Optimized co-fermentation of glucose-xylose mixtures by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture for mathematical modelling

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Submitted by

Varsha Singh
(2K15/IBT/15)
Delhi Technological University, Delhi, India

under the supervision of

Dr. Kriti Bhandari

(Assistant Professor)

&

Prof. D. Kumar

(Head of Department)

Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road,
Delhi - 110042, India

CERTIFICATE

This is to certify that the project entitled **"Optimized co-fermentation of glucose-xylose mixtures by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture for mathematical modelling"** submitted by **Varsha Singh (2K15/IBT/15)** in partial fulfilment of the requirement for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under our guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Date: 12-07-2017

Dr. Kriti Bhandari
Project Mentor
Department of Biotechnology
Delhi Technological University

Prof. D. Kumar
Co-Supervisor
Head of Department
Department of Biotechnology
Delhi Technological University

DECLARATION

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Place: **Varsha Singh**

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VARSHA SINGH

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LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

Abbreviation	Expansion
IRENA	International Renewable Energy Agency
C_5	5-Carbon sugar (pentose)
C_6	6- Carbon sugar (hexose)
GHG	Green House Gases
gpy	Gallons per year
FFV	Flexible Fuel Vehicle
LCB	Lignocellulosic Biomass
DDGS	Distiller's Dried Grains with Solubles
1G	First Generation
2G	Second Generation
VHG	Very High Gravity
EtOH	Ethanol
HMF	Hydroxyl-methyl Furfural
DO	Dissolved Oxygen
CDW	Cell Dry Weight
CFU	Colony Forming Units

ABSTRACT

Economical production of ethanol from bio-based processes requires the utilization of both hexose and pentose sugars. Although conversion of the sugar mixture (available in different lignocellulosic feedstock) into ethanol is being carried out by various strains of the same or different organisms in co-culture system, very few models are available to predict the fate and the interaction between these microorganisms during fermentation.

In this study, mathematical models are proposed to predict the dynamics involved in the co-culture of hexose fermenting *Saccharomyces cerevisiae*(wild strain) and pentose fermenting *Scheffersomyces stipitis*(NCIM 3507) in fermentation media containing glucose and xylose, under microaerophilic conditions. The models were statistically validated conducting t-test and evaluating indices of agreement. Wilmott's index of agreement for the models predicting the concentration profiles of *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, glucose, xylose, ethanol and oxygen concentrations were 0.867, 0.929, 0.980, 0.995, 0.910 and 0.850, respectively, satisfying the criteria for 'very high' agreement between model-predicted and experimental values.

The co-culture models described in this work can prove to be a valuable tool for quick optimization of *S. stipitis* and *S. cerevisiae* co-culture for ethanol production from lignocellulose efficiently and in a sustainable way so that it can be implemented to encounter the economic necessities of the lignocellulosic ethanol industry.

INTRODUCTION

Over the past few years, ethanol has enticed a great deal of attentiveness as a potential substitute to fossil fuels. Presently, most of the ethanol production is done from raw materials like corn, sugar cane and beets, and molasses in India. However, these raw materials create a situation of food and fuel competition and not considered ethical or economically sustainable. The negative effects of using food crops as raw material is not only that it competes with food production straight (like in the case of corn) but also it competes with food production for water and land. Therefore, lignocellulosics including agricultural residues, municipal wastes, waste paper, and energy crops can be considered as the most reliable source for ethanol production. Advantages like renewability, easy availability, low prices (comparative to grain or sugar) large quantities, and environmental friendliness has made lignocellulosic biomass second to none as a raw material for ethanol production.

Second generation ethanol production from lignocellulose consists of four steps: pretreatment, saccharification or hydrolysis, fermentation and purification. A limitation to the growth of bioethanol usage is its production cost. According to the IRENA report (2013) [1], fermentation step alone is responsible for 23% of the equipment costs and 16% of the total costs. An economical lignocellulosic ethanol production process is possible if 3 of the following requirements are fulfilled:

- Efficient methods of pretreatment of lignocellulose so that cellulose and hemicellulose can be exposed.
- Hydrolytic enzymes incorporated in saccharification step should be available at low cost (cost-effectiveness and availability of these enzymes like cellulases, amylases, etc.)
- Utilization of both hexose and pentose sugars either by a single organism that can convert both the sugars into ethanol or by using a combination of two or more strains, one strain capable of converting one sugar.

If the abovementioned objectives can be implemented into the process of ethanol production, the sugar can be converted to ethanol at high rates, high final ethanol concentrations and large quantities.

Hydrolysis of pre-treated lignocellulosic material yields a mixture of sugars: Hexoses (C6) and Pentoses (C5). Two leading sugars in lignocellulosic hydrolysates are glucose and xylose.

Fermentation of the hydrolysate obtained after saccharification of pre-treated lignocellulose is carried out by microorganisms; *Saccharomyces cerevisiae* (yeast) and *Zymomonas mobilis* (bacteria) being the most used ones. However these microbes naturally ferment hexoses only but not pentose sugars thereby resulting in a lower yield of ethanol from lignocellulose. Among various yeast strains such as *S. cerevisiae, S. uvarum, Schizhosaccharomyces pombe, Candida shehatae* and *Scheffersomyces stipitis; S. stipitis* gave maximum ethanol yield proving that it has the ability to ferment pentoses. Therefore, co-culture of hexose fermenting and pentose fermenting microorganisms can enhance the yield of ethanol.

Fermentation of hexoses and pentoses has been carried out together in a single process for a very long duration by the co-culture of various microorganisms. Hexose-fermenting *S. cerevisiae* and pentose-fermenting *S. stipitis* have been cultured separately in reactors in series to get complete conversion of glucose as well as xylose because it was observed that pentose intake in *S. stipitis* was competitively inhibited by hexoses. Therefore co-fermentation of xylose and glucose when done with immobilized *S. stipitis* and *S. cerevisiae*, was found to improve xylose conversion. Also, the diauxic growth found in *S. stipitis* and the competition for oxygen between *S. stipitis* and *S. cerevisiae* led to the development of a process where *S. cerevisiae* would be inactivated with heat prior to xylose fermentation [2]. However, co-culture of *S. cerevisiae* OVB11 and *S. stipitis* when carried out for ethanol fermentation of hydrolysate from rice straw, produced maximum ethanol concentration at 36 hours and declined after that. The productivity and yield were 0.33 g/l/h and 0.4 g/g respectively and the fermentation efficiency was 95% [3]. Suriyachi et al. (2013)[4] optimized the conditions for simultaneous saccharification and co-fermentation involving co-culture of *S. cerevisiae* and *S. stipitis* using design of experiments.

Several kinetic models are available for ethanol fermentation mediated by pure cultures of *S. cerevisiae* or *S. stipitis.* Since co-culture systems are complicated in terms of the dynamics involved, few literature are available for the same. Developing mathematical models that can predict the interactive behaviour between the fermenting microorganisms, as well as its kinetics can be advantageous for an efficient lignocellulosic ethanol production process. A dynamic flux balance model was created for the co-culture of *S. cerevisiae* and *E. coli* in a glucose-xylose mix by Hanly et al. in 2012[5]. A similar model was developed by Hanly and Henson (2013)[6] for the co-culture of *S. cerevisiae* and *S. stipitis*. Unrean and Khajeeram (2015)[7] have developed a kinetic model for the co-culture of *S. cerevisiae* and *S. stipitis* to optimize the initial biomass ratio for lignocellulose fermentation. These models, however, do not consider

the dependence of biomass growth on concentrations of dissolved oxygen. The growth of *S. stipitis* follows Monod model with dependence on the dissolved oxygen. The aim of this study is to develop and statistically validate mathematical models for predicting the dynamics of biomass, substrate and ethanol, for the co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in a glucose-xylose mix under microaerophilic batch fermentation conditions.

REVIEW OF LITERATURE

3.1. BIOETHANOL: A SUSTAINABLE FUEL

The world's present economy is highly dependent on various fossil energy sources such as oil, coal, natural gas, etc. These are being used for the production of fuel, electricity and other goods [8]. Presently, more than 80% of the total global energy demand is obtained from fossil fuels, of which 58% alone is spent by transport sector [9].

Excessive consumption of the conventional fuels have resulted in three major challenges: (1). the non-renewable fuels are being exhausted at a very fast rate in order to fulfil the increasing demand of all kinds of fossil fuels stemmed due to motorization and industrialization of the world [10]. But the fossil fuel reserves are inadequate and estimated to be exhausted in next 40-50 years, (2). Growing consumption of fossil fuels contribute to the emission of the greenhouse gases and global warming subsequent in the upsurge of sea levels, loss of diversity and urban pollution [11]. (3). Political crisis has led us to an imbalance in oil demand and supply resulting in import of fossil fuels. This has led us to a re-think of our dependence on fossil fuels, since such a crisis is unsettling to the energy sector of both the developed and the developing country [12]. Therefore, it is essential to find out a substitute of fossil fuels for our industrial economies and consumer societies. The substitute should be renewable, efficient and cost-effective with lesser emission of greenhouse gases [13].

Gasoline and petroleum have been the dominant transportation fuel for a century with a few noticeable deleterious effects. Biofuels are extensively seen as alternative to fossil fuels to offset forthcoming decline of oil production and to mitigate the nascent increase in GHG emissions. Ethanol can be a sustainable replacement of these conventional fuels because of its ease of production and lesser toxicity.

Ethanol as a fuel gained importance in the 1970's, when the cost of production dropped in line with the gasoline and oxygen addition to the fuel was required by the air quality regulations [14]. Ethanol production capacity has increased to 109% since 2000 and is growing at a rate of 3.5 billion gallons per year (gpy). Despite of this, the ethanol production by volume is only 2% of gasoline and 1.7% of petroleum in US [15]. Currently, there are five fuel ethanol producers across the country, producing 1.52 million tons of fuel ethanol annually from starch-based feedstock including corn, wheat and cassava.

3.1.1. Ethanol as Fuel

Ethanol, also called ethyl alcohol, is an organic compound and the simplest primary alcohol. 170 million gpy of industrial ethanol is produced via synthetic route in US which represents over 60% of ethanol used for manufacturing [16]. Ethanol can either be used as a fuel or as a blend with other fuels like gasoline. Without mutilation, unmodified engines can resist up to 30% ethanol blended in gasoline, but rubber and plastic components of engine can deteriorate over time at higher concentrations of ethanol in gasoline.

Ethanol has a low energy density when used in combustion because it is 30% oxygen by weight. Pure ethanol has an octane rating of 113 thereby increasing the horsepower and prevent knocking. Ethanol is added to fuels to add oxygen to the fuel because oxygenated fuel burns cleaner, producing lesser carbon mono oxide and particulates. Pipelines cannot be used for ethanol distribution because ethanol, being hydroscopic, can get contaminated with latent water. To avoid this problem, ethanol is splash blended in tankers before transport to service stations [17].

Ethanol has been shown to have potential of reducing many air pollutants like particulates and benzene [18]. Ethanol in blend with gasoline is traded at two different grades: E10 and E85. The former, E10, is required at locations where oxygenated fuel is needed and sold as reformulated gasoline. It has been considered to be ozone neutral because the fuel reduces carbon mono oxide emissions. E85 is 85% ethanol in gasoline and this can be used in "Flexible Fuel Vehicles" (FFVs) [19]. E85 blend is hard to find and hence most FFVs run on 0 to 10% ethanol blends without any damage. Ethanol has many advantages as a fuel over other conventional fuels and its production is sustainable at levels that demand widespread adoption. Bioethanol cannot be simply regarded as a renewable source of energy but many thermodynamic analysis has provided a comprehensive indication of energy related sustainability of the biofuel techno-system [20].

3.2. FEEDSTOCKS FOR ETHANOL PRODUCTION

The process of ethanol production was fully explained till the mid-18th century, however, the method of fermentation and distillation to produce ethanol has been used for thousands of years. The process of production of ethanol by biological route at industrial scale have been optimized in decades of research.

Anaerobic breakdown of sugars by yeast or bacteria to produce ethanol and carbon dioxide is called fermentation and represented by the reaction:

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$ …….. (1) ($\Delta G° = -227$ kcal/mol)

Fermentation must proceed in the lack of oxygen because the respiration of glucose releases much more energy and hence preferred by the organism.

$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2$ …….. (2) ($\Delta G° = -686$ kcal/mol)

The form of energy that is used by a cell is ATP (Adenosine Triphosphate), and only 15 kcal of energy released during fermentation is used by the cell as ATP (showing an efficiency of 6.6% as compared to 39.4% in respiration).

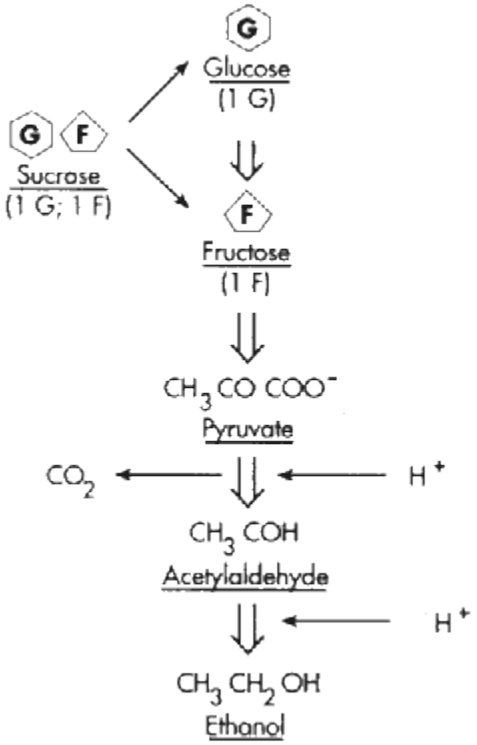

Figure 1. Ethanol production in living cells

Ethanol is produced by fermentation of sugars which can be obtained from biomass. Feed-stocks containing carbohydrates can be broken down into free fermentable sugars for the production of ethanol. These feedstocks can be divided into 3 major groups: Sugar (containing sugar crops, like sugarcane, and by-products of sugar refineries), Starchy crops (like corn) and Lignocellulosic biomass (2^{nd} generation ethanol). The abovementioned groups of feedstocks differ from each other significantly in terms of retrieval of sugar solutions [13].

3.2.1. Overall process of bioethanol production

Ethanol production is a multi-step process that depends on the raw material being used for the production. The three major steps involved in the production of ethanol can be summarized as follows:

1. Obtainment of fermentable sugars in a solution.
2. Fermentation – production of ethanol from sugars.
3. Purification of the ethanol obtained.

Figure 2. Overall process of ethanol from cellulose

One or more of the above mentioned steps can be combined in the process of ethanol production depending upon the type of raw material (feedstock) being used and the technology for conversion. Biomass is stored in warehouses in the ethanol plant where it is conditioned so that bacterial contamination can be avoided and early fermentation does not occur. Depending on the biomass, an additional step called pretreatment is used to extract the carbohydrates and make them more accessible to fermentation. The hydrolysate (liquid obtained after the saccharification or hydrolysis of the carbohydrates and containing the sugars) is subjected to fermentation along with the yeast and other nutrients for the growth of yeast.

The mode of fermentation can be either batch, fed-batch or continuous (in a very few cases). The cell densities can be made high by recycling or immobilizing the yeasts in order to enhance the fermentation productivity and make the process of ethanol production efficient [21].

Depending on the species of the yeast, composition of hydrolysate, cell density and other parameters, the fermentation lasts from 6 h to 72 h at temperatures between 25.8 to 30.8 degrees Celsius. At higher concentrations of ethanol (above 8-14% in broth) the activity of the yeast is

inhibited. The last step after the production is distillation which is used to purify and concentrate the ethanol. In this step, anhydrous ethanol containing 99.6% alcohol and 0.4% water is obtained from hydrated azeotropic mixture of 95.5% alcohol and 4.5% water. The remaining flow from the distillation is called vinasse, or stillage. The co-products obtained from the column may include products for feeding animals, fertilizers, etc. [22].

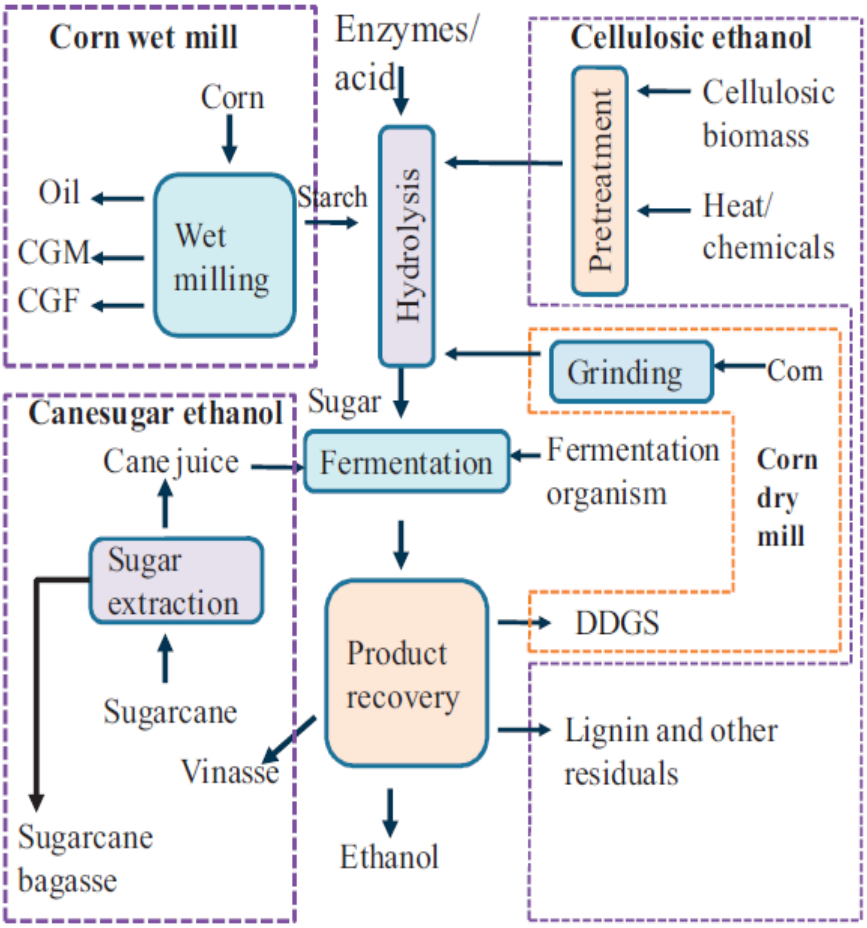

Fig. 3. Schematic representation of production of ethanol from cane sugar, corn, and cellulosic biomass.

Sugar can be directly extracted from sugarcane, and the residual bagasse is used as a boiler fuel to provide much of the energy for the extraction and ethanol production and recovery operations. In a corn dry mill, corn is ground, and enzymes and heat are added to hydrolyze

starch to sugars for conversion to ethanol, while the oil, protein, and fiber in corn are recovered after fermentation as an animal feed known as DDGS. Wet mills first fractionate corn to separate corn oil, corn gluten meal (CGM), and corn gluten feed (CGF) to capture value for food and animal feed, and the starch can then be hydrolyzed to sugars for fermentation to ethanol. For cellulosic biomass, heat and acids or enzymes hydrolyze the hemicellulose and cellulose portions to release sugars that can be fermented to ethanol, and the lignin and other remaining fractions can be burned to provide all the process heat and electricity for the conversion step with excess electricity left to export [21].

3.2.2 Ethanol from various sources

Ethanol is produced from raw materials that contain carbohydrates or other complex polysaccharides containing sugar. The agricultural raw material can be classified in different generations:

First Generation (1G) Bioethanol

When a raw material containing sugar (and starch) is used for ethanol production, it falls under the category of first generation biofuel. Sugars directly yield ethanol when subjected to fermentation. First generation raw material includes sugarcane, molasses, sugar beet, and fruits. These category of biofuels does not require processes like milling, pretreatment, hydrolysis and detoxification. Starchy materials like corn, wheat, cassava, etc. are also used as a raw material for the production of 1G ethanol.

Second Generation (2G) Bioethanol

Second generation of biofuels are produced by using agricultural raw material that contains complex polysaccharides like cellulose, hemi-cellulose which can be further broken down into simple sugars thereby yielding ethanol on fermentation. For the production of 2G ethanol, the raw material needs to undergo processes like milling, pretreatment, and hydrolysis are required. Sometimes an additional step of detoxification might be required, when a toxic substrate is fed into the bioreactors or a toxin is produced during the reaction. The step preceding fermentation process is the main difference between the production processes of different generations of ethanol from simple sugar, starch or lignocellulosic material [23].

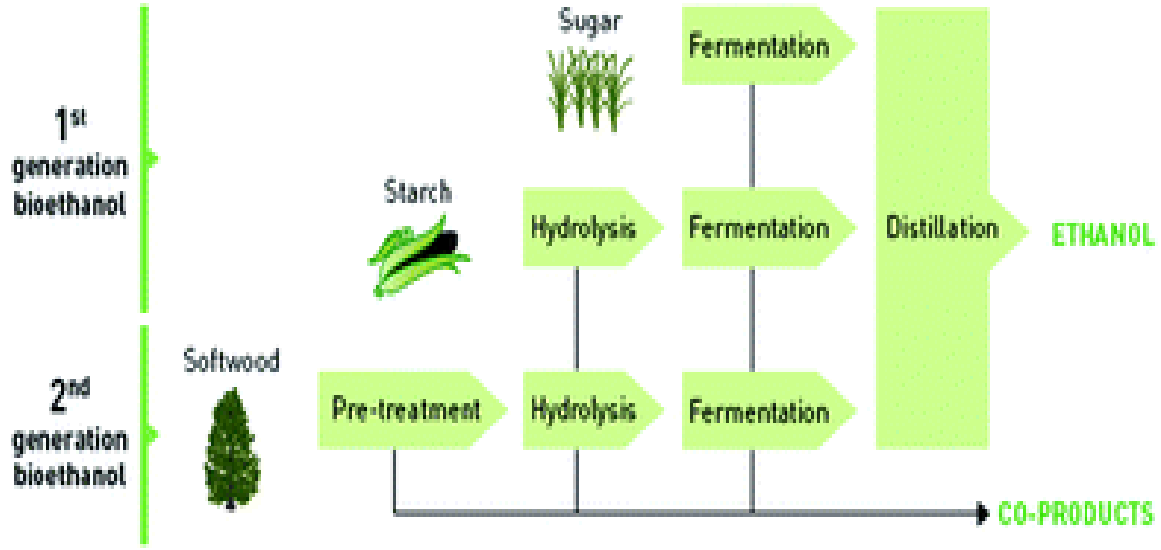

Figure 4. Different generations of bio-ethanol

3.2.2.1 Ethanol from Sugars

Main feedstocks for ethanol production from sugars are sugar cane, sugar beet and molasses (by-product of sugar mills). In Brazil, almost about 79% of the ethanol is produced from fresh sugar cane juice and the rest of it is produced by using the cane molasses [24]. Presently in India, sugar cane juice is not being used for ethanol production. Instead sugar cane molasses is majorly used as a raw material [25].

Saccharomyces cerevisiae has high capability to hydrolyse the cane sugar (sucrose) into glucose and fructose which can be further fermented to ethanol. Hence, *S. cerevisiae* is the most employed organism for ethanol fermentation. Although saccharomyces has the ability to grow under anaerobic conditions, aeration is an important factor for growth of the organism and ethanol production. Under anaerobic conditions, the salts and other compounds can pose a negative influence on the fermentation. The molasses is conditioned so as to neutralize the inhibitory effects of the medium component on fermentation. The fermentation media should be supplemented with nutritional factors that promote the growth of yeast. The conversion of non-fermentable substances into assimilable compounds for improving the alcoholic fermentation can be achieved with the addition of some commercial enzymatic complex of amylases, cellulases and amylopectinases. On the other hand, according to Maye (2006) [26], addition of a minimum inhibitory concentration of hop acids to molasses will stop bacteria growth and avoid the need of antibiotics along with the enhanced yield of ethanol.

Batch and semi-continuous processes

The Melle-Boinot process is the typical process for fuel ethanol production by batch fermentation. This process comprises the weight and sterilization of feedstock, followed by the adjustment of pH with H_2SO_4 and of the degrees Brix to values of 14–22. Obtained wort is fermented by yeasts. The produced wine is decanted, centrifuged and sent to ethanol separation stage, whereas the yeasts are recycled to the fermentation in order to reach high cell concentration during cultivation [27].

Fed-batch culture implies low levels of substrate concentration during the course of fermentation, while ethanol is accumulating in the medium. This type of cultivation regime, along with the cell recycling, is the most employed technology in Brazil for bioethanol production due to the possibility of achieving higher volumetric productivities.

Control of the flow rate of medium feed is quite advantageous because the inhibitory effect caused by high substrate or product concentrations in the fermentation broth can be neutralized. It was observed that the addition of sucrose in linear or exponentially decreasing way leads to 10–14% increase in ethanol productivity [28].

Continuous processes

The design and development of continuous fermentation systems have allowed the implementation of more cost effective processes. Continuous processes have several advantages compared to conventional batch processes mainly due to the reduced construction costs of the bioreactors, lower maintenance and operation requirements, better process control, and higher productivities (Table 1). For those very reasons, 30% of ethanol production facilities in Brazil employ continuous fermentation processes [29]. Most of these advantages are due to the high cell concentration found in these processes. Such high densities can be reached by immobilization techniques, recovery and recycling of cell biomass, or control of microbial growth. The major drawback is that yeasts cultivated under anaerobic conditions during long time diminish their ability to synthesize ethanol. In addition, at high dilution rates enabling elevated productivities, the substrate is not completely consumed and yields are reduced.

