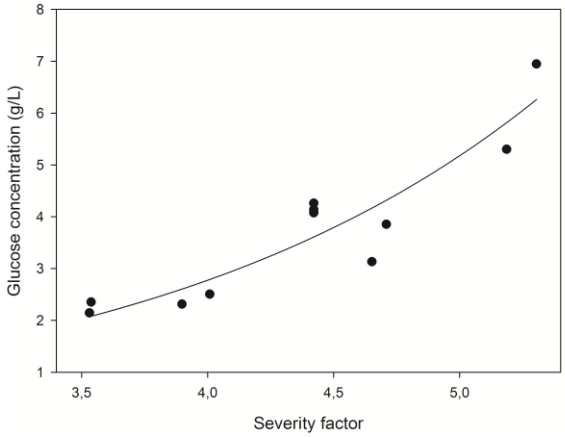

Figure 19: Relationship between glucose release after enzymatic hydrolysis for 8 h and severity factor

As discussed previously, the optimization of pretreatment was done by taking into account the cellulose conversion after 8 hours of hydrolysis. In industrial applications saccharification should preferably last less than 12 hours. The highest enzymatic hydrolysis yields were obtained at pretreatment conditions close to those of the upper values of temperature. In order to confirm this result, an additional experiment (run number 12) was included in the experimental design, as summarized in Table 2. This extra experiment, which was performed at the highest severity factor, resulted in lower yield in enzymatic hydrolysis which could be attributed to the destruction of cellulose present. The second order polynomial regression model that was obtained during this study, by applying the equation described in section 2.7, can be described by the following equation:

$$Eh = -165.9296 + 1.5420 \cdot x_1 + 0.9744 \cdot x_2 - 0.0035 \cdot x_1^2 - 0.0212 \cdot x_2^2 + 0.0018 \cdot x_1 \cdot x_2$$

where *Eh* represents the % enzymatic hydrolysis of cellulose, X_1 and X_2 the temperature and duration of the pretreatment respectively. After testing the model for adequacy by the ANOVA (ANalysis Of VAriance), it was shown that the probability *p*-value was very low (p=0.026) and the coefficient of determination (R^2=0.83) very high, indicating a high significance of the model with good ability to describe the experimentally obtained data.

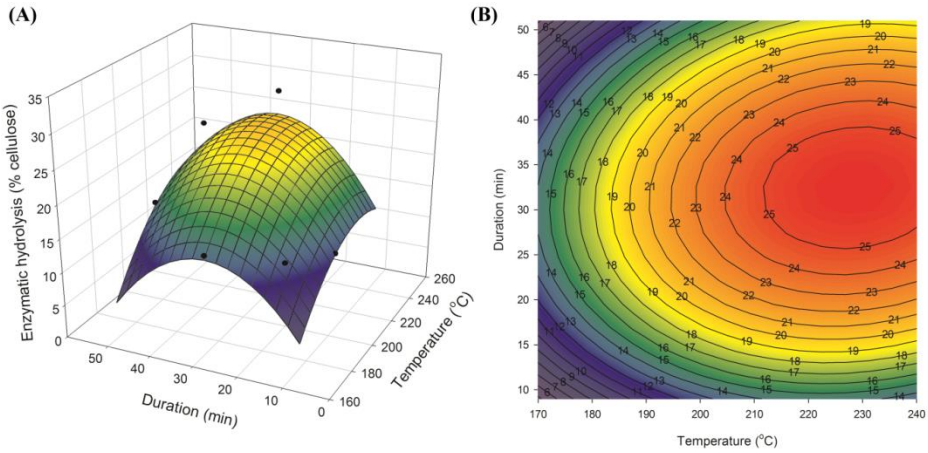

Figure 20: Response surface (A) and contour plot (B) of cellulose enzymatic hydrolysis yield at different pretreatment conditions

The resulting response surface and contour plot of the model can be seen in Figure 20, where the response surface presents a maximum point of cellulose hydrolysis. By solving the previous equation, the optimum combination of pretreatment temperature and duration was found to be 229°C and 33.8 minutes with a predicted cellulose hydrolysis of 26.3%. The optimum values are fairly close to the values of run 8 (230°C and 30 minutes), where the experimentally achieved cellulose hydrolysis reached 30.4%, indicating that the model underestimated this value. The results obtained during this run were very promising for subsequent ethanol production trial,

as both high cellulose content and high cellulose hydrolysis after only 8 h incubation were achieved.

3.1.2 Fermentation of saccharified bagasse at high solids content

In order to achieve high initial sugar concentrations and subsequent high ethanol production, the solids content was increased to 18% w/w. Saccharification was performed in the reactor described in section 2.2.3 at 50°C with an enzyme load equal to 10FPU/g solids. Saccharification took place for either 12h or 24h. Another set of experiments was also included, where extra enzymes (at the same enzyme loading) were added at the start-up of the fermentation in order to evaluate the efficiency of the saccharification step. In Figure 21, it can be observed that bagasse was totally liquefied after even 12h of hydrolysis. At the same time, glucose and total reducing sugars concentration reached 76.3g/L and 81.9g/L respectively, which is very promising for the subsequent ethanol fermentation. Since these values slightly increased to 77.4g/L and 82.8g/L respectively after 24h of hydrolysis, it was concluded that 12h of hydrolysis were enough for efficient cellulose conversion. Moreover, the low difference between glucose and reducing sugars indicates the high efficiency of Cellic® CTec2 to convert cellulose to glucose with no significant accumulation of cellobiose or other oligosaccharides. Cellulose hydrolysis (based on total reducing sugar, as no hemicellulose is present) reached 59.8% after 12h and 60.5% after 24h of enzymatic treatment which might indicate that glucose inhibition on cellulase has occurred. High levels of sugars concentration have also been reported from sugarcane bagasse (Zhao et al., 2013), agave bagasse (Caspeta et al., 2014) and corncob (Chen at al., 2007).

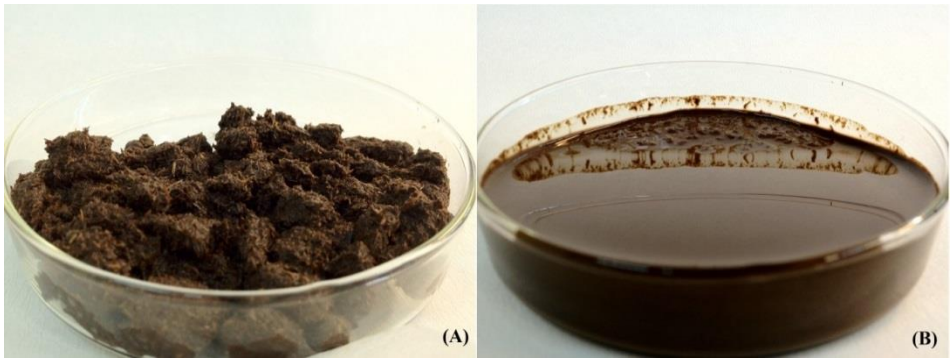

Figure 21: Pretreated bagasse at 18% w/v solids loading before (A) and after (B) enzymatic hydrolysis for 12h.

The highest ethanol production (in both 12h and 24h saccharification) was observed after 22h of fermentation (Figure 22) and reached 41.4g/L with a volumetric productivity of 1.88g/L·h (Table 4). The ethanol yield per bagasse reached 23g/100g bagasse, which was equivalent to 60.8% of the maximum theoretical yield (based on the total conversion of cellulose to ethanol) (Table 4). Addition of extra enzymes at the start-up of the fermentation enhanced the ethanol concentration by 16% and 17% after 12 h and 24 h saccharification, respectively.

Finally when SSF process was evaluated, the ethanol production and productivity were significantly lower (Table 4). Highest ethanol production reached only 24.44g/L with a volumetric productivity of 0.26g/L·h (Table 4 and Figure 22). Ethanol yield was 13.6g/100g of bagasse which is equivalent to 40% of the maximum theoretical yield.

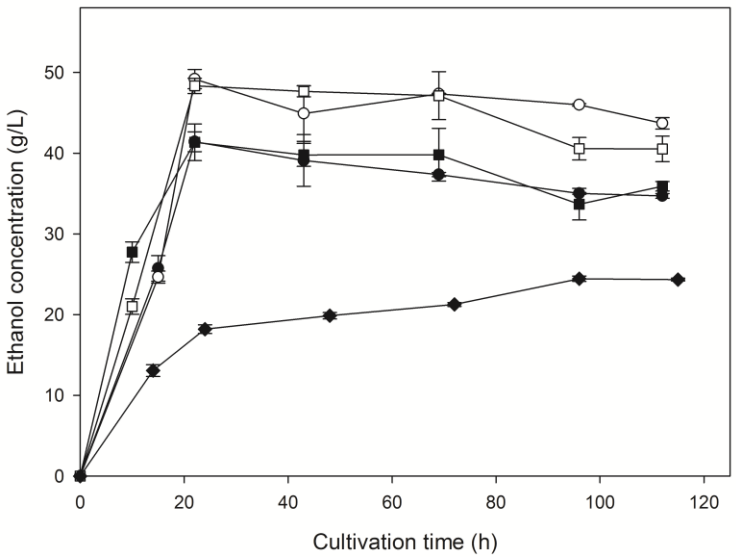

Figure 22: Time course of ethanol production after 12 hours liquefaction without (●) and with the addition of extra enzymes (○), 24 hours of liquefaction without (■) and with (□) the addition of extra enzymes and without separate liquefaction step (♦).

It was demonstrated that the application of a separate hydrolysis step (SHF process) resulted in an increase of 69.5% in ethanol production and a 7.2 times higher productivity compared to the SSF process. Despite the fact that there are several reports in the literature showing that a SSF process is more beneficial comparing to SHF (Alfani et al., 2000; Tomás-Pejó et al., 2008, Öhgren et al., 2007), as was also demonstrated by Cannela and Jørgensen (2013), the application of new generations of cellulolytic enzyme preparations makes SHF process more advantageous. On the other hand, SSF process is more suitable when older generation enzymatic solutions (like Celluclast) are applied. The application of saccharification prior to fermentation, especially at high solids content, has be proven to be beneficial for liquid hot water pre-

treated olive pruning (Manzanares et al., 2011), steam pre-treated spruce (Hoyer et al., 2013a,b) and sugarcane bagasse (de Souza et al., 2012).

Table 4: Effect of different saccharification conditions on ethanol production, productivity, yield and relative yield (calculated as percentage of maximum theoretical yield)

Saccharification conditions			Ethanol production		
Duration (h)	Addition of extra enzymes	Concentration (g/L)	Yield (g/100 g DM)	Productivity (g/L·h)	Relative ethanol yield
12	-	41.4 ± 1.2	23.0 ± 0.7	1.88 ± 0.06	60.8 ± 1.9
12	+	47.9 ± 3.5	26.6 ± 1.9	2.18 ± 0.16	70.4 ± 5.0
24	-	41.4 ± 2.3	23.0 ± 1.3	1.88 ± 0.10	60.8 ± 3.4
24	+	48.3 ± 2.4	26.9 ± 1.3	2.20 ± 0.10	71.2 ± 3.4
0	-	24.4 ± 0.3	13.6 ± 0.2	0.26 ± 0.01	40.0 ± 0.5

The ethanol production demonstrated during this work was higher than those reported by Ban et al. (2008), where only 5.4g/L of ethanol was achieved when the solid fraction of phosphoric acid pre-treated bagasse was fermented by *S. cerevisiae* and 14.5g/L when liquid fraction was fermented by *Pachysolen tannophilus* with the addition of 60FPU/g DM enzyme load. In another work, Dogaris et al. (2012) also achieved lower ethanol production (27.6g/L) by fermentation with *Neurospora crasse* of dilute-acid pretreated sweet sorghum bagasse. About the same ethanol production (42.3g/L) was reported by Li et al. (2010) from AFEX pretreated sorghum bagasse, with lower yield (15.9g/100g raw material) comparing to this work. Finally, Chen et al (2012) also achieved lower ethanol yields (21g ethanol/100g of raw material) during fermentation of ammonia hydroxide pretreatment sorghum

bagasse with the addition of higher enzyme load (60FPU/g of glucan) supplemented with high β-glucosidase (64CBU/g of glucan).

3.2 Ethanol production from high dry matter liquefied dry sweet sorghum stalks

As discussed in the introduction, the main technical challenge of sweet sorghum usage concerns its low storage stability. For this reason, sweet sorghum should be used in a short term after harvesting, which results in problems for an annual availability of the stalks. Moreover, the high volumes of the produced sorghum in a really short period of time require the use of big facilities which will work only temporarily.

Common practices of stalks utilization, such as separation of juice from the bagasse, sometimes suffer from the low sugar recovery yields and the storage stability of the produced juice. In order to preserve stalks or juice from contaminations, cooling units should be used which in turn increase the overall cost of the process. In most of the cases, if sugars are not separated from the lignocellulosic fraction, only solid-state fermentation can be applied presenting several problems including difficulties for scaling-up the process and ethanol recovery (Singhania et al., 2009).

