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Study on amino acids fermentability and inhibitor tolerance of *Corynebacterium glutamicum* strains using lignocellulose feedstock

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Abstract

Currently, fermentation medium accounts for large percentage of production cost of biochemicals. Utilization of lignocellulosic material as feedstock for amino acids production could be a proper solution to this difficulty. However, high inhibitors content in the pretreated lignocellulosic material is the major technical bottleneck when lignocellulose is used as feedstock of amino acid fermentation. Inhibitory compounds from pretreatment of lignocellulose feedstock negatively interfere with the consequent enzymatic hydrolysis and fermentation. In this thesis, the difficulty was overcome by several practical approaches on improving amino acids fermentability of *Corynebacterium glutamicum* using lignocellulosic feedstock. Two *Corynebacterium glutamicum* strains were used in this study. *Corynebacterium glutamicum* SIIM B460 was used for glutamic acid fermentation and *Corynebacterium glutamicum* SIIM B253 was employed for lysine fermentation using freshly pretreated corn stover feedstock without inhibitor removal.

L-Glutamic acid is commercially important amino acid that is mainly used as food additive and flavor enhancer in the form of sodium salt. In the first part of study, tolerance and degradation of lignocellulose derived inhibitors were analyzed for *C. glutamicum* SIIM B460 for glutamic acid production, and strain with enhanced tolerance and degradation capability was evolved by evolutionary adaptation. Inhibitor tolerance of *C. glutamicum* SIIM B460 for glutamic acid fermentation was analyzed for ten inhibitors in isolation using synthetic media. Strain demonstrated strong tolerance against furans (furfural, 5-hydroxymethylfurfural (HMF)), various organic acids (acetic acid, formic acid, levulinic acid), and some of the phenolic compounds (4-hydroxybenzaldehyde, vanillin), but sensitive to coniferyl aldehyde and syringaldehyde. Degradation capability of strain was analyzed for five inhibitors in isolation using synthetic media. *C. glutamicum* was able to degrade furfural, HMF, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde into the corresponding alcohols and acids with less toxicity to the strain. Evolutionary adaptation approach was developed using inhibitor containing hydrolysate. Adaptation improved the tolerance and degradation capability of the strain and consequently glutamic acid fermentation and cell growth were improved. Finally putative degradation pathways were proposed for furfural, HMF, acetic acid, vanillin, 4-hydroxybenzaldehyde and syringaldehyde on the basis of previous studies reported.

Lysine is an essential amino acid, is valuable as medicament, chemical agent, food material (food industry), and as feed additive (animal food). In the second part of study tolerance and degradation behavior of *C. glutamicum* B253 were analyzed and non-biodetoxified corn stover hydrolysate was used for lysine production. *C. glutamicum* SIIM B253 was employed for lysine production using corn stover hydrolysate. 10 typical inhibitors produced from lignocellulose pretreatment were selected and used for testing the tolerance of

strain for lysine fermentation. Strain revealed strong tolerance against nearly all pretreatment inhibitors when used in isolation in synthetic media, including furans (furfural, 5-hydroxymethylfurfural (HMF)), organic acids (acetic acid, formic acid, levulinic acid), and phenolic compounds (4-hydroxybenzaldehyde, vanillin, coniferyl aldehyde, syringaldehyde). Tolerance mechanism was investigated by examining the response of *C. glutamicum* SIIM B253 on biodegradation of furfural, HMF, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde and acetic acid. Lysine fermentation was compared using synthetic media, biodetoxified and non-biodetoxified hydrolysate. In contrast to various other strains, *C. glutamicum* B253 growth and lysine fermentability was better in terms of tolerance and degradation capacity and produced comparatively higher lysine concentration in non-biodetoxified hydrolysate compared to synthetic medium and biodetoxified hydrolysate. Furfural, HMF, vanillin, 4-hydroxybenzaldehyde and acetic acid present in non-biodetoxified hydrolysate were degraded very quickly. Different inoculum ratios did not have any significant difference for lysine production. While revealed strong tolerance of strain to grow and degrade the inhibitors present in non-biodetoxified corn stover hydrolysate even at 1% inoculum ratio. Results revealed that *C. glutamicum* is potential strain with strong tolerance against pretreatment inhibitors.

The research works presented in this thesis show the technical advantages of using *C. glutamicum* strains for amino acid production using lignocellulose feed stock. Evolutionary adaptation strategy and the inherently tolerant *Corynebacterium glutamicum* SIIM B460 could be applied for the biorefinery process of inhibitors enriched lignocellulosic material for glutamic acid production. *C. glutamicum* B253 due to its excellent tolerance and degradation capability, can be used in non-biodetoxified hydrolysate, reducing the biodetoxification step, with high lysine yield saving time and cost. The approach for amino acids production, proposed in this thesis, has benefits at certain range. These advancements certainly provide help on the commercialization of amino acids production from lignocellulosic material. Further efforts are required for development of fast, efficient, and cost effective amino acids production.

KEY WORDS: *Corynebacterium glutamicum*, inhibitor tolerance and degradation, lignocellulose, glutamic acid, evolutionary adaptation, lysine

Table of contents

Chapter 1

General Introduction

1. Background

Lignocellulosic biorefinery and biochemical production is one of the emerging research fields these days. However, utilization of lignocellulosic materials for production of biochemicals, for example, amino acids (glutamic acid and lysine), and organic acids (lactic acid, citric acid etc) is an expensive process. Biomass utilization as an energy source for biochemical production is a promising choice. For making overall process economical, lignocellulosic operational and processing cost should be reduced and the performance of fermenting strains should be enhanced.

The biotechnological production of amino acids by *C. glutamicum* needs a continuous improvement of production process, with special focus on optimization of the fermentation parameter and cost effective method. Besides the existing methods of industrial fermentations, many efforts are underway to improve the glutamic acid and lysine production, utilizing inexpensive available substrates to reduce production costs. So far, better and cost-effective methods of glutamic acid and lysine production have always been major issue for scientific community [1, 2]. Therefore, the main objective of this study is to use lignocellulosic biomass as a cheap energy source for amino acids production, using inhibitor tolerant strain, with the capability to degrade the inhibitors, and process optimization.

Lignocellulosic biomass is considered as a promising feedstock because it is cheap, plentiful, and renewable [3]. Its utilization in biofuels and other value added commodities could decrease environmental pollution, create job opportunities, and get rid of the controversial issues over land application in the 1st generation products. Lignocellulosic biomass is highly recalcitrant due to its physical and chemical structure, which confers rigidity to the plant cell wall. It cannot be directly utilized by most of the microorganisms, so it requires pretreatment [4, 5]. Pretreatment is essentially needed for utilization of lignocellulosic biomass for preparation of value-added products; including glutamic acid, lysine, lactic acid and ethanol. Pretreatment is generally performed under severe conditions such as high pressure, high temperature and addition of acids or bases to remove lignin and hemicellulose from cellulose. Due to harsh pretreatment condition, a broad range of toxic compounds are also discharged, which act as inhibitors for microbial growth and enzymatic saccharification. The pretreatment inhibitors are primarily grouped as furans, organic acids and phenolic compounds [5]. These inhibitors generally inhibit the cell growth and fermentability of the strain.

Lignocellulose biorefinery needs to achieve technical perfection before commercialization of the process. The lignocellulose bioconversion technology for obtaining the value added commodities has consistently been improved over the last few decades. As a result, dry dilute acid pretreatment (DDAP) [6-8], onsite enzyme preparation, designing the unique bioreactor with helical stirrer [9], and evolutionary adaptation are the key achievements [10-12].

Quick, cost effective, and efficient amino acid production from lignocellulose biomass, in the presence of these pretreatment inhibitors requires management of many technical aspects: (1) Using inhibitor tolerant strain. (2) Biochemical production using strain which can degrade the inhibitors and produce the biochemical simultaneously, saving cost and time (3) Perform evolutionary adaptation of amino acid producing strains to increase the tolerance and biodegradation capability of strain.

In present study, corn stover was used as cost effective carbon source for glutamic acid and lysine production from *Corynebacterium glutamicum* strains. Tolerance and biodegradation capability of the strains towards the pretreatment inhibitors was analyzed.

This thesis consists of four chapters including, a general introduction, research work, and a general conclusion. Chapter 1 consists of introduction to glutamic acid and lysine, biosynthetic pathway and applications, opportunities and challenges to lignocellulose biorefinery for amino acid production. The research work in chapters 2 and 3 is under preparation for submission in the academic journals. Chapter 2, describes the inhibitor tolerance and biodegradation capability of *Corynebacterium glutamicum* SIIM B460 in glutamic acid fermentation. The research work is important in terms of findings of high tolerance and degradation performance of *Corynebacterium glutamicum* SIIM B460 towards pretreatment inhibitors. Adaptation of the strains to the pretreatment inhibitors increased the tolerance and reduced the degradation time to pretreatment inhibitors and enhanced glutamic acid production. Chapter 3, Evaluation of tolerance and degradation of lysine producing *Corynebacterium glutamicum* SIIM B253 to pretreatment inhibitors. This study clearly revealed that strain can grow better and produce more lysine in corn stover hydrolysate without biodetoxification and can quickly degrade the inhibitors present in the hydrolysate. Chapter 4, general conclusion.

1.1. *Corynebacterium glutamicum*

Corynebacterium glutamicum is a non-motile, gram-positive, fast growing, non-spore former, saprophytic, facultative anaerobic bacterium, commonly used for amino acids production. It was first time isolated in Japan [13, 14]. It is found in the soil or on the skin of vegetables and fruits [15, 16]. *C. glutamicum* is different from other gram-positive bacteria due to its typical bilayer plasma membrane , with an inner mycolic acid and outer lipid layer, fixed and stabilized by an arabinogalactan–peptidoglycan polymer complex having a thick cell wall glycan core, with a crystalline surface S-layer, comprising of high molecular mass glycans

and arabinomannans having numerous proteins and lipids [17, 18]. The genome sequences of various strains of *C. glutamicum* and species closely related to *C. glutamicum* have been determined [19-22] and a complete molecular biology toolbox is available to manipulate it [23-25]. Beyond the industrial application of this organism to produce a variety of primary metabolites including amino acids nucleotides and vitamins [26-28], genetically engineered *C. glutamicum* can produce poly(3- hydroxybutyrate), organic acids (e.g., lactate and succinate), ethanol or isopropanol which are used in the transportation and plastic industries [29-33]. Remarkably, when stress is applied to this organism, for instance biotin limitation, penicillin G or moderate heat shock, cerulenin treatment or ethambutol [14], or oxygen deficiency in the non-availability of a terminal electron acceptor [29, 34], autolysis does not occur but rather secretion of amino acids takes place or stops growth but remain metabolically active, may be due to the presence of a persistence and resuscitation process [35-39].

C. glutamicum has also displayed strong inherent potential to produce recombinant proteins, owing to its industrial robustness and efficient protein secretion pathways. It is considered as a biotechnology workhorse of the emerging biorefinery industry. *C. glutamicum*-based processes have been developed or are being developed to produce amino acids including: L-threonine [13], L-phenylalanine [27], L-tryptophan [40], L-arginine [40-42], L-histidine [40], L-valine [40, 43, 44], L-alanine [45], L-cysteine [46], L-glutamine [47-49], L-isoleucine [50, 51], L-leucine [52], L-methionine [53, 54], L-proline [55, 56], L-serine [57, 58], L-tyrosine [59], L-citrulline [60], and L-ornithine [61, 62]. More than 30 biochemicals are produced by *C. glutamicum* in the largest amount by weight are: ethylene, formaldehyde, propylene, propylene oxide, ethylene dichloride, nitrobenzene, methanol, cyclohexane, vinyl chloride, benzene, vinyl acetate, ethylbenzene, styrene, acrylic acid, terephthalic acid, cumene, ethylene oxide, bisphenol-A, p-xylene, ethylene glycol, butadiene, phenol, acrylonitrile, acetic acid, a-olefins, acetone, n-butanol, adipic acid, aniline and caprolactam, [63, 64].

1.2. Glutamic acid production

Glutamate was recognized as a compound responsible for a unique taste, called “umami”, isolated from a hot water extract of seaweed by a Japanese chemist, Kikunae Ikeda, in 1908. Dr. Ikeda discovered the flavor enhancing property of monosodium glutamate ($C_5H_3NO_4Na.H_2O$) [65]. It is crystalline white, transparent and readily soluble in water (73% w/v). Glutamic acid, is a significant chemical primarily employed in the production of monosodium glutamate. It is also widely used in food, chemical, pharmaceutical and other sectors. Kinoshita with his coworkers in 1957, isolated *Corynebacterium glutamicum*, which was initially termed as *Micrococcus glutamicus*, as an L-glutamate-producing bacterium [66, 67]. This was an innovation in the biotechnological production of amino acids by microbial fermentation. In contrast to chemical synthesis, this ensures that only the biologically active L-forms are produced. L-Glutamate is now widely used as a flavor enhancer. Glutamic acid production has reached to about 2.1 million tons worldwide per year through fermentation

using *C. glutamicum* or other closely related species and its need is gradually increasing annually at a rate of 3–5% [68].

The glutamate production process using *C. glutamicum* is now a well-established technology [13, 27, 40, 65, 69]. Biotin is an essential cofactor (required by the enzyme acetyl CoA carboxylase) for the production of fatty acids but under conditions of excess biotin, the wild-*type C. glutamicum* type strain does not secrete glutamate. Due to a limited supply or deficiency of biotin, fatty acid biosynthesis and consequently phospholipid synthesis is drastically reduced. As a result, membrane formation (protein- phospholipid complex) is defective which alters permeability for an increased export of intracellular glutamic acid. It is found that there is an alteration in the membrane composition of phospholipids in oleic acid and glycerol auxotroph mutants. This facilitates release of intracellular glutamic acid. The knowledge on the membrane permeability of glutamic acid is successfully exploited for increased industrial production of glutamic acid [14, 68, 70]. The glutamic acid production pathway from glucose is shown in Fig. 1.1. Glucose is broken down to phosphoenol pyruvate and then to pyruvate. Pyruvate is converted to acetyl CoA. Phosphoenol pyruvate (by the enzyme phosphoenol pyruvate carboxylase) can be independently converted to oxaloacetate. Both these carboxylation reactions are quite critical, and require biotin as the cofactor. The next series of reactions that follow are the citric acid (Krebs) cycle reactions wherein the key metabolite namely α-ketoglutarate is produced. In the routine citric acid cycle, α-ketoglutarate is acted upon by the enzyme α- ketoglutarate dehydrogenase to form succinyl CoA. For the production of glutamic acid, α-ketoglutarate is converted to L-glutamic acid by the enzyme glutamate dehydrogenase (GDH). This enzyme is a multimer, each subunit with a molecular weight of 49,000. The reducing equivalents, in the form of NADPH + H^+, are required by GDH. They are generated in the preceding reaction of Krebs cycle (catalysed by the enzyme isocitrate dehydrogenase) while converting isocitrate to α-ketoglutarate. The supply and utilization of NADPH + H^+ occurs in a cyclic fashion through the participation of the two enzymes, namely isocitrate dehydrogenase and glutamate dehydrogenase. The essential requirement for glutamic acid production is the high capability for the supply of the citric acid cycle metabolites. This is made possible by an efficient conversion of phosphoenol pyruvate as well as pyruvate to oxaloacetate .Thus, there are two enzymes (phosphoenol pyruvate carboxylase and pyruvate carboxylase) to efficiently produce oxaloacetate, while there is only one enzyme (pyruvate dehydrogenase) for the formation of acetyl CoA. Certain microorganisms which have either phosphoenol pyruvate carboxylase (e.g., *E. coli*) or pyruvate carboxylase (e.g. *B. subtilis*) are not capable of producing glutamic acid to any significant extent. *C. glutamicum* has both the enzymes and therefore can replenish citric acid cycle intermediates (through oxaloacetate) while the synthesis of glutamic acid occurs. Another key enzyme that can facilitate optimal production of glutamic acid is α-ketoglutarate dehydrogenase of citric acid cycle. Its activity has to be substantially low for good synthesis of glutamic acid, as is the case in *C. glutamicum*. Further, exposing the cells to antibiotics (penicillin) and surfactants reduces the activity of α-ketoglutarate dehydrogenase while glutamate dehydrogenase activity remains unaltered. By this way, oxidation of α-ketoglutarate

via citric acid cycle can be minimized, while the formation of glutamic acid is made maximum possible [68]. Under standard conditions where sufficient quantities of ammonium are present in the fermentation medium, glutamate is produced from 2-oxoglutarate, catalyzed by the enzyme glutamate dehydrogenase (coded for by gdh)[14, 71].

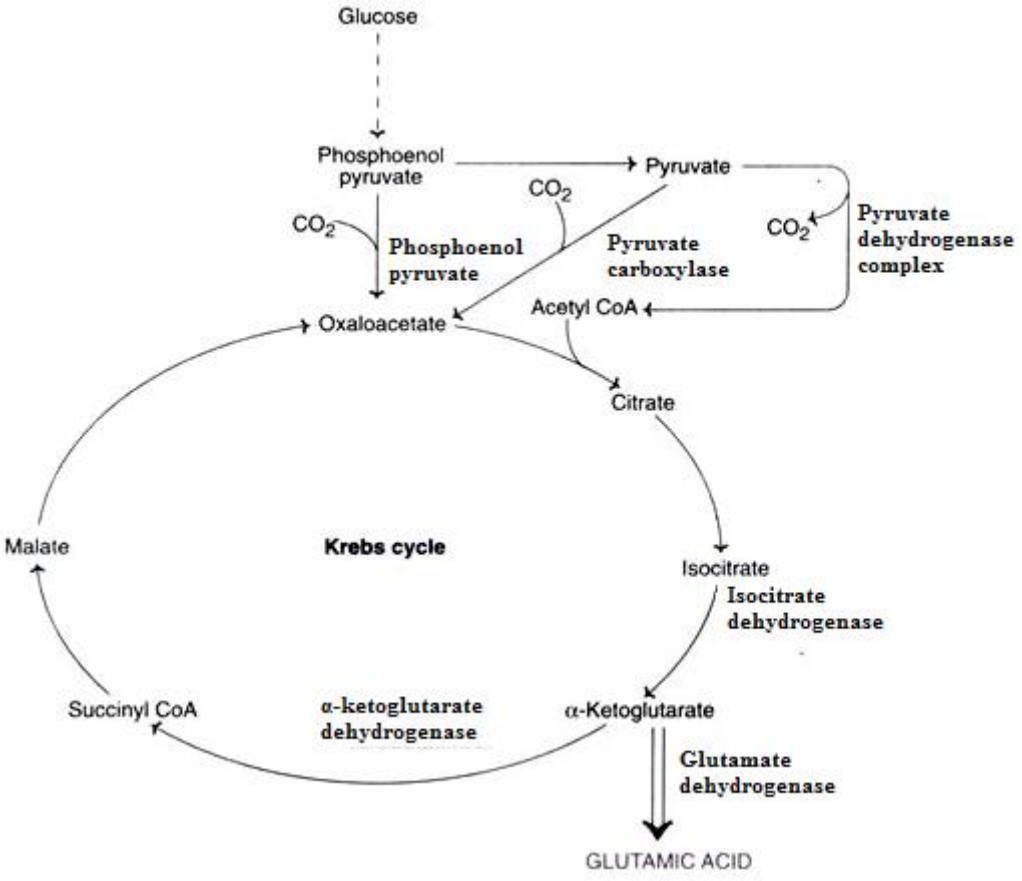

Fig. 1.1. Biosynthetic pathway of L-glutamate in *C. glutamicum*
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Glutamic acid is commercially used as flavor intensifier, to increase the taste in processed food such as noodles, soups, Chinese foods, biscuits, meat and vegetable processing etc. Out of three types of monosodium glutamate, the L, D, and LD, only the L-type has the flavor enhancing property. MSG is also employed as an intermediate in production of Folic acid. Glutamic acid mother liquor in MSG production is being employed in the making of sauce and fertilizer etc. MSG is naturally found in tomatoes, mushrooms and peas [72].
Although, glutamic acid is produced by several living organisms such as plants and microorganism but increasing demands of glutamic acid could be fulfilled by cost effective microbial sources.

1.3. Lysine production

Amino acids are the basic units of proteins which play the key role in biological functions. Lysine is an essential amino acid. Lysine is valuable as medicament, chemical

agent, food material (food industry), and it also is widely used as feed additive (animal food). Corn, barley and wheat which are commonly employed as the main ingredients for animal feed possess suboptimal concentration of lysine, due to this reason lysine becomes limiting for feed efficiency. Lysine demand is continuously increasing in recent years and annual production reached to 2,200,000 tons.

Lysine is produced either by seven or ten steps from its precursor oxaloacetate, depending on which route is employed. The first step in the lysine synthetic pathway is the conversion of oxaloacetate to aspartate by adding an amino-group from glutamate, catalyzed by the *aspB*-gene-product. Aspartate is phosphorylated to L-4-aspartyl phosphate by the reaction catalyzed by the *lysC*-gene-product, and then to L-aspartate 4-semialdehyde via *asd*. L-4-aspartyl phosphate is then converted to dehydrodipicolinate by *dapA*. In the next step L-piperidine 2,6-dicarboxylate is made by *dapB*. At the level of L-piperidine 2,6-dicarboxylate there are two possibilities for the conversion to meso-2,6-diaminopimelate, the last step before lysine. Either the direct reaction adding the second amino-group in one single step is employed (*ddh*), known as the dehydrogenase variant, or four successive reactions, named the succinyl variant, is employed. The succinylase variant involves the *dapD*-, *dapC*-, *dapE*- and *dapF*-gene-products, and using this variant the TCA intermediate succinyl-Coa is involved. The final step is the decarboxylation of meso-2,6-diaminopimelate to lysine (*lysA*), which finally is exported out of the cell by lysine permerase (*lysE*) [73]. Due to the commercial importance of lysine production in *C. glutamicum*, the lysine synthetic pathway has received a lot of attention (Fig. 1.2).

Lysine is known as a vital element for the health and nutrition because of its major importance in biological processes [74]. Lysine stimulates the cell division [75]. It is important for the conversion of fatty acids to simple compounds, energy production and helps in the lowering of blood cholesterol, absorption and storage of calcium, helps in the synthesis of collagen and connective tissues consisting of cartilage, tendons, bones and skin. Lysine helps to clean the arteries which prevents from the heart diseases like angina and cancer [76].

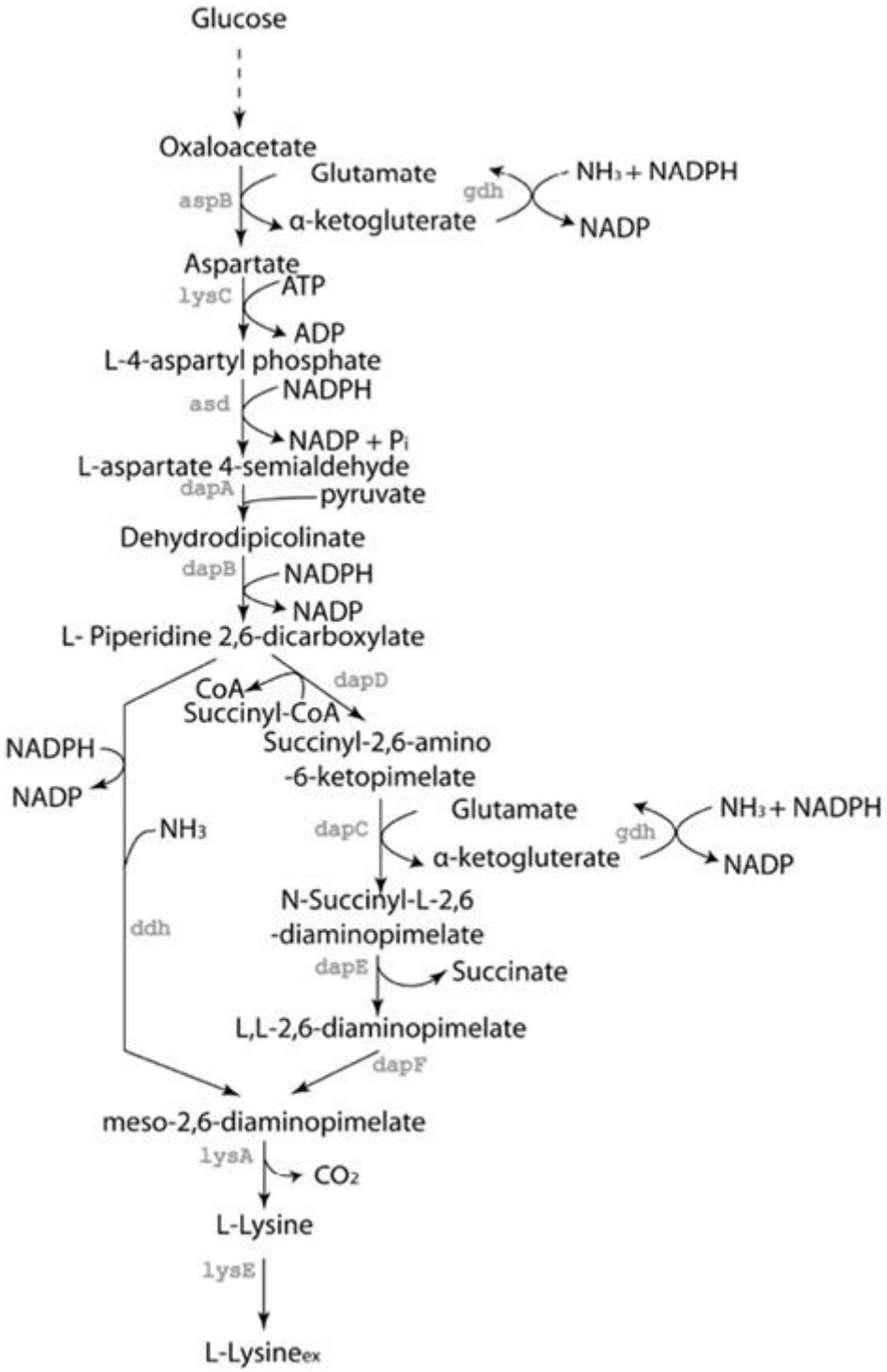


Fig. 1.2. Lysine biosynthetic pathway in *Corynebacterium glutamicum*, including gene-names (grey letters). This information was taken from [50, 73].

1.4. Lignocellulosic Biomass

Worldwide annual production of lignocellulose biomass has been estimated to approach 10–50 billion dry tons [77, 78], this being the most abundant available renewable carbon source. 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.

Generally, cellulose is the dominant structural polysaccharide of lignocelluloses followed by hemicelluloses and lignin [79]. Woody biomass has a relatively high content of cellulose and lignin, whereas grasses have a higher content of hemicellulose and less lignin [78]. Apart from these three major components, plant cell walls also contain minor amount of other substances such as pectins, proteins, extractives, several inorganic compounds, and ash [80], but they do not have significant impact in forming the lignocellulose structure [81]. It is generally believed that the lignin, hemicelluloses, pectin, etc., and their spatial interlinks with cellulose have formed rigid and compact structure (Fig. 1.3) (physical barriers) of plant cell wall which is recalcitrant for the microbial degradation [78, 82].

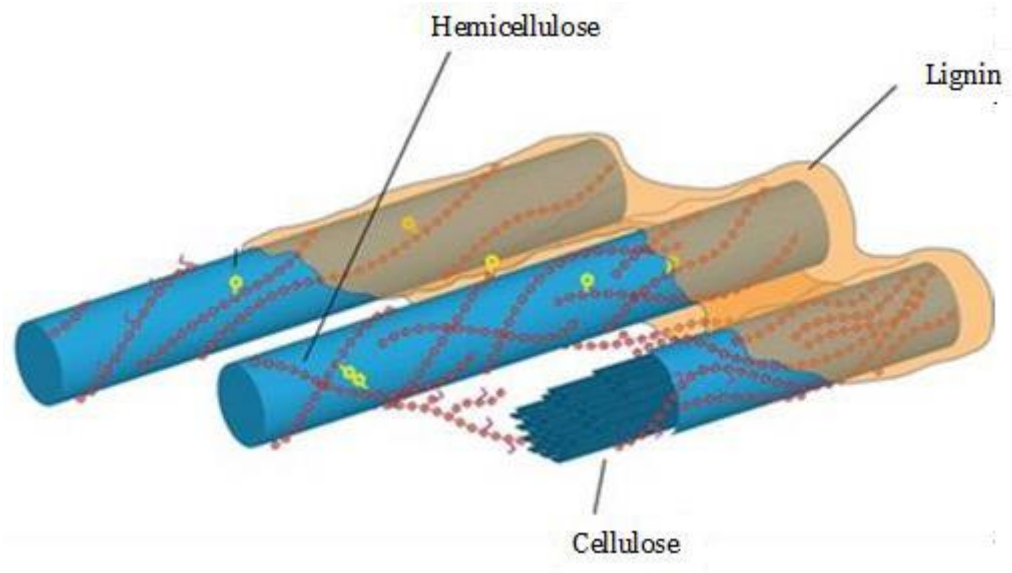


Fig. 1.3. Arrangement of the major structural components in plant cell walls [83, 84]

Lignocellulosic biomass is comprised of three different polymers namely cellulose, hemicellulose, and lignin [85]. Cellulose is most abundant polysaccharide on earth and composed of glucose monomers which are linked by β(1→4) glycosidic bonds, accounts for 35-50% of biomass dry matter basis [86]. Hemicellulose is composed of heteropolymers of hexoses and pentoses, this accounts for 20-35% of dry matter basis [86]. In contrast to cellulose, hemicellulose is highly branched and it has a lower degree of polymerization. A few hydroxyl groups of the sugar units in side chains have been replaced by acetyl groups. Hemicellulose on degradation discharges both monomeric sugars and acetic acid [87]. Hemicellulose is connected to cellulose and lignin by hydrogen bonds and covalent bonds, respectively [88]. Lignin is naturally hydrophobic and amorphous, a complex aromatic polymer resulting from the polymerization of three core 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 [85, 86, 89,

[90]. Lignocellulose provide rigidity and strength to plant cell walls, and protect them from the microbial and insects' attack.

Chemical interaction between cellulose, hemicellulose and lignin

As described earlier, in lignocellulose, cellulose acts as a core (skeleton) of the structure, hemicelluloses are arranged between the cellulose micro- and macrofibrils, whereby both cellulose and hemicelluloses are embedded in lignin (Fig. 1.3). Between these three components, mainly four interpolymer (in between different components) and intrapolymer (within individual components) linkages are identified: ether, ester, carbon-carbon, and hydrogen bonds [91]. A summary of the bonding position is given in Table 1.1.

Table 1.1. Overview of main bonds exist within and in between the three major components of lignocellulose [92].

Linkage	Intrapolymer	Interpolymer
Ether bond	Lignin, (hemi)cellulose	Cellulose-Lignin, Hemicellulose-lignin
Carbon-carbon bond	Lignin	NI
Hydrogen bond	Cellulose	Cellulose-hemicellulose Hemicellulose-Lignin Cellulose-Lignin
Ester bond	Hemicellulose	Hemicellulose-lignin

1.5. Lignocellulose conversion to different products

Lignocellulosic biorefinery for chemicals and biochemicals production involves following steps: pretreatment, conditioning or detoxification, enzymatic hydrolysis, fermentation, and purification or distillation. Fig. 1.4 shows the advanced dry lignocellulosic biorefinery processes developed by our group, and this process has no wastewater generation from pretreatment to fermentation [6-9].