Aeration also plays an important role during continuous cultivation. Cell concentration, cell yield from glucose, and yeast viability may be enhanced by increasing air supply whereas ethanol concentration decreases under both microaerobic and aerobic conditions. Cell growth inhibition by ethanol is reduced at microaerobic conditions compared to fully anaerobic cultivation and specific ethanol productivity is stimulated with the increase of oxygen percentage in the feed [30].

Regime	*Configuration*	*Ethanol conc. In broth, g/L*	*Productivity g/L/h*	*Yield %of Theor. Max.*
Batch	Reuse of yeast from previous batches; yeast separation by centrifugation	80-100	1-3	85-90
Fed-Batch	Stirred tank with variable feeding rate (exponential dependence with time)	53.7-98.1	9-31	73.2-89
Repeated Batch	Stirred tank; flocculating yeast; up to 47 stable batches	89.3-92	2.7-2.52	79.5-81.7
Continuous	CSTR; flocculating yeast; residence time 3-6 h; cell recycling by centrifugation	70-80	5-20	94.5
Continuous removal of EtOH	Removal by vacuum; cell recycling	50	23-26.7	----

Table 1. Some fermentation processes for ethanol production from sugar cane molasses using *S. cerevisiae*

3.2.2.2 Ethanol from starch

Starch is a high yield feedstock for ethanol production, but its hydrolysis is required to produce ethanol by fermentation. Information has been provided about the effects of operating conditions on the enzymatic hydrolysis of corn starch using commercial a-amylase. The product of this first step, called liquefaction, is a starch solution containing dextrines and small amounts of glucose.

Ethanol production from corn

Corn is milled for extracting starch, which is enzymatically treated for obtaining glucose syrup. Then, this syrup is fermented into ethanol. There are two types of corn milling in the industry: wet and dry. During wet-milling process, corn grain is separated into its components. Starch is converted into ethanol and the remaining components are sold as co-products. During dry-milling, grains are not fractionated and all their nutrients enter the process and are concentrated into a distillation co-product utilized for animal feed called Dried Distiller's Grains with solubles (DDGS).

Fermentation is performed using S. cerevisiae and is carried out at 30–32 °C with the addition of ammonium sulfate or urea as nitrogen sources. Proteases can be added to the mash to provide an additional nitrogen source for the yeast resulting from the hydrolysis of corn proteins [31].

Ethanol production from wheat

Although in France ethanol is mostly produced from beet molasses, it is also produced from wheat by a process similar to that of corn. Some efforts have been done for optimizing fermentation conditions. For example, Wang et al. (1999c) [32] have determined the optimal fermentation temperature and specific gravity of the wheat mash. Soni et al. (2003) [33] have optimized the conditions for starch hydrolysis using a-amylase and glucoamylase obtained by solid-state fermentation of wheat bran. The drawbacks of this technology include longer fermentation times, and sometimes incomplete fermentations probably caused by product inhibition, high osmotic pressures and inadequate nutrition [34].

Ethanol production from cassava

Cassava represents an important alternative source of starch not only for ethanol production, but also for production of glucose syrups. In fact, cassava is the tuber that has gained most interest due to its availability in tropical countries being one of the top ten more important tropical crops. Ethanol production from cassava can be accomplished using either the whole cassava tuber or the starch extracted from it. Starch extraction can be carried out through a high-yield large-volume industrialized process as the Alfa Laval extraction method [35], or by a traditional process for small- and mid-scale plants. This process can be considered as the equivalent of the wet-milling process for ethanol production from corn. The production of cassava with high starch content (85–90% dry matter) and less protein and minerals content is relatively simple.

Ethanol production from other starchy materials

Besides corn and wheat, ethanol can be produced from rye, barley, triticale [36], and sorghum [37]. For these cereals, some pretreatments have proven to be useful. Wang et al. (1997) [36] have employed the pearling of wheat, barley, rye and triticale grains for increasing starch content of the feedstock in an average of 12% obtaining a 6.5–22.5% enhance in ethanol yield during fermentation. In addition, VHG technology has been tested with successful results for oats, barley, rye and triticale [38]. It has been reported the ethanol production from other plant sources with high starch concentration.

One of the most promising crops for fuel ethanol production is the sweet sorghum, which produces grains with high starch content, stalks with high sucrose content and leaves and bagasse with high lignocellulosic content.

3.2.2.3 Ethanol from lignocellulosic biomass

It is evident the importance of lignocellulosic biomass as a feedstock for ethanol production. Lignocellulosic complex is the most abundant biopolymer in the Earth. It is considered that lignocellulosic biomass comprises about 50% of world biomass and its annual production was estimated in 10–50 billion ton [39]. Many lignocellulosic materials have been tested for bioethanol production. In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six main groups: crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (alfalfa hay, switch grass, reed canary grass, coastal Bermuda grass, thimothy grass), and municipal solid wastes (MSW).

Numerous studies for developing large-scale production of ethanol from lignocellulosics have been carried out in the world. However, the main limiting factor is the higher degree of complexity inherent to the processing of this feedstock. This is related to the nature and composition of lignocellulosic biomass. Two of the main polymers of the biomass should be broken down into fermentable sugars in order to be converted into ethanol or other valuable products. But this degradation process is complicated, energy-consuming and non-completely developed.

3.2.3 Comparison of the main types of feedstock

The selection of the most appropriate feedstock for ethanol production strongly depends on the local conditions. Evidently, North American and European countries have based their ethanol industry on the starchy materials due to their agro-ecological conditions. These conditions are not appropriate for cultivation of sugar cane, the highest yielding feedstock. The employ of starchy crops, specifically corn, for bioethanol production has provoked a hot debate on the suitability of these raw materials considering the energy input required for their production.

Lignocellulosic materials represent a promising option as a feedstock for ethanol production considering their output/input energy ratio, their great availability both in tropical and temperate countries, their low cost (primarily related to their transport), and their ethanol

yields. One of the advantages of the use of lignocellulosic biomass is that this feedstock is not directly related to food production.

33 mill ton per year of corn stover would be necessary to ensure the total ethanol production of 11,000 mill liters per year considering a more realistic yield of 330 L EtOH/dry ton. The above-mentioned demonstrates the vast possibilities of biomass taking into account that, in this case, there will be no competition for cultivable land with crops dedicated to food production.

3.3 LCB AS RAW MATERIAL

Asia is the major producer of rice straw and wheat straw, whereas corn straw and bagasse are mostly produced in America. These agro-residues are also utilized as animal fodder, as domestic fuel, and as fuel to run boilers. The utilization fraction of wheat straw, rice straw and corn straw is too low and varies with geographic region. Each year a large portion of agricultural residues is disposed of as waste. For instance, approximately 600-900 million tons per year rice straw is produced globally. The options for the disposal of rice straw are limited by the great bulk of material, slow degradation in the soil, harboring of rice stem diseases, and high mineral content. Only a small portion of globally produced rice straw is used as animal feed, the rest is removed from the field by burning, a common practice all over the world, increasing air pollution and affecting human health.

Agrowaste	*Africa*	*Asia*	*Europe*	*America*	*Oceania*
Rice straw	20.9	667.6	3.9	37.2	1.7
Wheat straw	5.34	145.20	132.59	62.64	8.57
Corn straw	0.00	33.90	28.61	140.86	0.24
Bagasse	11.73	74.88	0.01	87.62	6.49

Table 2. Quantities of agricultural waste (million tons) reportedly available for bioethanol production

Substrate (%)	*Cellulose (%)*	*Hemicellulose (%)*	*Lignin (%)*	*Protein (%)*	*Ash (%)*
Rice straw	32-47	19-27	5-24	__	12.4
Wheat straw	35-45	2030	8-15	3.1	10.1
Corn straw	42.6	21.3	8.2	5.1	4.3
Bagasse	65	(total carbohydrate)	18.4	3	2.4

Table 3. Chemical composition of agricultural wastes.

Agricultural residue	*Potential annual ethanol production (globally) (giga litres)*
Rice straw	205
Wheat straw	104
Corn straw	58.6
Sugar cane bagasse	51.3

Table 4. Worldwide potential bioethanol production from agricultural wastes.

Rice straw can potentially produce 205 billion litres bioethanol per year, which is the highest among these four mentioned agricultural wastes. However, bioethanol production from LCB on commercial scale still faces certain technical barriers that make it economically non-competitive when compared to the contemporary sugar and starch based ethanol. The major bottlenecks are the necessity of energy consuming pretreatment process, requiring a number of steps for overall conversion, great diversity in the nature and composition of biomass, inability of natural microorganisms to ferment both hexose and pentose sugars, and formation of inhibitors. As attempts to overcome these barriers and attain sustainability in lignocellulosic ethanol production, numerous research efforts have been devoted in recent years. The nature of biomass and its lignocellulosic content are the primary factors affecting the conversion efficiency and ethanol yield of LCB, which further differ in response to the source of biomass [40]. One of the most important technological aspects for commercialization of lignocellulosic ethanol is the scale up of the process from laboratory to industrial scale.

Despite the techno-economic challenges, lignocellulosic ethanol is very close to industrialization. However, inadequate knowledge on the composition of LCB, conversion techniques, technological approaches and microbial roles may limit research efforts, which are being continued to overcome the bottlenecks still need to address towards a sustainable commercialization of LCB based ethanol.

3.4 SOURCES OF LCB

As presented in Fig. 5, sources of LCBs can be divided into several groups, which include energy crops (perennial grasses and other dedicated energy crops), aquatic plants (water hyacinth), forest biomass and wastes (softwood and hard wood, sawdust, pruning and bark thinning residues), agricultural residues (cereal straws, stovers and bagasse), and organic fraction of municipal solid wastes (MSW) [41, 43]. These biomass resources seem to be the largest, promising, abundant and are available throughout the world. LCBs can be used as ethanol feedstocks without practically any extra land requirements or interference on food and fibre crop production.

Majority of the global agricultural residues are obtained from four crops, which are corn, wheat, rice and sugarcane, while the rest of agro-wastes make up only a little amount of the total world biomass. Biomass based ethanol industry requires a continuous and re- liable supply of raw materials to maintain a low cost production and keep a minimal use of water, fertilizer and cultivable land. Energy crops in this aspect are regarded as the most promising source of LCBs. Energy crops used as bioethanol feedstocks maybe either C3 or C4 plants.

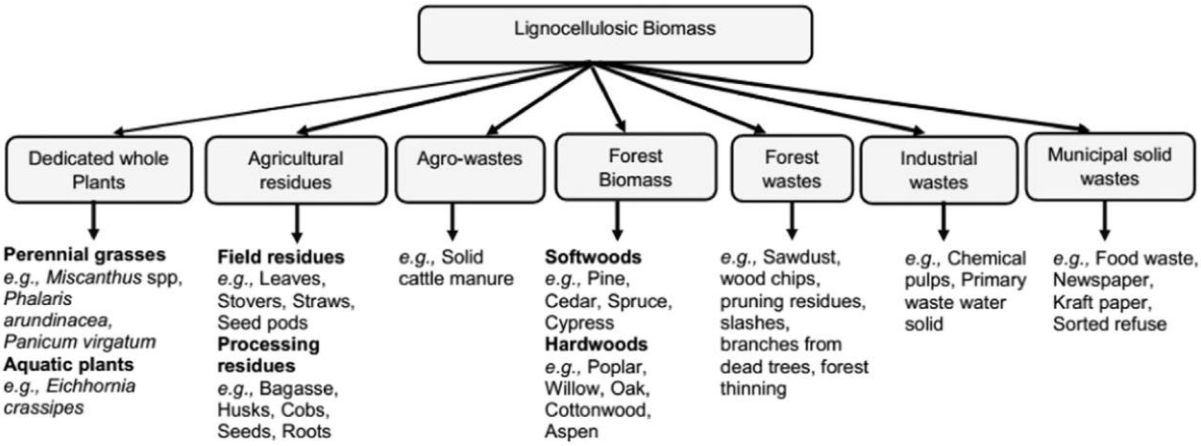

Figure 5. Sources of lignocellulosic biomass

3.5 COMPOSITION OF LCB

LCBs consist of mainly holocellulose and lignin, where holocellulose contains cellulose and hemicelluloses (Fig. 6). Holocellulose constitute roughly two-third of the total dry weight and is the exclusive substrates for ethanol [43]. The lignocellulosic contents in LCBs significantly vary based on the source and physical properties of the biomass.

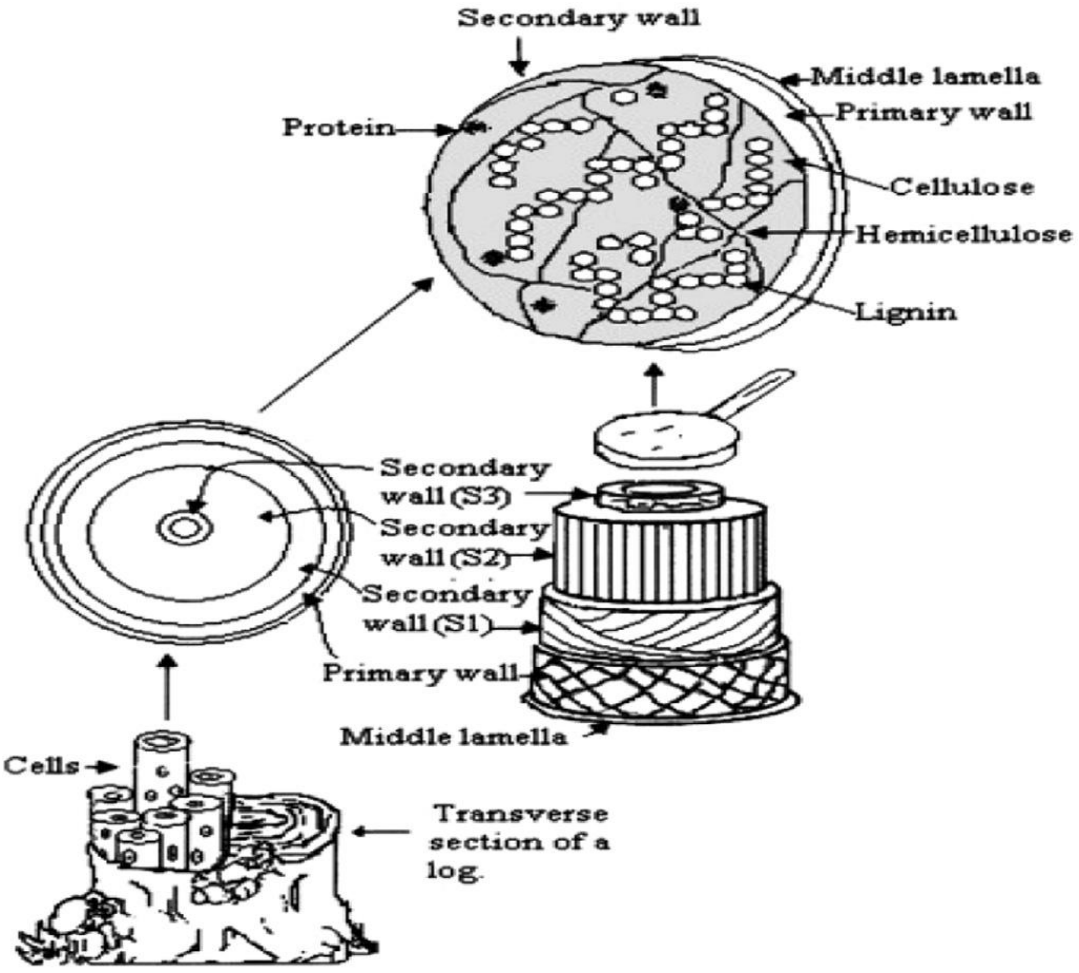

Fig. 6. Structure and composition of LCB

3.5.1 Chemical Composition of LCB

Lignocellulose is a composite material synthesised by plant cells, consisting mainly of polymeric carbohydrates (cellulose and hemicelluloses) and the aromatic polymer lignin (Fig. 8). It also contains smaller amounts of pectins, inorganic compounds, proteins and extractives, such as waxes and lipids, which also have potential value. The exact composition of lignocellulose depends on the species, the plant tissue and the growth conditions.

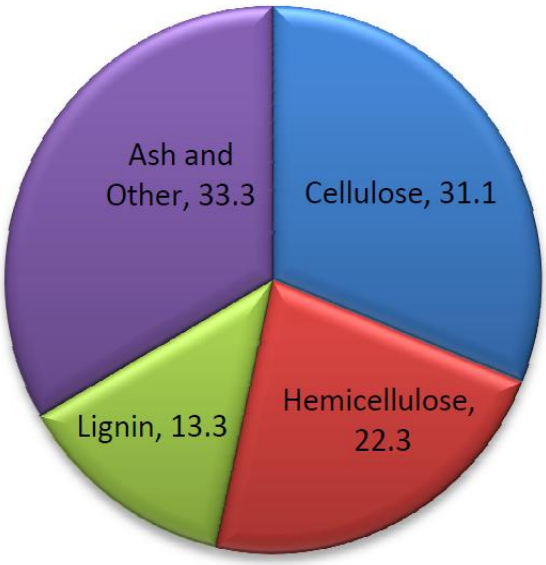

Figure 7. Composition of rice straw lignocellulose

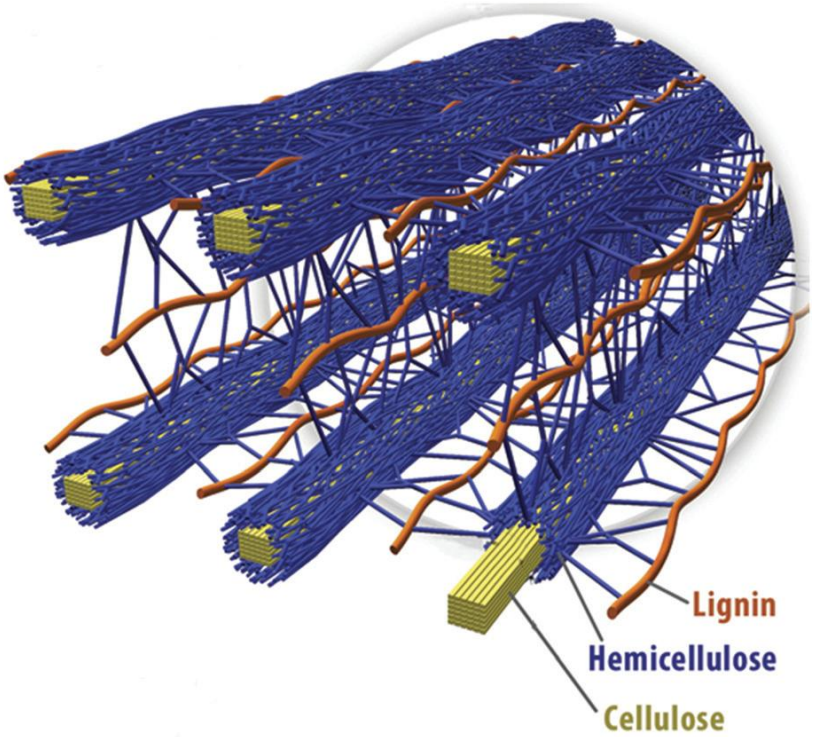

Figure 8. Spatial arrangement of cellulose hemicellulose and lignin in the cell walls of lignocellulosic biomass.

Cellulose

Cellulose is the largest single component of lignocellulose. Although the cellulose content of different biomass feedstocks varies significantly, it is typically in the range of 35–50 wt%. Cellulose is a linear polymer consisting solely of glucose units (Fig. 9a). The glucopyranosyl monomers are linked by 1-4-β glycosidic bonds. The β configuration at the anomeric carbons gives rise to a stretched chain conformation, with hydrogen bonds linking these chains into flat sheets. This is in contrast to starch, which has a helical shape due to α configuration at the anomeric carbon (Fig. 9b).

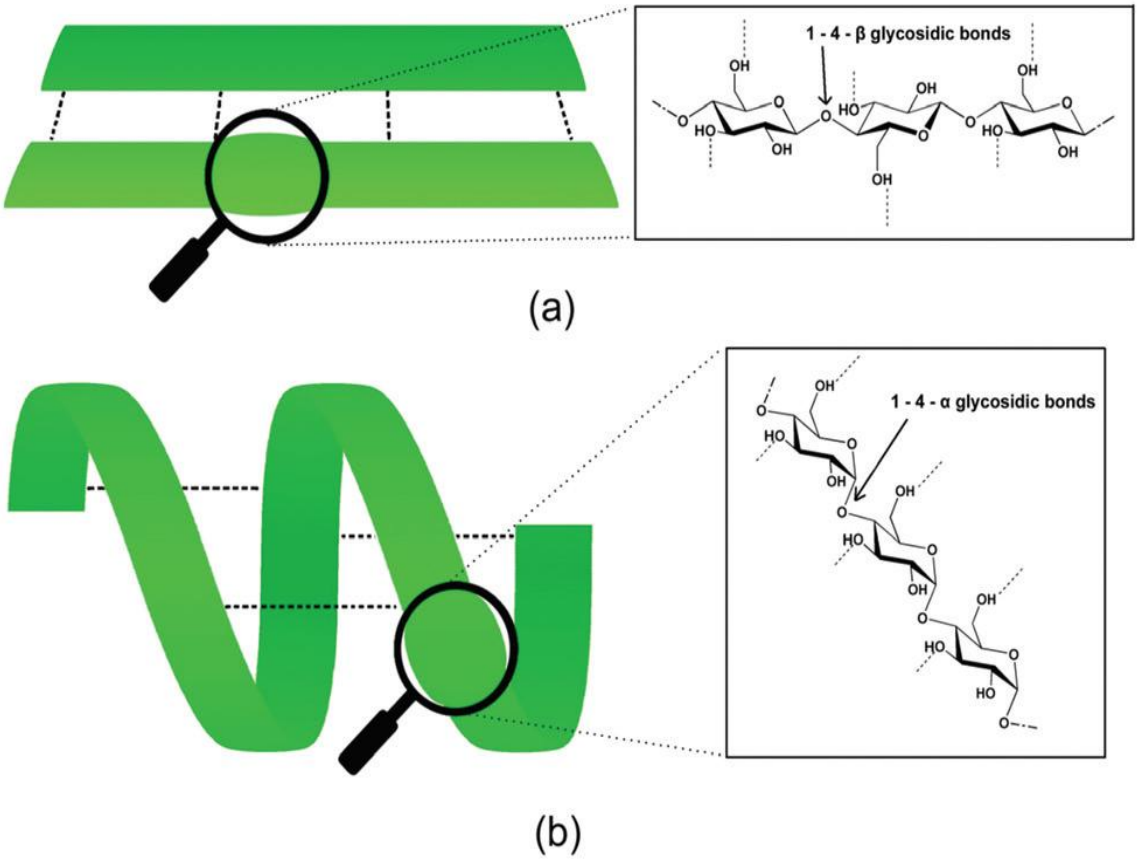

Figure 9. Impact of the geometry at the anomeric carbon on the polymer conformation.

(a) A stretched chain is observed for cellulose with β-configuration the biological conformer (cellulose I) being a flat sheet composed of several strands linked by hydrogen-bonds.

(b) Helical conformation of a starch molecule with α-conformation.

The linear conformation enables the packing of numerous cellulose strands into crystalline fibrils. In biosynthetic (native) cellulose (cellulose Ia and Ib), three hydrogen bonds per glucosyl unit occur: two intra-molecular hydrogen bonds and one intermolecular hydrogen

bond to a neighbouring cellulose molecule in the same sheet (Fig. 10b). The sheets are thought to interact mostly through van der Waals interactions which contribute significantly to the stabilisation of cellulose fibrils [45].

