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## High performance biorefinery processing of lignocellulosic agricultural residues for ethanol and L-lactic acid production

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## Abstract

Today, world is facing long-term energy shortage due to ever increasing energy demands and dwindling conventional fuel resources, plus environmental and global warming concerns. Lignocellulosic biorefinery is an important and sustainable alternative for renewable energy development to lessen the petroleum dependence and food crop usage for production of fuels and other value added commodities. Currently, high cost in lignocellulosic processing and operation is the major technical barrier in its commercialization. In this thesis, several practical solutions were developed to overcome the difficulties in lignocellulosic biorefinery processes for ethanol and lactic acid production, including the replacement of pure sugars by corn stover for yeast cell culture, the dried distillers' grains and solubles (DDGS) usage to replace expensive fermentation nutrients, the improved ethanol yield by evolutionary adaptation of yeast fermenting strains, open lactic acid fermentation under unsterilized conditions, and cofermentation of glucose and xylose to ethanol.

Dried distillers' grains and solubles (DDGS) is a major by-product of corn based ethanol production and generally used as animal feed due to its rich nutrient contents, including protein, fibers, oil, yeast cells, glucan, starch, and amino acids. This could be excellent cost effective nutrient for ethanol production. In this thesis, (Chapter 3 and 5) evaluated the utilization of DDGS as cost effective nutrient for ethanol production from adapted yeasts. In future, DDGS will be used in all SSF studies as inexpensive nitrogen source.

Solid seeds culture by using freshly pretreated corn stover as a carbon source instead of pure glucose reduced a 22% of yeast seeds culture preparation cost. In addition, yeast cell growth and ethanol fermentation performance did not show any difference when the yeast seeds were cultured by using glucose, the corn stover hydrolysate liquid, and the pretreated corn stover solids as carbon sources, respectively. The solid-liquid separation step, which is required in preparation of hydrolysate was eliminated. Moreover, additional bioreactor required for enzymatic hydrolysis was directly cut. During solid seeds culture, unused cellulase bound to solid materials could be recycled during ethanol fermentation step.

Pretreatment generates a wide range of toxic compounds, those reduce the cellulase activity, cell growth, and ethanol productivity. Therefore, evolutionary adaptation of fermenting strain could improve the fermentability and inhibitors tolerance. Evolutionary adaptation of a routine yeast strain *S. cerevisiae* DQ1 in corn stover hydrolysate was performed for consecutive 65 days that significantly improved ethanol fermentation performance in both corn stover hydrolysate and simultaneous saccharification and fermentation (SSF) at high solids content. About 50% more ethanol titer and yield were obtained from adapted strain over parental strain. The ethanol titer and yield of 71.40 g/L and 80.34% were obtained, respectively, at the optimum SSF conditions without any wastewater generation from pretreatment to fermentation. In addition, yeast extract replacement with cost effective DDGS also reduced the cellulosic ethanol production cost to large extent. The cost of DDGS was only 0.5% comparing to that of yeast extract.

A pediocin producing strain *Pediococcus acidilactici* was employed for open L-lactic fermentation under completely unsterilized condition in synthetic medium, corn stover hydrolysate, and in high solids content SSF. The added contaminant bacteria were also inoculated in the fermentation system to confirm the antibacterial function, and contaminant strain did not grow due to the existence of antibacterial pediocin produced by *Pediococcus acidilactici*. High lactic acid titer, productivity, and yield of 97.30 g/L, 1.47 g/L/h, and 69.34% were obtained, in high solids content SSF, respectively. The results provided a simple, robust and cost effective fermentation process for L-lactic acid using lignocellulose feedstock. Furthermore, labor consuming and energy intensive step was directly reduced.

A xylose fermenting strain *Saccharomyces cerevisiae* NAN-127 experienced evolutionary adaptation. Adapted strain produced 41.82 g/L ethanol corresponding to 91.01% yield by cofermentation of glucose and xylose in the fresh corn stover hydrolysate. More than 90% xylose was converted into the ethanol. Ethanol concentration and yield were reached to 74.14 g/L and 74.01% by adapted strain under industrial relevant conditions. Ethanol titer is very close to corn based ethanol 10% v/v (78.9 g/L). Further optimization of the strain is in process, we are pretty sure to achieve the target 10% v/v ethanol concentration from lignocellulosic biomass.

The research works presented in this thesis show considerable contributions towards cost reduction and technical advantages of lignocellulose biorefinery. Each approach proposal in this thesis reduced processing cost for ethanol and or L-lactic acid production at certain range. These advancements certainly provide helps on the commercialization of lignocellulosic biorefinery. Further efforts are required for development of fast, efficient, and cost effective biorefinery process.

**Keywords:** Lignocellulosic biorefinery; Evolutionary adaptation; Simultaneous saccharification and fermentation (SSF); Open L-lactic acid fermentation; Co-fermentation of glucose and xylose to ethanol.

# 农作物秸秆木质纤维素生产乙醇和乳酸的高效生物炼制过程研究

## 摘要

日益增长的能源需求、传统燃料的逐渐变少、环境和全球变暖等问题导致了当今世界面临能源短缺和转换的问题。以农作物秸秆木质纤维素为原料通过生物炼制技术生产液体生物燃料和高附加值生物基化学品，是减少石油和粮食用量，实现可持续发展的重要选择。目前，木质纤维素加工和操作的高昂费用是木质纤维素商业化的主要障碍。本文对几种实用的研究策略在生物炼制过程上的应用进行了测试，对降低乙醇和乳酸生产过程成本的方案进行了详细研究。这些策略包括用玉米秸秆原料替代葡萄糖培养酵母发酵菌种、用干酒醪及可溶物（DDGS）作为发酵营养添加剂替代昂贵的发酵营养物、通过适应性进化提高酿酒酵母发酵菌种的乙醇得率、以及在不灭菌的开放环境中进行乳酸发酵。

以预处理的玉米秸秆原料代替纯葡萄糖作为碳源，无需水解液制备的固液分离和酶水解过程而直接进行发酵菌种培养，以葡萄糖、玉米秸秆水解液和预处理玉米秸秆固体作为碳源时，酵母细胞的生长和乙醇发酵能力没有明显差异。该方法减少了 22%的酵母种子培养成本。

预处理过程产生的大量抑制物降低了纤维素酶酶活和发酵菌种的生长和发酵效率。本文以预处理后玉米秸秆水解液为驯化环境，连续驯化酿酒酵母 *Saccharomyces cerevisiae* DQ1 65 天，显著提高了玉米秸秆水解液和高固含量同步糖化与发酵（SSF）的乙醇发酵能力。本文使用廉价的 DDGS 替代昂贵的发酵营养添加物质，乙醇浓度和乙醇得率分别是 71.40 g/L 和 80.34%，而且预处理到发酵的全部过程无任何废水产生。这一结果达到了目前公开发表文献中的最高指标。

使用产细菌素的 L-乳酸发酵工程菌株乳酸片球菌 *Pediococcus acidilactici* TY112，使用不灭菌玉米秸秆水解液和高固含量玉米秸秆进行开放式的 L-乳酸发酵。向 *P. acidilactici* 发酵体系接种外来污染菌株，污染菌株因为 *P. acidilactici* 分泌的乳酸菌素而不能生长，这进一步证实了该工程菌株具备抗细菌的能力。在高固体含量同步糖化与发酵（SSF）过程中，乳酸浓度、乳酸产率和乳酸得率分别是 97.30 g/L、1.47 g/L 和 69.34%，达到了目前公开发表文献中的最高指标，为木质纤维素原料发酵产 L-乳酸提供了一个经济上可行的发酵技术。

对木糖发酵菌株 *Saccharomyces cerevisiae* NAN-127 进行驯化，驯化后菌种在含有葡萄糖及木糖的新鲜玉米秸秆水解液中进行共发酵，乙醇浓度及得率分别为 41.82 g/L、91.01%，其中超过 90%的木糖转化为乙醇。驯化菌种在工业条件高固含量下进行同步糖化与发酵，获得的乙醇浓度为 74.14 g/L，得率达 74.01%，该乙醇浓度与玉米发酵获得的 10% v/v (78.9 g/L) 浓度极为接近，后续菌种进一步优化将使得从木质纤维素原料发酵获得 10%(v/v)乙醇浓度。

本论文的各项研究结果具有现实可行的技术优势，其为降低木质纤维素生物炼制成本提供了重要策略，也推进了生物炼制产业化的实现。

**关键词：**木质纤维素生物炼制；驯化；同步糖化与发酵；开放式 L-乳酸发酵；葡萄糖木糖共发酵产乙醇

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# Chapter 1

# General Introduction

## 1.1. Background

Increasing concerns with instability in world oil prices, high energy demands, depletion of conventional fuel, and environmental pollution have compelled the world to switch away our dependency from current fossil fuels to renewable energy for transportation and biochemical products. The statistical analysis on conventional fuel usage and associated problems indicate that China's crude oil consumption steadily increased from 0.08 (2001) billion tons to 0.45 (2013) billion tons [1, 2]. In addition, substantial dependence on finite energy resources flare political, social, and economical problems in the world [1-4]. However, biofuels especially liquid fuel (bioethanol) can be a promising option to get rid of current energy crisis and alleviate pollution problems [5, 6]. Since the beginning of 1990s' considerable attention has been focused towards production of ethanol and other value added commodities from pure sugars. But, due to increasing bio-product demands, food prices, and food crisis; first generation products are no more economically sustainable. However, lignocellulosic biomass is abundantly available, cheap, and non-edible plant parts, and its utilization for biofuels and other value added commodities could reduce environmental pollution, create job opportunities, and get rid of the controversial issues over land use in the 1st generation products [7-9]. The biorefinery concept has been introduced as utilization of biomass for biofuels and bioproducts. According to the definition of biorefinery by the National Renewable Energy Laboratory (NREL), "biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass" [10]. Lignocellulose biorefinery is analogous to crude oil refinery in terms of product ranges but different in terms of raw material used. Biorefinery products are ethanol, lactic acid, amino acids, biodiesel, and biogas. While, conventional refinery products, include gasoline, diesel, kerosene, and liquefied petroleum gas etc. Recently, greenhouse gases' emission has been reduced due to biofuels consumption [5, 6]. Lignocellulose biorefinery faces many technical challenges and need a proper solution before commercialization of the process. In the last few decades, a considerable attention has been paid in improving the lignocellulose bioconversion technology for obtaining the value added commodities. In this context, dry dilute acid pretreatment (DDAP) [11-13], onsite enzyme production, designing the unique bioreactor with helical stirrer [14], and evolutionary adaptation are the key achievements [15-17].

However, we still need to handle many technical challenges for fast, cost effective, and efficient lignocellulose conversion, such as; (1) use freshly pretreated lignocellulosic materials as cost effective alternative of glucose for preparation of yeast seeds culture (2) utilize inexpensive nutrient (DDGS) instead of yeast extract in SSF (3) perform SSF at high solids content (4) reduce biodetoxification time (5) perform evolutionary adaptation of ethanol fermenting strains (6) cofermentation of glucose and xylose (7) open lactic acid fermentation to reduce operational cost by eliminating the sterilization step (8) perform pretreatment and SSF at continuous mode (9) decrease cellulase dosage and its cost. In this thesis, efforts are put together to solve some of the above difficulties, and details are discussed in the next chapters.

This thesis consists of six chapters including, a general introduction, research work, and a general conclusion. Chapter 1 consists of opportunities and challenges to lignocellulose biorefinery for ethanol and lactic acid production. The research work in chapters 2-5 has been published or is under review in the academic journal or in preparation. Chapter 2, Cellulosic ethanol production from yeast seeds culture prepared by using freshly pretreated corn stover materials (Qureshi et al.[18]), published in Applied Biochemistry and Biotechnology. The significance of this work is the utilization of freshly pretreated corn stover solids as a carbon source for yeast seeds culture preparation as an alternative to pure glucose, and this can save about 22% seeds culture preparation cost. Chapter 3, High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain (Qureshi et al. [19]), published in Bioresource Technology. This research paper describes the long term evolutionary adaptation of routine yeast *S. cerevisiae* DQ1 towards potent inhibitors present in corn hydrolysate (undetoxified) and DDGS was used as cost effective nutrient instead of yeast extract. In addition, wastewater generation and fresh water usage were reduced from pretreatment to fermentation. Chapter 4, Open fermentation of high titer L-lactic acid from corn stover by a pediocin producing *Pediococcus acidilactici* strain (In submission to Bioengineering and Biotechnology). This article describes the open L-lactic acid fermentation using corn stover at high solids content under completely unsterilized conditions, and this can save the feedstock, medium and bioreactor sterilization energy intensive step and cost. Chapter 5, Cofermentation of glucose and xylose by evolutionarily adapted xylose fermenting *Saccharomyces cerevisiae* under industrial relevant conditions (In preparation). This article describe the evolutionary adaptation of xylose fermenting strain and cofermentation of glucose and xylose to achieve highest ever possible ethanol concentration and yield. In the last chapter (6), a general conclusion follows by future work suggestions.

## 1.2. History of biofuels

Biofuels production was started in the late 19th century, when bioethanol was obtained from sugars (sugarcane) and starch of corn. Rudolf Diesels' first engine was ran with peanut oil. Until the 1940s, biofuels production was seen feasible for transportation, but decreasing world oil prices stopped their further development. Once again, biofuels production need was felt in the mid of 1970s largely due to increase in world fossil fuel prices.

In that time, Brazil started ethanol production from sugarcane and United States produced from corn. Brazil was the main producer of fuel ethanol since 1970 [20]. However, in 2005, the US surpassed Brazil as the world's leading producer of bioethanol and since then the production gap has increased. In most parts of the world, biofuel production increased in last decade. Energy security and sustainable agriculture concerns, and the emission of greenhouse gases have become an important driver for biofuel production. Recently, many countries have adopted ethanol-gasoline blending strategies to switch away dependency from current fossil fuels to renewable clean fuels, and worldwide biofuel production is increasing. However, biofuels production from sugarcane and corn enhances food and feed crisis. Thus, alternative renewable energy source and cost effective methodologies need to be developed. Currently, much focus has been directed towards biofuel and other value added commodities production from lignocellulosic biomass [21, 22]. United States and European Union both provides financial support for advancement of biofuels production processes. In February 2012, the US Department of Energy (DOE) granted a total funding of US$766 million to 16 cellulosic ethanol projects, ranging from pilot to commercial scale [23]. Although cellulosic ethanol commercialization has now started, lignocellulosic conversions are still regarded as costly. For making the lignocellulosic biorefinery economically feasible, further process development are certainly needed. With these efforts, cellulosic processing cost could be reduced, in the same way to for example, the Brazilian ethanol industry, where production cost have dropped 3-4 fold since the start [24], and more reduction can be expected.

## 1.3. Lignocellulosic Biomass

Globally, a substantial increase in ethanol production capacity could be achieved by utilizing the lignocellulosic materials as feedstock. Lignocellulosic biomass is highly abundant and diverse, and includes: trees, energy crops, and agricultural residues, for example, corn stover, sugarcane bagasse, wheat straw, spruce, barley straw, among others. Abundant availability and low cost of lignocellulosic biomass suggests that there is an almost unlimited supply of raw materials for production of biofuels and bioproducts. Composition of some potential lignocellulosic materials for ethanol and lactic acid production has been summarized in Table 1.1.

**Table 1.1 Composition of lignocellulosic materials used in biorefinery**

| Lignocellulosic Biomass | Cellulose % dry matter basis | Hemicellulose % dry matter basis | Lignin % dry matter basis | Sources |
|---|---|---|---|---|
| Aspen wood log | 45.61 | 17.8 | 20.20 | [25] |
| Barley straw | 37.1 | 26.3 | 16.9 | [26] |
| Bermuda grass | 47.8 | 13.3 | 19.4 | [27] |
| Corn cob residue | 72.09 | 4.63 | 7.18 | [28] |
| Corn stover | 36.1-41 | 17.1-34.4 | 12.6-26 | [29-33] |
| Cotton stalk | 30.58-36.6 | 16.85-18.1 | 26.4-29.99 | [34, 35] |
| Eastern redcedar | 34.2 | 21.3 | 32.2 | [36] |
| Forage sorghum | 32.2-35.6 | 16-22.8 | 18.2-25.7 | [27, 37, 38] |
| Palm oil waste | 32.7-39.8 | 17.3-23.04 | 26.77-34.37 | [39-41] |
| Pine | 41.9-42.9 | 18.9-20.1 | 28.6-33.7 | [42, 43] |
| Rapeseed straw | 27.6-44.6 | 20-22.9 | 16.8-20 | [27] |
| Reed | 39.5 | 29.8 | 24 | [27] |
| Rice husk | 21.50-35.86 | 18.20-23.06 | 14.62-24.52 | [44, 45] |
| Rice straw | 31.5-41.5 | 18-23 | 16.8-20.3 | [46-48] |
| Salix | 43 | 21.3 | 26.6 | [49] |
| Spruce | 43-44.9 | 19.7-21.4 | 27-31.1 | [50, 51] |
| Sugarcane bagasse | 42.1-42.8 | 25.8-27.5 | 22.1-23.7 | [52, 53] |
| Sweet sorghum bagasse | 35.1-43.5 | 18-22.2 | 18.6-19.7 | [54, 55] |
| Switch grass | 32-38.9 | 21.5-28.9 | 21.4-30.4 | [56, 57] |
| Wheat straw | 34.8-41.8 | 22.15-28 | 17.7-23.4 | [58-62] |

Lignocellulosic biomass is composed of three different polymers namely cellulose, hemicellulose, and lignin [63]. Cellulose: the most abundant polysaccharide on earth and composed of glucose monomers linked by β(1→4) glycosidic bonds, accounts for 35-50% of biomass dry matter basis [64]. Hemicellulose is composed of heteropolymers of hexoses and pentoses, this accounts for 20-35% of dry matter basis [64]. In contrast to cellulose, hemicellulose is highly branched and it has a lower degree of polymerization. Some of the hydroxyl groups of the sugar units in the side chains have been replaced by acetyl groups. Hemicellulose on degradation releases both monomeric sugars and acetic acid [65]. Hemicellulose is linked to cellulose and lignin by hydrogen bonds and covalent bonds, respectively [66]. Lignin is naturally hydrophobic and amorphous, a complex aromatic polymer resulting from the polymerization of three major phenolic components: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; and this accounts for 15-20% dry matter basis. Lignin is considered as the cellular glue that holds cellulose and hemicellulose fibers together. Lignocellulose also contains other components, including ash, extractives, waxes, proteins, and fatty acids [7, 63, 64, 67]. Lignocellulose provide rigidity and strength to plant cell walls, and protect them from the microbial and insects' attack.

## 1.4. Lignocellulose conversion to ethanol and lactic acids

Lignocellulosic biorefinery for ethanol and lactic acid production involves following steps: pretreatment, conditioning or detoxification, enzymatic hydrolysis, fermentation, and purification or distillation. Fig. 1.1 shows the advanced dry lignocellulosic biorefinery processes developed by our group, and this process has no wastewater generation from pretreatment to fermentation [11-14, 68].

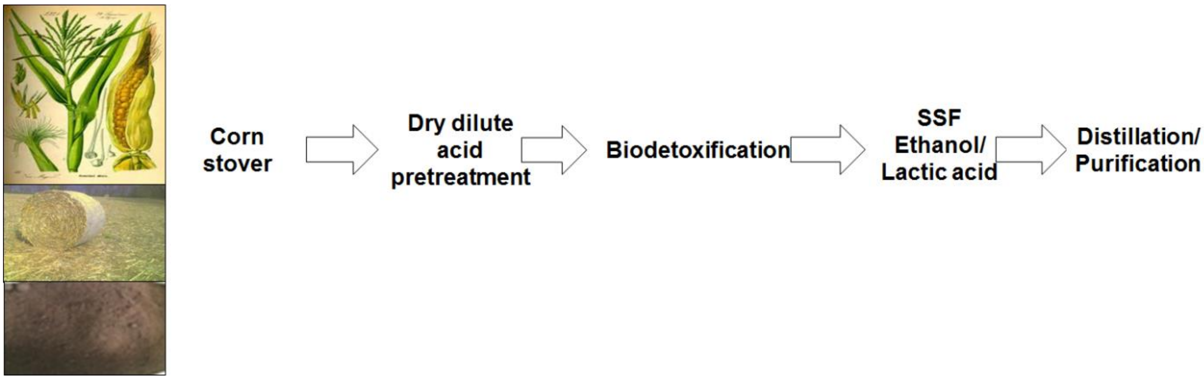


**Fig. 1.1**. Advanced dry lignocellulose biorefinery process scheme for ethanol and lactic acid production.

### 1.4.1. Pretreatment

Generally speaking, the objective of pretreatment is to disrupt the recalcitrant plant cell wall structure. Besides, pretreatment should help solubilizing the hemicellulose and lignin, and to make cellulose accessible to hydrolytic enzymes for conversion to fermentable sugars. Pretreatment is considered as the single most expensive process step in the lignocellulosic biorefinery. About 20 % of the lignocellulosic biomass conversion cost is attributed to pretreatment only. This is more than any single step cost in the whole lignocellulose conversion process [63].

In the last few decades, several pretreatment methods are developed, including physical, chemical, physico-chemical, and biological. Physical methods, for example, milling (wet, dry, ball, and vibro energy), microwave radiation, pyrolysis, extrusion, and freeze pretreatment. Physical methods open up the fiber structure and create a larger accessible surface area, these methods are energy intensive and not feasible for commercial applications. However, in combination with other pretreatment methods these can be useful [63, 64, 69-73]. Chemical pretreatment methods, include dilute acid (sulphuric, hydrochloric, and nitric acids), and dilute base (sodium, potassium, calcium, and ammonium hydroxide), oxidants, ionic liquid, organosolv treatment, and ozonolysis. Acid is commonly used to dissolve hemicelluloses, while lignin is typically dissolved by alkaline or organosolv pretreatments [12, 13, 63, 73-77]. Physico-chemical combined processes are advantageous over separate physical or chemical pretreatment method, and common combination, includes $SO_2$ steam explosion, and AFEX. A promising physiochemical pretreatment method is ammonia fiber explosion (AFEX). AFEX, however, does not remove either lignin or hemicelluloses to any greater extent, this method is considered to be promising for agricultural residues and herbaceous crops, but not very efficient against softwoods, perhaps due to high lignin content [73, 77]. Biological pretreatment either make use of microorganisms, including *Phanerochaete chrysosporium, Trametes* versicolor*, Ceriporiopsis. subvermispora,* and *Pleurotus ostreatus* to delignify or enzymes (laccase) to remove phenolic compounds. In general, brown and soft rots mainly attack cellulose while imparting minor modifications to lignin, white-rot fungi more actively degrade the lignin component. The biological pretreatment appears to be a promising technique, it has very evident advantages, including no chemical requirement, low energy input, mild environmental conditions, and environmentally friendly. However, its apparent disadvantages, include time consuming, requires careful control of growth conditions, and large space is required [63, 78-83].

All pretreatment methods have their advantages and disadvantages and choice of methods depends on the feedstock, available facilities, simplicity, and cost of operation. However, the best pretreatment method should meet the following criteria, increase cellulose recovery, increase hemicellulose and lignin removal, zero wastewater generation, less inhibitors formation, cost effective, and to be operated at high solids content [11-13, 67, 84, 85]. Dilute acid pretreatment method is commonly used. The major disadvantages of conventional dilute acid pretreatment include huge wastewater generation due to low solids content used in pretreatment, loss of fermentable sugars during the solids/liquid separation after pretreatment, and relatively high inhibitor compounds generation. Recently, our group has established a novel dry dilute acid pretreatment (DDAP) method [12, 13], in which the solids content in the pretreatment was fed to an extremely high level up to 70% of the total feedstock. DDAP corn stover was successfully applied in lignocellulosic processing for ethanol and lactic acid production under high solids content SSF [14, 68, 86, 87]. This pretreatment method is known as ‘dry’ method, because both the corn stover feedstock and the pretreated corn stover product were ‘dry’ with no free water generation during the pretreatment. In this fashion, three difficulties of conventional dilute acid pretreatment were solved: dry in and dry out, thus no wastewater was generated, dry pretreated product thus no solids/liquid separation was needed, and low pretreatment inhibitor generation.

### 1.4.2. Detoxification

During pretreatment various degradation products, including furan derivatives (furfural and 5-hydromethylfurfural (HMF)), organic acids (acetic acid, formic acid, and ferulic acid), and lignin derivatives (vanillin, 4-hydroxybenzaldehyde, guaiacol, and phenol) are generated. These pretreatment inhibitors severely reduce cellulase activity, microbial growth, and fermentability [88, 89]. However, detoxification or conditioning step cannot be skipped for obtaining the high ethanol titer and yield from high solids content of lignocellulosic biomass. Several methods are reported for removing pretreatment inhibitors, includes overliming, ion exchange, vaporization, and water washing [68, 88, 90-92]. However, these methods face many difficulties, including massive freshwater usage and wastewater generation, loss of the fine lignocellulose particles and fermentative sugars, and incomplete removal of inhibitors [68, 93]. A detailed comparison based on wastewater generation and fresh water usage during detoxification or conditioning with their advantages and disadvantages is presented in (Chapter 3; results and discussion section (Table 3.3)). However, a new method, biodetoxification could be a promising choice for inhibitors removal, this depends on microorganisms to degrade the toxins as part of their normal metabolism by secreting peroxidase or laccase enzymes into the hydrolysate [94-97]. Biodetoxification has many advantages, such as no loss of cellulose solids, reduce fresh water usage, and thus high solids contents are available for fermentation. However, most of biodetoxification method could be applied only to the liquid hydrolysate system, in which the cellulose was hydrolyzed under toxin inhibition to cellulase enzymes and the toxin concentrations had been diluted [68, 91, 94-97]. Unique biodetoxification system by using *Amophotheca resinae* was developed and applied in high solids loading of pretreated material for inhibitors removal [68]. Long biodetoxification time was only limitation of this method, and now onsite biodetoxification in the bioreactor is under development that could reduce biodetoxification time from 7 days to only 1.5-2 days.

### 1.4.3. Enzymatic hydrolysis

Cellulose comprises the largest fraction of the sugars in lignocellulosic materials (Table 1.1) and glucose is main carbon source for many microorganisms. However, development of microorganisms fermenting hemicellulose sugars is rapidly increasing. Most of the pretreatment methods also partially remove and degrade the hemicelluloses. Therefore, main focus has been put on improving cellulases activity, reducing its cost and dosage. However, some pretreatment methods leave the hemicelluloses in the material [98]. Hydrolysis involves several enzymes, for example, endoglucanase, exoglucanase or cellobiohydrolases, β-glucosidases, and xylanases [99]. High solids content in the enzymatic hydrolysis increases the product inhibition, which results in lower performance of the enzymes. Presence of lignin increases non-productive enzyme binding that reduces the availability of enzyme for hydrolysis. In addition, the activity of some enzymes might be lost due to denaturation or degradation. Other factors are closely linked to the substrate composition and thus the pretreatment method employed, including cellulose crystallinity, cellulose degree of polymerization, substrate’s available area, hemicellulose content, feedstock particle size, porosity, and cell wall thickness [77, 98]. Addition of surfactants polyethylene glycol, tween, and other proteins to the lignocellulosic biomass before hydrolysis have been shown to improve enzyme performance by reducing unproductive adsorption of enzymes onto lignin. Addition of these compounds reduce the enzyme dosage requirement and increases ethanol titer and yield [100]. PEG is proposed to bind to lignin by hydrophobic and hydrogen bonding and thereby prevent the lignin from binding of enzymes. The benefit achieved by the reduction of the enzyme loading should be compared to the costs of adding surfactants [98, 100, 101]. Development of more efficient hydrolytic enzymes is the subject of extensive research to reduce their costs. There exist many possible strategies for improving the enzyme mixtures, stability, and specific activity of the enzymes. Enzyme mixture should be designed based on substrate specificity. Other possibilities should be explored for further improving the enzyme activity, and reducing the enzyme cost and its enzyme dosage.

### 1.4.4. Fermentation

Pretreated lignocellulosic biomass could be converted into desired product either by using separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation. In SHF, enzymatic hydrolysis and fermentation are performed at optimum conditions, but separate bioreactor is required for each step. For reducing the capital investment and simplifying the fermentation process two steps are integrated in the simultaneous saccharification and fermentation (SSF), where enzyme and fermenting microorganisms are mixed together in same bioreactor. Enzymatic hydrolysis releases sugars and simultaneously converted into final products by fermenting strains. Fed-batch SSF has been shown on many occasions to be beneficial for various aspects including: (a) ease of mixing after partial saccharification, which allow to operate SSF at high solids content; (b) lower energy consumption due to lower viscosity; (c) low inhibitors concentration is maintained that facilitates the fermenting strain, *S. cerevisiae* to convert them to less inhibitory compounds, and (d) maintaining low glucose concentration in the medium, facilitating effective glucose and xylose cofermentation by engineered strains [102].

Consolidated bioprocessing (CBP) is more recent approach, which provides a promising opportunity for developing a simplified method. In CBP, three steps involved in the bioconversion of lignocellulosic biomass to ethanol are integrated in single unit [7, 74, 103]. (1) Production of auxiliary enzymes required for efficient saccharification, (2) hydrolysis of pentose and hexose sugars, and (3) fermentation of pentose and hexose sugars to ethanol and or lactic acid. It was estimated that total projected costs for bioconversion of lignocellulosic biomass to ethanol for an advanced process featuring onsite dedicated cellulase production in combination with simultaneous saccharification and cofermentation was 49.9$\$m^{-3}$ [103]. About 75% of that cost could be saved by CBP (11.1$\$m^{-3}$).

## 1.5. High solids content of lignocellulosic biomass from pretreatment to fermentation

High lignocellulosic processing cost is one of the technical barrier for its commercialization. From industrial point of view, all operational steps from pretreatment to fermentation should be operated at high solids contents [13, 14, 104-106]. Enzymatic hydrolysis performed at high solids contents offers several advantages over moderate solids contents, the main one is higher sugar concentrations [2, 3]. Theoretically, higher sugar concentrations translate into higher ethanol titer and consequently energy consumption at distillation stage could be reduced. In order to optimize the enzymatic hydrolysis at high-solids, various difficulties should be addressed. These factors, including biomass source, pretreatment method, enzyme source, enzyme mixture, enzyme cost and its dosage, and pretreatment inhibitors [107].

There are evident advantages of operating SSF at high solids concentrations. Capital and operational costs could be decreased due to reduced number of equipment and reduced energy consumption [76, 108]. Starch based ethanol industries could handle high sugars concentration for obtaining 10–15% (v/v) high ethanol titer. But, the implementation of high solids contents using lignocellulosic biomass, however, need several advances in knowledge and technology. The lignocellulosic biomass received from pretreatment unit differs in consistency depending on the origin of the material and pretreatment method used. The generation of toxic compounds during the pretreatment step is another difficulty that is more pronounced at high solids loading operation of enzymatic hydrolysis and fermentation. Pretreatment inhibitors difficulty could be solved by long term evolutionary adaptation of fermenting strain in the environmental where strains to be used [15, 16]. A detailed procedure of evolutionary adaptation has been described in the next sections and in Chapters (3). Another difficulty is the viscosity problem, which is more prominent at high solids operations. High viscosity leads to improper mass transfer problems due to paste like mixture. This difficulty could be solved by proper bioreactor design, and unique helical stirrer bioreactor designed has given practical solution to this difficulty [14]. In this thesis, a high solids contents were used in all operational steps: pretreatment, biodetoxification, enzymatic hydrolysis, and fermentation. Performing all operational steps at high solids loading of lignocellulosic biomass for production of bioethanol and value added commodities presents a need for understanding the physicochemical and biological challenges of each process in a systematic way for advancement of lignocellulosic biorefinery. The saccharification efficiency is decreased with increased solids loading largely due to the inhibitory effects of sugars, which influence on overall ethanol titer and yield. Improved feeding strategies, detoxification, and evolutionary adaptation could give partial and practical solutions to these difficulties. The increasing number of research papers on high solids loading in all operational stages reflects not only the desire to implement lignocellulosic biorefinery on a commercial scale, but also hopeful to achieve it.

## 1.6. Pretreatment inhibitors: An environmental stress

Pretreatment inhibitors are formed and released during pretreatment of lignocellulosic biomass and their composition depends on the feedstock, pretreatment methods, and pretreatment conditions (concentration of any chemical used, pH, temperature, and residence time). These toxic compounds severely inhibit cellulase activity, cell growth, and ethanol productivity and yield. Thus, it is important to remove these inhibitory compounds prior enzymatic hydrolysis and fermentation at high solids contents. Fermentation inhibitors are classified into three groups; furans, weak acids, and phenolic compounds. Furans are released during pretreatment from degradation of sugar moieties, with furfural from pentose sugars and 5-hydroxymethyl furfural (HMF) from hexose sugars. Furfural disturbs the cells' central metabolic pathways, including glycolytic, pentose phosphate pathway (PPP), and tricarboxylic acid cycle (TCA), as shown in Fig. 1.2 [109-111]. HMF and furfural also causes malfunctioning in the DNA repairing mechanism [112]. In addition, synergetic effect of furans in combination with other inhibitors, including phenolic compounds, acetic acid, formic acid , and levulinic acids is more toxic than single inhibitor [113, 114]. Furfural is converted to furfuryl alcohol by the action of NADH-dependent alcohol dehydrogenase (ADH) in the yeast [115]. Furfural reduce the glycerol formation in the yeast cell, which is necessary for regeneration of NADH. This suggests that inhibition function of furfural reduce the NADH regeneration that leads to accumulation of furfural. Influence of pretreatment inhibitors on the cell metabolism and on macromolecules is shown in Fig. 1.2.