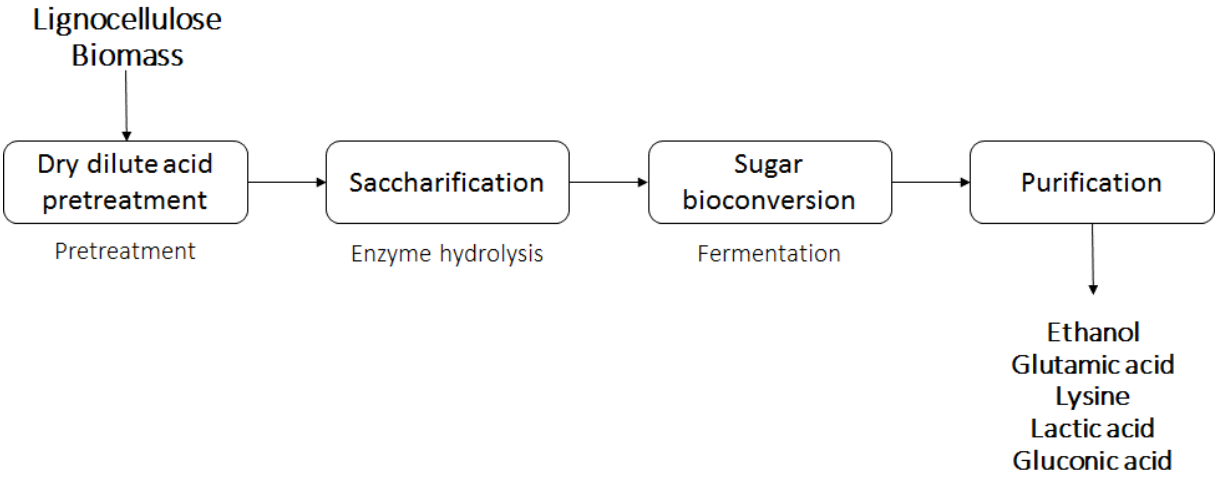


Fig. 1.4. Overview of biomass conversion pathways:

1.5.1. 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 availability of the cellulose to hydrolytic enzymes for translation 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 [85].

In the last few decades, multiple 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 [85, 86, 93-97]. 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 employed to dissolve hemicelluloses, while lignin is typically dissolved by alkaline or organosolv pretreatments [6, 8, 85, 96, 98-101]. 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 [96, 98]. Biological pretreatment either make use of microorganisms, including *Phanerochaete chrysosporium, Ceriporiopsis. subvermispora, Trametes* versicolor, and *Pleurotus ostreatus* to de lignify or enzymes (laccase) to remove phenolic compounds. The biological pretreatment seems to be a best technique, It has many advantages, for example low energy input, mild environmental conditions, no chemical requirement and environmentally friendly. But some of its disadvantages include, time consuming, need careful management of growth conditions, and requires more space [85, 102-107].

All pretreatment methods have some advantages and some 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, enhance cellulose recovery, enhance hemicellulose and lignin removal, zero wastewater generation, less inhibitors formation, cost effective, and to be operated at high solids content [6-8]. Dilute acid pretreatment method is commonly employed. The core disadvantages of dilute acid pretreatment are huge wastewater generation due to low solids content employed in pretreatment, the solids/liquid separation causes loss of fermentable sugars after pretreatment, and comparatively high inhibitor compounds generation. Recently, our group has established

a novel dry dilute acid pretreatment (DDAP) method [6, 8] 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 [9, 108-110]. This pretreatment method is known as 'dry' method, as the corn stover feed stock and product both were 'dry', no free water was generated during the pretreatment. In this fashion, three difficulties of conventional dilute acid pretreatment were solved: dry in and dry out, no wastewater generation, dry pretreated product thus no solids/liquid separation required because of dry pretreated product, and low concentration of pretreatment inhibitor generation.

In past several decades, a large number of pre-treatment techniques (some presented in Table 4 have been investigated and are comprehensively reviewed by [92, 96, 111-121]. In literature, lignocellulose pretreatment technologies are majorly classified into: physical & mechanical, chemical, physico-chemical, and biological. Some of the most commonly employed pretreatment techniques of lignocelluloses and their comprehensive comparison is presented in Table 1.2.

Table 1.2. Advantages and disadvantages of some lignocellulose pretreatment methods. Source: [84, 98, 117, 122].

Pretreatment method	**Advantages**	**Disadvantages**
Mechanical comminution	Reduces cellulose crystallinity	Power consumption is usually high
Alkali	Efficient removal of lignin Low inhibitor formation	High cost of alkali Alters lignin structure
Acid (dilute)	Solubilizes hemicellulose Low acid consumption Short processing time Acid recovery is not required	High pressure and temperature are needed Cellulose hydrolysis is not effective Formation of inhibitors Equipment corrosion
AFEX	Highly effective for agricultural residues Cellulose becomes more accessible Reduce the lignin content Low formation of inhibitors	High cost of ammonia Ammonia recycling is needed Alters lignin structure Less effective for the lignin rich materials e.g. softwoods
ARP	Removes most of the lignin Cellulose rich pulps are obtained after pretreatment Most suitable to herbaceouses	High energy costs and liquid loading Less satisfactory for softwoods

CO_2 explosion	Accessible surface area is increased Cost effective Does not cause formation of byproducts	Lignin or hemicelluloses are unaffected
Green solvents (ILs)	Lignin and hemicellulose hydrolysis Ability to dissolve high loadings of different type of lignocelluloses Mild processing conditions	Potentially high solvent costs Need for solvent recovery and recycle Unknown eco-toxicology of many formulations
LHW	Separation of nearly pure hemicellulose from rest of feedstock No need for catalyst Hydrolysis of hemicellulose	High energy/water input Solid mass left over will need to be dealt with (cellulose/lignin)
Ozonolysis	Reduces lignin content Does not produce byproducts	Large amount of ozone is required Expensive
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents recovery and recycle is needed Requires high energy High cost
SCF	Low degradation of sugars Cost effective Increases cellulose accessible area	High pressure requirements Lignin and hemicelluloses are unaffected
Steam	Cost effective Lignin transformation Solubilization of hemicelluloses	Hemicellulose degradation Acid catalyst is needed to improve the process performance Formation of byproducts
Biological	Degrades lignin and hemicelluloses Low energy requirement	Hydrolysis is very slow

AFEX: Ammonia Fiber Explosion; ARP: Ammonia Recycle Percolation; IL: Ionic liquid; LHW: Liquid hot water; SCF: Supercritical fluid

1.5.2. Enzymatic hydrolysis

Cellulose makes up the largest fraction of the sugars in lignocellulosic materials (Table 1.1), while glucose is main carbon source for numerous microorganisms. Nonetheless, development of microorganisms fermenting hemicellulose sugars is on the rise. Majority of

the pretreatment methods also eliminate and worsen the hemicelluloses, partly. For these reasons, main attention has been given to improving cellulases activity, reducing its cost and dosage. Though, some pretreatment methods leave the hemicelluloses in the material [123]. Hydrolysis involves multiple enzymes, for example, endoglucanase, exoglucanase or cellobiohydrolases, β-glucosidases, and xylanases [124]. High solids content in the enzymatic hydrolysis enhances product inhibition, thus lowering the enzymes' performance. Existence of lignin enhances non-productive enzyme binding that decreases the availability of enzyme for hydrolysis. In addition, some enzymes may lose the activity because of denaturation or degradation. Other reasons are closely related to the substrate composition and thus the pretreatment method employed. These include cellulose crystallinity, cellulose degree of polymerization, substrate's available area, hemicellulose content, feedstock particle size, porosity, and cell wall thickness [98, 123]. To cut their costs, development of more efficient hydrolytic enzymes is the hot topic of research. Many strategies exist for improving the enzyme mixtures, stability, and specific activity of the enzymes. Enzyme mixture should be designed based on substrate specificity. Other avenues should be explored to further improve the enzyme activity, and reduce its cost and dosage.

1.5.3. Fermentation

The conversion of pretreated lignocellulosic biomass into desired product can be accomplished either by using separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation. In SHF, enzymatic hydrolysis and fermentation are done in optimal conditions, but separate bioreactor is needed for each step. In SSF, two steps are integrated to reduce the capital investment and simplify the fermentation process. Enzyme and fermenting microorganisms are mixed together in same bioreactor. Enzymatic hydrolysis discharges sugars and simultaneously converts into final products by fermenting strains. Fed-batch SSF has been proven to be beneficial for various aspects. For example: (a) ease of mixing after partial saccharification, hence SSF operation at high solids content; (b) lower energy consumption thanks to lower viscosity; (c) maintaining low inhibitors concentration that helps the fermenting strain to convert them to less inhibitory compounds, and (d) retaining low glucose concentration in the medium, facilitating effective glucose and xylose cofermentation by engineered strains [125].

1.6. Degradation products of lignocellulose pretreatment - Inhibitors

Pretreatment of lignocellulose biomass may produce degradation products, mostly from sugars and lignin, such as furan aldehydes, aliphatic acids and phenolic compounds (Fig. 1.5). Presence of these compounds affects both saccharification and fermentation steps in the bio-conversion of lignocellulose. Pretreatment by-products generation however mainly dependents on type of raw material (due to the heterogeneity of biomass, plant cell walls differ in their easiness of degradation), pretreatment method, conditions, and the catalyst.

Thus, the composition of inhibitors in lignocellulosic hydrolysates will greatly vary. These toxic compounds severely inhibit cellulase activity, cell growth, and fermentation. Thus, it is important to remove these inhibitory compounds prior enzymatic hydrolysis. Fermentation inhibitors are classified into three groups; furans, phenolic compounds and weak acids.

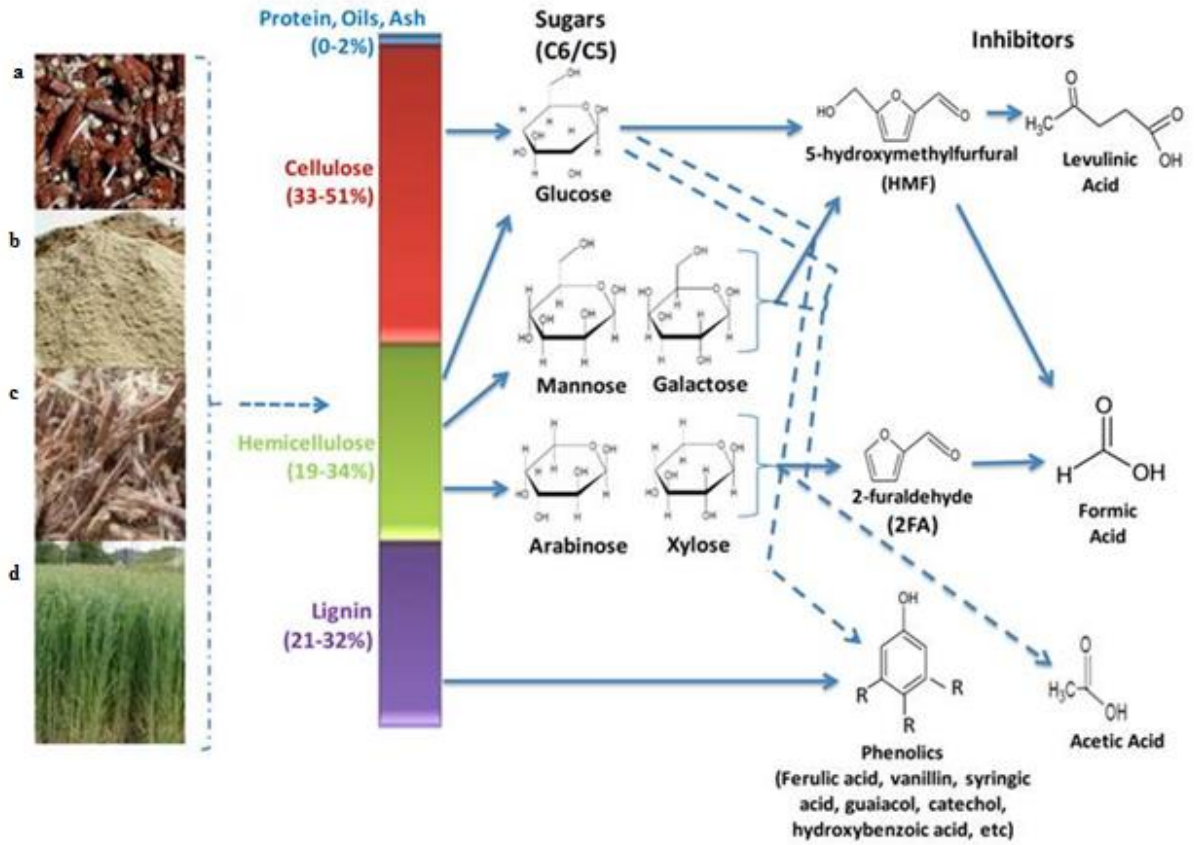


Fig. 1.5. Inhibitors generated during pretreatment of lignocellulose biomass. Lignocellulose biomass includes a: maize cobs, b: saw dust, c: sugar cane bagasse, d: fast growing grasses [126].

1.6.1. Furans

Extensive degradation of (hemi) celluloses are responsible for the formation of furan aldehydes, predominantly furfural and 5-Hydroxymethyl furfural (HMF) Fig. 1.5. [92]. Kinetic studies have shown that the biomass pretreatments (e.g. using acid catalyst) at high temperatures (>160 ℃) and a longer residence time have been reported to be significant for formation of furan aldehydes and their concentrations strongly enhance with increasing temperature and reaction time. Pentose sugars dehydrate to furfural, while hexose sugars degrade to HMF [127].

Furans are discharged 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. HMF and

furfural also causes malfunctioning in the DNA repairing mechanism [128]. In addition, synergetic effect of furans in combination with other inhibitors, including acetic acid, levulinic acids formic acid, and phenolic compounds is more toxic than single inhibitor [129, 130]. Furfural is converted to furfuryl alcohol by the action of NADH-dependent alcohol dehydrogenase (ADH) in the yeast [131]. Furfural decrease the glycerol formation in the yeast cell, which is necessary for regeneration of NADH. This suggests that inhibition function of furfural decrease the NADH regeneration that leads to accumulation of furfural. Influence of pretreatment inhibitors on the cell metabolism and on macromolecules is shown in Fig. 1.6.

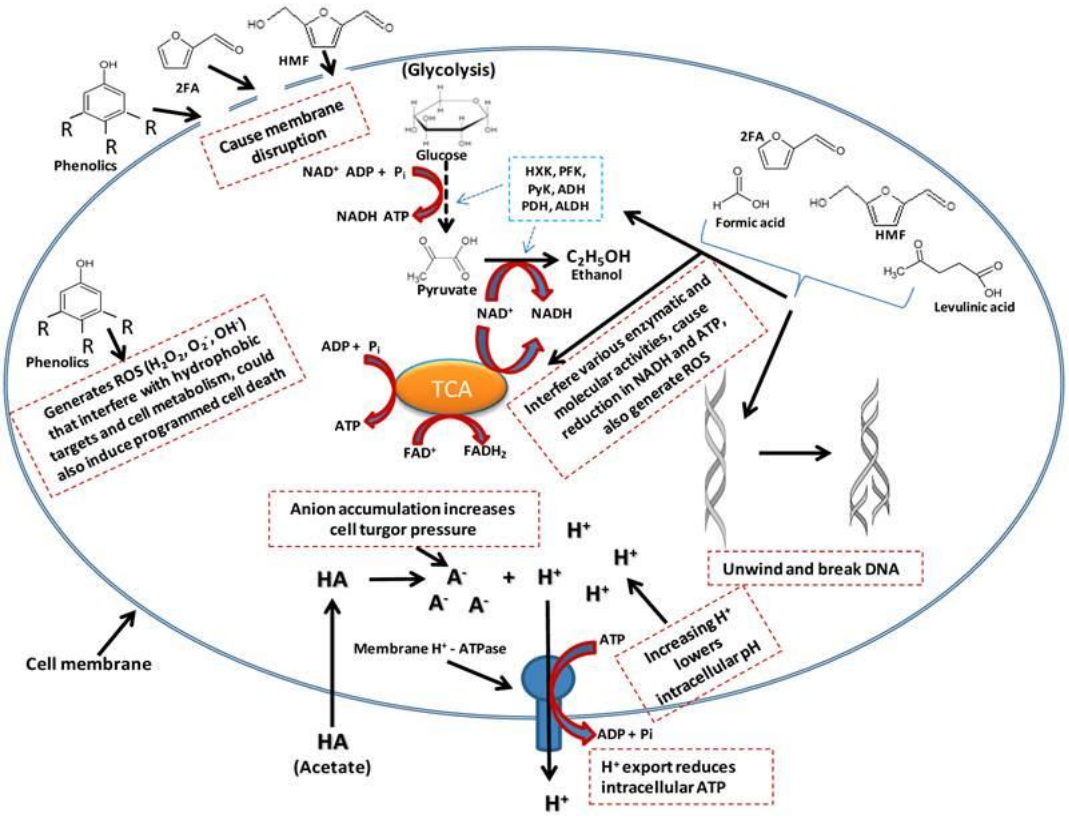


Fig. 1.6. Effect of pretreatment inhibitors on macromolecules and cell membrane [126].

1.6.2. Aliphatic acids

Weak acids, including acetic acid, levulinic acid and formic acid are commonly discharged during the pretreatment. Acetic acid is produced and discharged from hemicellulose de-acetylation, formic acid and levulinic acids are discharged from HMF degradation [5, 132]. Acetate diffuses across the cell membrane and discharge 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 maintained by pumping the proton out of cell by membrane ATPase. For this additional ATP to be generated, this is achieved by enhanced ethanol production under anaerobic conditions at the cost of biomass formation [126]. According to anion accumulation theory, the

intracellular pH is disturbed once undissociated form of the weak acid get diffuse through the plasma membrane from fermentation medium. Once undissociated acid enter in the cell, then dissociates within the cell, resulting lowering the cytosolic pH and generate protons and acid anions. The cell then need to correct the pH imbalance. In addition, accumulation of acids also enhances the free radicals formation that enhances the oxidative stress on fermenting strain. [133] investigated the effect of acetic acid concentration on ethanol productivity, this study suggest that moderate acetic acid concentration below 100 mM enhances ethanol production, while more than 100 mM acetic acid concentration decreases yeast growth and ethanol fermentability. Formic acid inhibits macromolecules (DNA and proteins), DNA synthesis, and DNA repairing mechanism [126].

1.6.3. Phenolic compounds

Lignin degradation may result in formation of a variety aldehydic, aromatic, phenolic, and polyaromatic compounds. Among others, phenols are well known by-products of lignin, and may also form from sugars [134] and also some extractives are phenols [80, 135]. Phenolic compounds are however diverse [136] and a large number of different phenols e.g. vanillin, vanillic acid, dihydroconiferyl alcohol, hydroquinone, coniferyl aldehyde, catechol, acetoguaiacone, 4-hydroxybenzoic acid, and homovanillic acid are produced by the plants [137]. The prime factors influencing formation of phenols are pretreatment temperature, residence time, type of catalyst and catalyst concentrations.

Phenolic compounds are discharged and produced from the lignin degradation and sugars dehydration during the pretreatment. Phenolic compounds are divided into three groups, includes acids (vanillic acid, ferulic acid, syringic acid and 4-hydroxybenzoic acid), alcohols (vanillyl alcohol, guaiacol and catechol,), and aldehydes (4-hydroxylbenzaldehyde, syringic aldehyde and vanillin) [134, 138-140]. Phenolic compounds are further catagorized into two groups according to their molecular weight: Low molecular weight compounds are more toxic than the high molecular weight [141]. Phenolic compounds concentration depends on lignin contents of feedstock and its linkage to cellulose and hemicellulose [142]. 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 discharges 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 [126, 139, 143]. In addition, reactive oxygen species (ROS) interact with proteins/enzymes, which result in malfunctioning of these proteins/enzymes and consequently induce programmed cell death [142]. Phenolic compounds are more toxic that furans even at low concentrations [126, 138, 139]. Mechanism and inhibition effect of lignocellulose inhibitors vary by changing the feedstock, operation conditions and microorganism. Generally speaking, all inhibitors decrease cell growth, enzyme activity, and fermentation performance at certain level.

1.6.4. Other inhibitors

A certain amount of ash components present mainly as inorganic salts, is contained in lignocellulosic materials. Moreover, inorganic ions are also added with chemicals used in pretreatment, during pH adjustment of the pretreated raw material, and are plausibly also leached off from process equipment [141, 144]. Despite *S. cerevisiae* being an osmotolerant microorganism, high concentrations of salts have been demonstrated to reduce the volumetric ethanol production rate, with more pronounced effects when xylose was used as the sole carbon source [145]. In addition, the amplitude of the inhibitory effect is dependent on the nature of the ion, with Ca^{2+} being the most inhibitory [146]. Ethanol is also a potential inhibitor of *S. cerevisiae*, as it has been reported to reduce the specific growth rate, viability, and specific fermentation rate [147]. However, since the concentration of ethanol in lignocellulosic bioethanol processes rarely exceeds 50 g L^{-1}, ethanol probably elicits a minor effect on its own. However, ethanol may act synergistically in combination with other inhibitors.

1.6.5. Combined effects of inhibitors

Synergism means that the combined effect of several inhibitors is greater than the summed effects exerted by individual inhibitors acting in isolation. Few studies report on the combined effects of several inhibitors in a robust experimental design. This is probably due to the great many experiments that would have to be carried out to systematically decipher synergistic effects. Thus, most studies of the effects of lignocellulose-derived inhibitors have been performed by examining the inhibitors in isolation, except for a few studies that will be mentioned here. [148] have performed a full 2^3 factorial design to elucidate the potential synergistic effects of acetic acid, furfural, and *p*-hydroxybenzoic acid on *S. cerevisiae*. It was found that furfural and acetic acid interacted synergistically by reducing the specific growth rate, ethanol yield, and biomass yield [148]. Furthermore, syringaldehyde and acetovanillone are known to interact synergistically in *S. cerevisiae* by reducing the volumetric ethanol productivity when added to a wheat straw hydrolysate pretreated by wet oxidation [149]. Furfural was also found to act synergistically with HMF and aromatic aldehydes such as vanillin, 4-hydroxybenzaldehyde, and syringaldehyde in *E. coli* by reducing the specific growth rate and ethanol yield, whereas all other binary combinations were additive [150]. In another study of *E.coli*, furfural acted synergistically with its conversion product furfuryl alcohol by reducing the specific growth rate and ethanol yield [151]. Finally, it was found that none of the weak acids commonly found in lignocellulosic hydrolysates acted synergistically on the specific growth rate of *E. coli* in binary combinations [130]. There is no doubt that inhibitory substances formed or released when pretreating lignocellulosic materials are challenging for microorganisms, acting as impediments to increasing the solids consistency in fermentation processes as the inhibitor concentration would increase accordingly. All inhibitor classes and combinations thereof are obviously important, as described above. However, the

furan aldehydes and, in particular, furfural have been identified as key mediators of hydrolysate toxicity in several investigations [152, 153].

1.7. Strategies to overcome inhibitory problems

Optimization of upstream processing i.e. use of pretreatment technique that do not result in formation of by-products or use of less severe pretreatment conditions to minimize the by-product formation is one way to avoid the inhibitory problems of lignocellulose hydrolysates. However, with the current technology, a harsh pretreatment is needed to overcome the obstacles related with the biomass recalcitrance. Therefore, preventing the formation of inhibitors is difficult. Another approach is that separation of solid and liquid fractions of the pretreated material, proper wash of solids, and hydrolysis of solids in an optimal environment would help to obtain high sugar yields. But, it would be economically expensive since more process units are needed. In addition, fermentation of sugar rich pretreatment liquid hydrolysates would anyway need of addressing inhibitory problems. Hence, removal of inhibitory compounds present in lignocellulose hydrolysates is an important research area of research.

Over the years, different techniques categorized as physical, chemical, biological and in their combination were developed to lighten the toxic effect of lignocellulose hydrolysates. These include the use of chemical additives, microbial treatments, enzymatic treatments, heating and vaporization, liquid-liquid extraction, and liquid-solid extraction [144]. However, many of these methods are effective only for a certain inhibitor or a class of inhibitors [108]. Also, some have been found to be more efficient than other, and some are more realistic in terms of industrial application [108]. Here we will discuss detoxification, and more specifically biodetoxification.

1.7.1. 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 produced. These pretreatment inhibitors severely decrease cellulase activity, microbial growth, and fermentability [154, 155]. However, detoxification or conditioning step cannot be skipped for obtaining the high titer and yield of any desired product from high solids content of lignocellulosic biomass. Multiple methods are reported for removing pretreatment inhibitors, includes overliming, ion exvariation, vaporization, and water washing [137, 154, 156, 157]. However, these methods face many difficulties, , mainly enormous freshwater usage and wastage, loss of the fine lignocellulose particles and fermentative sugars, and inadequate removal of inhibitors [157, 158].

1.7.2. Biological detoxification

Biodetoxification is excellent for inhibitors removal, this depends on microorganisms secreting peroxidase or laccase enzymes into the hydrolysate, as part of their normal metabolism, thereby degrading the toxins [159-161]. Some of the many plus points of biodetoxification are: no loss of cellulose solids, reduced fresh water usage, and thus increased availability of high solids contents for fermentation. But, most of biodetoxification method can only be applied to the liquid hydrolysate system, in which the cellulose has been hydrolyzed under toxin inhibition to cellulase enzymes and the toxin concentrations have been diluted [156, 157, 159-162]. Unique biodetoxification system by using *Amophotheca resinae* was developed and applied in high solids loading of pretreated material for inhibitors removal [157]. Long biodetoxification time was only limitation of this method, but this has been solved by onsite biodetoxification onsite biodetoxification in the bioreactor that decrease biodetoxification time from 7 days to only 1.5-2 days [163]. Micro-organisms, such as some species of yeasts [164-166], fungi [162, 167], and bacteria [162, 168] can detoxify furan aldehydes, aliphatic acids, and aromatic compounds [169-173]. However, the selectivity of degradation varies depending on microorganism employed. *T. reesei* was able to metabolize furan aldehydes, acetic acid, and benzoic acid derivatives of a willow hydrolysate [174]. *Desulfovibrio furfuralis* utilized furfural, furfuryl alcohol and 2-furoic acid as source of carbon and energy [175] and metabolized furfural to acetate, CO_2 and/or methane [176]. *E. coli* strains was shown to degrade furfural into furfuryl alcohol [170, 171]. However, conversion rates of these organisms were low and would require multiple days. During this period they also consumed sugars from hydrolysates, e.g. *T. reesei* consumed 35% of fermentable sugars [137].

Corynebacterium glutamicum strains were used in this study to determine the biodegradation capability of the strains and results suggest that strain has excellent capability to degrade almost all inhibitors and in very short time.

There are multiple other possible strategies to solve the pretreatment inhibitors problem, including fermentation approaches, genetic engineering, and evolutionary adaptation [10, 12].

1.7.3. Fermentation approaches

There are various methods to overcome the pretreatment inhibition problems. One of the approaches could be dilution of medium, which decreases the inhibitors concentration. Dilution of medium will also dilute the sugars concentration that leads to decreased ethanol concentration and enhanced ethanol distillation cost. Another similar approach could be, enhance the inoculum size of the fermenting microorganism. This could be economically feasible only if cells can be recycled in the fermentation process, as in SHF, while in SSF and CBP not possible. Additional possibility could be fed batch fermentation process. In this case, initially fermenting microorganisms will be exposed to lower inhibitors concentration. This will permit 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 employed by maintaining the lower glucose concentration.

1.7.4. 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 enhance the resistance against inhibitors. [177] 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 4x 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, boostd yeast performance and enhanced ethanol productivity in the presence of acetic acid and formic acid [178]. A potential disadvantage of genetic engineering is that the approach may work well if resistance against specific inhibitor is needed. In most of cases a wide range of inhibitors produced and discharged during pretreatment, and tolerance to each inhibitors need a specific gene to be introduced or overexpressed, sometimes more than one gene is needed. 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.

1.7.5. Evolutionary adaptation

Evolutionary adaption could be a promising strategy to enhance the inhibitor tolerance and fermentability of strains in the same culture conditions where strain will be employed [10, 12, 179, 180]. 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 boosts the inhibitors tolerance and fermentability of microorganism might be due to integrating certain variations at genomic level resulted from random mutations. These mutations enhance the expression of certain set of genes that enhance glucose consumption and inhibitors transformation [126].

Dose dependent adaptation show enhanced inhibitor tolerance towards furans, which consequently improved cell growth, glucose consumption, and ethanol fermentability. Evolutionary adaptation has many advantages; (1) enhance inhibitor tolerance, (2) increase fermentability, and (3) decrease detoxification time. *Corynebacterium glutamicum* was used in this study, experienced a long term evolutionary adaptation by using corn stover hydrolysate. (Chapter 2).

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 employed for the

specific lignocellulose feedstock should be matched closely, because a subtle variation 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 variations in any aspects, such as feedstock type, pretreatment method, conditioning (detoxification), or hydrolysis conditions. Selective pressure during the long term adaptation enhances 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 [181]. During evolutionary adaptation multiple 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 enhanced performances in the actual experimental conditions [130, 182]. [181] defined 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. A possible explanation for the better performances of the adapted strain might be due to the improvements of target enzymes that permit more efficient sugar utilization and ethanol production in presence of pretreatment inhibitors [183]. It can be concluded that evolutionary adaptation is the best choice to increase the fermentability and inhibitor tolerance of microorganisms, thus, all the strains employed in the lignocellulosic biorefinery should be experienced evolutionary adaptation.

1.8. Objectives of the thesis

The main objectives of this study were to develop fast, efficient, and cost effective lignocellulosic biomass conversion methods for glutamic acid lysine production. The detailed objectives of the thesis are shown in Fig. 1.7.

- Determination of inhibitor degradation capability of *Corynebacterium glutamicum* B460 and draw a hypothetical pathway for the inhibitors metabolism for *Corynebacterium glutamicum* B460 for glutamic acid production (Chapter 2).
- Evolutionary adaptation of *Corynebacterium glutamicum* SIIM B460 to inhibitors of pretreated corn stover hydrolysate to enhance the inhibitor tolerance and degradation capability of the strain for glutamic acid production and consequently the genome sequence analysis of the adapted strain. (Chapter 2).

- Tolerance analysis of *Corynebacterium glutamicum* SIIM B253 towards the pretreatment inhibitors for lysine production. (Chapter 3).
- Determination of inhibitor degradation capability of *Corynebacterium glutamicum* SIIM B253 and hypothesize a metabolic pathway. (Chapter 3).
- Lysine production using pretreated non-biodetoxified corn stover hydrolysate. (Chapter 3).
- Analysis of pretreatment inhibitor degradation capability of *C. glutamicum* using non-biodetoxified corn stover hydrolysate. (Chapter 3).
- Effect of different inoculum ratios on lysine production using non-biodetoxified hydrolysate. (Chapter 3).
- Effect of different inoculum ratios on inhibitors degradation using non-biodetoxified corn stover hydrolysate. (Chapter 3).