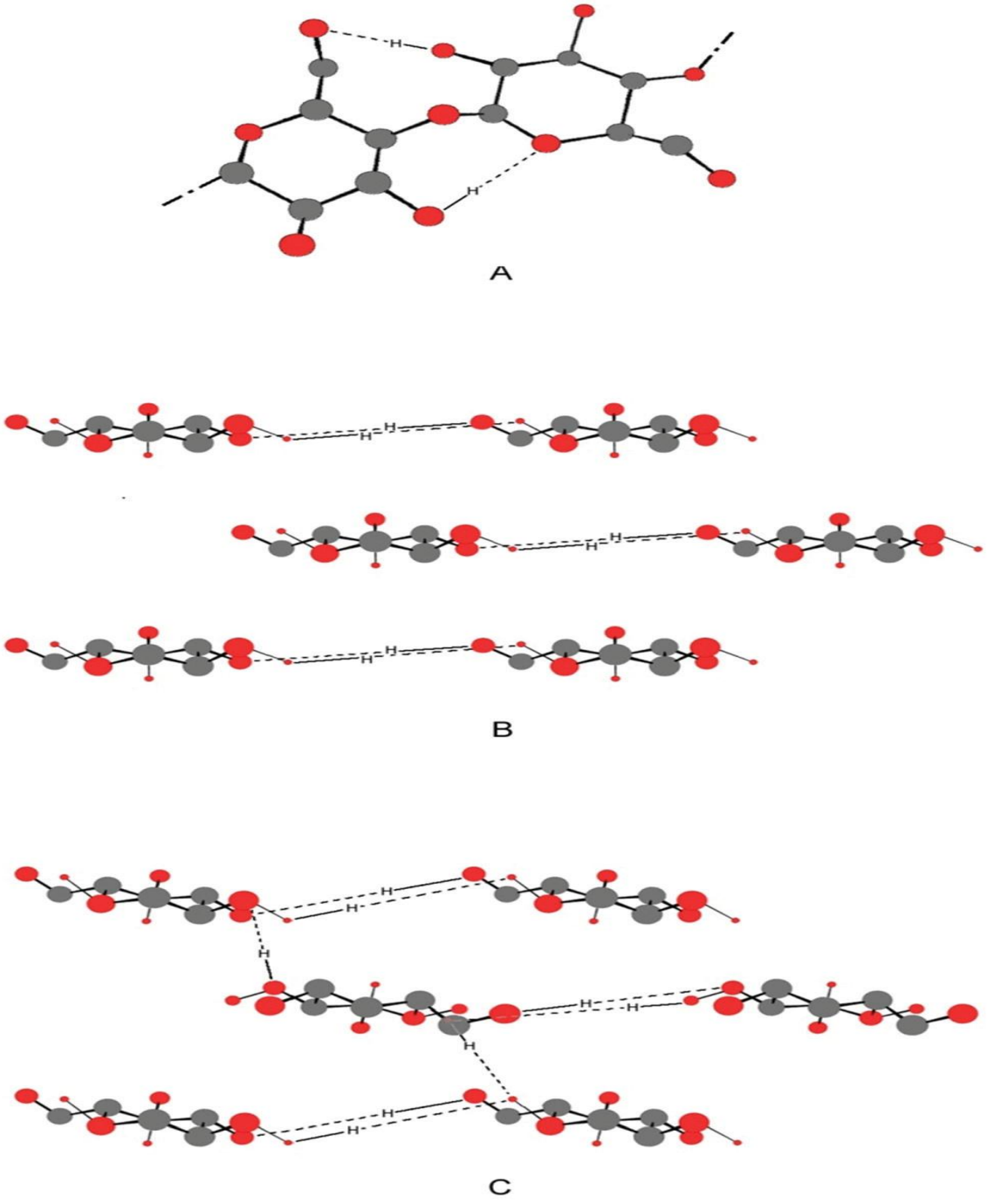

Figure 10. (A) Cellobiose, the repeating unit in crystalline cellulose I, with intra-molecular hydrogen bonds shown. Axial cross sections of 3 sheets of (B) cellulose I and (C) cellulose II, with intermolecular hydrogen bonds shown. Cellulose strands are represented by cellobiose units and hydrogen atoms have been omitted for clarity unless involved in hydrogen-bonds.

Celluloses Ia and Ib can be transformed into cellulose II, a non-natural but thermodynamically more stable form of cellulose, by swelling (mercerization) and dissolution/ regeneration; in this form, the crystal symmetry is changed and hydrogen-bonds between sheets occur (Fig. 10c) [44]. Cellulose has the highest degree of polymerisation among the lignocellulosic polymers. The number of glucosyl units in one polymer strand can be 10 000 or higher [47].

Hemicellulose

Hemicellulose is a group of polysaccharides and makes up around 25 wt% of the biomass. These carbohydrate polymers are of lower molecular weight than cellulose (degree of polymerisation around 100–200) [48]. Hemicellulose is composed of both hexose and pentose sugars; the C6 sugars glucose, mannose, galactose and the C5 sugars xylose and arabinose (Fig. 11).

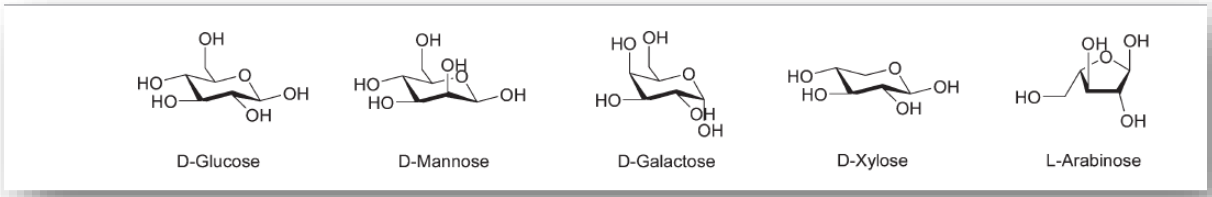

Figure 11. The hexoses and pentoses typically found in hemicellulose

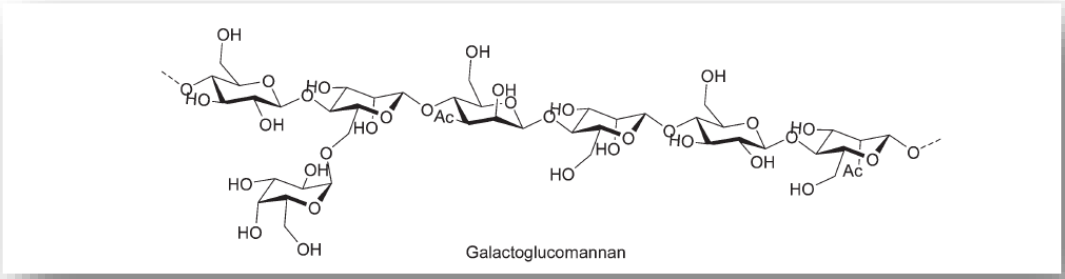

Figure 12. Galactoglucomannan, a branched hemicellulose found in softwood.

Hemicellulose polymers can be branched and may be decorated with functionalities such as acetyl and methyl groups, cinnamic, glucuronic and galacturonic acids. For example, the main chain of galactoglucomannan, a branched hemicellulose found in softwood, is built from (1→4)-linked β-D-glucopyranosyl and (1→4)-linked β-D-mannopyranosyl units. The mannosyl units are also substituted to some extent by both acetyl groups in the C-2 and C-3 position and by a (1→6)-linked α-D-galactopyranosyl units (Fig. 12) [49].

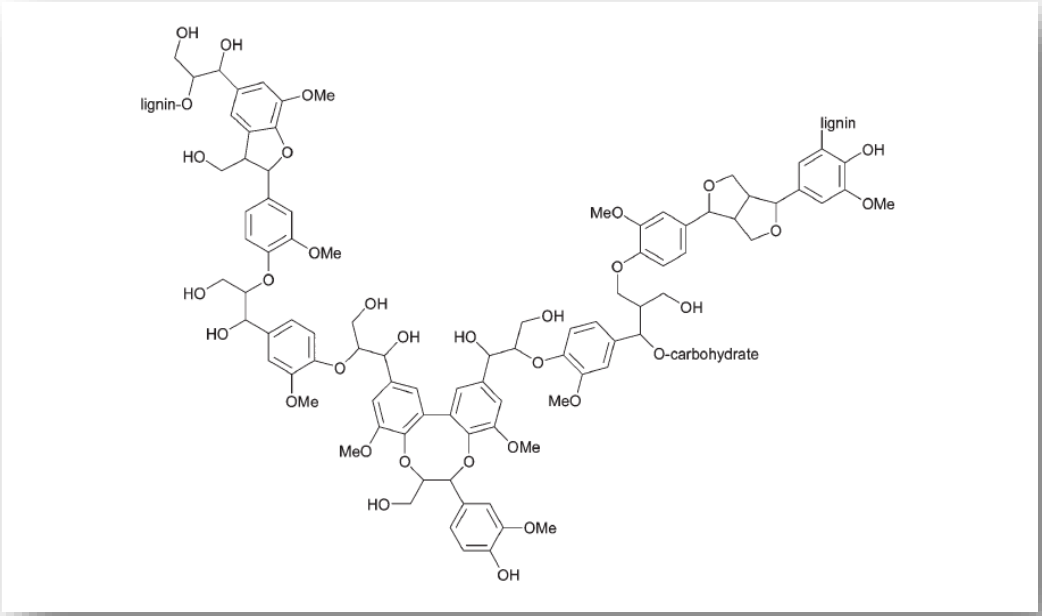

Figure 13. Lignin fragment with various C-O and C-C linkages (typically present in native lignin).

Lignin

Lignin is an aromatic, water-insoluble polymer and becomes part of the composite after plant growth has ceased. It provides water-proofing, structural reinforcement and resilience to biological and physical attack compared to the all-carbohydrate cell walls of immature plant tissues. It is biosynthesised from up to three monomers: coniferyl, sinapyl and pcoumaryl alcohols, in order of abundance. Once incorporated into the lignin polymer, the subunits are identified by their aromatic ring structure and therefore called guaiacyl, syringyl and p-hydroxyphenyl subunits, respectively.

The composition of lignin differs between softwood, hardwood and grasses, with softwood consisting almost exclusively of guaiacyl units while hardwood also contains a large number of syringyl units. This difference in composition has a great effect on the delignification chemistry and therefore on biomass deconstruction. Guaiacyl units are more likely to C–C cross-link at the C-5 position of the ring, these cross-links can form during lignification as well as during delignification [50]. The C5 position is substituted in the syringyl unit, which therefore cannot participate in substitution reactions. The C–C cross-links cannot be hydrolysed by acid or base, making delignification of softwoods more difficult than for hardwoods and grasses.

The lignin polymer contains a wide range of linkages. The most common linkage is the β-O-4 ether bond. Roughly 50% of all inter-subunit bonds are of this type. The β-O-4 ether bonds lead to a linear elongation of the polymer. Other C–O and C–C linkages are present in lower abundance, and branching occurs when lignification is advanced. The most common linkages are depicted in Fig. 13.

The lignin crust has been identified as one of the major obstacles for an energy-efficient biomass deconstruction process [51]. Native lignin not only prevents access of polysaccharide hydrolases to their substrates, but modified lignin adhering to the pulp after pretreatment also causes unproductive binding of hydrolases [52]. This leads to the need for higher enzyme loadings in the enzymatic carbohydrate hydrolysis and prevents efficient enzyme recycling.

3.5.2 Bonds between lignin and carbohydrates: lignin–carbohydrate complexes

Hemicellulose and lignin are not only entangled, but also covalently cross-linked. In grasses, these lignin–carbohydrate complexes contain ferulic acid (Fig. 14). Ferulic acid is initially bonded to hemicellulose (arabinoxylan) via ester bonds [53]. During lignification, its aromatic ring can be incorporated into the growing lignin network by participating in the radical polymerisation reaction [50]. Ferulic acid can also dimerise hemicellulose chains. The extent of cross-linking via lignin–carbohydrate complexes has been correlated with increased cell wall rigidity and resistance to enzymatic digestion. Thus, these cross-links must be broken by chemically hydrolysing the ester bonds in order to obtain an effective deconstruction process [54].

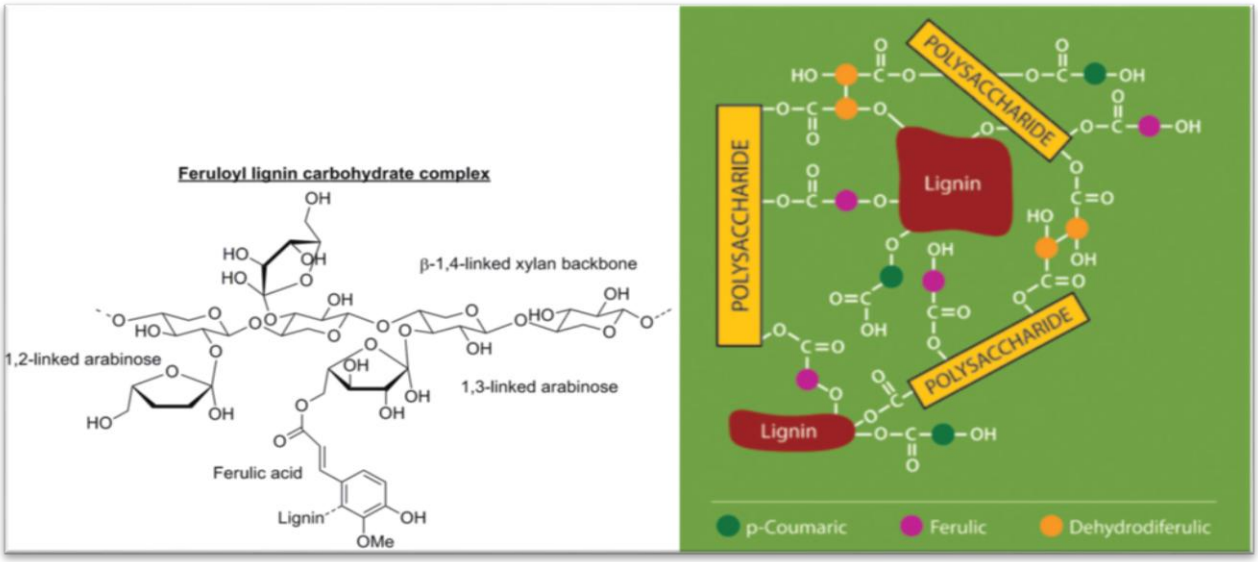

Figure 14. Grass lignin–carbohydrate complexes involving ferulic acid (left). The network that is formed, mediated by ferulic acid, is depicted on the right.

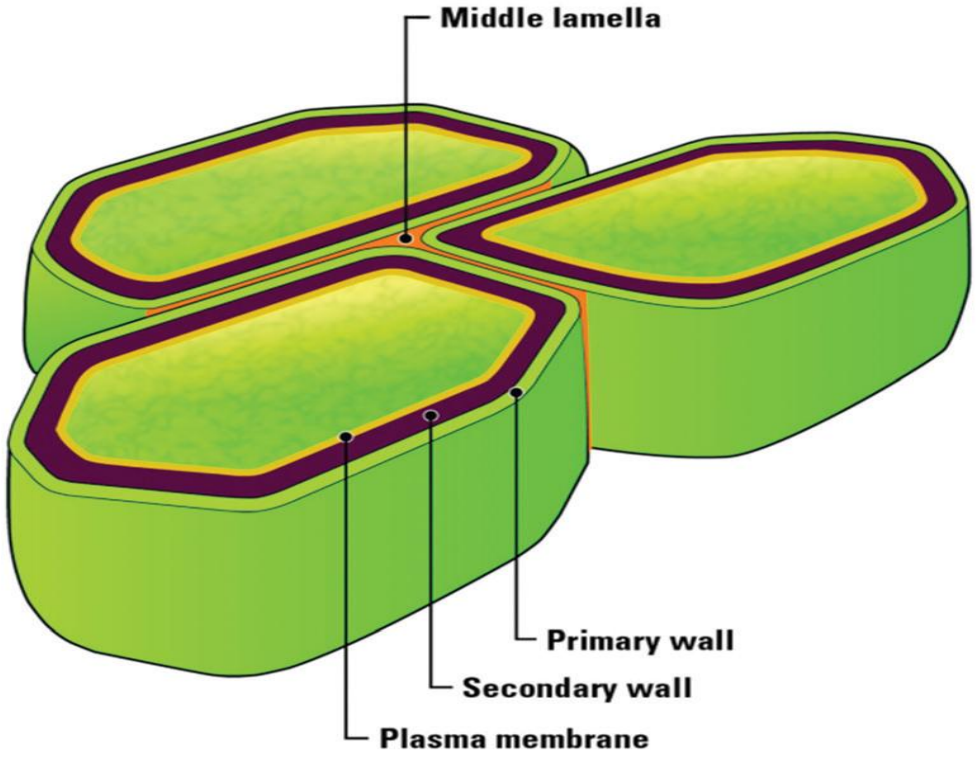

Figure 15. Porous structure of lignocellulosic tissue.

3.5.3 Ultrastructure

Lignocellulose is also structured beyond the assembly of the biopolymers into a composite. This ultrastructure is determined by the fact that lignocellulose is synthesised by plants as walls surrounding their cells to strengthen the plants' structures. Lignocellulosic plant material is therefore porous and light-weight. The cells in woody tissues are elongated and mostly orientated in the axial direction, interconnected via tiny holes in the walls (pits and perforation plates), making wood an anisotropic material. These long perforated cells create channels that enable transport of nutrients and water between roots and leaves. This structure also affects the diffusion of chemicals into and out of wood chips during chemical deconstruction. Several cell types can be found in lignocellulosic tissues, which differ in size and shape, cell wall polymer content and in function. In wood, a few cells are oriented in the radial direction to enhance structural rigidity and limit lateral expansion. Between adjacent cell walls is a lumen called the middle lamella, whose content holds the cell walls together (Fig. 15). It is devoid of cellulose fibrils and therefore rich in hemicellulose.

3.6 CONVERSION OF LCB INTO ETHANOL

Conversion of LCB into ethanol involves several steps as shown in Fig. 16. The major processing steps are pretreatment and/or detoxification, hydrolysis, fermentation and product recovery. The harvested or collected biomass is first dried to a suitable moisture level that subsequently undergoes a size reduction through grinding.

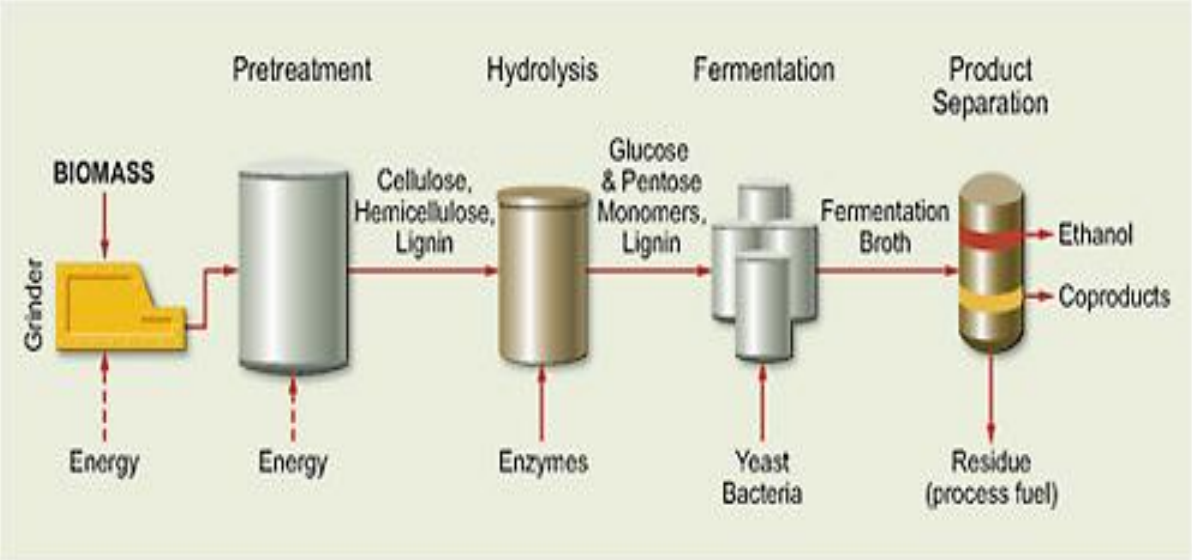

Figure 16. Schematic diagram of LCB into ethanol conversion.

3.6.1 Pretreatment of the biomass

Role of pretreatment

The most complicated and costly step in the conversion of LCB into ethanol is the pretreatment. Cellulose in LCB is usually coated or sheathed by hemicelluloses forming cellulose-hemicellulose complex that works as a chemical barrier and prevent the access of enzymes in the complex under its natural condition [55]. The cellulose-hemicellulose complexes are further encapsulated with lignin and produce a physical barrier to the hydrolysis of biomass to produce fermentable sugars. Therefore, pretreatment is required to remove lignin–cellulose–hemicellulose complexes through altering the macroscopic, sub-microscopic and microscopic structures of the biomass, making it accessible to hydrolytic enzymes that finally convert cellulose and hemicellulose into fermentable sugars. The alterations of biomass occurred during pretreatment basically include removal of lignin (as shown in fig. 17, decrease in the crystallinity of cellulose, and increase in the surface area and porosity of the biomass. Some pretreatment processes could also release fermentable sugars by hydrolysing hemicellulose fraction of the lignocellulose.

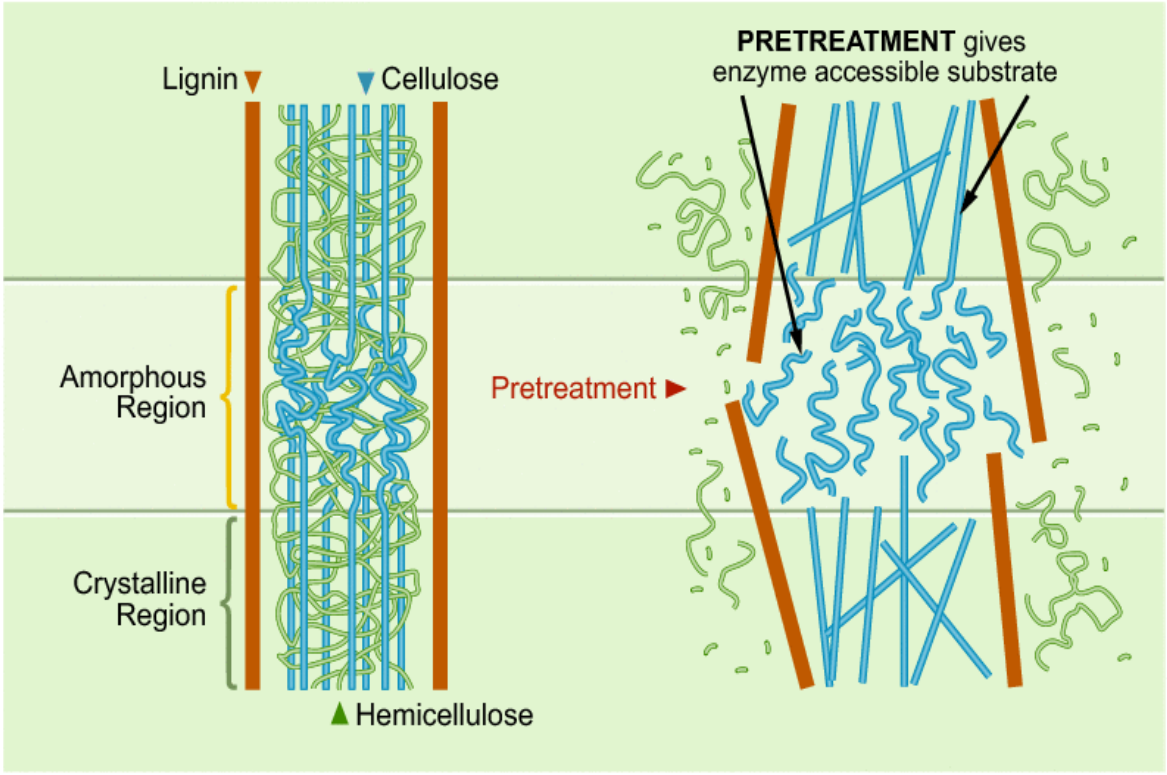

Figure 17. Schematic of pretreatment to disrupt the physical structure of biomass.

Since pretreatment is an energy consuming step, the energy efficiency of any pretreatment process should be evaluated. The energy efficiency for a pretreatment process can be determined from the ratio of total sugar yield and energy consumption during pretreatment as shown in the Equation (3) [56].

$$\eta = TSY / TEC \quad (3)$$

Where,

η is the energy efficiency (kg/MJ);

TSY is the total monomeric sugar yield (kg);

TEC is the total energy consumption during pretreatment.

Pretreatment methods: general concept

To date, many pretreatment methods have been investigated on a wide range of LCBs, which are either simple or technologically and logistically more intensive. The mode of action, reaction conditions and outcomes of the pretreatment processes vary significantly among each other [51]. Each pretreatment method has some advantages and disadvantages with regard to its techno-economic and process conditions. However, an effective pretreatment method should have some general criteria to be used in lignocellulosic ethanol production as pointed out below.

- It should release cellulose and hemicellulose from lignocellulosic complex, and alter these carbohydrate polymers with high digestibility to enzymes or other treatments applied in the subsequent steps to produce fermentable sugars.
- It should cause a minimum damage of hemicellulose and cellulose under the pretreatment conditions, with a maximum recovery of these polysaccharides.
- It should show no or little degradation of sugars, loss of sugars and production of sugar derived inhibitors.
- It should generate minimum toxic compounds under the pre- treatment conditions.
- It should require minimum size reduction of the biomass.
- It should have capability to operate in reasonable batch size and moderate cost reactors.
- It should produce a high yield of pretreated biomass with high fermentation compatibility.
- It should show a high lignin recovery for producing valuable co-products.
- It should require minimum heat and power to complete the process.
- It should require low capital and operational costs.

Pretreatment methods can be broadly classified into four groups, such as physical, chemical, physic-chemical and biological. Physical pretreatments work on the biomass through increasing the accessible surface area and pore volume, decreasing the degree of polymerization of cellulose and its crystallinity, hydrolysis of hemicelluloses, and partial depolymerisation of lignin.

The energy requirements for physical pretreatments depend on the final particle size and reduction in crystallinity of LCBs. In most cases when physical pretreatment is the only option, the required energy is higher than the theoretical energy content available in the biomass. Physical pretreatment methods are expensive and usually cannot be used on a large scale process.