Moderate amounts of furfural usually increase the ethanol production perhaps due to less glycerol formation, which is affected in presence of furfural, and more glucose is available for ethanol production [110]. HMF also have similar effects on the cell growth and ethanol fermentability [116]. HMF is converted into HMF alcohol by NADPH-dependent enzyme rather than NADP-dependent. (ADH6) alcohol dehydrogenase 6 converts HMF to HMF alcohol in yeast HMF [43] causes stronger inactivation of cell replication than is caused by furfural [115].

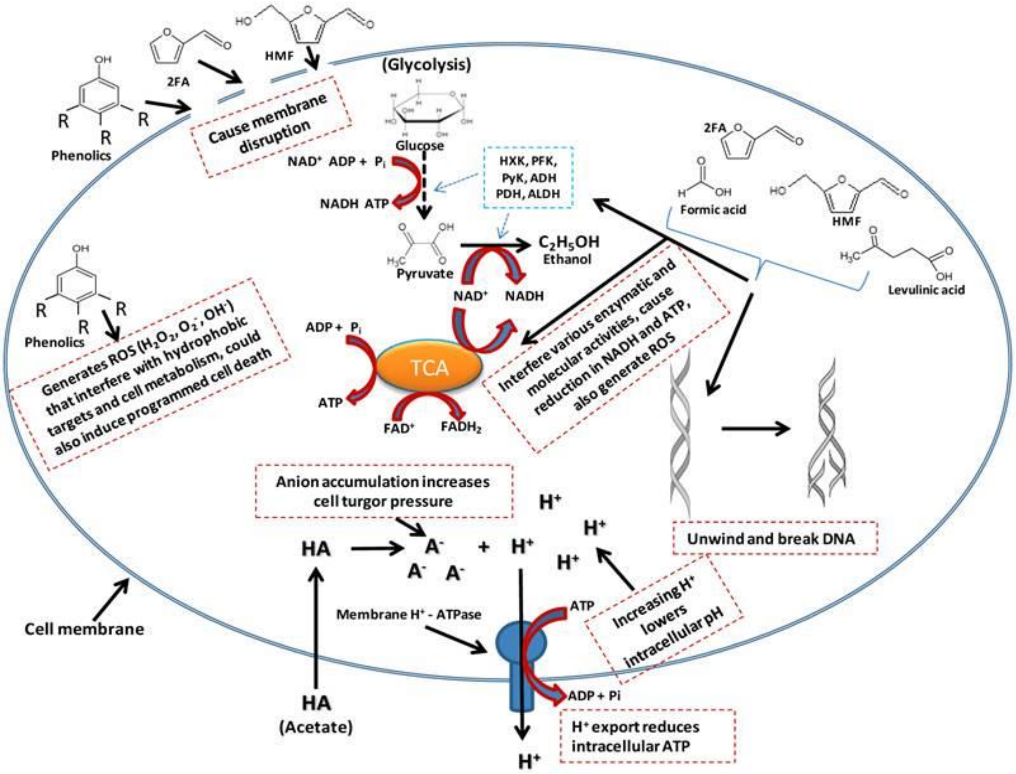


**Fig. 1.2.** Effect of pretreatment inhibitors on macromolecules and cell membrane. (http://www.ijbs.com/ms/getimage.php?name=ijbsv09p0598g03.jpg&type=thumb).

Weak acids, including acetic acid, formic acid, and levulinic acid are commonly released during the pretreatment. Acetic acid is formed and released from hemicellulose de-acetylation, formic acid and levulinic acids are released from HMF degradation [117, 118]. Acetate diffuses across the cell membrane and release protons on dissociation in the cytosol, which leads to lowering the intracellular pH, disturb transmembrane pH potential, and malfunctioning of proteins/enzymes. This pH imbalance is managed by pumping the proton out of cell by membrane ATPase. For this additional ATP to be generated, this is achieved by increased ethanol production under anaerobic conditions at the cost of biomass formation [119, 120]. According to anion accumulation theory, the intracellular pH is disturbed once undissociated form of the weak acid will diffuse across the plasma membrane from fermentation medium. Once undissociated acid enter in the cell, then dissociates inside the cell, thus decreasing the cytosolic pH and generate protons and acid anions. The cell must then correct this pH imbalance. In addition, accumulation of acids also increases the free radicals formation that enhances the oxidative stress on fermenting strain. Larsson et al. [121] investigated the effect of acetic acid concentration on ethanol productivity, this study suggest that moderate acetic acid concentration below 100 mM increases ethanol production, while more than 100 mM acetic acid concentration reduces yeast growth and ethanol fermentability. Formic acid inhibits macromolecules (DNA and proteins), DNA synthesis, and DNA repairing mechanism [119].

Phenolic compounds are released and formed from the lignin degradation and sugars dehydration during the pretreatment. Phenolic compounds are divided into three groups, includes acids (ferulic acid, vanillic acids, 4-hydroxybenzoic acid, and syringic acid), alcohols (guaiacol, catechol, and vanillyl alcohol), and aldehydes (vanillin, syringic aldehyde, and 4-hydroxylbenzaldehyde) [43, 119, 122, 123]. Phenolic compounds are further classified into two groups on the basis of their molecular weight: low and high molecular weight phenolic compounds. Low molecular weight compounds are more toxic than the high molecular weight [124]. Phenolic compounds concentration depends on lignin contents of feedstock and its linkage to cellulose and hemicellulose [65]. The inhibition mechanisms of phenolics is not fully explored perhaps due to wide range and minute quantity of each compound. A possible mechanism can be disturbance of cell membrane. Membrane disruption releases proteins, RNAs, ATP, and ions outside of the cell and this leads to reduction in ATP level, decrease in proton motive force, protein malfunctioning, and nutrient transport problems [43, 119, 125]. In addition, reactive oxygen species (ROS) interact with proteins/enzymes, which result in malfunctioning of these proteins/enzymes and consequently induce programmed cell death [126]. Phenolic compounds are more toxic that furans even at low concentrations [43, 119, 127]. Mechanism and inhibition effect of lignocellulose inhibitors vary by changing the feedstock, operation conditions and microorganism. Generally speaking, all inhibitors reduce cell growth, enzyme activity, and fermentation performance at certain level.

## 1.7. Evolutionary adaptation improves the inhibitor tolerance and fermentability of *Saccharomyces cerevisiae*

The inhibition of yeast and cellulase enzyme from furans, organic acids, and phenolic compounds is certainly a huge technical barrier for operating the enzymatic hydrolysis and fermentation at high solids content to reduce ethanol distillation and lactic acid purification cost. Several strategies have been developed to overcome the pretreatment inhibitors associated problems. These include, water washing, detoxification, microbial treatment, evaporation, and conditioning. Some of these methods are efficient than other, and other are not feasible at large scale. Another possibility could be, to reduce the pretreatment inhibitors formation during pretreatment but, this seems impractical. During severe pretreatment conditions more inhibitors are formed and at moderate conditions less sugars are released. There are several other possible strategies to solve the pretreatment inhibitors problem, including fermentation approaches, genetic engineering, and evolutionary adaptation [15, 16].

### 1.7.1. Fermentation approaches

There are various methods to overcome the pretreatment inhibitors problem. One of the approaches could be dilution of medium, which reduces the inhibitors concentration. Dilution of medium will also dilute the sugars concentration that leads to reduced ethanol concentration and increased ethanol distillation cost. Another similar approach could be, increase the inoculum size of the fermenting microorganism. This could be economically feasible only if cells are recycled in the fermentation process, as in SHF, while in SSF and CBP not possible. Another approach could be fed batch fermentation process. In this case, initially fermenting microorganisms will be exposed to lower inhibitors concentration. This will allow strain to slowly metabolize the sugars and adapt to inhibitors in the fermentation medium. In addition, this process can be efficient if pentose fermenting microorganism is used by maintaining the lower glucose concentration.

### 1.7.2. Genetic engineering

Genetic engineering introduces the heterologous or homologous genes into the fermenting strains, and new gene codes for novel protein/enzyme, which give tolerance to engineered strains against particular inhibitors. Overexpression of existing genes in the microorganisms can also increase the resistance against inhibitors. Petersson et al. [128] identified NADPH-dependent alcohol dehydrogenase (ADH6p), which is involved in HMF and furfural reduction in yeast. Overexpression of ADH6 gene generated a strain with at least 4-fold increased HMF degradation in defined medium under both aerobic and anaerobic conditions. Overexpression of gene encoding a PPP-related enzyme, transaldolase/transketolase, in the xylose-fermenting yeast, improved yeast performance and increased ethanol productivity in the presence of acetic acid and formic acid [129]. A potential disadvantage of genetic engineering is that the approach may work well if resistance against specific inhibitor is required. In most of cases a wide range of inhibitors produced and released during pretreatment, and tolerance to each inhibitors need a specific gene to be introduced or overexpressed, sometimes more than one gene is required. Therefore, genetic engineering may not be practical method for improving the inhibitor tolerance and fermentability of microorganisms to all pretreatment inhibitors present in hydrolysate or in pretreated material.

### 1.7.3. Evolutionary adaptation

Evolutionary adaption could be a promising strategy to increase the inhibitor tolerance and fermentability of strains in the same culture conditions where strain will be used [15, 16, 130, 131]. Evolutionary adaptation can be performed in the synthetic medium containing inhibitors simulating to hydrolysate, diluted hydrolysate, pure hydrolysate, and freshly pretreated solids. In the evolutionary adaptation process, cells are continuously transferred from previous medium to fresh medium containing the potent inhibitors. This transfer process is repeated for many generations to obtain a stable and adapted strain. Sometimes, a short evolutionary adaptation is performed for 1-10 days, or long term adaptation may be 100 to 1000 days or more. Evolutionary adaptation improves the inhibitors tolerance and fermentability of microorganism might be due to integrating certain changes at genomic level resulted from random mutations. These mutations enhance the expression of certain set of genes that increase glucose consumption and inhibitors transformation [119]. [116] performed adaptation of ethanologenic yeast by using synthetic medium with increasing furfural and HMF concentration from 10 to 120 mM. Furfural was found to be more toxic than HMF at same concentration, while the synergetic effect of furans significantly reduced the yeast growth [116]. Cell recycle batch fermentation was performed for evolutionary adaptation of the flocculent *S. cerevisiae* strain in the synthetic medium by gradually increasing inhibitors concentration for 39 consecutive days. Adaptation improved ethanol fermentability and growth over parental strain by 70% and 10%, respectively [16]. Recent review [132] mentioned that evolutionary engineering is potential approach for improving the performances of microorganisms used in lignocellulose biorefinery. Hawkins and Doran [43] developed adaptation strategies for *S. cerevisiae* strain XR 122N by using 17.5% w/v pretreated solids content of pine. Xylose fermenting *S. cerevisiae* strain was experienced long term adaptation for 15 days by using a series of culture medium by gradually increasing sugarcane bagasse hydrolysate concentration, strain with improved specific ethanol productivity 2.25 g/g/h in comparison to 1.15 g/g/h using 50% hydrolysate was evolved. Further increase of hydrolysate concentration had decreased ethanol productivity [133]. Heer and Sauer [134] carried out the adaptation of *S. cerevisiae* TMB3400 for over 300 generations. Cultured cells were continuously transferred in the synthetic medium by gradually increasing furfural concentration, which leads to reduced lag phase from 90 to 16 hours. Adaptation of natural xylose fermenting yeast strain, *Pichia stipitis* was conducted in steadily increasing wheat straw hydrolysate concentration from 20 to 80%, and improved ethanol performance was obtained from adapted strain over parental strain [135]. Adaptation of *S. cerevisiae* DQ1 was evaluated in corn cob residue hydrolysate, concentration was progressively increased that leads to improved ethanol titer and yield of 62.68 g/L and 55.70%, respectively [15]. *S. cerevisiae* 307-12H60 and 307-12H120, and *Pichia stipitis* 307-10H60 showed improved conversion rates for HMF and furfural at 30 and 60 mM over parental strain. A 100% biotransformation rates were observed for 30 mM furfural and 60 mM HMF to their respective alcohols by *S. cerevisiae* strains while *Pichia stipitis* showed 60% conversion of 60 mM HMF [136]. Keating et al. developed a strain of *S. cerevisiae* T1 by adaptation through CRBF in media supplemented with furfural, HMF and acetic acid, and concluded that tolerance level and fermentability were dose-dependent.

Dose dependent adaptation show increased inhibitor tolerance towards furans, which consequently improved cell growth, glucose consumption, and ethanol fermentability. Evolutionary adaptation has many advantages; (1) increase inhibitor tolerance, (2) improve fermentability, and (3) reduce detoxification time. *S. cerevisiae* DQ1 used in this study were experienced a long term evolutionary adaptation by using corn stover hydrolysate (Chapter 3).

The adaptation efficiency of a fermenting strain is highly dependent on the specific environment in which the strain is to live and ferment. Thus, the target strain used for the specific lignocellulose feedstock should be matched closely, because a subtle change in the hydrolysate such as carbon source, nutrient content, or inhibitory components may lead to a completely different outcome of the fermentation. A well-adapted strain with high fermentation performance in one hydrolysate may not be suitable for another hydrolysate with changes in any aspects, such as feedstock type, pretreatment method, conditioning (detoxification), or hydrolysis conditions. Selective pressure during the long term adaptation increases the rate of mutation and consequently beneficial mutants with enhanced fitness (evolved strain) could arise and expand in the population, these expansions are referred as adaptive events [137]. During evolutionary adaptation several phenotypes will appear and compete for natural selection to choose the fittest mutant. From large scale applications perspective, it is not necessary to obtain the fittest phenotype but to obtain increased performances in the actual experimental conditions [138, 139]. [137] described that relative tolerance of adaptive mutants changed to inhibitors with generation number and genes are up or down regulated. This might be due to loss of certain mutations during long term adaptation. Exact mechanism behind the evolutionary adaptation is not fully explored. During long term adaptation random mutation takes place and these mutations might be responsible for improved characteristics of fermenting stain. Certain genes involved in the biotransformation of pretreatment inhibitors are changed. When yeast cells are cultured in the hydrolysate medium or in the glucose (synthetic medium) gene expression is changed, this happens perhaps due to adaptation of the strain [140]. A possible explanation for the better performances of the adapted strain might be due to the improvements of target enzymes that allow more efficient sugar utilization and ethanol production in presence of pretreatment inhibitors [141]. It can be concluded that evolutionary adaptation is the best choice to improve the fermentability and inhibitor tolerance of microorganisms, thus, all the strains used in the lignocellulosic biorefinery should be experienced evolutionary adaptation. Evolutionary adaptation procedure is shown in Fig. 3.1. (Chapter 3).

## 1.8. Cost effective sources for glucose and yeast extract

Composition of the seeds and fermentation medium plays a critical role in the seeds culture quality and ethanol productivity. The development of a low-cost fermentation medium containing all essential nutrients is required for cell growth and ethanol fermentation. Standard medium for *Saccharomyces cerevisiae* contains glucose, minerals, and yeast extract. Apart from minerals, glucose and yeast extract (YE) are expensive component. Some inexpensive nutrients that could replace glucose are freshly pretreated corn stover solids and or hydrolysate. While, YE could be replaced with dry distillers' grains and solubles (DDGS), corn steep liquor (CSL), and rice bran. In this study, freshly pretreated solid material was used as cost effective carbon source for preparation of yeast seeds culture (Chapter 2) and dry distillers' grains solubles as nutrients in high solids content SSF (Chapter 3).

### 1.8.1. Freshly pretreated solid materials as a carbon source for preparation yeast seeds culture

Among the various cost related items, glucose consumption used in the preparation of yeast seeds culture is considerably high in the large scale commercial plant for ethanol fermentation [142]. All the seed fermentors require glucose as the carbon source thus the glucose consumption for seed culture is considerably high. However, glucose utilization in seeds culture may not be feasible at large scale. Therefore, glucose replacement with pretreated corn stover solids as a carbon source could improve financial viability of lignocellulosic ethanol. Previous studies have also evaluated the hydrolysate as a carbon source for yeast seeds culture preparation [14, 143]. Preparation of corn stover hydrolysate requires an independent saccharification bioreactor for hydrolysis of the pretreated corn stover, followed by the solid/liquid separation to obtain the hydrolysate liquid for seed culture use. This saccharification not only increased the fixed capital cost of equipment, but also led to the greater risk of contamination of the hydrolysate liquor in the relatively open operation of solids/liquid separation. The cost of centrifugation could also be saved by solid seeds culture preparation.

### 1.8.2. Dry distillers' grains solubles (DDGS)

The use of low-cost fermentation nutrients would certainly reduce the cellulosic ethanol production costs. Nitrogen source is second most important component of seeds culture and fermentation medium after carbon source and is required for a proper cell growth and ethanol productivity. Commonly used nitrogen sources, include yeast extract and peptone are expensive and contribute almost 50 % of the cost of ethanol production [144]. DDGS is nutrient rich contains (crude protein, crude fats, glucan, starch, amino acids and, yeast cells), a by-product of ethanol production using corn as the feedstock [145-148]. DDGS is generally used as animal feed; its annual production has increased upto 12 million metric ton. Cost of yeast extract cost is about $ 59.67/kg (http://www.biodee.net/ProductDetail.aspx?id=822&lang=en), while that of DDGS is $0.188/kg (http://www.grains.org/buyingselling/ddgs). Only 0.5% cost of yeast extract can give same ethanol concentration and yield. Considering the cost effectiveness of DDGS for obtaining high ethanol titer and yield, in this study DDGS was used as a nitrogen source for ethanol production from high solids content of corn stover. Supplementation of DDGS concurrently reduced the cellulosic ethanol production cost and increased the ethanol productivity and yield at high solids content SSF (Chapter 3).

## 1.9. Glucose and xylose cofermentation to ethanol at high solids content

Pretreated lignocellulosic biomass contains both pentose and hexose sugars. Lignocellulosic hydrolysate is composed of 60-70% of glucose and 30-40% of xylose. The fermentation of pentose sugars still remains a challenge. Therefore, efficient xylose conversion to ethanol and lactic acid is very important from economical view point. Although the obtained results (Chapter 3 and 4) were satisfactory from glucose fermentation to ethanol and lactic acid. But, efficient and complete lignocellulose sugars utilization for biofuels seems practical solution to reduce the processing cost and genetically engineered strains are essentially required to solve this difficulty [149, 150]. *Saccharomyces cerevisiae* is routine ethanol fermenting strain due to high ethanol conversion efficiency, high ethanol tolerance, and significant inhibitor tolerance [124]. However, *S. cerevisiae* could not utilize xylose due to lack of xylose to xylulose conversion enzymes. Only a few strains are available for fermentation of pentose sugars to ethanol. The most promising yeast species identified so far are *Candida shehatae*, *Pichia stipitis,* and *Pachysolen tannophilus*. The fermentation of hexose and pentose sugars is combined in single unit commonly known as co-fermentation to reduce process complexity. Slow fermentation rates of pentose sugars compared to hexose sugars, high sensitivity to inhibitors, carefully regulated oxygen requirement and low product tolerance prohibited commercial application of co-fermentation. The improvement of stains by genetic engineering is the key for effective utilization of pentose sugars to boost overall economy of lignocellulosic conversion to biofuel and other value added commodities.

Therefore, several metabolic engineering strategies were practiced to introduce xylose metabolism pathways in commonly used *S. cerevisiae* [99, 151-154]. Filamentous fungi, bacteria, and natural xylose fermenting strains can utilize xylose by two possible routes as shown in Fig. 1.3.

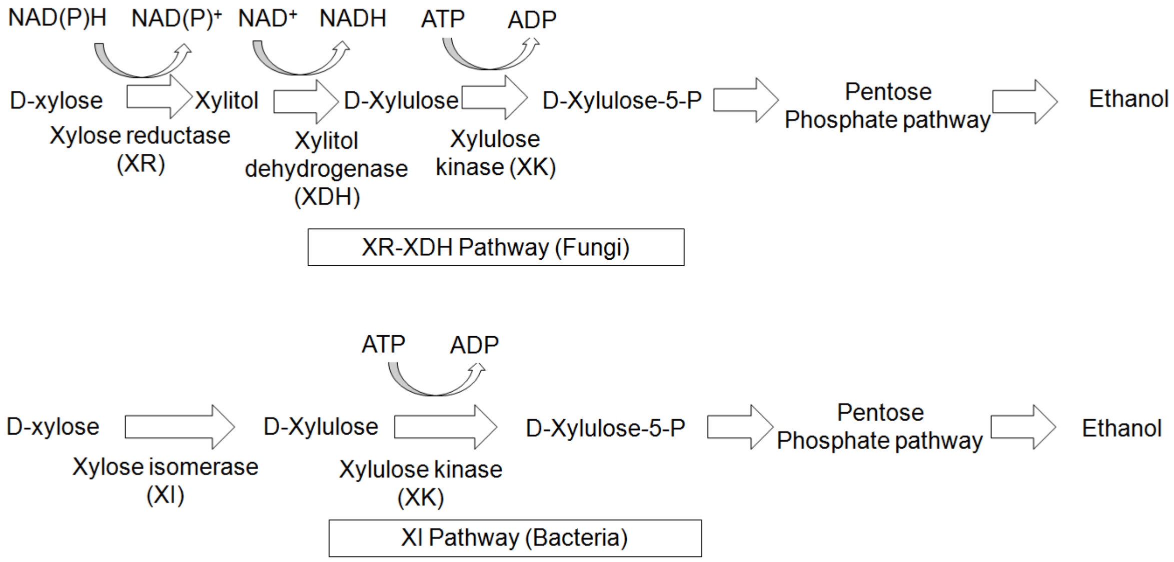


**Fig. 1.3.** Metabolic pathways of xylose conversion to ethanol in industrial fermenting microbes

One of these pathways introduce xylose isomerase (XI) and xylulo kinase, while in another pathway xylose reductase, xylitol dehydrogenase and xylulo kinase were introduced. Due to different xylose utilizing pathways and expressions patterns, there are various consideration to introduce them efficiently into the *S. cerevisiae* [154]. Several research groups are optimizing the each pathway, therefore, still it is hard to decide, which pathway is better than the other [154]. Engineered xylose fermenting strains can give highest possible ethanol titer and yield close to starch based technology. Lignocellulosic hydrolysate contains glucose and xylose, and the glucose fermentation rate is about 3-10 fold higher than that of xylose fermentation largely due to preference of transport system for glucose consumption. Low ethanol productivity due to sequential glucose and xylose consumption is one of the technical difficulties in lignocellulosic biorefinery. In last decade, rate of xylose consumption has been improved by using several strategies: metabolic engineering, protein engineering, and omic technologies. Recent studies show the improved rate of xylose conversion to ethanol in the range of 0.2 to 0.8 g ethanol/g cells/h by engineered *S. cerevisiae* [155-158]. Xylitol accumulation by engineered *S. cerevisiae* strains is another difficulty perhaps due to redox imbalance [152], and this problem is solved by proper pH regulation and micro aeration [159].

## 1.10. Open lactic acid fermentation from high solids content of lignocellulosic biomass under completely unsterilized conditions

Lactic acid is widely consumed in food, pharmaceutical, textile, cosmetic, and chemical industries [160]. One of the technical challenge in the lactic acid production is high operational cost (sterilization and purification) [161]. This difficulty could be solved by open lactic acid fermentation without sterilization. Here, open fermentation means, fermentation is performed without sterilization from bioreactor, feedstock, water used in preparation of medium and in SSF, nutrients, and sampling in open conditions. Generally, a step of autoclaving the medium and bioreactor before fermentation is unavoidable due to possible contamination chances. Sterilization step would enhance the lignocellulosic operational cost due to high energy consumption and labor intensive step. In addition, nutrients are lost due to heat treatment during sterilization. Open lactic acid fermentation under completely unsterilized conditions could be an excellent choice [162-166].

Several research groups have conducted open lactic acid fermentation by using thermophilic strains [161-172] as shown in Table 1.2. These studies face various difficulties, including low lactic acid titer from lignocellulosic materials [162-167, 170, 171] and low optical purity of produced lactic acid [165, 170]. Only few studies obtained higher lactic acid titer, productivity, and optical purity [161, 169, 172] under open fermentation conditions at the cost of pure sugars. Utilization of pure sugars increases socio-economic problems.

**Table 1.2. Open lactic acid fermentation studies**

| Strain | Carbon source | Lactic acid titer (g/L) | Lactic acid yield (%) | Lactic acid productivity (g/L/h) | Optical purity (%) | References |
|---|---|---|---|---|---|---|
| *Bacillus coagulans* NBRC 12583 | Tapoica starch | 90.8 | 90 | 1.26 | 99.5 | [173] |
| *Bacillus* sp. NL01 | Steam exploded corn stover | 75.03 | 74.5 | 1.04 | [a]100 | [163] |
| *Bacillus sp.* strain 2–6 | Synthetic medium (glucose) | 118.0 | 97.3 | 4.37 | 99.4 | [161] |
| *Mixed culture* | Kitchen refuse | 28 | [b] NM | 0.388 | 98-100 | [164] |
| *Mixed culture* | Kitchen refuse | 31.1 | [b] NM | 0.431 | Mixed isomer | [166] |
| *Bacillus coagulans* NBRC12583 | Kitchen refuse | 86 | 53 | 0.716 | 97 | [165] |
| Mixed culture | Kitchen refuse | 34.5 | [b] NM | 0.205 | 100 | [167] |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Bacillus coagulans* C106 | synthetic medium (Xylose) | 215.7 | 95.0 | 4.0 | 99.6 | [169] |
| *Bacillus coagulans* JI12 | Wheat straw hydrolysate | 80.6 | 49 | 3.4 | [a]100 | [168] |
| *Mixed culture* | Kitchen waste | 64 | 62 | 0.533 | Mixed L: D 83:17 | [170] |
| *Bacillus coagulans* IPE22 | Wheat straw hydrolysate | 54.55 | [b]94 | 1.01 | 100 | [171] |
| *Bacillus sp.* strain 2–6 | Synthetic medium (glucose) | 107 | 95 | 2.9 | 99.8 | [172] |
| *Pediococcus acidilactici* TY112 | Corn stover | 97.34 | 69.38 | 1.47 | 100 | This study |

[a] Not mentioned in the paper. Optical pure L-lactic acid produced so assumed to be 100%. [b] NM not mentioned.

## 1.11. Bacteriocin

Bacteriocins are small antimicrobial peptides produced by bacteria, and more specifically by lactic acid producing bacteria (LAB). Bacteriocin are often active against wide range of species (broad range) or specific species (narrow spectrum) [174-177]. Bacteriocins find potential application in food industry to prevent food spoilage, as antimicrobials in personal care formulations [178, 179]. Only bacteriocin that is approved for use as a food preservative by the Food and Drug Administration (FDA) is nisin from lactic acid bacteria (LAB) [180]. The fact that many bacteriocins are produced by food-grade LAB and possess potent antimicrobial activity means that they are ideally suited to controlling food spoilage and pathogenic bacteria [181-183].

Bacteriocins can be classified into four groups: Class I, small peptides includes nisin and lantibiotics (post-translationally modified peptides). Class II, containing unmodified peptides and size is less than 10 kDa, these bacteriocins are heat stable [184]. Class III, molecular weight larger than 10 kDa and heat labile. Class IV, the complex bacteriocin containing lipid or sugar moiety [183, 185].

More specifically, pediocins are produced by *Pediococcus* strains. Pediocin production, purification and characterization have been investigated from several *Pediococcus* strain, including *Pediococcus pentosaceous* NCDC 273, *Pediococcus damnosus* NCFB1832, *Pediococcus parvulus* 133, *Pediococcus acidilactici* NRRL B5627, *Pediococcus pentosaceus* Mees 1934 [186-191]. Pediocins were characterized on the basis of heating, freezing, proteolytic treatment and acid treatment and still can inhibit the growth of indicator bacterial strains [192]. Due to these properties, pediocin are used in food industry as a natural food preservative to improve the food quality [193-196]. Pediocin can also eliminate the growth of contaminating strain during the fermentative processes and this function has never been investigated. In this study, pediocin as a function of performing open lactic acid fermentation under completely unsterilized condition was evaluated (Chapter 4).

## 1.12. Objectives of the thesis

The main objectives of this study were to develop fast, efficient, and cost effective lignocellulosic biomass conversion methods for ethanol and lactic acid production. The detailed objectives of the thesis are as follow:

- Utilization of the freshly pretreated solids materials (corn stover) as a carbon source for preparation of yeast seeds culture for ethanol SSF (Chapter 2).
- Evolutionary adaptation of routine yeast strain of *Saccharomyces cerevisiae* DQ1 to corn stover hydrolysate inhibitors (Chapter 3).
- High solids content ethanol SSF by using cost effective nutrients; dry distillers' grains solubles (DDGS) instead of very expensive and common nutrient (yeast extract) from adapted DQ1 strain (Chapter 3).
- Open fermentation of high titer L-lactic acid from corn stover by a pediocin producing *Pediococcus acidilactici* strain (Chapter 4).
- Open L-lactic acid fermentation could reduce the operational cost of sterilization step. These results show significant development in new dry lignocellulosic biorefinery for ethanol and lactic acid production using lignocellulosic biomass. The overall experimental scheme is shown in Fig. 1.4.

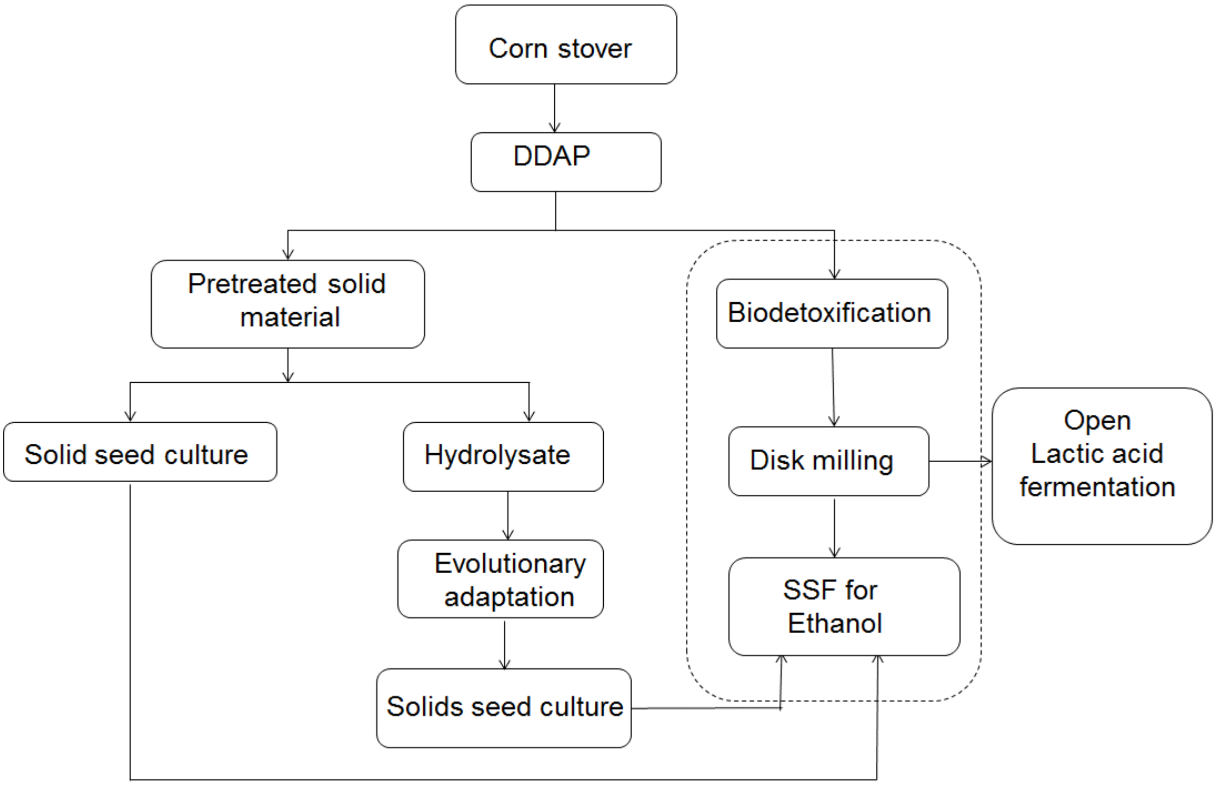


**Fig. 1. 4**. Overall experimental scheme of the research work presented in this thesis

# Chapter 2

# Cellulosic ethanol fermentation using *Saccharomyces cerevisiae* seeds cultured by pretreated corn stover material

## 2.1. Introduction

Cost reduction is the key issue for commercialization of cellulosic ethanol production from lignocellulosic feedstock [108, 197]. Among various cost effective items, glucose consumption used for growing fermenting microbe cells is considerably high in large scale plants [142]. For general ethanol fermenting strains such as *Saccharomyces cerevisiae*, the seeds inoculation ratio was approximately 10% (v/v) of the fermentation volume of the next stage fermentor. A typical design of cellulosic ethanol plant with 61 million gallons production annually in the NREL technical report in 2011 [142] requires two seeds trains of five sequentially aligned fermentors in each train before the seeds finally enter the ethanol production fermentor with the inoculation ratio of 11% (v/v). In all the fermentors of the seeds culture, glucose is as the carbon source. Only in the final production fermentor, the pretreated corn stover is used as the carbon source for ethanol production. Apparently, if the glucose used is replaced by the corn stover materials, the overall cost of cellulosic ethanol could be reduced by a large percentage, especially in the industrial scale plants.

In the previous studies, corn stover hydrolysate, a clear supernatant liquid prepared from enzymatic hydrolysis of the pretreated corn stover, was widely used in the seeds culture of fermenting strains such as *S. cerevisiae* [13, 14, 68, 143, 198]. However, the preparation of corn stover hydrolysate requires an independent saccharification bioreactor and a solid/liquid separation equipment to obtain the hydrolysate liquid. This step not only increases the fixed capital cost for equipment, but also leads to a high risk of contamination during the solids/liquid separation [199-201].