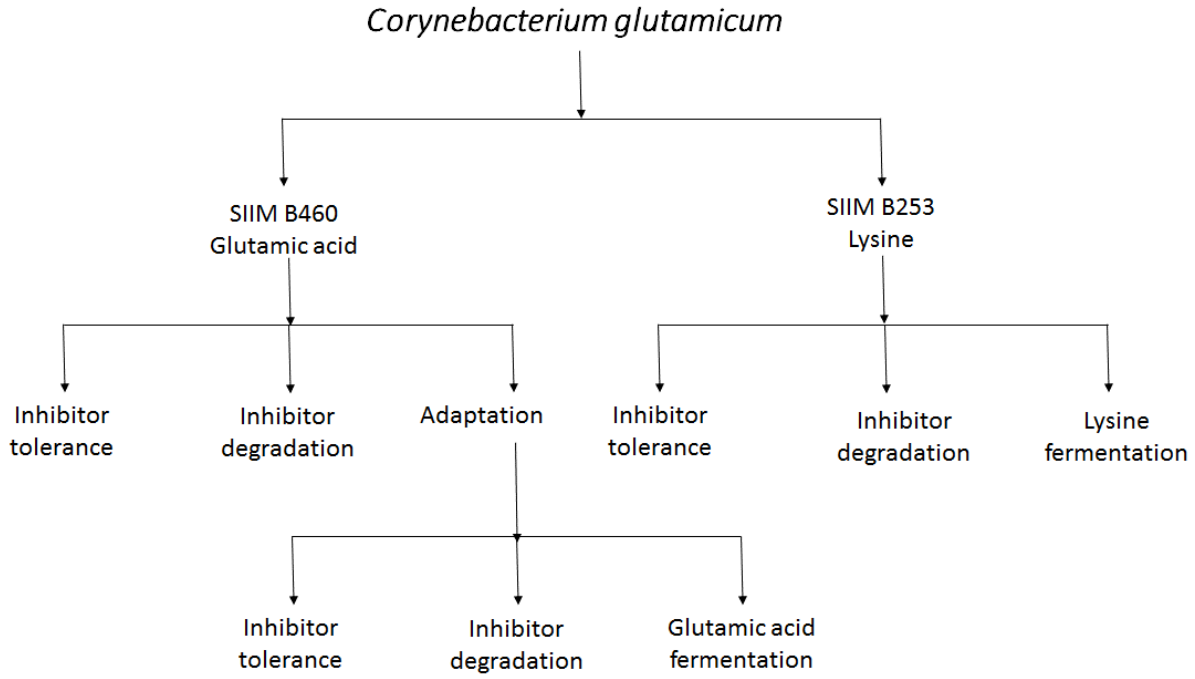


Fig. 1.7. Overall experimental scheme of the research work presented in this thesis

Chapter 2

Evolutionary adaptation of *Corynebacterium glutamicum* for enhanced inhibitor tolerance and degradation to lignocellulose-derived inhibitors

2.1. Introduction

Glutamic acid is commercially important amino acid that is used in food industry as additive and flavor enhancer in the form of sodium salt [184]. Its annual production reached over 2 million tons [185]. Currently, high glutamic acid production cost is one of the major technical barriers. Therefore, alternative cost-effective carbon sources are certainly needed to give a proper solution to reduce fermentation medium cost. Lignocellulosic biomass could be promising energy source due to its low cost, abundant availability and renewable nature [186]. Lignocellulosic biomass can not be utilized directly as carbon source due to its recalcitrant nature. For efficient utilization of lignocellulosic material, pretreatment is an essential step. Pretreatment breaks down the lignocellulosic structure to make cellulose accessible to hydrolyzing enzymes [96]. Due to severe pretreatment conditions, some by products are also generated, which inhibit enzyme activity and fermentability [5, 141]. These compounds are classified into three groups: organic acids, phenolic compounds, furans. In general, they affect overall cell physiology and often result in decreased cell viability, yield, and productivity [5]. Aldehydes and phenols are considered as more toxic than organic acids [187]. The composition and content of these inhibitors present in lignocellulosic hydrolysates depends on the type of raw materials used and the operational conditions during pretreatment and hydrolysis processes [188, 189]. It is also known that inhibitory effects on microbial cell growth and metabolism vary with their concentration and the nature of microorganisms [149, 190].

To address the negative effects of inhibitors, several strategies have been investigated. One possibility is to apply detoxification processes, including physical, chemical, or biological methods, prior to the fermentation [129, 137, 159, 191]. However, inhibitor detoxification tends to be complicated and it is an additional step and cost [192, 193].

Utilizing a combination of inhibitor-tolerant strains with desired properties for detoxification of cellulosic hydrolysates can be a better cost-effective approach for industrial scale fermentation [194, 195]. Several studies have been performed to understand the inhibitors effect, microbial tolerance and their degradation pattern. For example, *Escherichia coli* [106, 196, 197], *Saccharomyces cerevisiae* [148, 198-201], *Cupriavidus basilensis* HMF14 [202], *Amorphotheca resinae* ZN1 [156, 203], *Cryptococcus curvatus* [204], *Trichosporon* [205], *Pichia stipites* [206], *Rhodosporidium toruloides* [207] and *Zymomonas mobilis* [208, 209]. However, only few studies are reported for inhibitors tolerance and degradation by *Corynebacterium glutamicum*. Effect of various inhibitors on *C. glutamicum* growth and ethanol fermentation

[210], acetate metabolism [211-213] and furan degradation [214] were investigated. Biodegradation mechanisms for furfural and HMF was extensively investigated [170, 202, 215]. A putative degradation pathway of furfural in *Pseudomonas putida* F2 in 1969 was proposed by Trudgill [216], which was then verified by Koenig and Andreesen [217].Koopman et al. [202] extended the pathway to HMF in *Cupriavidus basilensis* HMF14. Ran et al. [156] and Wang et al. [203] proposed degradation pathways for furfural and HMF for *Amorphotheca resinae* ZN1. Metabolic pathways of phenolic compounds were studied for vanillin, 4-hydroxybenzaldehyde and syringaldehyde for *Amorphotheca resinae* ZN1 by [209].

However, many of the industrially interesting microorganisms, obtained so far, are not robust enough to withstand pretreatment inhibitors' stress conditions. Research studies suggest that evolutionary adaptation could be another possibility to enhance inhibitors tolerance and degradation and it is better than genetic engineering approach [12, 179]. In genetic engineering a specific gene is inserted for each inhibitor, whereas, adaptation is performed in same condition that will be applied in production unit.

The development and use of robust glutamic acid producing microorganisms, resistant to pretreatment inhibitors, is certainly required for commercial scale glutamic acid production from lignocellulosic material.

Detailed biodegradation behavior of *Corynebacterium glutamicum*, which is a potential microorganism for glutamic acid production, is unknown. In this study, inhibitors tolerance degradation and evolutionary adaptation performance of an industrial fermentation strain *C. glutamicum* SIIM B460 to the model inhibitors were systematically investigated and the putative degradation pathways were proposed based on the experimental data, the genome information of *C. glutamicum* and the previous studies on ethanologenic strains. Long term adaptation further improved the tolerance and degradation capability of C. *glutamicum* SIIM B460. This study provides key insights on inhibitors' tolerance and degradation of *C. glutamicum.* It is also useful for the future improvement of detoxification efficiency and metabolic modification of the strain.

2.2. Materials and Methods

2.2.1. Raw materials and chemicals

Corn stover was harvested from Dancheng County, Henan Province, China, in fall 2013. Corn stover was water washed to remove field dirt, stones and metals, then air-dried, milled by a beater pulverizer to pass through the 10-mm apertures in diameter. The virgin corn stover contained 37.2 % of cellulose and 19.9 % of hemicellulose determined according to a two-step H_2SO_4 hydrolysis method developed by the National Renewable Energy Laboratory (NREL) protocol [218].

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity was 135 FPU/g determined using the

NREL protocol LAP-006 [219], the cellobiose activity was 344 CBU/g using the method described by [220], and the cellulase protein content was 90 mg/g.

Furfural and HMF were purchased from Shanghai DEMO Medical Tech Co., Ltd, Shanghai, China. 4-Hydroxybenzaldehyde, syringaldehyde, and vanillin were purchased from Sangon Biotech Co., Shanghai, China. Acetic acid, lactic acid and formic acid were purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd, phenol from Shanghai Generay Biotech Co., Ltd, coniferyl aldehyde from Sigma-Aldrich Co., USA, and levulinic acid was purchased from Ruiteng chemical Co., Ltd, China. Yeast extract was purchased from Oxiod, Basingstoke, Hampshire, UK. All other standard chemicals including glucose, peptone, NaCl, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, Urea, $FeSO_4$, MnSO4, NaOH, and H_2SO_4 were of reagent grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Agar was purchased from New Probe, Beijing, China.

2.2.2. Strains and media

Glutamic acid fermenting strain *C. glutamicum* SIIM B460 was purchased from Shanghai Industrial Institute of Microorganisms (SIIM), Shanghai, China. One vial of *C. glutamicum* SIIM B460 was taken from the -80 ℃ freezer and culture was streaked on petri plate containing LB medium, and incubated for 36 h at 30 ℃, pH 7.0. A single colony was transferred into 30 mL of pre-culture medium in 250 mL Erlenmeyer flask and incubated in shaking incubator at 30 ℃, 200 rpm, pH 7.0. After 10 h, 10% (v/v) of the culture was transferred to the seeds culture medium and incubated for 8 h at 30 ℃, pH 7.0 in shaking incubator. Seeds culture was used as the inoculum in all fermentation experiments.

Culture media used included:

(1) LB agar medium (g/L): yeast extract: 5, peptone: 10, NaCl: 5, and agar: 17 (pH 7.0).

(2) Pre-culture medium (g/L): glucose: 25, KH_2PO_4: 1.5, $MgSO_4.7H_2O$: 0.6, urea: 2.5, $FeSO_4$: 0.002, $MnSO_4$: 0.002, and corn steep liquor: 25 (pH 7.0).

(3) Seeds culture medium (g/L): glucose: 25, KH_2PO_4: 1.5, $MgSO_4.7H_2O$: 0.6, urea 2.5, $FeSO_4$: 0.002, $MnSO_4$: 0.002, and corn steep liquor: 5 (pH 7.0).

(4) Synthetic medium (g/L): glucose: 60, KH_2PO_4: 1.0, $MgSO_4 \cdot 7H_2O$: 0.6, urea: 3.0, $FeSO_4$: 0.002, $MnSO_4$: 0.002, and corn steep liquor: 0.5 (pH 7.0).

(5) Adaptation medium: Corn stover hydrolysate prepared at solids loading of 15% (w/w), KH_2PO_4: 1.0, $MgSO_4 \cdot 7H_2O$: 0.6, urea: 3.0, $FeSO_4$: 0.002, $MnSO_4$: 0.002, and corn steep liquor: 0.5 (pH 7.0)

All media were sterilized at 115 ℃ for 20 min. $FeSO_4$ and $MnSO_4$ freshly prepared to avoid the oxidation and filter sterilized.

2.2.3 Pretreatment and hydrolysate preparation

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment (DDAP) according to method reported elsewhere [6, 8]. Briefly, 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 then pretreated at 175 ℃ for 5 min. The pretreated corn stover contained 50% (w/w) of solids and no free water was generated during pretreatment. The dry solid of the pretreated corn stover contained 39.89% of glucan and 3.04 % of xylan according to the two-step H_2SO_4 hydrolysis method [218].

Corn stover hydrolysate was prepared by enzymatic hydrolysis of dry dilute acid pretreated corn stover at the cellulase dosage of 15 FPU/g dry corn stover matter (DM) (equivalent to 10 mg protein/g DM) at 15% (w/w) of solids loading at 50 ℃ for 48 h. The hydrolysate slurry was centrifuged to remove the solids to obtain the clear hydrolysate containing 33.69 g/L of glucose, 2.54 g/L of acetic acid, 0.379 g/L of furfural, and 0.209 g/L of HMF. Additional glucose was added to corn stover hydrolysate to maintain the glucose concentration to 60 g/L. Before use, the nutrient composition, including 1 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4 \cdot 7H_2O$, 3 g/L of urea, 0.002 g/L of $FeSO_4$, 0.002 g/L of $MnSO_4$, and 0.5 g/L of corn steep liquor were added.

2.2.4. Evolutionary adaptation

Evolutionary adaptation of *C. glutamicum* SIIM B460 was carried out by continuously transferring the cultured bacterial cells into the fresh corn stover hydrolysate. In details, 5% (v/v) of the culture broth from the last culture was transferred every 24 h into the fresh hydrolysate and incubated at 30 ^oC in the shaking incubator at 200 rpm. At the end of each transfer, the sample was collected and used for glucose, glutamic acid, and growth (OD_{600}) analysis. This successive transfer process was repeated for 128 days until the fermentation performance maintained stable.

2.2.5. Inhibitor tolerance and degradation

Inhibitor tolerance of *C. glutamicum* SIIM B460 was carried out by using different concentrations of each inhibitor using synthetic medium. 5% (v/v) of inoculum ratio was used and incubated at 30 ^oC in the shaking incubator at 200 rpm. pH was adjusted at 7 using 20 % urea throughout the course of fermentation up to 36 h.

Inhibitor degradation of *C. glutamicum* was analyzed by using each inhibitor in the synthetic medium. 10% of inoculum ratio was used and incubated at 30 ^oC at 200 rpm for 72 h. pH was adjusted at 7 with 20 % urea.

2.2.6. Glutamic acid fermentation

Glutamic acid fermentation of *C. glutamicum* SIIM B460 was carried out by using synthetic medium and corn stover hydrolysate. 20 % (v/v) of inoculum ratio was used and incubated at 30 ℃ at 200 rpm in the shaking incubator for 72 h. pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. 0.1 g/L of penicillin was added at 8^{th} and 16^{th} h.

2.2.7. Analytical methods

Samples were periodically collected, centrifuged to obtain the supernatant. Glucose and glutamic acid were measured on SBA Biosensor 40D (Shandong Academy of Sciences, Jinan, China).

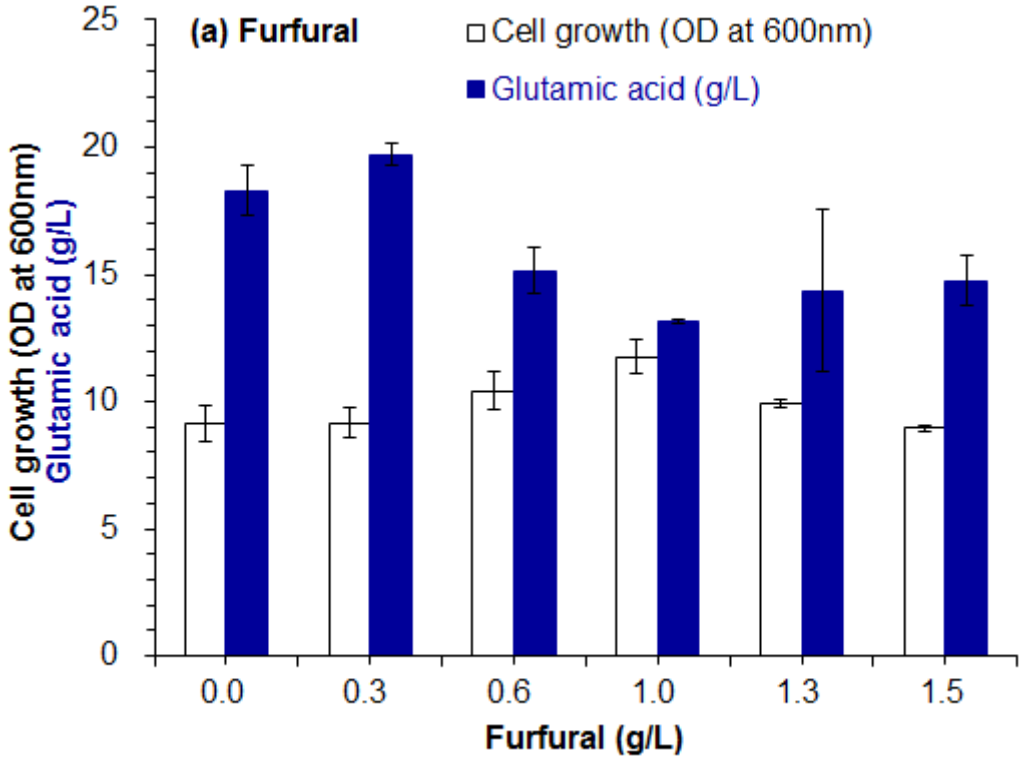
Furfural, furfuryl alcohol, HMF, 5-hydroxymethylfurfuryl alcohol (HMF alcohol), syringaldehyde, syringic alcohol, syringic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydeoxyebenzoate, vanillin, vanillyl alcohol and vanillic acid were analyzed on reverse-phase HPLC (LC-20AT, Japan), equipped with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan). Furfural and furfuryl alcohol 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 and HMF alcohol 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. Syringaldehyde, syringic alcohol, syringic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydeoxyebenzoate, vanillin, vanillyl alcohol, and vanillic acid were analyzed using 100% acetonitrile solution (pump A) and 0.1% formic acid (pump B) at a ratio of 90% to 10% as the mobile phase at 1.0 mL/min at the column temperature of 35 ℃ and the detection wavelength of 270 nm. Acetic acid, 2-furoic acid (furoic acid), and 5-hydroxymethylfuroic acid (HMF acid) were determined on HPLC 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.

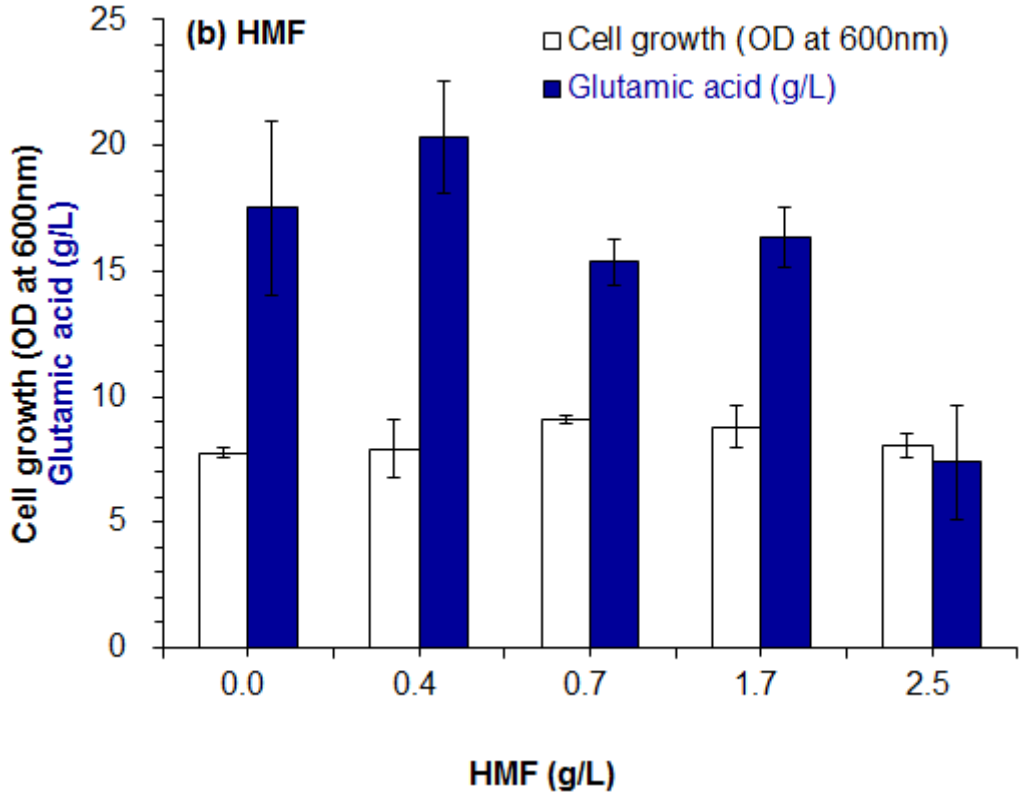
2.3. Results and Discussion

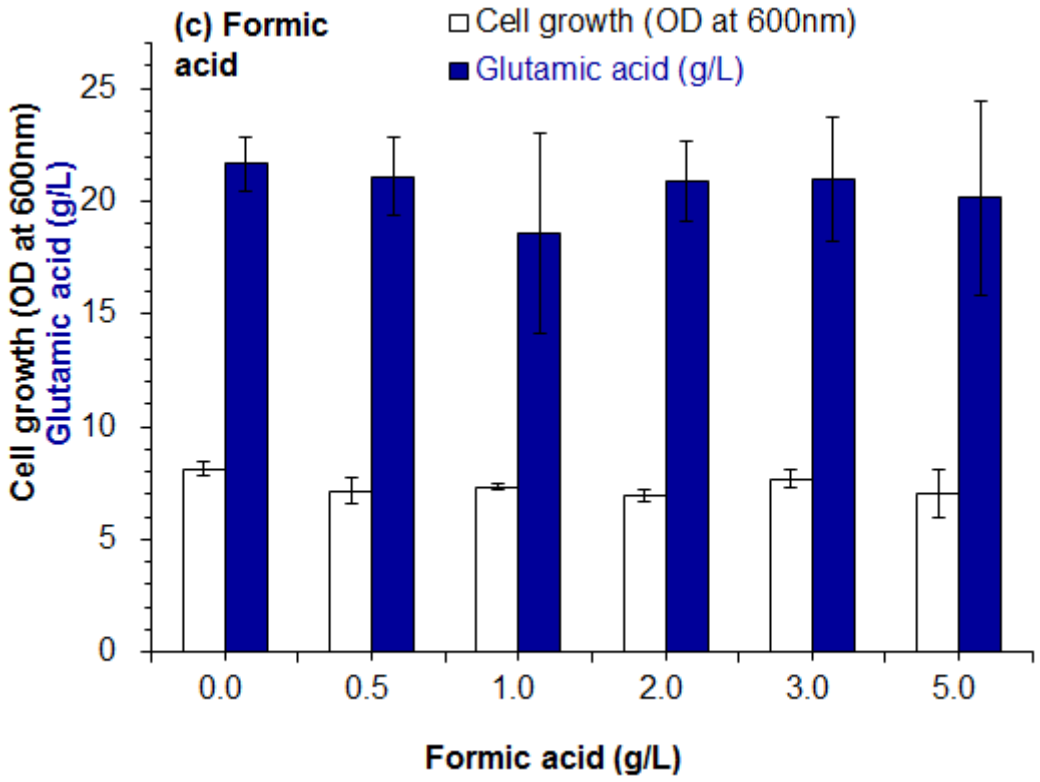
2.3.1. Inhibitor tolerance and evolutionary adaptation of *C. glutamicum*

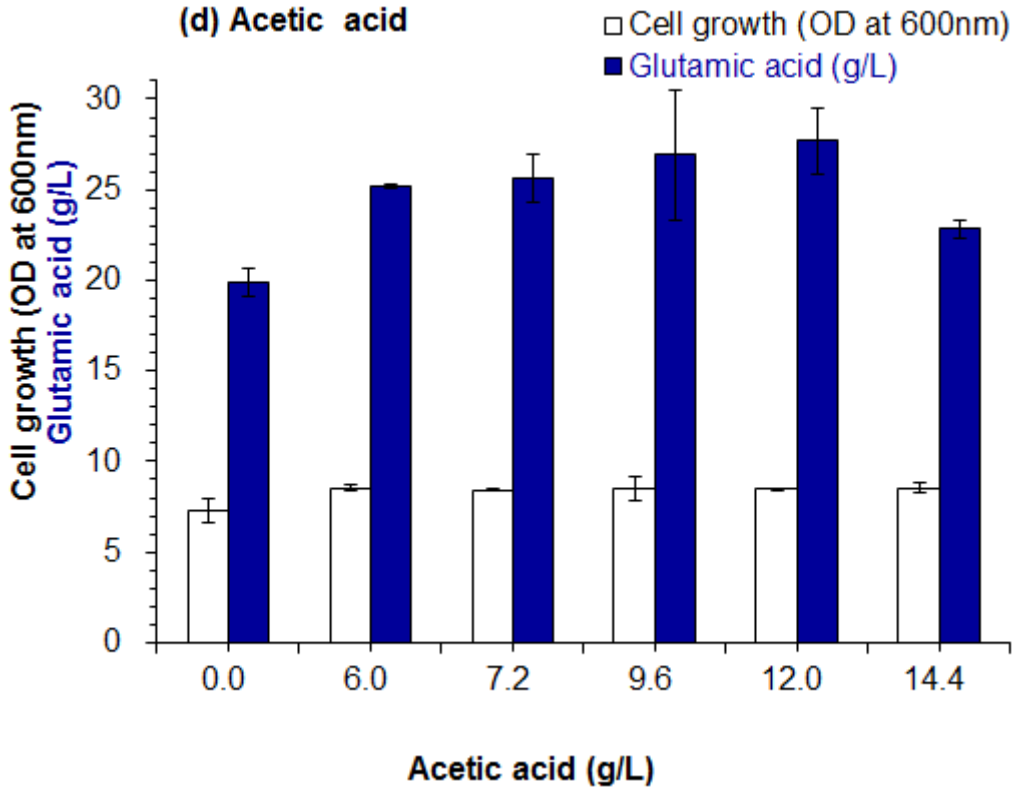
Tolerance of *C. glutamicum* against various inhibitors were assayed by adding inhibitor compounds into synthetic medium based on the inhibitor concentrations in the hydrolysate [6] (Fig. 2.1). Furfural and HMF are generally regarded as the most toxic inhibitors to majority of fermenting strains, but *C. glutamicum* showed the strong tolerance to furans (Figs. 2.1a and 2.1b). Only at high concentration of furfural and HMF, the cell growth

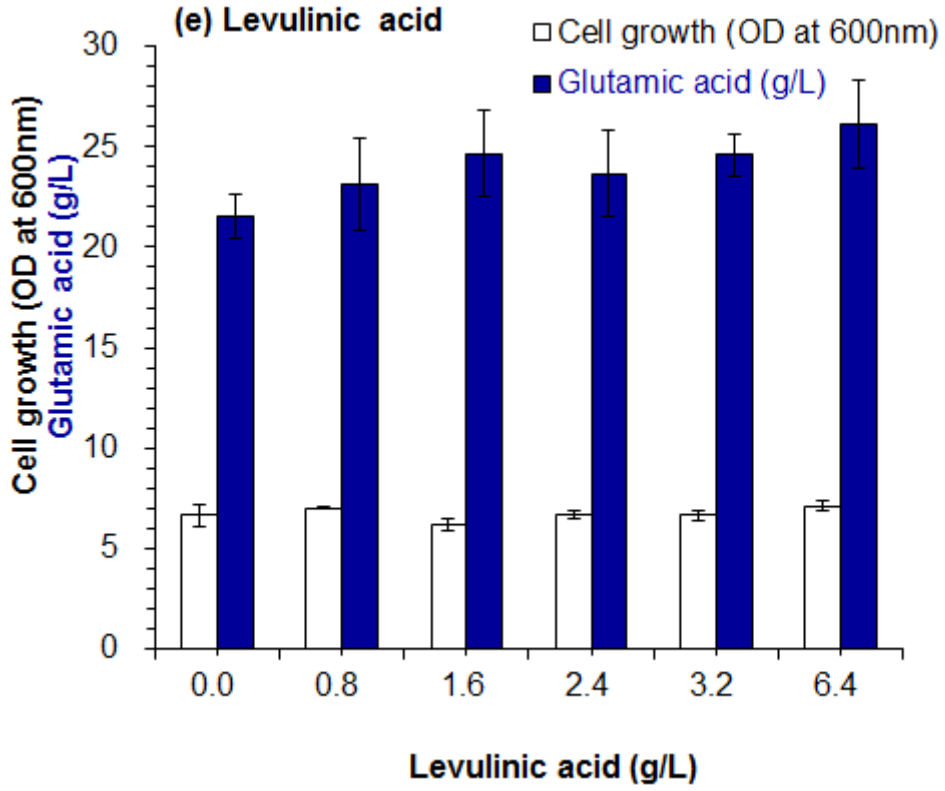
and glutamic acid generation were negatively affected. *C. glutamicum* showed excellent tolerance to formic acid, acetic acid, and levulinic acid even at high concentration range (Figs. 2.1c-2.1e). For phenolic aldehyde inhibitors, vanillin and 4-hydroxybenzaldehyde did not show the obvious inhibition on *C. glutamicum* (Figs. 2.1f and 2.1i), but syringaldehyde and coniferyl aldehyde significantly reduced the cell growth and glutamic acid generation (Figs. 2.1g and 2.1h) in the range tested. Conclusively, *C. glutamicum* has very strong tolerance to furans (furfural and HMF), organic acids (acetic acid, formic acid and levulinic acid, and phenolic compounds (4-hydroxybenzaldehyde and vanillin). Based on the obtained results, it is concluded that strain is tolerant to most of the pretreatment inhibitors, present in typical hydrolysate and pretreated material.

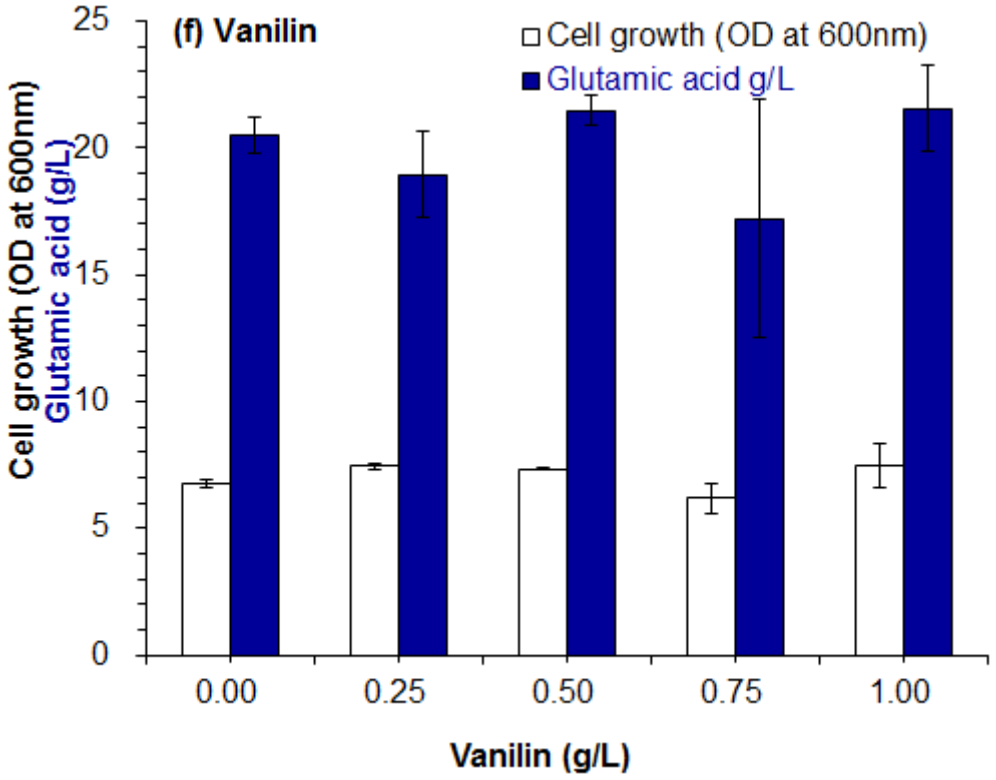


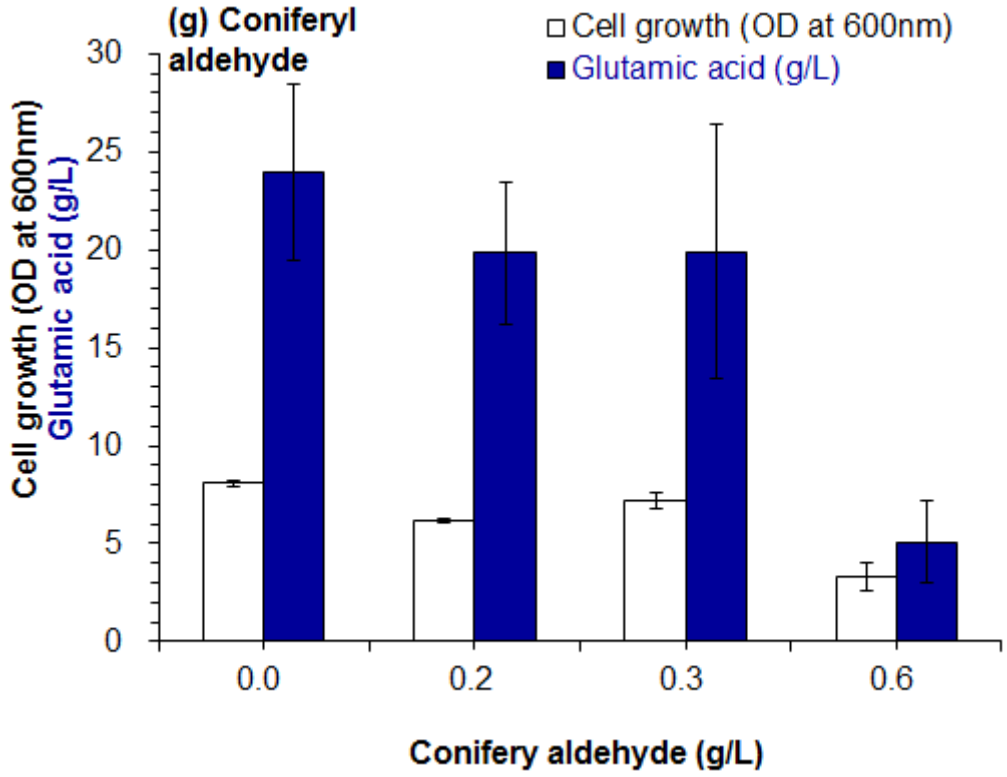

(b) HMF
Cell growth (OD at 600nm)
Glutamic acid (g/L)
Cell growth (OD at 600nm)
Glutamic acid (g/L)
25
20
15
10
5
0
0.0
0.4
0.7
1.7
2.5
HMF (g/L)


(c) Formic acid
Cell growth (OD at 600nm)
Glutamic acid (g/L)
Cell growth (OD at 600nm)
Glutamic acid (g/L)
25
20
15
10
5
0
0.0
0.5
1.0
2.0
3.0
5.0
Formic acid (g/L)