Chemical pretreatments include primarily alkalis and acids that work on the biomass through delignification, decreasing the degree of polymerization and crystallinity of cellulose. H_2SO_4, HCl, H_3PO_4 and HNO_3 are used during acid pretreatment of bio-mass. Among these chemicals, H_2SO_4 is the most commonly used acid, while NaOH is the major alkali. Acid pretreatment is applied to solubilise the hemicellulosic fraction in the biomass and make cellulose more accessible to enzymes. Organic acids such as fumaric or maleic acids are also used as alternative acids that enhance cellulose hydrolysis and reduce production of inhibitors [51, 58]. Cellulose solvents are another type of chemical additives that include alkaline H2O2, ozone, organosolv, glycerol, dioxane, phenol or ethylene glycol, which are known to disrupt cellulose structure and promote hydrolysis. Concentrated mineral acids (H2SO4, HCl), ammonia based solvents (NH3, hydrazine), aprotic solvents (DMSO), metal complexes (ferric sodium tartrate, cadoxen, and cuoxan), and wet oxidation have also been reported to reduce cellulose crystallinity and disrupt the association of lignin with cellulose, as well as dissolve hemicelluloses [57]. Physico-chemical pretreatment exploit the use of conditions and compounds that affect physical and chemical properties of biomass. It includes a good number of technologies and includes steam explosion, ammonia fiber explosion (AFEX), ammonia re-cycling percolation (ARP), soaking aqueous ammonia (SAA), wet oxidation, CO2 explosion etc. Like other pretreatment methods, physico-chemical technologies also increase accessible surface area of the biomass for enzyme accessibility, decrease cellulose crystallinity and remove hemicelluloses and lignin during pretreatment [56,51].

Biological pretreatment of LCB can be carried out using microorganisms, particularly fungi such as white rot, brown rot and soft rot fungi, where white rot fungi are most efficient for this purpose [1]. This kind of treatment alter the structure of lignin and cellulose and separate from the lignocellulosic matrix. Brown rot fungi attack cellulose while white and soft rot fungi work

on both cellulose and lignin [59]. The biological conversion of different lignocellulosic feedstocks such as forest and agricultural residues, or lignocellulosic crops dedicated to ethanol offers numerous benefits but its development is still hampered by economic and technical obstacles [60]. Lignin degradation occurs during biological pretreatment of biomass through the action of lignin degrading enzymes secreted by the fungi. Although biological pretreatment involves mild conditions and are of low costs, the disadvantages are the lower rates of hydrolysis and longer pretreatment times required compared to other technologies. Current efforts in biological pretreatments are to combine this technology with other pretreatments and develop novel microorganisms for rapid hydrolysis [61].

Since different LCBs have different physicochemical properties, there will not be a standalone method for all biomass, rather pretreatment methods and their conditions vary depending on the nature of the biomass. Moreover, it should also be taken into consideration that certain pretreatment methods significantly affect subsequent conversion steps of the biomass with regard to the cellulose digestibility, generation of toxic compounds, energy demand in the downstream process and wastewater treatment demands [62].

3.6.2 Detoxification

Although pretreatment is an essential step for converting LCB into ethanol, it has a major side effect on the overall process due to generation of lignocellulose-derived by-products under the pretreatment conditions that act as inhibitors for enzymes and fermenting microorganisms in the subsequent steps if their accumulation is sufficiently high [43]. The by-products produced during pretreatment include sugar acids, acetic acid, formic acid, levulinic acid, hydroxyl-methylfurfural (HMF) and furfural [63]. Most of the lignocellulose-derived inhibitors are produced when hemicelluloses and lignin degraded during pretreatment, whereas, cellulose and extractives of the biomass may be the source of inhibitors being affected unintentionally by the pretreatment conditions (Fig. 18). The pretreatment inhibitors can be categorized into three major groups based on their origin, such as aliphatic acids, furan derivatives, and phenolic compounds [64, 65].

Certain strategies have been suggested to counteract the problems with inhibitors that include both alternative measurements for the conversion process and treatment of the pretreated biomass to remove or neutralize inhibitors using biological, physical, and chemical methods. However, type and quantity of inhibitors depend on the pretreatment methods applied, and it is taken into consideration prior to selecting a detoxification method. The detoxification

methods should selectively remove inhibitors, and be cheap and easy to integrate into the process [64].

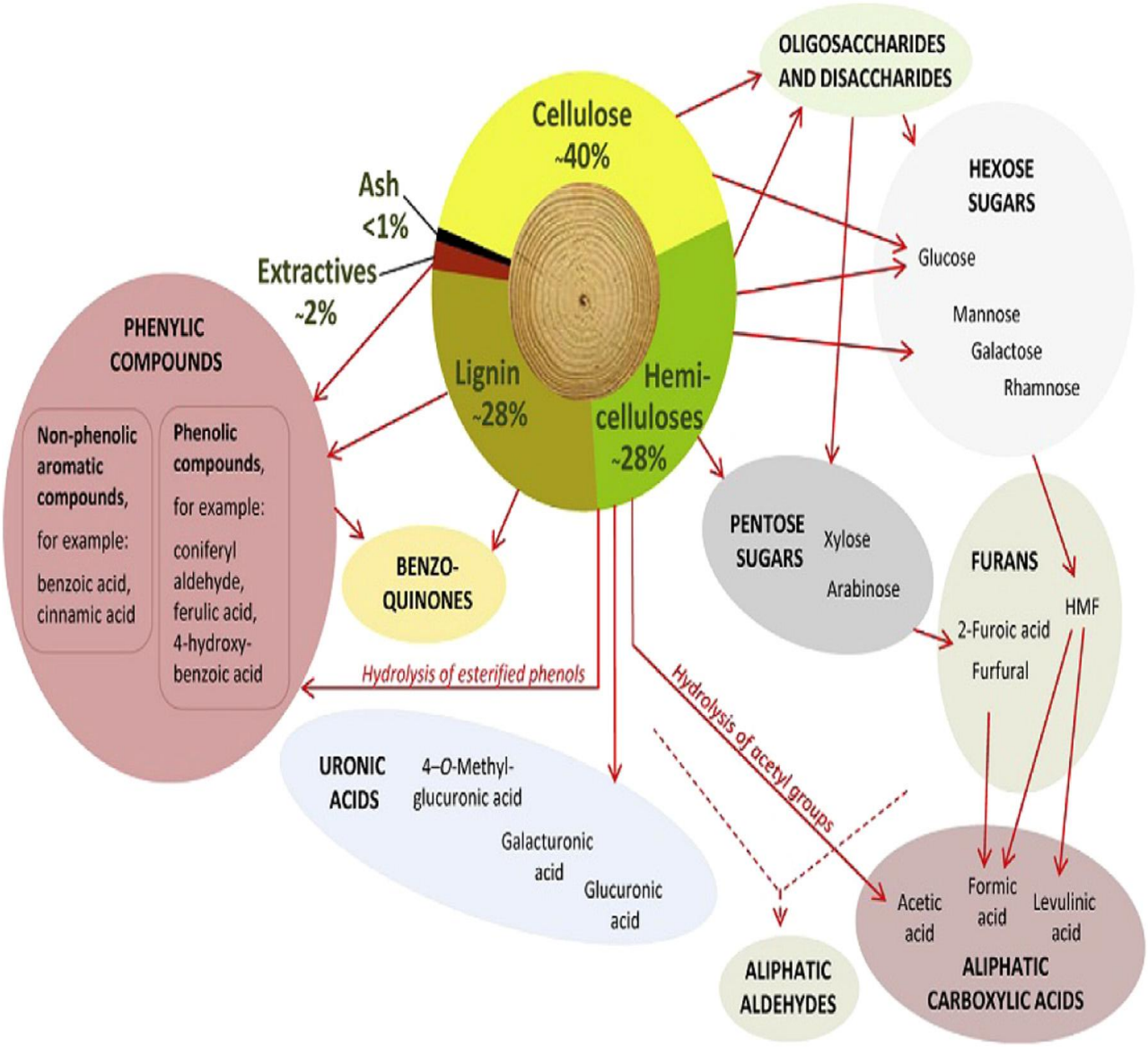

Figure 18. Inhibitors generated from degradation of different compounds in LCBs after pretreatment under acidic conditions. Arrows indicate tentative formation pathways.

3.6.3 Hydrolysis

Cellulose and hemicellulose present in LCBs are required to convert into fermentable sugars through a process called hydrolysis [66]. In most cases, the outcomes of the pretreatment process are two fractions such as water-insoluble solids containing mainly cellulose and lignin, and a liquid fraction containing hemicellulose. Based on the pretreatment method and conditions,

hemicellulose maybe either almost completely hydrolyzed to its monomeric sugars and can be converted into ethanol during fermentation without further hydrolysis process; or it is converted into oligosaccharides if undergoes incomplete depolymerization and require further hydrolysis prior to subjecting to fermentation [67]. On the other hand, liquid fraction contains monomeric sugars or oligosaccharides of hemicellulose. In general, conversion of the hemicellulose and cellulose into their monomeric sugars involves either acid or enzymatic hydrolysis.

Acid hydrolysis

Acid hydrolysis can be done via two approaches, such as dilute acids treatment at high temperature and pressure with a short reaction time ranging between seconds and minutes, and concentrated acids treatments at low temperature [68, 69].

Enzymatic hydrolysis

Enzymatic hydrolysis of LCB is usually done either by using enzyme producing microorganisms directly that secretes enzymes during their growth in the media or commercially available enzyme system, where the latter is more feasible and widely used. Enzymatic hydrolysis has been considered the most promising and effective processes due to enzyme's specificity to the substrate, working at relatively lower temperatures and generation of minimum inhibitors. However, cost of enzymes is a major challenge for generating cost-effective ethanol on a commercial scale [70].

The conversion of lignocellulosic biomass into fermentable sugars depends on hydrolysis conditions and biomass types. The sugar yield during enzymatic hydrolysis may be affected by a good number of factors, which are loosely divided into two groups:

(1) Enzyme and process related factors and

(2) Substrate related factors, which may be inter-linked with each other.

Enzyme and process–related factors are the kind and nature of pretreatment methods applied in early stages of the biomass treatments, type of enzymes, and dosage, sources & efficiency of the enzymes. Among the substrate–related factors, following can be mentioned [58]:

- Composition and structure of the feedstock.
- Crystallinity of cellulose.
- Degree of polymerization of cellulose.
- Available surface area on the substrate.

- Presence of lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier.

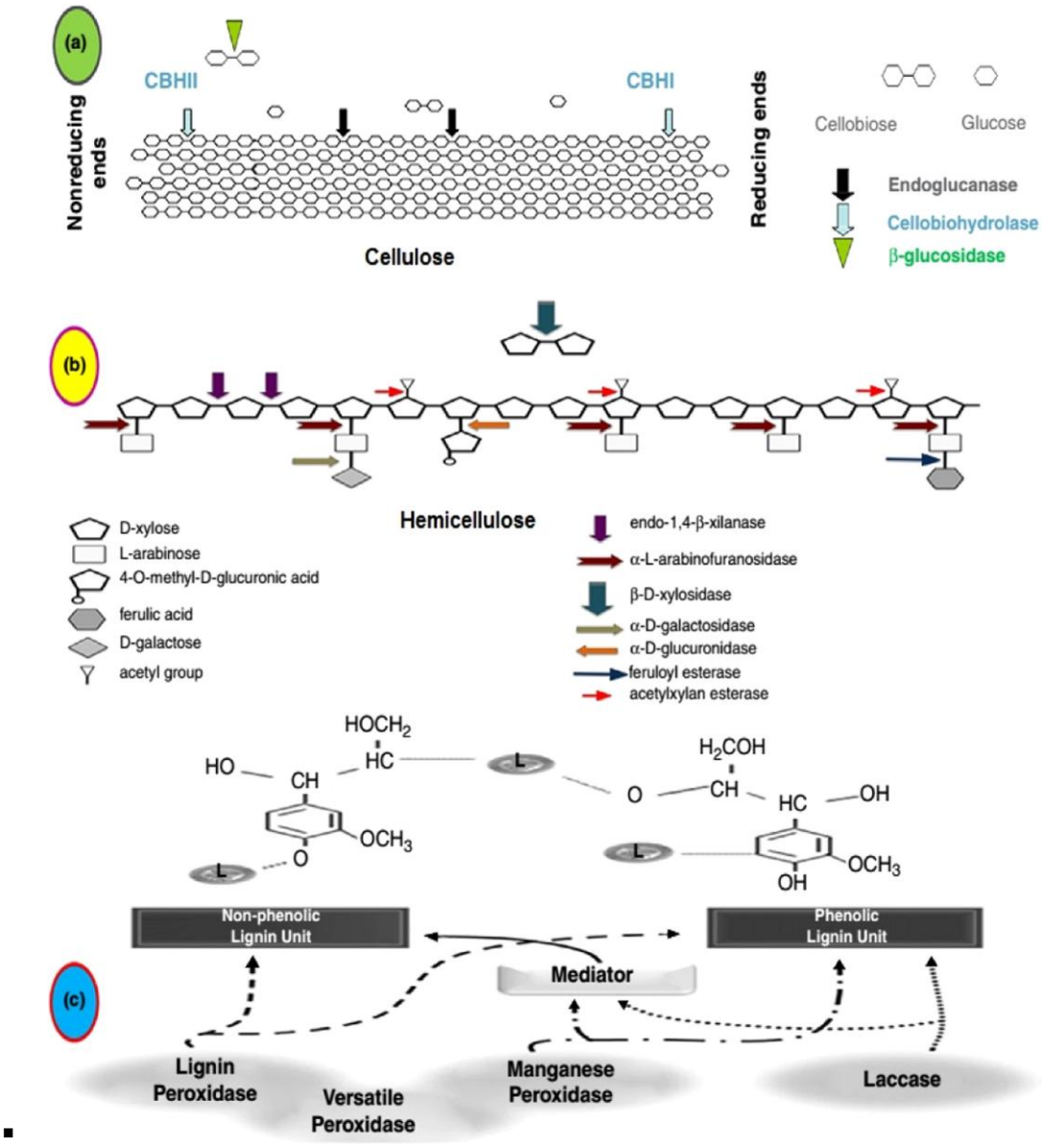

Figure 19. Enzymes involved in the enzymatic hydrolysis of cellulose (a), hemicellulose (b) and lignin(c)

- Hemicellulose content (Removal of hemicellulose increases the mean pore size of the substrate and therefore increases the accessibility and the probability of the cellulose to become hydrolyzed).
- Feedstock particle size (reduction of particle size increases specific surface area and subsequently the accessibility of cellulose to the enzymes).

- Porosity (pore size of the substrate in relation to the size of the enzymes is the main limiting factor in the enzymatic hydrolysis of LCB).
- Cell wall-thickness (coarseness) that limits liquid penetration.
- Changes in accessibility to enzymes.

3.6.4. Fermentation

After pretreatment and hydrolysis of LCBs, simple sugars are produced as a result of depolymerisation of cellulose and hemi-cellulose that are then fermented by the relevant microorganisms and converted into ethanol. The overall process is referred to as fermentation. Ethanol fermentation can be done either by submerged or solid state fermentation. In submerged fermentation, water is an important liquid that is used to make fermentation mash by mixing a pre-defined solid with water. On the other hand, solid state fermentation is the bioconversion of the LCB in its natural state. In a solid state fermentation, LCBs are moistened with a thin layer of water on the surface of the biomass, using weight ratios of water to lignocellulose are typically between 1:1 and 10:1 [71]. The expected advantages of solid state fermentation over submerged fermentation are [72]:

(a) Smaller fermenter volume, because there is no excess water in the fermenter;

(b) Lower sterilization energy costs, because less water needs to be heated;

(c) Easier aeration, because air can circulate freely between the substrate particles, and because the liquid film covering the substrate has a large surface area compared to its volume;

(d) Reduced or eliminated capital and operating costs for stirring and for effluent treatment;

(e)Lower costs for product recovery and drying;

(f)A more natural environment for lignin-degrading fungi: many of these fungi grow and perform better under SSF than under submerged conditions; and

(g)A less favourable environment for many bacteria, lowering the risk of contamination [72].

However, exploitation of solid state fermentation on commercial ethanol production from LCBs is far away due to techno-economic limitations of this process. As a result, submerged fermentation is the most common practice and used in almost all of the ethanol production facilities.

Fermentation can be performed by three modes such as batch, fed–batch and continuous fermentation. The choice of most suitable process depends on the kinetic properties of fermenting microorganisms and type of feedstock, in addition to aspects of process economics.

Batch culture is usually done in a closed culture system by inoculating microorganism into an initial fermentation media containing defined amount of nutrients and allowed to ferment until the finishing of the nutrients [69]. It is the simplest mode of fermentation where nothing is added after inoculation except possibly acid or alkali for pH control. In batch fermentation, the microorganism works in high substrate concentration initially and a high product concentration finally [73]. Fed–batch system is widely used to produce ethanol on commercial scale due to the fact that offers the advantages from both batch and continuous processes [74]. In a fed–batch fermentation system, microorganism works at low substrate concentration with an increasing ethanol concentration during the course of fermentation process. Fed-batch cultures often provide better yield and productivity than batch cultures for the production of microbial metabolites. The major advantage of fed–batch over batch system is the ability to reach to the maximum viable cell concentration, prolong culture lifetime, and allow product accumulation to a higher concentration. This system allows maintenance of critical process variables (e.g. temperature, pH, and dissolved oxygen) at specific levels through feedback control [69]. However, the productivity of fed-batch fermentation is limited by the feed rate, which in turn limited by the cell-mass concentration [75]. Continuous fermentation can be performed in different kind of bioreactors–stirred tank reactors (single or series) or plug flow reactors. It often gives a higher productivity than batch fermentation, but at low dilution rates it offers the highest productivities. In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel [69].

During fermentation, microbial conversion of pentoses, hexoses and disaccharides derived from cellulose and hemicellulose occurred based on the following reactions (Eqs.4–7) as described by [76]. The theoretical ethanol yield can also be calculated from these equations. As per equations (4) and (5), ethanol yield from the monosaccharides (hexoses and pentoses) is 510g/kg, while ethanol yield from the disaccharides (C10 and C12) is 540g ethanol per kg of disaccharides (Eqs.6 and 7). In general, ethanol yield, productivity and percent theoretical ethanol yield can be calculated for any substrate in the fermentation system using the Eqs.8-9 reported in literatures [21, 76].

$$C_6H_{12}O_6 \text{ (glucose)} \longrightarrow 2C_2H_5OH + 2CO_2 \quad (4)$$

$$3C_5H_{10}O_5 \text{ (arabinose; xylose)} \longrightarrow 5C_2H_5OH + 5CO_2 \quad (5)$$

$$C_{12}H_{22}O_{11} \text{ (cellobiose)} + H_2O \longrightarrow 4C_2H_5OH + 4CO_2 \quad (6)$$

$$3C_{10}H_{18}O_9 \text{ (xylobiose)} + 3H_2O \longrightarrow 10C_2H_5OH + 10CO_2 \quad (7)$$

Ethanol yield is calculated as the amount of ethanol produced per unit of substrate utilized as

$$\mathrm{YEtOH} = \mathrm{CV/m} \quad (8)$$

Where, Y_{EtOH} is ethanol yield (g/kg), C is ethanol concentration (g/ L), V is initial volume of liquid medium (L), and m is the mass of the substrate (kg).

Ethanol productivity is estimated as the amount of ethanol produced per unit of substrate utilized per unit of time. It is typically determined when ethanol concentration is maximum. Ethanol productivity is calculated as

$$PEtOH = CV/mt = 1000\, YEtOH/t \quad (9)$$

Where, P_{EtOH} is ethanol productivity (mg/kgh), and t is the time at which the ethanol concentration produced on substrates is maximum (h).

The achievements of fermentation, particularly ethanol yield vary considerably depending on the biomass, type of sugars, concentration of sugars in the hydrolysates produced after pre-treatment and hydrolysis, microorganisms, and process conditions of the fermentation. For this reason, it is difficult to draw a conclusion on the potential of different biomass and ranking the biomass based on their ethanol yield as fermentation of a wide range of LCBs is not possible to carry out under the same condition.

3.6.5 Product recovery

Ethanol is recovered from the fermentation broth by distillation or distillation combined with adsorption [51]. Distillation is a mature process for ethanol recovery. The first part is distilling the fermentation broth to separate ethanol from water to reach an ethanol concentration of 95%, and the bottom liquid containing residual lignin, unreacted cellulose and hemicellulose, ash, enzyme, organisms, and other components are send to the waste- water treatment [77]. These materials may be concentrated, and burned as fuel to power the process, or converted to various co–products.

3.7 ROLE OF MICROORGANISMS

Numerous microorganisms from different groups, including bacteria, yeast, fungi and actinomycetes have the potential roles in all the major conversion steps (pretreatments, detoxification, hydrolysis and fermentation) of lignocellulosic ethanol production. The roles and potential features of some natural and engineered microorganisms are summarized in Table 5.

Species/Strain	*Characteristics*	*Contribution*	*Major feature/role*
S. cerevisiae	Facultative anaerobic yeast	Fermentation	Robust and well suited to the fermentation of LCB hydrolysates; tolerant to inhibitors; high yield of 0.45 g/g
S. cerevisiae (Recombinant)	Facultative anaerobic yeast	Fermentation	Enhanced robustness to inhibitors
Z. mobilis	Ethanologenic G-negative bacteria	Fermentation	High specific ethanol productivity Lower biomass yield
E. coli (Recombinant)	Mesophilic G-negative bacteria	Fermentation	Easy genetic manipulation; Novel metabolic engineering strategies
S. stipitis	Xylose fermenting yeast	Fermentation	Efficiently metabolize glucose; Improves volumetric productivity
Pichia segobiensis	Xylose fermenting yeasts	Fermentation	Long-term adapted for xylose fermentation and tolerance to hydrolysate inhibitors
Clavispora NRRL Y-50464	Natural Isolate	Hydrolysis and fermentation	Robust to inhibitors and produce cellobiase
Klebsiella oxytoca	Recombinant bacterium	Fermentation	Capable of growing at low pH; High Temperature as 308 K; Rapid fermentation; can grow on wide variety of sugars
Pachysolen tannophilus	Gram- negative bacteria	Fermentation	Hexose and pentose fermenters
Kluveromyces marxianus	Thermophilic yeast	Fermentation	Capable to ferment a large spectrum of sugars
Thermoascus auranticus	Thermophilic fungus	Hydrolysis	Cellulase and xylanase producer
Trichoderma viridae	Filamentous fungus	Hydrolysis	Cellulase producer
Caldibacillus cellulovorans	Thermophilic aerobic bacterium	Hydrolysis	Mannanase producer
Caldocellum saccharolyticum	Extreme thermophilic bacterium	Hydrolysis	Cellulase producer

Bacillus subtilis	Gram- positive bacterium	Hydrolysis	Cellulase producer
Cellulomonas biazotea	Gram-positive bacterium	Hydrolysis	Xylanase producer
Clostridium	Anaerobic bacteria	Hydrolysis	Cellulase and hemicellulase producer
Sulfolobus solfataricus	Aerobic bacteria	Hydrolysis	Cellulase and hemicellulose producers
Methylobacterium extorquens	Natural bacterial isolates	Detoxification	Removal of ferulic acid, furfural, 5-HMF
Trichoderma reesie	Soft rot fungi	Detoxification	Removal of phenols, furans, and weak acids
Phanerochaete chrysosporium	White rot fungi	Detoxification	Detoxification of phenolic inhibitors through releasing enzymes
Aspergillus terreus	Filamentous fungi	Pretreatment	Delignification and cellulose degradation
Pseudomonas sp.	Aerobic bacteria	Pretreatment	Ligninase producers
Pleurotus florida	White-rot fungi	Pretreatment	Some selective lignin degradation but sugar losses may occur

Table 5. Potential of some selected microorganisms in lignocellulosic ethanol production.

3.7.1 Role of microorganisms in pretreatment and detoxification

Microorganisms (soft rot or white rot fungi) used during biological pretreatment of LCBs degrade lignin or delignify the lignocellulosic network through direct metabolism or producing lignin peroxidase [72], which partially release hemicellulose and cellulose from the matrix. Likewise, microbial detoxification of the pretreated biomass produced enzymes (peroxidase and laccase) that work on the inhibitors and help to remove or neutralize them [75]. With regard to in situ microbial detoxification, bacteria and yeasts have been considered more effective than soft rot fungi. Five bacterial strains (*Methylobacterium extorquens, Pseudomona ssp., Flavobacterium indologenes, Acinetobacter sp., and Arthrobacter aurescens*) and one fungus (*C. ligniaria* C8 NRRL30616) have been reportedt o remove toxic compounds from a defined mineral medium containing a mixture of ferulic acid, HMF, and furfural as carbon and energy sources [75,78]. Acetic acid, furfural and benzoic acid derivatives have been removed from the hydrolysate by *Trichderma reesei* [64].

3.7.2 Role of microorganisms in hydrolysis

Although hydrolysis of carbohydrate polymers, particularly cellulose can be done both chemically and enzymatically, the latter has been used widely as it requires less energy and mild environment conditions compared to acid hydrolysis [79]. Hydrolysis of cellulose requires cellulases that are normally produced by various microorganisms including bacteria and fungi under aerobic or anaerobic, mesophilic or thermophilic conditions [80]. Bacterial genera includes *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbiospora,* and *Streptomyces*, while fungal genera include *Trichoderma, Aspergillus, Schizophyllum, Sclerotium, Fusarium, Pha- nerochaete, Humicola, Schizophillum* and *Penicillium* [69,150]. Unlike cellulose, hemicellulose (xylan) is chemically quite complex, and its degradation requires multiple enzyme systems, which are produced from a wide variety of fungi and bacteria such as the species of *Trichodrema, Penicillium, Talaromyces, Aspergillus,* and *Bacillus* [69]. Several filamentous fungi are the source of commercial hemicellulases that are used to degrade hemicellulose, where some of these microorganisms can convert hemicellulose and its oligosaccharides and monosaccharides into ethanol [81].