In this study, a simple approach of seeds culture using the pretreated corn stover materials as carbon source was tested. The freshly pretreated corn stover solids were directly fed into the fermentor as the carbon source without enzymatic hydrolysis step, together with the cellulase enzyme for seeds cultivation. Whole culture slurry (liquid and solids phase) was sequentially transferred in the seed fermentors after a suitable culture time, and the same operation was repeated until the production fermentor. The SSF seed culture developed could be advantageous in terms of complete cellulase utilization, glucose replacement, and contamination risk free. The practicability of this method highly depends on whether the seed quality (cell viability) and quantity (cell number) meet the requirement in the multiple seeds culture and production fermentor from the relatively inexpensive carbon source (pretreated corn stover solids). The objectives include to evaluate cell growth and ethanol production of SSF seeds culture, to compare ethanol production efficiency using yeast seeds cultured by different carbon sources (glucose, corn stover hydrolysate, and pretreated corn stover solids), and to estimate the economic feasibility of the pretreated corn stover solids as carbon source in industrial scale. The method offers a simple and practical approach of cost reduction for the large scale ethanol fermentation from lignocellulosic biomass.

## 2.2. Materials and Methods

### 2.2.1. Raw materials and chemicals

Corn stover (CS) without pretreatment is defined as virgin corn stover. It was grown in Dancheng County, Henan Province, China, and harvested in fall 2012. The corn stover was milled coarsely on a beater pulverizer and screened through mesh with the circle diameter of 10 mm. The milled corn stover was washed to remove field dirts, stones, and metals, then dried till constant weight and sealed in plastic bags for use. The cellulose and hemicellulose contents of corn stover were determined according to a two-step $H_2SO_4$ hydrolysis method developed by the National Renewable Energy Laboratory (NREL) [202]. The virgin corn stover contained 37.2 % of glucan and 19.9 % of xylan. The pretreated corn stover contained 41.4 % of glucan and 4.0 % of xylan. Dry dilute acid pretreated material contains, acetic acid 14.98 mg/g DM, HMF 3.33 mg/g DM, and furfural 7.09 mg/g DM.

Yeast extract was purchased from Oxiod (Basingstoke, Hampshire, England). All other standard chemicals including glucose, peptone, $KH_2PO_4$, $(NH_4)_2SO_4$, $MgSO_4$, NaOH, and $H_2SO_4$ were of reagent grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Agar was purchased from New Probe Bioscience Technology Co., Beijing, China.

### 2.2.2. Strains and enzymes

The ethanol fermenting strain *Saccharomyces cerevisiae* DQ1 was stored at Chinese General Microorganisms Collection Center, Beijing, China, with the registration number of CGMCC 2528. ) was used as [14, 143].

The biodetoxification strain *Amorphotheca resinae* ZN1 was stored at Chinese General Microorganisms Collection Center, Beijing, China, with the registration number of CGMCC 7452 for the removal of inhibitors from the pretreated corn stover [68].

The cellulase enzyme Youtell #6 was kindly provided by Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell #6 was 135 FPU/g determined using the NREL protocol LAP-006 [203], and the cellobiose activity was 344 CBU/g using the method of Sharma et al. [204].

The culture media used included:

(1) Synthetic medium: 20.0 g/L of glucose, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, and 1.0 g/L of yeast extract;

(2) Adaptation medium: 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4 .7H_2O$, and 1.0 g/L of yeast extract (pH 6.0);

(3) Fermentation medium: 2 g/L $KH_2PO_4$, 1 g/L $MgSO_4$, 1 g/L $(NH_4)_2SO_4$ and 1 g/L yeast extracts;

(4) YPD medium: glucose 20g/L; peptone 20 g/L; yeast extract 10 g/L and agar 20 g/L. All media were sterilized at 115 ℃ for 20 min.

### 2.2.3. Pretreatment

Corn stover was pretreated using the dry dilute acid pretreatment procedure as described in Zhang et al. [13]. The corn stover feedstock was firstly presoaked with diluted sulfuric acid solution (5.0%, w/w) with the ratio of the solid (dry corn stover) to the liquid (sulfuric acid solution) of 2:1 (w/w), then was pretreated at 190 ℃ for 3 minutes. The pretreated corn stover was withdrawn from the reactor and the solids content was 50% (w/w).

The pretreated corn stover was biologically detoxified to remove the fermentation inhibitors by using *A. resinae* ZN1 strain according to the procedure in Zhang et al. [68]. The detoxified corn stover was used as the final ethanol production fermentation, and was not used in the seeds culture steps.

### 2.2.4. Corn stover hydrolysate preparation

Freshly pretreated corn stover solid was hydrolyzed as described in Huang et al. [198]. Briefly, 15% (w/w) freshly pretreated corn stover solids were hydrolyzed using Youtell # 6 at 50 ℃ for 48 hours with the dosage of 15 FPU/g DM (dry pretreated corn stover). The water insoluble solids were separated from corn stover hydrolysate by centrifugation at 10,000 rpm for 10 minutes. The clear hydrolysate contained 61.05 g/L of glucose, 32.09 g/L of xylose, 2.23 g/L of acetic acid, 0.32g/L of furfural, and 0.16 g/L of 5-hydroxymethylfurfural (HMF). Hydrolysate was used as carbon source for yeast seed culture.

### 2.2.5. Cell cultivation using freshly pretreated corn stover as carbon source

The schematic diagram of the ethanol fermenting strain *S. cerevisiae* DQ1 culture using the freshly pretreated corn stover (without detoxification treatment) as carbon source is shown in Fig. 2.1. The adaptation procedure used was to allow the yeast gradually adapting the inhibitors containing hydrolysate and assure the reproductively of the fermenting strain. The details are described as follows:

Step 1: A vial of *S. cerevisiae* DQ1 stock culture was inoculated into a 100 mL Erlenmeyer flask containing 20 mL of the synthetic medium (20 g/L of glucose contained) and cultured for 18 hours in a shaking incubator at 30 ℃, 150 rpm.

Step 2: 5 ml of the cell culture from Step 1 was inoculated into a 250 mL flask containing 50mL of the adaptation medium at 10% (v/v) inoculation ratio, then cultured for 12 hours at 37 ℃ and 150 rpm. In this step, 2.5 grams (dry base) of the pretreated corn stover solids (5% of the total medium by weight percentage) and 0.34 grams of cellulase Youtell #6 at 15 FPU/g DM were added into the adaption medium before the culture started.

Step 3: 5 ml of the cell culture from Step 2 was inoculated into the 50 ml of the adaptation medium cultured for 12 hours at 37 ℃ and 150 rpm. In this step, 5 grams (dry base) of the same solid corn stover (10% of the total medium by weight percentage) and 0.68 grams of cellulase at 15 FPU/g DM were added into the adaption medium before the culture started.

Step 4: The process was repeated for further adaptation purpose. 5 ml of the cell culture from Step 3 was inoculated into the 50 ml of the adaptation medium cultured for 12 hours at 37 ℃ and 150 rpm, and 5 grams (dry base) of corn stover (10%, v/v) and 0.68 grams of cellulase at 15 FPU/g DM were added into the adaption medium before the culture started.

Step 5: The whole suspension from Step 4 was used as seeds culture of the simultaneous saccharification and fermentation (SSF) in the final SSF bioreactor for production of ethanol using the detoxified corn stover.

The cell cultivation using the hydrolysate liquid prepared from the pretreated corn stover followed the same procedure as reported in Zhang et al. [14].

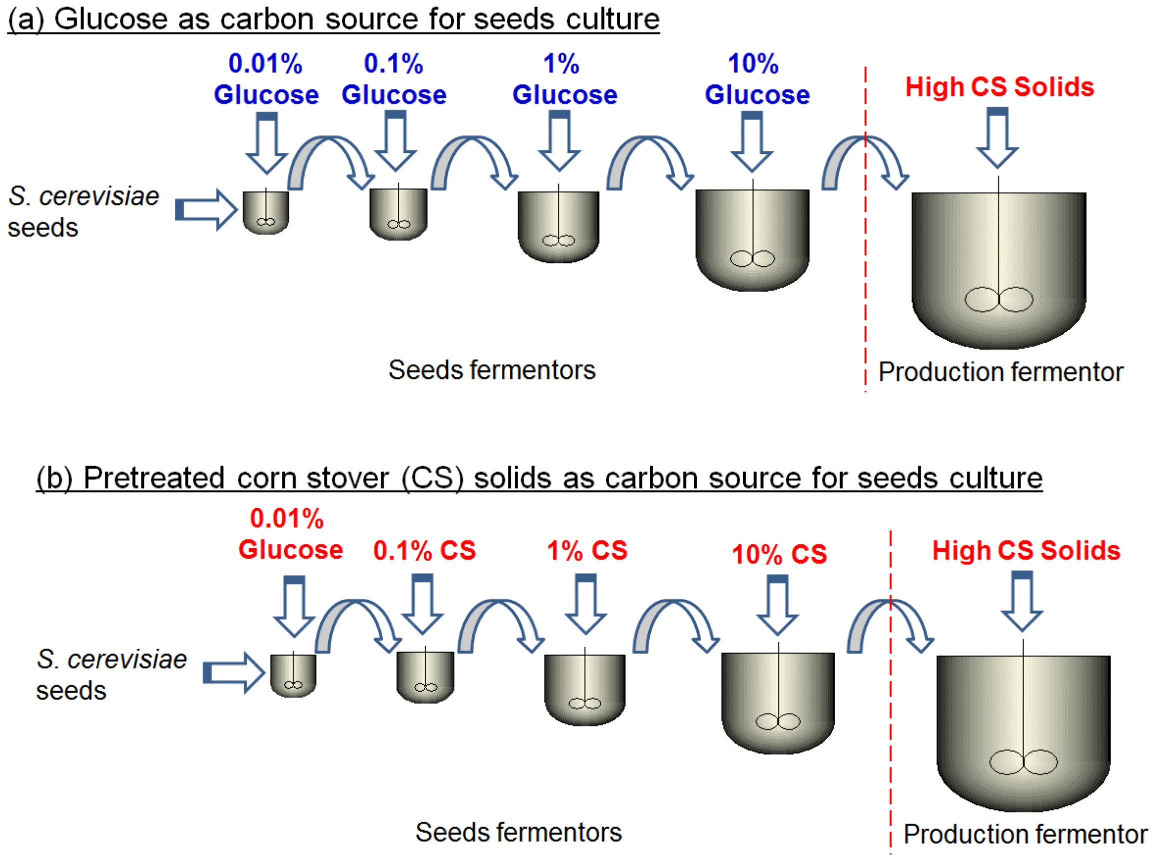


**Fig. 2. 1**. Schematic diagram of yeast seeds culture using freshly pretreated corn stover solids as carbon source.

### 2.2.6. Simultaneous saccharification and ethanol fermentation (SSF) operation

All SSF experiments were performed in a 5L bioreactor under the solids loading of the pretreated corn stover up to 25% (w/w). A unique helical ribbon stirrer was installed for well mixing of the solids with enzyme and nutrients as described in Zhang et al. [14]. The SSF process was operated at two stages:

Stage 1 (prehydrolysis): 6 hours at 50 ℃, pH 4.8 after cellulase was added at 15 FPU/g DM. The pretreated, detoxified and disk milled corn stover feedstock was fed into the bioreactor in semi-continuous mode during 6 hours.

Stage 2 (SSF): The prehydrolyzed slurry was cooled down to 37 ℃, and then the fermentation medium nutrients were added to reach the fermentation medium composition. The yeast seed culture from step 5 of "Cell Cultivation Using Freshly Pretreated Corn Stover as Carbon Source" section was inoculated to start the SSF operation. The SSF continued for 72 h, and samples were withdrawn with regular intervals. The pH was maintained at 4.8 during the overall operation by addition of 5 M NaOH or 2 M H2SO4 solutions. Each experiment was duplicated, and the mean value was calculated.

### 2.2.7. Analysis

The samples were centrifuged to remove the solids and the supernatant was collected for analysis on HPLC to obtain the periodic concentration of glucose, ethanol, as well as acetic and lactic acid. All samples were filtered through a 0.22 µm filter before injection. The HPLC used consisted of LC-20AD pump, RI detector RID-10A (Shimadzu, Kyoto, Japan), and a Bio-Rad Aminex HPX-87H column at 65℃ with 0.6 mL/min of 5 mM $H_2SO_4$ as the mobile phase.

The optical density of yeast cells in the fermentation slurry was not able to measure because of the high solids inside. An alternative method was used by counting the colony forming unit (CFU/mL). The CFU number of the yeast cells was counted by spreading the each sample collected at regular interval (SSF) after dilution on to the YPD agar plates, and incubated at 30 ℃ for 48 hours; the single colony numbers grown on the agar plates were counted.

### 2.2.8. Ethanol yield calculation

Ethanol yield was calculated according to the modified method by taking into account the ethanol formation and the water loss in the hydrolysis [205].

$$\text{Ethanol yield} = \frac{[C_1]\times W}{976.9-0.804\times[C_1]}\cdot\frac{1}{0.511\times f\times[Biomass]\times m\times 1.111}\times 100\%$$

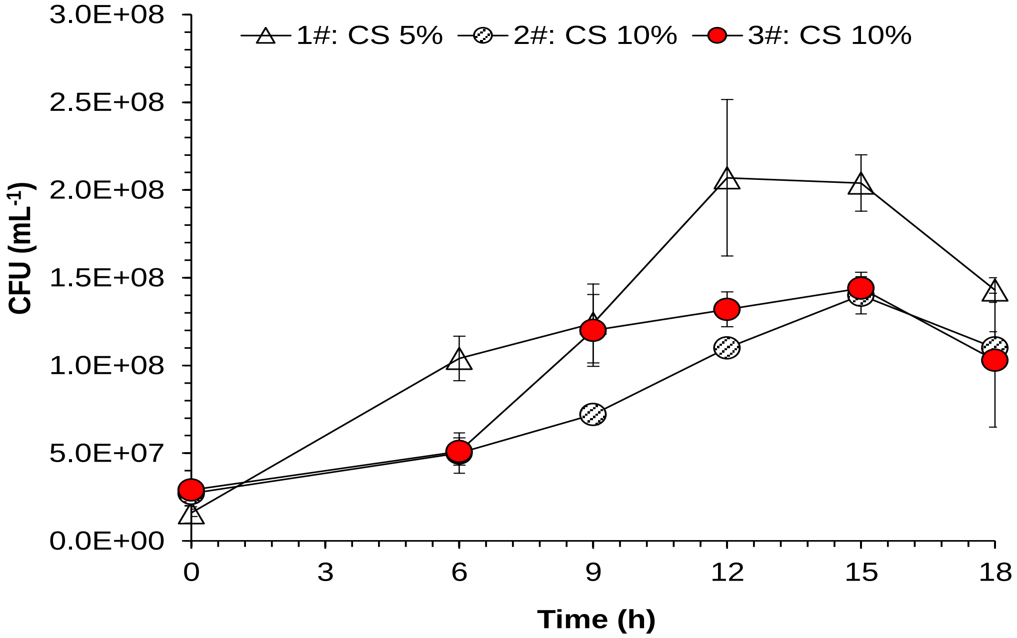
where $[C_1]$ was the ethanol concentration in the culture broth (g/L), $W$ was the total water input of the SSF (g), $f$ was the cellulose fraction of corn stover feedstock, $[Biomass]$ was the dry corn stover concentration at the beginning of the SSF (g/g), *m* was the total weight of the SSF (g), 0.511 was the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, and 1.111 is the conversion factor for cellulose equivalent to glucose, 976.9 and 0.804 are constants of the formula derived from the method development in [205].

## 2.3. Results and Discussion

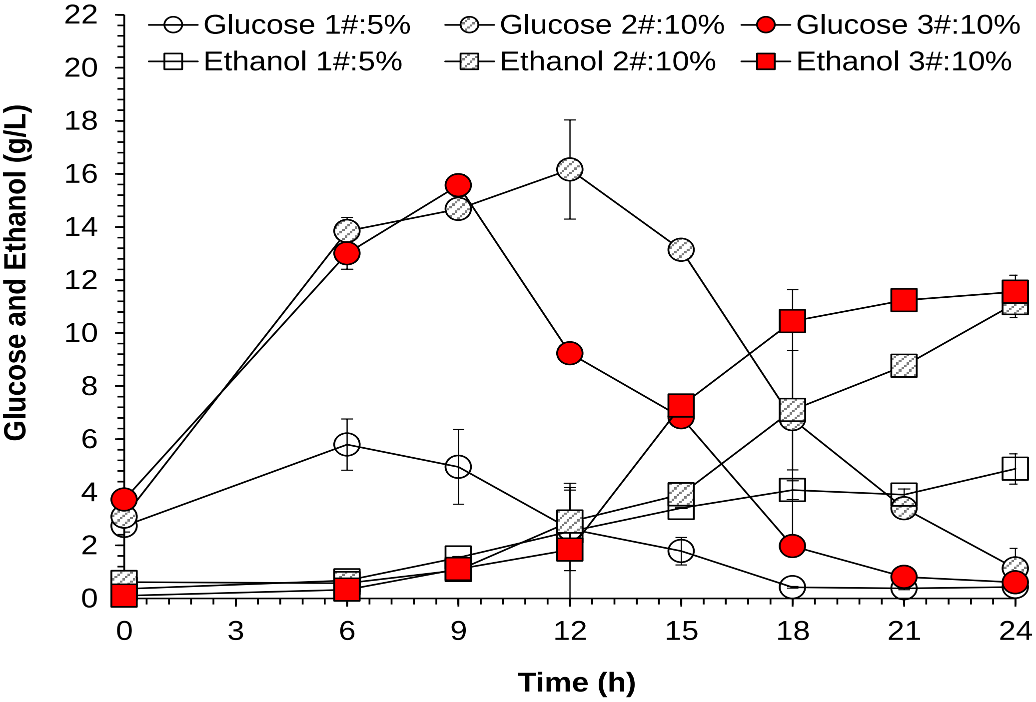
### 2.3.1. Seeds culture using freshly pretreated corn stover solids as carbon source

The *S. cerevisiae* DQ1 seed culture was prepared by using the freshly pretreated corn stover solids (without any detoxification treatment) as the carbon source. Fig. 2.2 (a) shows the *S. cerevisiae* DQ1 cell growth indicated by CFU number. In the first seed culture, the yeast cell broth from the synthetic medium was consecutively transferred to the SSF culture containing 5% (w/w) pretreated corn stover solids; the second seed culture was carried out by transferring the first seeds culture broth into the SSF culture containing 10% (w/w) pretreated corn stover solids; the third seeds culture again was carried out by transferring the second culture broth into the SSF culture containing 10% (w/w) pretreated corn stover (repeating the second seeds culture). The cell CFU number ($2.0\times10^8$) reached its maximum in the period of 12-15 hours' culture time. In the second culture, the CFU number was relatively decreased and reached approximately 70% of the maximum CFU number in the first culture after 15 hours' cultivation ($1.5\times10^8$). The CFU number in the third culture was similar to that in the second culture, with fast growth rate and a slightly higher CFU number. The overall cell growth in the three cultures was in a satisfactory condition and met the requirement of the consequent SSF operation.

Fig. 2.2 (b) shows the time course of glucose consumption and ethanol formation in the seeds culture. The glucose released and the ethanol produced were low because of the low pretreated corn stover solids loading (5%, w/w); with the increased solids loading to 10% (w/w) in the other two cultures, the glucose released and consumption, as well as the ethanol production increased and the third culture gave the best performance, although the CFU number was slightly decreased in the second and third seeds culture. The reason might come from the adaptation function of yeast cells to the inhibitors in the pretreated corn stover, similar to the adaptation function in the seeds culture in the corn stover hydrolysate liquid [143]. Also, although the glucose released from corn stover and ethanol produced in the seeds culture did not make a significant contribution to the final ethanol titer in the production fermentor, it still added a surplus on the ethanol titer and yield in the SSF using the pretreated corn stover by replacing glucose as the carbon source.



(a) Cell viability



(b) Time courses of glucose and ethanol

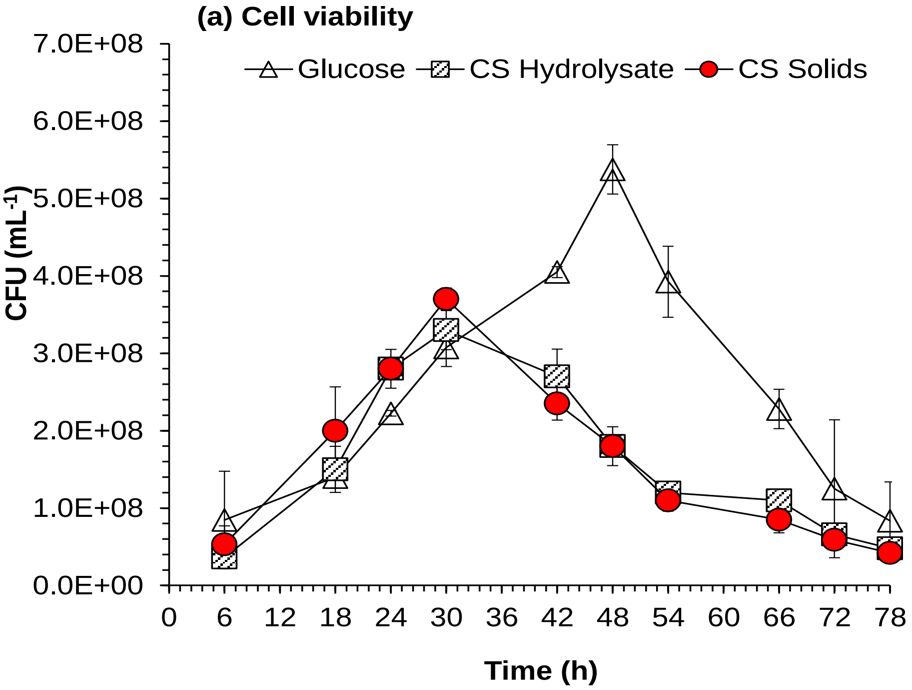
**Fig. 2.2.** Solids seeds culture using freshly pretreated corn stover. Seeds culture of *S. cerevisiae* DQ1 using the freshly pretreated corn stover solid as carbon source. Conditions: cellulase 15 FPU/g DM, 37 ℃, pH 6.0 and shaked at 150 rpm. (a) Yeast cell viability (CFU/mL); (b) Time course of glucose utilization and ethanol production. 1#: *S. cerevisiae* DQ1 seeds culture from the 1st step in Section 2.4 were transferred to the culture medium containing 5.0% (w/w) of the pretreated corn stover solids for 12 hours (the Step 2 in Section 2.4); 2#: the seeds culture from the 1# was transferred into the medium containing 10% of the pretreated corn stover for 12 hours (the Step 3 in Section 2.4); 3#: the seeds culture from 2# was transferred into the culture medium containing 10% of the pretreated corn stover for 12 hours (Step 4 in Section 2.4).

### 2.3.2. SSF using yeast seeds cultured by pretreated corn stover solids

The seeds culture prepared by using the pretreated corn stover materials as carbon source was inoculated into the simultaneous saccharification and fermentation (SSF) of corn stover at the volume ratio of 10%. Three SSF cases were carried out using the seeds cultured by the synthetic medium (glucose), the clear corn stover hydrolysate liquid, and the freshly pretreated corn stover, respectively. The SSF results were shown in Fig. 2.3.

Fig. 2.3 (a) shows that the CFU number in the SSF increased from the start and reached at maximum, then declined. In the first case using the synthetic medium (glucose) for seeds culture, the CFU number reached $5.4\times10^8$ after 48 hours then declined. In the second case using the clear corn stover hydrolysate for seeds culture, the CFU number reached $3.2\times10^8$ in the earlier culture time of 30 hours, then declined. In the third case using the pretreated corn stover solids as the carbon source, the CFU number reached $3.6\times10^8$ at the same culture of 30 hours, similar to that using the hydrolysate liquid and as twice as that in the seeds culture ($1.5\times10^8$), suggesting that the cell growth in the seeds culture using the corn stover solids was at least as good as that using hydrolysate for seeds culture. The high CFU number of *S. cerevisiae* DQ1 indicates that the corn stover materials provided sufficient energy and nutrients, and neglected the effect of inhibitors for cell growth.

Fig. 2.3 (b) shows the time courses of glucose consumption and ethanol production in the SSF of pretreated and detoxified corn stover at high solids loading of 25% (w/w). In the three SSF cases, the ethanol titer and yield were 47.48 g/L and 72.07% when the seeds were cultured using glucose, 52.34 g/L and 80.17% by clear pretreated corn stover hydrolysate, and 49.99 g/L and 76.10% by freshly pretreated corn stover solids, respectively.



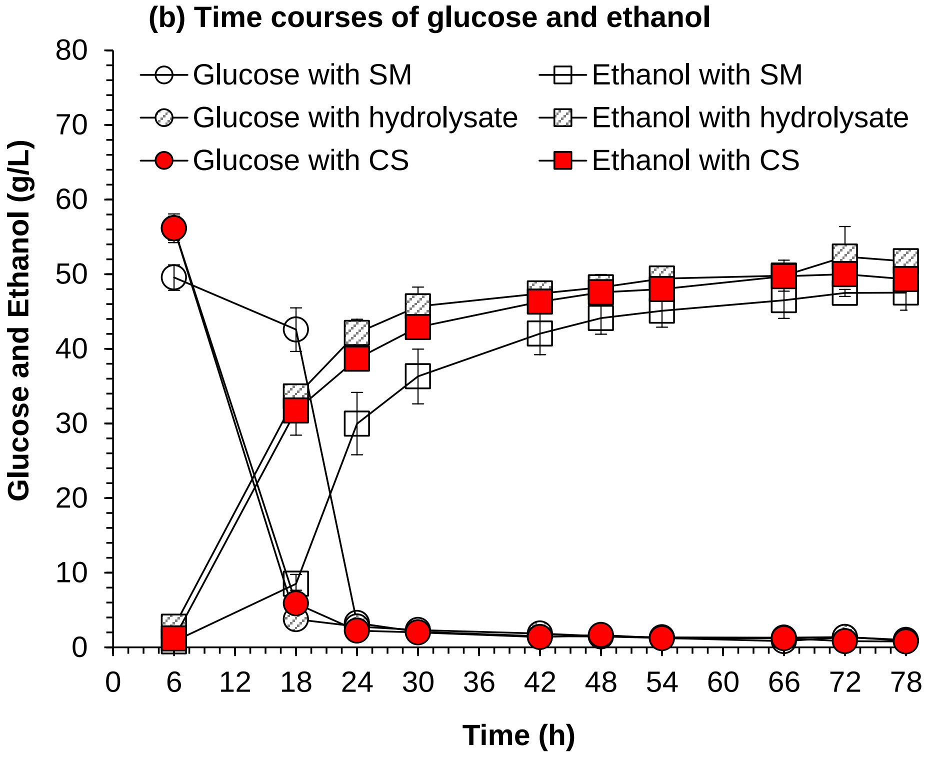


**Fig. 2.3.** SSF performance of corn stover using different carbon source cultivated seeds culture. SSF of corn stover using seeds cultured with different carbon sources. (a) Cell viability (CFU/ml); (b) Time course of glucose utilization and ethanol production. *S. cerevisiae* DQ1 were cultured using the synthetic medium (glucose), the clear corn stover hydrolysate, and the freshly pretreated corn stover solids as carbon sources. The SSF was carried out in a 5L helically stirred bioreactor. Conditions: 25% (w/w, dry base) solids loading, cellulase 15 FPU/g DM; the prehydrolysis was lasted for 6 hours at 50 ℃ and pH 4.8, then the SSF was operated for 72 hours at 37 ℃, pH 4.8.

Although the CFU number in the first case was greater than that in the other two cases, the ethanol titer and yield in both the second and third cases were higher than that in the first case, suggesting the yeast cells were went through the adaptation treatment in the corn stover hydrolysate or the corn stover solids containing system. In the cases using the corn stover hydrolysate and solids as the carbon source, both the glucose consumption and ethanol production were close, indicating the seeds culture using the pretreated corn stover solids could be effectively applied for the consequent SSF without separate hydrolysis step.

### 2.3.3. Cost estimation on using pretreated corn stover for seeds culture

Preliminary cost estimation of seeds culture in the two cases using glucose and corn stover solids was calculated based on an industrial scale of 100,000 ton/annum ethanol production. Only the cost of seeds culture materials (glucose or corn stover) in the two cases was compared as shown in the Table 2.1. Two operational cases were selected based on the results of this study in Fig. 2.3 and applied on the 100,000 ton/annum fuel ethanol production scale, without considering the changes in the scale-up processes. Case 1 was based on the use of synthetic medium containing 20 g/L of glucose for seeds culture; Case 2 was based on the use of corn stover solids containing 100 g/L of the pretreated corn stover solids (dry basis). The cost estimation process of the two seeds culture cases were summarized in Table 2.1.

In the operational cases of Table 2.1, a 4th-stage seeds culture system was assumed for the 100,000 ton/annum ethanol production before the final production fermentor. The inoculation ratio of the single transfer between two seeds fermentors, or between the last seeds fermentor and the final production fermentor was set to 10%. Thus the overall seeds inoculation ratio was the summary of the four stages 11.11%. The estimation results in Table 1 show that approximately 22% of the seeds culture materials cost was saved by using corn stover solids ($2.09 million) to replace glucose ($2.69 million) in the production scale of 100,000 ton/a cellulosic ethanol plant, besides the cost reduction of equipment and operations by using the pretreated corn stover materials. Although the estimation was preliminary on a conceptual process, the cost reduction data still sufficiently demonstrated the advantages of the seeds culture method using the pretreated corn stover solids as the seeds culture materials.

When corn stover hydrolysate liquid was used for seeds culture instead of corn stover solids, the ethanol production performance was similar as shown in Fig. 2.3. However, the cost for using hydrolysate liquid certainly increased due to the existence of additional processes in corn stover saccharification to obtain the hydrolysate, and the solids/liquid separation to obtain clear hydrolysate liquor. Furthermore, the contamination risk was also significantly increased if the corn stover hydrolysate liquor was used because the solids/liquid separation was hard to be processed in a strictly closed circle. Another possible advantage of corn stover solids usage was that the cellulase enzyme absorbed onto the corn stover residues were not lost by feeding the overall slurry in the next stage seeds fermentor or production fermentor for recycled use on corn stover saccharification.

**Table 2. 1. Cost estimation based on 100,000 ton/annum cellulosic ethanol production scale using corn stover solids as carbon source for seeds culture**

| | Case 1 | Case 2 |
|---|---|---|
| Carbon source dosage in seeds culture media (g/L) [a] | 20.0 (glucose) | 100.0 (CS) |
| Ethanol titer in the production fermentor (g/L) [a] | 47.5 | 50.0 |
| Fuel ethanol production annually (ton/a) | 100,000 | 100,000 |
| Required total ethanol fermentation broth volume ($m^3$) [b] | 2,105,000 | 2,000,000 |
| Required minimum seeds fermentor stages [c] | 4 | 4 |
| Single stage inoculation ratio (%, v/v) [a] | 0.1000 | 0.1000 |
| Overall inoculation ratio (%, v/v) [d] | 0.1111 | 0.1111 |
| Required total seeds culture volume ($m^3$) [e] | 234,000 | 222,200 |
| Required carbon source (ton, glucose or CS) [f] | 4,678 (glucose) | 22,200 (CS) |
| Carbon source cost: feedstock price ($/ton) [g] | 570.6 | 64.4 |
| Carbon source cost: enzyme ($/ton) [h] | 0 | 29.6 |
| Overall carbon source costs on seeds culture ($, Million) [i] | 2.69 | 2.09 |

Conditions: 25% (w/w) solids loading in production fermentor with cellulase 15 FPU/g DM; 6 hours' prehydrolysis at 50 ℃ and pH 4.8, followed by 72 hours' SSF at 37 ℃ and pH 4.8.

[a] This study, Fig. 2.3. The extra ethanol produced in Case 2 using pretreated corn stover materials for seeds culture (50 g/L) than that in Case 1 using glucose for seeds culture (47.5 g/L) was neglected.

[b] Ethanol fermentation broth volume ($m^3$)
= Ethanol production annually (ton/a) / [Ethanol titer in the production fermentor (g/L) × 1000]
= 100,000/(47.5×1000) = 2,105,000 ($m^3$) (Case 1)
or
= 100,000 / (50.0×1000) = 2,000,000 ($m^3$) (Case 2)

[c] Humbird et al., NREL technical report [142]: Page 26-35.