Storage stability problems could be solved by drying the stalks until the water content is low enough. On the other hand, the necessity of using high solids concentration of sweet sorghum particles in order to achieve high sugar and ethanol concentrations, results in a high viscosity mash where the insoluble materials are very difficult to be separated from the juice without addition of water. Under these conditions, only solid state fermentation is allowed leading to several problems that previously were mentioned. In order

to rapidly decrease the viscosity of the high solid content mash, an enzymatic hydrolysis step, prior to the fermentation, was evaluated and optimized during this work. This step also enables a better mixing and an easier ethanol recovery due to the submerged cultivation of the fermenting microorganisms.

3.2.1 Inhibition of glucose, fructose and sucrose on the commercial enzyme solutions

The liquefaction of the lignocelluloses present in the stalks of sweet sorghum is different from the non-sugar crops. This difference has to do with the presence of high concentration of soluble sugars (glucose, fructose and sucrose) in the stalks, which results in the inhibition of cellulases and in turn in hindering the enzymatic hydrolysis. From these sugars, only glucose is known to have an inhibitory effect on cellulases including those from *T. reesei* (Celluclast® 1.5L) and the β-glucosidase from *A. niger* (Novozym® 188) that were used during this work (Xiao et al., 2004). Glucose acts as an inhibitor on β-glucosidase leading to cellobiose accumulation. This phenomenon results in a strong inhibition of cellobiohydrolases and endoglucanases. It was mentioned that the constant of glucose inhibition (K_{IG}) on β-glucosidase could be as low as 0.3g/L to 0.5g/L (Oh et al., 2000) which indicates the great importance of keeping glucose levels as low as possible.

For this reason the inhibitory effect of fructose and sucrose on the mixture of Celluclast® 1.5L and Novozym® 188 was investigated. Inhibition was studied during hydrolysis of Whatman N°1 filter paper at a substrate concentration of 35g/L. It was found that neither sucrose nor fructose with concentration increasing up to 200g/L had an inhibitory effect on cellulases. It can thus be concluded that keeping sucrose intact and avoiding its hydrolysis to fructose and glucose are very important. In this way, it is possible to

prevent from a strong inhibition to commercial cellulases. As a consequence, it was interesting to investigate the existence of invertase activity both in commercial enzyme solutions and as an endogeny activity in sweet sorghum. It was found that Novozym® 188 contained a significant invertase activity (0.83Units mg^{-1} of protein) in contrast with Celluclast® 1.5L which had none. Moreover, sweet sorghum was also found to contain some endogenous invertase activity (3.73U/g solids). To prevent sucrose hydrolysis, enzymatic liquefaction was performed only by Celluclast® 1.5L, whereas Novozym® 188 was added at the start-up of fermentation when the yeast starts consuming the glucose.

The presence of soluble sugars in sweet sorghum stalks reduces the possibility of applying a pretreatment, especially if it involves high temperatures. Indeed, these sugars can undergo degradation and caramelisation reactions which can result in the formation of inhibitory compounds, such as furfural and hydroxyl-methyl-furfural, which in turn can inhibit the fermentation process.

Through this work, in order to reduce the water content and in turn making stalks more stable during storage, stalks were air dried at 85°C for 10 to 12h. In this way, endogenous invertase was also deactivated. Another benefit of drying the stalks includes the reduction in total volume and weight, making storage and transportation easier. It was also demonstrated that sweet sorghum stalks are stable for up to 8 months when the water content is less than 13% w/w (Shen and Liu, 2008).

3.2.2 Effect of the liquefaction step on ethanol production

During this work, an enzymatic liquefaction step was included in the process in order to evaluate the ability to rapidly decrease the viscosity of the

slurry and permitting submerged fermentation. Enzymes can also be added at the start-up of the fermentation but this can have a negative impact on ethanol productivity. This is result that during the initial stages of fermentation the high viscosity of the slurry could result into partial inhibition of the yeast. The evaluation of the effect of enzyme liquefaction was performed at an initial solid content of 35% w/v to evaluate the performance of the enzyme under high gravity conditions. The enzyme load applied was equal to 13FPU/g solids.

Table 5: Results of ethanol yield and productivity of fermentations at different liquefaction duration. Initial solids were 35% w/v and enzyme loading 13FPU/g solids.

Liquefaction duration (h)	Ethanol yield (g/kg of solids)	Productivity (g/L·h)
12	191.2 ± 0.90	2.5 ± 0.0
10	191.0 ± 14.5	2.3 ± 0.2
6	192.5 ± 12.3	1.3 ± 0.1
2	198.9 ± 1.10	1.3 ± 0.2
0	181.7 ± 21.1	1.2 ± 0.1
No enzymes	153.3 ± 13.8	1.0 ± 0.0

As can be seen in Table 5, the addition of enzymes when combined with a separate liquefaction step enhanced the ethanol production by 29.76% and the ethanol productivity by 250%. More specifically, after 12h of liquefaction the productivity was estimated to be 2.5 g/L·h and the ethanol concentration to

60g/L. It can therefore be concluded that the addition of enzymes is necessary to increase both ethanol yield and productivity.

Finally, it can be noticed that higher productivities can be achieved if the enzymes are left for at least 2h to act on the dried stalks prior to inoculation.

3.2.3 Evaluation of the combined effect of liquefaction duration and enzyme load on ethanol productivity

Liquefaction duration and enzyme load are two very important factors during enzymatic hydrolysis of lignocellulose. These two factors interact with each other and affect the efficiency of hydrolysis. In an ideal process, both enzyme load and duration should be as low as possible and the hydrolysis of cellulose as high as possible, in order to reduce the process cost. Efficient cellulose hydrolysis affects both the ethanol yield and the productivity during the subsequent fermentation. For this reason, an optimum combination of these two factors should be found. This optimum will lead to reduced cost of liquefaction and will enable high ethanol yield and productivity.

A RSM regression model was used in order to estimate the interactive effects of these two important variables. These factors varied from 1.8 to 10.2h and from 2.8 to 11.2FPU/g of solids for liquefaction duration and enzyme load respectively. Ethanol volumetric productivity was considered as the response of this design, as it was found that ethanol yield was not affected by these variations (data not shown). The different treatment combinations are shown in Table 6. Preliminary fitting of the equation described in section 2.7 indicated that the combined effect factor of these two variables ($x_1 \cdot x_2$) was not statistically significantly. For this reason, this factor was removed in order to obtain the second regression model described by the following equation:

$$P = -2.2716 + 0.579 \cdot x_1 + 0.6743 \cdot x_2 - 0.0337 \cdot x_1^2 - 0.0405 \cdot x_2^2$$

where x_1 represents the liquefaction duration and x_2 the enzyme load. This model was tested by ANOVA for adequacy and it was found that the *p*-value was very low (p=0.0086) and the coefficient of determination (R^2=0.867) was high. These values indicate the good correlation between the experimental and predicted values and support the significance of the model.

The resulting response surface and contour plot which shows the effect of liquefaction duration and enzyme loading on ethanol productivity is shown in Figure 23. From the response surfaces it can be observed that there is a maximum value of ethanol volumetric productivity. After solving the previous equation the optimum combination of liquefaction duration and enzyme load was found to be 8.6h and 8.32FPU/g solids, resulting to a predicted volumetric productivity of 3.03g/L·h. In order to verify these values, an extra fermentation was carried out on liquefied sweet sorghum stalks from this combination. Under these conditions, sweet sorghum stalks totally liquefied at the end of the hydrolysis (Figure 24).

Table 6: Treatment combination from the experimental design and the experimental responses of ethanol volumetric productivity.

Treatment	Coding setting level (X_1= Liquefaction duration, X_2=FPU/g DM)		Actual level (X_1=Liquefaction duration, X_2=FPU/g DM)		Productivity (g/L·h)
	X_1	X_2	X_1	X_2	P
1	-1	-1	3	4	1.19
2	1	-1	9	4	2.74
3	-1	1	3	10	1.60
4	1	1	9	10	2.76
5	0	0	6	7	2.74
6	0	0	6	7	2.61
7	-1.414	0	1.8	7	1.60
8	1.414	0	10.2	7	2.65
9	0	-1.414	6	2.8	1.25
10	0	1.414	6	11.2	2.76
11	0	0	6	7	2.85

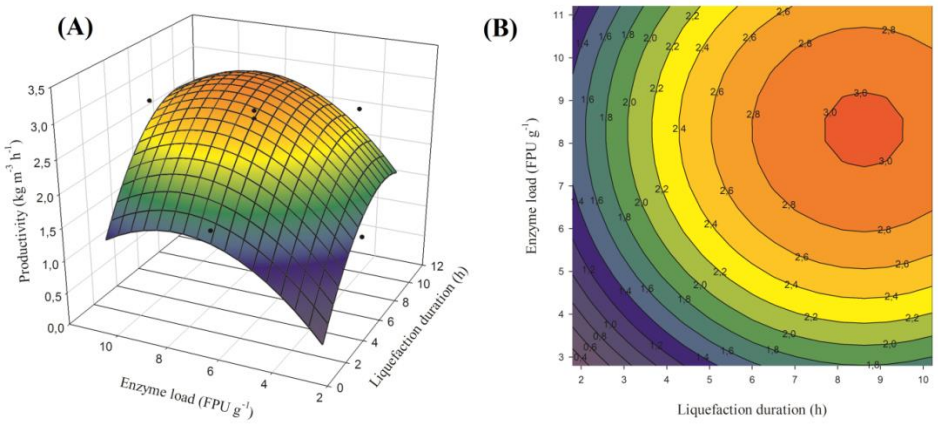

Figure 23: (A) Response surface and (B) contour plot of ethanol productivity resulted from fermentations of dried sweet sorghum at 35% w/v solids content.

At the subsequent fermentation, ethanol reached high levels of production (62.53g/L) after 21h of fermentation, resulting in an ethanol volumetric productivity of 2.98g/L·h (Figure 25), which was fairly close to the model prediction. Under these conditions, ethanol yield reached 19.85g/100g of solids, which is equivalent to 76.75% of the maximum theoretical yield (based on the soluble sugar fraction). Cellulose hydrolysis after the liquefaction and at the end of fermentation was found to be 17% and 21% respectively, based on total cellulose in the raw material.

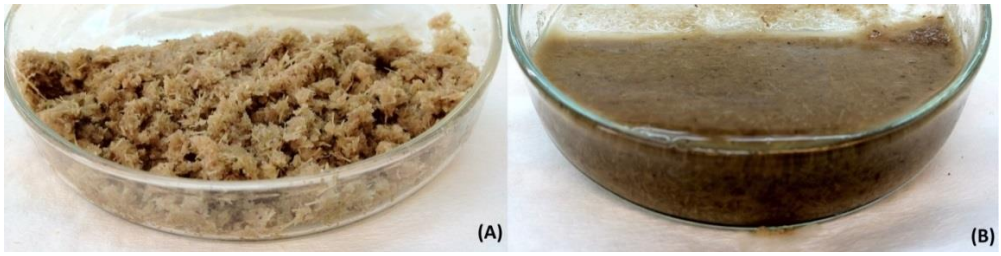

Figure 24: Sweet sorghum at 35% w/v solids concentration, (A) before liquefaction and (B) after liquefaction at optimum conditions.

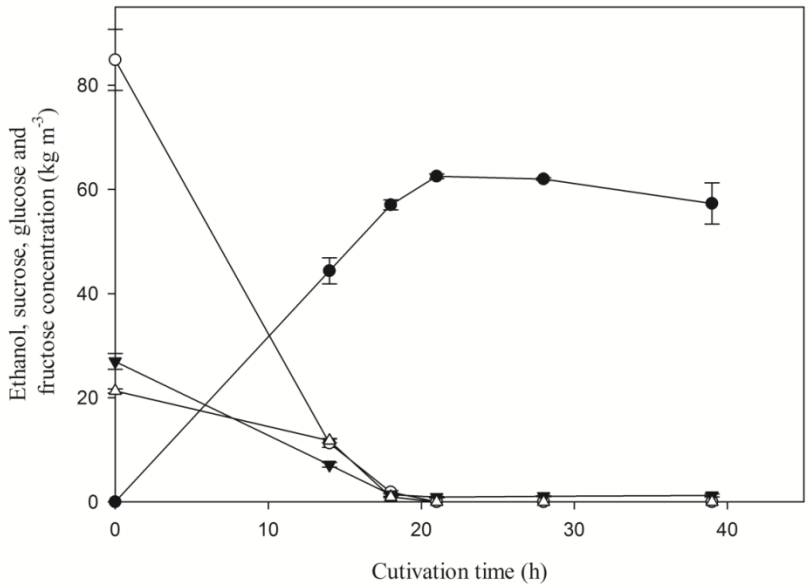

Figure 25: Time course of ethanol production (●) and sucrose (○), glucose (▼) and fructose (□) utilization during fermentation of *S. cerevisiae* MAK2 on liquefied stalks at optimum conditions and solids concentration 35% w/v.

3.2.4 Effect of the solids concentration on ethanol production

At the last stage of this work, the effect of initial solids concentration on ethanol production was evaluated in a range from 10% to 50% w/v. High solids concentrations are desired to achieve higher concentrations of ethanol. On the other hand, as the concentration of solids is increased, the inhibition of glucose to commercial enzyme solutions will be more severe and some problems might occur during the fermentation step.