3.7.3 Role of microorganisms in fermentation

Many bacteria and yeasts can ferment soluble sugars in the absence of oxygen, which results in the production of ethanol. These microorganisms can be obtained from yeast, bacteria and filamentous fungi [82]. However, filamentous fungi produce ethanol at low rates and have limited tolerance to ethanol [81]. Although a good number of microorganisms can play roles in the fermentation, the efficiency and feasibility of all microorganisms as ethanol fermenter are not equal. Commercial exploitation of these microorganisms is also limited and only a few are used on large scale ethanol production. The most common and widely used microorganism for ethanol fermentation is a yeast (*S. cerevisiae*), which has been proved to be robust and well suited to the fermentation of lignocellulosic hydrolysates [83, 84]. It can efficiently ferment six carbon sugars, but hardly pentoses due to the lack of enzymes that convert xylose to xylulose [85]. The common bacterial species used for ethanol fermentation is a gram-negative bacterium, *Z. mobilis* [69, 86]. Some thermophilic anaerobic bacteria such as, *Thermoanaerobacter ethanolicus, Clostridium thermohydrosulfuricum, Thermoanaerobacter mathranii, Thermoanaerobium brockii, and Clostridium thermosaccharolyticum* have been investigated for lignocellulosic ethanol production [69]. Even though most bacteria have a broad substrate range, ethanol is rarely the single product of their metabolism that creates difficulties in the

downstream processing of ethanol recovery [87]. The performance of any microorganism as ethanol fermenter can be evaluated based on their efficiency under different process conditions including wide temperature range, pH range, ethanol tolerance, growth rate, ethanol productivity, osmotic tolerance, specificity, ethanol yield, genetic stability, and inhibitor tolerance [69]. The characteristics of an ethanologenic microorganism to be involved in lignocellulosic ethanol production include capability to utilize multiple sugars, high ethanol yield, tolerance to high ethanol concentration, high ethanol productivity, good growth in simple and inexpensive media, capability to grow in undiluted fermentation broth with resistance to inhibitors, and ability to retard contaminants under the growth condition.

3.8 TECHNOLOGICAL PROGRESS IN LIGNOCELLULOSIC ETHANOL

3.8.1 Process integration

Although generation of ethanol from LCB is considered advantageous and promising, still there are some technical and economic challenges, despite the fact that substantial research efforts have been made to improve the conversion process. Requirement of different technological steps, such as pretreatment, detoxification, hydrolysis and fermentation has made the conversion process more complicated and economically non-competitive, thereby confining it into pilot or demonstration plants. In recent years, integration of processing steps at different configuration has been studied as attempts to reduce steps as well as capital investments, energy consumption and process time. Typically, enzymatic hydrolysis of cellulose and fermentation of simple sugars are done separately, which is known to separate hydrolysis and fermentation (SHF). After as light modification, separate hydrolysis and co-fermentation (SHCF) process comes out when both pentose and hexose sugars are produced in a separate hydrolysis of hemicellulose and cellulose, and both sugars are fermented together [55]. The most promising process integration in ethanol production is the simultaneous saccharification and fermentation (SSF), which is widely used both industrial and laboratory scales. SSF is referred to as the performance of both cellulose hydrolysis and fermentation of hexose sugars in a single reactor. SSF is considered as an economic solution for its reduced process time and less equipment requirement, even though still there has some bottlenecks with this technique.

Another emerging and promising technique is the consolidated bioprocessing (CBP), which is an alternative to technique to SSF. It incorporates not only hydrolysis and fermentation of all kinds of soluble sugars but also produce hydrolysing enzymes for converting biomass into ethanol in a single step [89]. The objective of CBP is to generate ethanol from LCB direct and

sustainably that could lead to more competitive and economically viable technology for bringing bioethanol dream into reality, as shown in figure 20 [88].

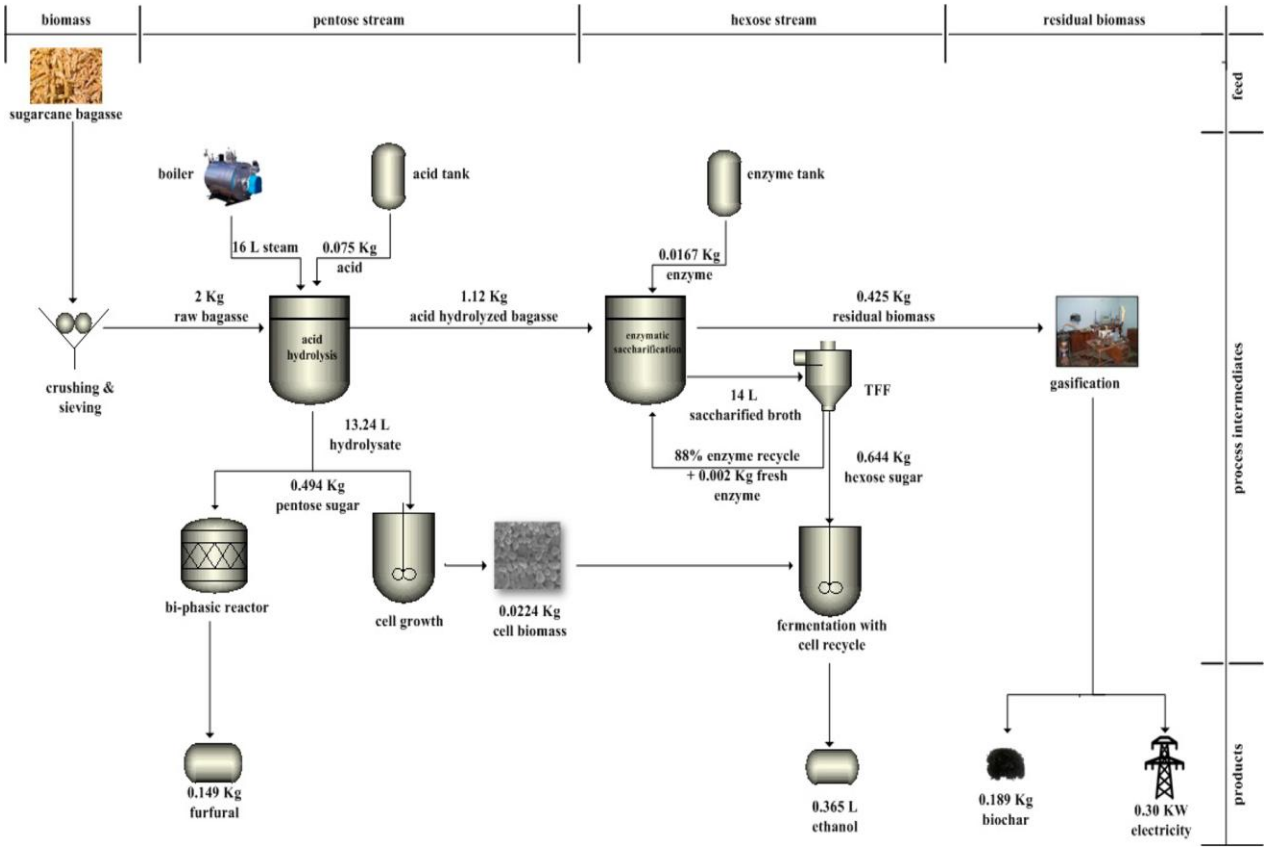

Figure 20. Flow diagram of the consolidated process scheme.

3.9 CO-FERMENTATION OF XYLOSE AND GLUCOSE

Compared to starch based ethanol production that use hydrolysate of starch as the fermentation media containing mostly glucose, the hydrolysate of LCB often contain mixers of sugars, pentoses and hexoses, particularly xylose and glucose, respectively. Glucose is readily fermented to ethanol by many naturally occurring microorganisms with high yield. Although natural microorganisms, especially *S. cerevisiae* have been widely used in fermentation over many years, there are some limitations with this yeast in lignocellulosic ethanol production. One of the major hindrances is the incapability of fermenting xylose, which are produced together with glucose as a result of hydrolysis of hemicelluloses and cellulose. The hydrolysates of hardwood and agricultural residues contain 5–20% xylose, which cannot be utilized by the most commonly used yeast [90]. On the other hand, there are several xylose-fermenting yeasts, such as *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae*.

However, low conversion efficiency has made them un-attractive for lignocellulosic ethanol production on industrial scale. In addition, oxygen uptake rate limits metabolism of these microorganisms that results in low ethanol yield [91]. As attempts to overcome the oxygen limiting problems and ferment both xylose and glucose, a co-culture system has been investigated using xylose and glucose fermenting microorganism (e.g., *Pichia stipitis* and *Saccharomyces cerevisiae*, respectively) in either one single or two reactors [92].

Substantial research efforts have been made in recent years to ferment both glucose and xylose that focus on the development of novel microorganisms altering the genetic pattern of the natural microorganisms with desired traits through either recombinant DNA technology [55, 92] or evolution and adaptation techniques. Recombination of bacteria and yeast makes capable of the strains to co-ferment both xylose and glucose to ethanol. Genetic engineering for heterologous metabolic pathways in a natural strain include oxidoreductase-based pathway along with xylose reductase and xylitolde hydrogenase, and the isomerase-based pathway with xylose isomerase [93, 94]. The genetic modifications have been widely done in three micro-organisms, such as *S. cerevisiae* (yeast), *Z. mobilis* (bacterium) and *E. coli* (bacterium). Although the concept of developing recombinant strains brings some advantages in lignocellulosic ethanol production, several challenges exist with this technological approach, in addition to the fact that commercial exploitation of recombinant strains is still difficult due to some technical barriers. Despite the engineered strains contain both xylose and glucose metabolic pathways, glucose is rapidly and preferentially consumed by these strains in a mixture of sugars due to the lack or low affinity of a xylose transporter protein in the cells [95]. Furthermore, the sequential metabolism of mixed sugars consequences in decreased ethanol productivity and final ethanol yield [96]. As attempts to solve this bottleneck and to attain simultaneous utilization of xylose and glucose during SSF, it has been suggested to maintain a low glucose level through addition of a controlled level of cellulases [97]. An alternative way is to insert and express the gene for *P. stipitis* xylose transporter SUT1in a genetically modified, xylose-assimilating strain that utilize 50/50 of these two sugars during fermentation [98].

According to a US Department of Energy (DOE) study, co-culture bioconversion is a very plausible and potentially high-payoff opportunity for ethanol production [99]. Research has been conducted to study utilization of coculture for ethanol production by co-fermentation of glucose and xylose or various biomass hydrolysates. Some research efforts have shown promising results. These include co-culture of immobilized Z. mobilis and free cells of Pichia stipitis (reclassified as Scheffersomyces stipitis) [100], co-culture of ethanologenic E. coli strain KO11 with S. cerevisiae [101], co-culture of Z. mobilis and Candida tropicalis for ethanol

production from hydrolyzed agricultural wastes [102], co-culture of S. cerevisiae and Pachysolen tannophilis [103], and coculture of restricted catabolite repressed mutant P. stipitis and respiratory-deficient mutant S. cerevisiae [104].

3.10 CURRENT CO-CULTURE SYSTEMS FOR ETHANOL PRODUCTION

Co-cultures are industrially applied to wastewater treatment, biogas production, biological soil remediation, and production of traditional foods, such as cheese, yoghurt, pickles, whisky, and so on [105]. Moreover, many environmental bioconversions are catalyzed by mixed microbial cultures in an apparently stable fashion in natural systems. Based on insights into how commercial and natural systems function, the idea of using a co-culture approach for production of ethanol is to combine a xylose-fermenting microorganism and a glucose-fermenting microorganism to ferment glucose and xylose simultaneously.

3.10.1 Interactions between microorganisms in co-culture systems

To have a stable co-culture, certain requirements must exist. One is that the two strains must be compatible and able to grow together. Laplace et al. [106] have studied the compatibility aspects of various strains through Petri plate assay. According to the study, none of the six tested *S. cerevisiae* strains were found to inhibit growth of *P. stipitis* or *C. shehatae*, and none of the five tested *C. shehatae* strains were found to have an inhibitory effect on growth of Saccharomyces species. Thus, each strain of *C. shehatae* could be used with Saccharomyces species. Among the six tested P. stipitis strains, five demonstrated killer activity against Saccharomyces species, and three of these five strains showed killer activity against *S. cerevisiae*.

Another requirement is that the fermentation conditions, such as pH, temperature, and oxygen supply, for the two strains should be compatible. For example, *Z. mobilis* ferments glucose at pH 7 and temperature of 37 ^{o}C, but these conditions are not compatible with those of xylose-fermenting yeasts (*P. stipitis* and *C. shehatae*), which need pH 5 and temperature 30 ^{o}C. In contrast, the pH and temperature at which S. cerevisiae ferments glucose to ethanol are compatible with those of xylose-fermenting yeasts. Therefore, a combination of *P. stipitis* or *C. shehatae* and *S. cerevisiae* could and has been used in co-culture processes.

Compared with pure culture, interactions between the different microorganisms play a critical role in co-culture systems. The interactions can occur either through direct cell-to-cell communications or by signal substances in the fermentation broth [105]. Specifically, stable co-culture could be controlled by metabolic interactions (i.e., syntrophic relationships, or

competition for substrates) and other interactions (i.e., growth promoters or inhibitors such as antibiotics) [107]. The possible interactions between two microbial species can be categorized as positive, negative, and neutral. For example, positive interaction in cocultures may take place through reduction of available oxygen by aerobic microbes, creating anaerobic conditions that promote growth of anaerobic or microaerophilic strains. This kind of microbial mixed culture provides protection from environmental influences. Negative interaction could happen when two microorganisms compete for the same resource, such as space or a limiting nutrient. Neutral interaction means that there is no effect when the two populations are present together. For a desirable coculture system, positive interactions between the two microorganisms are expected. However, the interactions between microorganisms in mixed culture environments may not always lead to desirable consequences. Therefore, understanding the interactions between associated strains in a co-culture system is very important. However, very little research has been done so far, primarily due to the complex nature of systems containing multiple microorganisms. This offers new avenues for future research.

3.10.2 Strains used in current co-culture systems

The principal naturally ethanologenic microorganisms include a number of yeasts and bacteria. Yeasts include *S. cerevisiae, Kluyveromyces marxianus*, *P. stipitis, C. shehatae, P. tannophilius*, and so on. Bacteria that can produce ethanol from biomass include *E. coli, Z. mobilis, Zymobacter palmae, Clostridium cellulolyticum, Clostridium thermocellum, Clostridium thermosaccharolyticum* (now classified as *Thermoanaerobacterium thermosaccharolyticum*), and *Bacillus stearothermophilus*. Among these microorganisms, *S. cerevisiae* is normally used for ethanol fermentation, but most wild strains of S. cerevisiae are not able to metabolize xylose; *P. stipitis*, *C. shehatae*, and *P. tannophilius* are found to be capable of fermenting xylose [104]. When selecting combined microbial species for a co-culture system, the first step is to choose a glucose-fermenting microorganism and a xylose-fermenting microorganism, then test their compatibility and study their co-fermentation performance.

The yeast genus Saccharomyces is preferably used as the glucose-fermenting strain with a xylose-fermenting strain. Many researchers prefer to use *P. stipitis* as the xylose-fermenting strain with a glucose-fermenting strain in their co-culture systems. The most commonly used strain combination is *P. stipitis* and *S. cerevisiae* or its respiratory-deficient mutant. The reason for this preference is that the pH and temperature at which *S. cerevisiae* ferments glucose to ethanol are compatible with those of *P. stipitis*. Low levels of oxygen (approximately 2 mmol $l\ h^{-1}$) are necessary for efficient ethanol formation from xylose by the xylose-fermenting yeasts

in order to maintain cell viability and nicotinamide adenine dinucleotide (NADH) balance [107, 108]. However, *S. cerevisiae* does not require oxygen to ferment glucose. Respiratory-deficient mutant strains of *S. cerevisiae* have been utilized in co-culture systems to solve this conflict problem of oxygen supply, since a respiratory-deficient Saccharomyces mutant can generate an oxygen profile favorable to xylose-fermenting yeast [110]. The other issue with using the combination of *P. stipitis* and *S. cerevisia*e is that rapid formation of ethanol from glucose in the co-culture scheme may induce inhibition of xylose fermentation due to the low ethanol tolerance of *P. stipitis* [108]. Delgenes et al. applied continuous culture conditions to address this problem [110]. Under continuous fermentation, glucose concentration can be kept sufficiently low so as not to repress xylose utilization by the xylose-fermenting yeast. Apart from *S. cerevisiae*, *S. diastaticus*, *Z. mobilis*, *K. marxianus*, and *C. thermocellum* have served as the glucose-fermenting microorganism in some co-culture systems [110, 106]. On the other hand, *P. tannophilus*, *C. tropicalis*, *C. shehatae*, *K. fragilis*, and recombinant *E. coli* have been used in co-cultures systems as the xylose-fermenting microorganism in place of *P. stipitis* [101, 102, 103].

3.10.3 Fermentation modes

Three fermentation modes can be used in co-culture systems: batch, continuous, and fed-batch. Abbi et al. conducted fermentation of xylose and rice straw hydrolysate by *C. shehatae* in batch, fed-batch, and continuous culture conditions and found that fed-batch or continuous cultures exhibited higher ethanol yields and volumetric productivities [111]. For co-culture systems, the selection of fermentation mode depends on the microbes in the system. Laplace et al. compared batch and continuous fermentation of glucose/xylose mixture by a respiratory-deficient mutant of *S. cerevisiae* co-cultivated with *C. shehatae*. Their results showed that xylose was poorly utilized in batch condition (only 6%), but continuous condition provided simultaneous conversion of glucose and xylose, because the high fermentative potential of *S. cerevisiae* generated glucose concentrations low enough to allow xylose conversion.

Most current co-culture systems were operated in batch mode, and some co-cultures were operated in continuous mode, but no systems used a fed-batch approach. Although batch mode is simple and easily controlled, it has some limitations. One is that the glucose can suppress xylose fermentation, especially at the initial stage, because xylose conversion is completely inhibited at glucose concentration of 2.3 g/l and higher [109]. In a continuous fermentation co-culture system, glucose concentration can be kept sufficiently low so as not to repress xylose utilization by the xylose-fermenting yeast [109, 110]. For example, control can be obtained by adjusting the dilution rate so the glucose concentration can be kept below 2.3 g/l; hence, fast

and simultaneous conversion of glucose and xylose in co-cultured systems can be easily achieved by continuous fermentation. Another way to achieve low glucose concentration is by use of *S. cerevisiae*; its high fermentative potential allows fast xylose conversion by generating a low glucose concentration environment. This approach, however, can be limited if the amount of ethanol produced from glucose exceeds the ethanol tolerance of the xylose-fermenting organism. When the glucose is close to being depleted, the high ethanol concentration (around 30 g/l) can inhibit the xylose fermentation process [112]. Continuous fermentation with medium outflow can avoid accumulation of ethanol and other inhibiting metabolites in the system. This, however, is potentially subject to instabilities if the two organisms show biphasic growth or very different growth rates. Wash out is one of the critical issues for this mode, even with a very low dilution rate.

So far, fed-batch mode has not been utilized in any co-culture system. However, this mode can affect culture growth and avoid overflow metabolism (such as acetate for *E. coli*) due to the feeding of a growth-limiting nutrient substrate. In addition, fed-batch mode can solve the problem that arises when the concentration of inhibitors begins to impact on ethanol production for pentose-fermenting yeasts by maintaining an optimum dilution rate [113]. Therefore, it has advantages for use in co-culture systems.

3.10.4 Fermentation conditions

The fermentation conditions for a co-culture system depend mainly on the selection of the two microorganisms. Each selected pair of microorganisms will have its own optimum values for temperature, pH, aerobic or anaerobic environment, and inoculum size. All of the co-culture fermentations tried thus far have been conducted at laboratory scale with working volume of 1.5 l or less. For simplicity, most researchers use a synthetic medium (a mixture of glucose and xylose). For co-fermentation of glucose and xylose, both the initial total sugar concentration and the proportion of glucose and xylose play important roles in affecting fermentation performance. However, very limited studies have been performed to understand exactly how these two factors interact in co-culture systems. The only known research on the effects of initial total sugar concentration is by Laplace et al. [106], but this effort did not address the question of how different sugar compositions could affect the system. In addition, Laplace et al. investigated the effects of initial total sugar concentration on the fermentative performance of pure cultures for *P. stipitis*, *C. shehatae*, *S. cerevisiae*, and *Z. mobilis*.

Most current co-culture systems used fermentation temperature of 30 ^{o}C. This is especially true for co-culture systems that use the combination of *P. stipitis* and *S. cerevisiae*, since 30 ^{o}C is

optimum for *S. cerevisiae* to ferment glucose and for *P. stipitis* to ferment xylose. The optimal fermentation temperature for *C. thermocellum* and *C. thermosaccharolyticum* is approximately 60 °C [72, 107]. The co-culture system with the combination of *C. thermocellum* and *C. thermohydrosulfuricum* also needed 60°C due to the application of thermophilic anaerobic species.

Since most researchers are familiar with the combination of *S. cerevisiae* and *P. stipitis*, and pH 5.0 is the optimal value for fermentation by these two yeast strains, this value was used in most co-cultures systems. Usually, the pH of the fermentation medium is controlled at 4.5 –7.5 by adding sodium/potassium hydrate or hydrogen chloride.

In addition to the effects of fermentation medium, temperature, and pH, oxygen is an important parameter for some co-culture systems, such as those using *P. stipitis* for xylose fermentation. Fermentation and growth of these yeasts occur simultaneously in a low-oxygen environment [72]. For example, *P. stipitis* induces fermentative activity in response to oxygen limitation [114].

The final parameter for consideration is inoculum size. However, there is little information published on how relative inocula volumes influence fermentation performance. the inoculum volume in current co-culture systems ranged from 2% to 10% (v/v), and most co-culture systems selected inocula size of 3% (v/ v). As to the ratio of the glucose-fermenting microorganism to the xylose-fermenting microorganism, Laplace et al. [106], and Kordowska-Wiater and Targon ´ski [104] chose to use the same amount of the two species in their systems.

3.10.5 Fermentation performance

Several parameters such as ethanol yield (Yp/s), volumetric ethanol productivity (Qp), specific ethanol production rate (q_p), efficiency of substrate utilization (E) or other measures are used to evaluate fermentation processes. Among these, ethanol yield (g ethanol/g consumed sugar substrate) is most useful. The theoretical ethanol yield for glucose/ xylose fermentation is 0.51 g/g [53]. However, neither sugar yields this much ethanol in practice.

The overall ethanol yield of different co-culture systems ranged from 0.25 to 0.50 g/g. The highest overall ethanol yield was obtained at 0.49–0.5 g/g, which is about 98% of the theoretical yield [92]. By employing the strain combination of P. stipitis and respiratory-deficient mutant S. cerevisiae in batch fermentation, Taniguchi et al. [92] successfully achieved complete sugar utilization in 40 h at volumetric ethanol productivity of 0.94 g/l/h and ethanol yield of 0.50 g/g.

3.10.6 Potential benefits and challenges to using co-culture for co-fermentation of hexose and pentose at industrial scale

One of the major challenges to the co-culture process is the apparently low ethanol tolerance of xylose-fermenting yeasts [115]. Delgenes et al. [110] reported that ethanol inhibition of *P. stipitis* occurs at ethanol concentration of 30 g/l.

Another major challenge is finding optimal operating ranges for process parameters (pH, temperature, and oxygen demand) and the acceptable ranges of substrate that can enable optimal activity of each strain in co-culture. Unlike pure culture, co-culture organisms can differ with respect to pH, temperature, and oxygen requirements. Therefore, compromises in process parameters are sometimes necessary; for example, competition for oxygen resulted in low xylose conversion by co-immobilized *S. cerevisiae* and *P. stipitis* [109].

Lignocellulosic hydrolysates contain a broad range of inhibitory and toxic compounds, the composition and concentration of which depend upon the type of lignocellulosic materials and the pretreatment and hydrolysis processes. These inhibitory and toxic compounds have a significant and negative impact on pure culture fermentation, and even co-culture fermentation. Due to use of synthetic medium, most laboratory co-cultures did not encounter these problems.

3.11 KINETIC MODEL FOR CO-CULTURE SYSTEMS

Diverse kinetic models have been proposed for pure cultures on co-fermentation of glucose and xylose in the literature [73]. However, very little investigation has been done on modelling of such a co-culture system to describe the dynamics of the system quantitatively. This is due to the complex nature of the dynamics, the difficulty in analysing the dynamics, and control of systems containing two microorganisms.

Another problem associated with developing kinetic models for co-culture systems is the lack of kinetic parameters for each strain in a co-culture system. Due to the interactions between the two strains in co-culture systems, the behaviour of each strain is different from that seen in pure culture, and therefore the kinetic parameters of each strain in co-culture and pure culture will be different. In theory, by capturing the dynamic properties of systems, a kinetic model could be used as a powerful tool to help obtain optimum operating conditions, achieve sufficient profitability, and reduce tests by eliminating extreme possibilities. For these reasons, it would be worthwhile to develop a kinetic model for ethanol-production co-culture systems.