[d] Overall seeds inoculation ratio (%, v/v)
= 0.1 (Stage 4) + 0.01 (Stage 3)+ 0.001 (Stage 2) + 0.0001 (Stage 1) = 0.1111

[e] Total seeds culture volume ($m^3$)
= Total ethanol fermentation broth volume ($m^3$) × Overall seeds inoculation ratio (%, v/v)
= 2,105,000 × 0.1111 = 234,000 ($m^3$) (Case 1)
or
= 2,000,000 × 0.1111 = 222,300 ($m^3$) (Case 2)

[f] Required carbon source (ton)
= Total seeds culture volume ($m^3$)  [Carbon source dosage in seeds culture media (g/L) / 1000]
= 234,000 / (20.0 /1000) = 4678 (ton) glucose (Case 1)
or
= 222,300 / (100.0 / 1000) = 22,220 (ton) CS (Case 2)

[g] Carbon source: feedstock price
Case 1: Glucose = \$0.2579/lb = \$570.6/ton ([142]: Page 63);
Case 2: Corn stover = \$0.741/gal ethanol = \$0.741/gal × 87 gal/ton = \$64.4 /ton ([142]: Page 4)

[h] Carbon source: Enzyme cost
Case 1: Glucose = 0 assumed for glucose;
Case 2: Corn stover = \$0.34/gal ethanol = \$0.34/gal × 87 gal/ton = \$29.6/ton ([142]: Page 4)

[i] Overall carbon source costs on seeds culture
Case 1: Required carbon source (ton) × (Carbon source prices + processing cost) (\$/ton)
= 4678 (tons) × (\$570.6/ton + 0) = \$2.69 million
Case 2: Required carbon source (ton) × (Carbon source prices + processing cost) (\$/ton)
= 22,220 (tons) × (\$64.4/ton+ \$29.6/ton) = \$2.09 million

## 2.4. Conclusion

The *S. cerevisiae* DQ1 strain was sequentially transferred in the culture systems containing 5-10% of freshly pretreated corn stover solids, then inoculated as the seed into the production fermentor. The ethanol titer and yield of the SSF using yeast seeds cultured by synthetic medium (glucose), corn stover hydrolysate liquid, and corn stover solids as carbon sources reached to 47.48 g/L and 71.07%, 52.34 g/L and 80.17%, 49.99 g/L and 76.10%, respectively. Approximately 22% of the cost for yeast cell culture was saved by using the pretreated corn stover solids as carbon source and the contamination risk using hydrolysate liquid was avoided.

# Chapter 3

# High ethanol fermentation performance of dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain

## 3.1. Introduction

High ethanol titer in fermentation broth is crucially important for reducing the energy demand of the consequent distillation step [76]. When cellulosic ethanol fermentation is conducted using lignocellulose feedstock, the simultaneous saccharification and fermentation (SSF) technology is frequently used to lessen the strong inhibition of high sugar concentration on cellulase enzyme catalyzed cellulose hydrolysis. During SSF, high solid feedstock content is certainly required to reach the high ethanol titer. Consequently, two difficulties emerged in such a high solids content fermentation: the poor mixing of the solid feedstock majority with the liquid minority of cellulase enzyme solution and fermenting seeds broth, as well as the accumulation of inhibitory compounds with the increased pretreated solids content.

The first difficulty could be efficiently solved by proper bioreactor design, such as the horizontal rotating reactor [98] or the helical ribbon stirring bioreactor [14]. For the second difficulty, development of an inhibitor tolerant fermenting strain by evolutionary adaptation may provide a partial but practical solution [15, 16, 134]. Evolutionary adaptation allows the microorganism to grow in the inhibitors containing environment with gradually enhanced tolerance to the inhibitors, and finally the random mutations of the relevant genes at the genomic level may occur to generate a stable strain with improved ethanol fermentability. Martin et al. [133] adapted a genetically engineered xylose-utilizing strain *S. cerevisiae* by cultivating the cells for 15 days in the synthetic medium with increasing phenolics, furaldehydes and aliphatic acids concentrations, and the improved ethanol production was observed. Heer and Sauer [134] carried out the adaptation of *S. cerevisiae* TMB3400 for 30 transfers in the minimal medium containing 17 mM furfural, and the lag phase of the cell growth in the hydrolysate was reduced from 90 hours to 16 hours. Landaeta et al. [16] adapted a flocculent *S. cerevisiae* strain for 39 days using synthetic medium with gradual increased inhibitors concentration, and the cell growth and ethanol productivity were increased by 70 % and 10 %, respectively. Gu et al. [15] developed a detailed evolutionary adaptation method for *S. cerevisiae* DQ1 in the corncob residue hydrolysate, and the ethanol titer was increased to a high level of 62.68 g/L.

Adaptation efficiency of a certain fermenting strain highly depends on the specific environment where the strain is to be used. A well-adapted strain with high fermentation performance in one specific hydrolysate may not be suitable for another hydrolysate in terms of different feedstock types, pretreatment methods, conditioning (detoxification), or hydrolysis conditions, because of the variation in the inhibitors type and concentration. In this study, a long term evolutionary adaption of a highly robust yeast strain *S. cerevisiae* DQ1 was conducted in the dry dilute sulfuric acid pretreated (DDAP) corn stover hydrolysate. After 130 transfers (taking 65 days and equivalent to 780 generations of the cell growth), a stable strain was obtained and applied to the simultaneous saccharification and fermentation at high corn stover solids content [13, 14, 68]. Not only the high ethanol titer (71.40 g/L) and the yield (80.34%) were obtained, but also the minimum water usage and wastewater generation were reached by using the DDAP pretreatment and biodetoxification methods. The present study provides a practical approach to obtain the robust strains suitable for lignocellulose biorefinery system.

## 3.2. Materials and Methods

### 3.2.1. Raw materials

Corn stover (CS) was obtained from Dancheng County, Henan Province, China, in fall 2013. Corn stover was water-washed to remove the impurities and air-dried. The dry dilute sulfuric acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan according to the two-step $H_2SO_4$ hydrolysis method [206]. The moisture content of the corn stover after drying was 7.0% (w/w). This moisture content had been taken into account for calculations of solids/liquid ratio and sulfuric acid concentration before the pretreatment operation. The corn stover was milled using a beater pulverizer to pass through the 10-mm apertures in diameter, then sealed in plastic bags and stored at room temperature until used.

### 3.2.2. Strains and enzymes

The ethanol fermenting strain *S. cerevisiae* DQ1 (CGMCC 2528) was used as the parental strain at the starting point of evolutionary adaption [143]. *Amorphotheca resinae* ZN1 (CGMCC 7452) was used as the biodetoxification strain for degrading inhibitors exists in the dry dilute sulfuric acid pretreated corn stover via solid state fermentation [68].

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell #6 was 135 FPU/g (cellulase protein equivalent to 90 mg/g DM) determined using the NREL protocol LAP-006 [203], and the cellobiase activity was 344 CBU/g using the method described by Sharma et al. [204].

### 3.2.3. Pretreatment and biodetoxification operations

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment (DDAP) according to Zhang et al. [13] and He et al. [12]. Briefly, dry corn stover and dilute sulfuric acid solution at 5.0% (w/w) were co-currently fed into the reactor at a solid/liquid ratio of 2:1 (w/w) with helically stirring mixing of 50 rpm, then pretreated at 175 ℃ for 5 min. The solids content of the pretreated slurry was around 50% (w/w) and no wastewater was generated.

The pretreated corn stover slurry was detoxified using *A. resinae* ZN1 according to Zhang et al. [68]. Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ to pH value of 5-6, and then inoculated with *A. resinae* ZN1 at 10% (w/w) ratio as the seeds and incubated at 28 ℃ for several days to degrade the inhibitory compounds. No additional fresh water was used during biodetoxification, and the solids content of the biodetoxified corn stover material was about 50% (w/w).

### 3.2.4. Evolutionary adaptation of *S. cerevisiae* DQ1

The hydrolysate used for evolutionary adaptation was prepared by enzymatic hydrolysis of DDAP pretreated corn stover (without biodetoxification) at 15% (w/w) solids content, 15 FPU/g DM (dry matter) cellulase (equivalent to 10 mg protein per gram of dry matter), 50 $^{o}$C, pH 4.8 and 150 rpm for 48 hours in the helically stirring bioreactor [14]. The hydrolysate was centrifuged at 16,125 g for 10 min to remove the solid residues and the supernatant was used as adaption medium. The hydrolysate contained 60.99 g/L of glucose, 23.23 g/L of xylose, 2.28 g/L of acetic acid, 0.36 g/L of furfural and 0.21 g/L of 5-hydroxymethylfurfural (HMF). Evolutionary adaptation scheme is shown in Fig. 3.1.

The evolutionary adaptation of *S. cerevisiae* DQ1 was conducted by continuously transferring the cultured yeast cells from previous medium into the fresh corn stover hydrolysate. 100 mL conical flasks were used for the continuous cell transfer. In details, 10% (v/v) of the culture solution from last culture was transferred every 12 hours into the fresh hydrolysate and incubated at 37 $^{o}$C in the shaking incubator. At the end of each transfer, the sample was collected and used for glucose and ethanol analysis. This successive transfer process was repeated for 65 days until the fermentation performance maintained stable.

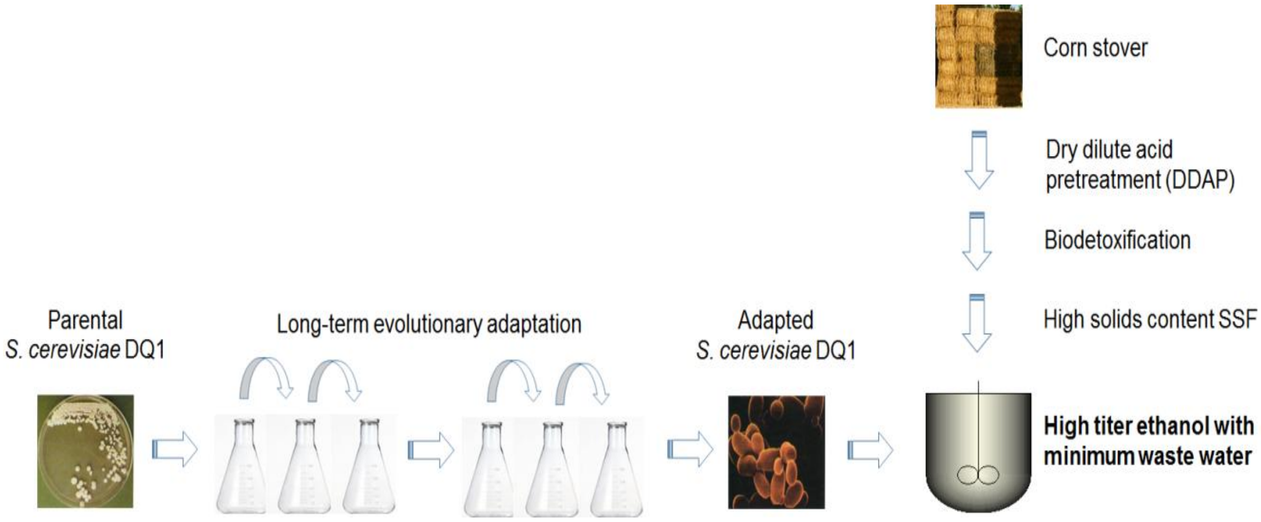


**Fig. 3.1.** Long term evolutionary adaptation of *S. cerevisiae* DQ 1 in corn stover hydrolysate

### 3.2.5. Simultaneous saccharification and ethanol fermentation (SSF)

SSF was performed in the 5-L helical ribbon stirring bioreactor using the DDAP pretreated and biodetoxified corn stover feedstock. The SSF started with 6 hours prehydrolysis at 50 $^{o}$C, then followed by 66 hours SSF at 37 $^{o}$C. Unless otherwise stated, the cellulase dosage was 15 FPU/g DM and the pH was maintained at 4.8 by automatic regulation with 5 M NaOH. The parental *S. cerevisiae* DQ1 strain was used as the control after a simple three-step treatment [18]. The samples were withdrawn at regular intervals, centrifuged at 11,167 g for 5 min and the supernatant was analyzed. The yeast cell viability during SSF was determined by counting the colony forming units (CFU) on the petri dish of the diluted fermenting broth [18].

### 3.2.6. Analysis

The cellulose and hemicellulose contents of the corn stover were analyzed using a two-step $H_2SO_4$ hydrolysis method [206]. The raw corn stover contained 37.2% of glucan and 19.9% of xylan on the dry weight basis. The dry dilute sulfuric acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan. The glucose and xylose concentrations in the liquid portion were 3.05 g/L and 12.56 g/L, respectively.

Glucose, ethanol, acetic acid, and lactic acid contained in the taken samples were analyzed on HPLC (LC-20AD pump, RI detector RID-10A, Shimadzu, Kyoto, Japan) with Bio-Rad Aminex HPX-87H column operated at 65 ℃ and 0.6 mL/min of 5 mM $H_2SO_4$ as the mobile phase. Furfural, furfuryl alcohol and furoic acid were analyzed using 50% acetonitrile solution as the mobile phase at 1.0 ml/min at the column temperature of 35 ℃ and the detection wavelength of 220 nm. HMF, HMF alcohol and HMF acid were analyzed using the following gradient: the initial flow phase was composed by pure water (pump A) and acetonitrile (pump B) at a ratio of 95% to 5%; first, acetonitrile was increased from 5% to 100% over 0 to 15 min then acetonitrile was decreased from 100% to 5% over 15 to 20 min. Finally, acetonitrile was used at 5% over 20 to 30 min. The flow rate was 0.6 mL/min, the column temperature was 35 ℃, and the detector wavelength was 230 nm.

Ethanol yield was calculated using the formulation deduced by [205] specifically for the high solids and high ethanol titer SSF process:

$$\text{Ethanol yield} = \frac{[C_1]\times W}{976.9 - 0.804\times[C_1]}\cdot\frac{1}{0.511\times f\times[Biomass]\times m\times 1.111}\times 100\%$$

where [$C$1] was the ethanol concentration in the culture broth (g/L), $W$ was the total water input of SSF (g), $f$ was the cellulose fraction of corn stover feedstock, [*Biomass*] was the dry corn stover concentration at the beginning of SSF (g/g), $m$ was the total weight of SSF (g), 0.511 was the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, and 1.111 is the conversion factor for cellulose equivalent to glucose.

## 3.3. Results and Discussion

### 3.3.1. Evolutionary adaptation of *S. cerevisiae* to enhance its fermentability

The thermo- and inhibitor tolerant strain *S. cerevisiae* DQ1 was selected as the starting strain for the long-term evolutionary adaptation using the freshly prepared corn stover hydrolysate as the culture medium. The yeast cells were transferred into the fresh corn stover hydrolysate every 12 hours successively until the glucose utilization rate and ethanol production rate were stable as shown in Fig. 3.2. The adaptation of *S. cerevisiae* DQ1 was conducted for 1,560 hours with 130 cell transfers (equivalent to 780 generations). The glucose consumption and ethanol production fluctuated in the first 50 transfers (approximately 300 generations) and then maintained stable with only random shifts. The ethanol yield from the glucose in the corn stover hydrolysate in Fig. 3.2 was relatively low (~80%), while the average ethanol yield is above 90%. The reasons for the low yield include: (1) short fermentation time in the adaptation cell transfer (every 12 hours), and (2) the existence of inhibitors in the corn stover hydrolysate used evolutionary adaptation (2.28 g/L of acetic acid, 0.36 g/L of furfural, and 0.21 g/L of HMF).

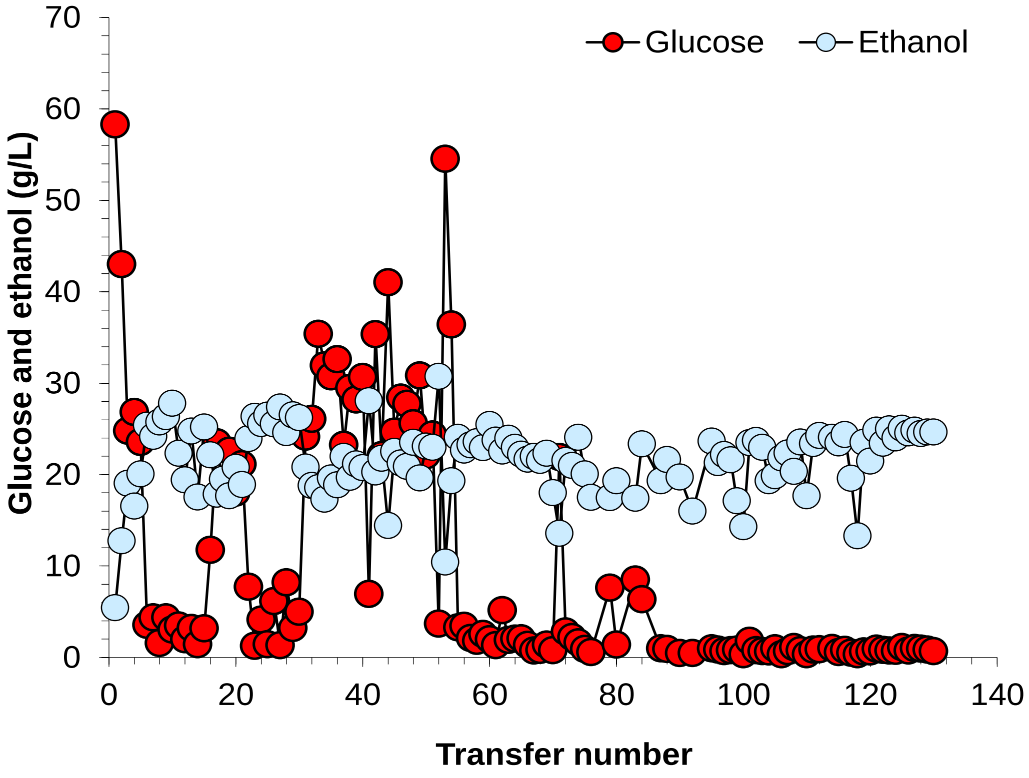
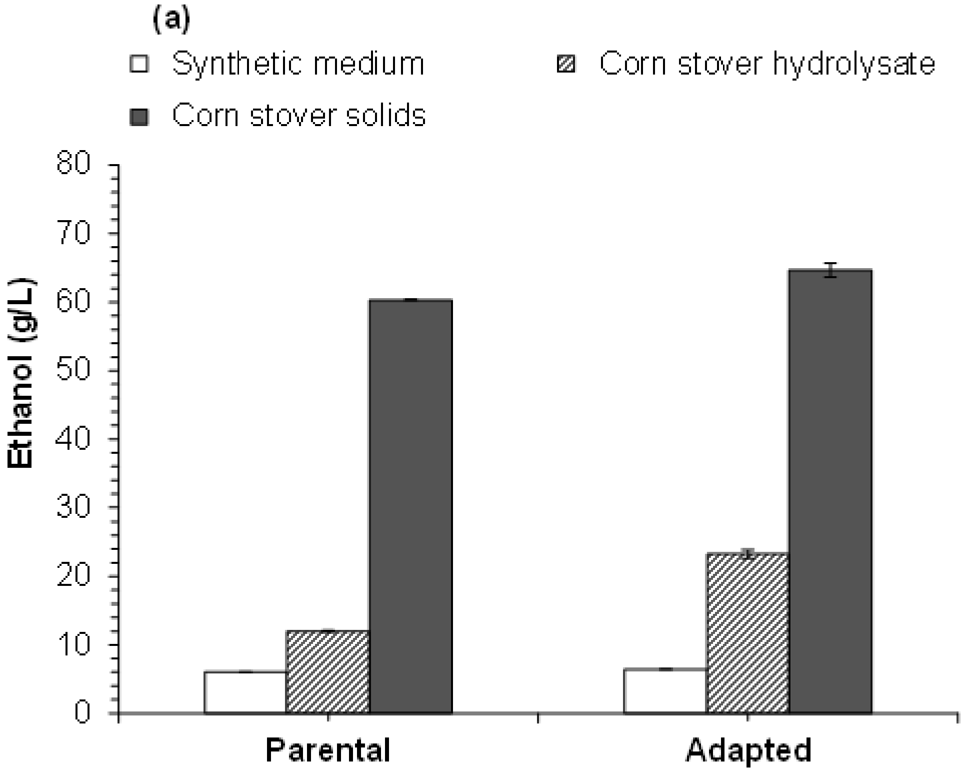


**Fig. 3.2.** Time course profile of the evolutionary adaptation of *S. cerevisiae* DQ1. The evolutionary adaptation process was conducted at 37 °C in flasks containing the corn stover hydrolysate. It was transferred every 12 hours with 10% (v/v) culture liquid and lased for 1560 hours.

Ethanol fermentation performance of the adapted *S. cerevisiae* DQ1 was compared with the parental strain in the pure glucose solution (synthetic medium), the liquid corn stover hydrolysate, and the SSF, respectively. Fig. 3.3 (a) shows that the ethanol titer of the adapted strain was almost same to that of the parental strain in the synthetic medium, but increased to 23.25 g/L from 12.2 g/L of the parental strain in the corn stover hydrolysate. The ethanol titer of the adapted strain was also slightly increased to 64.68 g/L from 60.33 g/L of the parental strain in the SSF, approximately 7.2% increase comparing to the parental strain. The reason for the limited improvement between the adapted and parental strains is that the inhibitors in the pretreated corn stover were almost completely removed by biodetoxification treatment. The adapted strain could play more significant roles in the case of inhibitor containing hydrolysate systems, which commonly exist in various lignocellulose biorefinery processes. The cell viability of the adapted in the corn stover hydrolysate and in SSF system was elevated by almost 50% comparing to the parental strain as shown in Fig. 3.3 (b). This performance could also enhance the tolerance of fermenting strains in the inhibitors containing lignocellulose systems for improved ethanol fermentability.



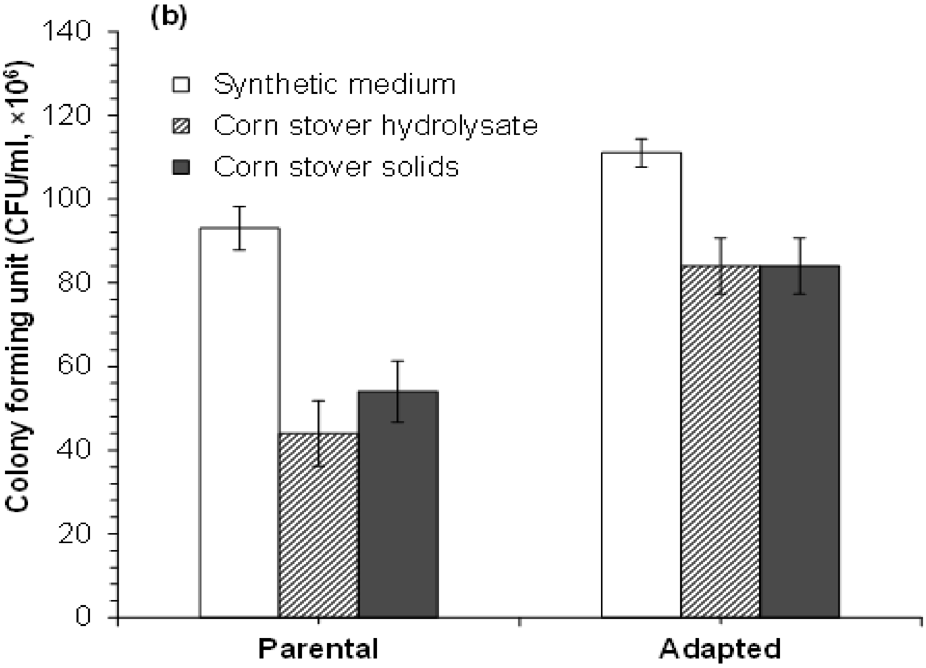


**Fig. 3. 3**. Fermentation performance evaluation of the adapted *S. cerevisiae* DQ1. (a) Comparison of ethanol fermentation performance; (b) Comparison of cell viability during fermentation. Fermentation conditions with synthetic medium and corn stover hydrolysate: 37 ℃, 150 rpm for 12 hours in flasks. Conditions for SSF were the same as those in Materials Section. (The SSF conditions were; 6 hours prehydrolysis at 50 °C, then followed by 66 hours SSF at 37 °C, cellulase dosage was 15 FPU/g DM and the pH was maintained at 4.8 by automatic regulation with 5 M NaOH). The experiments were performed in duplication and data presented in figures are average of two parallel experiments. Error bars are shown for standard deviation.

### 3.3.2. Optimal SSF performance of the evolutionary adapted *S. cerevisiae* DQ1

Ethanol fermentation using the adapted *S. cerevisiae* DQ1 strain at the high solids content SSF was optimized with changing process parameters, including the biodetoxification time, solids content, cellulase dosage, and nutrient addition as shown in Table 1.

Table 3.1 indicates that the inhibitor concentration in the pretreated feedstock including furfural, HMF and acetic acid decreased with the prolonged biodetoxification time, leading to the increased ethanol titer, yield, and productivity. Higher solids content enhanced the ethanol titer and productivity but the ethanol yield was decreased. Cellulase dosage enhanced the ethanol titer, yield and productivity at the price of increased enzyme cost. In order to obtain optimal ethanol fermentation performance, the adapted *S. cerevisiae* DQ1 was applied to the SSF of the dry dilute acid pretreated corn stover under various fermentation parameters in terms of biodetoxification level (0, 3, 5, 7 and 14 days), solids corn stover content (20, 25, 30 and 35%), cellulase dosage (7, 15 and 30 FPU/g DM), the nutrients addition.

Table 3.2 and Fig 3.4 (a) shows that the impact of the biodetoxification level on the ethanol concentration and yield. Poor fermentation performance was observed, when freshly pretreated corn stover without any treatment was utilized in SSF perhaps due to high concentration of toxic compounds in pretreated feedstock. Adaption improved inhibitor tolerance but, inhibitors removal (biodetoxification) could not be skipped to reach high ethanol concentration and yield. Therefore, removal of pretreatment inhibitors was crucially important for obtaining higher ethanol titer and yields under high solids content SSF. Table 3.1 shows the effect of biodetoxification levels on the inhibitors degradation, during the biodetoxification, furfural and HMF were degraded to their relative alcohols and acids (less toxic compounds) as described in Ran et al. [91]. In the first step, furfural and HMF were converted into their relative alcohols to release cells from severe stress then further degradation to acids took place. When furfural concentration reached zero after 5 days, a significant increase in glucose utilization and ethanol production was observed Almeida et al. [122], biodetoxification of pretreated corn stover for 7 days were chosen for the following researches. Ethanol concentration and yield increased from 45.05 g/L to 58.84 g/L and 66.44% to 82.39% when biodetoxification level increased from 3 to14 days.

The influence of solids loading and enzyme dosage on ethanol production were also conducted. Table 3.2 and Fig 3.4 (b) shows that ethanol titer increased from 46.69 to 68.21 g/L with increasing solids loading from 20% to 35%, while the ethanol yield decreased from 88.60% to 60.36%. In agreement with our previous study, feeding time also increased from 2 h to 12 h with increasing solids loading from 20% to 35% Zhang et al. [14]. Effect of cellulase dosage was tested at 7 FPU, 15 FPU and 30 FPU/g DM under solids loading of 30% (w/w). Both final ethanol titer and ethanol yield were increased with the cellulase dosage, while the enzyme cost increased accordingly as shown in Table 3.2 and Fig 3.4 (c).

**Table 3.1. Effect of biodetoxification time on inhibitors concentration in the pretreated corn stover**

| Biodetoxification time (Day) | Furfural (mg/g DM) | HMF (mg/g DM) | Acetic acid (mg/g DM) |
|---|---|---|---|
| 0 | 7.09 | 3.33 | 14.98 |
| 3 | 2.05 | 2.04 | 13.78 |
| 5 | ND | 0.88 | 1.48 |
| 7 | ND | 0.21 | ND |
| 14 | ND | ND | ND |

ND= not detected. Biodetoxification conditions: 50% (w/w) solids loading, 10% (w/w) inoculation ratio of *Amorphotheca resinae* ZN1 as the seeds, pH 5-6, 28 ℃ for 0, 3, 5, 7, 14 days, respectively.

**Table 3.2. Optimization of the SSF of the pretreated corn stover using the adapted *S. cerevisiae* DQ1**

| Parameters | Values | Ethanol titer (g/L) | Ethanol yield (%) | Ethanol productivity (g/L/h) |
|---|---|---|---|---|
| Biodetoxification time (days) | 0 | 2.54 | 3.75 | 0.03 |
| | 3 | 45.36 | 66.44 | 0.63 |
| | 5 | 50.86 | 71.65 | 0.70 |
| | 7 | 55.68 | 79.43 | 0.77 |
| | 14 | 58.84 | 82.39 | 0.82 |
| Solids content (%, w/w) | 20 | 46.33 | 88.60 | 0.65 |
| | 25 | 55.68 | 79.46 | 0.77 |
| | 30 | 64.68 | 72.35 | 0.90 |
| | 35 | 68.21 | 60.36 | 0.95 |
| Cellulase dosage (FPU/g DM) | 7 | 54.71 | 60.90 | 0.76 |
| | 15 | 64.68 | 72.35 | 0.90 |
| | 30 | 71.52 | 79.42 | 0.99 |
| Nutrients addition | 0 | 56.05 | 61.79 | 0.78 |
| | 1 g/L YE | 64.68 | 72.35 | 0.90 |
| | 1 g/L DDGS | 70.52 | 79.25 | 0.98 |
| | 5 g/L DDGS | 71.40 | 80.34 | 0.99 |

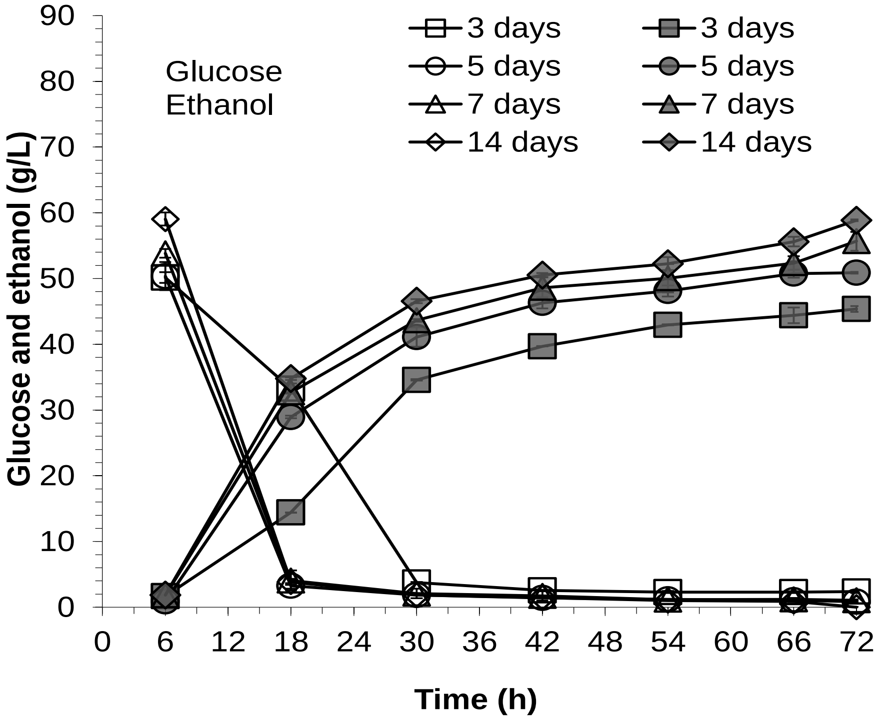
Conditions for “biodetoxification time”: Prehydrolysis at 25% solids content, 15 FPU/g DM cellulase dosage equivalent to 10 mg protein/g DM, 50 ℃, pH 4.8 for 6 hours; then SSF with 10% (v/v) yeast inoculation at 37 ℃ for 66 hours.

Conditions for “solids content”: Biodetoxified for 7 days; Prehydrolysis at 20, 25, 30, and 35 % solids content, respectively, 15 FPU/g DM cellulase, 50 ℃, pH 4.8 for 6 hours, 6 hours, 7 hours, and 12 hours, respectively; then SSF with 10% (v/v) yeast inoculation at 37 ℃ for 66 hours, 66 hours, 65 hours, and 60 hours, respectively.

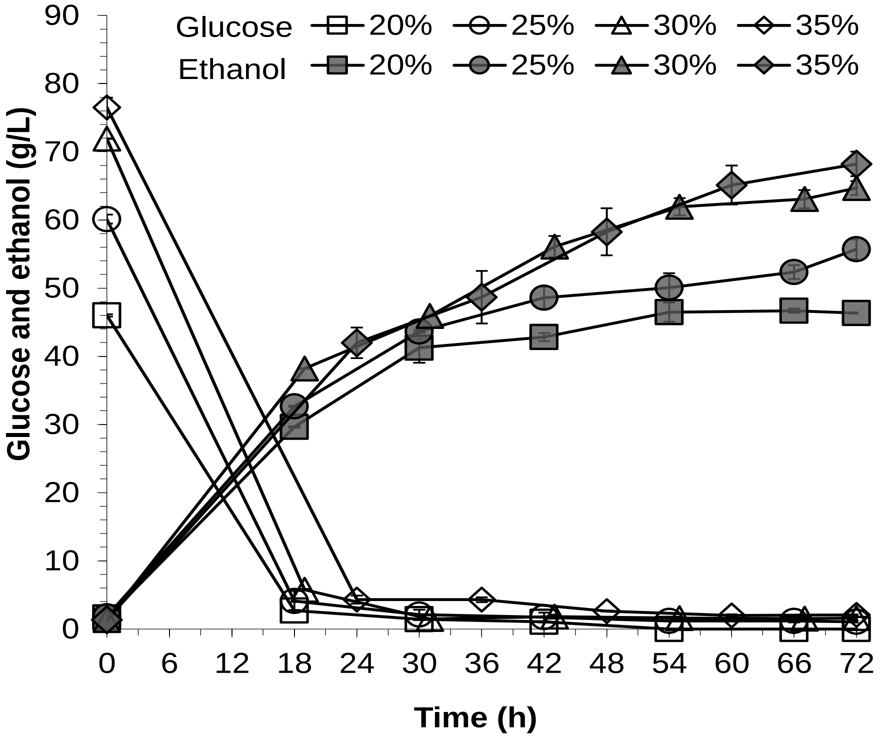
Conditions for “cellulase dosage”: Biodetoxified for 7 days; Prehydrolysis at 30% solids content, 7 FPU/g DM, 15 FPU/g DM, and 30 FPU/g DM cellulase, 50 ℃, pH 4.8 for 12 hours, 7 hours, and 6 hours, respectively; then SSF with 10% (v/v) yeast inoculation at 37 ℃ for 60 hours, 65 hours and 66 hours, respectively.