Results of ethanol production under different solids contents are shown in Table 7. Ethanol production gradually increased with the increase in the solids

content up to 40% w/v. At higher solid content, the ethanol concentration was notably low because of glucose inhibition which was very strong and no liquefaction observed. Under these conditions, a part of the soluble sugars remained intact, indicating problems during the fermentation step. Although the ethanol concentration in 40% w/v solids was slight higher than on 35% w/v, the volumetric productivity decreased to half when the solids content increased from 35% to 40% w/v. This increase also had a negative impact on the ethanol yield. It was therefore concluded that the optimal solid concentration was 35% w/v since the increase in ethanol concentration above this value was not worth compared to the decline in productivity and yield.

Table 7: Fermentation parameters of ethanol production by *S. cerevisiae* MAK2 on liquefacted dried sweet sorghum stalks at different solid contents. Productivity, Yield and Relative Yield were calculated at maximum ethanol concentration. Relative Yield was calculated on basis of maximum Yield ($Y_{ethanol/sugars}$=0.511) based on soluble sugars concentration. Liquefaction was performed at optimum conditions as described earlier.

Initial Dry Matter (w/v)	Maximum ethanol concentration (g/L)	Productivity (g/L·h)	Yield (g ethanol/100g solids)	Relative yield (%)
10%	20.21 ± 0.95	1.19 ± 0.06	22.45 ± 1.06	86.81 ± 4.00
20%	36.33 ± 3.22	2.14 ± 0.19	20.18 ± 1.79	78.04 ± 6.90
30%	55.37 ± 0.95	2.21 ± 0.05	20.51 ± 0.35	79.29 ± 1.40
35%	62.53 ± 0.46	2.98 ± 0.02	19.85 ± 0.14	76.75 ± 0.56
40%	64.50 ± 3.60	1.29 ± 0.07	17.91 ± 1.00	69.28 ± 3.90
50%	38.3 ± 0.51	0.39 ± 0.01	5.11 ± 0.11	19.75 ± 0.40

Compared to other works using the whole stalks of sweet sorghum (without separation of the juice) for ethanol production, process configuration suggested in this work was more advantageous for higher ethanol production. Shen and Liu (2008) performed solid state fermentation of dried sweet sorghum stalks and they achieved a yield of 27.02g/100g of soluble sugars, which was equivalent to 10.55g/100g of solids, after supplementation of sweet sorghum with external nutrients. Mamma et al. (1996) achieved 49g/L of ethanol with fermentation by mixed cultures of *Fusarium oxysporum* and *S. cerevisiae* of alkali pretreted stalks, which has a negative impact on the process cost. Mizuro et al. (2009) suggested the use of the basidiomycete *Flammulina velutipes* for the fermentation of sorghum, with the addition of Celluclast® 1.5L, supplemented with β- glucosidase and xylanase. Despite the fact that ethanol yield (19.3g/100g) was close to the one achieved in this work, fermentation needed 10 days to be completed, resulting in a very low productivity. Yu et al. (2010) suggested to pretreat fresh sweet sorghum with H_2SO_3 (0.25g/g of solids) at 100°C for 120 min. With the addition of high enzyme load (60FPU/g), they achieved only 44.5g/L. Finally, Siwarasak et al. (2011) used crude enzyme powder from *Trichoderma reesei* to hydrolyze fresh sweet sorghum stalks. They achieved an ethanol concentration of only 35g/L with a corresponding productivity of 0.18g/L·h.

When the whole stalks were used, ethanol production was higher than when bagasse was used. Moreover, fewer process steps were necessary resulting in a more simple process. This also has an impact on the cost of ethanol production. For this reason, the use of dried stalks instead of bagasse was applied during the next part of this thesis.

3.3 Evaluation of lipid production by the yeast *Lipomyces starkeyi* CBS 1807 from dried sweet sorghum stalks

In this part of this work, the potential of using dried sweet sorghum stalks for the cultivation and lipids accumulation of the yeast *L. starkeyi* was evaluated. Apart from the technical challenges of using sweet sorghum previously discussed, another challenge is the low carbon to nitrogen (C:N) ratio, estimated around 60-65 (Economou et al., 2010) which is a result of the protein contained in the stalks. On the other hand, not all the proteins are readily available for the yeast and this could result in a higher actual C:N ratio. The incorporation of the enzymatic hydrolysis could also result in the increase of this ratio as sugars will be released from the structural carbohydrates.

3.3.1 Evaluation of lipid production from synthetic media

At the initial stage of this work the cell growth, sugar utilization and lipid production patterns of *L. starkeyi* CBS 1807 were characterized on synthetic media with glucose, fructose and sucrose, either as single carbon and energy source or in mixtures at the ratio present in sweet sorghum stalks (glucose, 16%; fructose, 16% and sucrose 68%). All 3 sugars were suitable for the growth of the yeast and sufficient for the lipid production (Figure 26). The highest lipid production was achieved when glucose was used as carbon source. 5.71g/L of lipids were obtained with a lipid content of 49% w/w. The lowest lipid production was observed when cultivated in sucrose. However, in the sugar mixture 4.49g/L of lipids were produced with a lipid content of 41.3% w/w were achieved, indicating that the sugar composition of sweet sorghum stalks is suitable for the cultivation and lipid production of *L. starkeyi*.

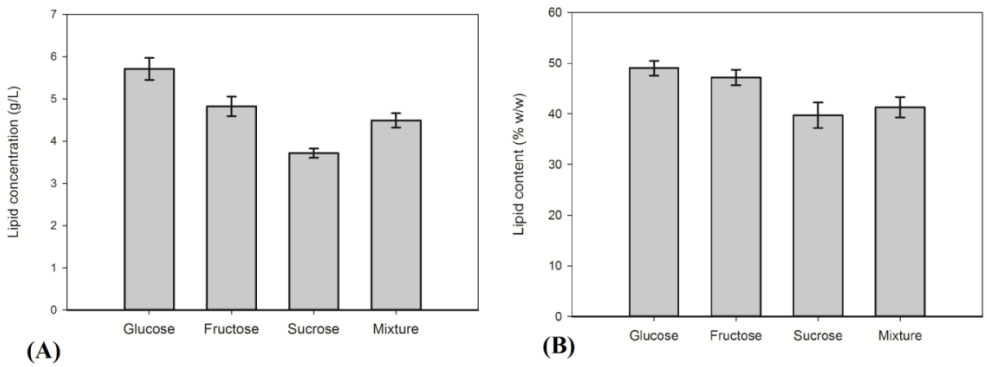

Figure 26: Effect of different sugars on the lipid concentration (A) and lipid content (B), under constant C:N ratio of 100 by the addition of a mixture of yeast extract and ammonium sulfate.

In order to further evaluate and improve the lipid production from the sugar mixture, other factors affecting lipid accumulation were studied and optimized. Generally, lipid accumulation is influenced by several factors such as nitrogen, temperature etc (Zhu et al., 2008). Among these factors, nitrogen plays a very important role as the accumulation of lipids starts after the depletion of nitrogen from the cultivation broth (Papanikolaou et al., 2007). The form of the nitrogen source (organic or inorganic) can also affect the ability of the microorganisms to accumulate lipids (Papanikolaou and Aggelis, 2011b). For these reasons, it is of great importance to evaluate the nitrogen source as well as the ratio of the carbon to nitrogen concentrations (C:N).

In the first part, the effect of organic (yeast extract, meat peptone and urea) and inorganic (NH_4Cl, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$) nitrogen sources on lipid production was evaluated at a constant C:N ratio equal to 100. It was observed that complex organic nitrogen sources (yeast extract and peptone) were more beneficial for lipid accumulation (Figure 27). The highest lipid production was observed in the presence of yeast extract and reached 5.23g/L with a lipid content of 43.7% w/w. The same positive effect of the presence

of organic nitrogen source was reported for different microorganisms like *Cunninghamella echinulata* (Certik et al., 1999), *Trichosporon fermentans* (Zhu at el., 2008) and *Rhodosporidium toruloides* (Evans and Ratledge, 1984b).

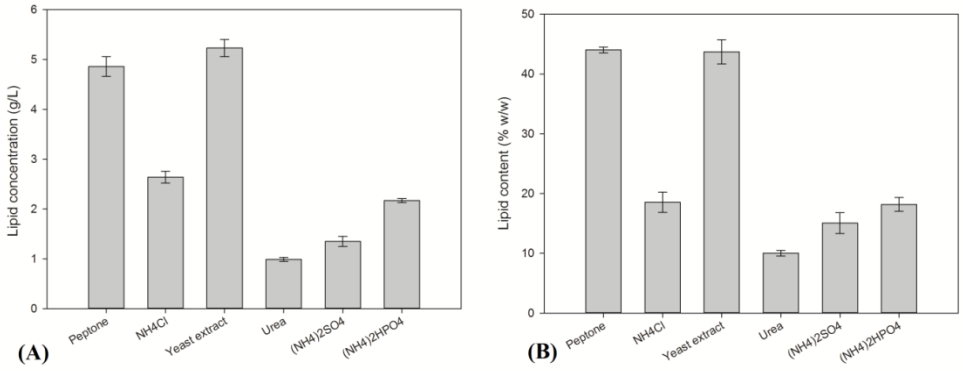

Figure 27: Effect of nitrogen source on lipid concentration (A) and lipid content (B) using sugar mixture for the yeast cultivation under constant C:N ratio of 100.

Finally, the effect of different C:N ratios in a wide range (40-250) was investigated by using yeast extract as a nitrogen source (Figure 28). The lipid production increased with the increase of C:N ratio up to 190, where 5.81g/L and 47.3% w/w of lipid production and lipid content, respectively, were achieved. A further rise of the C:N ratio resulted in a slight drop of both lipid production and lipid content. Similar results concerning the % w/w lipid accumulation at low C:N ratios were reported by Wild et al. (2010) for the same microorganism. High lipid production can be achieved in a quite large range of C:N ratios. To conclude, high lipid production can be achieved with C:N ratios above 100.

Under the optimum conditions for the yeast cultivation in synthetic media, the yield of lipid production per gram of consumed sugars was $Y_{L/S}$ =

0.131g/g, the productivity 1.162g/L·day and the yield of biomass formation $Y_{X/S}$ = 0.276g/g (Figure 29).

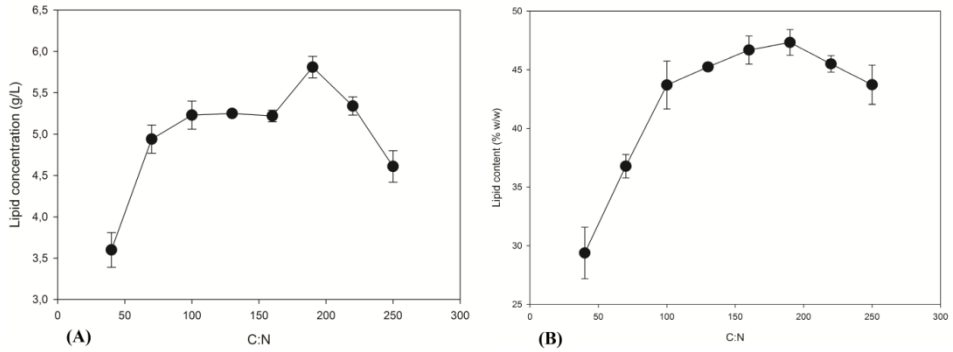

Figure 28: Effect of different C:N ratios on lipid concentration (A) and lipid content (B), when cultivation was performed using the sugar mixture with the addition of yeast extract as nitrogen source.

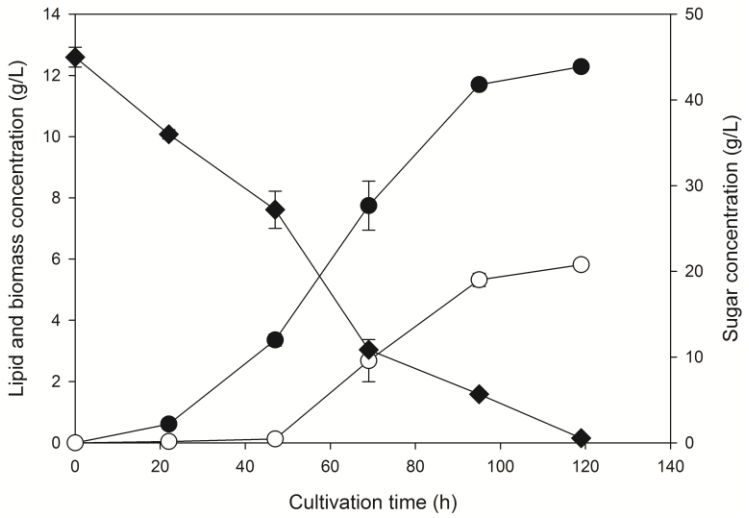

Figure 29: Time course of biomass (●) and lipid (○) concentration and sugars consumption (♦) when *L. starkeyi* was cultivated on the sugar mixture, at a C:N ratio of 190 and yeast extract as nitrogen source.

3.3.2 Lipid production from *L. starkeyi* cultivated on juice obtained from dried sweet sorghum stalks

During the preliminary experiments it was observed that *L. starkeyi* was not able to grow in the presence of solids from sweet sorghum stalks and as a result the solids were removed after the enzymatic saccharification. This phenomenon could be a result of damages caused by the solids to the cells through shear forces, as it was previously suggested during ethanol production from chopped sweet sorghum stalks in rotary drum fermentor (Whitfield et al., 2012).