3.11.1 Kinetic Parameters

In fermentation processes, the determination of specific rates of growth (μ_X), production (μ_P), and consumption (μ_S) was taken into consideration and calculated according to Equations. 10 to 12:

$$\mu_x = \frac{1}{X}\left(\frac{dX}{dt}\right) \tag{10}$$

$$\mu_s = \frac{1}{S}\left(-\frac{dS}{dt}\right) \tag{11}$$

$$\mu_p = \frac{1}{X}\left(\frac{dP}{dt}\right) \tag{12}$$

To evaluate the influence of substrate in microorganism growth, the values of K_S and μ_{max} were calculated according to Monod equation (Eq. 13), applying the linearization method of Lineweaver–Burk (Eq. 14).

$$\boldsymbol{\mu = \frac{\mu_{max}.S}{K_s + S}} \tag{13}$$

$$\boldsymbol{\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max}}.\frac{1}{S}} \tag{14}$$

The biomass yield based on substrate ($Y_{X/S}$) and ethanol yield based on substrate ($Y_{P/S}$) were defined by Eqs. 15 and 16:

$$Y_{\frac{X}{S}} = \frac{X_f - X_0}{S_0 - S_f} \tag{15}$$

$$Y_{\frac{P}{S}} = \frac{P_f - P_0}{S_0 - S_f} \tag{16}$$

3.11.2 Mathematical Modelling

The dynamic description of ethanol fermentation using unstructured models can be carried out basically with three differential equations for microorganism growth, substrate uptake, and ethanol formation (Eqs. 17-19), which can be obtained from the mass balance in the reactor.

$$r_x = \frac{dX}{dt} \tag{17}$$

$$r_s = \frac{dS}{dt} \tag{18}$$

$$r_p = \frac{dP}{dt} \tag{19}$$

Where, X, S, and P are the concentrations of cells, substrate, and ethanol, respectively. The rates of cell growth, r_x (g L−1 h−1), substrate uptake, r_S (g L−1 h−1), and product formation, r_P (g L−1 h−1), can be expressed by a non-structured model for the process in batch mode.

r_x is determined by using Andrews and Levenspiel's model and product by Leudking-Piret equation. The parameters thus determined (using the above equations with the data obtained in fermenter runs) were used to predict the models [116].

MATERIALS AND METHODS

4.1. Microorganisms

4.1.1. Growth and maintenance of strains used

Saccharomyces cerevisiae (wild strain) and *Scheffersomyces stipitis* (NCIM 3507) were used in this study. *S. cerevisiae* ferments hexoses anaerobically and *S. stipitis* ferments hexoses and pentoses under microaerophilic conditions. The cultures were preserved for a longer period in 10% (vv^{-1}) glycerol stock by refrigeration at -20^0C. Revival of stock was done by overnight incubation of 500 µl culture in 50 ml YPD broth in 100 ml Erlenmeyer flask at 30^0C on an orbital shaker (180 rpm). The strains were maintained in YPD agar (composition (wv^{-1}):- 1% yeast extract, 2% peptone, 2% dextrose and 2% agar) slants and plates for short term storage at 4^0C.

S. cerevisiae and *S. stipitis were* acclimatized to glucose and xylose respectively by sub culturing in YPM supplemented with glucose or xylose. The strains were stored in YPM plates at 4^0C until further use. All media components were obtained from Himedia (Mumbai, India).

4.1.2. Glycerol stock preparation

20% (v/v) solution of glycerol was prepared. This solution and overnight culture were taken in 1:1 ratio in a cryopreservation vial. This stock of culture was kept at -20^o C deep freezer.

4.2. Fermenter and fermentation media

4.2.1. Media

YPD (Yeast extract Powder Dextrose) was used for growth and maintenance of yeast cultures of both *S. cerevisiae* and *S. stipitis*; the composition of YPD is as mentioned (wv^{-1}): 1% yeast extract, 2% peptone and 2% dextrose.

YPM media was used for acclimatization of yeast on glucose or xylose. YPM-G (glucose) was used for maintaining stock of *S. cerevisiae* and YPM-X was used for *S. stipitis*. The composition of YPM – 5g/L peptone, 3g/L yeast extract, 3g/L malt extract, 0.2g/L calcium chloride, 2.5g/L potassium dihydrogen phosphate, 0.5g/L magnesium sulphate septahydrate, 1g/L ammonium sulphate, 10g/L glucose/xylose.

Ethanol fermentation was carried out in YE media containing 0.1% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.01% $CaCl_2$, 0.01% NaCl, 0.05% $MgSO_4$, 0.1% KH_2PO_4 and 2% sugars (glucose

or xylose) (all in (wv^{-1}))of pH 5.6 [7]. The same media (YEG/X) was used for inoculum development and all the further experiments.

4.2.2. Growth curve

S.cerevisiae and *P.stiptis* were inoculated in the YPD culture media and kept for 24 hrs at 30 degree Celcius. Samples were taken every 2 hrs and biomass was evaluated at OD_{600} nm. A graph for growth was plotted for *S. cerevisiae* and *S. stipitis* as shown below:

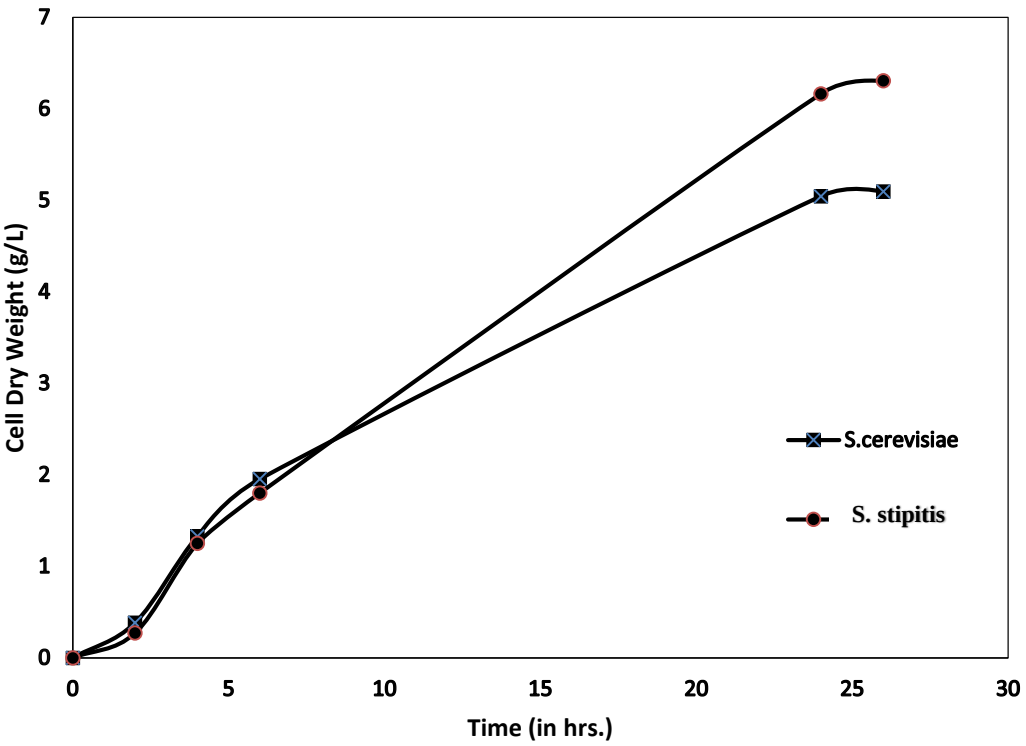

Figure 21. Growth curves for S. stipitis and S. cerevisiae

4.2.3. Correlation between OD and CDW

Media was prepared for *S. cerevisiae* and *S. stipitis* separately, autoclaved and inoculated with respective strains. The cultures were kept at 30 degree Celsius for 24 hrs and samples were taken after every 2 hrs. The samples were analysed for OD at 600 nm and then the dry weight was determined. A graph between OD at 600nm and Cell dry weight was plotted for both the strains.

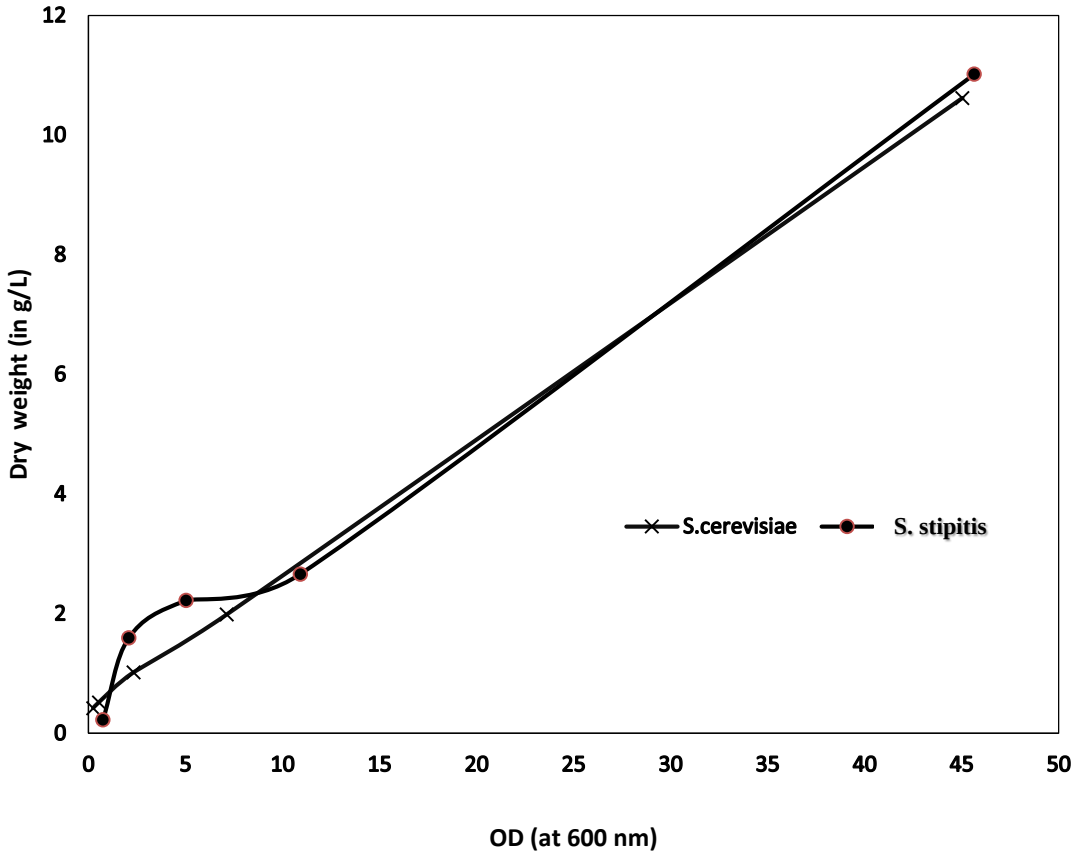

Figure 22. Graph for OD600 vs. Cell Dry weight

4.2.4. Fermenter

All batch fermentation experiments (anaerobic and microaerophilic) were carried out in 7l jacketed bioreactor vessel (Applikon Bio Console ADI 1025 Bio Controller 1010); working volume of 4 l. The vessel was autoclaved along with the fermentation media (121^0C, 15psi). Sugars and phosphates were separately sterilized. DO and pH probes were calibrated before the reactor was sterilised.

The controllers were set to maintain pH (5.6±0.2), temperature (30±1 ^{0}C), agitation speed (180±20 rpm). The pH was adjusted and maintained throughout the fermenter run at 5.6 with 1N HCl (Hydrochloric Acid) and 1 N NaOH (Sodium Hydroxide). Foam was controlled by injecting 1-2 drops of antifoam (sterile 5% (vv^{-1}) suspension of silicon oil in poly propylene glycol (PPG) solution), occasionally.

4.3. k_La estimation

For microaerophilic fermentations, k_La measurement was done by static method. Very low oxygen concentrations were maintained in the reactor by sparging air at a flow rate of 0.25 vvm. For anaerobic fermentation, the media was sparged with nitrogen maintaining DO concentration at 0%.

The following equation was used for the estimation:

$$\frac{dCox}{dt} = kLa\ (Cox * -Cox) - q0xb \qquad (20)$$

Where,

k_La is volumetric oxygen transfer coefficient in the fermenter,

C_{ox}* is the saturation concentration of oxygen,

C_{ox} is the dissolved oxygen concentration,

$q_{0x}b$ is the oxygen uptake rate by microorganisms, which is zero for the static method.

4.4. Batch fermentation for kinetic studies

The kinetic parameters of batch fermentation were quantified separately for *S. cerevisiae* and *S. stipitis*. Therefore, fermenter runs of pure cultures were carried out for 48 hours for the following cases:-

i) Anaerobic fermentation with *S. cerevisiae* and glucose

ii) Microaerophilic fermentation with *S. stipitis* and glucose

iii) Microaerophilic fermentation with *S. stipitis* and xylose

Seed cultures were raised in 250 ml of fermentation media containing glucose or xylose by inoculating a colony of the yeast and incubating it overnight at 30^0C, 180 rpm. Inoculum was prepared in such a way that the initial biomass concentration in the reactor was equal to 0.1 g cell/l.

4.5. Co-culture batch fermentation

Microaerophilic co-culture fermentation was carried out for 48 hours with 50% each of *S. cerevisiae* and *S. stipitis* in YE fermentation media containing both glucose and xylose (1% (wv^{-1}) each), so that the total initial biomass concentration in the reactor was equal to 0.1 g/l.

4.6. Analytical methods

5 ml sample was collected from the fermenter aseptically every 2 hours for 48 hours, out of which, 1 ml was used for the analysis of biomass. The remaining 4ml was centrifuged at a speed of 8500 rpm for 10 min. The pellet was kept and the supernatant was stored in 1 ml aliquots at 20^0C until further analysis of sugars and ethanol.

4.6.1 Cell biomass concentration

Cell biomass concentration of pure culture batch fermentations was determined from a correlation between OD_{600} and cell dry weight. OD_{600} 1 corresponds to cell dry weight (CDW) of 0.6 g/l and 0.9 g/l respectively, for *S. cerevisiae* and *S. stipitis*.

Cell biomass concentration of *S. cerevisiae* and *S. stipitis* in mixed culture was determined by differential plating, based on the ability of *S. stipitis* to grow on glycerol plates containing 0.1% (wv^{-1}) erythromycin(Laplace et al., 1992, 1993). The number of colony forming units (CFUS) was enumerated from YEP + glucose (*S. cerevisiae* and *S. stipitis*) and YEP + glycerol plates (*S. stipitis* alone) (in triplicates). The individual CDWs were then calculated from a correlation made between CDW and the number of CFUs from pure cultures of *S. cerevisiae* and *S. stipitis*, separately.

4.6.2 Analysis of sugars and ethanol

Glucose, xylose and ethanol were determined by HPLC (Agilent 1100 series, Agilent Technologies) with an Aminex® HPX-87 H column (Bio-Rad Hercules, CA, USA)) and a refractive index detector (RID). 5 mM H_2SO_4 was used as mobile phase at 50^0C and a flow rate of 0.7 ml/min. Culture supernatant aliquots after thawing were filtered through 0.22μm nylon syringe filters (Axiva), prior to HPLC. Ethanol and sugar concentrations were calculated from correlations between peak area and concentration of standard solutions.

4.6.3 Colony Counting and Replica plating

When doing co-culture, the *S. stipitis* and *S.cerevisiae* are hard to be differentiated. So, this can be done by doing replica plating and counting colonies by using 0.1 percent erythromycin in the medium. *S.cerevisiae* is inhibited by the action of erythromycin but *S. stipitis* survives on the erythromycin plate and thus the two strains can be distinguished from one another. If these two strains can be grown on same plate and replica plating is done, then only *S. stipitis* survives and thus we can count the number of colonies of both individually.

Composition of media for replica plating:

Erythromycin	- 1g/L	Peptone	- 2%
Glycerol or Glucose	- 2%	Yeast extract	- 1%

4.7. Model Development

Monod model was assumed for microbial growth. Modifications were made to Monod equation accounting for ethanol inhibition and glucose repression. Non-competitive inhibition was assumed. Following Slininger et al. (2014) [117], dissolved oxygen was assumed to be a substrate. Model equations were put together for rate of change of biomass (*S. cerevisiae* and *S. stipitis*), product (ethanol) and substrates (glucose, xylose and dissolved oxygen).

4.8. Model equations

All the terms and constants used in the equations are described in table 6.

Table 6. Nomenclature used in modelling

Symbol	Parameter
$\mu_{\max 1,G}$	maximum specific growth rate of 1 on glucose (l/h)
$\mu_{\max 2,G}$	maximum specific growth rate of 2 on glucose (l/h)
$\mu_{\max 2,Xy}$	maximum specific growth rate of 2 on xylose (l/h)
$K_{G,1}$	saturation constant for growth of 1 on glucose(g/l)
$K_{G,2}$	saturation constant for growth of 2 on glucose (g/l)
$K_{Xy,2}$	saturation constant for growth of 2 on xylose (g/l)
α	conversion factor for the specific growth rate from anaerobic to microaerophilic conditions
K_{Ox}	saturation constant for oxygen limited growth(g/l)
$K_{iE,G}$	ethanol inhibition constant on glucose uptake(g/l)
$K_{i,G}$	glucose repression constant on xylose (g/l)
$K_{iE,Xy}$	ethanol inhibition constant on xylose uptake (g/l)
$Y_{X_1/G}$	yield of biomass 1 on glucose (g/g)
$Y_{X_2/G}$	yield of biomass 2 on glucose (g/g)
$Y_{X_2/G}$	yield of biomass 2 on xylose (g/g)
$Y_{E_1/G}$	yield of ethanol by 1 on glucose (g/g)

$Y_{E_2/G}$	yield of ethanol by 2 on glucose (g/g)
$Y_{E_2/Xy}$	yield of ethanol by 2 on xylose (g/g)
$Y_{X_2/Ox}$	yield of biomass 2 on oxygen (g/g)
k_{la}	volumetric oxygen transfer coefficient (h^{-1})
C_{Ox}^{*}	equilibrium oxygen concentration (g/l)
C_{Ox}	concentration of glucose(g/l)
C_{Xy}	concentration of xylose (g/l)
C_{Ox}	concentration of oxygen (g/l)
C_E	concentration of ethanol (g/l)
X_1	biomass concentration of 1 (g/l)
X_2	biomass concentration of 2 (g/l)

1- *Saccharomyces cerevisiae* 2- *Scheffersomyces stipitis*

The specific growth rate of *S. cerevisiae* on glucose substrate under anaerobic conditions is indicated by equation 21. Growth of *S. cerevisiae* is inhibited by higher concentration of ethanol, which is accounted for in equation 21.

$$\mu_{1,G} = \mu_{\max 1,G} \frac{C_G}{K_{G,1} + C_G} \frac{1}{1 + \frac{C_E}{K_{iE,G}}} \tag{21}$$

The specific growth rate of *S. stipitis* on glucose and xylose as substrates under microaerophilic conditions are also affected by higher concentrations of ethanol as indicated in equations 22 and 23.

$$\mu_{2,G} = \mu_{\max 2,G} \frac{C_G}{K_{G,2} + C_G} \frac{1}{1 + \frac{C_E}{K_{iE,G}}} \tag{22}$$

$$\mu_{2,Xy} = \mu_{\max 2,Xy} \frac{C_{Xy}}{K_{Xy,2} + C_{Xy}} \frac{1}{1 + \frac{C_E}{K_{iE,Xy}}} \tag{23}$$

S. cerevisiae tends to have a higher growth rate under aerobic conditions [118, 119]. Hence the growth rate of *S. cerevisiae* in anaerobic culture had to be multiplied by a factor α to get its growth rate under microaerophilic conditions. Therefore, the rate of change of concentration of *S. cerevisiae* under microaerophilic conditions is given by equation 24.

$$\dot{X}_1 = \alpha \mu_{1,G} X_1 \tag{24}$$

According to Slininger [117], the specific growth rate of *S. stipitis* is dependent on dissolved oxygen concentration. Also, the growth of *S. stipitis* is affected by glucose repression in presence of glucose [120, 121, 4]. Therefore, the rate of change of concentration of *S. stipitis* is given by equation 25.

$$\dot{X}_2 = \left(\mu_{2,G} + \mu_{2,Xy}\right) \frac{C_{Ox}}{K_{Ox} + C_{Ox}} \frac{1}{1 + \frac{C_G}{K_{i,G}}} X_2 \tag{25}$$

S. stipitis can also assimilate hexoses. Hence the rate of glucose consumption is the sum of the glucose consumption rates of *S. cerevisiae* and *S. stipitis* (equation 26). Also, xylose gets consumed by *S. stipitis* only (equation 27).

$$\dot{C}_G = -\frac{1}{Y_{X_1/G}} \mu_{1,G} X_1 - \frac{1}{Y_{X_2/G}} \mu_{2,G} \frac{C_{Ox}}{K_{Ox} + C_{Ox}} X_2 \tag{26}$$

$$\dot{C}_{Xy} = -\frac{1}{Y_{X_2/Xy}} \mu_{2,Xy} \frac{C_{Ox}}{K_{Ox} + C_{Ox}} \frac{1}{1 + \frac{C_G}{K_{i,G}}} X_2 \tag{27}$$

Since the growth of *S. stipitis* is dissolved oxygen limited, the rate of oxygen depletion is given by equation 28.

$$\dot{C_{ox}} = k_{la}(C_{Ox}^* - C_{Ox}) - \frac{1}{Y_{X_2/Ox}}\left(\mu_{2,G} + \mu_{2,Xy}\right) \frac{C_{Ox}}{K_{Ox} + C_{Ox}} X_2 \tag{28}$$

Ethanol is produced by *S. cerevisiae* as well as *S. stipitis* by consumption of glucose and xylose, the rate of production is given by equation 29.

$$\dot{E} = \frac{Y_{E_1/G}}{Y_{X_1/G}}\,\mu_{1,G}\,X_1 + \frac{Y_{E_2/G}}{Y_{X_2/G}}\,\mu_{2,G}\,\frac{C_{Ox}}{K_{Ox}+C_{Ox}}\,X_2 + \frac{Y_{E_2/Xy}}{Y_{X_2/Xy}}\,\mu_{2,Xy}\,\frac{C_{Ox}}{K_{Ox}+C_{Ox}}\,\frac{1}{1+\frac{C_G}{K_{i,G}}}\,X_2 \quad (29)$$

4.9. Solving the model

The kinetic parameters like maximum specific growth rates, glucose and xylose saturation constants and yield co-efficients of biomass and ethanol on glucose and xylose were determined from biomass, sugar and ethanol concentrations of each pure culture. The other constants (α, K_{ox}, $K_{i,\,G}$, $K_{iE,\,G}$, $K_{iE,\,and\,Xy}$) were determined by trial and error from a range of values tested.

The mathematical models were solved using MATLAB R2014b (The MathWorks Inc., Natick, Massachusetts). Solver ode45 which utilises Runge-Kutta method for integration was used for solving the ordinary differential equations 24-29. The concentration profiles for biomass, substrates and ethanol were then plotted.

4.10. Validation of the model

Model simulations were done by simultaneously testing the ranges of values for those parameters that could not be obtained from pure culture fermentation studies. The concentration profiles from these simulations were compared with experimental results and those tested values of kinetic constants that gave the best agreement with the experimental results were accepted. Model-predicted and experimental values were subjected to t- test for statistical comparison using Minitab®17.1 (Minitab Inc., Pennsylvania, US). Model validation was done by evaluating and analysing various indices of agreement.

Statistical validation of the models were carried out by evaluating mean absolute error (MAE), root mean square error (RMSE), refined index of agreement (d_r), index of agreement (IA), relative error (RE), Nash-Sutcliffe coefficient of efficiency (E) and Legates-McCabbe's index (E) [122, 123]. Square of the correlation coefficient (R^2) was also calculated.

1. Mean Absolute Error (MAE) is given by equation S-1

$$MAE = \frac{\sum_{i=1}^{n}|P_i - O_i|}{n} \quad (S\text{-}1)$$

Where,

n represents the number of time points,

P_i signifies the predicted value of concentration at each time point, and

O_i is the experimental value.

MAE was divided by the mean of observed values ($\bar{O}$) and reported as relative percentage (MAE/$\bar{O}$ X 100).

2. Root Mean Square Error (RMSE) is given by equation S-2

$$RMSE = \left[\frac{\sum_{i=1}^{n}(P_i - O_i)^2}{n}\right]^{0.5} \quad \text{(S-2)}$$

3. Relative error (RE) is given by equation S-3

$$RE = \frac{RMSE}{\bar{O}} \quad \text{(S-3)}$$

4. Wilmott's refined index of agreement (d_r) is given by equation S-4

$$d_r = \begin{cases} 1 - \frac{\sum_{i=1}^{n}|P_i - O_i|}{c\sum_{i=1}^{n}|O_i - \bar{O}|}, when \\ \sum_{i=1}^{n}|P_i - O_i| \leq c\sum_{i=1}^{n}|O_i - \bar{O}| \\ \frac{c\sum_{i=1}^{n}|O_i - \bar{O}|}{\sum_{i=1}^{n}|P_i - O_i|} - 1, when \\ \sum_{i=1}^{n}|P_i - O_i| > c\sum_{i=1}^{n}|O_i - \bar{O}| \end{cases} \quad \text{(S-4)}$$

Where, $\bar{O}$ is the mean of experimental values and c=2.