Conditions for “nutrients”: Biodetoxified for 7 days; Prehydrolysis at 30% solids content, 15 FPU/g DM cellulase, 50 ℃, pH 4.8 for 7 hours; then SSF with 10% (v/v) yeast inoculation at 37 ℃ for 65 hours. The “0” indicates no additional nutrients added into the SSF; “1 g/L YE” indicates 1.0 g/L of yeast extract, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4 .7H_2O$. Other SSF was the same to “1 g/L YE” unless stated elsewhere. The nutrients in the “1 g/L DDGS” indicate 1.0 g/L of DDGS, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4 .7H_2O$; “5 g/L DDGS” indicate 5.0 g/L of DDGS, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4 .7H_2O$.

(a) Detoxification time



(b) Solids loading



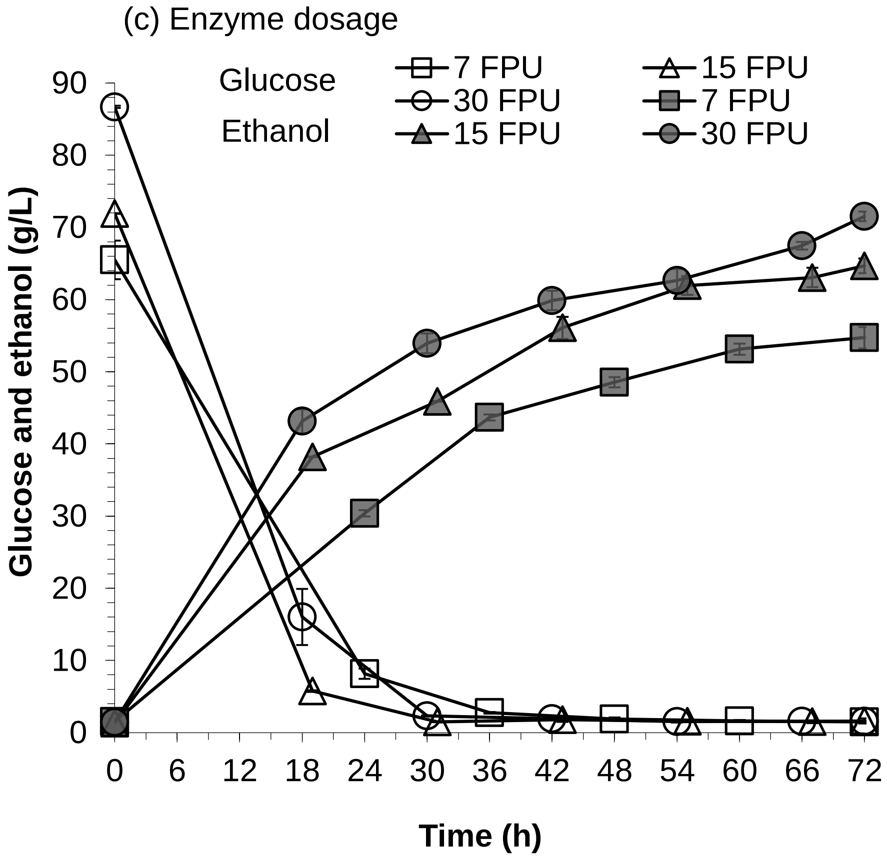
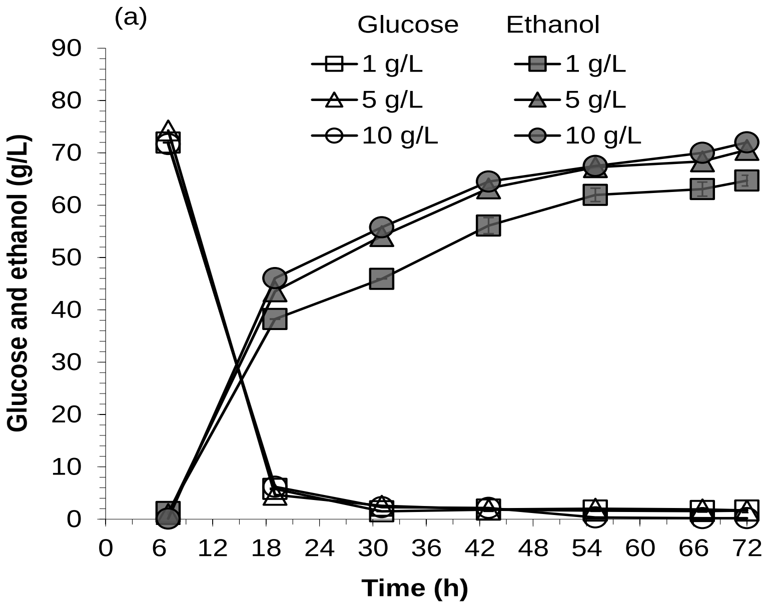


**Fig. 3.4.** Optimization of fermentation conditions for high ethanol titer and yield from adapted *Saccharomyces cerevisiae* DQ1. SSF was performed in 5 L helical stirring bioreactor. (a) Biodetoxification level (3, 5, 7 and 14 days). Conditions: Prehydrolysis stage: 25% (w/w, dry base) solids loading, cellulase 15 FPU/g DM (equivalent to 10 mg protein/g DM), 50 ℃, 150 rpm, pH 4.8 for 6 h. SSF stage: strain was inoculated after prehydrolysis stage at 37 ℃. (b) Effect of solids loading on ethanol production and yield. Prehydrolysis stage: 20%, 25%, 30%, and 35% (w/w dry base) solids loading, cellulase 15 FPU/g DM (equivalent to 10 mg protein/g DM). Prehydrolysis lasted for 6 h when 20% and 25%, 7 h when 30% and 12 h when 35% solids loading, 50 ℃, 150 rpm, 4.8 pH. SSF stage: strain was inoculated after prehydrolysis stage at 37 ℃. (c) Effect of cellulase dosage on ethanol production and yield. Prehydrolysis stage: 30% (w/w) solids loading, cellulase dosage 7, 15 and 30 FPU/g DM (equivalent to 4.7, 10, 20 mg protein/g DM). Prehydrolysis was performed for 6 h, 7 h and 12 h for 7FPU/g DM, 15FPU/g DM and 30FPU/g DM, 50 ℃, 150 rpm, 4.8 pH. SSF stage: strain was inoculated after prehydrolysis stage at 37 ℃. The experiments were performed in duplication and data presented in figure is average of two parallel experiments. Error bars are shown for standard deviation.

Fig. 3.5 shows that ethanol concentration and yield increased from 64.68 g/L to 71.99 g/L and 72.35 % to 81.05 % with increasing yeast extract concentration from 1 g/L to 10 g/L. Glucose consumption and ethanol generation rate were lower when YE or both basal medium and YE were deleted from SSF medium comparing with the full nutrients supplementation. This occurred perhaps due to lower yeast viability (shown in Fig. 3.7) in absence of sufficient nutrients.


(a)

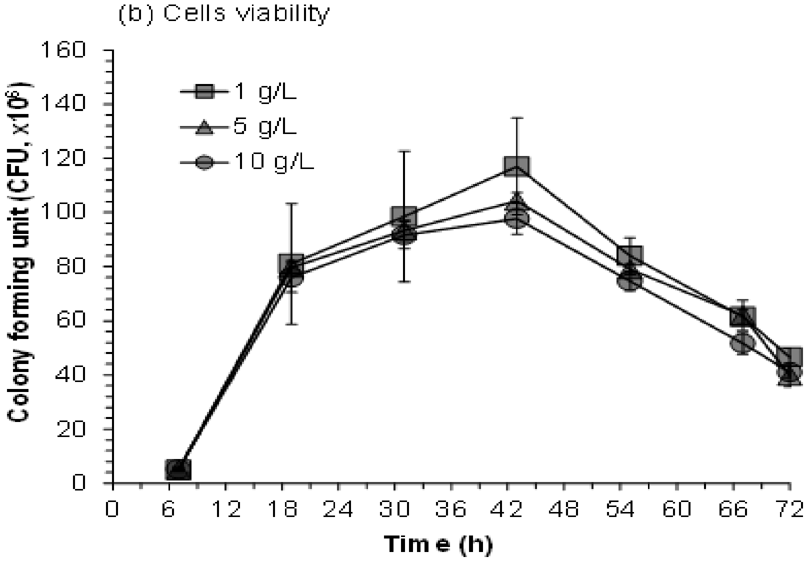
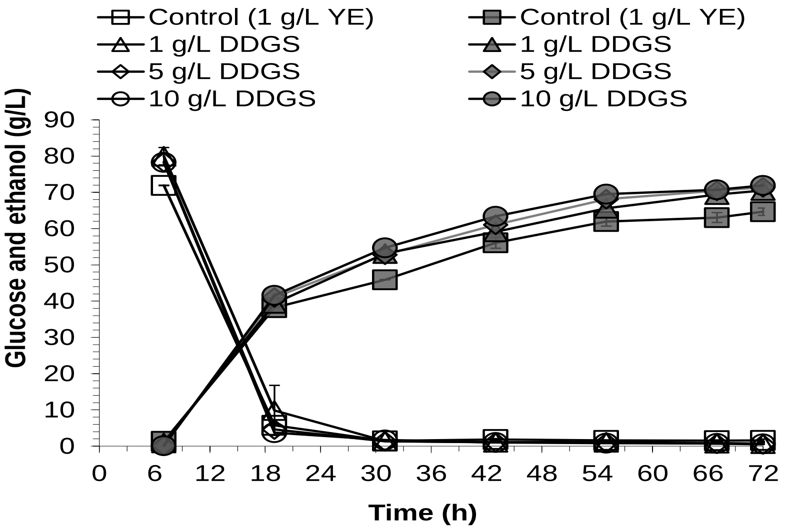

(b) Cells viability

**Fig. 3.5.** Effect of nutrients yeast extract (YE) on ethanol titer and yield under high solids loading and deletion of nutrients from SSF medium. SSF was performed in 5 L helical stirring bioreactor. (a) Fermentation profile of glucose utilization and ethanol production. (b) Yeast viability (CFU/ml). Conditions: Prehydrolysis stage: 30% (w/w) solids loading, cellulase dosage 15 FPU/g DM (equivalent to 10 mg protein/g DM), YE 1, 5 and 10 g/L 50 ℃, 150 rpm, pH 4.8 for 7 h. SSF stage: strain was inoculated

after prehydrolysis stage at 37 ℃. The experiments were performed in duplication and data presented in figure is average of two parallel experiments. Error bars are shown for standard deviation.

An important cost reduction measure was tested by replacing yeast extract (YE) with a cheap nutrient alternative usually used for animal feed, distillers' dried grains with solubles (DDGS). A better performance of DDGS usage than YE was observed, although its price was only 0.5% of the yeast extract additive [145]. The time course of the SSF operation with DDGS addition was illustrated in Fig. 3.6 under the optimal biodetoxification time, solids content, and cellulase enzyme dosage. The glucose was consumed out within 12 hours after yeast inoculation and ethanol steadily increased to 71.40 g/L within 72 hours. The viable cells increased in the first 36 hours, and then gradually decreased, probably because of the osmotic pressure of the high ethanol formation.

(a) Glucose and ethanol time profile



(b) Yeast viability

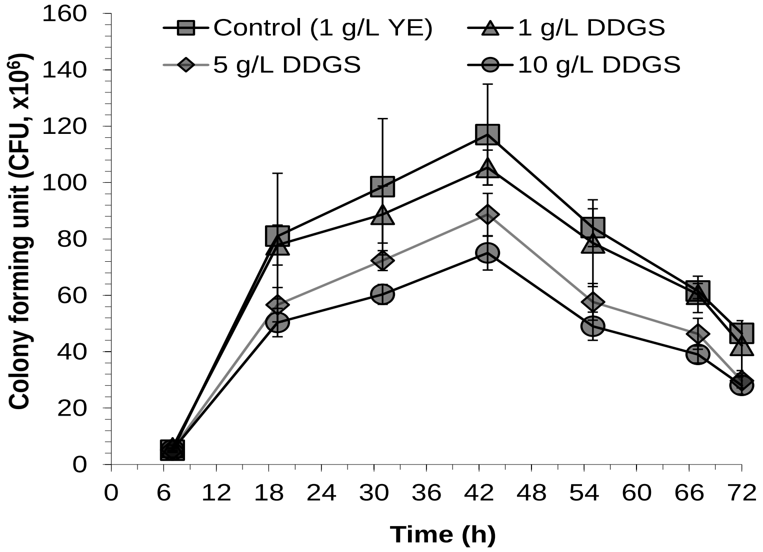
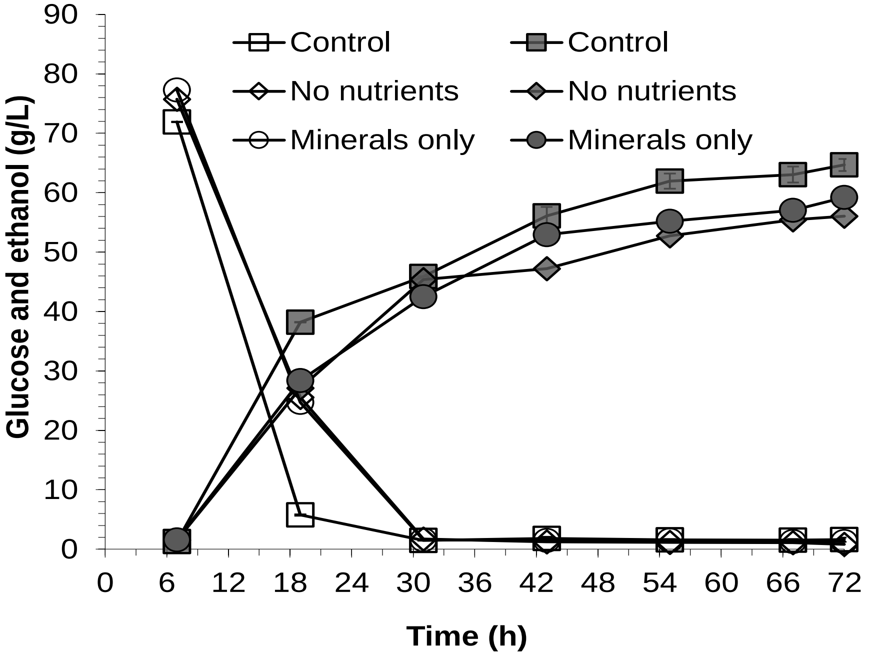


**Fig. 3.6.** Effect of dry distiller's grain soluble (DDGS) on ethanol titer and yield under high solids loading. SSF was performed in 5 L helical stirring bioreactor. (a) Fermentation profile of glucose utilization and ethanol production (b) Yeast viability (CFU/ml).Conditions: Prehydrolysis stage: 30% (w/w) solids loading, cellulase dosage 15 FPU/g DM (equivalent to 10 mg protein/g DM), DDGS 1, 5 and 10 g/L, 50 ℃, 150 rpm, pH 4.8 for 7 h. SSF stage: strain was inoculated after prehydrolysis stage at 37 ℃.

Fig 3.7 shows the ethanol titer, glucose consumption, and cell viability (CFU/mL). Ethanol titer and glucose consumption increased with passage of time. Ethanol titer remained lower when no nutrients and no nitrogen source were added comparing to fully nutrient SSF. Nitrogen source was more effective than minerals. Although, cells viabilities were enough to obtain ethanol titer upto 56 and 59 g/L for no nutrient and minerals only, respectively.

(a) Glucose and ethanol time profile



(b) Cells viability

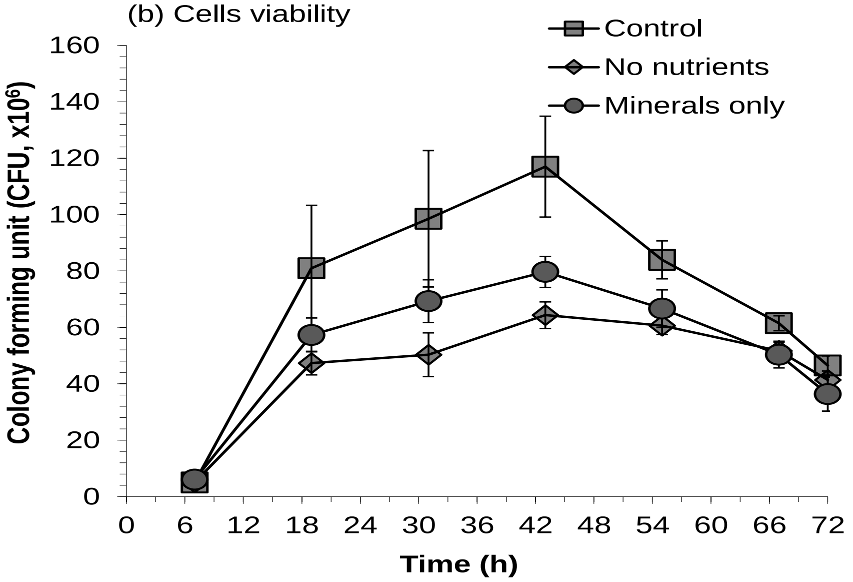


**Fig. 3.7.** Deletion of nutrients from SSF medium. (a) Comparison of glucose utilization and ethanol concentration in absence of YE and absence of all nutrients. SSF was performed in 5 L helical stirring bioreactor. (b) Cells viability (CFU/ml).Conditions: Prehydrolysis stage 30% (w/w) solids loading, cellulase dosage 15 FPU/g DM (equivalent to 10 mg protein/g DM, without YE, without external nutrients (YE and mineral salts), 50 ℃, 150 rpm, pH 4.8 for 7 h. SSF stage: strain was inoculated after prehydrolysis stage at 37 ℃.

### 3.3.3. Evaluation of cellulosic ethanol potentials

The optimal ethanol fermentation by the adapted *S. cerevisiae* DQ1 strain using the dry dilute sulfuric acid pretreated (DDAP) corn stover as the feedstock was 71.40 g/L in the ethanol titer, 80.34% in the yield, and 0.99 g/L/h in the productivity (within the overall 72 hours). More importantly, no any wastewater was generated starting from the pretreatment step to the fermentation step. This result was compared to the current high titer ethanol fermentation cases published in the academic journals as summarized in Table 3.3.

These cases were divided into three categories: (1) all the solids and pretreatment liquid were utilized for ethanol fermentation (the whole pretreated slurry), thus no wastewater was generated from pretreatment to fermentation; (2) the solids and the partial pretreatment liquid were utilized for fermentation, and the majority of the pretreatment liquid was released as waste water; and (3) only the solids was utilized for fermentation, and a massive amount of wastewater was generated. It should be noted that the fermentation cases were not operated at exactly same conditions such as the nutrient addition, therefore the comparison results could be considered as the reference only.

In the first category with the whole pretreated slurry used for SSF, Hoyer et al. [207] using the steam explosion pretreated spruce as feedstock, and obtained 47.8 g/L in ethanol titer and 72% in ethanol yield at 25% solids content (13.7% water insoluble solids content). This is obviously low comparing to the present result of 71.40 g/L in ethanol titer and 80.34% in ethanol yield. Furthermore, the fresh water usage in the pretreatment step (3.1 kg/kg dry matter) was more than three folds greater than that of the present case (1 kg/kg dry matter).

In the second category with the utilization of all the solids and the partial pretreatment liquid, Varga et al. [33] pretreated the corn stover with alkaline wet-oxidation pretreatment, and the cellulose rich solids were obtained after solid/liquid separation and thoroughly washed with fresh water. The washed solids fraction was used to SSF and partial pretreatment liquid was re-used to adjust the solids content. 52.3 g/L of the ethanol titer with the yield of 78.0% were obtained with 16.7 kg fresh water usage and nearly 24.7 kg wastewater generation for 1 kg of corn stover used. Lan et al. [42] pretreated the Lodgepole wood chips with SPORL at 25% solids content, and 2.3 folds of fresh water to the original dry material (in weight) was used in the disk-milling process, then the solids and partial liquid were used for SSF, leading to 47.4 g/L of ethanol and nearly 5.3 kg wastewater generation per kg of dry feedstock. Obviously, the ethanol fermentation performance in the two cases of the second category was far below that of the present case (71.4 g/L of ethanol and zero waste water generation).

For the third category with the utilization of the solids portion only, the majority of the published high ethanol fermentation studies fall into this category, not only for the solid feedstock contains high percentage of cellulose, but also the inhibitors are already removed by thoroughly washing. The routine procedure includes the following steps: different pretreatment methods with a high liquid/solid ratio and followed by a solid/liquid separation to obtain the solid fraction; then the solid fraction was thoroughly water-washed to remove the inhibitory compounds; again the solids fraction was obtained after a solid/liquid separation step and only the solid part was used for ethanol fermentation. In this procedure, the feedstock sometimes was almost “pure cellulose” while the hemicellulose and lignin fractions were completely removed. Several studies demonstrated high ethanol fermentation performance: 80.0, 75.0, 69.3, and 58.7 g/L of ethanol with the yield of 82.7, 89.4, 74.7, and 73.4 % were obtained, but the high wastewater of 36, 28, 176, and 33 kg were also generated per kg dry feedstock solid utilized [27, 28, 46, 53].

**Table 3.3. Comparison of high ethanol titer production under high solids content**

| Feedstock | Pretreatment methods | Solids content (%, w/w) | Ethanol titer (g/L) | Ethanol yield (%) | Ethanol productivity (g/L/h) | Wastewater generation [a] (kg/kg) | Sources |
|---|---|---|---|---|---|---|---|
| **Whole package of the pretreated materials (solids and liquid) were used in the ethanol fermentation [b]** | | | | | | | |
| Corn stover | Dilute acid with biodetoxification | 30 | 71.4 | 80.3 | 0.99 | 1.0 + 0 | This study |
| Spruce | Steam explosion | 25 | 47.8 | 72.0 | 0.34 | 3.1 + 0 | [207] |
| **Solids and partial pretreatment liquid were used in the ethanol fermentation [c]** | | | | | | | |
| Corn stover | Wet oxidation with washing | 17 | 52.3 | 78.0 | 0.43 | 16.7 + 8.0 [c] | [33] |
| Lodgepole wood | SPORL with wet-disk milling | 20 | 47.4 | 77.9 | 0.33 | 3.0 + 2.3 | [42] |
| **Solids only (after solids/liquid separation) were used in the ethanol fermentation** | | | | | | | |
| Sugarcane bagasse | Formiline with washing | 20 | 80.0 | 82.7 | 0.55 | 28.0[d] + 8.0 [e] | [53] |
| Corn cob residue | Dilute-alkaline pretreatment with washing | 20 | 75.0 | 89.4 | 1.04 | 20.0 + 8.0 [c] | [28] |
| Reed | Phosphoric acid-acetone with washing | 36 | 69.3 | 74.7 | 0.57 | 104.0 + 72.0 | [27] |
| Rice straw | Dilute acid and dilute alkaline with washing | 16 | 58.7 | 73.4 | 0.49 | 25.0 + 8.0 [e] | [46] |
| Palm empty fruit bunches | Alkali with washing | 30 | 62.5 | 70.6 | 0.66 | 4.0 + 8.0 [e] | [40] |
| Aspen wood logs | SPORL with wet disk milling | 18 | 60.0 | 77.0 | 0.50 | 3.0 + 40.0 | [25] |
| Corn stover | Steam explosion with washing | 20 | 59.8 | 77.2 | 0.31 | 2.3 + 15.0 | [29] |
| Wheat straw | Steam explosion with washing | 25 | 58.6 | 56.9 | 0.73 | 3.0 + 8.0 [e] | [58] |
| Wheat | Dilute acid with | 20 | 57.0 | 70.0 | 0.40 | 9.0 + 8.0 [e] | [208] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| straw | washing | | | | | | |
| Eastern Redcedar | Acid bilsulfite with washing | 20 | 52.0 | 75.6 | 0.27 | 5.0 + 5.0 | [36] |
| Forage sorghum | Steam explosion with washing | 16 | 50.0 | 85.0 | 0.52 | 1.0 [f] + 8.0 [e] | [37] |
| Corn stover | Steam explosion with washing | 30 | 49.5 | 68.2 | 0.34 | 1.0 + 8.0 | [30] |
| Sweet sorghum bagasse | Hydrothermal | 18 | 47.9 | 70.4 | 0.37 | 10.0 + 0 | [209] |
| Spruce | $SO_2$ with steam explosion [g] | 20 | 40.0 | 53.0 | 0.42 | 4.0 + 0 | [102] |
| Rapeseed straw | Dilute acid with washing | 20 | 39.9 | 57.9 | 0.42 | 16.7 + 8.0 [e] | [210] |

[a] Waste water generation is the sum of two portions: (1) the acid or alkali or solvent solutions generated from the liquid addition or steam condensation during the pretreatment, and (2) the waste water solution generated from the washing of the pretreated lignocellulose biomass.

[b] Although the whole pretreated materials (solids and liquid) were used in the ethanol fermentation, fresh water was added into the fermentation system to adjust the solids content.

[c] Here the partial pretreatment liquid refers to the liquid generated during pretreatment, not including the wastewater generated from water-washing step.

[d] The liquid volume of the $Ca(OH)_2$ used for deformylation was unknown, so the total liquid used during pretreatment might be more than 28 kg/kg dry solid material.

[e] Because the water used for washing the solid fraction was not given. The authors just mentioned “washed to neutral pH” or “thoroughly washed”, according to Lu et al. [30]], the water used for washing was at least 8.0 kg/kg dry solid material.

[f] The detailed information on steam explosion pretreatment was not mentioned in the study, so the liquid or water used was assumed to be 1 kg/kg dry solid material according to Lu et al. [30].

[g] The pretreated slurry was solid/liquid separation, and the pretreatment liquid was used for yeast cultivation and the solids fraction was used to ethanol fermentation. Additional fresh water was added to adjust the solids content during hydrolysate preparation.

Other studies in the third category did not completely remove hemicellulose and lignin from the feedstock, but still only the water washed solid fraction was used for ethanol fermentation, left the waste water behind. Park et al. [40] reached the ethanol titer and yield of 62.5 g/L and 70.6%, respectively, with 12 kg/kg wastewater generation; Zhu et al. [25] produced 60 g/L ethanol with 43 kg wastewater per kg of feedstock; Liu et al. [29] consumed 17.3 kg/kg water to produce 59.8 g/L ethanol; Manzanares et al. [37] produced 50.0 g/L ethanol while 9 kg/kg wastewater was generated; Lu et al. [30] obtained 49.5 g/L ethanol with 9 kg/kg wastewater generation; Matsakas and Christakopoulos [209] obtained 47.9 g/L ethanol with 10 kg/kg wastewater generation; Ramachandriya et al. [36] produced 52.0 g/L ethanol at 20% solids content, and 5 kg/kg liquid at both pretreatment and washing steps was used; Alvira et al. [77] produced 58.6 g/L ethanol with 11 kg/kg water; Koppram and Olsson [102] produced 40 g/L ethanol at 53% ethanol yield at the cost of 4 kg/kg wastewater generation; Lopez-linares et al. [210] obtained the ethanol titer of 39.9 g/L and the yield of 57.9% with 16.7 kg/kg wastewater generation from pretreatment and 8 kg/kg from washing.

It is crucially important to reduce the wastewater generation in the large scale plants of cellulosic ethanol processes, because the wastewater treatment system in the farmland is not always available especially in the developing countries. In this study, the high ethanol fermentation performance by the adapted *S. cerevisiae* DQ1 strain was demonstrated.

Meanwhile, the significantly less fresh water usage and wastewater generation were achieved due to the applications of new technologies such as the dry dilute sulphuric acid pretreatment, biodetoxification, and high solids content SSF in helical ribbon stirring reactors. The present study provides a practical approach for improving ethanol fermentability at high solids content and we suggest that all the microorganisms used in lignocellulose biorefinery should be experienced an evolutionary adaptation process in order to enhance their fermentation performance.

## 3.4. Conclusion

A long term evolutionary adapted yeast strain was obtained after 65 days' adaptation (equivalent to 780 generations) by successively transferring in the corn stover hydrolysate containing high inhibitor substances. The adapted yeast strain demonstrated significantly improved ethanol fermentation performance during SSF at high solids content. The ethanol titer and yield of 71.40 g/L and 80.34% were obtained, respectively, at an optimum SSF condition without any wastewater generation from pretreatment to fermentation. The present study provides a practical approach for improving fermentability of microorganisms used in lignocellulose biorefinery.

# Chapter 4

# Open fermentation of high titer L-lactic acid from corn stover by a pediocin producing *Pediococcus acidilactici* strain

## 4.1. Introduction

Lactic acid is an important commodity widely used in food, pharmaceutical, textile, cosmetic, and chemical industries [160]. Recently, much attention has been focused towards optically pure L-lactic acid production due to its application as starting material to produce polylactic acid (PLA). PLA, is biodegradable and environment-friendly plastic, and resulting dependency from petrochemicals will be shifted away to renewable resources [86, 87, 161, 211-215]. Lactic acid could either be produced from chemical synthesis or microbial fermentation. Fermentative process is advantageous over chemical synthesis due to certain reasons, (1) optically pure L- or D-lactic acid instead of racemic mixture (chemical synthesis), (2) utilization of lignocellulosic material, and (3) environment-friendly. Currently two technical challenges are faced in the microbial lactic acid production, the first one is the high pure sugars cost, and the other one is the operating cost, such as sterilization and downstream separation and purification [213]. First difficulty is solved by using lignocellulosic biomass as cost effective substrates for lactic acid fermentation [86, 87, 171] and second issue could be solved by open lactic acid fermentation without sterilization.

Generally, a step of autoclaving the medium and bioreactor before fermentation is unavoidable to minimize the possible contamination chances, and this would enhance the lignocellulosic processing cost, it is energy intensive and labor consuming step. In addition, the nutrients are also damaged due to heat treatment during sterilization. Open lactic acid fermentation, without sterilization could be promising strategy to solve this problem [163-166]. However, previous studies have conducted open lactic acid fermentation by using thermophilic strains, including *Bacillus coagulans*, *Bacillus* sp. 2–6, and *Bacillus licheniformis* to conduct lactic acid fermentation without sterilization [161, 163, 171, 173, 216]. Several difficulties were faced by previous open lactic acid fermentation studies, including lower optical purity of accumulated lactic acid or mixed lactic acid production D and L-lactic, lower lactic acid titer, and sterilized conditions were used for fermentation at moderate temperature 37 ℃, and 45 ℃ in Ma et al.[162]. Therefore, still there is plenty of room for further research to improve the open lactic acid fermentation from lignocellulosic material for increasing the lactic acid titer and yield, and maintain high optical purity of accumulated lactic acid. Here, open conditions means no sterilization from bioreactor, nutrients, feedstock, water used in medium preparation or in SSF, and sampling in the open conditions. However, *P. acidilactici* TY112 engineered strain that could produce optically pure L-lactic acid, and also possess potential to produce pediocin. Pediocin is an antimicrobial peptide that could inhibit the growth of contaminating strains, and consequently, open lactic acid fermentation could be performed. Pediocin production and characterization from several lactic acid bacteria (LAB) has been investigated and experimentally analyzed for growth inhibition of food spoiling pathogen to solve the food spoilage issues. In addition, pediocin activity has never been investigated to perform open lactic acid fermentation under completely unsterilized conditions.

Therefore, in this study the open L-lactic acid fermentation was performed from *Pediococcus acidilactici* TY112 in the synthetic medium, in the detoxified corn stover hydrolysate, and in high solids content SSF under completely unsterilized conditions. In addition, *Bacillus subtilis* or *Staphylococcus aureus* (5.0% v/v) were manually added as contaminant to further confirm the efficiency of pediocin producing strain TY112 to eliminate the contamination in the synthetic medium (MRS) and in the detoxified corn stover hydrolysate. *P. acidilactici* TY112 show pretty satisfying results in the synthetic medium, in detoxified corn stover hydrolysate, and in high solids content SSF for obtaining high lactic acid titer and yield in completely unsterilized conditions, and no major difference in the final lactic acid was observed with or without sterilization. Lactic acid titer, productivity and yield reached to 97.30 g/L, 1.47 g/L/h, and 69.38 %, respectively. These results show great practical approach in the development of cost effective lignocellulosic biorefinery.

## 4.2. Materials and Methods

### 4.2.1. Raw materials and enzymes

Corn stover (CS) was obtained from Dancheng County, Henan Province, China, in fall 2013. Corn stover was water-washed to remove the impurities and air-dried, then milled using a beater pulverizer to pass through 10 mm screen. The milled corn stover was sealed in plastic bags and stored at room temperature until used. Yeast extract was purchased from Oxiod, Basingstoke, Hampshire, England. All other standard chemicals, including glucose, peptone, tryptone, $MnSO_4.H_2O$, $C_6H_{14}N_2O_7$, $MgSO_4.7H_2O$, $K_2HPO_4$, $CH_3COONa$, NaOH, NaCl and $H_2SO_4$ were received from Lingfeng Chemical Reagent Co., Shanghai, China. All chemicals were of analytical grade.

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell #6 was 135 FPU/g (cellulase protein equivalent to 90 mg/g DM) determined using the NREL protocol LAP-006 [203], and the cellobiase activity was 344 CBU/g using the method described by Sharma et al. [204].

### 4.2.2. Strains and cultivation conditions

*Pediococcus acidilactici* TY112 an engineered strain was used as L-lactic acid and pediocin producing strain. *Staphylococcus aureus, Bacillus subtilis* W800, and *Bacillus coagulans* 179 were used as indicator strain. *Amorphotheca resinae* ZN1 (CGMCC 7452) was used as the biodetoxification strain to remove the potent inhibitors from the dry sulphuric acid pretreated corn stover via solid state fermentation [68].