(d) Acetic acid
Cell growth (OD at 600nm)
Glutamic acid (g/L)
Cell growth (OD at 600nm)
Glutamic acid (g/L)
30
25
20
15
10
5
0
0.0
6.0
7.2
9.6
12.0
14.4
Acetic acid (g/L)


(e) Levulinic acid
Cell growth (OD at 600nm)
Glutamic acid (g/L)
Cell growth (OD at 600nm)
Glutamic acid (g/L)
30
25
20
15
10
5
0
0.0
0.8
1.6
2.4
3.2
6.4
Levulinic acid (g/L)


(f) Vanilin
Cell growth (OD at 600nm)
Glutamic acid g/L
Cell growth (OD at 600nm)
Glutamic acid (g/L)
25
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
Vanilin (g/L)


(g) Coniferyl aldehyde
Cell growth (OD at 600nm)
Glutamic acid (g/L)
Cell growth (OD at 600nm)
Glutamic acid (g/L)
30
25
20
15
10
5
0
0.0
0.2
0.3
0.6
Conifery aldehyde (g/L)

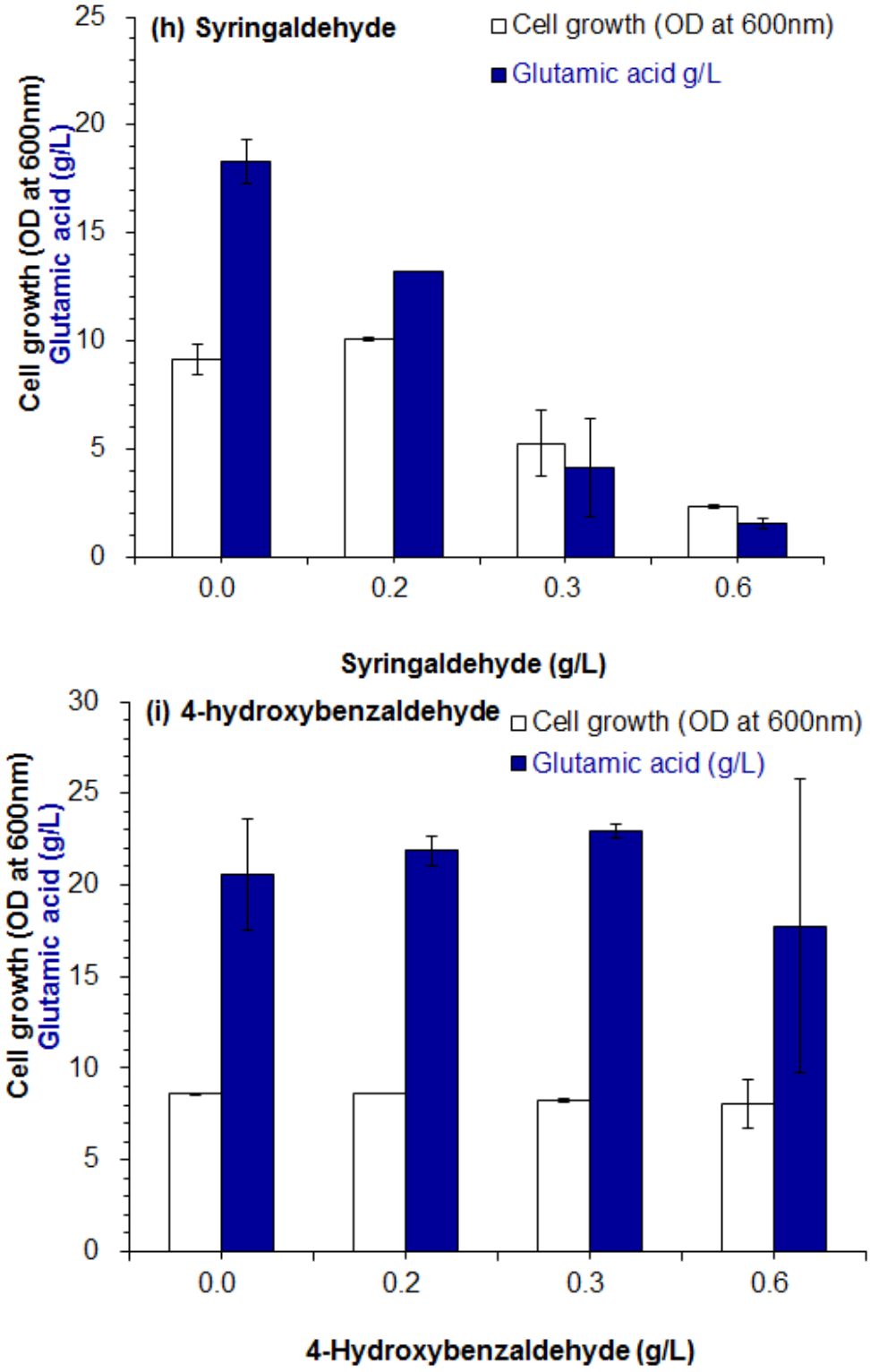


Fig. 2.1. Inhibitor tolerance and glutamic acid fermentability of *Corynebacterium glutamicum* SIIM B460 against different concentrations of various inhibitors. (a) Furfural (b) HMF (c) Formic acid (d) Acetic acid (e) Levulinic acid (f) Vanillin (g) coniferyl aldehyde (h) Syringaldehyde (i) 4-Hydroxybenzaldehyde. Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 36 hours in flasks, 5% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

For improving the tolerance of *C. glutamicum* SIIM B460 to inhibitors in the real lignocellulose hydrolysate, the long term evolutionary adaptation was carried out in the hydrolysate of dry dilute acid pretreated corn stover, by periodic transfer of cells into the new hydrolysate every 24 h and repeated. Totally, 128 cell transfers (equivalent to 3,072 hours) of *C. glutamicum* SIIM B460 were conducted until the cell growth and glucose consumption were stable. The glucose consumption and cell growth varied up to 70 transfers, and then maintained stable with only random fluctuations (Fig. 2.2).

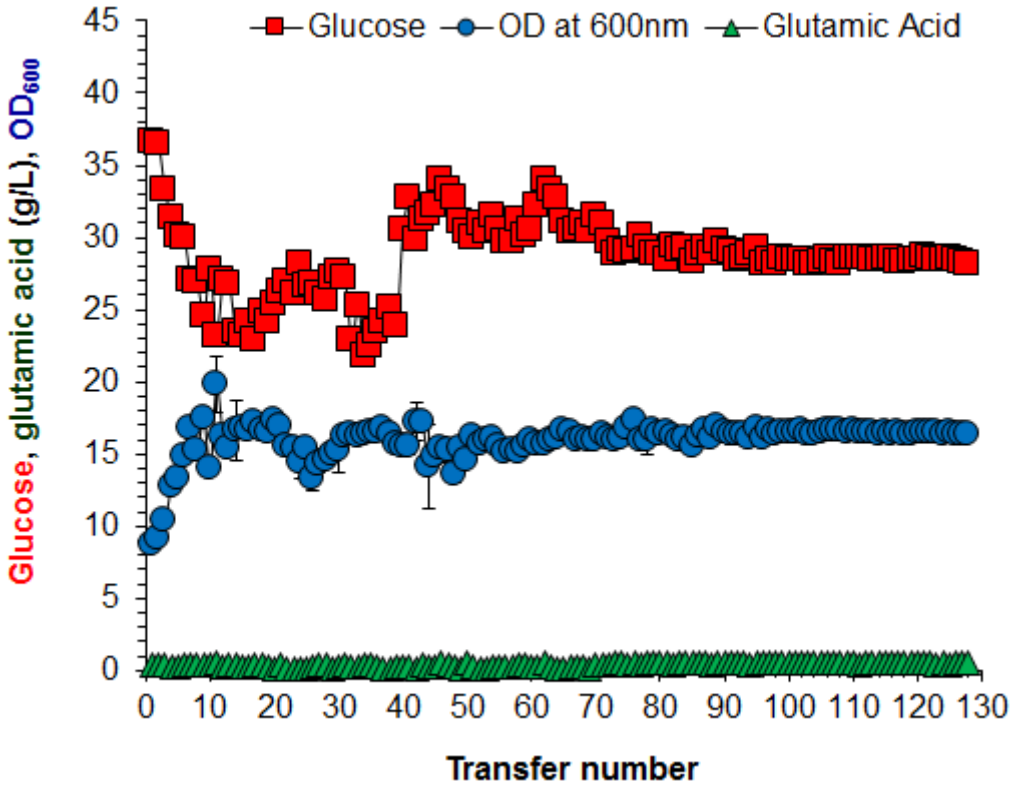


Fig. 2.2. Time course profile of the evolutionary adaptation of *Corynebacterium glutamicum* SIIM B460. The evolutionary adaptation process was conducted at 30 ^{o}C in flasks containing the corn stover hydrolysate. It was transferred every 24 hours with 5% (v/v) culture liquid and lasted for 3072 hours. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

Adapted *C. glutamicum* strain in the inhibitors containing synthetic medium showed the significantly improved cell growth at the existence of each inhibitor compound (Fig. 2.3). Improved performance of strain was probably due to adaptation behavior of strain in the presence of inhibitory compounds. Evolutionary adaptation increases inhibitor tolerance and degradation by altering the gene expression. Therefore, adapted strain performs better than parental strain in the presence of inhibitory compounds in the synthetic medium. The glutamic acid generation of the adapted strain was also improved compared to the parental strain. Inhibitors tolerance and glutamic acid fermentability were significantly improved by long term evolutionary adaptation of strain in corn stover hydrolysate.

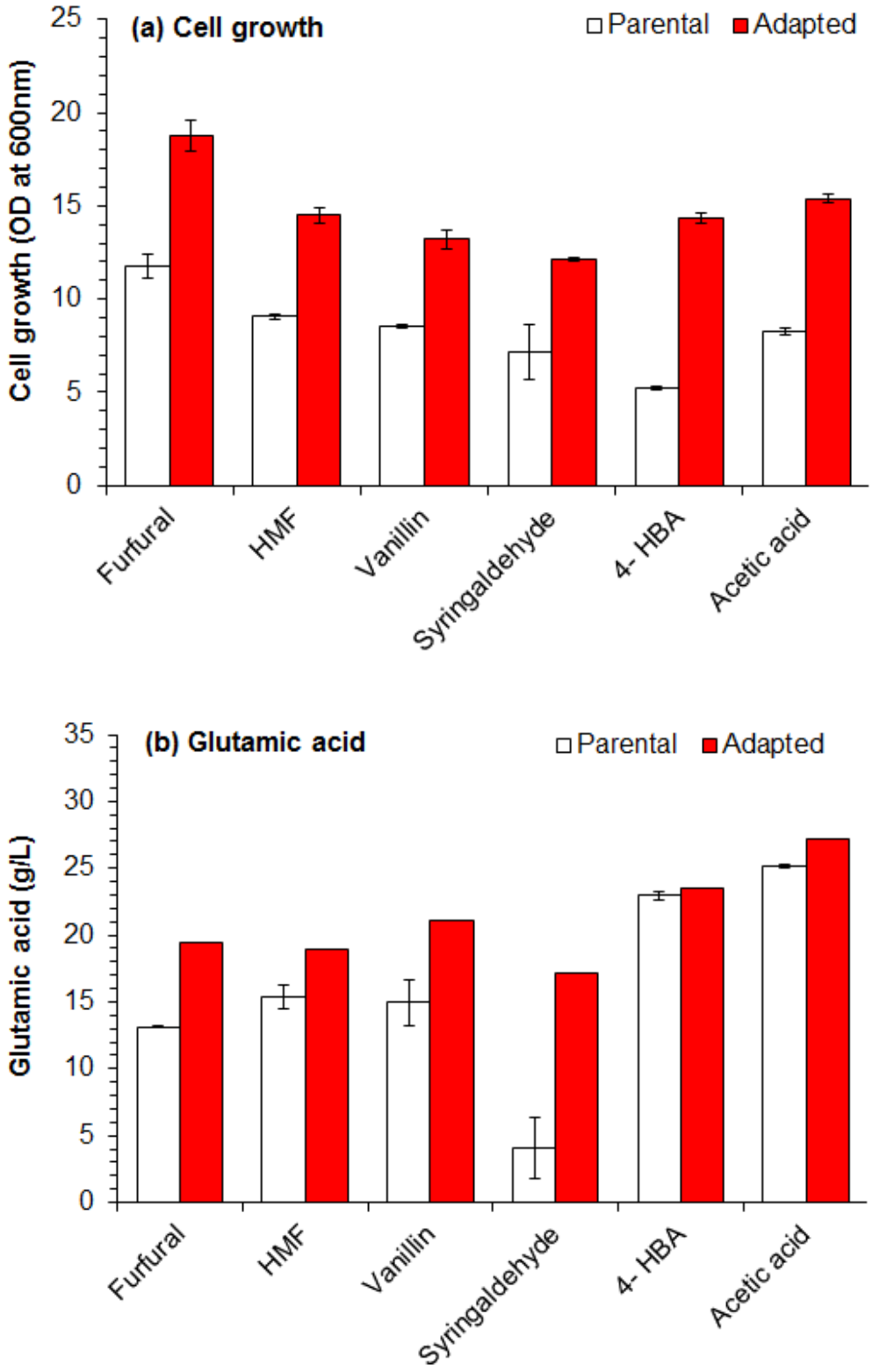
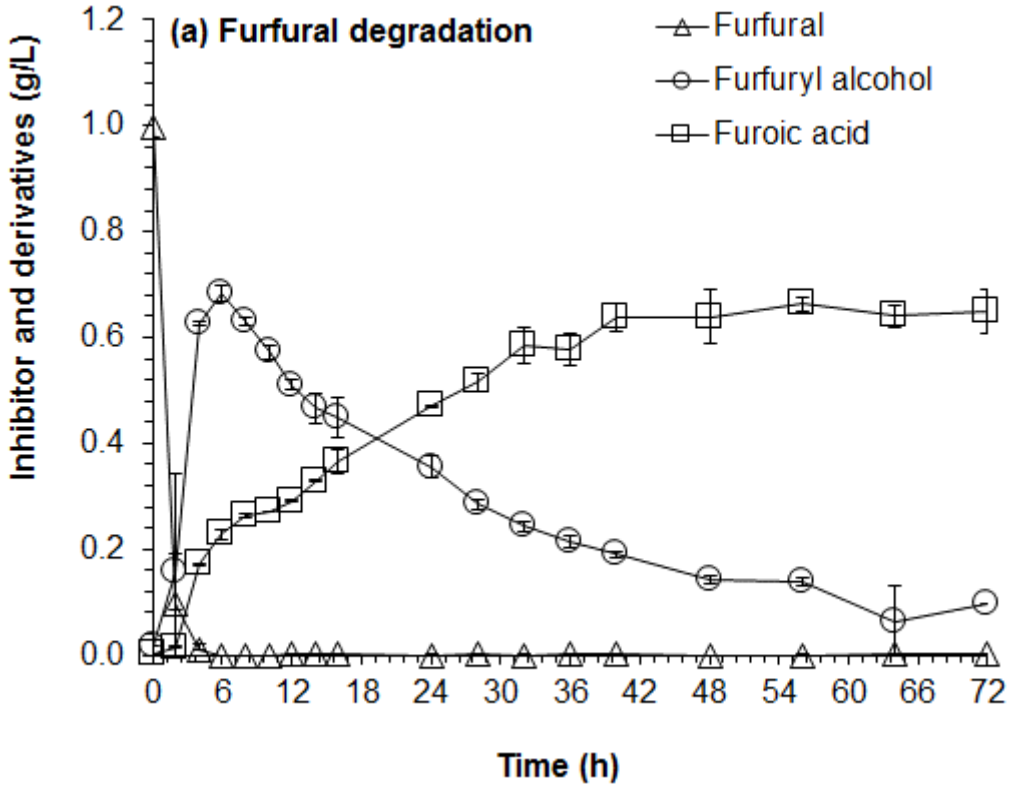


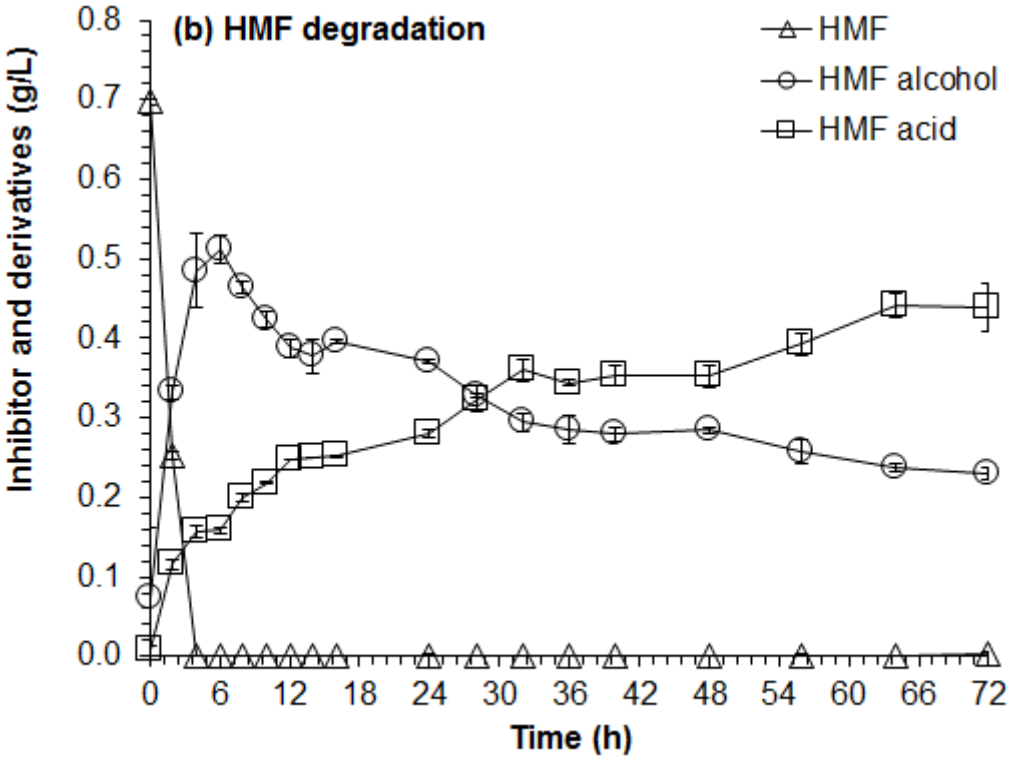
Fig. 2.3. Comparison of inhibitor tolerance and glutamic acid fermentability of parental and adapted *Corynebacterium glutamicum* SIIM B460 against different concentrations of various inhibitors. (a) Cell growth (OD_{600nm}), (b) Glutamic acid (g/L). Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 36 hours in flasks, 5% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

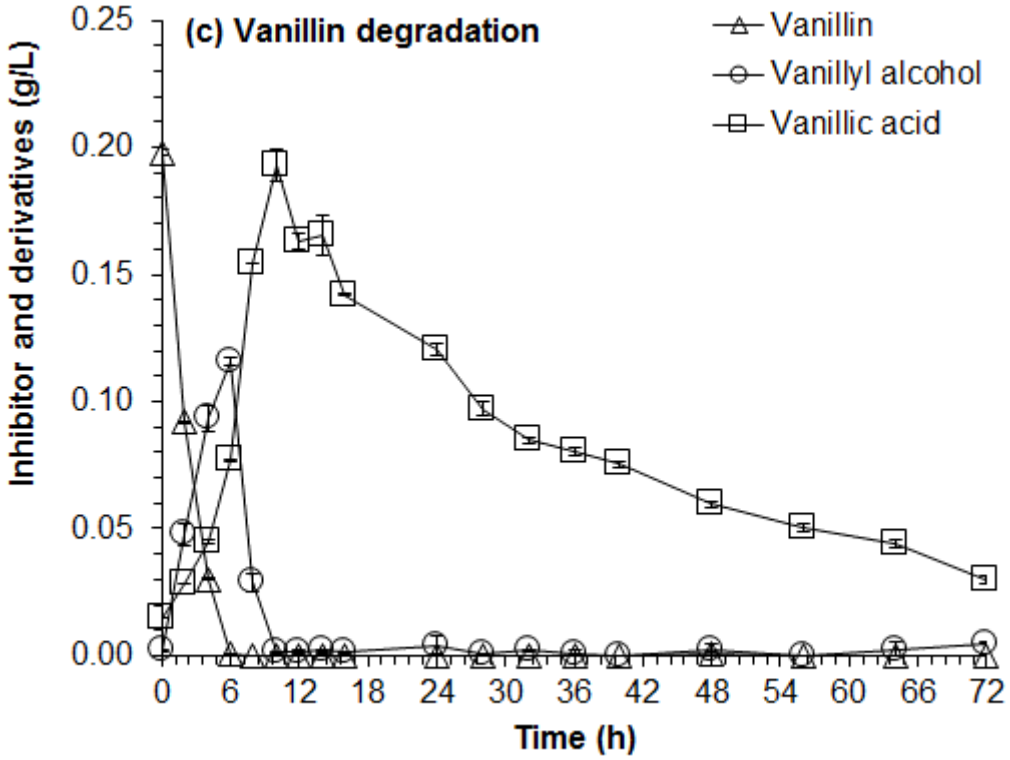
2.3.2. Inhibitors degradation and pathway analysis by *C. glutamicum*

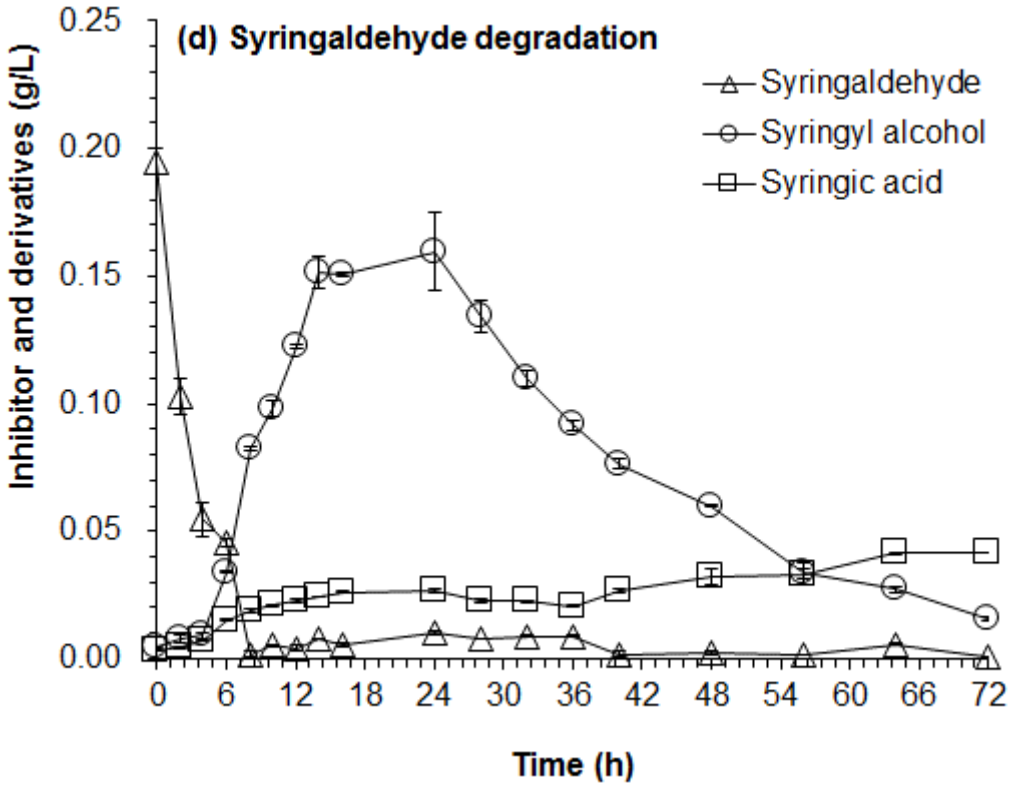
Inhibitor tolerance of microorganisms is generally considered as the capability of converting toxic compounds into less toxic compounds [208, 221]. Six model inhibitors were selected for testing the degradation capacity of *C. glutamicum*, including furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid.

Furfural was quickly converted to furfuryl alcohol then converted into furoic acid with the approximate mass balance among furfural and its derivatives (Fig. 2.4a). HMF was converted into HMF alcohol first then to HMF acid with the similar mass balance among the three compounds (Fig. 2.4b). Vanillin was converted into vanillyl alcohol as an unstable intermediate then quickly and completely converted into vanillic acid followed by the further degradation of vanillic acid (Fig. 2.4c). Syringaldehyde was converted into syringyl alcohol and the alcohol was converted into syringic acid (Fig. 2.4d). 4-hydroxybenzaldehyde was converted into 4-hydroxybenyl alcohol but only minimum 4-hydroxybenzoic acid was detected (Fig. 2.4e). For the acetic acid degradation, it was slowly assimilated completely at 48 hr without obvious intermediate formation (Fig. 2.4f). Adapted *C. glutamicum* strain significantly reduced the degradation time of all the inhibitors comparing to that of the parental strain (Fig. 2.5). Most inhibitors degradation time was decreased by more than 30 % using adapted strain in comparison to the parental strain. Adapted strain speed up the degradation process and decreased the required time of furfural, HMF, acetic acid, vanillin, syringaldehyde and 4-hydroxybenzaldehyde to 4, 4, 48, 6, 8 and 12 h, respectively, in comparison to many other strains, such as 5mM of furfural was degraded by *E.coli* after 72 h [106] , 17 mM by *Clostridium acetobutylicum* ATCC 824 after 12 h [107], 10 mM by *Amorphotheca resinae* ZN1 after 64 h [156], 5 mM by *Corynebacterium glutamicum* after 6 h [214]. 16 mM of HMF was degraded by *Clostridium acetobutylicum* ATCC 824 after 24 h [107]. Vanillin (3.96 mM) was degraded after 48 h, Syringaldehyde (4.91 mM) after 36 h and 4-hydroxybenzaldehyde (4.62 mM) after 48 h by *S. cerevisiae* [208].


(a) Furfural degradation
Furfural
Furfuryl alcohol
Furoic acid
Inhibitor and derivatives (g/L)
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
6
12
18
24
30
36
42
48
54
60
66
72


(b) HMF degradation
HMF
HMF alcohol
HMF acid
Inhibitor and derivatives (g/L)
Time (h)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
6
12
18
24
30
36
42
48
54
60
66
72


(c) Vanillin degradation
Vanillin
Vanillyl alcohol
Vanillic acid
Inhibitor and derivatives (g/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)


(d) Syringaldehyde degradation
Syringaldehyde
Syringyl alcohol
Syringic acid
Inhibitor and derivatives (g/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)