5. Wilmott's Index of Agreement (IA) is given by equation S-5

$$IA = 1 - \frac{\sum_{i-1}^{n}(P_i - O_i)^2}{\sum_{i=1}^{n}(|P_i - \bar{O}| + |O_i - \bar{O}|)^2} \quad \text{(S-5)}$$

6. Nash-Sutcliffe's coefficient of efficiency (E) is given by equation S-6

$$E = 1 - \frac{\sum_{i=1}^{n}(P_i - O_i)^2}{\sum_{i=1}^{n}(O_i - \bar{O})^2} \quad \text{(S-6)}$$

7. Legates McCabbe's index (E_1) is given by equation S-7

$$E_1 = 1 - \frac{\sum_{i=1}^{n} |P_i - O_i|}{\sum_{i=1}^{n} |O_i - \bar{O}|} \qquad \text{(S-7)}$$

8. Squares of the co-efficient of correlation (R^2) for experimental and predicted values of concentration were evaluated using Minitab® 17.1 and reported as percentage. The results of statistical validation are given in table 7.

	Saccharomyces cerevisiae	***Scheffersomyces stipitis***	**Glucose**	**Xylose**	**Ethanol**	**Dissolved Oxygen**
MAE(%)	20.8	22.5	27.4	10.9	19.5	9.9
RMSE	0.115	0.248	0.858	0.597	0.772	0.001
RE	0.252	0.308	0.665	0.149	0.280	0.130
d_r	0.673	0.769	0.912	0.944	0.712	0.547
IA	0.867	0.929	0.980	0.995	0.910	0.850
E	0.589	0.742	0.910	0.981	0.603	0.272
E_1	0.347	0.538	0.823	0.888	0.425	0.094
R^2 (%)	60.4	75.4	94	98.2	76.4	68.9

Table 7. Results of the statistical validation of co-culture mathematical models

The percentage of MAE was less than 25% for all cases except for glucose data. The relative error was also the highest for glucose. However, R^2 and other indices were very high (close to 1) for glucose data, indicating good performance of the model. According to the supplementary material provided by Bonifacio et al.(2017) [122], agreement between model predicted and experimental data is perfect, very high, high, moderate, low or very low if IA falls in the range 1, 0.8-1.0, 0.6-0.8, 0.4-0.6, 0.2-0.4, 0-0.2, respectively. All the values for IA, as given in the table, fall in the range satisfying the criteria for 'very high' agreement between the experimental and simulated data. Therefore, the models are statistically valid.

RESULTS AND DISCUSSION

5.1. Kinetic parameters from batch fermentation of pure culture

The concentration profiles of biomass, sugars and ethanol for pure cultures of *S. cerevisiae* on glucose, *S. stipitis* on glucose and xylose are given in figure respectively. Glucose depleted within 10 and 16 hours of fermentation for *S. cerevisiae* and *S. stipitis* whereas, ethanol concentration reached maximum at 20 hours and peaked at 40 hours for *S. cerevisiae* and at 24 hours for *S. stipitis*. When xylose was the substrate, it took 30 hours for *S. stipitis* to consume xylose and 34 hours to maximize ethanol titre. However, biomass concentration remained low for *S. cerevisiae* as anaerobic conditions did not favour biomass build up. Microaerophilic fermentation of *S. stipitis* favoured biomass increase. The maximum volumetric productivities were 0.16, 0.23 and 0.2 ($gl^{-1}h^{-1}$) respectively for *S. cerevisiae* and *S. stipitis* on glucose and xylose. The overall volumetric productivities were 0.11, 0.07 and 0.07 ($gl^{-1}h^{-1}$) respectively for *S. cerevisiae* on glucose and *S. stipitis* on glucose and xylose. The rest of the kinetic parameters relevant to the model simulation as well as the tested range of unknown parameters are summarised in table. It is evident from the results that the growth rate of *S. cerevisiae* on glucose under anaerobic conditions is lower than that of *S. stipitis* on glucose or xylose. Even though the overall yield of biomass of *S. cerevisiae* is considerably lower, overall ethanol yield of the same was better than *S. stipitis* fermented on either glucose or xylose.

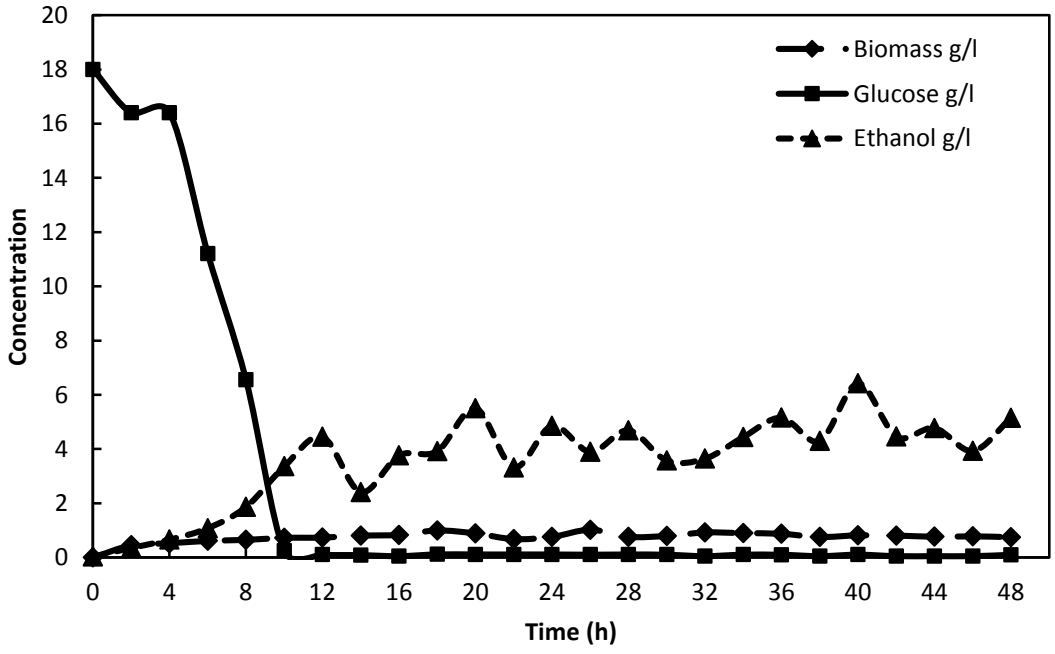

Figure 23. Concentration profiles of pure culture fermentation (a) Saccharomyces cerevisiae on glucose substrate

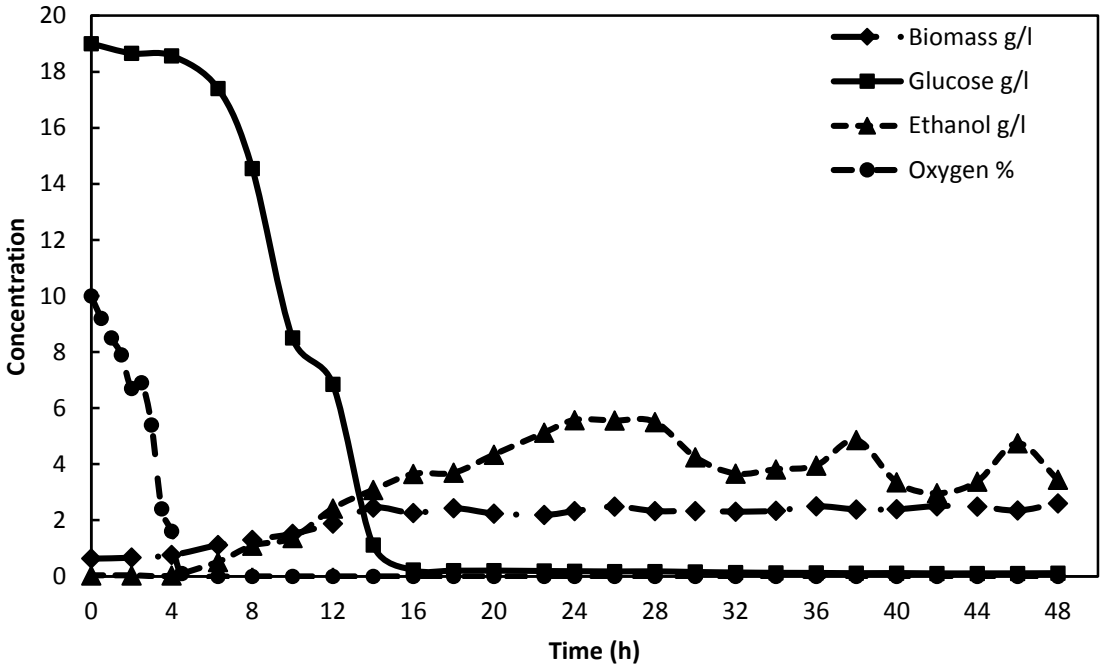

Figure 24. Concentration profiles of pure culture fermentation (b) Scheffersomyces stipitis on glucose substrate

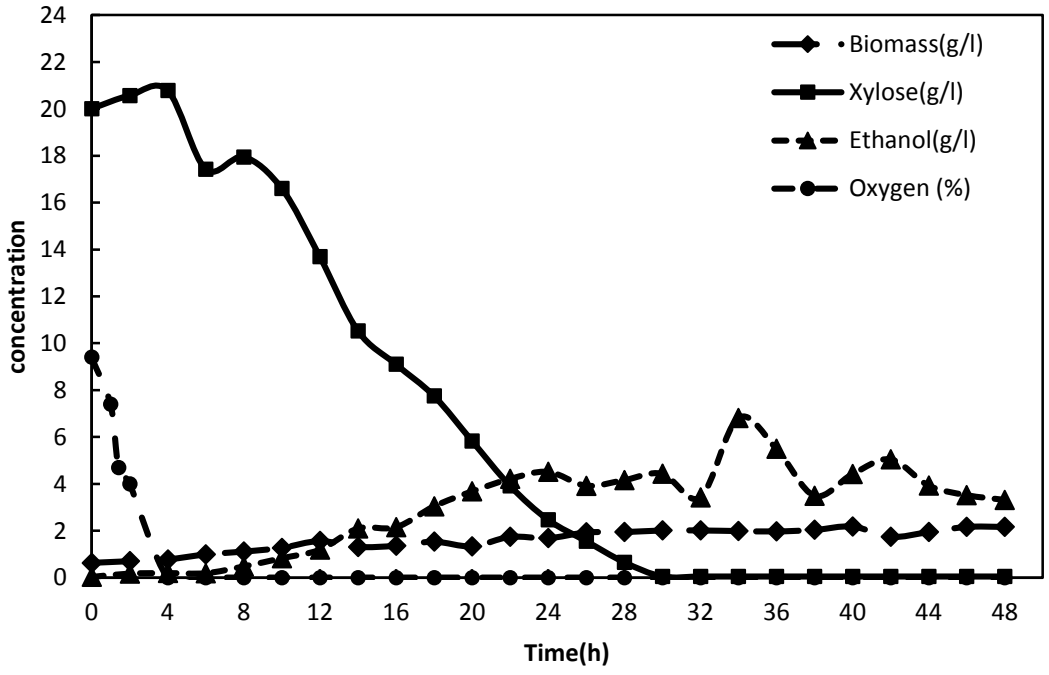

Figure 25. Concentration profiles of pure culture fermentation (c) Scheffersomyces stipitis on xylose substrate.

Table 8. Kinetic parameters obtained or range tested in this study

Parameter	Value obtained	Range tested
$\mu_{\max 1,G}$	0.08	--
$\mu_{\max 2,G}$	0.1	--
$\mu_{\max 2,Xy}$	0.1	--
$K_{G,1}$	0.5	--
$K_{G,2}$	0.7	--
$K_{Xy,2}$	0.3	--
α	--	1-5
K_{Ox}	--	0.0001-0.01
$K_{iE,G}$	--	1-1000
$K_{i,G}$	--	1-1000
$K_{iE,Xy}$	--	1-1000
$Y_{X_1/G}$	0.05	--
$Y_{X_2/G}$	0.14	--
$Y_{X_2/G}$	0.11	--
$Y_{E_1/G}$	0.29	--
$Y_{E_2/G}$	0.18	--
$Y_{E_2/Xy}$	0.17	--
$Y_{X_2/Ox}$	--	0-5
k_{la}	15.2	--

5.2. Concentration profiles of co-culture ethanol fermentation

The concentration profiles of *S. cerevisiae*, *S. stipitis*, glucose, xylose, ethanol and dissolved oxygen during co-culture fermentation are given in fig. Glucose was consumed within 10 hours of fermentation. Xylose began to be utilized only after that and was consumed within 32 hours, when the ethanol concentration also peaked. Viable cell concentration of *S. cerevisiae* decreased after 16 hours after which the biomass concentration of *S. stipitis* began to increase. The increase of *S. stipitis* concentration until then confirms that both yeasts consumed glucose. The maximum and overall ethanol productivities were 0.14 $gl^{-1}h^{-1}$ and 0.05 $gl^{-1}h^{-1}$, lower than

the productivities of pure culture. Co-culture with equal initial concentrations of biomass and substrate did not favour high ethanol titres. Dissolved oxygen concentration decreased in the first few hours of fermentation and remained low thereafter.

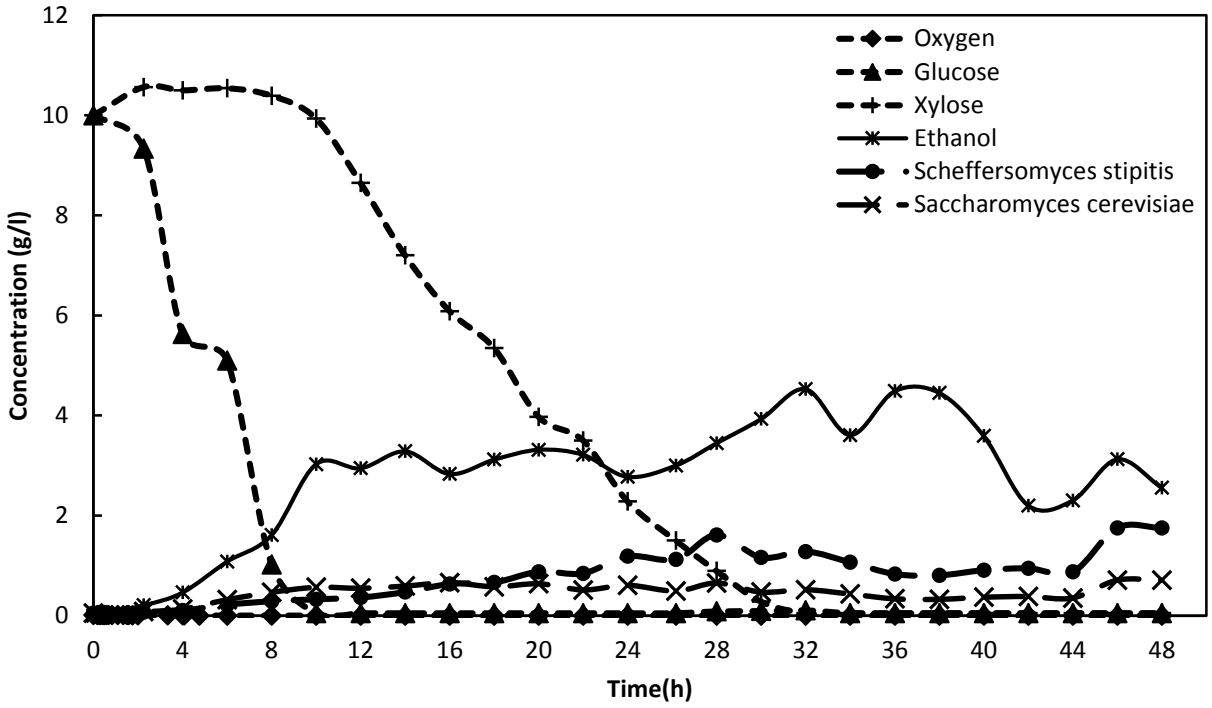

Figure 26. Concentration profiles of co-culture fermentation.

5.3. Simulation and model validation

Simulations were done for the parameter ranges tested, simultaneously making parity plots between experimental and simulated values. Those values of the parameters that gave the best parity were selected, summarized in table 10. The parity plots for biomass, substrates and ethanol considering an error margin of 25% is given in fig. The concentration profiles from the model prediction are given in fig. The experimental values stayed within the error margin for xylose, glucose and dissolved oxygen concentrations. A few points are outside the error margin in the parity plots of biomass and ethanol concentrations. This observation is substantiated by fig, plotted to draw a comparison between experimental and predicted data. It has to be noted that a deviation from the ideal profile is observed after 10 hours for *S. cerevisiae* and 28 hours for *S. stipitis*, the time points when glucose and xylose are exhausted. It is also around the same

time that ethanol concentrations deviate from its ideal profile. Plating technique enumerated the number of viable cells in the bioreactor. Therefore, naturally, after glucose or xylose exhaustion, the number of viable cells should decrease. But, instead, the growth rate showed an oscillatory behaviour, consistent with an oscillatory behaviour in ethanol titre. These results support the fact that both yeasts consumed ethanol under aerobic conditions after sugar exhaustion [6, 124]. This also explains why the ethanol productivity in co-culture is lower than that of pure cultures. Hence, the models apply well until the sugars are exhausted. To rectify this issue the model can be used to optimize the concentration of the biomass and the substrates such that the sugars are exhausted simultaneously. According to Unrean and Khajeeram (2015)[7], optimisation of the ratio of *S. cerevisiae* to *S. stipitis* in co-culture improved the ethanol titre and productivity by 26% and 29% respectively, when compared to pure culture. Table 10 also compares the kinetic parameters obtained from this study with the literature. Introduction of the term 'α' in the model equation makes up for the increase in the maximum specific growth rate of *S. cerevisiae* in the presence of dissolved oxygen. The inhibition constants ($K_{iE,\,G}$, $K_{i,\,G}$, $K_{iE,\,Xy}$) adopted in the literature for co-culture were obtained from pure culture studies. Higher values of inhibition constants obtained in this study accounts for the interaction between the yeasts in the co-culture. Also, yield of *S. stipitis* on oxygen is higher in this study.

Parameter	Value obtained in this study	Value in literature	Reference
α	3.13	--	--
K_{Ox}(g/l)	0.0001	0.0001	[117]
$K_{iE,G}$(g/l)	100	10	[6]
$K_{i,G}$(g/l)	100	0.5	[6]
$K_{iE,Xy}$(g/l)	100	4.5	[6]
$Y_{X_2/Ox}$(g/g)	1	0.0027	[117]

Table 9. Kinetic parameter values that gave the best parity and comparison with literature

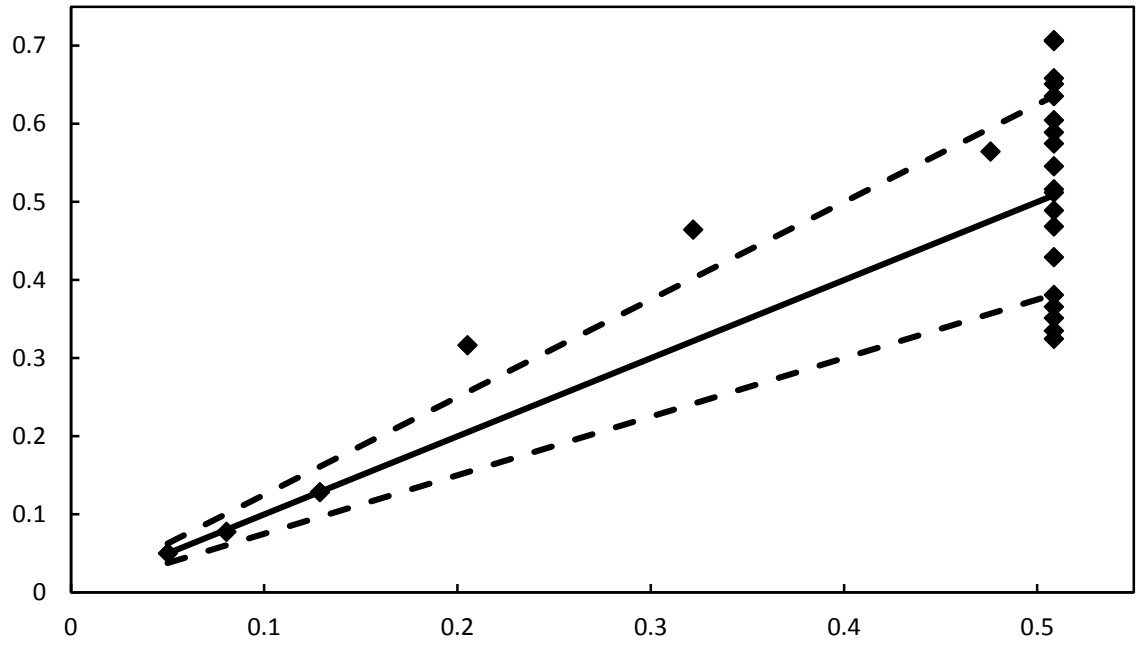

Figure 27. *Parity plots considering 25% error margin for concentrations of (a) Saccharomyces cerevisiae. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.*

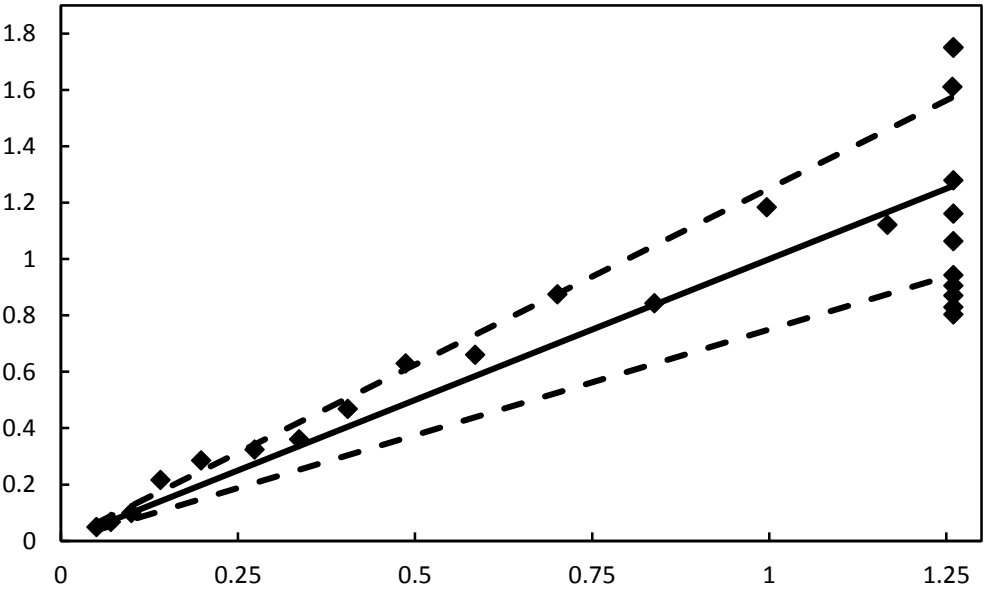

Figure 28. Parity plots considering 25% error margin for concentrations of (b) *Scheffersomyces stipitis*. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.

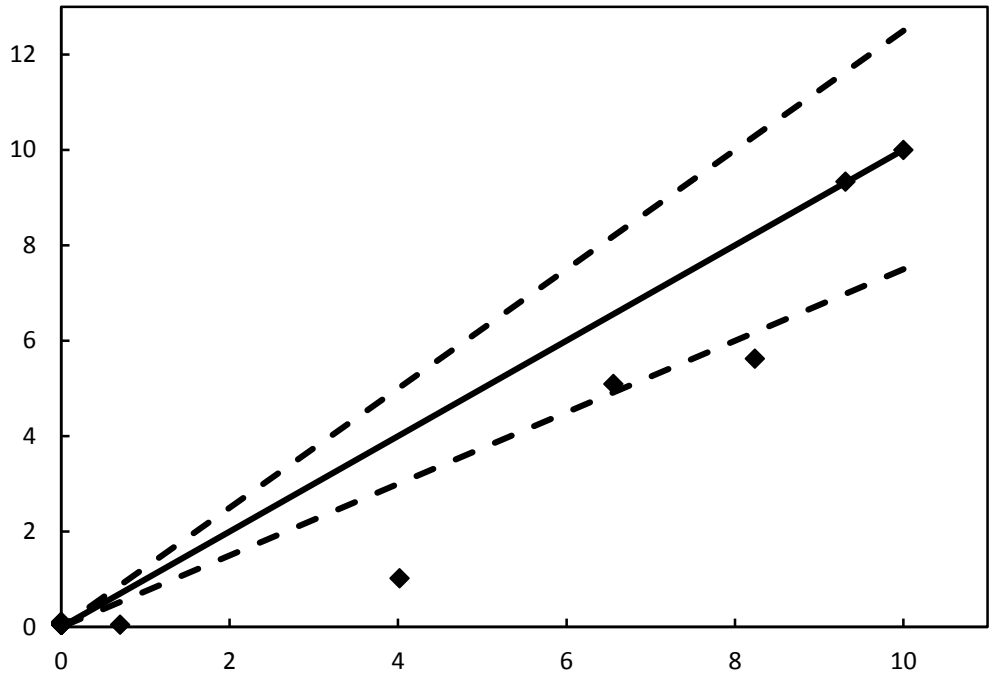

Figure 29. Parity plots considering 25% error margin for concentrations of (c) glucose. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.