*P. acidilactici* TY112 was grown in simplified MRS medium containing (g/L) tryptone 10.0, yeast extract 10.0, $MnSO_4$. $H_2O$ 0.25, $C_6H_{14}N_2O_7$ 2.0, $MgSO_4.7H_2O$ 0.58, $K_2HPO_4$. $3H_2O$ 2.0, $C_6H_{12}O_6$. $H_2O$ 22.0, $CH_3COONa$ 5.0 in shaking incubator at 42 ℃ for 12 hours’. *B. subtilis* W800 and *S. aureus* were grown in LB medium containing; tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L in the shaking incubator at 42 °C for 12 hours’. *B. coagulans* 179 was grown in the medium containing (g/L) glucose 20.0, yeast extract 10.0, peptone 10.0, $MgSO_4.7H_2O$ 0.25 in the shaking incubator at 42 °C for 12 hours’. Agar 8.0 g/L was added for preparation of soft agar medium, and 20 g/L agar were added for normal agar medium. Media were sterilized at 115 ℃ for 20 min.

### 4.2.3. Pretreatment and biodetoxification

Corn stover was pretreated using the dry dilute acid pretreatment (DDAP) method according to Zhang et al. [13] and He et al. [12]. Briefly, corn stover solids and the dilute sulfuric acid solution at 5.0% (w/w) were co-currently fed into the reactor at a solid/liquid ratio of 2:1 (w/w) under the helically stirring mixing. Then the corn stover was pretreated at 175 ℃ for 5 min to obtain the pretreatment product. The solids content of the pretreated corn stover was around 50% (w/w), and no wastewater was generated during DDAP.

The pretreated corn stover material was detoxified using the fungal strain *Amorphotheca resinae* ZN1 according to the method in Zhang et al. [68]. Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ to pH value of 5-6 and then inoculated with spores of fungus *A. resinae* ZN1 for 7 days at 28 ℃. The well grown spores and mycelia of *A. resinae* ZN1 on the corn stover solids was used as the seeds culture, then was inoculated onto the pretreated corn stover feedstock in a large static plastic box at 10% (w/w) to remove the inhibitors from the feedstock for 7 days as optimized by Qureshi et al.[19], Chapter 3. After the biodetoxification, the corn stover solids were autoclaved at 115 °C for 20 min and then stored at -20 °C for enzymatic hydrolysis and lactic acid fermentation.

### 4.2.4. Determination of pediocin activity

First of all, one vial of *P. acidilactici* TY112 was inoculated in 20 mL of MRS medium at 42 °C for 12 hours’. Culture was serially diluted; 10 µL of serially diluted ($10^{-6}$ dilution) culture were mixed with 10 mL of MRS soft agar (0.8% w/v) when the temperature of soft agar was less than 50 ℃. Well mixed culture was poured in petri plate, and allowed to solidify at room temperature, then incubated at 37 °C for 48 hours’. Single colonies were inoculated in fresh MRS plates for 12 hours’. 5.0 µL culture broth of (indicator strain) was mixed with 5 mL of soft agar LB medium and mixture was poured onto the TY112 colonies grown in plates, then incubated at 37 °C for 12 hours'. In the next step, pediocin activity was determined from cell free supernatant (CFS). *P. acidilactici* TY112 was grown in MRS broth for 12 hours' at 42 °C. Culture was centrifuged at 16,125g for 10 min to obtain CFS, and then CFS was filtered through 0.2 µm filter. A 20 % v/v of CFS was added in the fresh medium, then indicator strains, including *S. aureus, B. coagulans, and B. subtilis* were inoculated in separate flasks, and incubated in shaking incubator at 42 °C for 12 hours'. Growth (OD) was recorded after 12 hours' against control (without CFS).

In another set of experiment, the CFS was treated with 1 mg/mL of trypsin at 37 ℃, pH 7.5 for 1 hour. A 20 % v/v enzyme treated CFS was added in the fresh medium, and then inoculated with indicator strains for 12 hours' and growth OD was recorded.

### 4.2.5. Effect of initial pH on the growth of indicator strains

Initial pH of medium was adjusted in the range of 2.7-7.0 with addition of 1 M $H_2SO_4$ or 5 M NaOH. Indicator strains were inoculated and cultures were incubated in shaking incubator at 42 ℃ for 12 hours, and growth (OD) was recorded after 12 hours against control.

### 4.2.6. Open L-lactic acid fermentation in synthetic medium

L-lactic acid fermentation was performed in 3 L fermentor by using MRS medium with and without trypsin, and the ratio of contaminant strains was upto 5.0 % v/v. Negative control (without contamination and without trypsin), and negative control (without contamination with addition of trypsin). *B. subtilis* and *S. aureus* without and with trypsin, and open fermentation (without contaminants). Except, open fermentation all other experiments were performed under sterilized conditions to eliminate the external contamination effect. Fermentation conditions were; 150 rpm, 37 ℃ or 42 ℃, pH was adjusted to 6.5 with automatic addition of 5 M NaOH, and 48 hours'.

### 4.2.7. Corn stover hydrolysate preparation and open L-lactic acid fermentation

Corn stover hydrolysate was prepared by enzymatic hydrolysis of the biodetoxified corn stover. Briefly, the biodetoxified corn stover was hydrolyzed with 10 mg protein/g of dry corn stover (DM) at 15 % w/w corn stover solids content at 50 ℃ for 48 hours'. The slurry was centrifuged at 16,125g for 10 min to obtain the clear supernatant. The obtained clear hydrolysate contained 69.14 g/L of glucose, 12.01 g/L of xylose, 0.81 g/L of acetic acid, 0.05 g/L of 5-hydroxymethylfurfural (HMF), and furfural 0 g/L. Nutrients were supplemented with 10 g/L of tryptone, 10 g/L of yeast extract, 0.25 g/L of $MnSO_4.H_2O$, 2.0 g/L of $C_6H_{14}N_2O_7$, 0.58 g/L of $MgSO_4.7H_2O$, 2.0 g/L of $K_2HPO_4$. $3H_2O$, and 5.0 g/L of $CH_3COONa$ in the hydrolysate before it was used as fermentation medium. Experiments were designed as; negative control (without contamination and without trypsin), *B. subtilis* and *S. aureus* (without trypsin), and open fermentation (without contaminants). Except, open fermentation all other experiments were performed under sterilized conditions to eliminate the external contamination effect. Fermentation conditions were; 150 rpm, 42 ℃, pH was adjusted to 6.5 with automatic addition of 5 M NaOH, and incubation time 48 hours'.

### 4.2.8. Open L-lactic acid fermentation in SSF at high solids content

All SSF experiments using the DDAP pretreated and biodetoxified corn stover were performed in the 5-L helical ribbon stirring bioreactor [14]. The SSF process was operated at two stages. Stage 1: prehydrolysis was started with addition of the Youtell #6 cellulase into the tank at the 15 FPU/g DM dosage and the biodetoxified feedstock was fed gradually to maintain the liquid slurry into the bioreactor to a final 30 (% w/w) solids content within 6 hours' at 50 ℃, 150 rpm, 4.8 pH. Stage 2: SSF was started by decreasing the system temperature to 42 ℃ and inoculating the three-step adapted *P. acidilactici* TY112 seeds into the SSF system. The whole SSF operation lasted for 72 hours' and the samples were withdrawn at regular interval, centrifuged at 11,167g for 5 min and the supernatant was stored frozen until analysis. The cell viability during SSF was determined using the plate count method [18]. The pH was maintained at 5.5 during SSF process by addition of 5 M NaOH solution.

### 4.2.9. Analytical methods

The cellulose and hemicellulose contents of the corn stover were determined using a two-step $H_2SO_4$ hydrolysis method [206]. The raw corn stover contained 37.2% of glucan and 19.9% of xylan on the dry weight basis. Dry dilute acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan. The samples collected at different intervals were determined for glucose, lactic acid, acetic acid, and ethanol. The HPLC was equipped with LC-20AD pump, RI detector RID-10A (Shimadzu, Kyoto, Japan) and a Bio-Rad Aminex HPX-87H column operated at 65 ℃ with 0.6 mL/min of 5 mM $H_2SO_4$ as the mobile phase. Growth (OD) was recorded on DU800 spectrophotometer. Lactic acid yield was calculated using the following equation as described in Zhao et al. [87];

$$\text{Lactic acid yield} = \frac{[\text{Lac}]\text{f} \times \text{Vf} - [\text{Lac}]0 \times \text{V0}}{\text{f} \times \text{W CS} \times 1.111 \times 1.0} \times 100\%$$

where [Lac]f and [Lac]0 are the final and initial lactic acid concentrations g/L), Vf and V0 are the total liquid volume at the end and the start of the fermentation (L), WCS is the total weight of the biodetoxified corn stover (dry basis) fed into the SSF system (g), f is the cellulose fraction of the dry CS (g/g), 1.111 is the conversion factor for cellulose to equivalent glucose, 1.0 is the conversion factor for glucose to lactic acid on the mass basis of stoichiometric biochemistry. The unit g/L refers to the liquid fraction volume calculated on the basis of water mass balance of the SSF system.

## 4.3. Results and Discussion

### 4.3.1. Assay of pediocin activity of *Pediococcus acidilactici* TY112

Antimicrobial property of *Pediococcus acidilactici* TY112 was experimentally analysed towards growth inhibition of indicator strains, including *B. subtilis* W 800, *S. aureus*, and *B. coagulans* 179. Antimicrobial activity was determined by clear zone formation surrounding the TY112 colonies. Fig. 4.1 shows the clear zone on agar plates where TY112 colonies were inoculated, and normal growth in the control (without TY112 cells). Antimicrobial activity was further determined by using cell free supernatant (CFS) of TY 112.

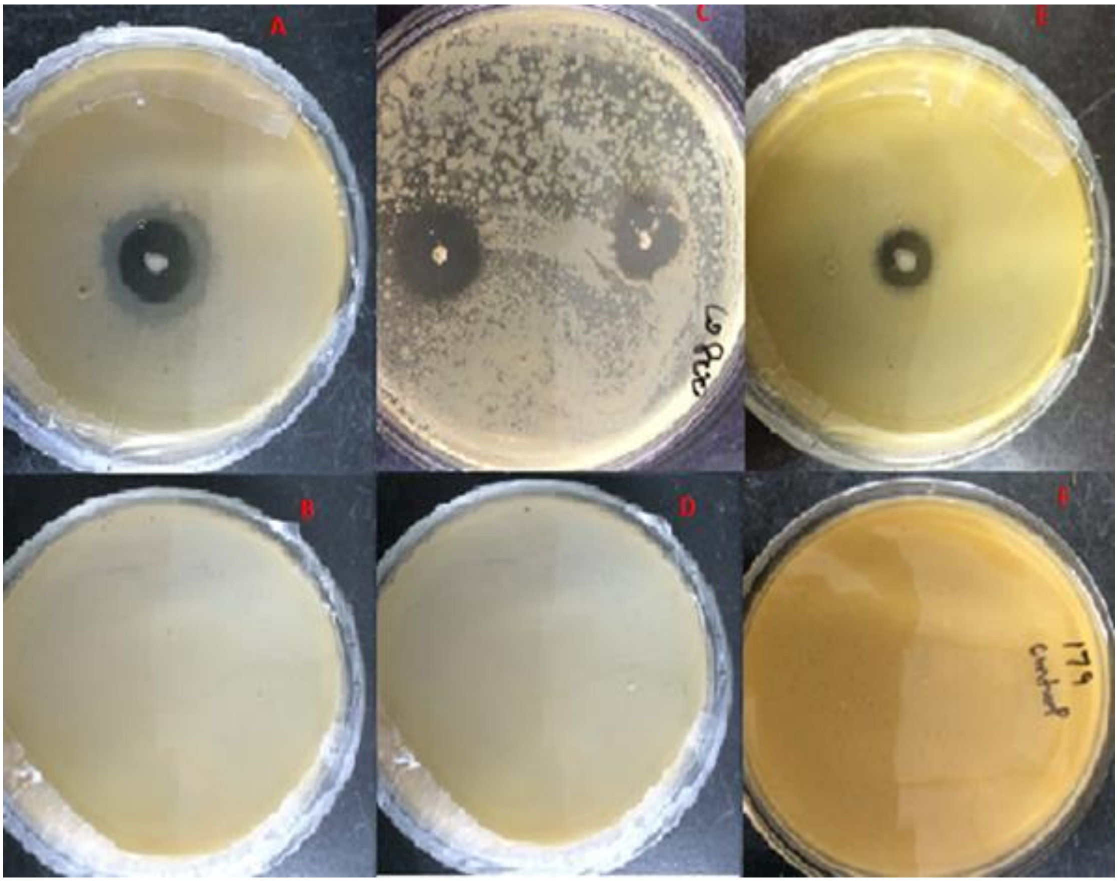
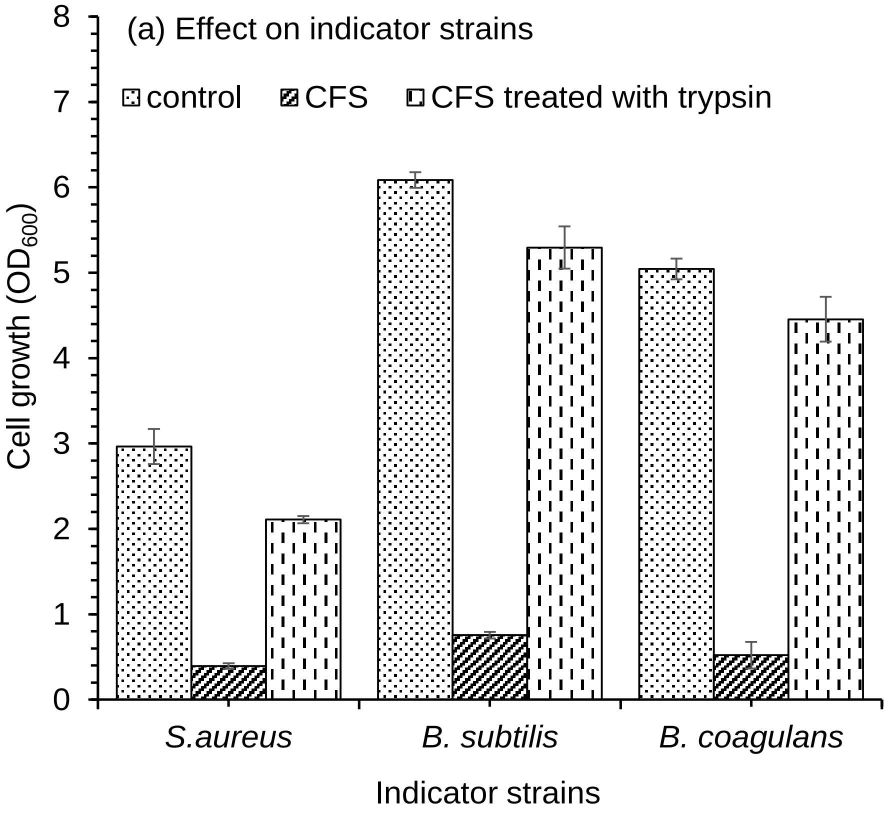
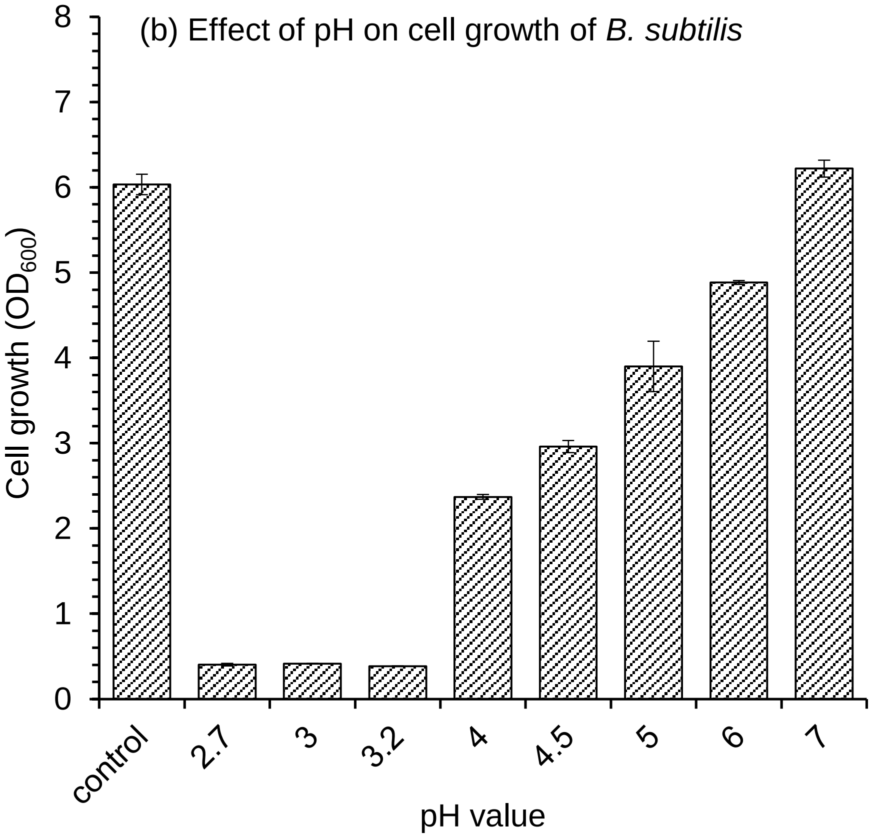
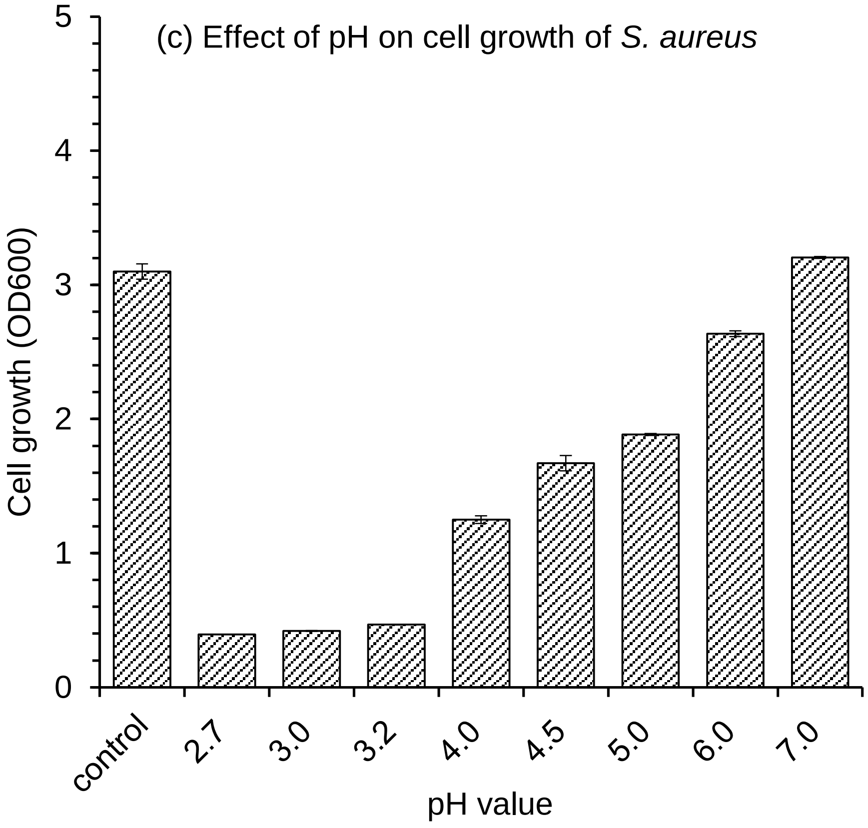


**Fig. 4.1.** Pediocin activity. (a) *Staphylococcus aureus* in presence of TY112, (b) control, (c) *Bacillus subtilis* W800, (d) control, (e) *Bacillus coagulans* 179, and (f) control.

Fig 4.2. (a) shows the comparison of indicator strains growth, (1) without CFS (control), (2) addition of 20% v/v CFS, and (3) CFS treated with trypsin. Growth of all strains was sensitive to CFS as shown in the Fig 4.2 (a). When CFS was treated with trypsin the growth of all indicator strains was not inhibited. These results suggest that trypsin hydrolyzed the pediocin that resulted the loss of antimicrobial property of CFS. Previous studies suggest that treatment of CFS with proteolytic enzymes, including proteinase K, pepsin, α-chymotrypsin, trypsin, and papain results in partial or complete inactivation of antimicrobial activity depending on the enzyme and pediocin concentration. Therefore, the function of pediocin produced by TY112 also confirmed by CFS treatment with trypsin, and almost complete inactivation of pediocin was observed. Another possible reason of growth inhibition could be lower pH due to accumulated lactic acid. Therefore, effect of pH on the growth of all three indicator strain was analyzed, results are shown in Fig. 4.2 (b-d). The growth of all indicator strains decreased with decreasing pH, and maximum growth was observed at the pH values of 6-7. These finding suggest that growth of all indicators strains was sensitive to lower pH and pediocin but pediocin is the main factor, and it was further confirmed during lactic acid fermentation when trypsin was added.




(b) Effect of pH on cell growth of *B. subtilis*
Cell growth ($OD_{600}$)
0
1
2
3
4
5
6
7
8
control
2.7
3
3.2
4
4.5
5
6
7
pH value


(c) Effect of pH on cell growth of *S. aureus*
Cell growth (OD600)
0
1
2
3
4
5
control
2.7
3.0
3.2
4.0
4.5
5.0
6.0
7.0
pH value

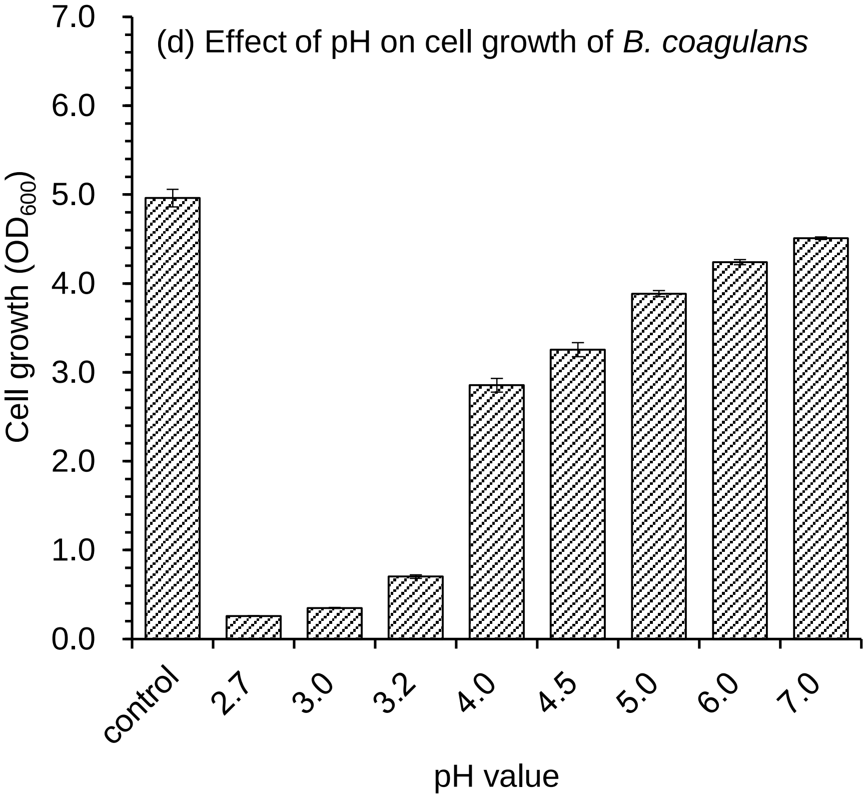
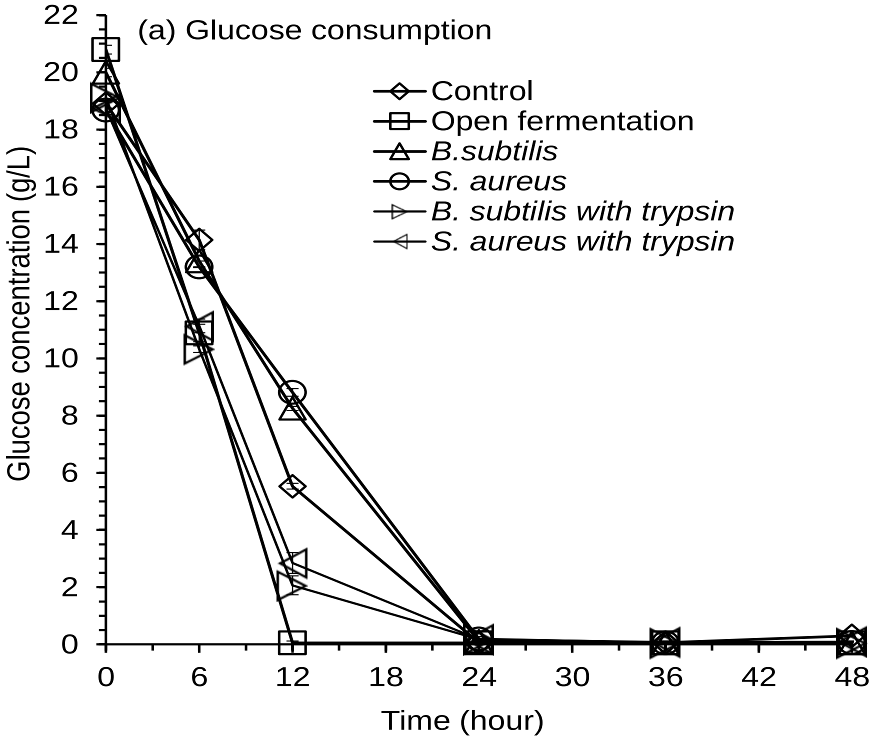


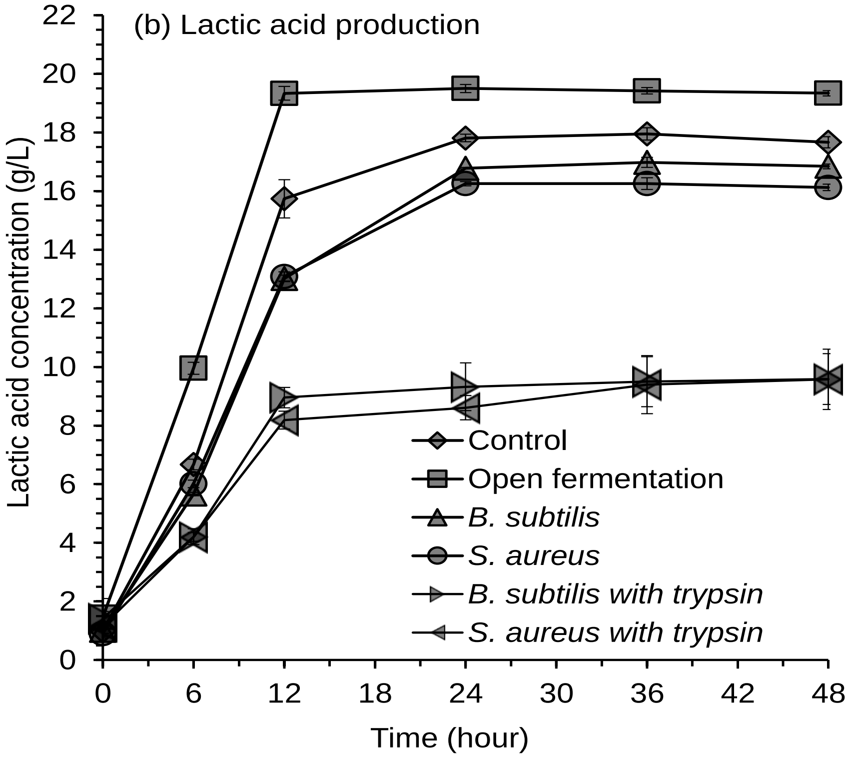
**Fig. 4. 2.** Effect of *P. acidilactici* TY112 cell free supernatant (CFS) and trypsin treated CFS on the growth ($OD_{600\ nm}$) of indicator strains in the synthetic medium. CFS was added at 20% v/v dosage in the fresh medium before inoculation of indicator strain, then cultures were inoculated, and incubated at 42 ℃ for 12 hours' in shaking incubator. *S. aureus* grown in LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L). *B. subtilis* W800 grown in LB medium. *B. coagulans* 179 was grown in medium containing glucose 20.0 g/L, yeast extract 10.0 g/L, peptone 10.0 g/L, $MgSO_4.7H_2O$ 0.25 g/L. Trypsin treatment conditions: CFS was treated with 1mg/mL of trypsin at 37 ℃, pH 7.5 for 1 hour. Enzyme treated CFS (20% v/v) was added in the fresh medium, then cultures were inoculated, and incubated at 42 °C for 12 hours' in shaking incubator. Conditions for 4.2. (b-d); Effect of initial pH on the growth of indicator strains. (b) *B. subtilis* W 800 , (c) *S. aureus*, (d) *B. coagulans* 179.

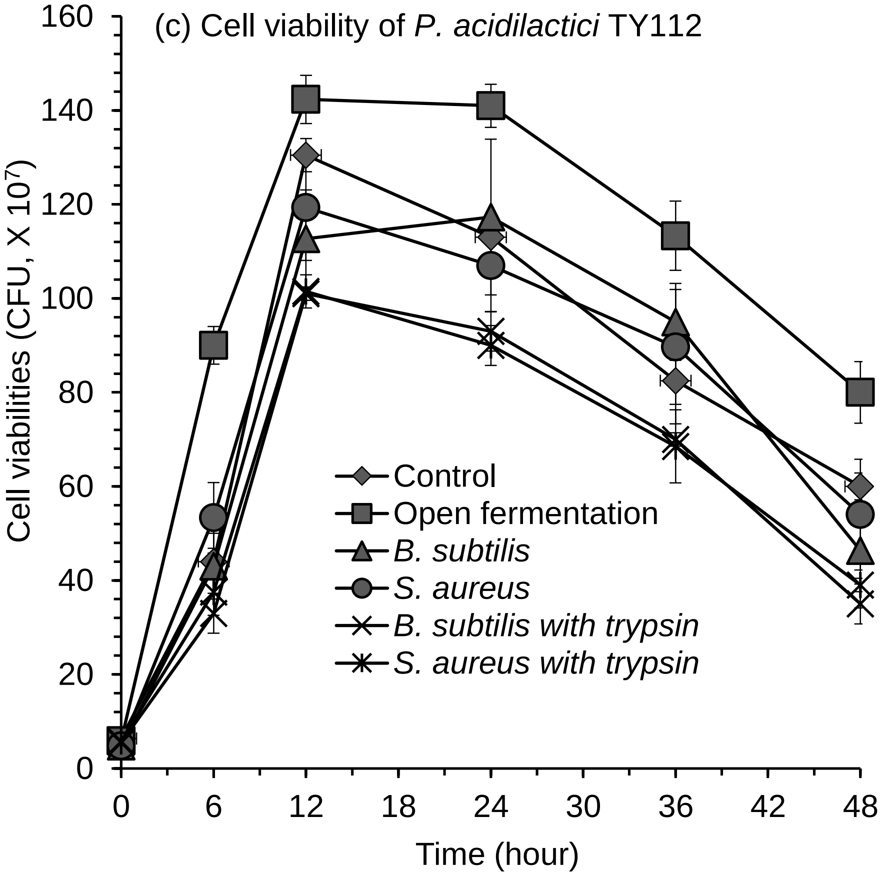
### 4.3.2. Open L-lactic acid fermentation of synthetic medium

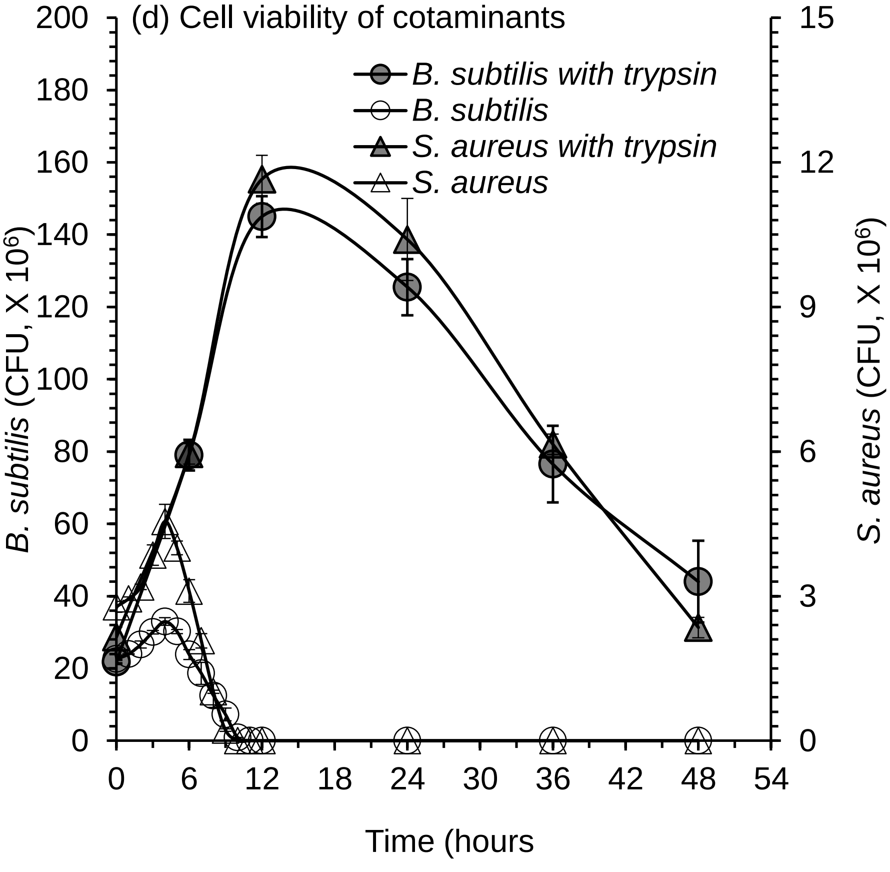
Fig. 4.3 shows the results of open L-lactic acid fermentation performed in 3 L fermentor by using the synthetic medium (MRS), the inoculation ratio of the contaminant strains was 5.0% v/v. Detailed experimental scheme is described in materials and methods (section 2.6). Fig. 4.3 (a, b) shows the glucose utilization and lactic acid formation profile. Lactic acid concentration gradually increased and reached upto 19 g/L after 48 hours and no residual glucose was detected after 48 hours. There was no major difference in the final lactic acid titer in all tested conditions, including control, *B. subtilis* and *S. aureus* without trypsin, and open fermentation conditions. For the confirmation of antimicrobial function of TY112, 1 mg/mL of trypsin was added in the beginning of experiment. Lactic acid titer was significantly reduced in these experiments perhaps due to loss of antimicrobial activity that leads to normal growth of contaminant strains (4.3. d). Fig. 4.3 (c) shows the cells viability (CFU/mL), growth steadily increase and reached maximum after 12 hours and remained almost stable upto 24 hours, then declined. Fig. 4.3 (d) shows that in the absence of trypsin, growth of contaminating strain increased upto 4 hours', and then declined. After 12 hours' no growth was observed in all tested conditions, while in presence of trypsin normal cell viability was counted. Several studies show that pediocin production was growth dependent, once growth of lactic acid producing strain reaches to exponential phase pediocin activity was higher and remained stable during stationary phase, and then declined in the death phase [187, 217-219]. This might be reason for initially increase in the growth of indicator strains, then decreased with fermentation time and after 12 hours no colonies was detected. TY112 cells could not grow in LB medium plates perhaps due to insufficient nutrients, and contaminant strains were not growing well in presence of TY112 cells on MRS medium plates. Therefore, TY112 viable cells were counted on MRS plates and *B. subtilis* and *S. aureus* viable cells were counted on LB plates, random samples were analyzed under microscope and no contamination was observed in open and control conditions.