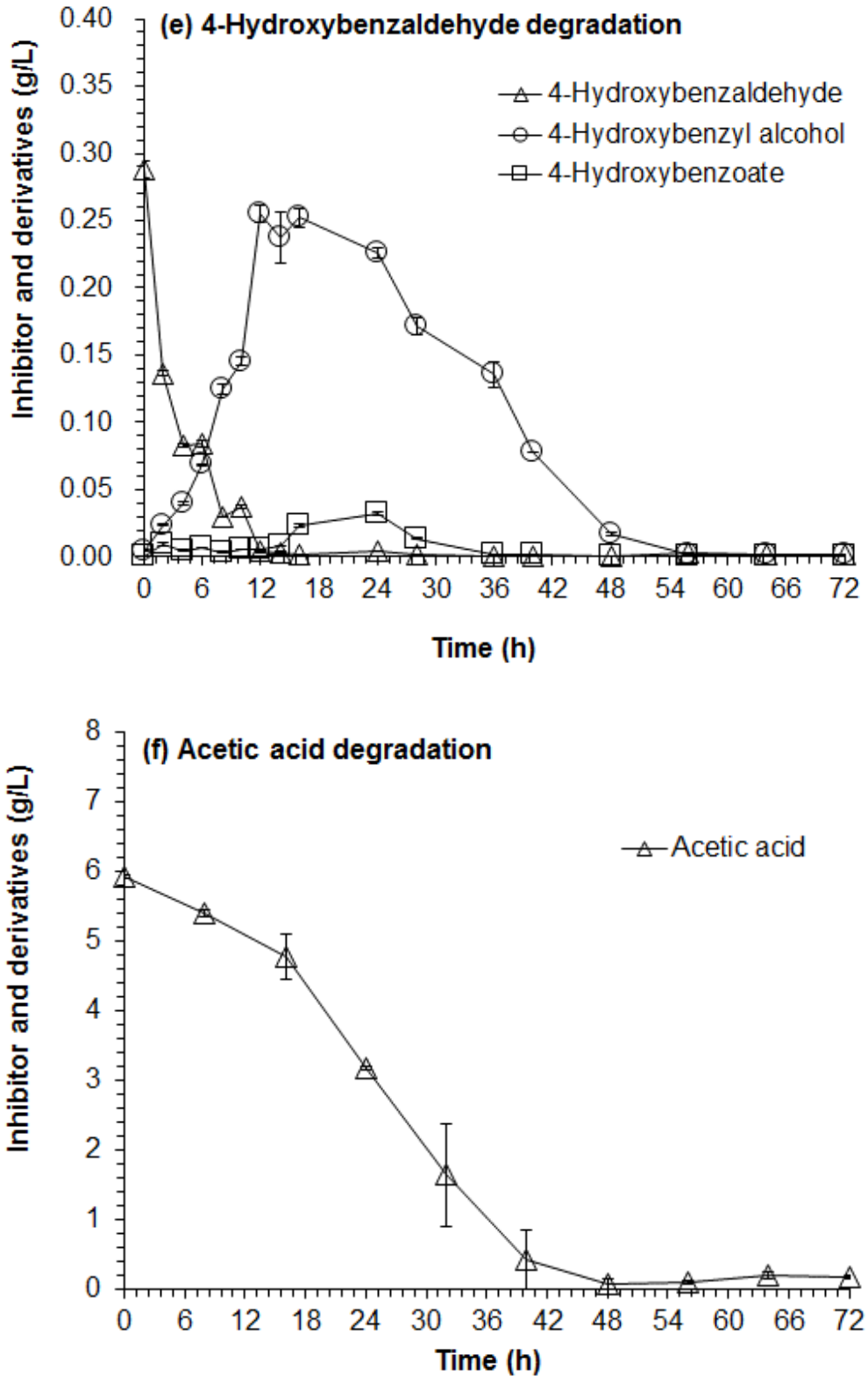
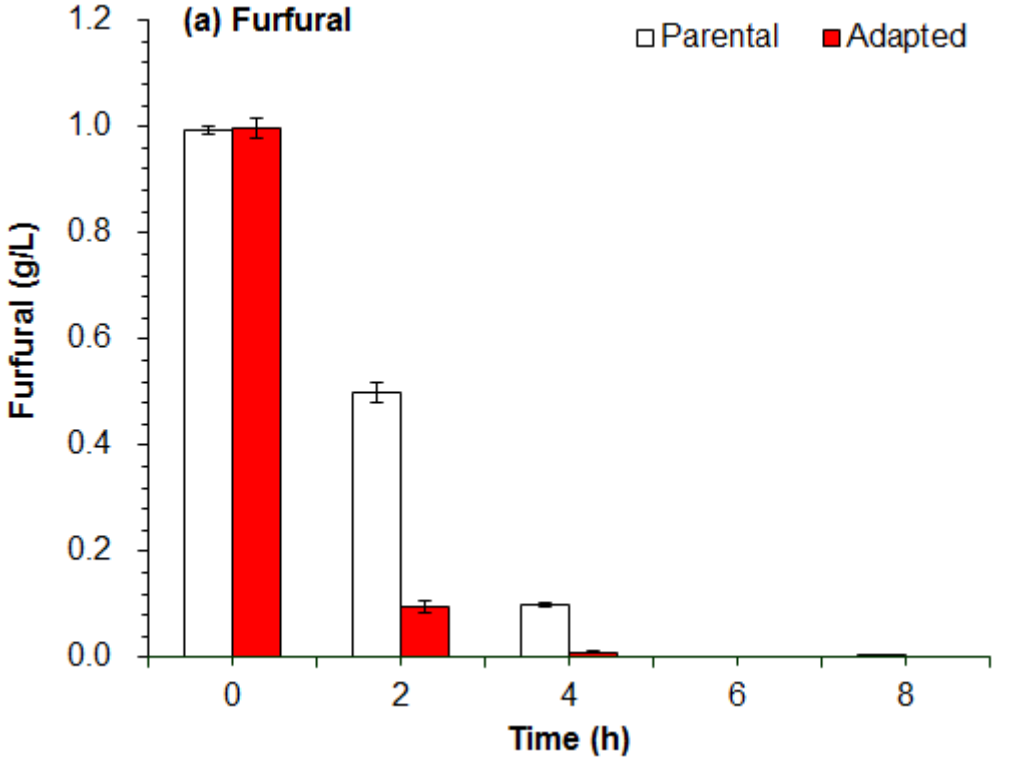
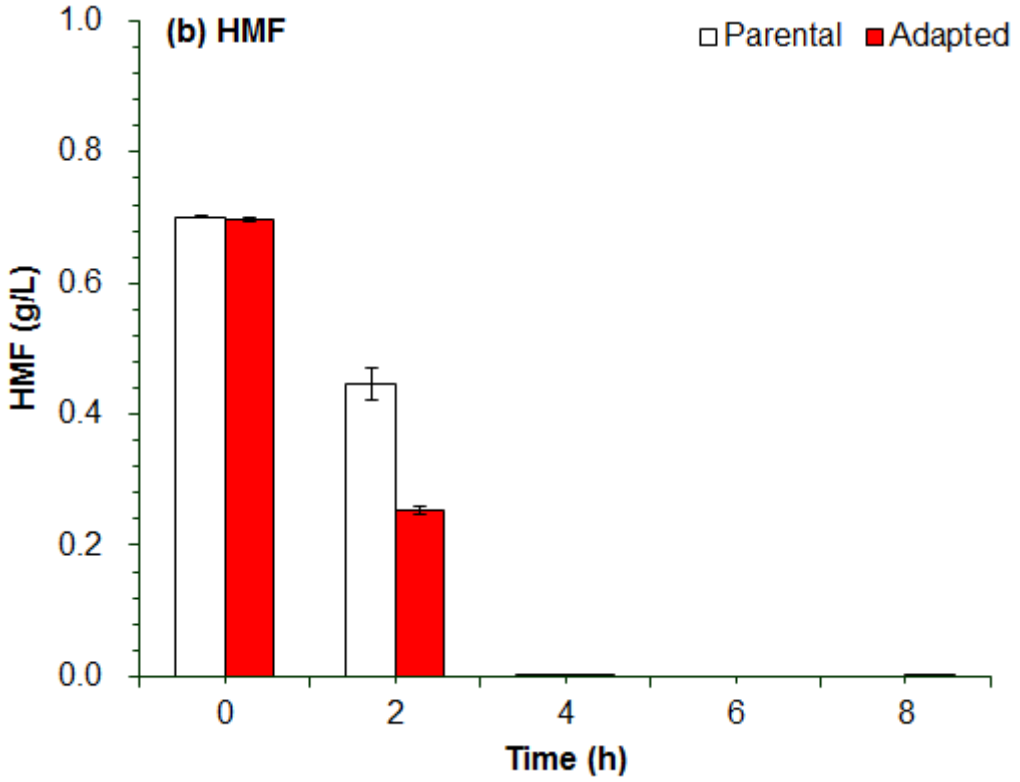
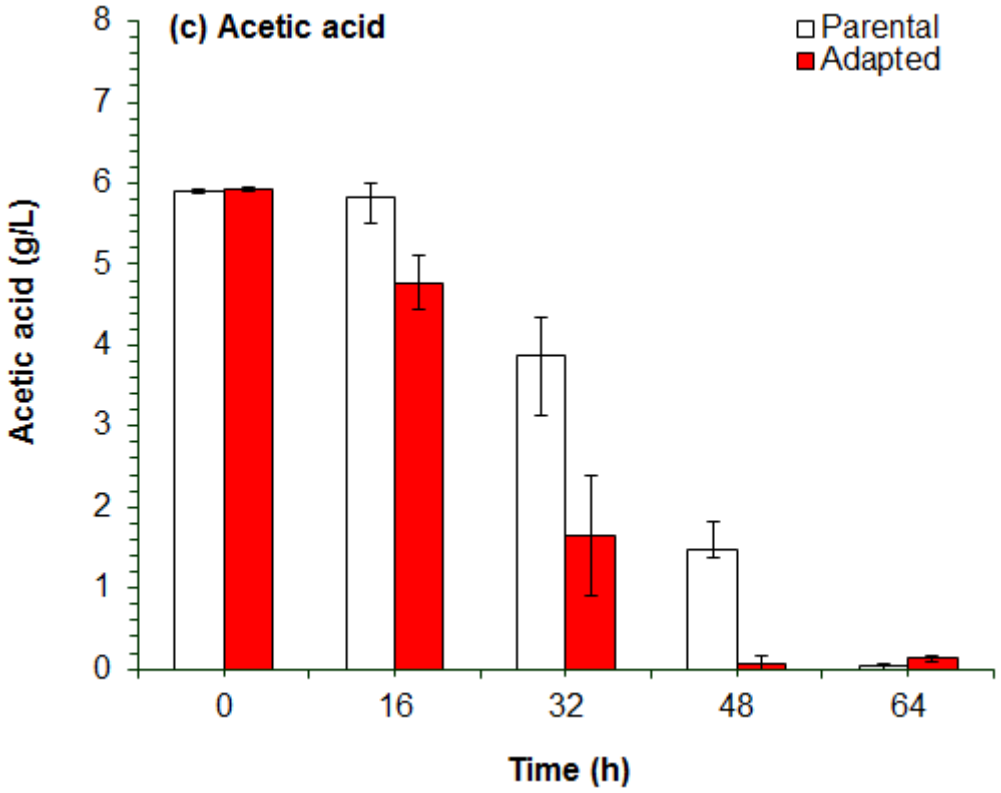
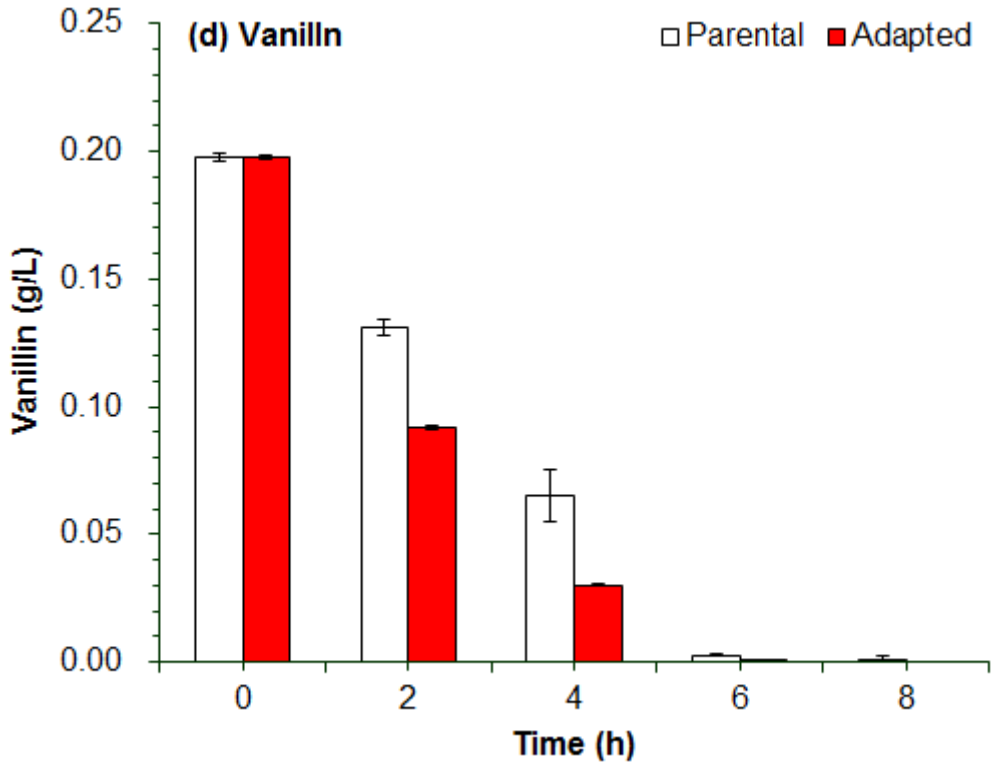


Fig. 2.4. Biodegradation by adapted *Corynebacterium glutamicum* SIIM B460 using various inhibitors. Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 72 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. (a) Furfural 1.0 g/L, (b) 5-HMF 0.7 g/L, (c) Vanillin 0.2g/L, (d) Syringaldehyde 0.2 g/L, (e) 4-hydroxybenzaldehyde 0.3 g/L, (f) Acetic acid 6 g/L. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.


(a) Furfural
Parental
Adapted
Furfural (g/L)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Time (h)


(b) HMF
Parental
Adapted
HMF (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Time (h)


(c) Acetic acid
Parental
Adapted
Acetic acid (g/L)
0
1
2
3
4
5
6
7
8
0
16
32
48
64
Time (h)


(d) Vanilln
Parental
Adapted
Vanillin (g/L)
0.00
0.05
0.10
0.15
0.20
0.25
0
2
4
6
8
Time (h)

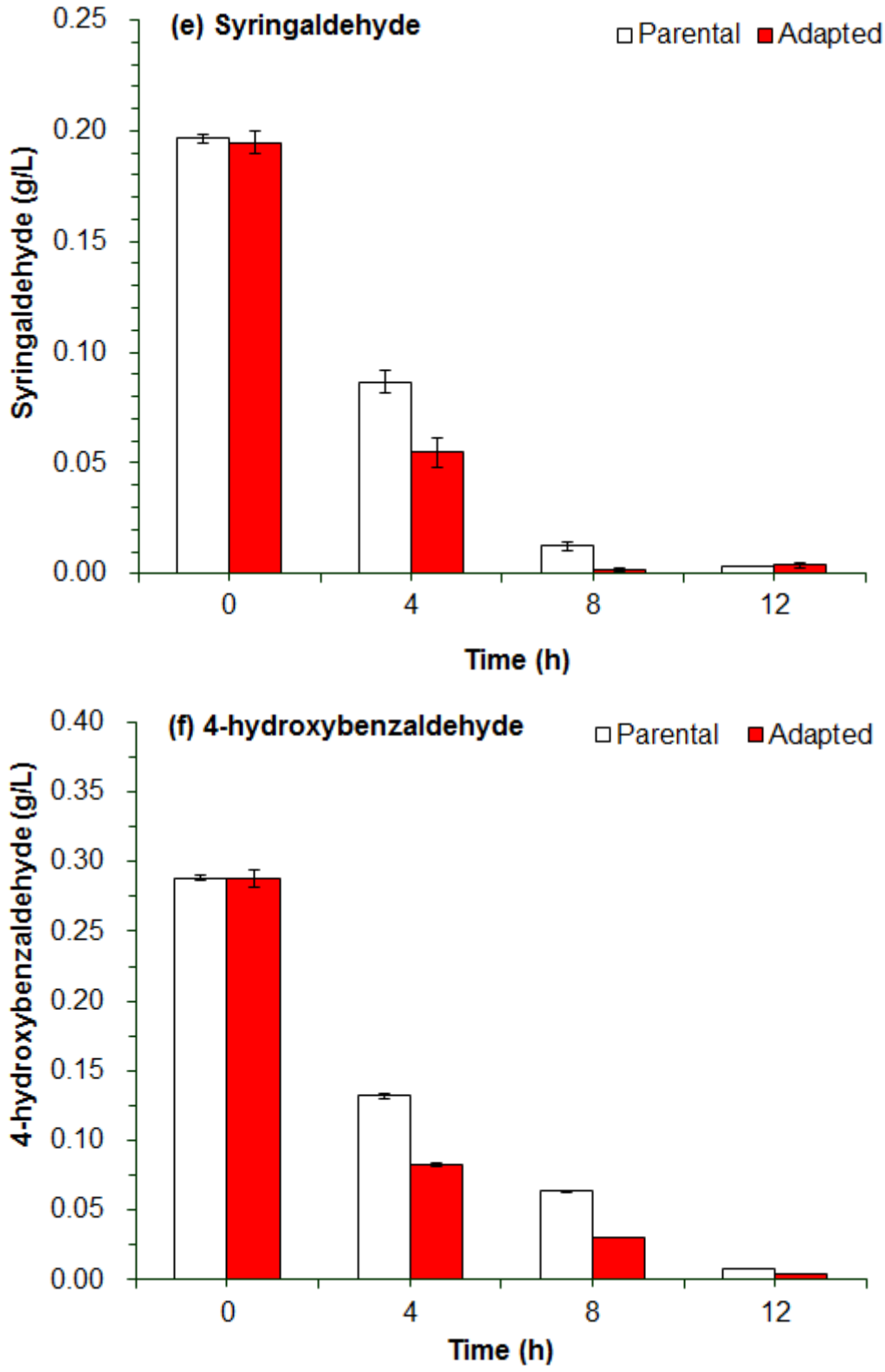


Fig. 2.5. Comparison of biodegradation by parental and adapted *Corynebacterium glutamicum* SIIM B460 using various inhibitors. Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 72 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. (a) Furfural 1.0 g/L, (b) 5-HMF 0.7 g/L, (c) Acetic acid 6 g/L (d) Vanillin 0.2g/L, (e) Syringaldehyde 0.2 g/L, (f) 4-hydroxybenzaldehyde 0.3 g/L,. The experiments were performed in

duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

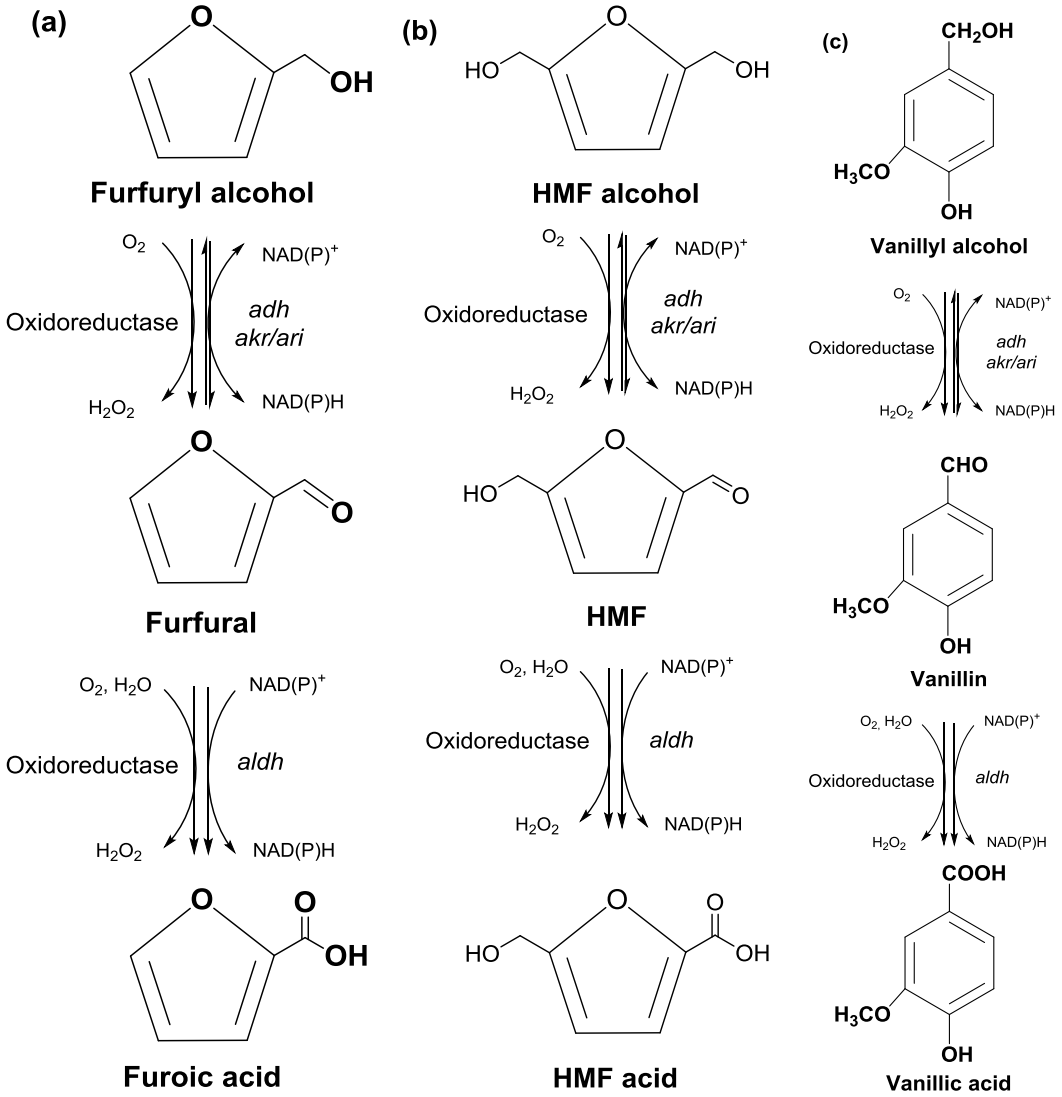
Based on the above experimental results and the previous pathway studies [156, 160, 203, 209, 222], the putative degradation pathways of inhibitors by *C. glutamicum* were proposed as shown in Fig. 2.6. For the furan aldehyde degradation, we proposed that furan aldehydes were reduced quickly into furfuryl alcohol by NAD(P)H dependent alcohol dehydrogenases (*adh*) or aldo-keto reductase/aldehyde reductases (*akr/ari*) consuming NAD(P)H. Furan alcohol oxidized to furan aldehydes again by alcohol dehydrogenase (*adh*) or aldo keto reductase/aldehyde reductase (*akr/ari*) with the formation of NAD(P)H, and then oxidized to furan acids consequently by aldehyde dehydrogenases (*aldh*) genes with the formation of NAD(P)H. Alcohol dehydrogenase, aldo/keto reductase and aldehyde dehydrogenase have been reported to degrade furans to furan alcohols and acids by *Saccharomyces cerevisiae* [177, 198, 200, 201, 223, 224], *E.coli* [196] and *Amorphotheca resinae* ZN1 [156]. It was presumed that for HMF and furfural reduction, nonspecific dehydrogenase was responsible for the upper pathway oxidation reactions in *Cupriavidus basilensis* HMF14 [202]. Tsuge et al. [214] described the degradation of furfural into furfuryl alcohol and furoic acid, and utilization of both NADH and NADPH as co-factors by *Corynebacterium glutamicum*. Wang et al. [203] identified Zn dependent alcohol dehydrogenase genes and *AKR*/*ARI* genes to be responsible for the furfural and HMF degradation to their corresponding alcohols in *Amorphotheca resinae* ZN1.

Phenolic aldehydes are proposed to be reduced quickly to phenolic alcohol by NAD(P)H dependent alcohol dehydrogenases (*adh*) or aldo-keto reductase/aldehyde reductases (*akr/ari*) utilizing NAD(P)H. Phenolic alcohols were oxidized to phenolic aldehydes at much lower concentration by *adh* genes or *akr/ari* genes with the formation of NAD(P)H, as well as aldehyde dehydrogenases (*aldh*) genes with the formation of NAD(P)H responsible for the further oxidation into phenolic acids. *Sphingomonas sp.*SYK6 converts vanillin to vanillic acid by vanillin dehydrogenase [222]. The genes encoding oxidoreductase, alcohol dehydrogenase, oxidoreductase/NADH oxidase, aldo/keto reductase, and a member of short-chain dehydrogenase/ reductase (SDR), respectively, involved in the conversion of phenolic aldehydes into the corresponding phenolic alcohols [209].

Acetic acid is hypothesized to be degraded to acetyl phosphate by acetate kinase (*ack*) gene then to acetyl-CoA by phosphotransacetylase (*pta*) gene then further entered into TCA cycle (Fig. 2.6f), which was also described by [225] and Cramer et al. (2007)[213] for acetic acid metabolism in *Corynebacterium glutamicum.*

In our *C. glutamicum* SIIM B460 genome http://www.ncbi.nlm.nih.gov/nuccore/417969368?report=genbank), there are many corresponding genes which highly possible functioned in the degradation pathways, such as *adh* genes (CGS9114_RS00260, CGS9114_RS04130, CGS9114_RS05970, CGS9114_RS08205, CGS9114_RS08420, CGS9114_RS10690 et al.). *akr/ari* genes (CGS9114_RS02805, CGS9114_RS06730 et al.), *aldh* genes (CGS9114_RS00305, CGS9114_RS00855, CGS9114_RS08210, CGS9114_RS08415, CGS9114_RS12030 et al.), *ack* gene CgS9114_00490 and *pta* gene CGS9114_RS00460.

These results indicated that *C. glutamicum* has an excellent capability to degrade almost all the tested inhibitors, including furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid. The adapted strain accelerated the degradation speed compared to parental strain [106, 107, 157, 208, 214]. The inhibitor degradation pathways and many functional genes were existed in our *C. glutamicum* (Fig. 2.6). These results clearly indicated that the adapted strain probably could be applied for glutamic acid production from non-detoxified corn stover hydrolysate or pretreated materials.



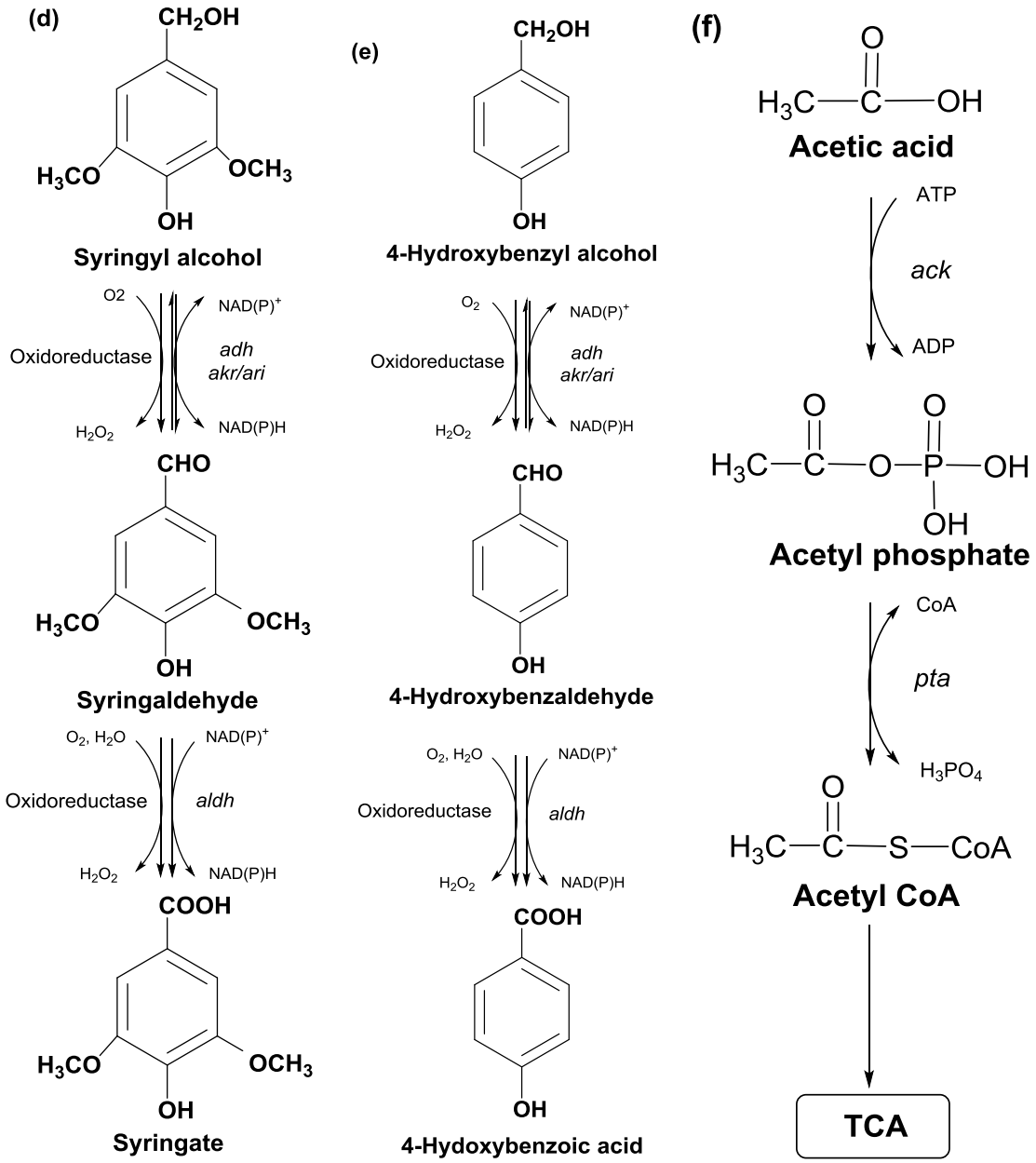


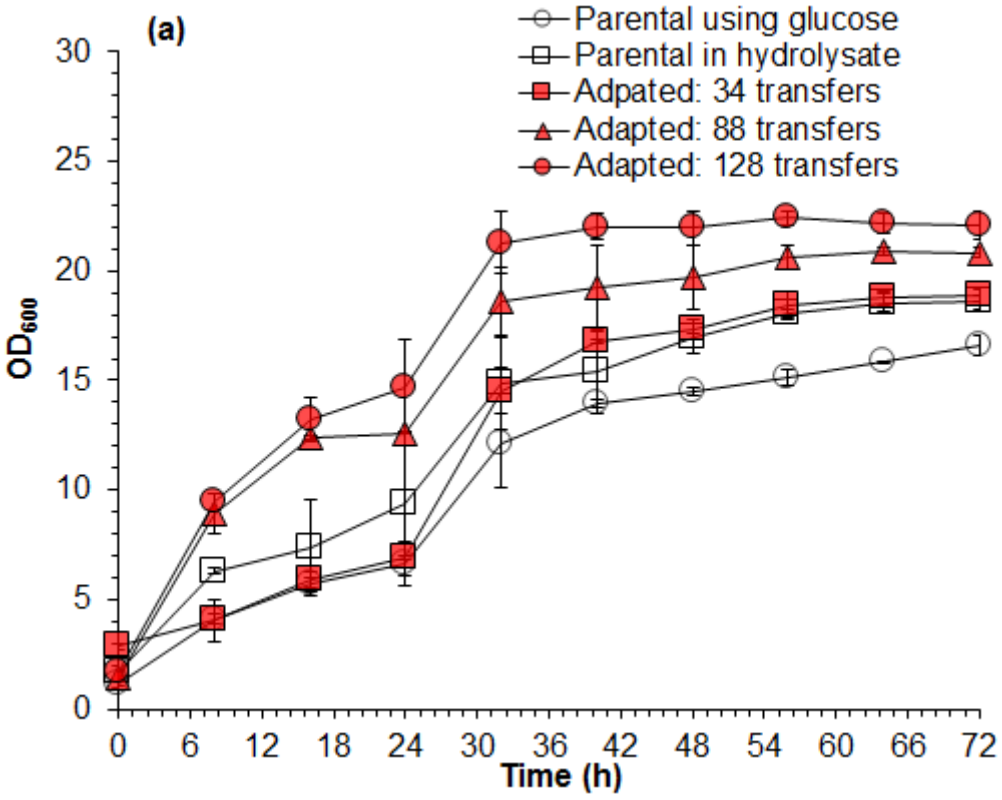
Fig. 2.6. Putative degradation pathways of (a) Furfural, (b) HMF, (c) Vanillin, (d) Syringaldehyde, (e) 4-Hydroxybenzaldehyde, and (f) Acetic acid in *C. glutamicum* SIIM B460. Furan aldehydes reduced into furfuryl alcohol by *adh* or *akr/ari* and oxidized back to furan aldehydes by *adh* or *akr/ari* genes. Further oxidized to furan acids by *aldh* genes. Phenolic aldehydes reduced into phenolic alcohol and oxidized back to phenolic aldehydes by *adh* or *akr/ari* genes. Further oxidized into phenolic acids by *aldh* genes. Acetic acid degraded to acetyl phosphate by *ack* gene and to acetyl-CoA by *pta* gene then entered into TCA cycle. Abbreviations: *adh*: alcohol dehydrogenase; *akr*: Aldo-keto reductase; *ari*: aldehyde reductase; *aldh*: aldehyde dehydrogenase, *ack*: acetate kinase; *pta*: phosphotransacetylase

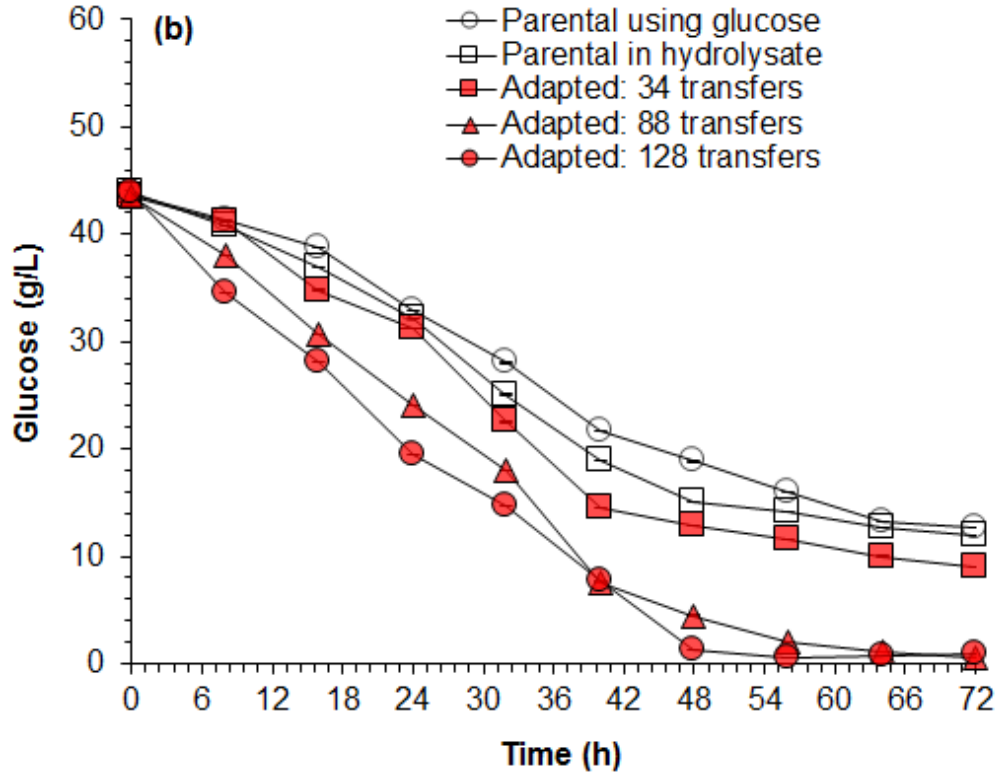
2.3.3. Glutamic acid fermentation of *C. glutamicum* using corn stover hydrolysate

Glutamic acid fermentation using corn stover hydrolysate as feedstock was performed by the parental and adapted *C. glutamicum* strains. Corn stover hydrolysate contained 33.69

g/L of glucose, 0.24 g/L of lactic acid 2.54 g/L of acetic acid, 0.379 g/L of furfural and 0.209 g/L of 5-hydroxymethylfurfural (HMF).

Fig. 2.7(a) shows the cell growth of different cultures up to 72 h. Results clearly show that the tolerance of *C. glutamicum* against pretreatment inhibitors increased during the whole course of evolutionary adaptation till 128 transfers, which is reflected by increase in the cell growth from parental to the last adapted strain. In the Cg 34, glutamic acid productivity did not improve to large extent, while in Cg 88 and Cg 128 prominent difference was observed. Glutamic acid concentration also increased with increasing number of transfers as shown in Fig. 2.7(b). Glutamic acid concentration increased by 39.73% and 32. 41% comparing to control (synthetic medium) and in corn stover hydrolysate. Evolved strain quickly exited the lag phase than parental strain perhaps due to adaption function toward inhibitors. Results indicate that the evolutionary adaptation significantly improved the glutamic acid fermentability of the parental *C. glutamicum* strain when the liquid corn stover hydrolysate was used. Evolutionary adaptation improved inhibitors tolerance and degradation, growth, and fermentability. Adapted strains could be utilized for industrial glutamic acid production from high solids content/ hydrolysate prepared from high solids content because this strain is adapted in same environment where it will be utilized, adapted strain will certainly perform better than parental strain. Previous studies on ethanol production [10, 226] have investigated and proved that adaptation improved the ethanol fermentation performance due to adaptation function and similar results were obtained in present study for glutamic acid production. In addition, fermentation performance of the strain could further be investigated by utilizing high solids content of pretreated corn stover or hydrolysate. Results shown in Fig. 7 (b) clearly indicate that glutamic acid production and growth was increased by increasing adaptation time or duration.