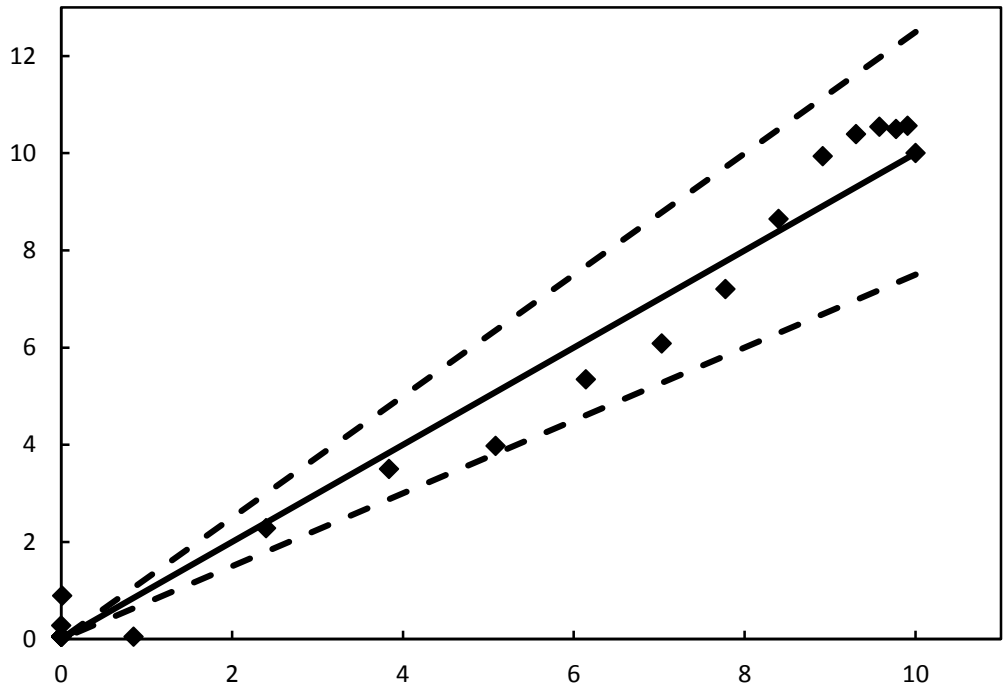

Figure 30. Parity plots considering 25% error margin for concentrations of (d) xylose. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.

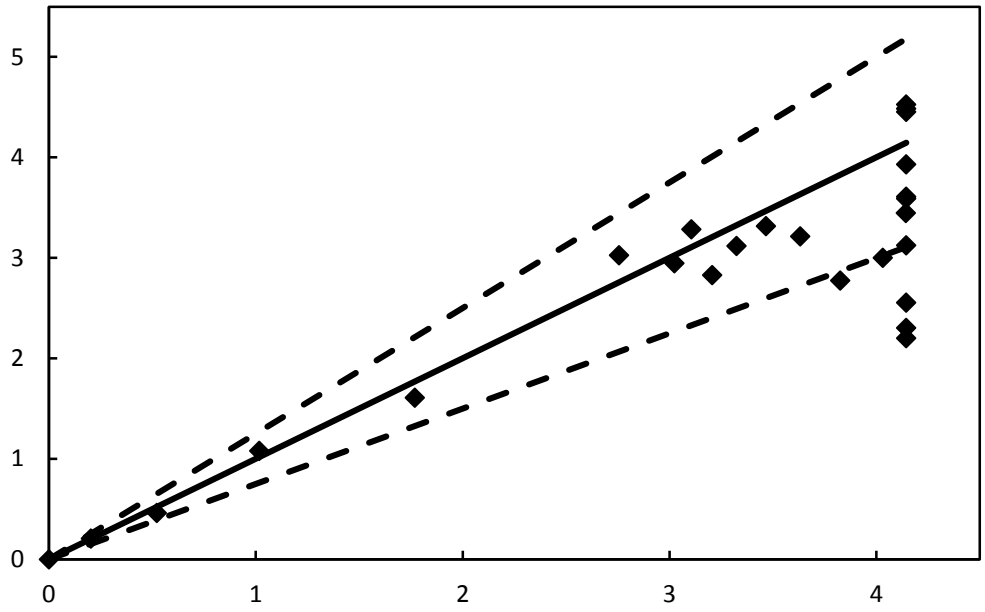

Figure 31. Parity plots considering 25% error margin for concentrations of (e) ethanol. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.

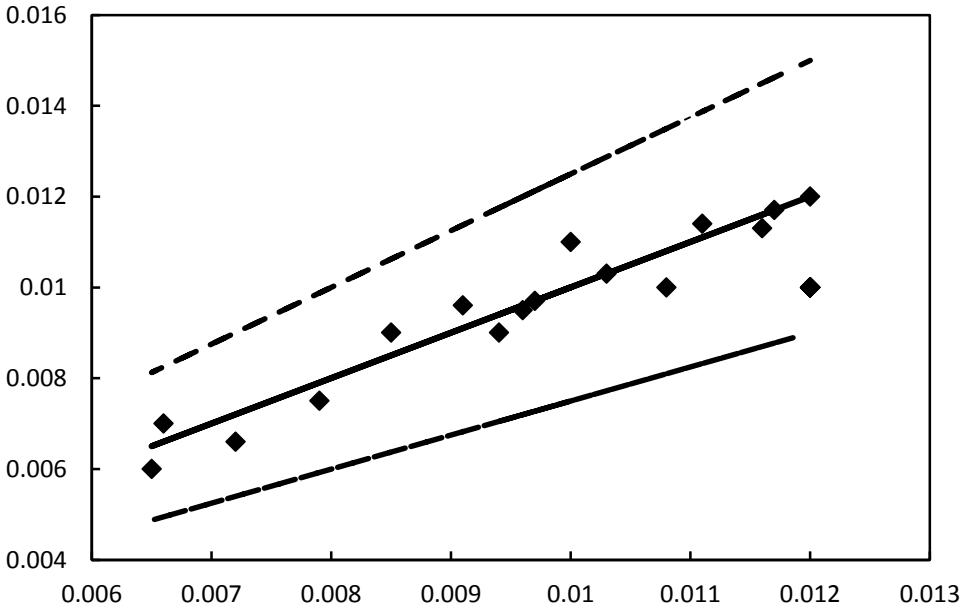

Figure 32. Parity plots considering 25% error margin for concentrations of (f) dissolved oxygen. The dotted lines represent upper and lower error margins, diamonds indicate experimental values.

Model Simulation Concentration Profile

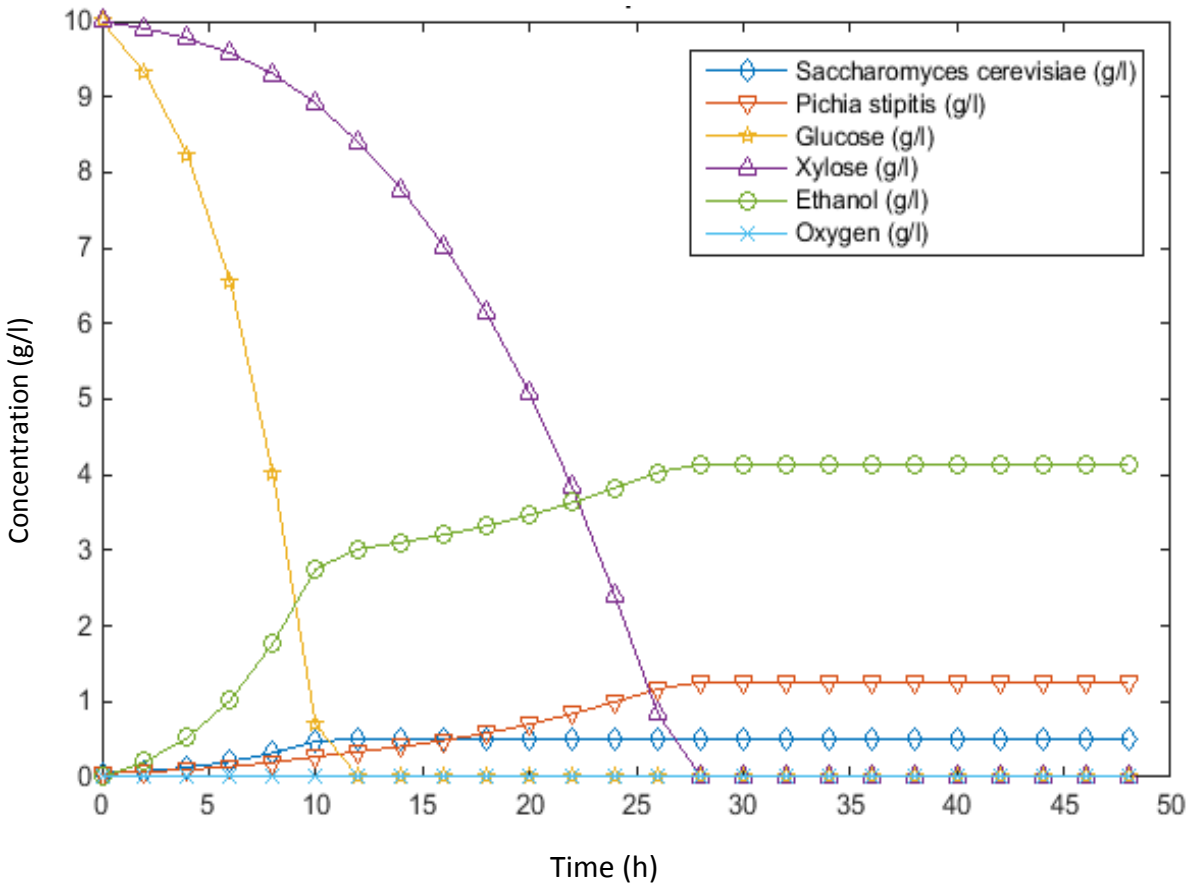

Figure 33. Concentration profile obtained from model prediction. The plot was created using MATLAB R2014b.

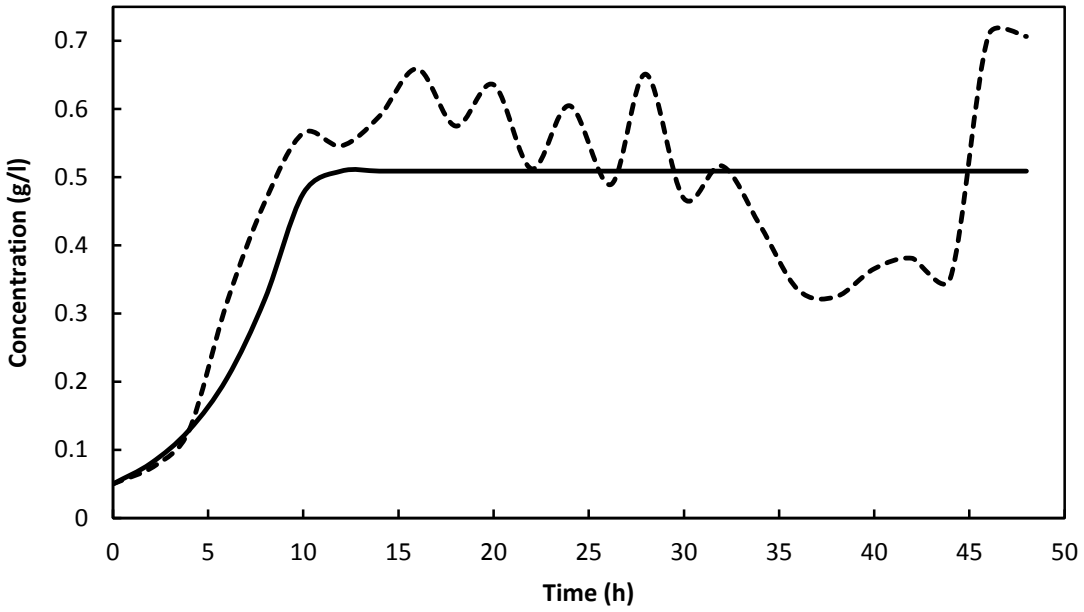

Figure 34. Comparison between experimental and model predicted results of (a) *Saccharomyces cerevisiae*. The dotted lines represent experimental data and the bold lines represent simulation data.

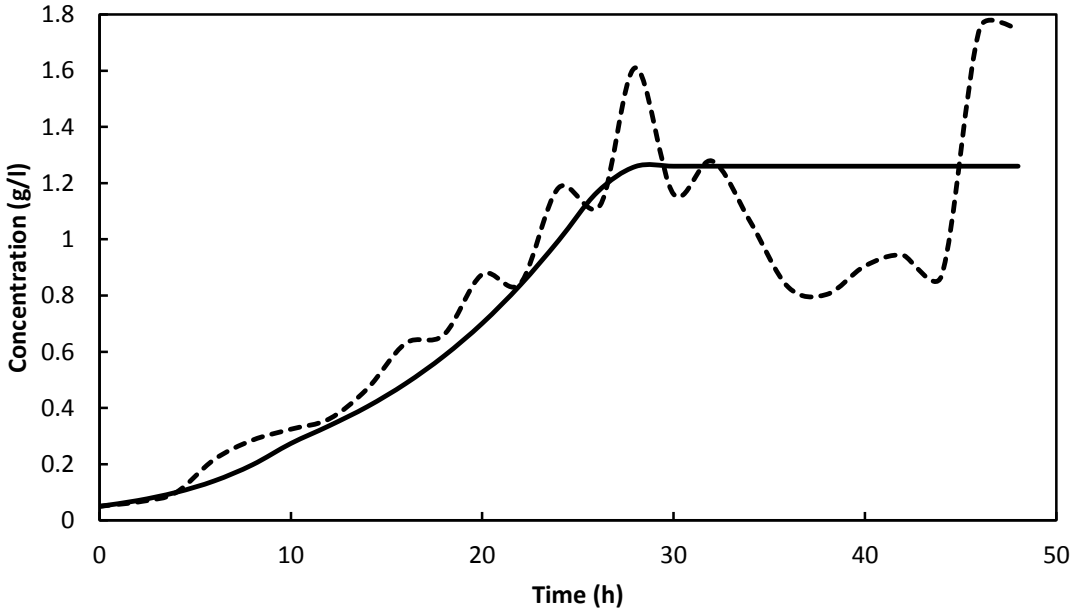

Figure 35. Comparison between experimental and model predicted results of (b) *Scheffersomyces stipitis*. The dotted lines represent experimental data and the bold lines represent simulation data.

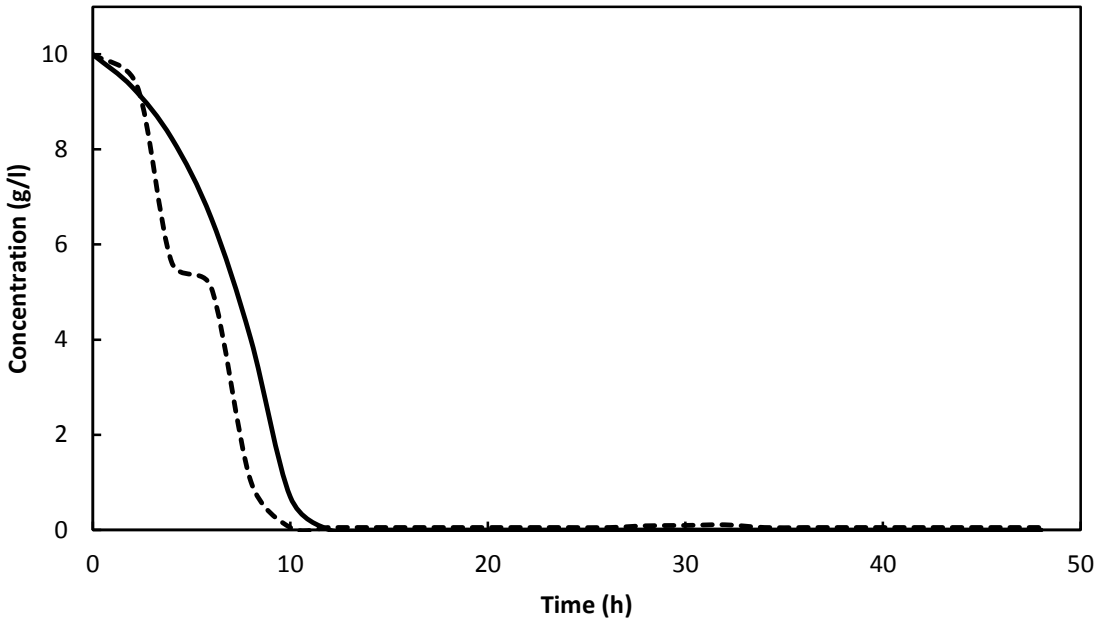

Figure 36. Comparison between experimental and model predicted results of (c) glucose. The dotted lines represent experimental data and the bold lines represent simulation data.

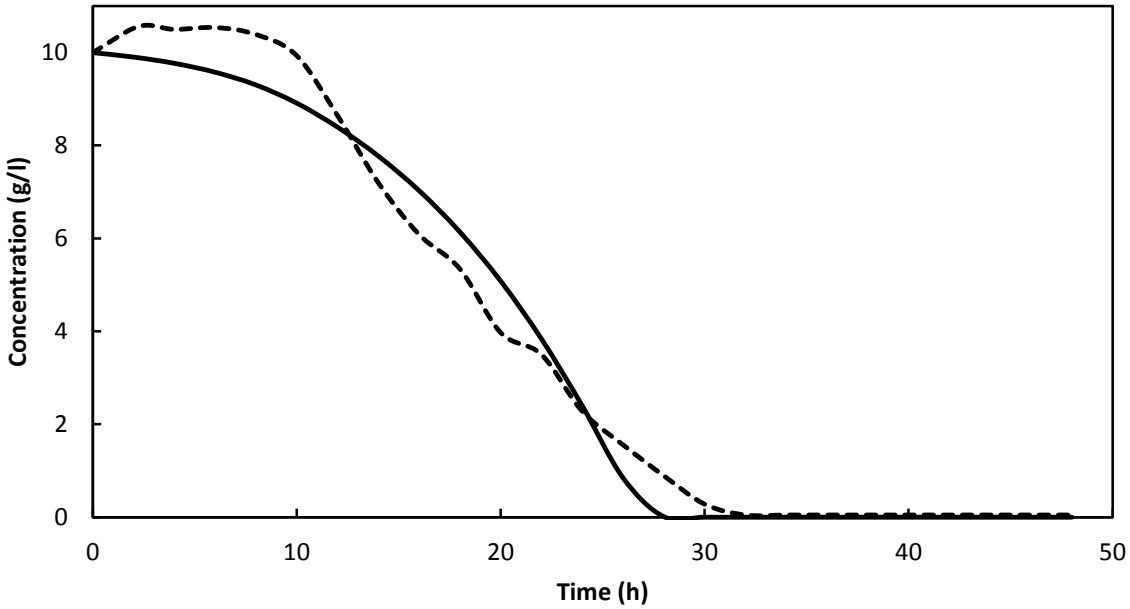

Figure 37. Comparison between experimental and model predicted results of (d) xylose. The dotted lines represent experimental data and the bold lines represent simulation data.

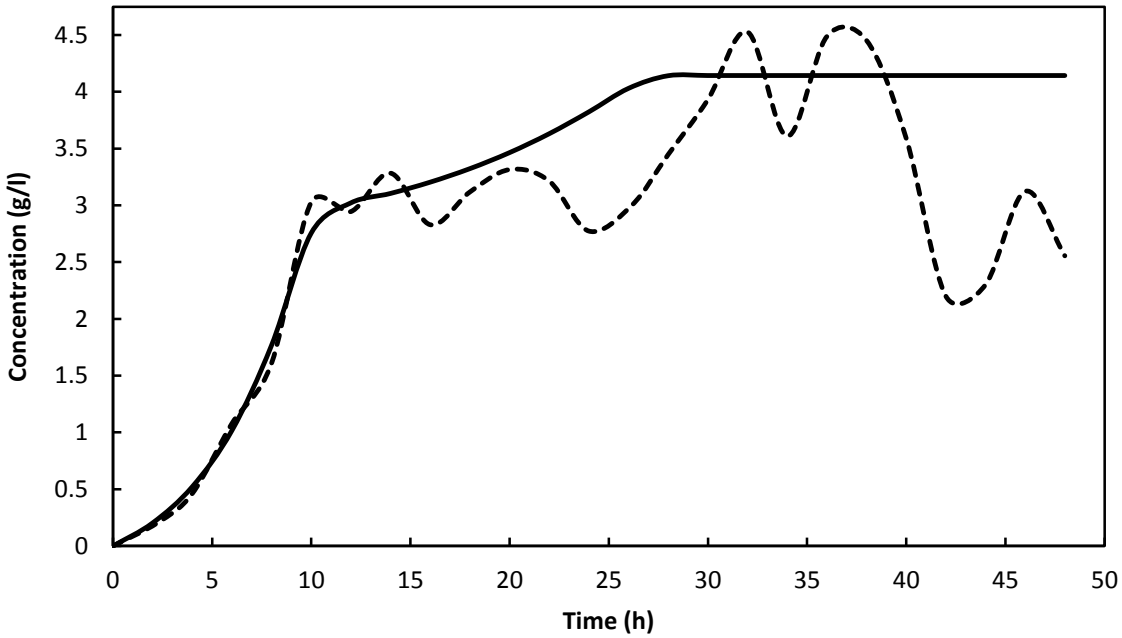

Figure 38. Comparison between experimental and model predicted results of (e) ethanol. The dotted lines represent experimental data and the bold lines represent simulation data.

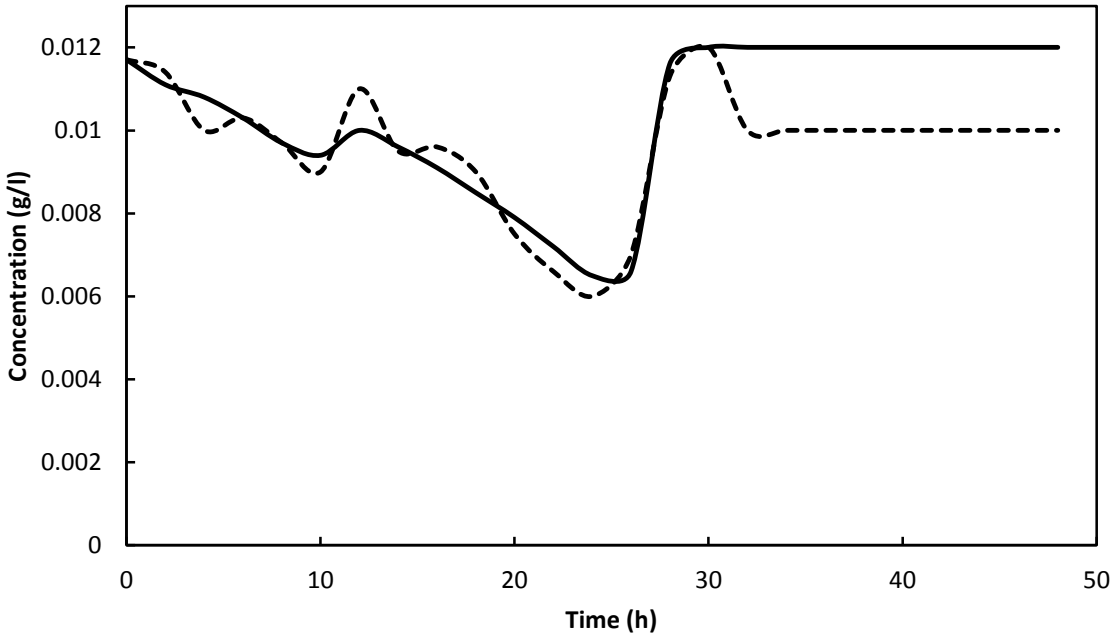

Figure 39. Comparison between experimental and model predicted results of (f) dissolved oxygen. The dotted lines represent experimental data and the bold lines represent simulation data.

The means of experimental and predicted values were subjected to t-test to see if they are statistically similar. Equal variances were assumed and a confidence interval of 95% was considered. All the results are summarized in table. The results confirm that the confidence interval has the value zero and that the P-value is greater than the level of significance α (0.05) for all cases. Therefore, there is no significant difference between the means of experimental and model simulated data. Various indices of agreement were calculated to validate the model. The values of Wilmott's Index of Agreement (table 10) evaluated for *S. cerevisiae*, *S. stipitis*, glucose, xylose, ethanol and dissolved oxygen satisfied the criteria for a 'very high' agreement (0.8-1) between experimental and simulated values [122, 123].

	Saccharomyces cerevisiae	*Scheffersomyces stipitis*	Glucose	Xylose	Ethanol	Dissolved Oxygen
Difference between means (predicted-experimental)	-0.0208	0.002	0.264	-0.06	0.415	0.000736
95% Confidence Interval for difference	-0.1162, 0.0745	-0.278, 0.282	-1.493, 2.021	-2.51, 2.38	-0.329, 1.160	-0.000226, 0.001697
T- value	-0.44	0.01	0.30	-0.05	1.12	1.54
P-value	0.663	0.990	0.764	0.958	0.267	0.130
Wilmott's Index of Agreement (Wilmott et al., 2012)	0.867	0.929	0.980	0.995	0.910	0.850

Table 10. Statistical validation of the co-culture models

CONCLUSION

Mathematical models were developed to predict the dynamics of the co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in glucose/xylose mixture. The models were statistically validated with a very high agreement between experimental and simulation values. Therefore these models can be extended to the co-culture of the yeasts in lignocellulose hydrolysates provided there are no fermentation inhibitors in the hydrolysates. Adopting a biological pretreatment of lignocelluloses can minimize the effect of fermentation inhibitors in the hydrolysates for better model performance. The ethanol productivity from the co-culture experiments was lower than that of pure cultures due to the consumption of ethanol by the yeasts, following sugar exhaustion. Hence, these models can be applied to predict the co-culture conditions to maximize the ethanol productivity and to minimize re-assimilation of ethanol.

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