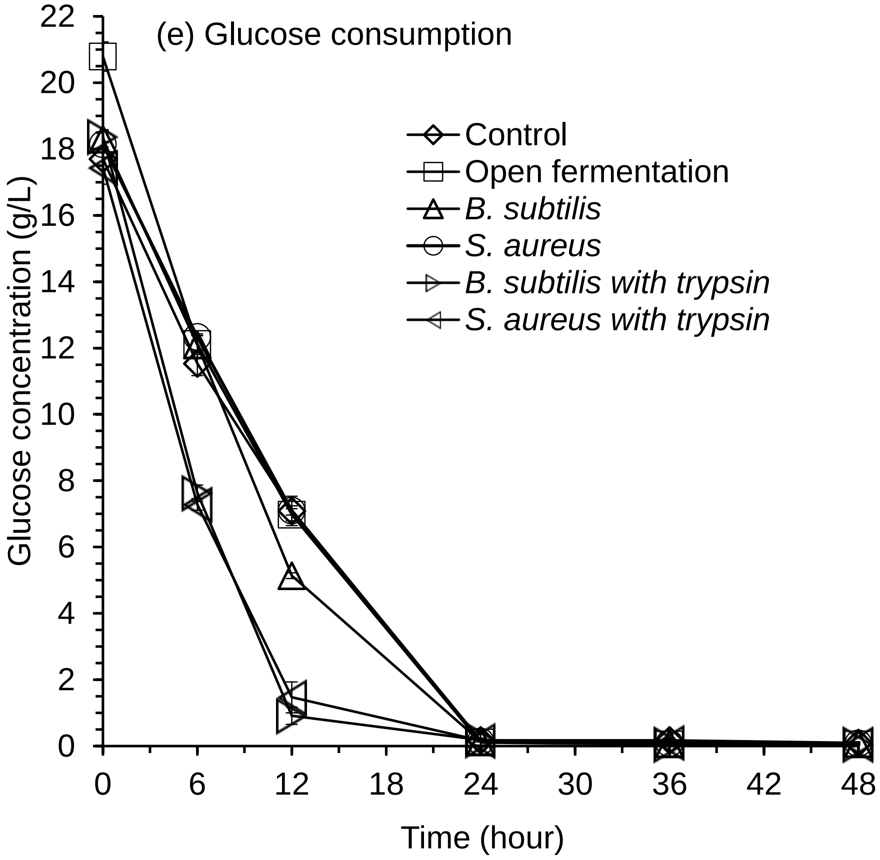
Open L-lactic acid fermentation was also performed at 37 ℃ and all fermentation conditions were same to previous experiment at 42 ℃. Results are shown in Fig. 4.3 (e-g). There was no any difference in overall glucose consumption, lactic acid titer, and cells viability in all tested conditions. No contamination was observed at moderate temperature, which is clear indication for the antimicrobial property of the TY112 strain to eliminate the possible contamination. In previous studies, where thermophilic strains were used in open lactic acid fermentation, sterilized conditions were certainly required for lactic acid fermentation at moderate temperature 37 ℃ and 45 ℃ [162]. These results show great potential for open lactic acid fermentation at moderate and high temperature under completely unsterilized conditions. Glucose consumption, lactic acid formation, and growth of indicator strains in absence of pediocin producing strains were compared as control experiment (data not shown). All the glucose was consumed by both indicator strains.


(a) Glucose consumption
Control
Open fermentation
*B.subtilis*
*S. aureus*
*B. subtilis with trypsin*
*S. aureus with trypsin*
Glucose concentration (g/L)
0
2
4
6
8
10
12
14
16
18
20
22
Time (hour)
0
6
12
18
24
30
36
42
48

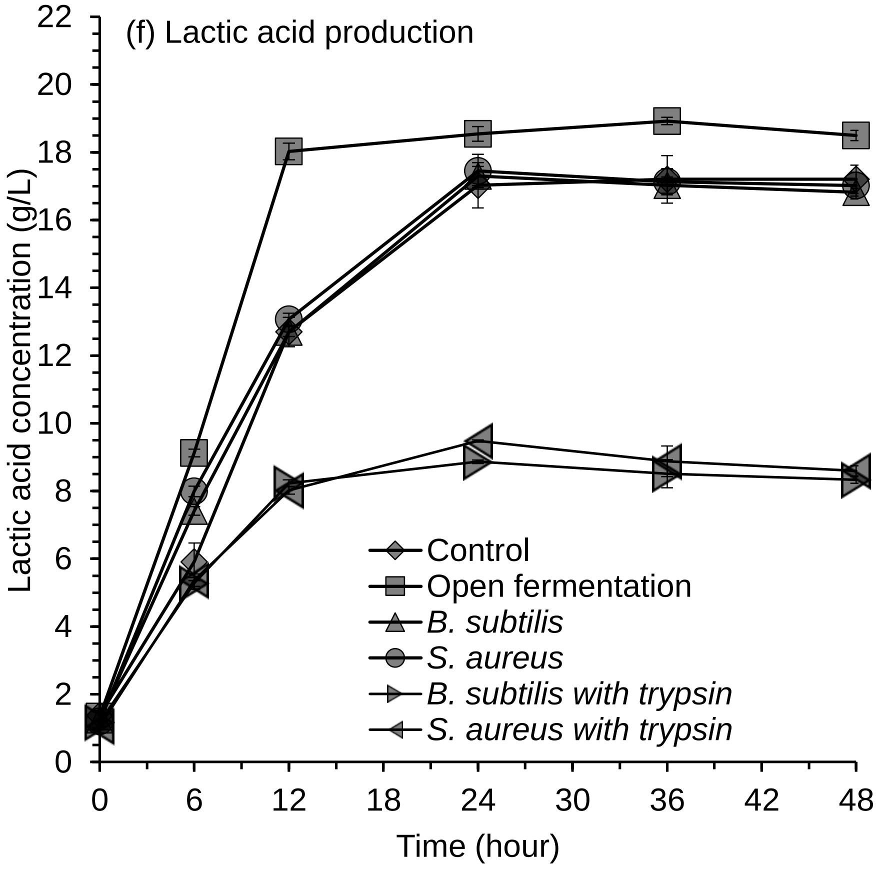

(b) Lactic acid production
Control
Open fermentation
*B. subtilis*
*S. aureus*
*B. subtilis with trypsin*
*S. aureus with trypsin*
Lactic acid concentration (g/L)
0
2
4
6
8
10
12
14
16
18
20
22
Time (hour)
0
6
12
18
24
30
36
42
48


(c) Cell viability of *P. acidilactici* TY112
Cell viabilities (CFU, X $10^7$)
0
20
40
60
80
100
120
140
160
Time (hour)
0
6
12
18
24
30
36
42
48
Control
Open fermentation
*B. subtilis*
*S. aureus*
*B. subtilis with trypsin*
*S. aureus with trypsin*


(d) Cell viability of cotaminants
*B. subtilis* (CFU, X $10^6$)
0
20
40
60
80
100
120
140
160
180
200
*S. aureus* (CFU, X $10^6$)
0
3
6
9
12
15
Time (hours
0
6
12
18
24
30
36
42
48
54
*B. subtilis with trypsin*
*B. subtilis*
*S. aureus with trypsin*
*S. aureus*



(e) Glucose consumption



(f) Lactic acid production

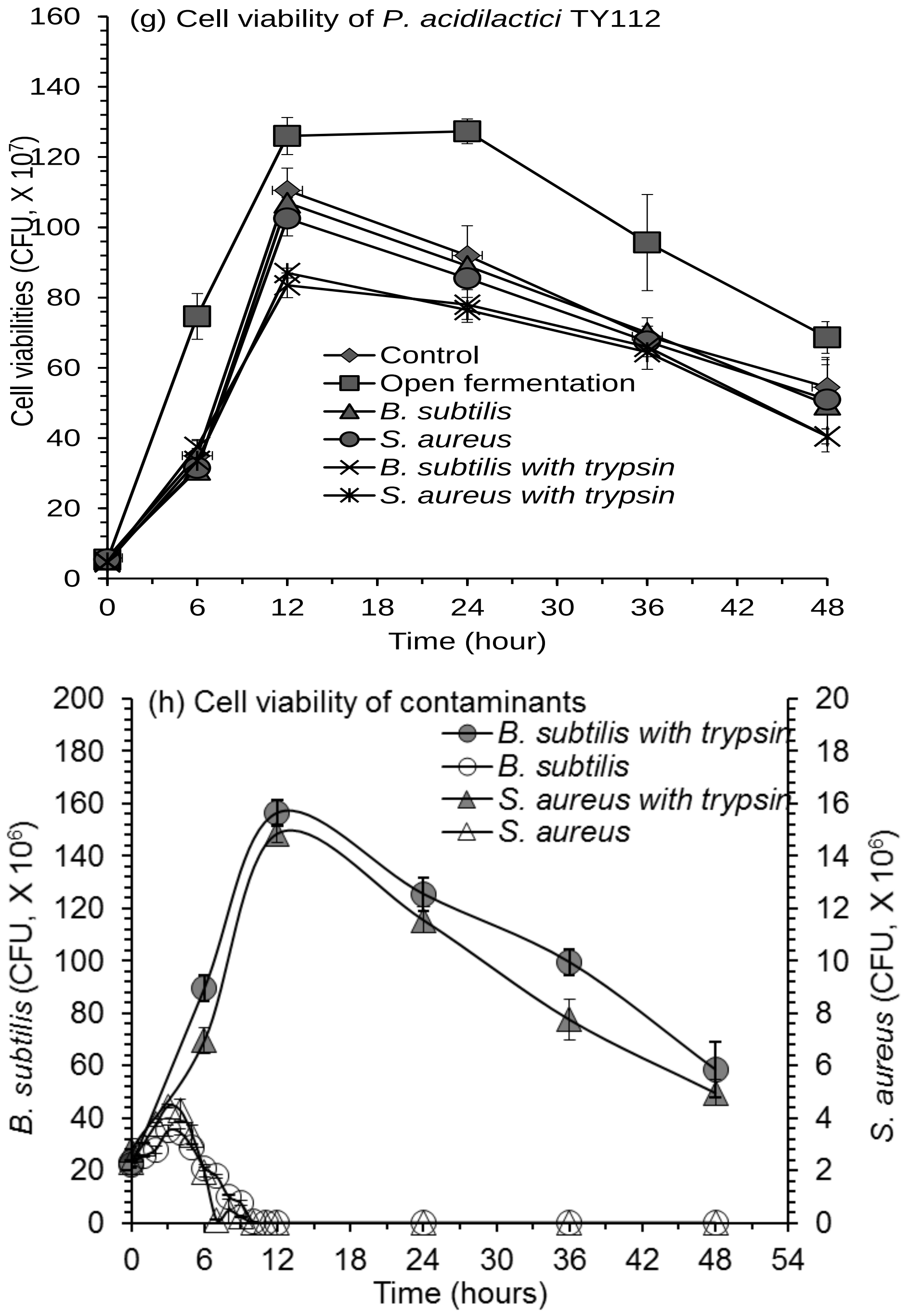
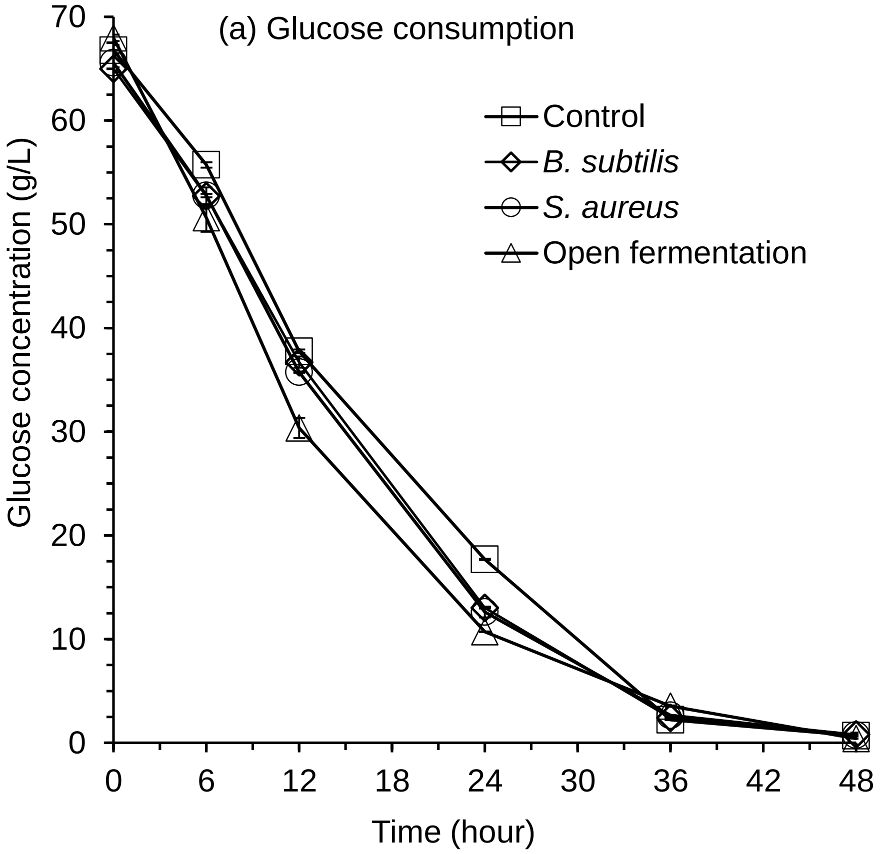
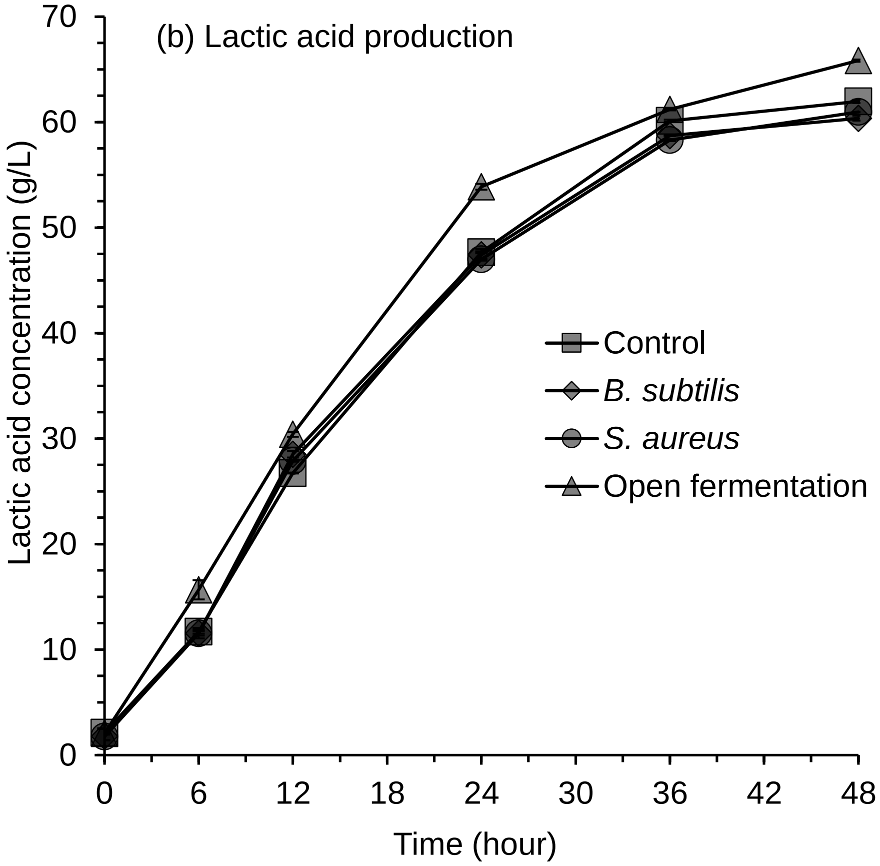


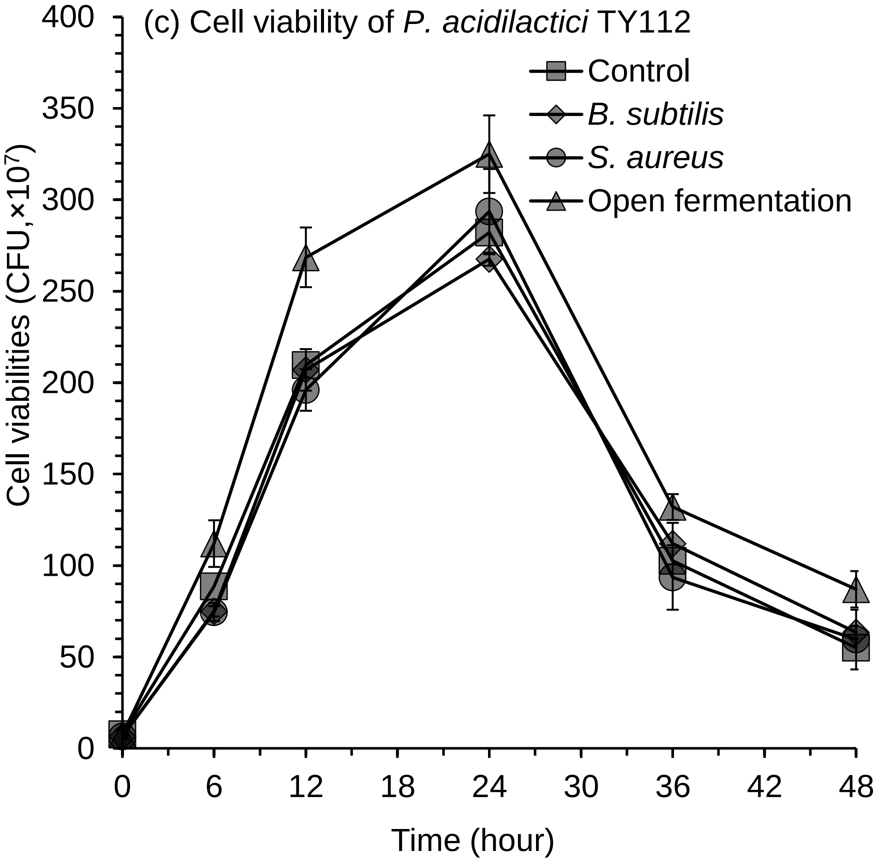
**Fig. 4.3.** Open L-lactic acid fermentation by using synthetic medium in 3 L fermentor. Conditions were: simplified MRS medium, 150 rpm, 37 ℃ or 42 ℃, pH was adjusted to 6.5 with 5 M NaOH automatic regulation, and 48 hours'. *P. acidilactici* TY 112 inoculation ratio was 10.0% v/v. 1. Control (without contaminants and without trypsin); 2. Open (without contaminants under completely unsterilized conditions); 3. *B. subtilis* (5.0% v/v was added as manual contaminant); 4. *S. aureus* (5.0% v/v was added as contaminant); 5. *B. subtilis* + trypsin (manual contaminants with 1.0 mg/mL of trypsin); 6. *S. aureus* + trypsin (manual contaminants with 1.0 mg/mL of trypsin). Results of 42 ℃ fermentation (a-d); a) glucose time profile , (b) lactic acid time course (c) cells viability of TY112, (d) cells viability of *B. subtilis* and *S. aureus* in presence an absence of trypsin, Results of 37 ℃ fermentation (e-g); (e) glucose time profile, (f) lactic acid time profile, (g) cells viability of TY112, and (h) cells viability of *B. subtilis* and *S. aureus* in presence and absence of trypsin.

### 4.3.3. Open L-lactic acid fermentation of detoxified corn stover hydrolysate

L-lactic acid fermentation was performed by using detoxified corn stover hydrolysate prepared from 15% w/w solids content of biodetoxified corn stover. Experimental design as described in materials and methods section (2.7). Manually added contamination ratio was 5.0% v/v. Fig. 4.4 shows that lactic acid concentration gradually increased and reached above 60 g/L after 48 hours' in all tested conditions. There was no any difference in the final lactic acid titer among the tested conditions, including control, with contaminants, and open fermentation conditions, and slightly higher lactic acid titer was obtained in case of open fermentation conditions. This might be due to increased cells viability (availability of more nutrients because sterilization causes loss of nutrients). Growth of contaminating strain increased upto 5 hours', then declined, and after 12 hours' no growth of contaminating strains was observed. The decrease in the cell viabilities of *B. subtilis*, and *S. aureus* depends on the pediocin concentration, as shown in Fig. 4.4 (d). At lowest concentration (in early growth hours), no detrimental effect was observed, and once sufficient pediocin was produced, then cell viabilities were reduced to undetectable limit.




(b) Lactic acid production
Lactic acid concentration (g/L)
0
10
20
30
40
50
60
70
Time (hour)
0
6
12
18
24
30
36
42
48
Control
*B. subtilis*
*S. aureus*
Open fermentation


(c) Cell viability of *P. acidilactici* TY112
Cell viabilities (CFU,×10⁷)
0
50
100
150
200
250
300
350
400
Time (hour)
0
6
12
18
24
30
36
42
48
Control
*B. subtilis*
*S. aureus*
Open fermentation

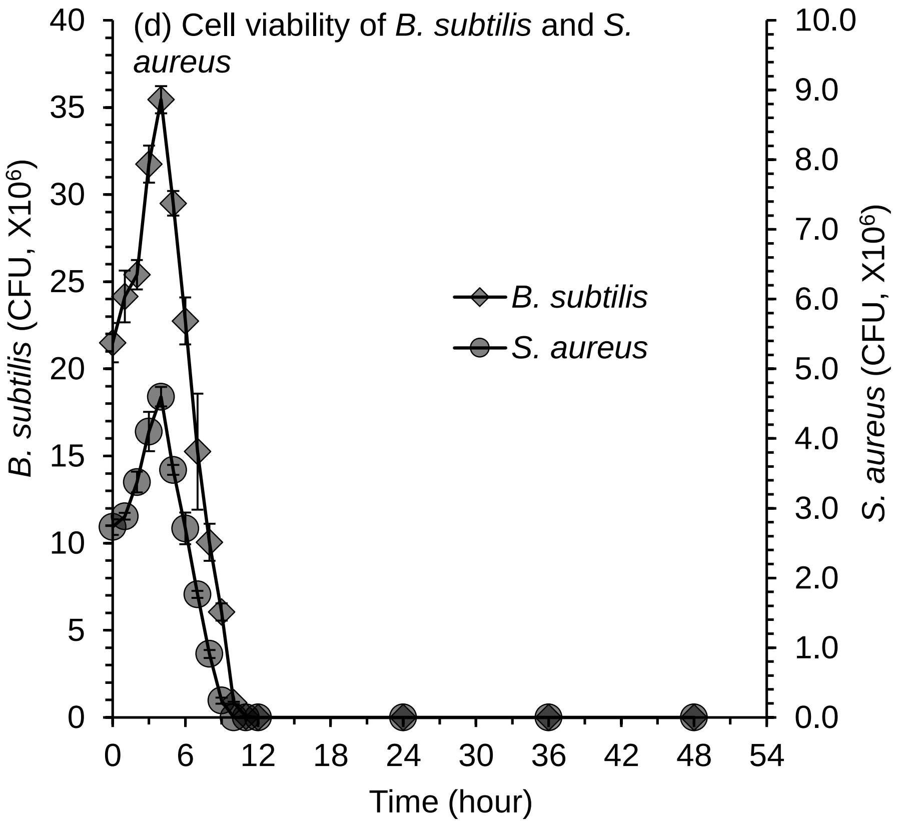


**Fig. 4.4.** Open L-lactic acid fermentation by using detoxified corn stover in 3 L fermentor. Conditions were: 100% detoxified corn stover hydrolysate, 150 rpm, 37 ℃ or 42 ℃, pH was adjusted to 6.5 with 5 M NaOH automatic regulation, and 48 hours'. TY112 inoculation ratio was 10.0% v/v. 1. Control (without contaminants) 2. Open (without contaminants under completely unsterilized conditions). 3. *B. subtilis*; 5.0% v/v was added as manual contaminant. 4. *S. aureus*, 5.0% v/v was added as contaminant. (a) Glucose (b) lactic acid time profile, (c) cells viability of TY112, and (d) cells viability of contaminating strain.

### 4.3.4. Open L-lactic acid fermentation of high solids content in SSF

At the start, main objective of this study was the development of cost effective strategy for obtaining the high lactic acid titer and yield under industrial relevant conditions. Simultaneous saccharification and lactic acid fermentation was performed at high solids content of biodetoxified corn stover under completely unsterilized conditions. In open lactic acid fermentation (medium, fermentor, water used in SSF, and nutrients were not sterilized, and during sampling no care was taken). Fig. 4.5 shows that lactic acid titer increased steadily and reached upto 97.30 g/L after 72 hours', and no any difference was observed in the final lactic acid titer and yield with or without sterilization fermentation. *P. acidilactici* cells viability increased with incubation time and reached upto ($433\times10^7$) after 42 hours', and then declined as shown in Fig. 4.5. Randomly samples were collected for microscopy, and no contamination was observed. Lactic acid titer, productivity and yield reached to 97.30 g/L, 1.47 g/L/h, and 69.38 %, respectively.

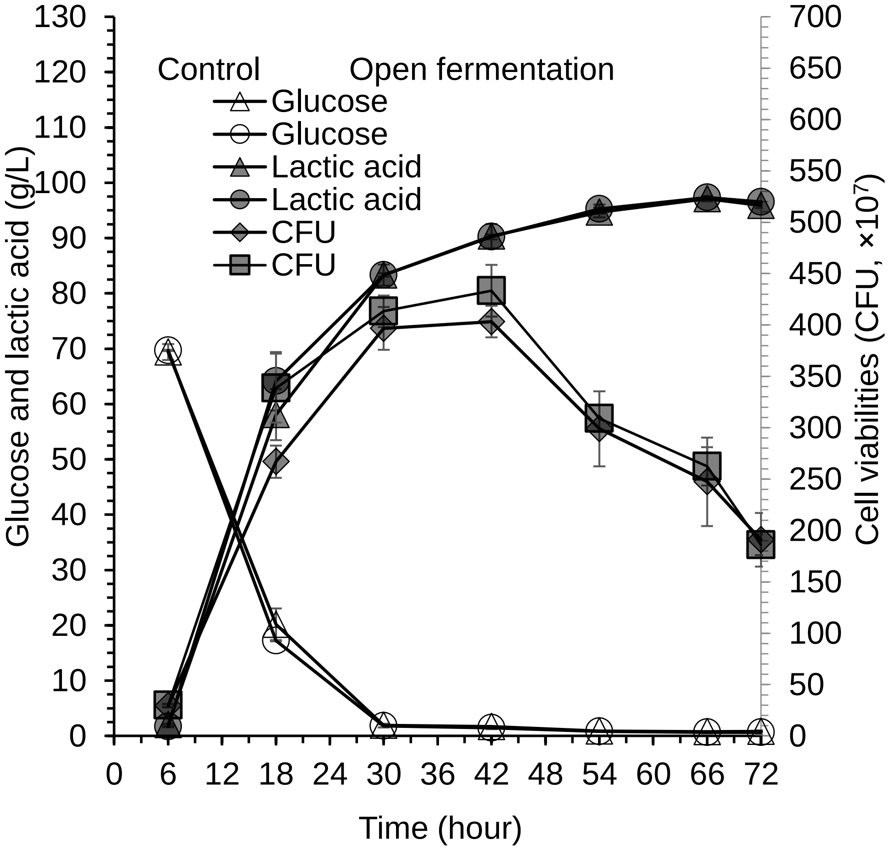


**Fig. 4.5.** SSF performance of biodetoxified corn stover at high solids content for L-lactic acid fermentation under completely unsterilized conditions. All SSF experiments were performed in 5 L helical ribbon bioreactor. SSF conditions were; 30% w/w solids content, 15 FPU/g DM cellulase dosage, with simplified MRS nutrients except glucose. Prehydrolysis was started by addition of cellulase in the bioreactor and fed-batch feeding of solid material to maintain liquid slurry during 6 hours at 50 ℃, 4.8 pH and 150 rpm. Three step adapted *P. acidilactici* TY112 seeds were inoculated to start real SSF process at 42 ℃, pH was controlled at 5.5 by automatic regulation of 5 M NaOH, 150 rpm.

## 4.4. Conclusion

In this study, pediocin producing strain *P. acidilactici* TY112 was used to perform open L-lactic acid fermentation under completely unsterilized conditions in synthetic medium, detoxified corn stover hydrolysate, and in high solids content SSF. TY112 performed pretty satisfactory, and no any difference was observed in the final lactic acid titer under sterilized and unsterilized conditions. Lactic acid titer, productivity and yield reached to 97.30 g/L and 95.81 g/L, 1.47 g/L/h, and 69.38%, respectively, under industrial relevant conditions. These results show great practical approach in the development of lignocellulosic biorefinery by reducing the operational cost of lignocellulosic materials.

# Chapter 5

# Cofermentation of glucose and xylose by evolutionarily adapted xylose fermenting *Saccharomyces cerevisiae* NAN-127 under industrial relevant conditions

## 5.1. Introduction

Bioethanol production from lignocellulosic biomass is of significant importance due to increasing energy demands and environmental concerns [4, 220]. High lignocellulosic processing cost is the major hindrance in the commercialization of second generation biofuels [221]. Undoubtedly, lignocellulosic materials at high solids content are essentially required for obtaining high ethanol concentration and yield. Despite wide variation in the carbohydrate composition of lignocellulosic material, glucose and xylose are always the main sugars components [220], and xylose accounts for 20-35% on the dry material basis. However, routine yeast, *Saccharomyces cerevisiae* could convert only glucose to ethanol, while xylose remains unutilized in the fermentation slurry. Therefore, xylose fermenting engineered strain is essentially required for proper and practical solution to incomplete and inefficient sugars conversion to ethanol, this could increase ethanol concentration and yield close to corn based biorefinery. Currently, lignocellulosic biorefinery faces three main issues: incomplete sugars utilization, pretreatment inhibitors, and high nutrients cost (yeast extract).

First difficulty of incomplete sugars utilization could be solved by engineered *Saccharomyces cerevisiae* for cofermentation of glucose and xylose [159]. Until now, metabolic engineering approaches have introduced either xylose reductase (XR)-xylitol dehydrogenase (XDH)-xylulose kinase (XK) pathway or xylose isomerase (XI)-xylulose kinase (XK) pathway [153, 154, 220]. First of all, XR converts xylose to xylitol, then xylitol is oxidized to xylulose by the action of XDH, and then xylulose is converted into xylulose 5-phosphate by XK. Whereas, in another pathway, xylose is converted into xylulose by the action of xylose isomerase, then xylulose is phosphorylated to xylulose 5-phosphate. This end product enters in the non-oxidative pentose phosphate pathway (PPP) and glycolysis to produce ethanol. Engineered *S. cerevisiae* strains have been applied for cofermentation of glucose and xylose for ethanol production. However, engineered strains could not be used under industrial relevant conditions due to pretreatment inhibitors problem.

Second difficulty of pretreatment inhibitors could be solved by long term evolutionary adaptation of engineered strain in the hydrolysate medium [15, 16, 19]. Evolutionary adaptation allows the microorganism to grow in the inhibitors containing environment, and improve the ethanol fermentability and inhibitors tolerance. This happens perhaps due to random mutation in certain genes. Inhibitors tolerance and fermentability of strain largely depends on the specific environment where the strain is to be used. This xylose fermenting strain has never experienced evolutionary adaptation towards pretreatment inhibitors, and a well-adapted strain improves ethanol fermentability in high contents SSF for obtaining the highest possible ethanol concentration and yield.

Third difficulty of high nutrients cost could be solved by replacing the yeast extract with cost effective nutrient: dry distillers' grains and solubles (DDGS). DDGS, is used as animal feed and has shown great potential as nutrient for ethanol production, the cost of DDGS is less than 0.5% that of yeast extract [145].