(a)
Parental using glucose
Parental in hydrolysate
Adpated: 34 transfers
Adapted: 88 transfers
Adapted: 128 transfers
OD600
Time (h)
0
5
10
15
20
25
30
0
6
12
18
24
30
36
42
48
54
60
66
72


(b)
Parental using glucose
Parental in hydrolysate
Adapted: 34 transfers
Adapted: 88 transfers
Adapted: 128 transfers
Glucose (g/L)
Time (h)
0
10
20
30
40
50
60
0
6
12
18
24
30
36
42
48
54
60
66
72

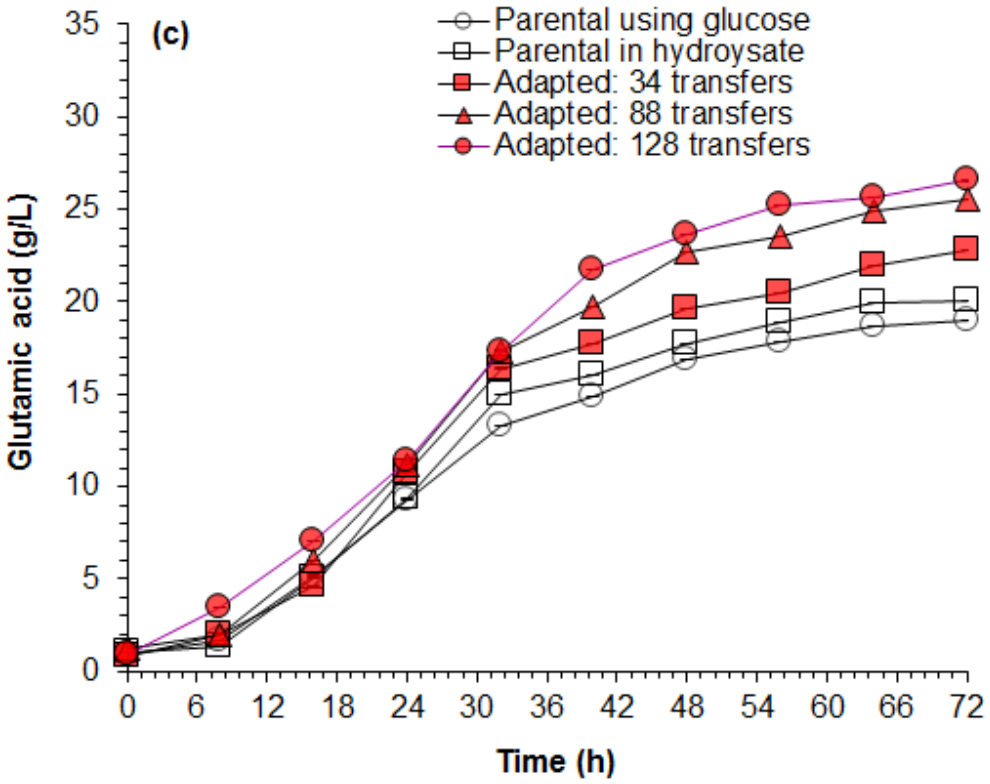


Fig. 2.7. Fermentability of parental and adapted *C. glutamicum* (a) cell growth (OD_{600nm}), (b) residual glucose (g/L), (c) glutamic acid (g/L). Fermentation conditions with synthetic medium and corn stover hydrolysate: 30 ℃, 200 rpm for 72 hours in flasks, 20% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 20% urea at regular interval. Penicillin 0.1 mL/L. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

During the long term evolutionary adaptation, random mutation take place due to selective pressure of the pretreatment inhibitors, and resulting rate of mutation increases, which might be responsible for generation of evolved strains with improved tolerance and fermentability comparing to parental strain [181, 227, 228]. In the course of adaptation continuously genes are up and down regulated based on the medium composition for survival of fermenting cells [182, 229].

2.4. Conclusion

Inhibitor tolerance and biodegradation capability of *C. glutamicum* was investigated and experimentally analyzed. *C. glutamicum* has strong tolerance to most of the pretreatment inhibitors, present in typical hydrolysate and pretreated material. Tested concentrations of inhibitory compounds were higher than those present in corn stover hydrolysate. *C. glutamicum* degrades furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid in very short time. Evolutionary adaptation increased the tolerance and degradation capability of the parental *C. glutamicum* strain. Cell growth and glutamic acid production were increased by the evolutionary adaptation of *C. glutamicum*. The results provide

important information for process intensifications of inhibitors removal from the pretreated materials and strain modifications for enhancing the inhibitor tolerance and degradation capacity.

Chapter 3

Tolerance and degradation evaluation of lysine producing C*orynebacterium glutamicum* to lignocellulose-derived inhibitors

3.1. Introduction

Corynebacterium glutamicum is a Gram positive, non-motile, aerobic soil bacterium [1]. Lysine is an essential amino acid, is valuable as medicament, chemical agent, food material (food industry), and as feed additive (animal food). Corn, barley and wheat which are commonly employed as the main ingredients for animal feed possess suboptimal concentration of lysine. Due to this reason, lysine becomes limiting factor for feed efficiency. Annual lysine production from *C. glutamicum* reached 1.5 million tons [185]. Lysine production from *C. glutamicum* certainly needs further improvement with a special attention on optimization of the production process using cost effective materials. High amino acid production cost from pure sugars is major technical barrier to meet the industrial needs [68]. However, inexpensive carbon source for lysine production could be alternative to current situation. Agro-industrial residues, such as corn fiber, corn stalks, corn stover, wheat straw, rice husk, rice straw could be potential biomass for biochemicals (amino acid, organic acids) and biofuel production [230-232]. Lignocellulosic biomass cannot be utilized directly as carbon source due to its recalcitrant nature. For efficient utilization of lignocellulosic material, pretreatment is an essential step. Pretreatment breaks down the lignocellulosic structure to make cellulose accessible to hydrolyzing enzymes [96]. Pretreatment generates wide range of toxic compounds, which inhibit enzyme activity and fermentability [5, 141]. These compounds are classified into three groups: organic acids, phenolic compounds, furans. Recently, many researches have investigated the influence of single inhibitor, formed by lignocellulosic biomass degradation, on the growth and fermentation [148, 233-235]. Presence of pretreatment inhibitors in fermentation medium can reduce the specific growth rate [236, 237], decrease biomass production [233, 237], decrease specific [233, 238] and volumetric productivity [236, 239].

The synergistic effect of inhibitory compounds is beyond a simple sum of the individual compound effects. This can perhaps be best exemplified by observing the tolerance level of any strain. [240] has shown that synergistic effect of inhibitors on yeast cells was more severe than individual inhibitors. Most of microorganisms, including industrial strains, are susceptible to pretreatment inhibitors [190, 233, 240, 241]. These inhibitors are reported to reduce enzymatic biological activities, to break down DNA, and to inhibit protein and RNA synthesis [242, 243]. Cell walls and membranes of microbial cells are also challenged when grown in presence of furfural and HMF [244, 245]. As a result, cell growth is delayed and productivity significantly reduced.

The formation of inhibitors during biomass pretreatment could be prevented by modifying the pretreatment conditions. But, these conditions might not be suitable for

releasing highest concentration of cellulose. Thus, inhibitory compounds should be removed prior to the fermentation process. There are several biodetoxification methods, including physical, chemical, or biochemical detoxification. However, these additional steps add cost and complexity to the process and generate extra waste products [129].

Alternatively, microbial fermentation hosts may be selected to tolerate or even metabolize the toxic compounds from lignocellulose hydrolysates. Researchers have made efforts to obtain inhibitor-tolerant strains. This approach has resulted in host organisms that can tolerate higher concentrations of furfural [152, 177] and metabolize acetate [246], furfural, and HMF [162, 202]. However, most of these strains can only tolerate single inhibitor. The strains tolerant to combined inhibitors are urgently required. In order to introduce or optimize such properties in a targeted manner, detailed insight into inhibitor tolerance and metabolism is the key. The latter has been the subject of intensive study over the last decade.

Corynebacterium glutamicum is a potential microorganism for lysine production. In this study, inhibitors' tolerance of *C. glutamicum* SIIM B253 was experimentally investigated for nine model inhibitors and degradation performance towards six model inhibitors. Non-biodetoxified corn stover hydrolysate was used for lysine production. This study provides important information on inhibitors tolerance and degradation of *C. glutamicum* for the future improvement of detoxification efficiency and metabolic modification of the strain. This study clearly indicates that *C. glutamicum* has excellent capability to tolerate and degrade pretreatment inhibitors and lignocellulose biomass can be used without prior biodetoxification for lysine production.

3.2. Material and Methods

3.2.1. Raw materials and reagents

Corn stover was harvested from Dancheng County, Henan Province, China, in fall 2014. Field dirt, stones and metals of corn stover was removed by water washing, then air-dried, milled by a beater pulverizer to pass through the 10-mm apertures in diameter. The virgin corn stover contained 37.2% of cellulose and 19.9% of hemicellulose determined according to a two-step H_2SO_4 hydrolysis method developed by the National Renewable Energy Laboratory (NREL) protocol [218].

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity was 135 FPU/g determined using the NREL protocol LAP-006 [219], the cellobiose activity was 344 CBU/g using the method described by [220], and the cellulase protein content was 90 mg/g.

Furfural and HMF were purchased from Shanghai DEMO Medical Tech Co., Ltd, Shanghai, China. 4-Hydroxybenzaldehyde, syringaldehyde, and vanillin were purchased from Sangon Biotech Co., Shanghai, China. Acetic acid, lactic acid and formic acid were purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd, phenol from Shanghai Generay Biotech Co., Ltd, coniferyl aldehyde from Sigma-Aldrich Co., USA, and levulinic acid was purchased

from Ruiteng chemical Co., Ltd, China. Yeast extract was purchased from Oxiod, Basingstoke, Hampshire, UK. All other standard chemicals including glucose, peptone, NaCl, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, Urea, $FeSO_4$, MnSO4, NaOH, and H_2SO_4 were of reagent grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Agar was purchased from New Probe, Beijing, China.

3.2.1. Strain and Medium

Lysine fermenting strain *C. glutamicum* SIIM B253 was purchased from Shanghai Industrial Institute of Microorganisms (SIIM), Shanghai China. One vial of *C. glutamicum* SIIM B460 was taken from the -80 ℃ freezer and culture was streaked on petri plate containing LB medium, and incubated for 48 h at 30 ℃, pH 7.0. A single colony was transferred into 30 mL of pre-culture medium in 250 mL Erlenmeyer flask and incubated in shaking incubator at 30 ℃, 200 rpm, pH 7.0. After 12 h, 10% (v/v) of the culture was transferred to the seeds culture medium and incubated for 8 h at 30 ℃, pH 7.0 in shaking incubator. Seeds culture was used as the inoculum in all fermentation experiments. The medium used for C. glutamicum SIIM B253 included:

1. LB agar medium contained 5 g of yeast extract, 10 g of peptone, 5 g of NaCl, and 17 g of agar in one liter of deionized water. pH 7.0 was adjusted using 5 M NaOH.

2. Pre-culture medium contained 25 g of glucose, 1.5 g of KH_2PO_4, 0.6 g of $MgSO_4.7H_2O$, 2.5 g of urea, 0.002 g of $FeSO_4$, 0.002 g of $MnSO_4$, and 25 g of corn steep liquor in one liter of deionized water. pH 7.0 was adjusted using 5M NaOH.

3. Seeds culture medium contained 25 g of glucose, 1.5 g of KH_2PO_4, 0.6 g of $MgSO_4.7H_2O$, 2.5 g of urea, 0.002 g of $FeSO_4$, 0.002 g of $MnSO_4$, and 25 g of corn steep liquor in one liter of deionized water. pH 7.0 was adjusted using 5M NaOH.

4. Synthetic medium contained 60 g of glucose, 1.0 g of KH_2PO_4, 0.6 g of $MgSO_4{\cdot}7H_2O$, 10 g of $(NH_4)_2SO_4$, 0.002 g of $FeSO_4$, 0.002 g of $MnSO_4$, and 20 g of corn steep liquor in one liter of deionized water. pH 7.0 was adjusted using 5 M NaOH.

5. Hydrolysate medium: Corn stover hydrolysate prepared at solids loading of 15 % (w/w), 1.0 g of KH_2PO_4, 0.6 g of $MgSO_4{\cdot}7H_2O$, 10 g of $(NH_4)_2SO_4$, 0.002 g of $FeSO_4$, 0.002 g of $MnSO_4$, and 20 g of corn steep liquor in one liter of corn stover hydrolysate. pH 7.0 was adjusted using 5M NaOH.

All media were sterilized at 115 ℃ for 20 min. $FeSO_4$ and $MnSO_4$ freshly prepared to avoid the oxidation and filter sterilized by bacterial filter.

3.2.2. Pretreatment and hydrolysate preparation

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment (DDAP) according to [6, 8]. Briefly, 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 then pretreated at 175 ℃ for 5 min. The pretreated corn stover contained 50% (w/w) of solids and no free water was generated during pretreatment. The dry solid of the pretreated corn stover contained 39.89% of glucan and 3.04% of xylan according to the two-step H_2SO_4 hydrolysis method [218].

In case of biodetoxified hydrolysate the slurry of pretreated corn stover was detoxified by *A. resinae ZN1* according to [157]. Pretreated corn stover was neutralized to pH value of 5–6 with 20% (w/w) $Ca(OH)_2$, and then *A. resinae ZN1*was inoculated at 10% (w/w) ratio and incubated at 28 ℃ for few days up till the degradation of the inhibitory compounds. During biodetoxification no additional fresh water was added, and the solids content of the biodetoxified corn stover material was about 50% (w/w).

Biodetoxified and non-biodetoxified corn stover hydrolysate was prepared by enzymatic hydrolysis of dry dilute acid pretreated corn stover at the cellulase dosage of 15 FPU/g dry corn stover matter (DM) (equivalent to 10 mg protein/g DM) at 15% (w/w) of solids loading at 50 ℃ for 48 h. The hydrolysate slurry was centrifuged to remove the solids to obtain the clear hydrolysate containing 70.6 g/L of glucose, 3.12 g/L of acetic acid, 0.56 g/L of furfural, and 0.353 g/L of HMF. Before use, the nutrient composition, including 1 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4 \cdot 7H_2O$, 3 g/L of urea, 0.002 g/L of $FeSO_4$, 0.002 g/L of $MnSO_4$, and 20 g/L of corn steep liquor were added.

3.2.3. Inhibitor tolerance and degradation

Inhibitor tolerance of *C. glutamicum* SIIM B253 was analyzed by using different concentrations of each inhibitor using synthetic medium. 10 % (v/v) of inoculum ratio was used and incubated at 30 ^oC in the shaking incubator at 200 rpm. pH was adjusted at 7 using 5M NaOH throughout the course of fermentation up to 48 h.

Inhibitor degradation of *C. glutamicum* was analyzed by using each inhibitor in the synthetic medium. 10% of inoculum ratio was used and incubated at 30 ^oC at 200 rpm for 72 h. pH was adjusted at 7 with NaOH.

3.2.6. Lysine fermentation

Lysine fermentation of *C. glutamicum* SIIM B253 was carried out by using synthetic medium and corn stover hydrolysate. 10% (v/v) of inoculum ratio was used and incubated at 30 ℃ at 200 rpm in the shaking incubator for 72 h. pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval.

3.2.7. Analytical methods

Samples were periodically collected, centrifuged to obtain the supernatant. Glucose and lysine were measured on SBA Biosensor 40D (Shandong Academy of Sciences, Jinan, China).

Furfural, furfuryl alcohol, HMF, 5-hydroxymethylfurfuryl alcohol (HMF alcohol), syringaldehyde, syringic alcohol, syringic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydeoxyebenzoate, vanillin, vanillyl alcohol and vanillic acid were analyzed on reverse-phase HPLC (LC-20AT, Japan), equipped with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan). Furfural and furfuryl alcohol 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.

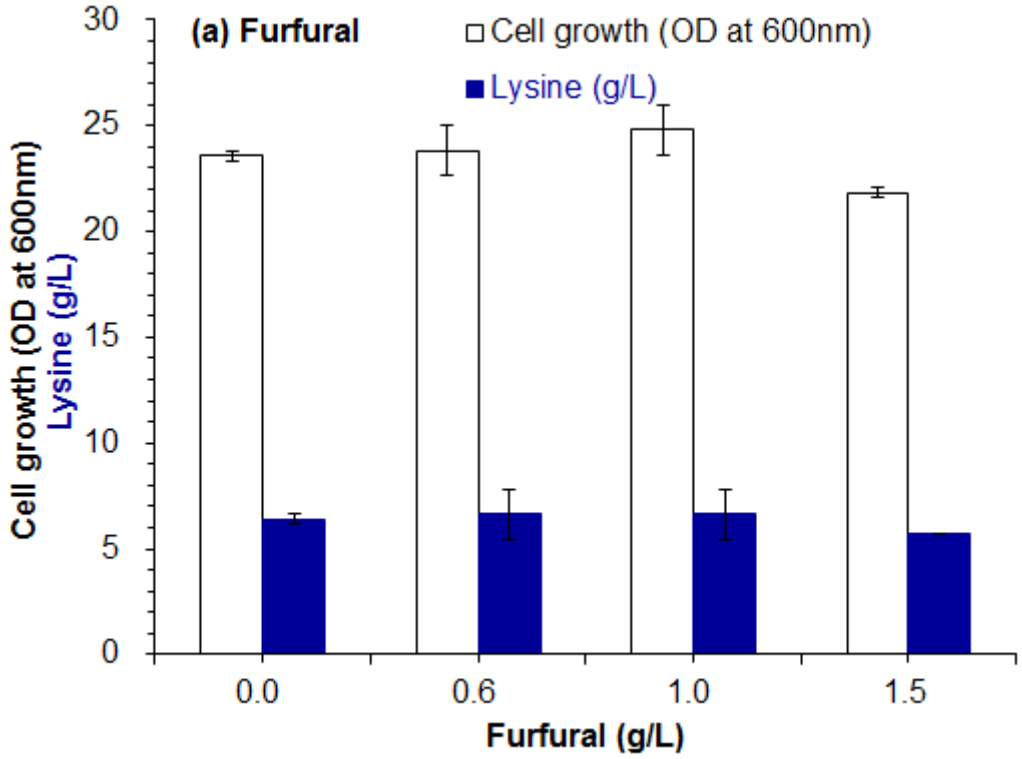
Syringaldehyde, syringic alcohol, syringic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydeoxyebenzoate, vanillin, vanillyl alcohol, and vanillic acid were analyzed using 100% acetonitrile solution (pump A) and 0.1% formic acid (pump B) at a ratio of 90% to 10% as the mobile phase at 1.0 mL/min at the column temperature of 35 ℃ and the detection wavelength of 270 nm. Acetic acid, 2-furoic acid (furoic acid), and 5-hydroxymethylfuroic acid (HMF acid) ware determined on HPLC 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.

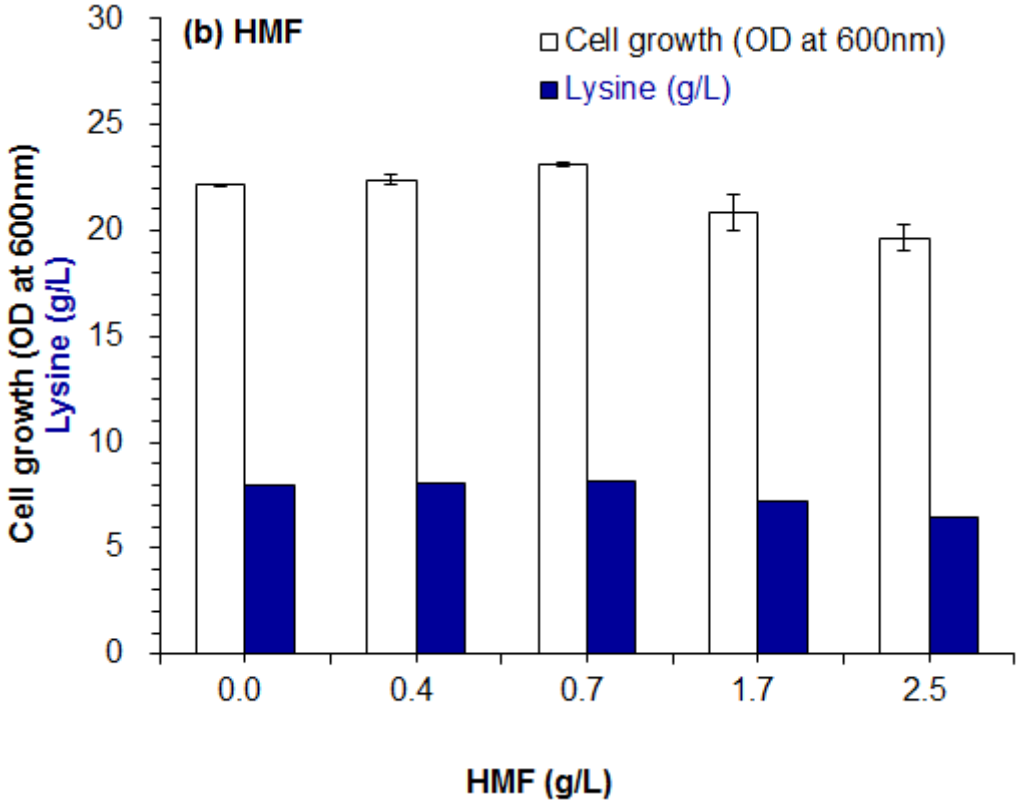
3.3. Results and Discussion

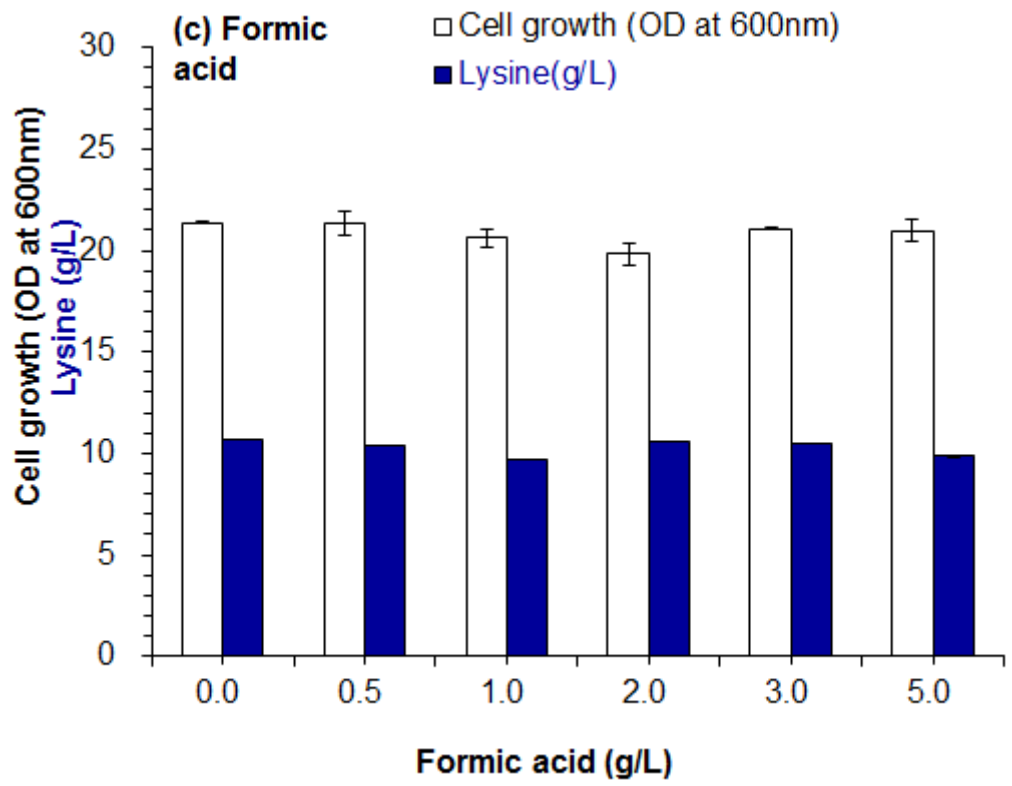
3.3.1. Inhibitor tolerance of *C. glutamicum*

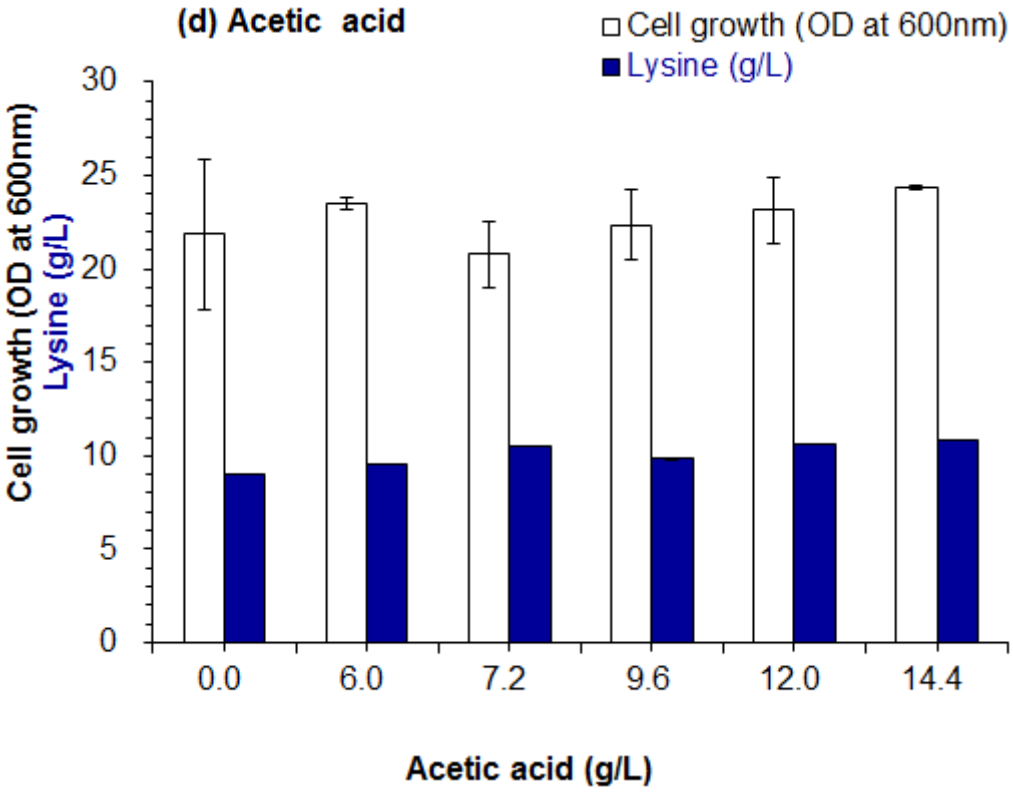
Tolerance of *C. glutamicum* against various inhibitors was assayed by adding inhibitor compounds into synthetic medium based on the inhibitor concentrations in the hydrolysate [6] (Fig. 3.1). *C. glutamicum* showed the strong tolerance to both furfural and HMF, which are generally regarded as the most toxic inhibitors to most fermenting strains (Figs. 3.1a and 1b). High concentration of furfural and HMF somehow negatively affected the cell growth and lysine production. *C. glutamicum* showed excellent tolerance to formic acid, acetic acid, and levulinic acid even at high concentration range (Figs. 3.1c-1e). For phenolic aldehyde inhibitors, vanillin, 4-hydroxybenzaldehyde, syringaldehyde and coniferyl aldehyde did not show the obvious inhibition on *C. glutamicum* growth and lysine production (Figs. 3.1f -1i) in the tested range. Conclusively, *C. glutamicum* has very strong tolerance to furans (furfural and HMF), organic acids (acetic acid, formic acid and levulinic acid), and phenolic compounds (4-

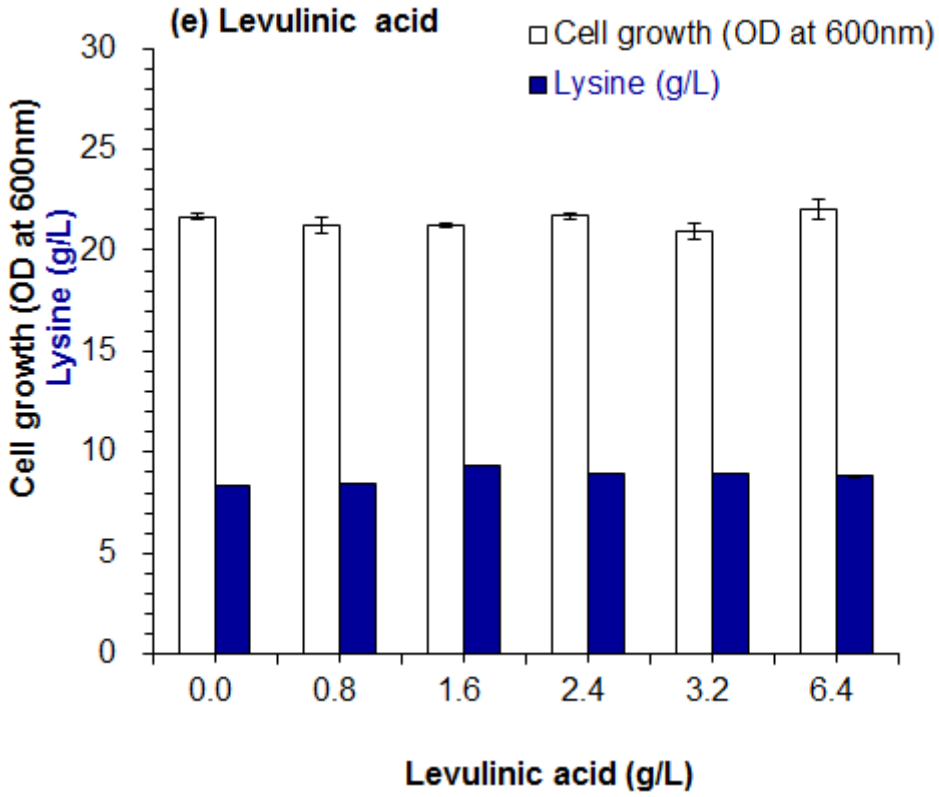
hydroxybenzaldehyde, vanillin, syringaldehyde and coniferyl aldehyde). Based on the obtained results, it is concluded that strain is tolerant to almost all of the pretreatment inhibitors present in typical hydrolysate and pretreated material.