During the initial experiments, the ability of *L. starkeyi* to exploit sweet sorghum's proteins as nitrogen source was evaluated by studying the effect of the addition of an external nitrogen source (more specifically yeast extract) at a concentration equivalent to 0.2g/100g of sweet sorghum on lipid production (initial sweet sorghum content was 8.7% w/w). This resulted in a significant decrease in lipid production from 4.69g/L (lipid content of 28.3% w/w) to 3.46g/L (lipid content of 20.8% w/w) (Figure 30) probably caused by the substantial decrease of the initial C:N ratio of the medium after the addition of yeast extract. Moreover, even without the addition of an external nitrogen source, the lipid production was lower compared to those obtained in synthetic media, underpinning the importance of increasing the C:N ratio by increasing the initial sugars concentration.

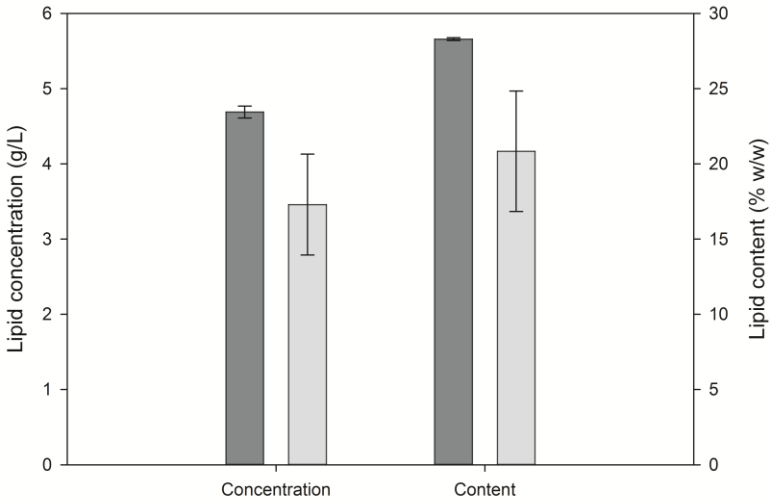

Figure 30: Effect of addition of external nitrogen source (0.2 g yeast extract per 100g of sorghum) on lipid concentration and content, when *L. starkeyi* was cultivated on sweet sorghum juice from a sweet sorghum initial concentration of 8.7% w/w. Experiment with the addition of external nitrogen source is represented by the light gray bar and the control by the dark gray bar.

It can be concluded that the incorporation of the enzymatic saccharification step can facilitate the increase of the sugar content and in turn the C:N ratio. For this reason, the effect of a distinct enzymatic saccharification step on lipid production was evaluated at a range of solids concentrations (8.7, 12 and 16% w/w). The incorporation of a distinct saccharification step resulted not only in an increase of the initial concentration of sugars (approximately 3 to 12%) but also in a better recovery of the liquid and in turn also of the sugars by reducing the viscosity of the slurry. As it was previously discussed this is an effect of the loss of the water-binding capacity due to the degradation and collapse of the structure of cellulose (Szijártó et al., 2011). It is also important to mention that the presence of a distinct enzymatic saccharification step resulted in the increase of lipid production in all the solids concentrations

(Figure 31). The highest lipid production was observed when the yeast was cultivated in the juice obtained from 12% w/w initial sweet sorghum concentration with the incorporation of a distinct saccharification step and without any additional nitrogen source (Figure 32). Under these conditions the lipid production reached 6.40g/L with a lipid content of 29.5% w/w. The lipid yield per gram of consumed sugars was $Y_{L/S}$ = 0.077g/g, while the corresponding yield for biomass formation was $Y_{X/S}$ = 0.262g/g which is comparable to the one obtained when using the synthetic media (C:N ratio of 190). Moreover the lipid productivity was 0.8g/L·day and the lipid yield per sweet sorghum solids reached 5.33g/100g of sweet sorghum. Lipid production obtained during this work was higher than most of the works reported in the literature when *L. starkeyi* was cultivated on renewable raw materials or on sweet sorghum (Table 8).

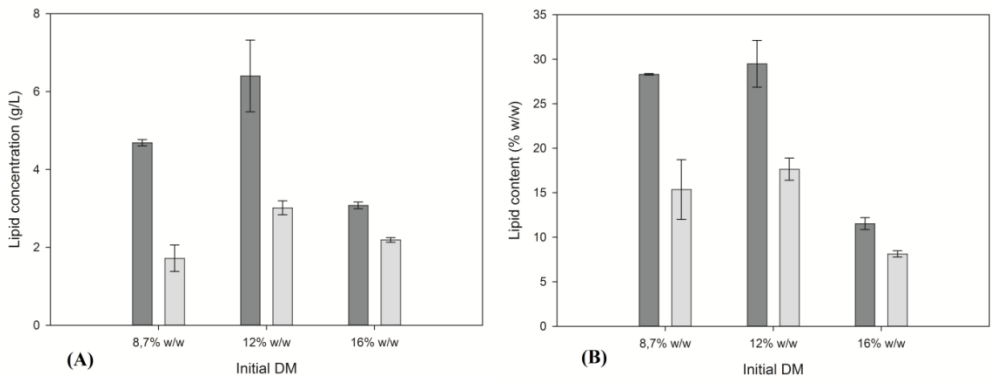

Figure 31: Effect of different initial sweet sorghum contents on lipid concentration (A) and content (B), with the presence (dark gray bars) or absence (light gray bars) of a distinct enzymatic saccharification step.

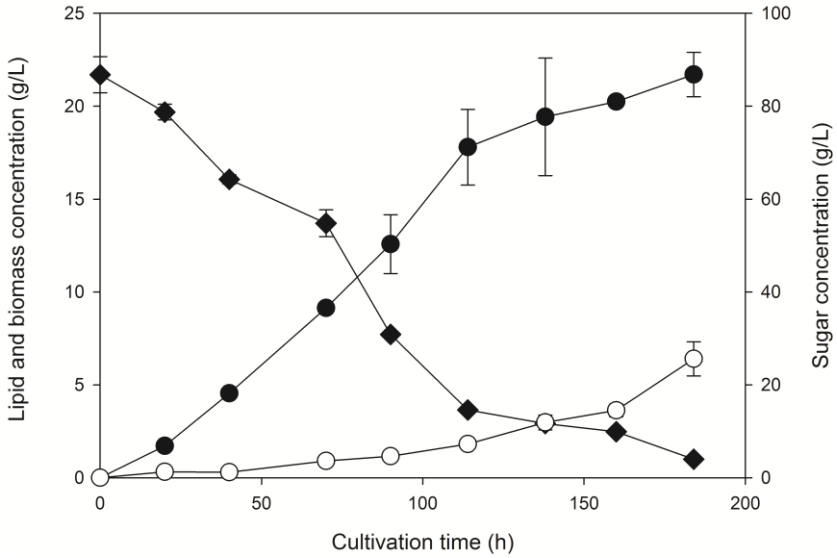

Figure 32: Time course of biomass (●) and lipid (○) concentration and sugars consumption (♦) when *L. starkeyi* was cultivated on sweet sorghum juice that came from 12% w/w sweet sorghum concentration without the addition of an external nitrogen source.

The profile of the lipids obtained at the optimum conditions were further analyzed (Table 9) in order to evaluate their suitability to be used as raw material for biodiesel production. The predominant fatty acid is oleic acid (49.85% w/w) following by palmitic acid (42.90% w/w). The high concentration of oleic acid is considered to be beneficial for the subsequent biodiesel production (Sitepu et al., 2013). Other fatty acids present in smaller quantities in the obtained lipids include palmitoleic and stearic acid. A similar fatty acid composition was also obtained when *L. starkeyi* was cultivated on starch (Wild at al., 2010), mixtures of glucose and xylose (Zhao et al., 2008) and various mixtures of glucose, cellobiose and xylose (Gong et al., 2012).

Table 8: Comparison of lipid concentrations obtained during this work with other results reported in the literature.

Microorganism	Raw material	Lipid concentration (g/L)	Productivity (g/L·day)	Reference
L. starkeyi	Non-detoxified dilute sulfuric acid pretreated wheat straw	4.6	n.a.	Yu et al. (2011)
L. starkeyi	Detoxified dilute sulfuric acid pretreated wheat straw	3.7	n.a.	Yu et al. (2011)
L. starkeyi	Ultrasonic treated sewage sludge	1.0	n.a.	Angerbauer et al. (2008)
L. starkeyi	Sweet potato starch	4.8	2.40	Wild et al. (2010)
L. starkeyi	Glucose-enriched fishmeal wastewater	2.7	0.45	Huang et al. (2011)
L. starkeyi	Detoxified corncob hydrolyzates treated with dilute sulfuric acid	8.1	1.01	Huang et al. (2014)
C. curvatus	Sweet sorghum bagasse	2.6	0.87	Liang et al. (2012)
Chlorella protothecoides	Sweet sorghum juice	2.9	0.59	Gao et al. (2010)
Schizochytrium limacinum	Sweet sorghum juice	6.9	1.38	Liang et al. (2010)
L. starkeyi	Juice from saccharified sweet sorghum	6.4	0.80	Present work

n.a. = not available

Table 9: Fatty acid composition of the lipids produced during cultivation of *L. starkeyi* on juice from 12% w/w liquefied sweet sorghum stalks.

Fatty acid	% concentration (w/w)
C16:0	42.90
C16:1	2.15
C18:0	4.90
C18:1 (n-9)	49.85
C20:4 (n-6)	0.17

3.4 Evaluation of lipid production by the yeast *Trichosporon fermentans* CBS 439.83 from dried sweet sorghum stalks

During this part of the work, the cultivation of the oleaginous yeast *T. fermentans* on liquefied sweet sorghum stalks in order to produce lipids will be discussed. Saccharification of dried sweet sorghum stalks was performed at the optimum conditions that were defined during ethanol production from stalks.

3.4.1 Evaluation of lipid production from synthetic media

During the initial stage of this work, the growth, sugar utilization and lipid production patterns of *T. fermentans* were characterized in the same sugars as for the case of *L. starkeyi*. From the initial experiments, it was found that *T. fermentans* cannot grow when it is cultivated on sucrose, probably due to the lack of invertase activity. For this reason, in all the experiments were sucrose

was present, Novozym® 188 was added to the cultivation broth at the same volumes that would normally be added when sweet sorghum stalks were used. *T. fermentans* was able to grow in all the sugars (Figure 33) and the higher lipid production was observed when it was cultivated in glucose, reaching 1.98g/L and 11.36% w/w lipid content. During the cultivation of the yeast in the mixture, the lipid concentration and content reached 1.05g/L and 7.59% w/w respectively. As for the lipid production, it remained at lower levels than those obtained from *L. starkeyi* on the same synthetic medium, indicating that further optimization is necessary.

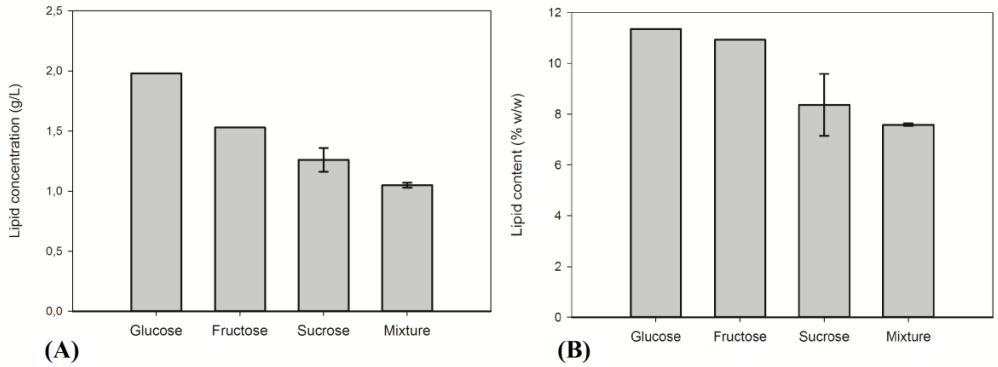

Figure 33: Effect of different sugars on the lipid concentration (A) and lipid content (B), under constant C:N ratio of 100 by the addition of a mixture of yeast extract and ammonium sulfate.

Subsequently, the effect of the nitrogen source was examined. The different organic nitrogen sources used involve peptone, yeast extract and urea and the inorganic sources include ammonium phosphate and ammonium sulphate. As for *L. starkeyi*, it was found that complex organic sources were more favourable for lipid accumulation with peptone giving the highest concentration (2.06g/L) with the lipid content equal to 11.73% w/w (Figure 34). A slightly higher lipid content was achieved when yeast extract was used

(12.15% w/w), but the concentration of lipids was much lower (1.42g/L), which was a result of the lower biomass formation when yeast extract was used.