In this study, an engineered strain for glucose and xylose cofermentation was used in the synthetic medium, in corn stover hydrolysate, in high solid content SSF for obtaining the highest ethanol concentration and yield were evaluated. For further improving the ethanol concentration and yield, xylose fermenting strain *Saccharomyces cerevisiae* is used in long term evolutionary adaptation to pretreatment inhibitors. An evolutionary adapted strain was applied to the simultaneous saccharification and fermentation at high corn stover solids content for obtaining the highest ever possible ethanol concentration and yield [13, 14, 68]. The ethanol concentration is close to corn based ethanol titer. Research work is going on to reach the highest ever ethanol concentration and yield and reduce the processing cost.

## 5.2. Materials and Methods

### 5.2.1. Raw material

Corn stover (CS) was obtained from Dancheng County, Henan Province, China, in fall 2013. Corn stover was water-washed to remove the impurities and air-dried. The dry dilute sulfuric acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan according to the two-step $H_2SO_4$ hydrolysis method [206]. The corn stover was milled using a beater pulverizer to pass through the 10-mm apertures in diameter, then sealed in plastic bags and stored at room temperature until used.

### 5.2.2. Strains and enzymes

An engineered xylose fermenting *Saccharomyces cerevisiae* NAN-127 was gifted by Shandong University, China. This strain was used as parental strain and in long term evolutionary adaptation experiments [222]. *Amorphotheca resinae* ZN1 (CGMCC 7452) was used as the biodetoxification strain for degrading inhibitors exists in the dry dilute sulfuric acid pretreated corn stover via solid state fermentation [68].

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell #6 was 63 FPU/g (cellulase protein equivalent to 49.5 mg/g DM) determined using the NREL protocol LAP-006 [203], and the cellobiase activity was 102 CBU/g using the method described by Sharma et al. [204].

### 5.2.3. Pretreatment

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment (DDAP) according to Zhang et al. [13] and He et al. [12]. Briefly, dry corn stover and dilute sulfuric acid solution at 5.0% (w/w) were co-currently fed into the reactor at a solid/liquid ratio of 2:1 (w/w) with helically stirring mixing of 50 rpm, then pretreated at 175 ℃ for 5 min. The solids content of the pretreated slurry was around 50% (w/w) and no wastewater was generated.

### 5.2.4. Onsite biodetoxification

Onsite biodetoxification was performed in bioreactor using *A. resinae* ZN1. Pretreated material was fed in the bioreactor and feedstock was neutralized with 20% (w/w) $Ca(OH)_2$ to pH value of 5-6, and then inoculated with *A. resinae* ZN1 at 10% (w/w) ratio as the seeds and incubated at 28 ℃ for 48 h, 150 L/h aeration rate. No additional fresh water was used during biodetoxification, and the solids content of the biodetoxified corn stover material was about 50% (w/w).

### 5.2.5. Preparation of corn stover hydrolysate

The hydrolysate used for evolutionary adaptation was prepared by enzymatic hydrolysis of DDAP pretreated corn stover (without biodetoxification) at 15% (w/w) solids content, 15 FPU/g DM (dry matter) cellulase, 50 $^{o}$C, pH 4.8 and 150 rpm for 48 hours in the helically stirring bioreactor [14]. The hydrolysate was centrifuged at 16,125 g for 10 min to remove the solid residues and the supernatant was used as adaption medium. The hydrolysate contained 60.79 g/L of glucose, 27.21 g/L of xylose, 3.24 g/L of acetic acid, 0.28 g/L of furfural and 0.21 g/L of 5-hydroxymethylfurfural (HMF).

### 5.2.6. Evolutionary adaptation of *S. cerevisiae* NAN-127

The evolutionary adaptation of *S. cerevisiae* NAN-127 was conducted by continuously transferring the cultured yeast cells from previous medium into the fresh corn stover hydrolysate. 100 mL conical flasks were used for the continuous cell transfer. In details, 10% (v/v) of the culture solution from last culture was transferred every 24 h into the fresh hydrolysate and incubated at 37 $^{o}$C in the shaking incubator. At the end of each transfer, the sample was collected and used for glucose and ethanol analysis. This successive transfer process was repeated for 85 consecutive days. The strain was stored at -80 ℃ in 30% glycerol and used as adapted strain.

### 5.2.7. Simultaneous saccharification and ethanol fermentation (SSF)

SSF was performed in the 5-L helical ribbon stirring bioreactor using the DDAP pretreated and biodetoxified corn stover feedstock. The SSF started with 7 hours prehydrolysis at 50 $^{o}$C, then followed by 89 hours SSF at 37 $^{o}$C. Cellulase dosage was 15 FPU/g DM and the pH was maintained at 5.5 by automatic regulation with 5 M NaOH. The parental *S. cerevisiae* strain was used after a simple three-step treatment [18]. The samples were withdrawn at regular intervals, centrifuged at 11,167 g for 5 min and the supernatant was analyzed. The yeast cell viability during SSF was determined by counting the colony forming units (CFU) on the petri dish of the diluted fermenting broth [18].

### 5.2.8. Analysis

The cellulose and hemicellulose contents of the corn stover were analyzed using a two-step $H_2SO_4$ hydrolysis method [206]. The raw corn stover contained 37.2% of glucan and 19.9% of xylan on the dry weight basis. The dry dilute sulfuric acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan.

Glucose, ethanol, acetic acid, lactic acid, furfural, and HMF contained in the taken samples were analyzed on HPLC (LC-20AD pump, RI detector RID-10A, Shimadzu, Kyoto, Japan) with Bio-Rad Aminex HPX-87H column operated at 65 ℃ and 0.6 mL/min of 5 mM $H_2SO_4$ as the mobile phase. Ethanol yield was calculated using the modified equation for xylose conversion [205] specifically for the high solids and high ethanol titer SSF process:

$$\text{Ethanol yield} = \frac{[C1]*W}{976.9-0.804*[C1]}\cdot\frac{1}{(0.511*f*[Biomass]*m*1.111)+(0.511*xylose*total\ liquid)}\times 100\%$$

where $[C1]$ was the ethanol concentration in the culture broth (g/L), $W$ was the total water input of SSF (g), $f$ was the cellulose fraction of corn stover feedstock, $[Biomass]$ was the dry corn stover concentration at the beginning of SSF (g/g), $m$ was the total weight of SSF (g), 0.511 was the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, 1.111 is the conversion factor for cellulose equivalent to glucose, xylose (g/L) after prehydrolysis in the SSF system, and total liquid, volume of liquid (L).

## 5.3. Results and Discussion

### 5.3.1. Effect of temperature on the *Saccharomyces cerevisiae* growth and ethanol fermentability

Fig. 5.1. shows the effect of temperature on parental yeast growth and ethanol fermentability in the synthetic medium. Temperature is one of important factor to be optimized for yeast growth and ethanol fermentation from lignocellulosic material. Temperature ranges of 30, 35 and 37 ℃ were tested, cell growth reduced with increasing temperature. But, there was no major difference in the ethanol fermentability at different temperature ranges.

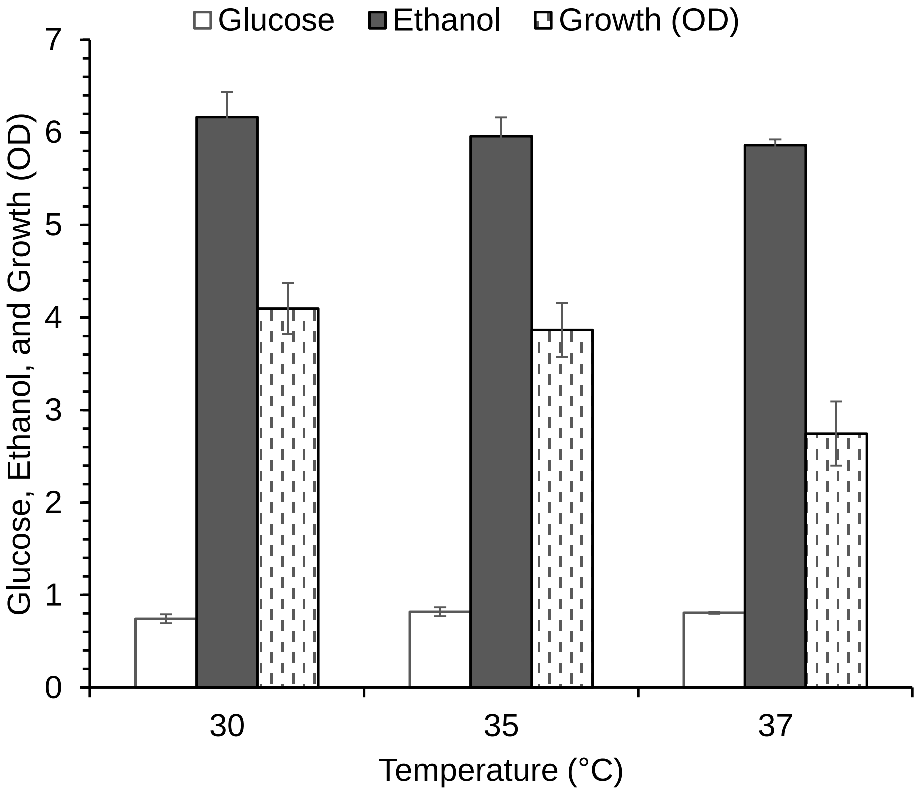


**Fig. 5.1.** Effect of temperature on yeast growth and ethanol fermentability in synthetic medium. Fermentation conditions: 20 mL of synthetic medium containing (20.0 g/L of glucose, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, and 1.0 g/L of yeast extract) incubated for 24 h, at different temperature (30, 35, 37 ℃), 200 rpm.

### 5.3.2. Long term evolutionary adaptation of xylose fermenting *S. cerevisiae* NAN-127

An engineered xylose fermenting yeast *Saccharomyces cerevisiae* NAN-127 was selected as the starting strain [222] for the long-term evolutionary adaptation using the freshly prepared corn stover hydrolysate as the culture medium. The yeast cells are transferred into the fresh corn stover hydrolysate every 24 hours successively, and process was repeated for 85 consecutive days and once glucose utilization rate and ethanol production rate was stable, adaptation was stopped, results are shown in Fig. 5.2.

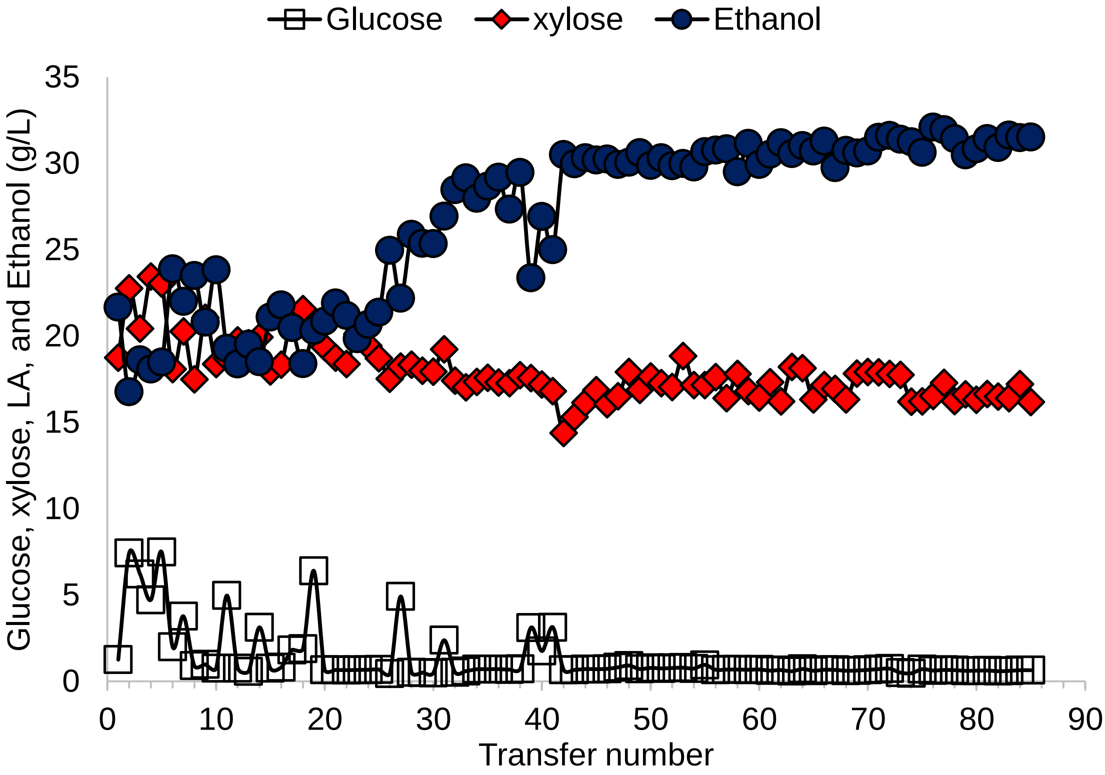


**Fig. 5.2.** Evolutionary adaptation of xylose fermenting *Saccharomyces cerevisiae*. Long term evolutionary adaptation conditions were; 50 % diluted corn stover containing (2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, and 1.0 g/L of yeast extract). Glucose and xylose concentration were adjusted to 60 g/L and 27 g/L with pure sugars. The culture was incubated for 24 h, at 37 ℃, 200 rpm, 5.5 pH.

### 5.3.3. Fermentation of glucose (synthetic medium) by parental and adapted strains

Fig. 5.3 shows the fermentability of glucose (synthetic medium) by parental and adapted strains. There was no significant difference in ethanol titer and glucose consumption by both strains. Slightly improved growth by adapted strain was observed comparing to that of parental strain. No difference in ethanol fermentability is attributed to no inhibitors present in the synthetic medium.

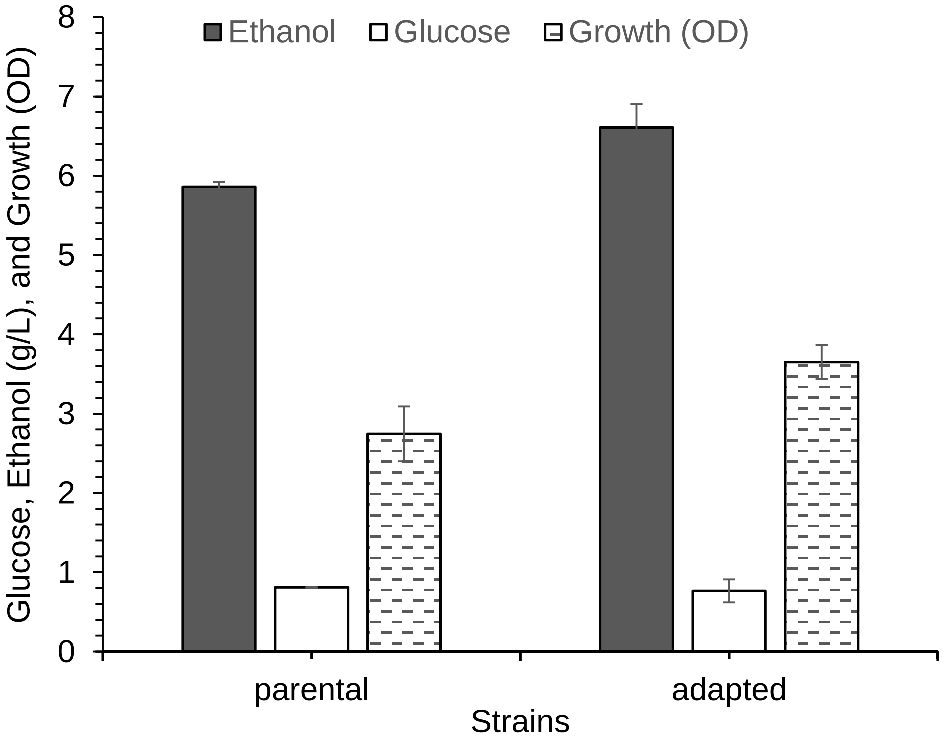
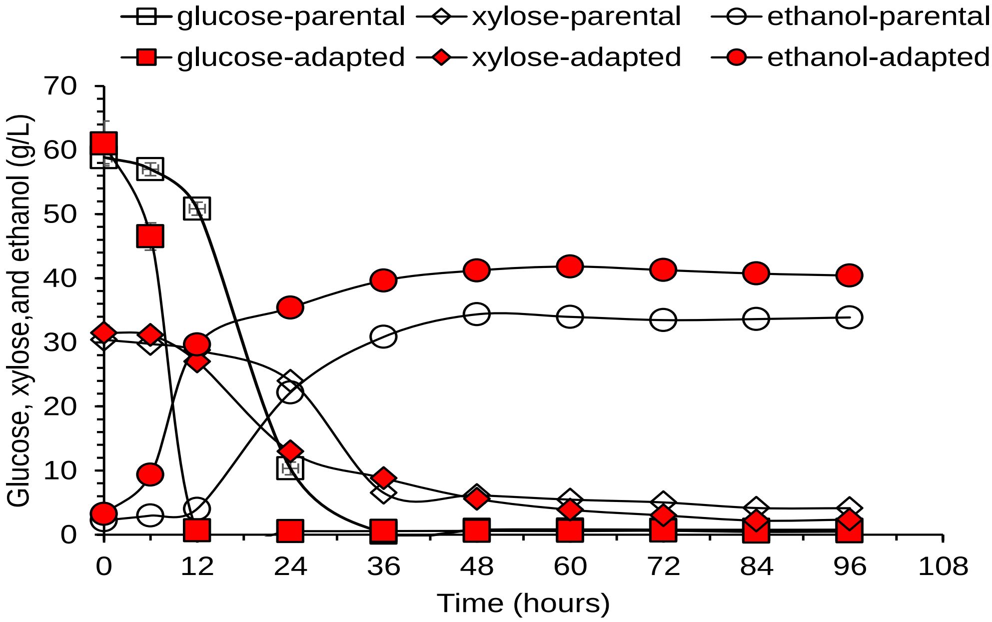


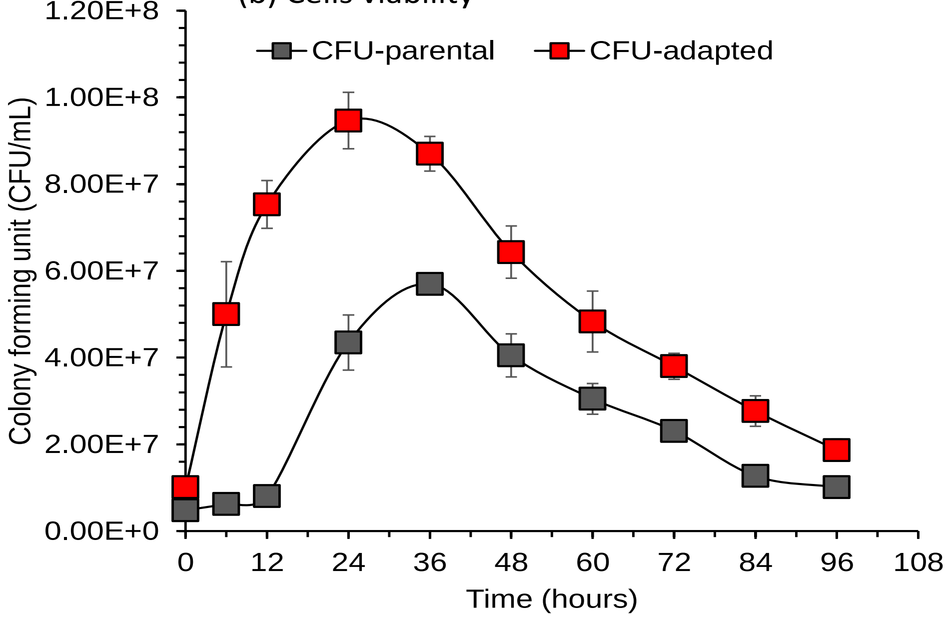
Fig 5.3. Fermentation in synthetic medium by parental and adapted strains. Fermentation conditions: synthetic medium containing (20 g/L of glucose, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, and 1.0 g/L of yeast extract). The culture was incubated for 24 h, at 37 ℃, 200 rpm, pH was adjusted to 5.5.

### 5.3.4. Evaluation of cofermentation of glucose and xylose in corn stover hydrolysate

Fig. 5.4 (a) shows the glucose and xylose cofermentation in the corn stover hydrolysate. Ethanol concentration increased from 33.88 g/L to 41.82 g/L corresponding to 78.99% to 91.01% by adapted strain over parental *S. cerevisiae* NAN-127. More than 90% xylose was consumed during the cofermentation. Fig 5.4 (b) demonstrated the cells viability of parental and adapted strains. Cells viability increased with the passage of time and reached maximum after 24 hours and 36 hours for adapted and parental strains, respectively. Results clearly show the improved ethanol fermentability of adapted strain that was largely due to high inhibitors tolerance as function of adaption.



(a) Ethanol formation, glucose and xylose consumption

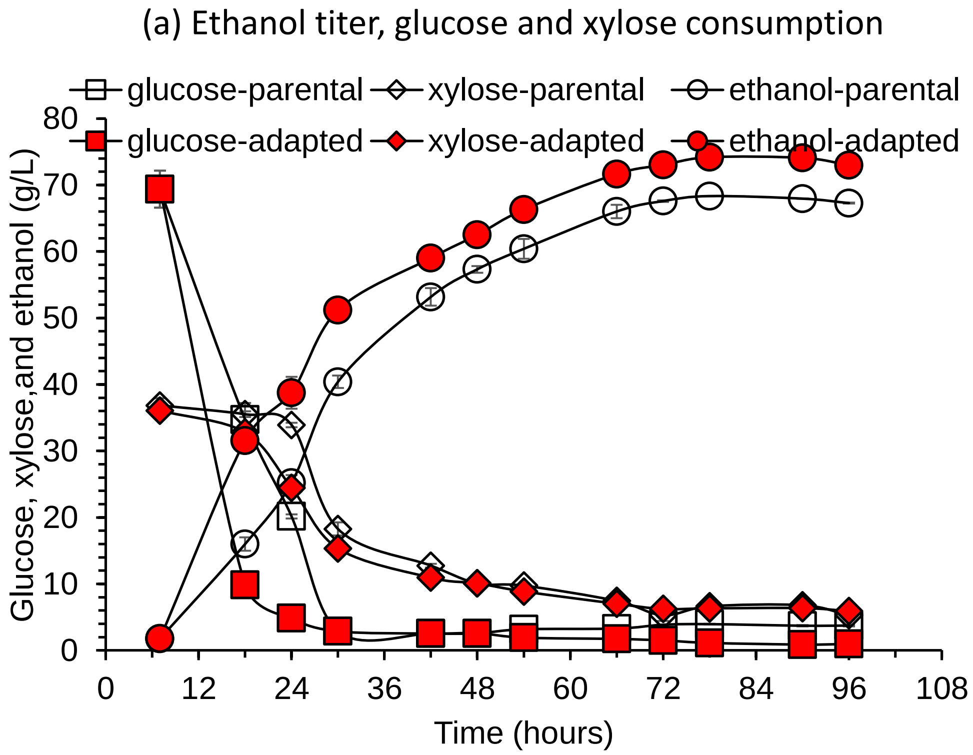


(b) Cells viability

**Fig. 5.4.** Glucose and xylose cofermentation of corn stover hydrolysate. Fermentation conditions: 50 % diluted corn stover containing (2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, and 1.0 g/L of yeast extract). Glucose and xylose concentration were adjusted to 60 g/L and 27 g/L with pure sugars. The culture was incubated for 24 h, at 37 ℃, 200 rpm, pH was adjusted to 5.5 by automatic regulation of pH with 5 M NaOH.

### 5.3.5. Cofermentation of glucose and xylose under industrial relevant conditions

Fig. 5.5 (a) shows the glucose and xylose cofermentation in the high solids content SSF. Ethanol concentration and yield reached to 74.14 g/L and 74.01% through cofermentation of glucose and xylose by evolutionarily adapted *S. cerevisiae*. Ethanol titer and yield of 68.33 g/L and 71.11% was obtained by parental strain. There was not much difference in ethanol titer and yield of parental and adapted strain perhaps due to biodetoxified corn stover was used as feedstock. Adapted strain possess great potential to perform better when potent inhibitors are present in the medium. Xylose concentration was maintained less than 5 g/L in both conditions. Fig 5.5 (b) shows the cells viability of parental and adapted strains. Cells viability increased with the passage of time and reached maximum after 42 hours for adapted and parental strains. Results clearly show the improved ethanol fermentability of adapted strain that was largely due to high inhibitors tolerance as function of adaption.


(a) Ethanol titer, glucose and xylose consumption

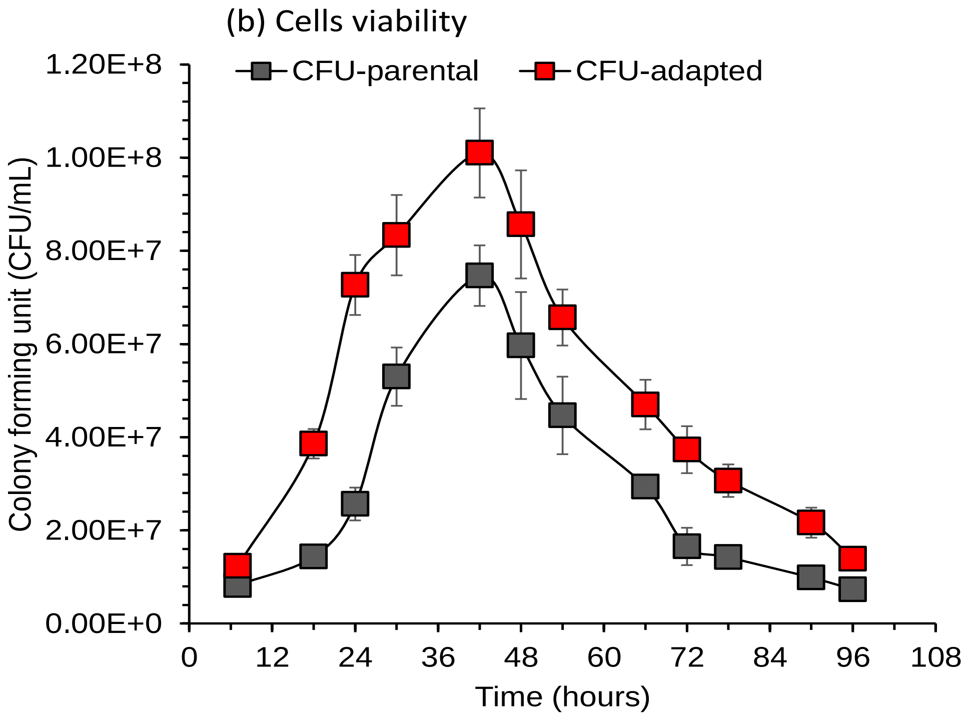


**Fig. 5.5.** Glucose and xylose cofermentation of high solids content in SSF. Fermentation conditions: Prehydrolysis at 30% solids content, 15 FPU/g DM cellulase, 50 ℃, pH 4.8 for 7 hours; then SSF with 10% (v/v) yeast inoculation at 37 ℃ for 89 hours. Nutrients were added as, 1.0 g/L of yeast extract, 2.0 g/L of $KH_2PO_4$, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4 .7H_2O$. pH was adjusted to 5.5 by automatic regulation of pH with 5 M NaOH.

## 5.3. Conclusion

Xylose fermenting *Saccharomyces cerevisiae* NAN-127 was evolutionary adapted in the corn stover hydrolysate for 85 consecutive days. Adapted strain improved the ethanol titer and yield from 33.88 g/L and 78.99% to 41.82 g/L and 91.01%. Ethanol concentration and yield reached to 74.14 g/L and 74.01 when adapted strain was applied in high solids content SSF by cofermentation of glucose and xylose. This ethanol titer is very close to corn based ethanol concentration 78.9 g/L (10.0v/v). Research work going on and main purpose of this study is to reach 10% v/v yield from lignocellulosic material by using DDGS and onsite produced enzyme instead of yeast extract and purchased enzyme.

# Chapter 6

## General conclusions and future perspectives

The objectives of the present study were to reduce the lignocellulosic processing and operational cost for making the lignocellulosic biorefinery economically feasible and environmentally sustainable to produce ethanol and lactic acid. In this study, several strategies were developed and evaluated by improving the ethanol and lactic titer and yield by reducing ethanol distillation/lactic acid purification cost. In addition, no wastewater generated from pretreatment to fermentation process. Various conclusions can be drawn from the results and many suggestions can be made for future perspective.

Solid seeds culture (SSC) method described in (Chapter 2), was developed as simplified method for yeast seeds culture preparation by using freshly pretreated lignocellulosic material as a carbon source. Generally, glucose is used as a carbon source for preparation of yeast seeds culture, which is not feasible for industrial applications. In SSC process, freshly pretreated corn stover solids, enzyme, and yeast cells were mixed in a flask to operate seeds culture in SSF mode. No any difference in the yeast seeds quality and ethanol fermentation performance was observed when the yeast seeds were cultured by glucose, the corn stover hydrolysate liquid, and the pretreated corn stover solids as carbon sources, respectively. Some of the studies have utilized hydrolysate as carbon source for yeast seeds culture, this pose several difficulties, including separate bioreactor for enzymatic hydrolysis, separation of clear liquid from solids, and chances of contamination during separation. These difficulties were directly cut by solids seeds culture. In addition, unused cellulase was also recycled.

Solid seed culture saved about 22 % of yeast seeds culture preparation cost comparing to the other studies where pure glucose is used as a carbon source for preparation of yeast seeds culture, a detailed cost calculation is shown in Table 2.1 (Chapter 2). In addition, centrifugation cost and additional bioreactor required for preparation of clear hydrolysate solution were also saved by developing the simple seeds culture method.

Pretreatment inhibitors pose technical challenge, especially at high solids content in SSF, long term evolutionary adaptation of *Saccharomyces cerevisiae* DQ1 improved ethanol titer and yield over parental strain. Adaptation was performed for 65 consecutive days in corn stover hydrolysate. The ethanol fermentation performance improved with ethanol titer of 71.40 g/L and the high yield of 80.34% in high solids content SSF by using adapted yeast and cost effective nutrient (DDGS) (Chapter 3). DDGS cost was only 0.5% compared to that of yeast extract.

Open L-lactic acid fermentation of corn stover by a pediocin producing *Pediococcus acidilactici* TY112 strain was evaluated to save sterilization cost from bioreactor to feedstock and nutrients. In the open L-lactic acid fermentation; feedstock, medium, water, and bioreactor were not sterilized and sample was also taken in open conditions. In addition, contaminant bacteria were manually added in the fermentation system, pediocin eliminated the growth of contaminants. There was essentially no difference in lactic acid titer and yield under sterilized or unsterilized conditions. For further confirmation of pediocin function, trypsin was added in the fermentation medium that hydrolyzed the pediocin, and resulting contaminant strains were growing normally. While, in absence of trypsin growth of contaminant strain was eliminated. High L-lactic acid titer, productivity and yield of 97.30 g/L 1.47 g/L/h, and 69.38%, respectively, were obtained in high solids content SSF under completely unsterilized conditions. Open L-lactic acid fermentation not only reduce the sterilization cost and step but, this developed a robust and simplified fermentation process for its commercial applications.

Xylose fermenting *Saccharomyces cerevisiae* NAN-127 was evolutionary adapted in the corn stover hydrolysate for 85 consecutive days. Adapted strain improved the ethanol titer and yield from 33.88 g/L and 78.99% to 41.82 g/L and 91.01%. Ethanol concentration and yield reached to 74.14 g/L and 74.01 when adapted strain cofermented the glucose and xylose under industrial relevant conditions. This ethanol titer is very close to corn based ethanol concentration 78.9 g/L (10.0% v/v). Research work going on and main purpose of this study is to reach 10% v/v yield from lignocellulosic material by using DDGS and onsite produced enzyme instead of yeast extract and purchased enzyme.

Research presented in this thesis shows great potential in improving the lignocellulosic biorefinery and making possibilities to commercialize it. Freshly pretreated corn stover materials as carbon source and dry distillers' grains and solubles as nutrient are of special significance. Furthermore, the high solids content SSF process with zero wastewater generation from pretreatment to fermentation is of significant importance. In the evolutionary adaptation studies, only ethanol fermentability and inhibitors tolerance were studied. However, molecular changes due to random mutation during long term evolutionary adaptation could be investigated for further understanding the changes at genomic scale. Besides these advancements, several challenges, including pretreatment and SSF at continuous mode and high enzyme cost still need a proper solution. Thus, more efforts are certainly required for making process economically sustainable at commercial scale.

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## List of Publications

- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. Cellulosic ethanol fermentation using *Saccharomyces cerevisiae* seeds cultured by pretreated corn stover material. **Applied Biochemistry and Biotechnology,** 2015, 175:3173-3183.
- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain. **Bioresource Technology,** 2015, 189:399-404.
- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. Open fermentation of high titer L-lactic acid from corn stover by a pediocin producing *Pediococcus acidilactici* strain. In submission to **Biotechnology and Bioengineering**.
- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. Cofermentation of glucose and xylose by evolutionarily adapted xylose fermenting *Saccharomyces cerevisiae* under industrial relevant conditions (In preparation).

## Presentations

- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. Yeast seeds culture using the pretreated corn stover and the consequent cellulosic ethanol fermentation in 6th International Symposium on Biocatalysis and Biosynthetic Engineering held on November 11-13, 2013 at East China University of Science and Technology.
- **Abdul Sattar Qureshi**, Jian Zhang, Jie Bao. SSF for high titer ethanol production by adapted *Saccharomyces cerevisiae* DQ1 under high solids loading of corn stover in AFOB Bioenergy and Biorefinery Division Annual Meeting and Bioenergy and Biorefinery Summit 2014 held on August 24-27th, 2014 at Shandong University, Jinan, China.

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