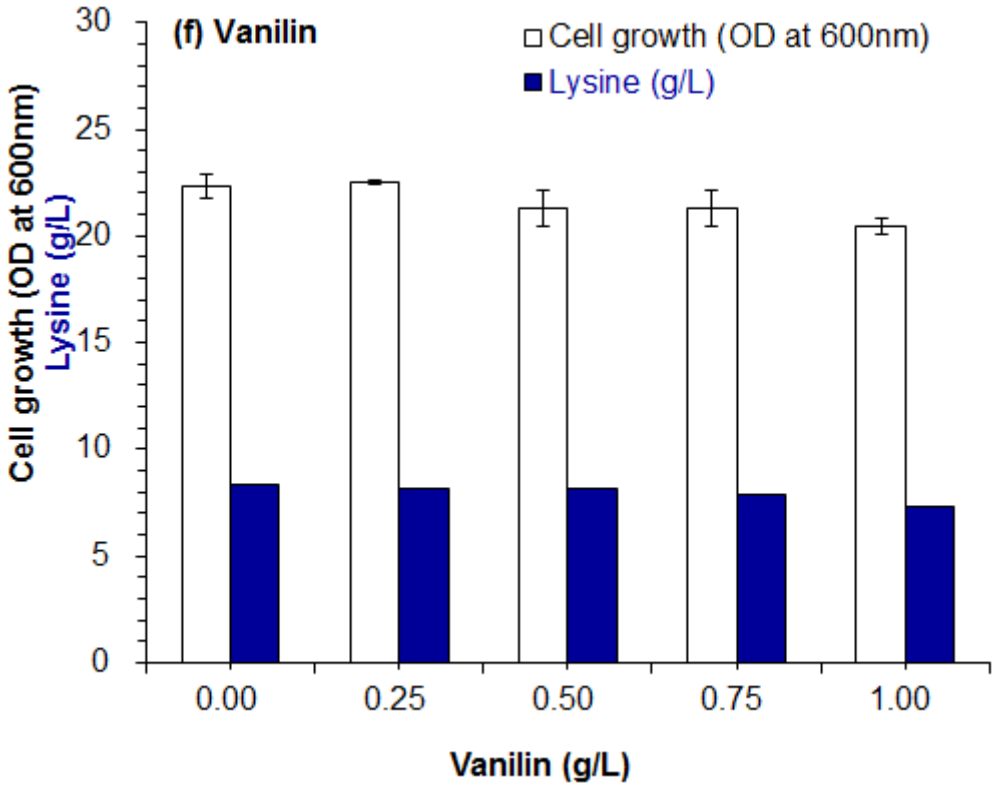


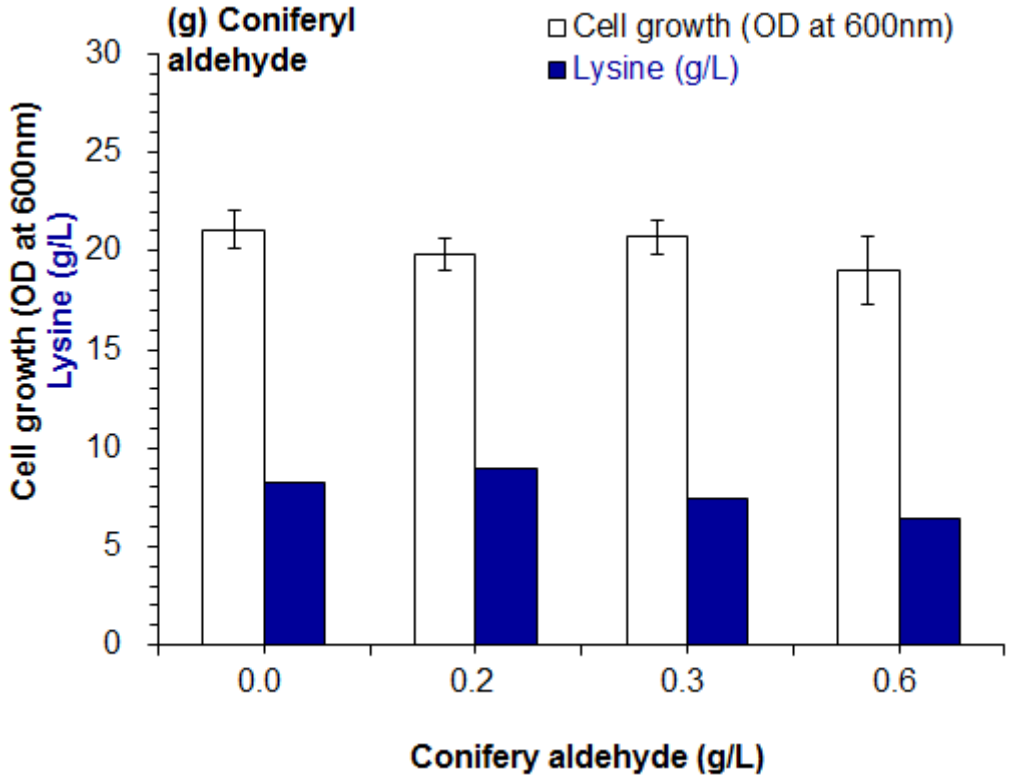


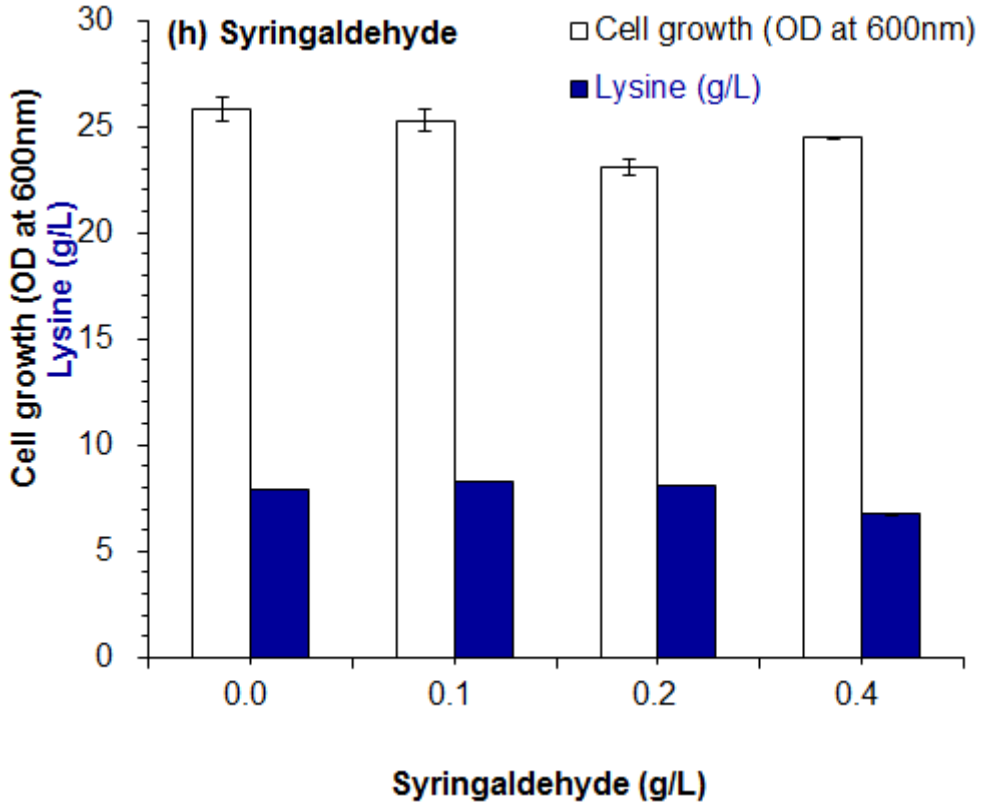

(c) Formic acid
Cell growth (OD at 600nm)
Lysine(g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.0
0.5
1.0
2.0
3.0
5.0
Formic acid (g/L)


(d) Acetic acid
Cell growth (OD at 600nm)
Lysine (g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.0
6.0
7.2
9.6
12.0
14.4
Acetic acid (g/L)


(e) Levulinic acid
Cell growth (OD at 600nm)
Lysine (g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.0
0.8
1.6
2.4
3.2
6.4
Levulinic acid (g/L)


(f) Vanilin
Cell growth (OD at 600nm)
Lysine (g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
Vanilin (g/L)


(g) Coniferyl aldehyde
Cell growth (OD at 600nm)
Lysine (g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.0
0.2
0.3
0.6
Conifery aldehyde (g/L)


(h) Syringaldehyde
Cell growth (OD at 600nm)
Lysine (g/L)
Cell growth (OD at 600nm)
Lysine (g/L)
30
25
20
15
10
5
0
0.0
0.1
0.2
0.4
Syringaldehyde (g/L)

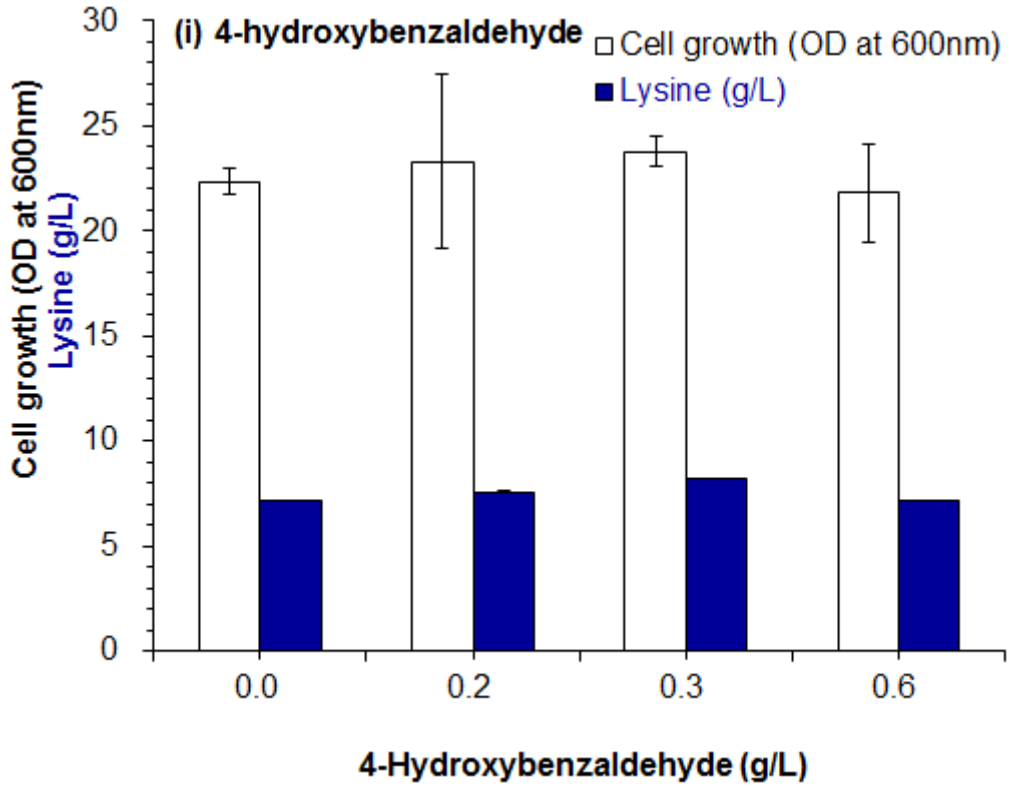


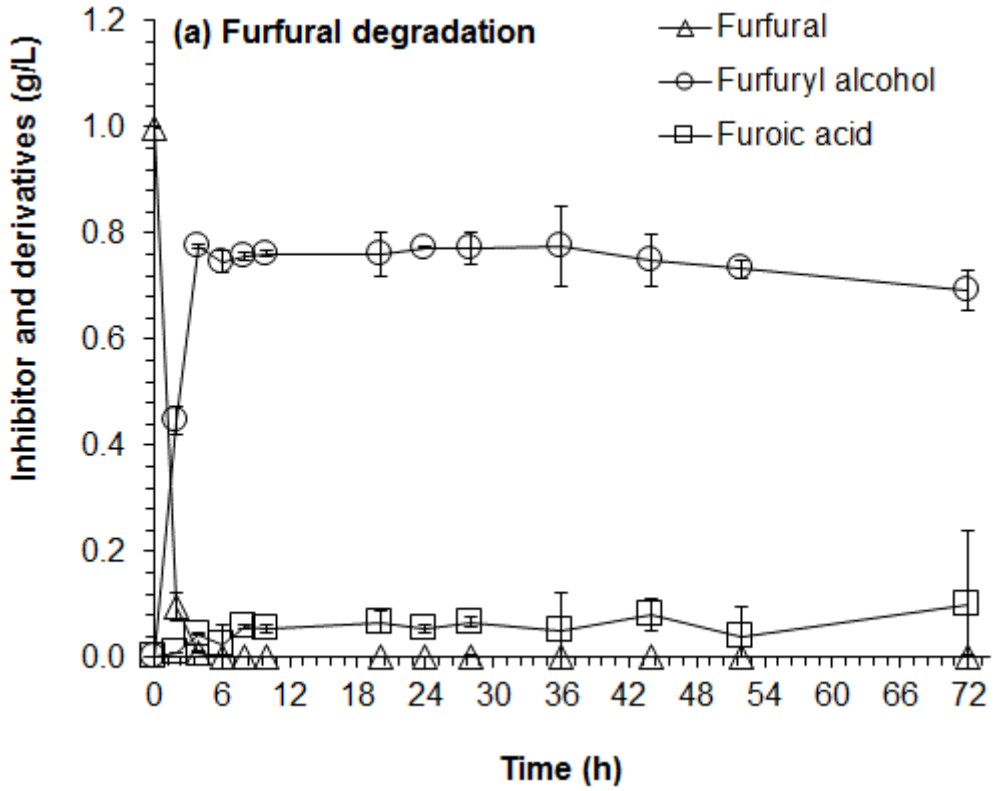
Fig. 3.1 Inhibitor tolerance and glutamic acid fermentability of *Corynebacterium glutamicum* SIIM B253 against different concentrations of various inhibitors. (a) Furfural (b) HMF (c) Formic acid (d) Acetic acid (e) Levulinic acid (f) Vanillin (g) coniferyl aldehyde (h) Syringaldehyde (i) 4-Hydroxybenzaldehyde. Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 48 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

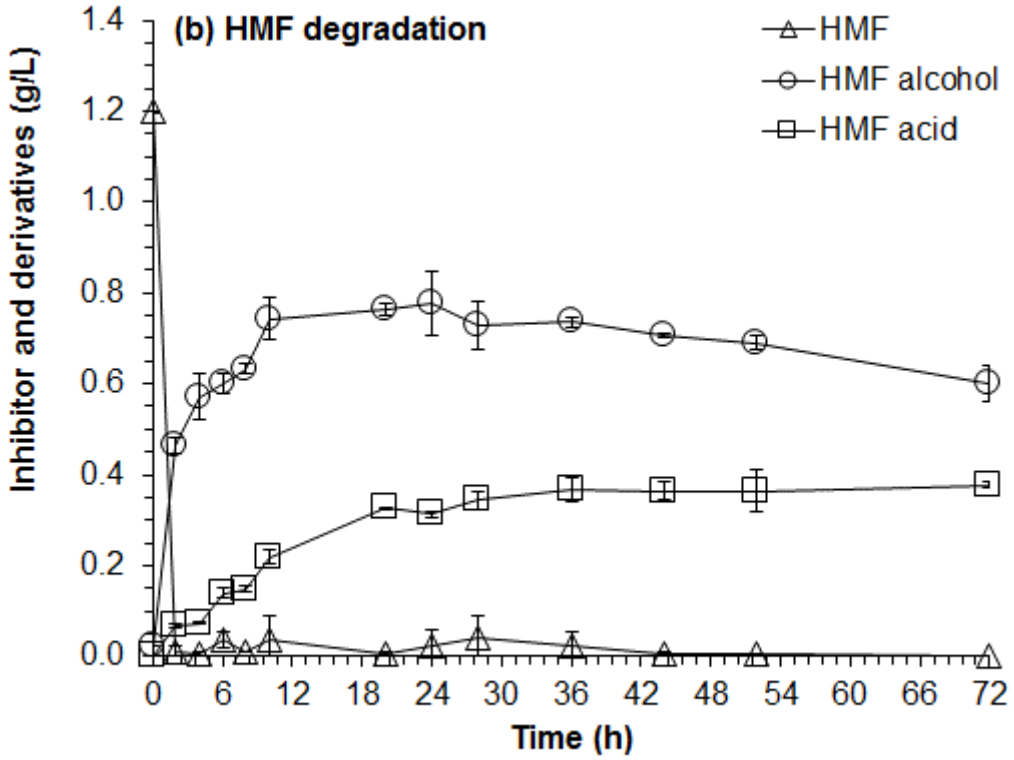
3.3.2. Inhibitor degradation by *C. glutamicum*

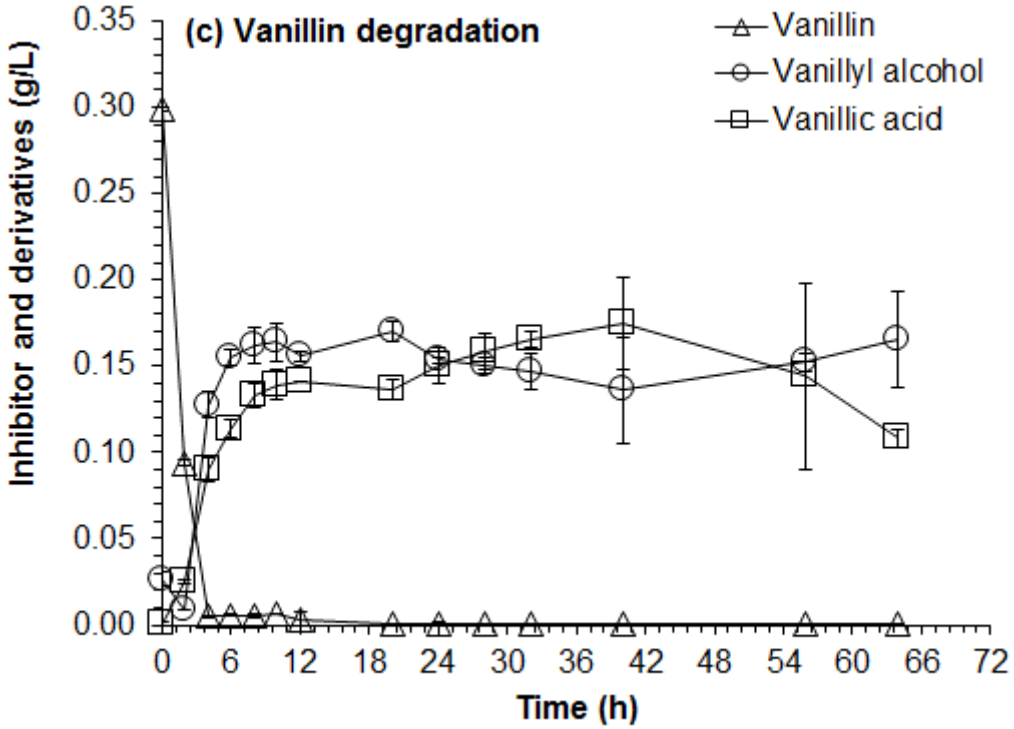
Inhibitor tolerance of microorganisms is generally considered as the capability of converting toxic compounds into less toxic compounds [208, 221]. Six model inhibitors were selected for testing the degradation capacity of *C. glutamicum*, including furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid.

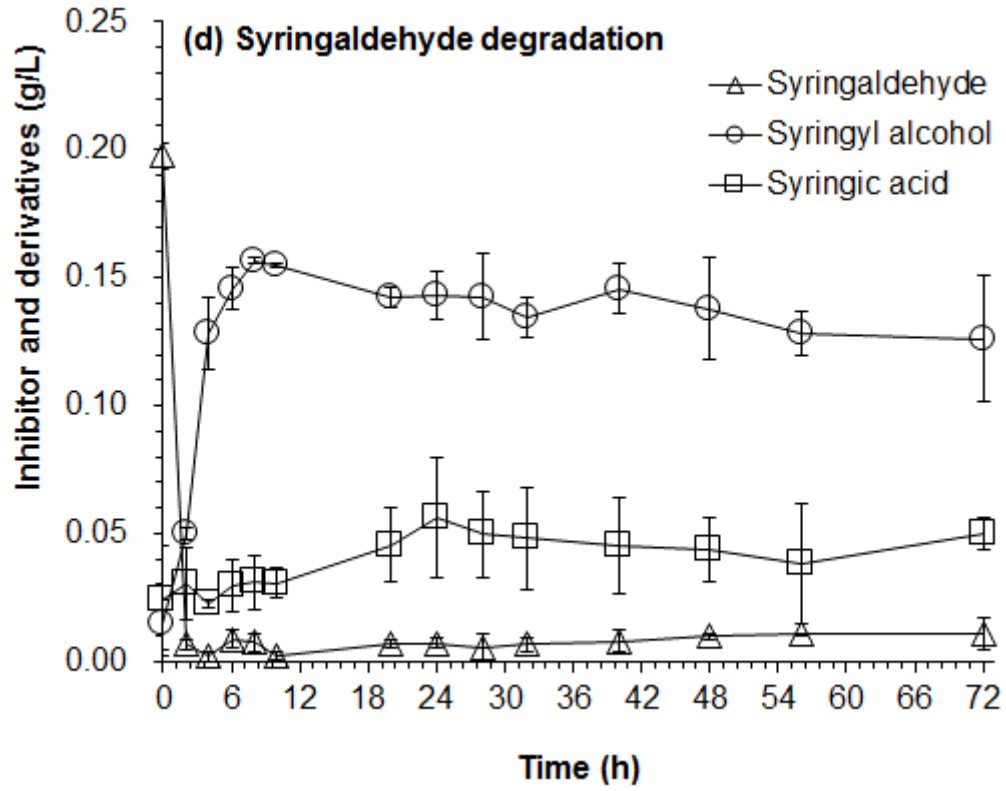
Furfural was quickly converted into furfuryl alcohol first then some of the furfuryl alcohol but minute quantity of furfuryl alcohol was degraded to furoic acid as only minimum furoic acid was detected, with the similar mass balance among the three compounds (Fig. 3.2a). HMF was quickly converted to HMF alcohol then converted into HMF acid with the approximate mass balance among HMF and its derivatives (Fig. 3.2b). Vanillin was converted into vanillyl alcohol then converted into vanillic acid approximately with the similar mass balance among the three compounds (Fig. 3.2c). Syringaldehyde was converted into syringyl alcohol followed by the further degradation of syringyl alcohol into syringic acid (Fig. 3.2d). 4-hydroxybenzaldehyde was converted into 4-hydroxybenyl alcohol and then quickly

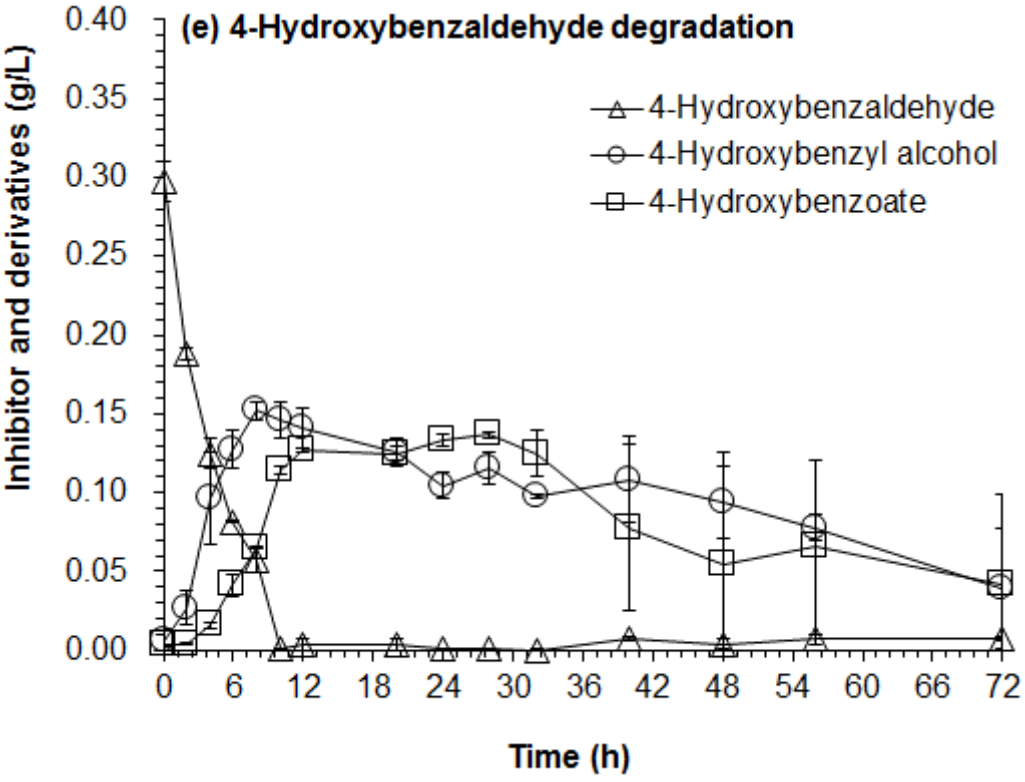
converted into 4-hydrobenzoic acid. (Fig. 3.2e). For the acetic acid degradation, it was slowly assimilated completely at 42 h without obvious intermediate formation (Fig. 3.2f). Furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid, were degraded with in very short period of 4, 2, 4, 4, 10 h, respectively in comparison to many other strains. Micro-organisms, such as some species of yeasts [164-166], fungi [162, 167], and bacteria [162, 168] can detoxify furan aldehydes, aliphatic acids, and aromatic compounds [169-173]. However, the selectivity of degradation varies depending on microorganism employed. *T. reesei* was able to metabolize furan aldehydes, acetic acid, and benzoic acid derivatives of a willow hydrolysate [174]. *Desulfovibrio furfuralis* utilized furfural, furfuryl alcohol and 2-furoic acid as source of carbon and energy [175] and metabolized furfural to acetate, CO_2 and/or methane [176]. *E. coli* strains was shown to degrade furfural into furfuryl alcohol [170, 171]. However, conversion rates of these organisms were low and would require multiple days.




(b) HMF degradation
HMF
HMF alcohol
HMF acid
Inhibitor and derivatives (g/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)


(c) Vanillin degradation
Vanillin
Vanillyl alcohol
Vanillic acid
Inhibitor and derivatives (g/L)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)


(d) Syringaldehyde degradation
Syringaldehyde
Syringyl alcohol
Syringic acid
Inhibitor and derivatives (g/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)


(e) 4-Hydroxybenzaldehyde degradation
4-Hydroxybenzaldehyde
4-Hydroxybenzyl alcohol
4-Hydroxybenzoate
Inhibitor and derivatives (g/L)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)

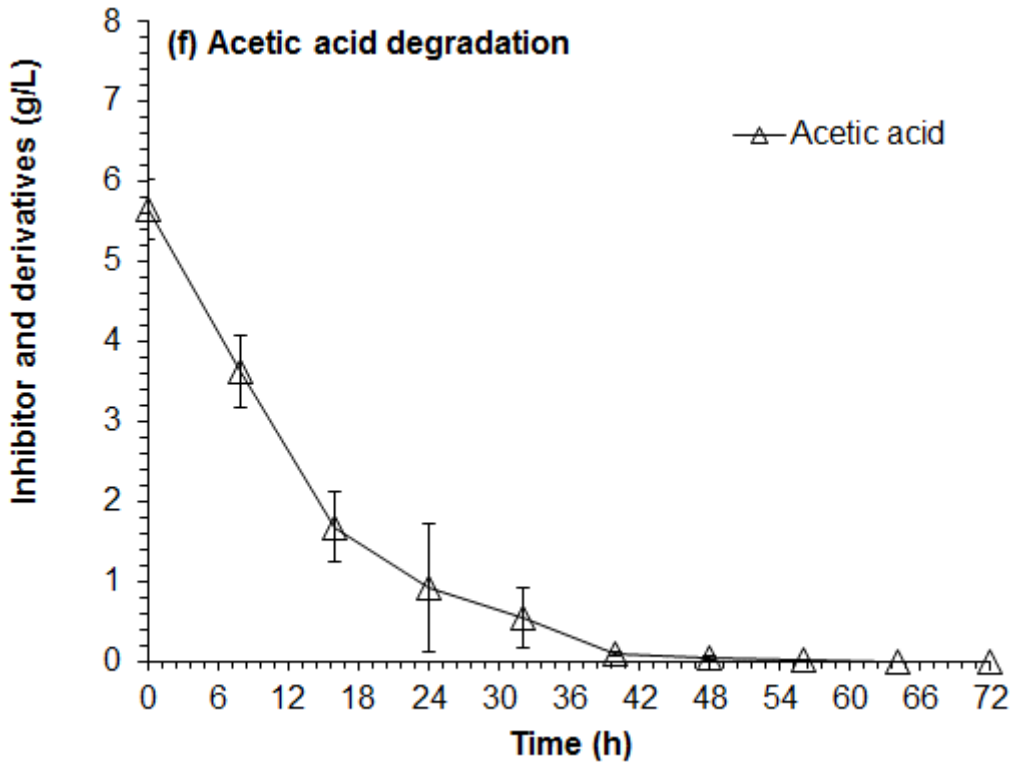


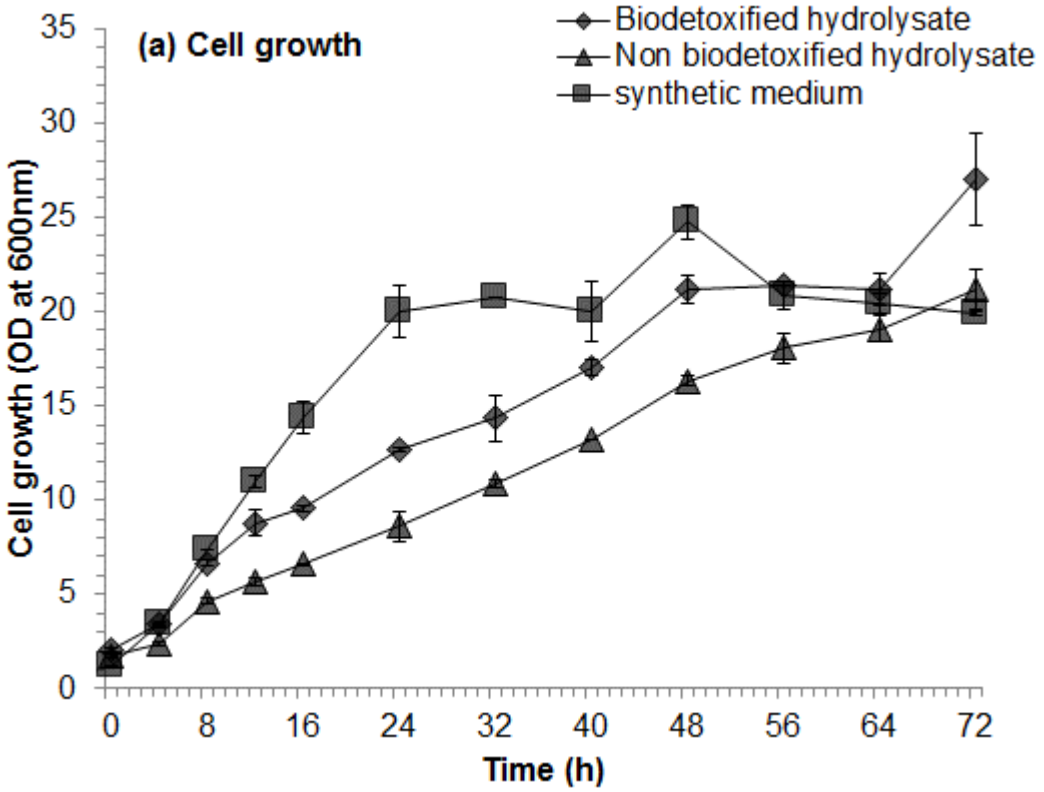
Fig. 3.2. Biodegradation by *Corynebacterium glutamicum* SIIM B253 using various inhibitors. Fermentation conditions with synthetic medium: 30 ℃, 200 rpm for 72 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval. (a) Furfural 1.0 g/L, (b) 5-HMF 1.2 g/L, (c) Vanillin 0.3g/L, (d) Syringaldehyde 0.2 g/L, (e) 4-hydroxybenzaldehyde 0.3 g/L, (f) Acetic acid 6 g/L. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

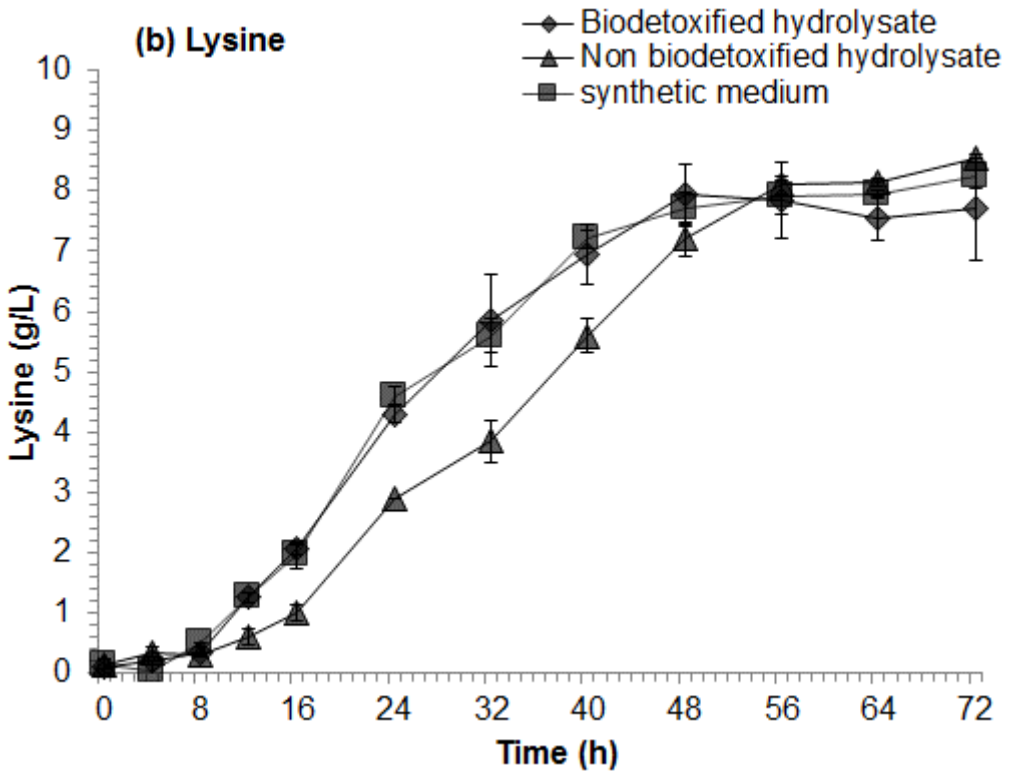
These results indicated that *C. glutamicum* has an excellent capability to degrade almost all the tested inhibitors, including furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid. The strain can degrade the inhibitors with short time compared to other strains [106, 107, 156, 208, 214]. These results clearly indicated that the strain could be applied for lysine production from non-detoxified corn stover hydrolysate or pretreated materials.