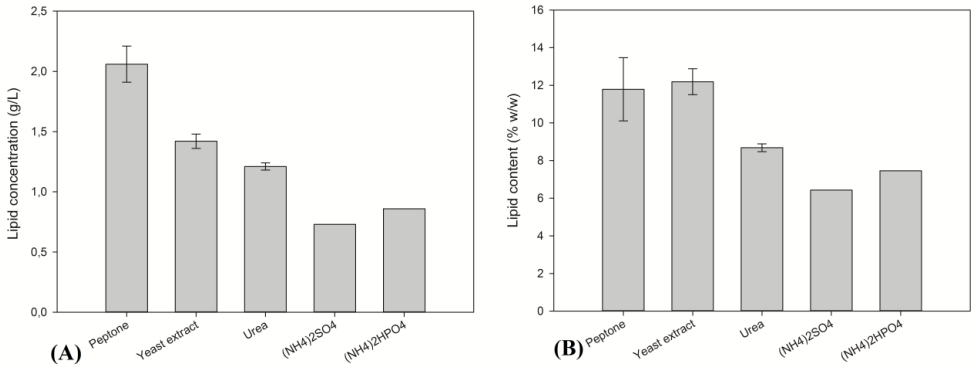

Figure 34: Effect of nitrogen source on lipid concentration (A) and lipid content (B) using sugar mixture for the yeast cultivation under constant C:N ratio of 100.

Finally, the effect of the C:N ratio varying between 40 and 220 on lipid production in the presence of peptone as nitrogen source was evaluated. With increasing the C:N the lipid production is increased up to ratio equal to 160, where 3.66g/L of lipids were produced corresponding to a lipid content of 21.91% w/w (Figure 35). Despite the fact that lipid production increased after optimizing the nitrogen source and the C:N ratio, the lipid content remained quite low due to the high biomass formation. The optimum C:N ratio found during this work was very close to the one reported by Zhu et al. (2008) who mentioned an optimal ratio of 163 for the strain CCIC 1368, and to the one reported by Huang et al. (2012) for the same strain (ratio 165).

Under the optimum conditions for the cultivation of the yeast in synthetic media, the yield of the lipid production per gram of consumed sugars was found to be $Y_{L/S}$ = 0.091g/g, the yield of biomass formation $Y_{X/S}$ = 0.418g/g and the productivity 0.523g/L·day (Figure 36). The obtained lipids by *T.*

fermentans were lower than those produced by *L. starkeyi*. On the other hand, *T. fermentans* presented higher biomass formation compared to *L. starkeyi* which resulted in the lower lipid content in the cells.

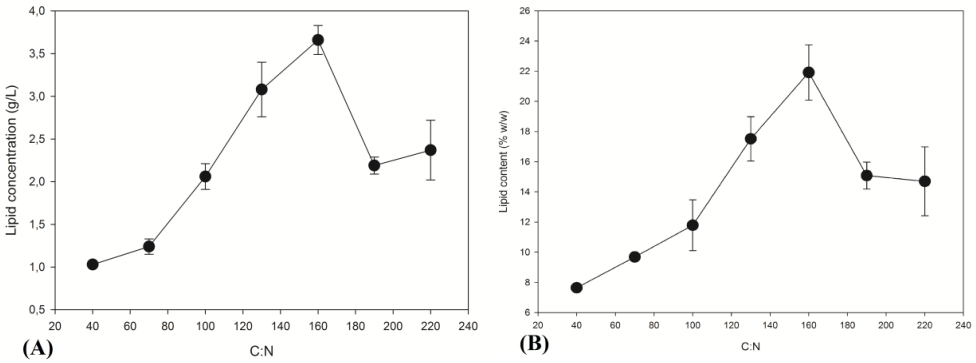

Figure 35: Effect of different C:N ratios on lipid concentration (A) and lipid content (B), when the cultivation was performed using sugar mixture with the addition of peptone as nitrogen source.

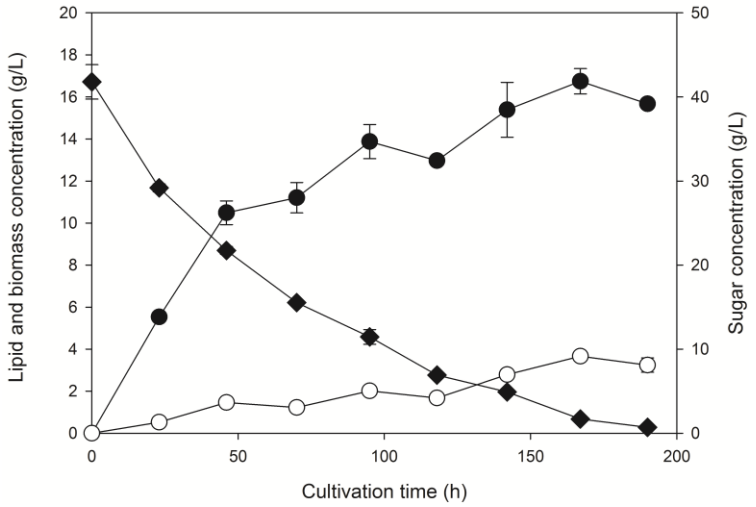

Figure 36: Time course of biomass (●) and lipid (○) concentration and sugars consumption (♦) when *T. fermentans* was cultivated on sugar mixture, at a C:N ratio of 160 and peptone as nitrogen source.

3.4.2 Evidence of Crabtree effect

In an attempt to increase the accumulation of lipids in synthetic media, the effect of the initial sugar concentration in a range from 40 to 200g/L was examined. During the investigation, it was observed that the pH rapidly decreased during the first day of cultivation, as the sugar concentration was increasing. More specifically, for 40g/L initial concentration of sugar, the pH after the first day of cultivation decreased to 6.04 where it remained more or less constant, whereas for 200g/L initial concentration of sugars it decreased to 3.69. The same trend of the decrease of pH was observed in the other sugar concentrations with the values after one day of cultivation being 4.49 (80g/L), 4.32 (120g/L) and 3.70 (160g/L).

In order to determine the reason for this decrease in the pH values, an HPLC analysis was run and it was found that ethanol was produced. The presence of ethanol was also verified with GC (Gas Chromatography) analysis. The highest ethanol production was observed at 200 g/L initial sugar concentration and reached 25g/L after 116 h of incubation (Figure 37). When the lowest concentration of 40g/L was used, only traces of ethanol (0.33g/L) could be detected. After a certain point of the cultivation ethanol started to get consumed indicating a 'diauxic growth' which was also observed by other researchers (Sarris et al., 2013; Sarris et al., 2014). All these phenomena are evidences of the existence of Crabtree effect on *T. fermentans*. As far as we know, it is the first time that Crabtree effect is found on *T. fermentans*. Crabtree effect is a biochemical phenomenon where ethanol can be produced in the presence of air. More specifically, there is a critical value of glucose concentration above which the cellular metabolism is shifted towards ethanol formation despite the presence of oxygen (Sarris et al., 2013). When glucose concentration decreases below this value, the yeast starts consuming ethanol in

order to form cell biomass. This phenomenon is a result of catabolite repression caused by glucose to some enzyme involved in Krebs cycle and oxidative phosphorylation chain, which in turn result in repression of the oxidative pathway (Ratledge, 1991). During the lipid production, Crabtree effect is not desirable due to its effect on shifting the carbon flow from biomass and lipid formation to ethanol. On the other hand, *T. fermentans* could be proven to be an interesting candidate for ethanol production. For this reason the ability of the yeast to produce ethanol under anaerobic conditions should be further evaluated.

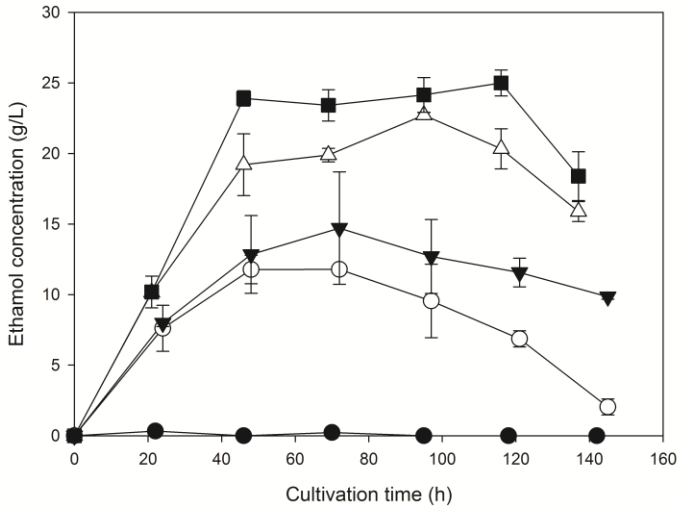

Figure 37: Ethanol production when initial concentration of sugar mixture was 40 g/L (●), 80 g/L (○), 120 g/L (▼), 160 g/L (Δ) and 200 g/L (■). Peptone was used as nitrogen source and the C:N ratio was equal to 160.

3.4.3 Lipid production from *T. fermentans* when cultivated on dried sweet sorghum stalks

Initial trials were performed in the presence of solids to evaluate the capability of the stalks to support yeast growth both as carbon and nitrogen source. For this reason the effect of the addition of peptone at a concentration equal to 0.4g/100g sorghum solids on lipid production was evaluated. The addition of even such small amount of external nitrogen source had a negative impact on lipid production, which decreased from 1.97g/L to 0.79g/L (Table 10). As it was observed during the cultivation of *L. starkeyi* that addition of external nitrogen source has a negative impact on lipid production which might be a result of the further decrease in C:N ratio. As was previously discuss, the ability of the stalks to support the yeast both as carbon and as nitrogen source has a positive impact on process economics, as the complex organic sources have a high cost. In contrast to *L. starkeyi*, *T. fermentans* growth was not inhibited by the presence of solids in the broth.

Table 10: Effect of enzymatic saccharification and external nitrogen addition (peptone at a concentration equal to 0.4g/100g sorghum) on lipid production.

Enzymatic saccharification	External nitrogen source	Lipid concentration (g/L)
+	-	1.97 ± 0.00
+	+	0.79 ± 0.12
-	-	1.05 ± 0.10

Subsequently, the effect of the presence of a distinct enzymatic saccharification step on lipid production was examined. As can be seen in Table 10, absence of the enzymatic saccharification step had a negative impact

on the lipid production, verifying that the presence of a saccharification step facilitates to the increase of sugars and in turn improves the C:N ratio. Finally, no attempt to increase solids concentration (and in turn sugars concentration) was made as the increased sugars would result in Crabtree effect and thus in lower lipid production.

At the last stage of the evaluation of *T. fermentans* as candidate for lipid production when cultivated on sweet sorghum stalks, the effect of solids removal was examined. As it was previously found that the presence of solids could inhibit the growth of *L. starkeyi* and the same negative effect could occur during growth of *T. fermentans*. In contrast with *L. starkeyi*, lipid accumulation of *T. fermentans* was not inhibited by the solids and the lipid production was even slight lower when the solids were removed. This could be possibly attributed to the action of the enzymes during cultivation and further hydrolysis of insoluble polysaccharides when solids are present. Once again the absence of the enzymatic saccharification had a negative impact on lipid production (Table 11).

Table 11: Effect of enzymatic saccharification in the absence of solids on lipid production.

Enzymatic saccharification	Lipid concentration (g/L)	Lipid content (%w/w)
+	1.80 ± 0.29	11.51 ± 2.31
-	1.45 ± 0.06	8.61 ± 0.21

Taking into account all the above, it can be concluded that the highest lipid production achieved without solids removal in the presence of enzymatic saccharification. Under these conditions, lipid production reached 1.97g/L

with a productivity of 0.493g/L·day. The yield of lipid production was $Y_{L/S}$ = 0.067g/g. The fatty acid profile of the obtained lipids is presented in Table 12. The most abundant was oleic acid (37.9% w/w) followed by palmitic (23% w/w) and linoleic acid (19.5% w/w). Siilar composition of fatty acids was found by Zhu et al. (2008), were the obtained lipids mainly consisted of palmitic, stearic, oleic and linoleic acid. Moreover, they reported that the unsaturated fatty acids were 64% of the total lipids, a value that is very close to the one obtained during this work (61.3%). Same fatty acid composition was also reported by Huang et al. (2009) with a concentration of unsaturated fatty acids equal to 65%. The similar composition of the obtained lipids to the vegetable oils makes a very promising raw material for biodiesel production.

Table 12: Fatty acid composition of the lipids produced during cultivation of *T. fermentans* on 8.7% w/w liquefied sweet sorghum stalks.

Fatty acid	**% concentration (w/w)**
C8:0	1.6
C11:0	6.0
C16:0	23.0
C16:1	2.0
C18:0	4.6
C18:1 (n-9)	37.9
C18:2 (n-6)	19.5
C18:3 (n-3)	1.9

3.5 Evaluation of lipid production by the yeast *Rhodosporidium toruloides* CCT 0783 from dried sweet sorghum stalks

At the last part of the work concerning biodiesel production, the yeast *R.toruloides* CCT 0783 was evaluated as a possible candidate. Saccharification of the sweet sorghum stalks was done as previously described.

3.5.1 Effect of nitrogen addition on lipid production

In order to evaluate the ability of *R. toruloides* to use sweet sorghum proteins as a nitrogen source, the effect of the addition of external nitrogen (yeast extract) at a concentration equivalent to 0.3g per 100g of stalks was evaluated at an initial sweet sorghum concentration of 8.7% w/w. As it was previously demonstrated, organic nitrogen sources are more favorable for lipids accumulation by *R. toruloides* (Evans and Ratledge, 1984a,b) and this was the reason why yeast extract was chosen. Addition of even this low amount of external nitrogen had a negative impact on lipid production resulting to a decrease from 6.12g/L to 2.60g/L (Figure 38).

This result is in good correlation with those obtained when both *L. starkeyi* and *T. fermentants* were cultivated on sweet stalks when external nitrogen source was added. It can be concluded that *R. toruloides* is also capable of efficiently using the proteins present in the stalks as nitrogen source and there is no need of the addition of external nitrogen source, fact that has a positive impact on the process economics.

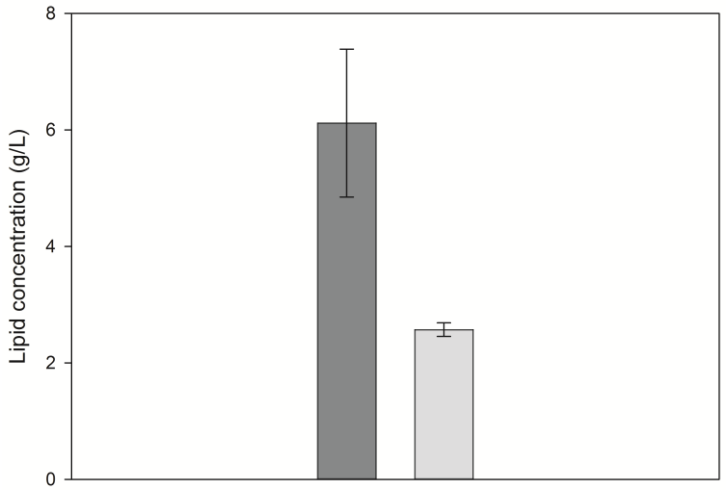

Figure 38: Effect of external nitrogen addition (0.3g of peptone per 100g sorghum on lipid production. Dark and light gray bars represents the cultivation without and with the addition of external nitrogen, respectively.

3.5.2 Effect of the initial sweet sorghum concentration, in the presence or absence of a distinct saccharification step, on lipid production yields

In a next step, the effect of the initial sweet sorghum content on the production of lipids was examined in the 8.7–16% w/w range. Lipid production increased with increasing the solid content up to 12% w/w, where 7.2g/L of lipids were produced (Figure 39). Further increase in the solids content resulted in a decrease in lipid production probably due to inefficient air-transfer properties of the high-solids mash. In all the solid contents absence of an enzymatic saccharification step resulted in significantly lower lipid production. At the highest solids content of 16% w/w no yeast growth was observed in the absence of the enzymatic saccharification step, as the water content was low and submerged fermentation could not take place. The

higher lipid production in the presence of the enzymatic saccharification step underscores once again the great importance of saccharification. As can be seen in Figure 40, at the highest solids content of 16% w/w enzymatic saccharification resulted in liquefaction of the material and consequently better mixing of the yeast. The viscosity of lignocellulosic substrates is known to decrease as a result of cellulolytic activity, the most probable reason being the collapse of structure and subsequent loss of water-binding capacity upon degradation of cellulose (Szijártó et al., 2011). Moreover, enzymatic saccharification facilitates the release of soluble sugars from cellulose and hemicellulose, and consequently improves the C:N ratio.

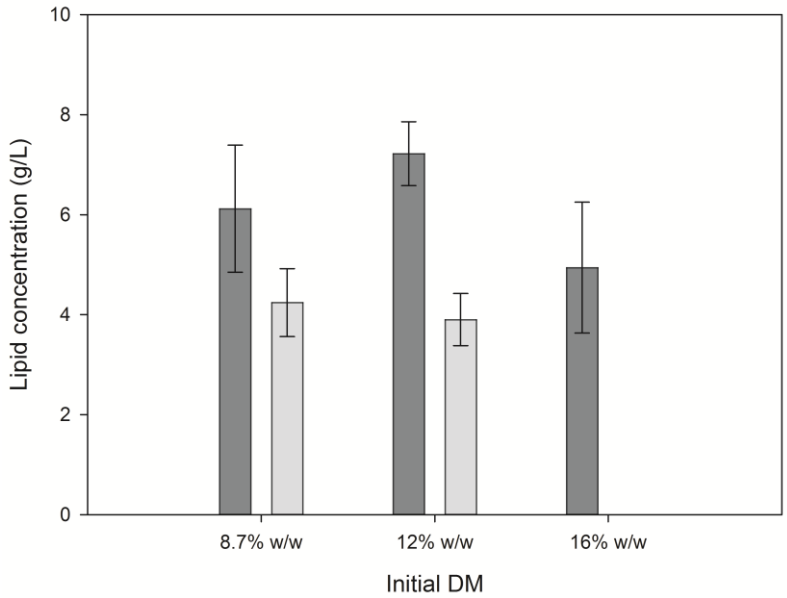

Figure 39: Effect of initial sweet sorghum concentration at a range from 8.7% w/w to 16% w/w in the presence (dark grey) or absence (light grey) of enzymatic saccharification on the production of lipids.

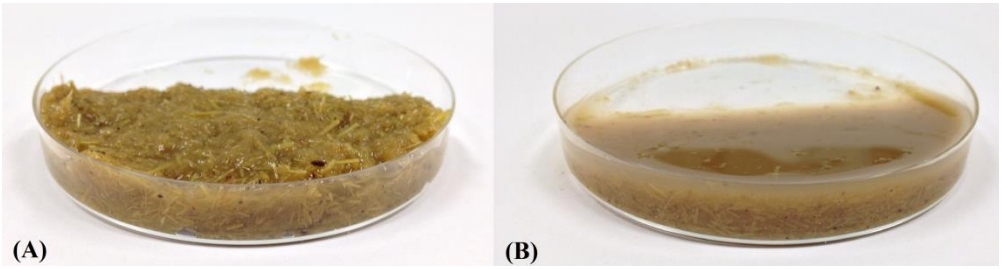

Figure 40: Sweet sorghum stalks at 16% w/w concentration (A) without the application of enzymatic saccharification and (B) after enzymatic saccharification.

3.5.3 Effect of removal of solids on higher lipid production

As mentioned previously the presence of solids had a negative impact on the ability of the yeast *L. starkeyi* to grow and accumulate lipids even at low solids content. Removal of solids resulted in efficient growth and lipid production of the yeast with the most probable reason being that the presence of solids reduced the oxygen transfer efficiency. On the other hand when *T. fermentans* was employed, removal of solids resulted in a slight decrease in lipid production at low levels of solids consistencies. As a consequence, during the last part of this work the effect of solids removal on the lipid production by *R. toruloides* was evaluated both with and without a separate saccharification step.

It was found that removal of solids after the saccharification step resulted in enhanced lipids production even at concentrations of sweet sorghum as high as 20% w/w (Figure 41). It was observed that lipid production increases with increasing the solids content both in the presence and in the absence of enzymatic saccharification. Enzymatic saccharification had a positive impact on lipid production on all the solids content. The maximum lipid concentration was obtained on 20% w/w solids content and was 13.77g/L with lipid content of 33.1% w/w. Cell growth, sugar consumption, and lipid production under this solid concentration are shown as a function of time in

Figure 42. The yield of lipid formation per gram of consumed sugars was equal to 0.105g/g, while the same yield for biomass formation was 0.318g/g. The obtained lipid productivity reached 1.377g/L per day and the incorporation of a distinct enzymatic saccharification step resulted in an increase in lipid production of 15.9% relative to the experiment without enzymatic saccharification.

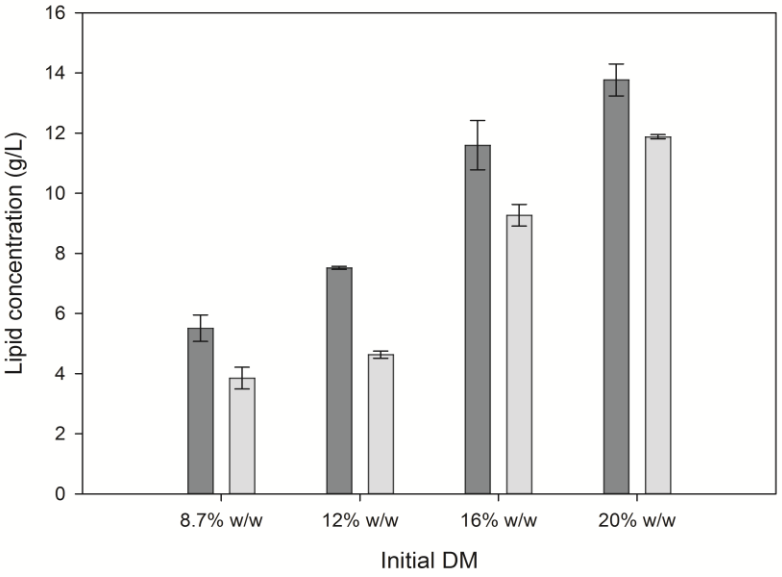

Figure 41: Effect of sweet sorghum concentration at a range between 8.7% w/w and 20% w/w on lipid production in the presence (dark grey) or absence (light grey) of enzymatic saccharification. Solids were removed prior to cultivation.

The lipid production obtained with *R. toruloides* during this work was higher than most of the lipid production reported in the literature when renewable resources have been used as raw materials (Table 13). Moreover during this work an enzymatic treatment of an energy crop was used which offers advantages over the acid hydrolysis of lignocellulosic materials (which is commonly used to prepare lignocellulosic hydrolysates), such as low energy

consumption due to the mild process requirements, high sugar yields, no requirement for detoxification and no unwanted wastes.

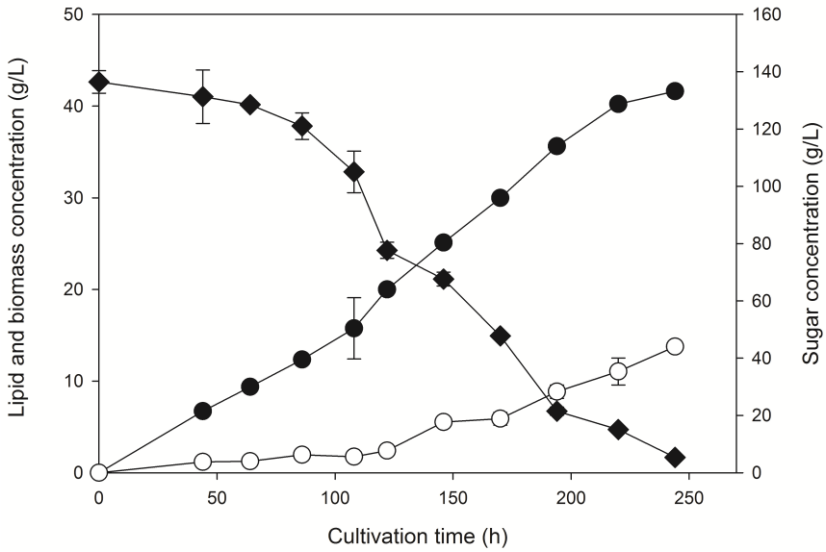

Figure 42: Time course of sugars consumption (♦), biomass (●) and lipid (○) concentration when *R. toruloides* was cultivated on sweet sorghum juice coming from 20% w/w sweet sorghum concentration.

Finally fatty acid profile of the lipids obtained when *R. toruloides* was cultivated on the juice obtained from 20% w/w sweet sorghum is shown in Table 14. The predominant fatty acid was oleic (55.78% w/w) followed by palmitic acid (29.18% w/w). As discussed previously, high concentrations of oleic acid are considered beneficial for the production of biodiesel. The percentage of unsaturated fatty acids is high (63.5% w/w) which makes the obtained lipids similar to the vegetable ones. The lipid composition obtained during this work had higher percentage of unsaturated fatty acids compared to the one reported by Hu et al. (2009), as the concentrations of oleic and

linoleic acid were lower and those of stearic and palmitic acid were higher. Slight differences were also observed with the composition obtained by Li et al. (2007) during the last stages of fed-batch cultivation. The lipids obtained had a slight lower concentration of unsaturated fatty acids compared to this work.

Table 13: Comparison of SCO production from renewable raw materials.

Microorganism	Raw material	Lipid concentration (g/L)	Reference
Rhodosporidium toruloides	Detoxified dilute sulfuric acid pretreated wheat straw	2.4	Yu et al., 2011
Yarrowia lipolytica	Detoxified hydrochloric acid pretreated sugarcane bagasse	6.7	Tsigie et al., 2011
Rhodotorula glutinis	Sulfuric acid hydrolyzed tree leaves	4.7	Dai et al., 2007
Trichosporon fermentant	Detoxified dilute sulfuric acid pretreated rice straw	11.5	Huang et al., 2009
Rhodotorula glutinis	Monosodium glutamate wastewater	5.0	Xue et al., 2008
Chlorella protothecoides	Sweet sorghum juice	2.9	Gao et al., 2010
Schizochytrium limacinum	Sweet sorghum juice	6.9	Liang et al., 2010
Trichosporon fermentans	Detoxified Sulphuric acid-treated sugarcane bagasse hydrolysate	15.8	Huang et al., 2012
Cryptococcus curvatus	Lime pretreated sweet sorghum bagasse	2.6	Liang et al., 2012
Cryptococcus curvatus	Dilute sulphuric acid pretreated sweet sorghum bagasse	4.3	Liang et al., 2014
Rhodosporidium toruloides	Juice from enzymatically saccharified sweet sorghum	13.8	This work

Table 14: Fatty acids of the obtained lipids during cultivation of *R. toruloides* on juice from 20% w/w liquefied sweet sorghum stalks.

Fatty acid	**% concentration (w/w)**
C14:0	0.71
C16:0	29.18
C18:0	6.56
C18:1 (n-9)	55.78
C18:2 cis12	7.68

3.6 Properties of the biodiesel that could come from the obtained lipids.

In order to evaluate the suitability of the yeast oils to serve as raw materials for biodiesel production, the properties of the corresponding biodiesel were predicted.

Cetane number (CN) is considered an important parameter of biodiesel and it is related to the ignition delay time and the combustion quality, with high values indicating better ignition properties (Meher et al., 2006). Also high values in CN are correlated with reduced NO_X exhaust emissions (Knothe et al., 2003) and helps to ensure good cold start properties (Ramos et al.,2009). CN should be above 47 in order to fulfill the requirements of the standard ASTM D 6751 for use in USA and above 49 to be used in E.U. (e.g. standard E DIN 51606) (Knothe et al., 2003). Finally, according to the UNE-EN 14214 standard CN should be above 51. In order to calculate the CN of the

obtained lipids, the CN of each fatty acid methy ester was calculated according to Krisnangkura (1986) by the following equation:

$$CN_{ME} = 58.1 + 2.8\left[\left(\frac{nc-8}{2}\right)\right] \times nDB$$

where *nc* represents the number of carbon atoms and *nDB* the number of double bonds of the each fatty acid. The CN of the lipids was determined as the weighted percentage of each of the methyl esters and its individual cetane number. All the three obtained oils had a CN above the minimum required by the international standards, with the one obtained from *L. starkeyi* presenting the highest value (Table 15).

Table 15: Predicted biodiesel properties of the obtained lipids.

Property	*L. starkeyi*	*T. fermentans*	*R. toruloides*
CN	62	55	60
DU	52	83	71
LCSF	6.7	4.6	6.2
CFPP (°C)	5	-2	3

Another important factor is the iodine number. Iodine number is the amount of I_2 consumed per 100g of substrate to achieve complete saturation (Lapuerta et al., 2009) and is used in order to calculate the degree of unsaturation of fatty acids. High iodine number correlates with high tendency in oxidation of the biodiesel. In order for an oil to serve as raw material for biodiesel, according to the EU standard UNE-EN 14214 iodine number should be below 120g I_2/100g biodiesel. In order to predict the iodine

number, the degree of unsaturation (DU) was calculated according to Ramos et al. (2009) by the follow equation:

$$DU = (wt.\%\ of\ monounsaturated) + 2(wt.\%\ of\ polyunsaturated)$$

As the same authors stated, there is a linear correlation between DU and iodine number which is presented in the Figure 42. Taking into account the degree of unsaturation (Table 15) all the 3 oils are below the standard and are suitable for the production of biodiesel (Figure 43).

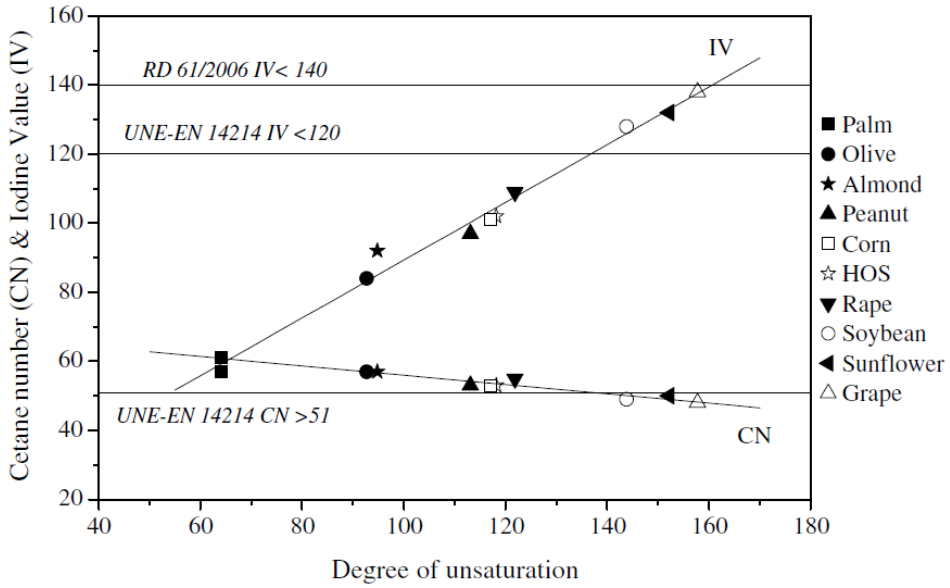

Figure 43: Correlation between DU with iodine number and CN. Values of oils from different sources were used to calculate this correlation.
Source: Ramos et al., 2009.

Finally, CFPP (Cold Filter Plugging Point) is an important property of biodiesel, indicating the ability of the biodiesel to be used at low temperatures. CFPP is calculated as the highest temperature where a certain volume of biodiesel, under standardized cooling conditions, fails to pass within a specific time frame through a standardized filtration device (Baptista et al., 2008).

Different countries have different standards depending on the environmental conditions. Moreover, CFPP values differ between winter and summer. In EU, during winter it can vary between -26°C (e.g. Estonia) to -5°C (e.g. Greece,) and during summer between -5°C (e.g. Estonia) to +5°C (e.g. Austria) (www.biofuelsystems.com). In order to calculate CFPP, the LCSF (Long Chain Saturated Factor) was calculated according to Ramos et al. (2009) by the following equation:

$$LCSF = 0.1 \times C16(wt.\%) + 0.5 \times C18(wt.\%) + 1 \times C20(wt.\%) + 1.5 \times C22(wt.\%) + 2 \times C24(wt.\%)$$

where wt.% is the amount of the specified methyl ester (in percentage). Finally, the CFPP values were calculated by the following equation (Ramos et al., 2009):

$$CFPP = 3.1417 \times LCSF - 16.477$$

The predicted values for the oils obtained during this work varied between -2°C and +5°C (Table 15). On the other hand CFPP can be improved with the addition of some commercial additives that disrupt the macrocrystalline formation or even with the addition of fatty acid ester derivatives (Torres et al., 2011). For example the commercial product Wintron Synergy® (Biofuel Systems Group LTD, Lancashire, England) is claimed to reduce the CFPP of rapeseed methyl esters, even at low concentration equal to 2% v/v, from -6°C to -24°C (http://www.biofuelsystems.com/other/wintron_synergy.pdf).

From all the above, it can be concluded that all the derived yeast oils could serve as raw materials for the production of biodiesel.

3.7 Evaluation of methane production from dried sweet sorghum stalks

It was previously demonstrated that sweet sorghum stalks are a suitable substrate for the production of both ethanol and microbial lipids that could be used for the production of biodiesel. During the last part of this work, the possibility of producing a gaseous fuel, i.e. methane, from dried sweet sorghum stalks through anaerobic digestion was evaluated as well. Thermophilic sludge was chosen for the digestion due to the advantages that it presents comparing to the mesophilic one (as was discussed in section 1.4).

3.7.1 Evaluation of different treatments on methane potentials

Both the soluble and insoluble carbohydrates present in sweet sorghum stalks can be used during anaerobic digestion for the production of methane. Despite the fact that the methane producing consortia can produce enzymes to hydrolyze insoluble carbohydrates (such as cellulose), in most cases the obtained methane yields are lower when the lignocellulosic materials are utilized without any kind of treatment (Jeihanipour et al., 2013; Vivekanand et al., 2013; Zheng et al., 2014). Application of a pretreatment (such as hydrothermal, dilute acid and steam explosion) could increase the digestibility of insoluble carbohydrates. On the other hand pretreatment of sugar crops like sweet sorghum, which contain high amounts of soluble sugars, can result in degradation of the sugar and in turn formation of inhibitors (such as furfural and HMF). For this reason the application of a physicochemical pretreatment at harsh conditions (for example high temperature or presence of acids) is not feasible. On the other hand, addition of hydrolytic enzymes could facilitate the hydrolysis of both cellulose and hemicellulose and in turn increase the methane yield.

During this work the enzymatic treatment was evaluated by employing a mixture of Celluclast 1.5L and Novozyme 188 at a ratio of 5:1 volumes, at the concentration that was previously found optimum for ethanol production. In order to evaluate the effect of enzymatic treatment, two different process configurations were evaluated, namely the 'one-step' and the 'two-steps' processes which resemble the SSF and SHF processes during bio-ethanol production from lignocelluloses.

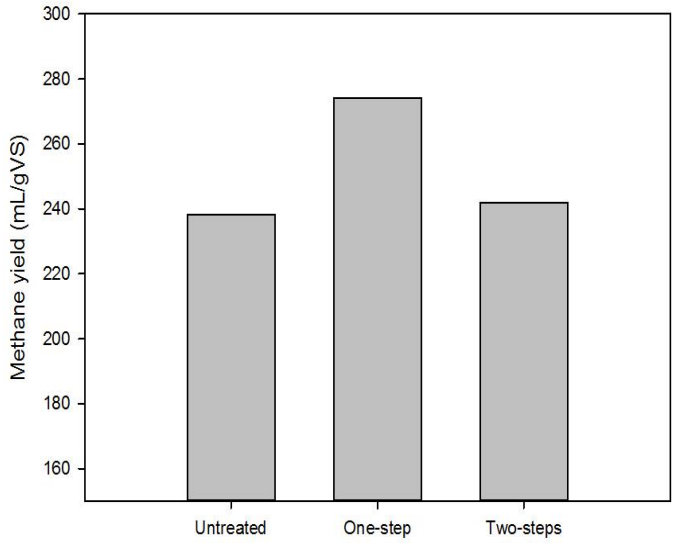

Figure 44: Effect of enzymatic treatment on methane yields. Enzymatic treatment was performed either in one step or in two steps.

When no treatment was applied to sweet sorghum stalks methane yield reached 238mL/g VS. In contrast, addition of enzymes improved the overall methane production yields (Figure 44).It is worth noticing that when a two-step process configuration was applied the increase of methane yield was only 1.7%, whereas during the one-step process the increase was 15.1% reaching a methane production of 274mL/g VS. This is most likely due to the higher

initial sugar concentration in the start-up of anaerobic digestion stage during the two-step process, which could result in the production of higher amounts of volatile fatty acids (VFAs). The presence of VFAs could result in lowering the pH below optimal which has a negative impact in methane production (Zhang et al., 2013; Chen et al., 2008).

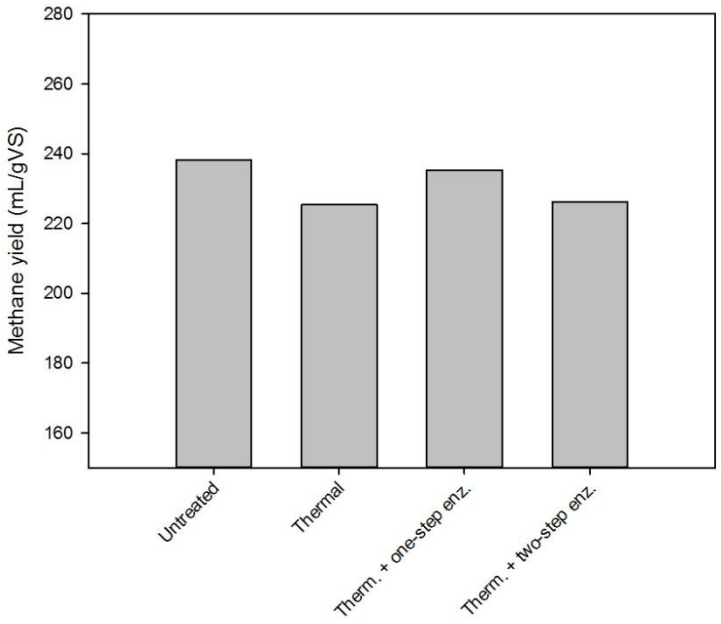

Figure 45: Effect of thermal treatment on methane yield with or without the combination of enzymatic treatment.

In the next stage the possibility of applying a hydrothermal pretreatment was evaluated. As previously mentioned high temperatures could result in degradation of sugars and formation of inhibitory compounds. For this reason a mild thermal pretreatment (1h at 105°C) was applied, without the addition of any acid or basic catalyst which could lead to severe degradation of soluble sugars. It was previously reported that a thermal pretreatment under mild conditions could enhance methane yield from sweet sorghum stalks

(Antonopoulou and Lyberatos, 2013). However the methane yield obtained during this work was 5.46% less compared to the untreated one, resulting in a methane production of 225mL/gVS. This could be attributed to a minor degradation of soluble sugars and formation of inhibitors. The addition of enzymes improved the methane production but the overall yield was less compared to the yield obtained with the untreated sweet sorghum (Figure 45). Finally, the same negative effect of the two-step process was also observed during utilization of thermally pretreated sweet sorghum stalks.

3.7.2 Evaluation of the combined effect of enzyme loading and I/S ratio on methane production

From all the above, it can be concluded that enzymatic treatment at 'one-step' process configuration of non-thermally treated sweet sorghum is necessary to achieve high yield of methane production. The I/S (Inoculum to Solid) ratio is considered to be a very important parameter during anaerobic digestion (Liu et al., 2009; Neves et al., 2004). Low ratio could result in inhibition of anaerobic digestion due to the accumulation of VFAs (Kafle et al., 2014), which is a result of the imbalance between the acidogenic and methanogenic stage (Adu-Gyamfi et al., 2012). On the other hand, low ratios means that the solids content is higher which in turn results in higher total methane production per volume of sludge, which is very important for the economic viability of the process. Therefore it is of great importance to determine the lowest I/S ratio at which the methane yield is not decreasing and at the same time the total production of methane is high.

In order to evaluate the limits of solids that the sludge is able to digest at high yield, microcrystalline cellulose was used as substrate at different I/S ratios (2, 0.67 and 0.33). Figure 46 represents the results of methane yield and

total methane production per L of sludge. With decreasing I/S ratio down to 0.67, the methane is increasing reaching a value of 341mL/gVS. Further decrease of the ratio results in slight decrease of the methane yield, which still remains higher than with an I/S ratio 2. It can be concluded that the sludge is capable of digesting materials at low I/S ratios resulting in higher overall total methane production, which during these experiments increased from 2.1L CH_2/L to 10.2L CH_2/L when I/S ratio is decreased from 2 to 0.33.

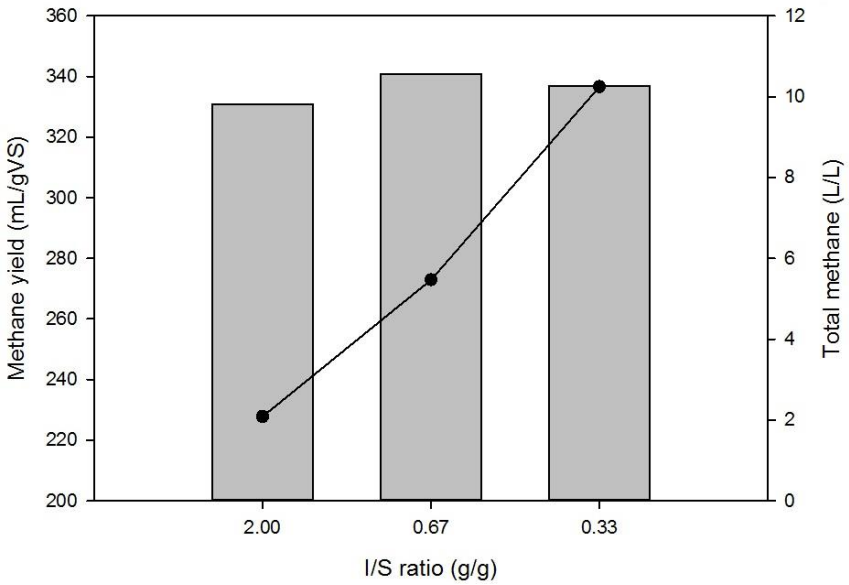

Figure 46: Effect of I/S ratio in methane yield and total methane production when avicel cellulose was used as substrate. Bars represents the yield, whereas dots the total methane.

Subsequently the combined effect of enzyme load and I/S ratio was evaluated by response surface methodology according to Circumscribed Central Composite (CCC) design. The 11 experimental combinations from

the experimental design are represented in Table 16 and were performed in duplicates.

Table 16: Codded and actual values of the experimental design.

Treatment	Coding values (X_1= Enzyme load, X_2= I/S ratio)		Actual values (X_1= Enzyme load, X_2= I/S ratio)	
	X_1	X_2	X_1	X_2
1	-1	-1	3	0.7
2	1	-1	13	0.7
3	-1	1	3	3.3
4	1	1	13	3.3
5	-1.414	0	0.93	2
6	1.414	0	15.07	2
7	0	-1.414	8	0.16
8	0	1.414	8	3.83
9	0	0	8	2
10	0	0	8	2
11	0	0	8	2

The results of the duplicate experiments are presented in Table 17. During the initial fitting of the quadratic model to the obtained results it was found that the R^2 was 0.495, whereas the Q^2 was -0.067, values that indicate that the model was not adequate enough to describe the experimental values and predict values of new experimental combinations. For this reason the values of the experiments at the combination 8FPU/g sweet sorghum and 0.16g/g I/S

ratio were excluded, as the methane production was inhibited (Table 17). The obtained model is described by the equation:

$$Met = 278.708 + 0.716472 \cdot \chi_1 - 16.9907 \cdot \chi_2 + 0.014611 \cdot \chi_1^2 - 1.58786 \cdot \chi_2^2 + 0.594616 \cdot \chi_1 \cdot \chi_2$$

The R^2 was improved to 0.886 and the Q^2 to 0.762 indicating that the model is capable of fitting the experimental data and efficiently predicting new data. Two additional factors that describe the efficiency of a model are the model validity and the reproducibility. For the model obtained during this work both of them were high, i.e. 0.735 and 0.849 respectively. Finally, two diagnostics tools were employed to verify the adequacy of the model to fit experimental data, namely the normal probability plot of residuals and the relationship between predicted and experimental data (Figure 47). Normal probability plot of residuals is made by plotting the observed residuals are plotted against the expected values (Prakash Maran et al., 2013) and is used to evaluate the normality of the residuals as well as to detect outliers. Whereas plot of experimental obtained data versus predicted ones indicates the efficiency of the model to describe the obtained experimental results. The model obtained during this work is sufficiently describing the experimental result, as the values of the data are fairly close to the linear line. This can also be observed in Table 17 where the experimental and predicted values for the duplicate experiments are given.

Table 17: Experimental obtained and predicted methane yields.

Treatment	Met (Methane yield, mL CH_4/gVS) experimental		Met (Methane yield, mL CH_4/gVS) predicted
	A	B	
1	267.15	273.02	269.57
2	277.92	290.82	283.23
3	210.68	225.40	213.51
4	256.48	239.09	242.64
5	229.42	246.11	240.16
6	274.20	260.08	270.41
7	47.11	49.86	-
8	201.86	216.86	215.03
9	242.05	256.17	254.56
10	257.15	249.21	254.56
11	259.60	263.16	254.56

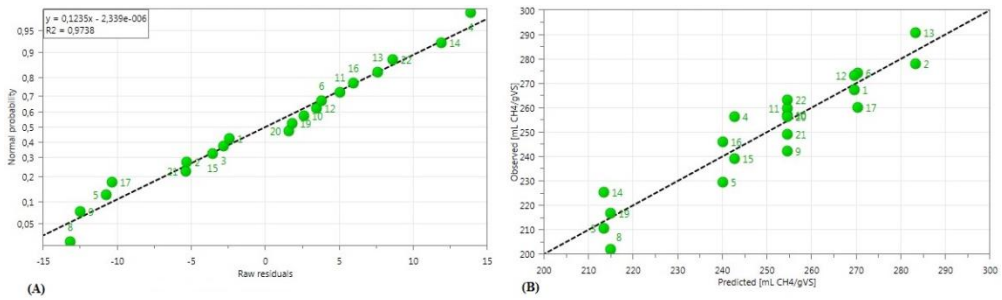

Figure 47: Diagnostic tools for model evaluation. (A) Residual normal probability and (B) Plot of observed values against predicted.

The resulting response surface and contour plot of the model is shown in Figure 48. It can be observed that low I/S ratio in combination with higher enzyme loadings lead to increased methane yields, where the yields are more affected by the I/S ratio than by the enzyme load. As previously discussed it is important to find the lowest I/S ratio at which the methane yield remains high, in order to increase the total methane production. During this work the highest methane yield (284.37mL CH_4/gVS) was achieved at a low I/S ratio equal to 0.7 with the addition of 13FPU/g resulting in a total production of 4.7L CH_4/L.

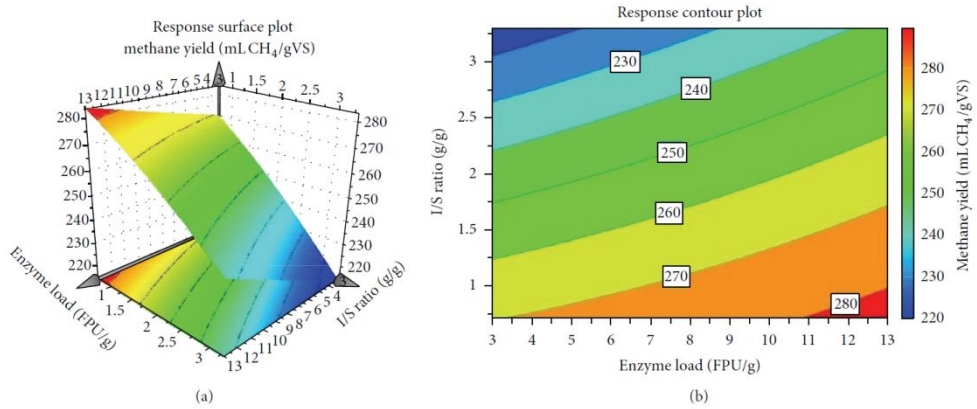

Figure 48: Response surface (A) and contour (B) plots of the methane yield at different combinations of enzyme load and I/S ratio.

4. CONCLUSIONS

The main purpose of this thesis was the evaluation of the potential uses of sweet sorghum stalks for the production of high added value products. Initially, an approach where soluble and insoluble carbohydrate fractions were separate utilized was evaluated. The potential of using high dry matter sweet sorghum bagasse for the efficient production of ethanol was demonstrated. Prior to fermentation, the optimization of the hydrothermal pretreatment of sweet sorghum bagasse resulted to a material with high cellulose content (66.84% w/w). During hydrolysis trial 30.42% of the initial cellulose was converted to glucose. The high cellulose composition of the material and the high yield in hydrolysis is very beneficial for the subsequent ethanol fermentation processes. Two different approaches were evaluated for ethanol fermentation (SSF and SHF) where during SHF configuration ethanol production was almost double compared to the SSF. Concerning the duration of the hydrolysis, 12h was proven to be adequate for efficient cellulose conversation, as the extension of the hydrolysis to 24h did not increased the glucose and ethanol concentrations. Finally, addition of extra enzyme load favors cellulose hydrolysis and ethanol production.

In a next step, a different approach of ethanol production was evaluated. The ability of using high dry material content of dried stalks for the efficient ethanol production was demonstrated. Incorporation of a distinct enzymatic hydrolysis step is necessary in order to increase both ethanol concentration and productivity. This step resulted in the rapid decrease of slurry's viscosity which in turn permitted the application of submerged fermentation. The combined effect of enzyme load and the duration of saccharification of 35% w/w solids were optimized, resulted in high ethanol production (62.53 g/L) with high

ethanol productivity (2.98 g/L·h). Compared with the approach where only the lignocellulosic fraction is used for ethanol production; utilization of whole stalks is proven to be more efficient, as more ethanol was produced with high volumetric productivity. For this reason, the possibility of using dried sweet sorghum stalks for the production of other forms of biofuels was evaluated.

The potential of producing microbial lipids from dried sweet sorghum stalks was evaluated. Three different yeasts, namely *L. starkeyi* CBS 1807, *T. fermentans* CBS 439.83 and *R. toruloides* CCT 0783, were evaluated for their ability to grow on saccharified stalks and accumulate lipids. Due to the presence of nitrogen (in form of proteins) in the stalks, the C:N ratio is not high enough for efficient lipid production. On the other hand, due to the form of the nitrogen source (proteins) not all of it is available for the yeast. Nevertheless, supplementation of the stalks with low amounts of external nitrogen source had a negative effect on lipid production from all the yeasts, indicating that the amount of proteins in the stalks is sufficient to support yeast growth. This fact also underpins the importance of rising the C:N ratio by increasing the sugar concentration. Incorporation of a distinct enzymatic saccharification step resulted in increased lipid production at all initial solids contents with all the yeasts. Removal of solids also had a positive effect in the lipid production in all the yeasts, except from *T. fermentans*. The highest obtained lipids were 13.77g/L when *R. toruloides* was cultivated in sweet sorghum juice that came from 20% w/w solids. From the fatty acid profile analysis of the obtained lipids, it was concluded that all of them present the appropriate characteristics to be suitable for the production of biodiesel. As a general conclusion, the ability of dried sweet sorghum stalks to be used as raw material for the cultivation of several yeast species was demonstrated. Moreover, stalks could serve both as carbon and nitrogen source, decreasing this way the cost of biodiesel production.

Apart from the liquid biofuels, during the last part of this work the potential of producing a gaseous biofuel (methane) was also demonstrated. Enzymatic treatment resulted in increased methane yields, whereas when a mild thermal treatment was incorporated it had a negative effect on the yields. One-step enzymatic treatment configuration resulted in higher methane production comparing to the two-step. Finally, the combined effect of enzyme load and I/S ratio was evaluated resulting in higher yields and total methane production.

As a general conclusion of this thesis, the ability of using sweet sorghum as a flexible substrate for the production of biofuels was demonstrated. Both liquid (ethanol and biodiesel) and gaseous (methane) biofuels were produced. A flexible process that can be switched between different kinds of biofuels is very favourable, as the production can be focused in the specific needs of the market at that time.

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