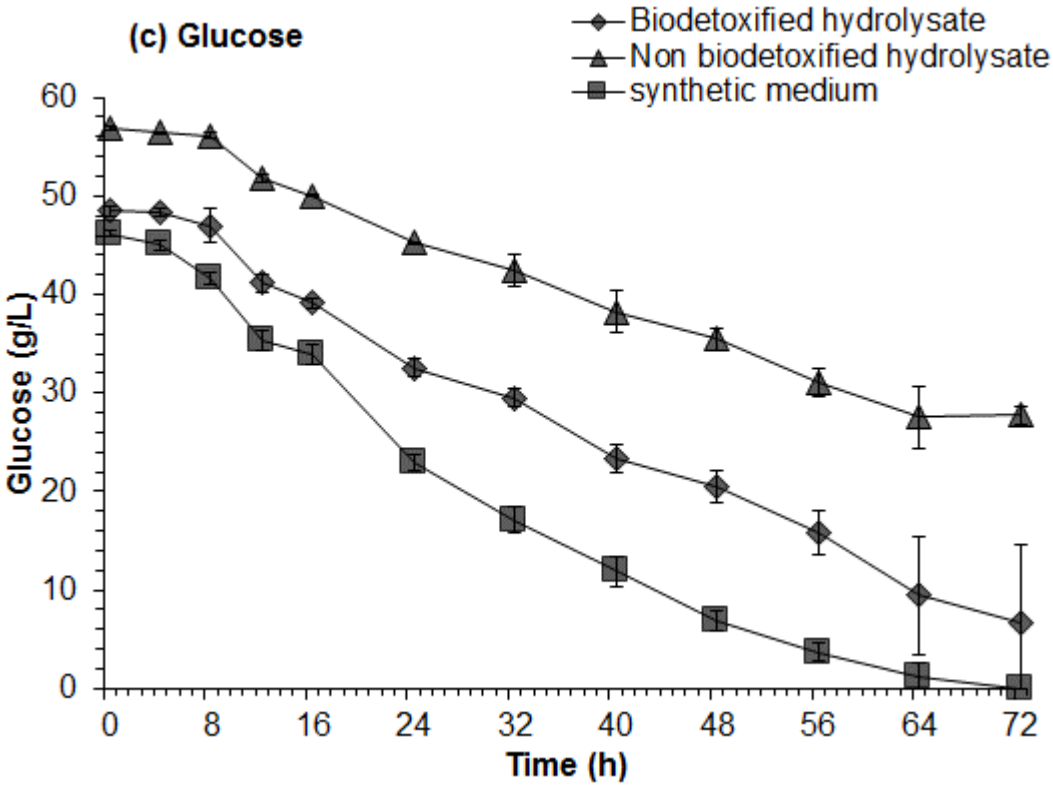
3.3.6. Lysine fermentation of *C. glutamicum* using corn stover hydrolysate

Lysine fermentation using synthetic medium, biodetoxified and non-biodetoxified corn stover hydrolysate was performed by the *C. glutamicum*. Non-biodetoxified corn stover hydrolysate contained 70.6 g/L of glucose, 3.12 g/L of acetic acid, 0.56 g/L of furfural and 0.353 g/L of 5-hydroxymethylfurfural (HMF). Biodetoxified corn stover hydrolysate contained 59.78 g/L of glucose, 0.78 g/L of acetic acid, 0 g/L of furfural and 0.007 g/L of 5-hydroxymethylfurfural (HMF).

Fig. 3.3(a) shows the cell growth of different cultures up to 72 h. Results clearly show that the cell growth of *C. glutamicum* in non-biodetoxified hydrolysate was nearly same as of biodetoxified hydrolysate and synthetic medium after 72 h. Higher lysine was generated by strain in non-biodetoxified hydrolysate in comparison to other media after 72 h as shown in Fig. 3.3(b). % yield of lysine by synthetic media, biodetoxified hydrolysate and non-biodetoxified hydrolysate was 21.62%, 22.42%, and 35.52% respectively as depicted in Fig. 3.3(d). Results indicate that the *C. glutamicum* has strong tolerance against pretreatment inhibitors and strain could be utilized for industrial lysine production from corn stover hydrolysate without any prior biodetoxification. This will save time and is cost effective. Strong tolerance of the strain using non-biodetoxified hydrolysate may be due to its capability to degrade the pretreatment inhibitors during lysine fermentation.




(b) Lysine
Biodetoxified hydrolysate
Non biodetoxified hydrolysate
synthetic medium
Lysine (g/L)
Time (h)
0
1
2
3
4
5
6
7
8
9
10
0
8
16
24
32
40
48
56
64
72


(c) Glucose
Biodetoxified hydrolysate
Non biodetoxified hydrolysate
synthetic medium
Glucose (g/L)
Time (h)
0
10
20
30
40
50
60
0
8
16
24
32
40
48
56
64
72

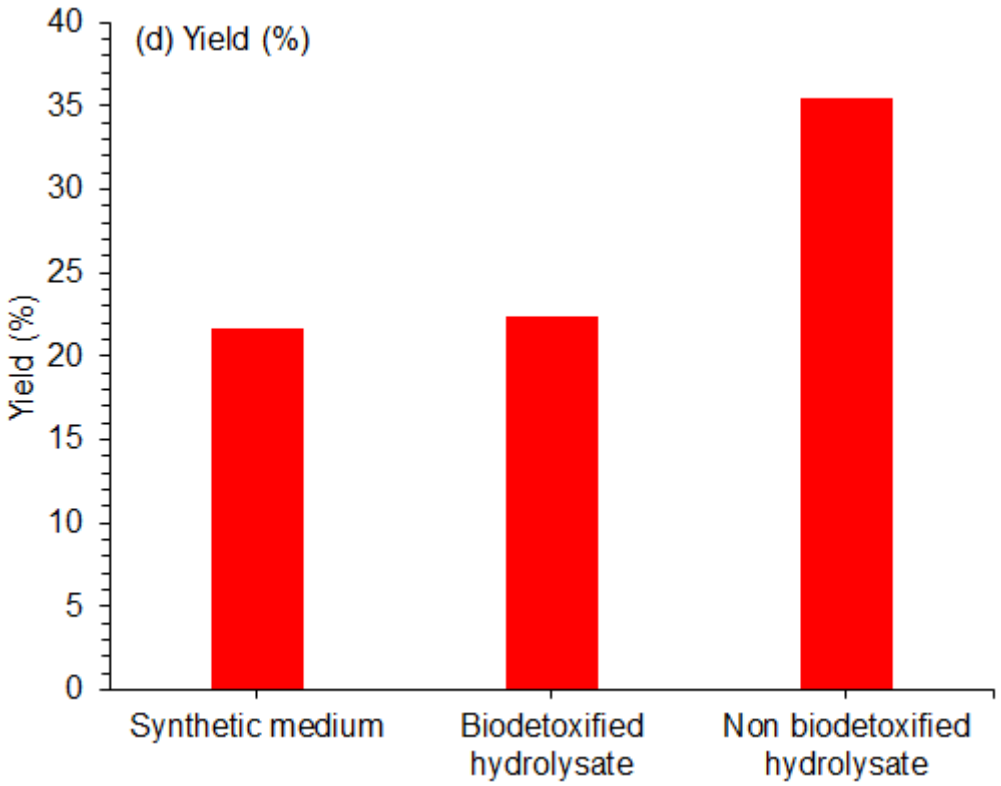
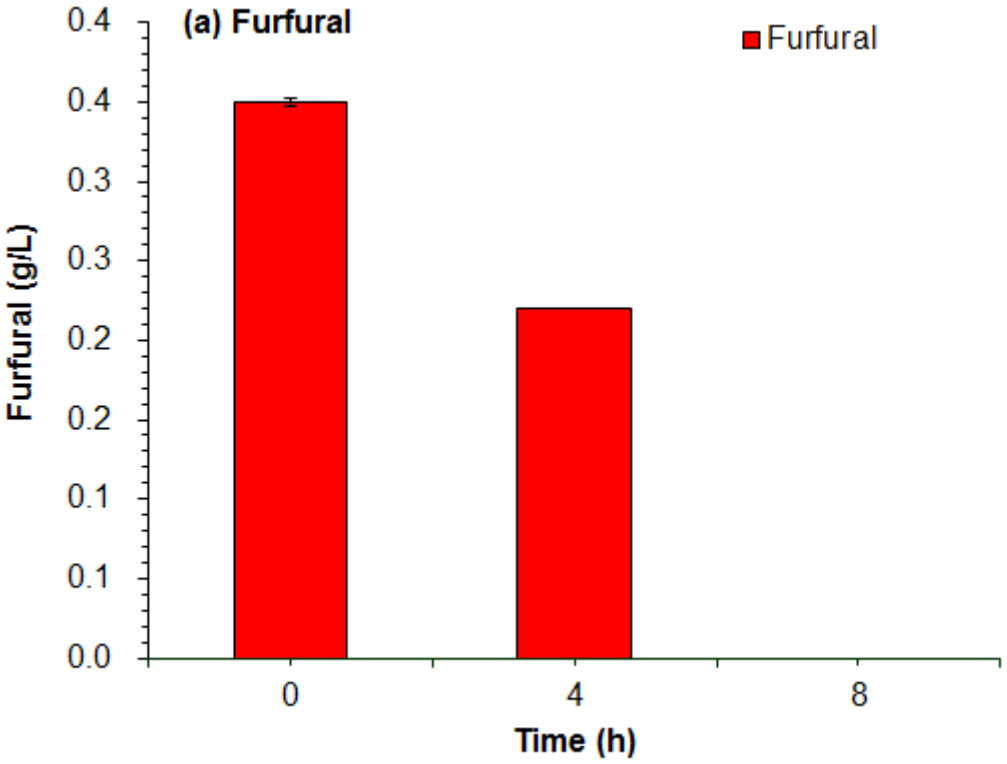


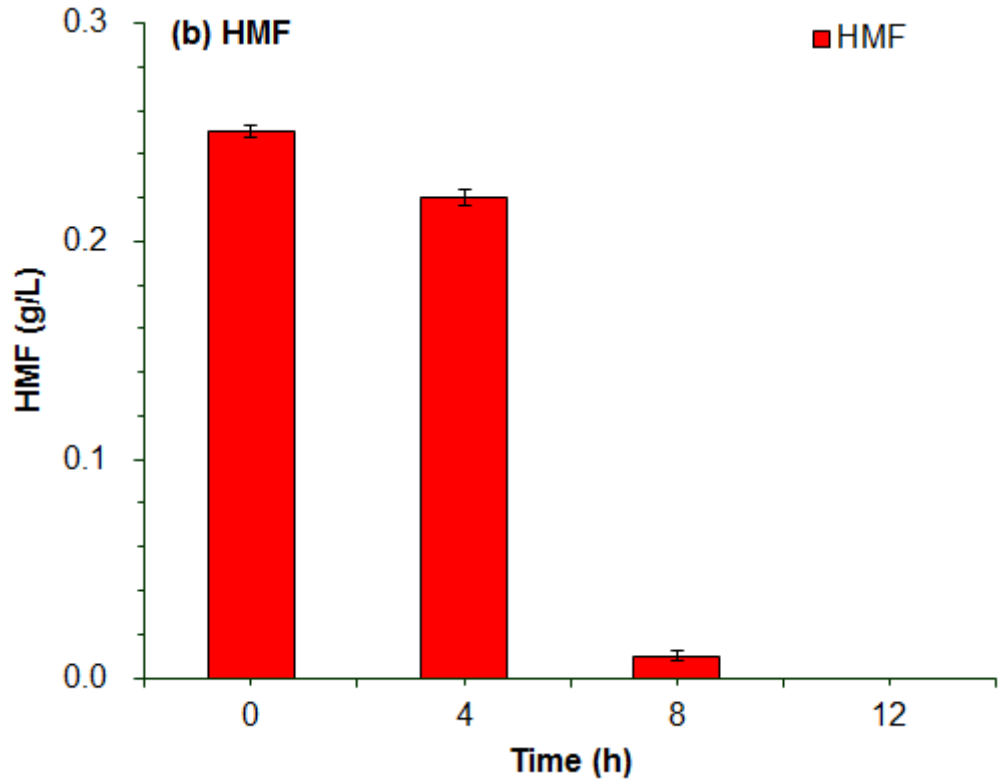
Fig. 3.3. Fermentability of *C. glutamicum* SIIM B253 in synthetic medium, biodetoxified hydrolysate and non-biodetoxified hydrolysate. (a) Cell growth (OD_{600nm}), (b) Lysine (g/L), (c) Residual glucose (g/L). Fermentation conditions with synthetic medium and corn stover hydrolysate: 30 ℃, 200 rpm for 72 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

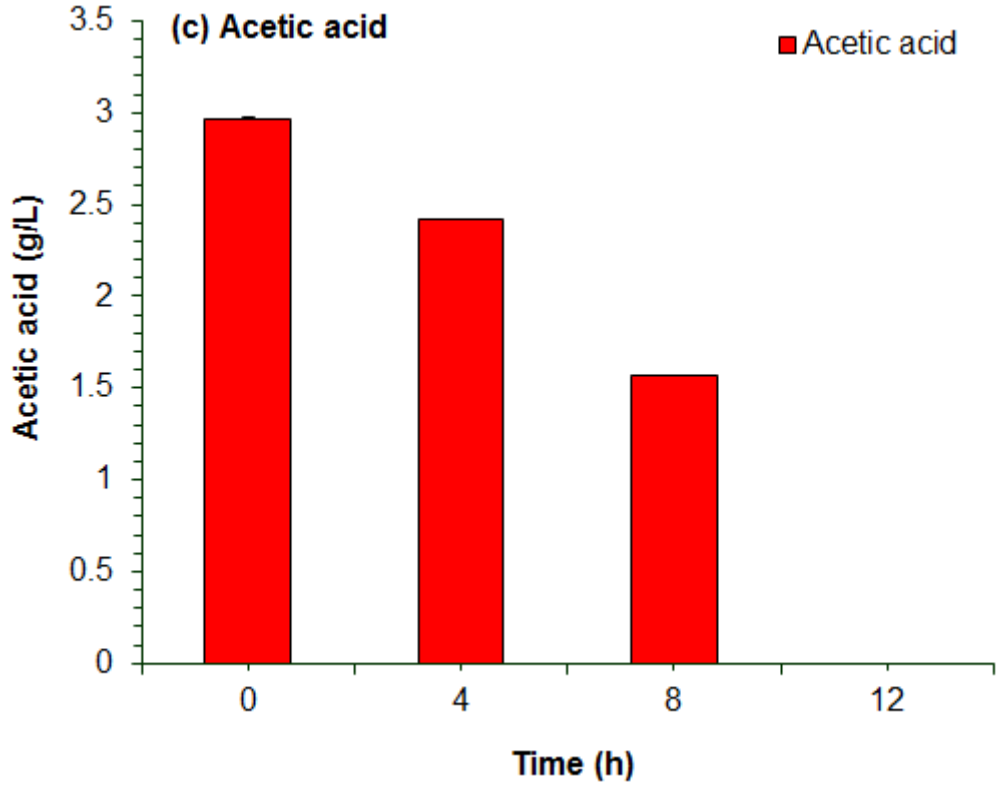
3.3.7. Inhibitor degradation by *C. glutamicum* using non-biodetoxified corn stover hydrolysate

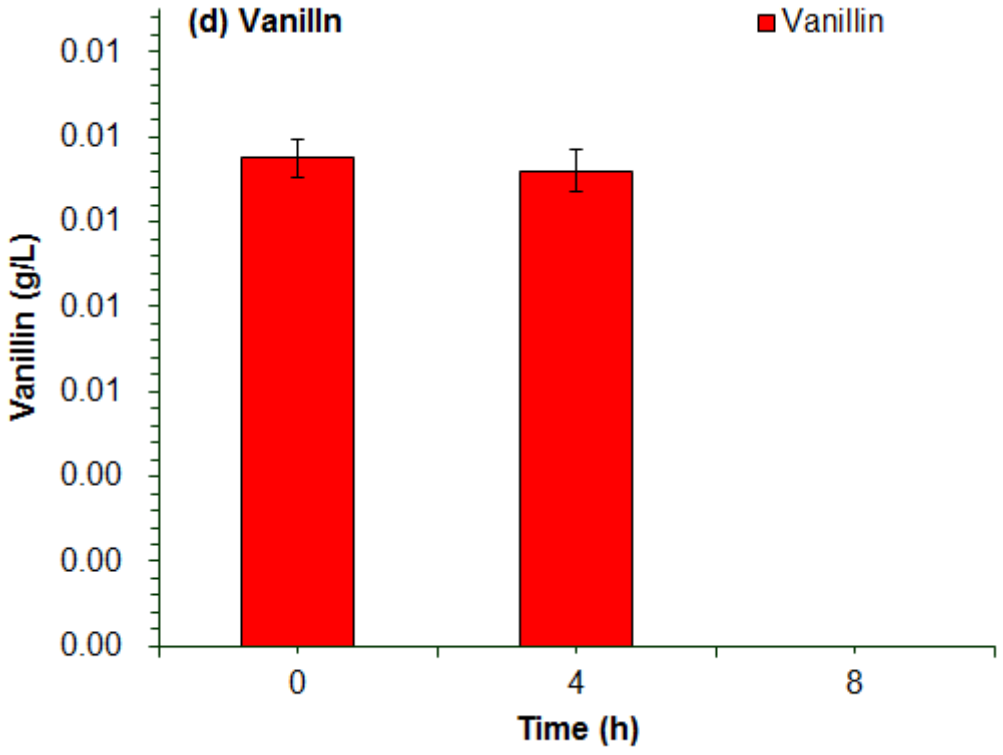
Previous experiment indicated the strong tolerance of the strain using non-biodetoxified corn stover hydrolysate. Tolerance of strain towards pretreatment inhibitors is generally due to its capability to degrade the pretreatment inhibitors. Inhibitors degradation by *C. glutamicum* was analyzed during fermentation using non-biodetoxified corn stover hydrolysate.

Using *C. glutamicum* in non-biodetoxified hydrolysate, furfural which is regarded as most toxic inhibitor was degraded within very short period of 8 h (Fig. 4.3a). HMF was all metabolized by the strain within 12 h (Fig. 3.4b). *C. glutamicum* degraded acetic acid within 12 h. Strain showed strong potential to degrade vanillin and 4-hydroxybenzaldehyde as both inhibitors were degraded within 12 h (Fig. 3.4d, f). Syringaldehyde degradation was slower in comparison to other inhibitors and strain could not degrade syringaldehyde up to 16


(a) Furfural
Furfural
Furfural (g/L)
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0.0
0
4
8
Time (h)


(b) HMF
HMF
HMF (g/L)
0.3
0.2
0.1
0.0
0
4
8
12
Time (h)


(c) Acetic acid
Acetic acid
Acetic acid (g/L)
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
Time (h)


(d) Vanilln
Vanillin
Vanillin (g/L)
0.01
0.01
0.01
0.01
0.00
0.00
0.00
0
4
8
Time (h)

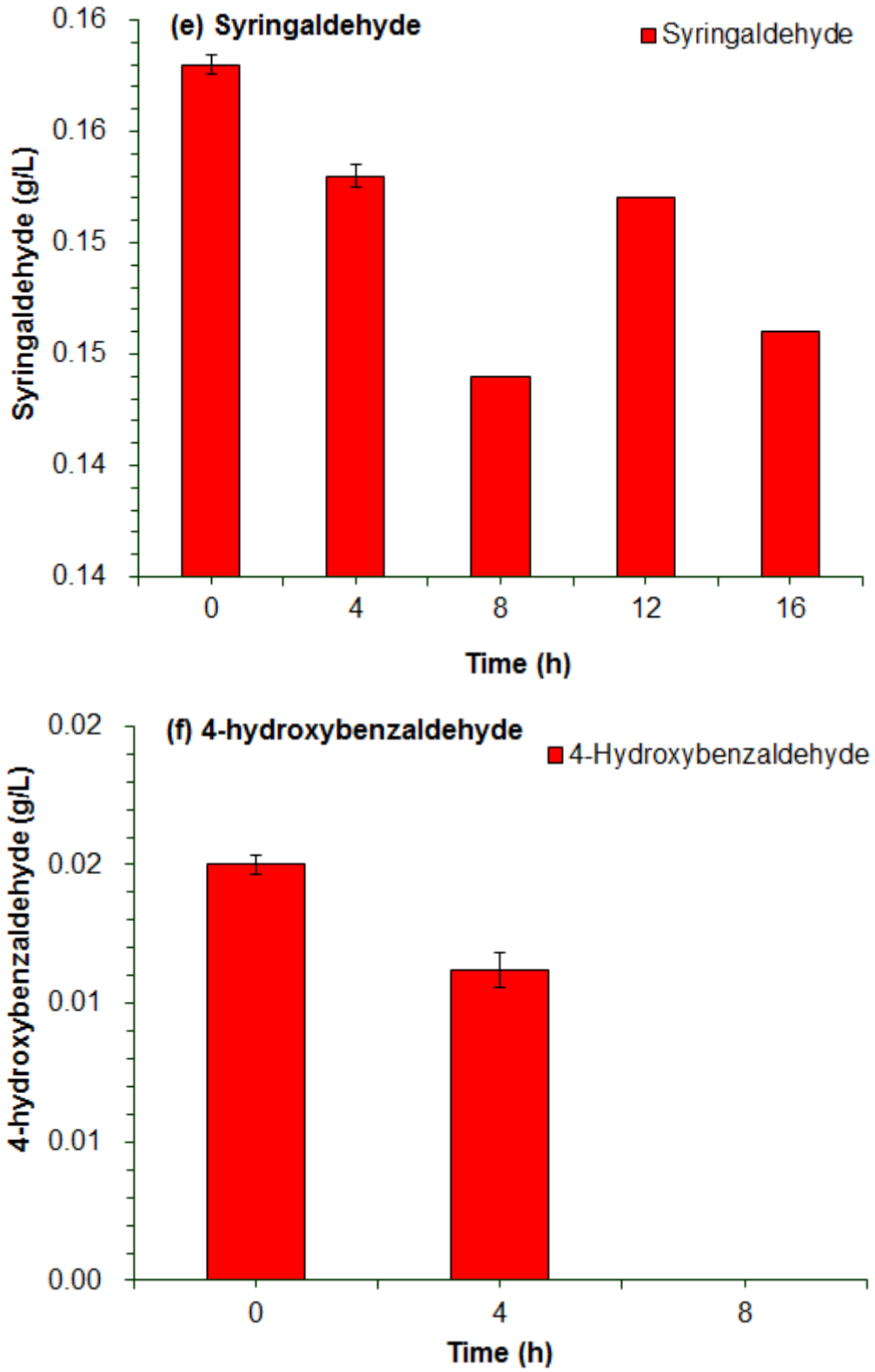
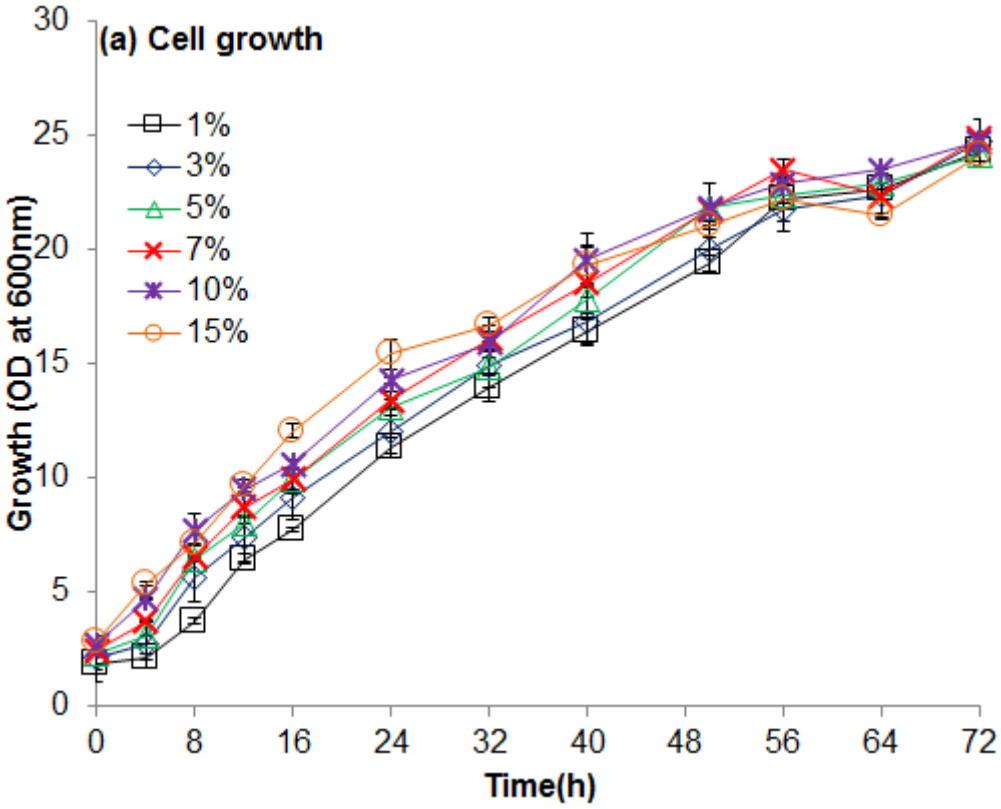


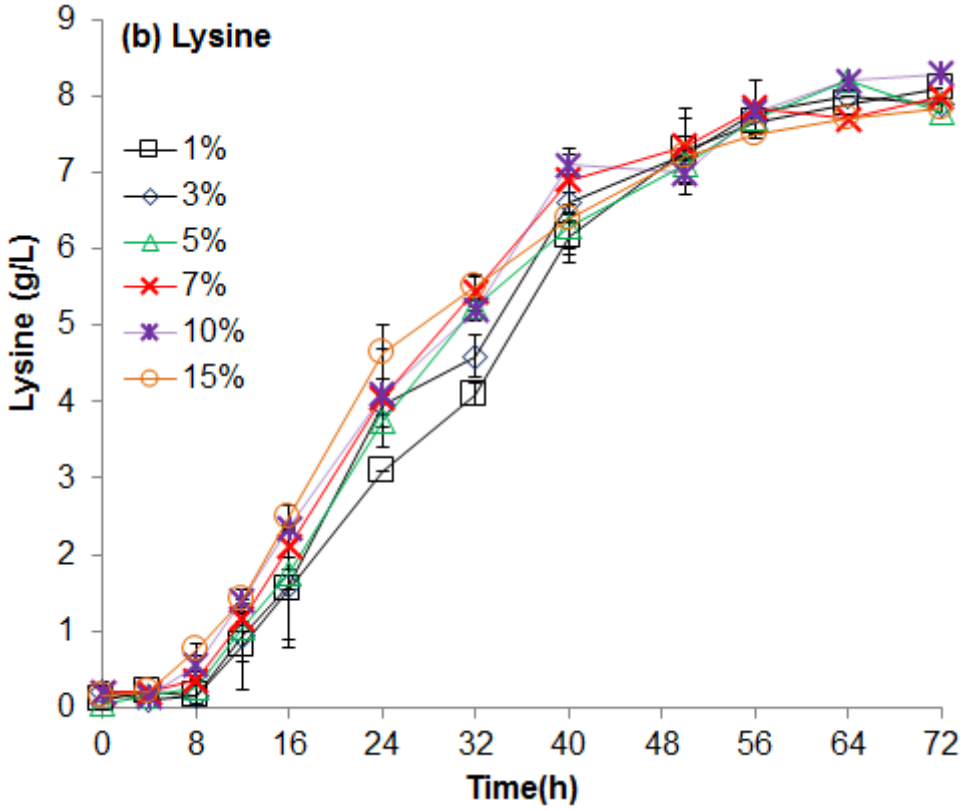
Fig. 3.4. Biodegradation by *Corynebacterium glutamicum* SIIM B253 using non-biodetoxified hydrolysate. Fermentation conditions: 30 ℃, 200 rpm for 72 hours in flasks, 10% (v/v) of inoculation ratio, pH was adjusted to value of 7.0 with addition of NaOH at regular interval. (a) Furfural, (b) 5-HMF, (c) Acetic acid, (d) Vanillin, (e) Syringaldehyde, (f) 4-hydroxybenzaldehyde. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

h. Usually the inhibitory compounds present in the hydrolysate have a synergistic toxic effect. *S. cerevisiae* can grow in the presence of either furfural or 5- HMF, but cannot grow in a mixture of both [241]. A similar synergistic effect has been found for ethanologenic *E. coli* strains [130]. In contrast present results clearly reveals that strain is capable to grow in non-biodetoxified corn stover hydrolysate, and no significant synergistic effects were observed on growth and lysine production.

3.3.8. Effect of different inoculum ratios on lysine fermentation of *C. glutamicum* using non-biodetoxified corn stover hydrolysate

It is generally necessary to optimize inoculum density. Too low density may provide insufficient biomass while too high density may generate too much biomass which can deplete the nutrients necessary for lysine fermentation. Inoculum size of 1-15% v/v was studied in shake flask using non-biodetoxified corn stover hydrolysate. Initially cell growth with 10% inoculum was best but after 72 h nearly same cell growth was noted on different inoculum size except at 1% inoculum probably due to low density gave insufficient biomass (Fig. 3.5a). Lysine fermentation was found better in higher inoculum ratios (7-15%) in the initial time but latter on no obvious difference was observed at 72 h, except with the inoculum ratio of 1%, in which lesser lysine was produced (Fig. 3.5b). Glucose utilization was observed in the exponential trend from 1-15% of the inoculum ratio (Fig. 3.5c). Maximum % yield of 43.67 % was observed with inoculum size of 3%, for lysine production from non-biodetoxified hydrolysate followed by 39.82% with 7% inoculum ratio (Fig. 3.5d). Increased inoculum size is an important conventional method that has been applied to overcome the inhibitory effects of furfural and HMF, since no strains have been available to grow in the presence of these inhibitors [177, 242, 247, 248], while *C. glutamicum* has demonstrated strong tolerance to grow in the media with the least inoculum ratio of even 1%. This clearly indicates the strong tolerance of the strain production prior biodetoxification towards pretreatment inhibitors and the potential of the strain to be used for lysine.


(a) Cell growth
1%
3%
5%
7%
10%
15%
Growth (OD at 600nm)
Time(h)
0
5
10
15
20
25
30
0
8
16
24
32
40
48
56
64
72


(b) Lysine
1%
3%
5%
7%
10%
15%
Lysine (g/L)
Time(h)
0
1
2
3
4
5
6
7
8
9
0
8
16
24
32
40
48
56
64
72

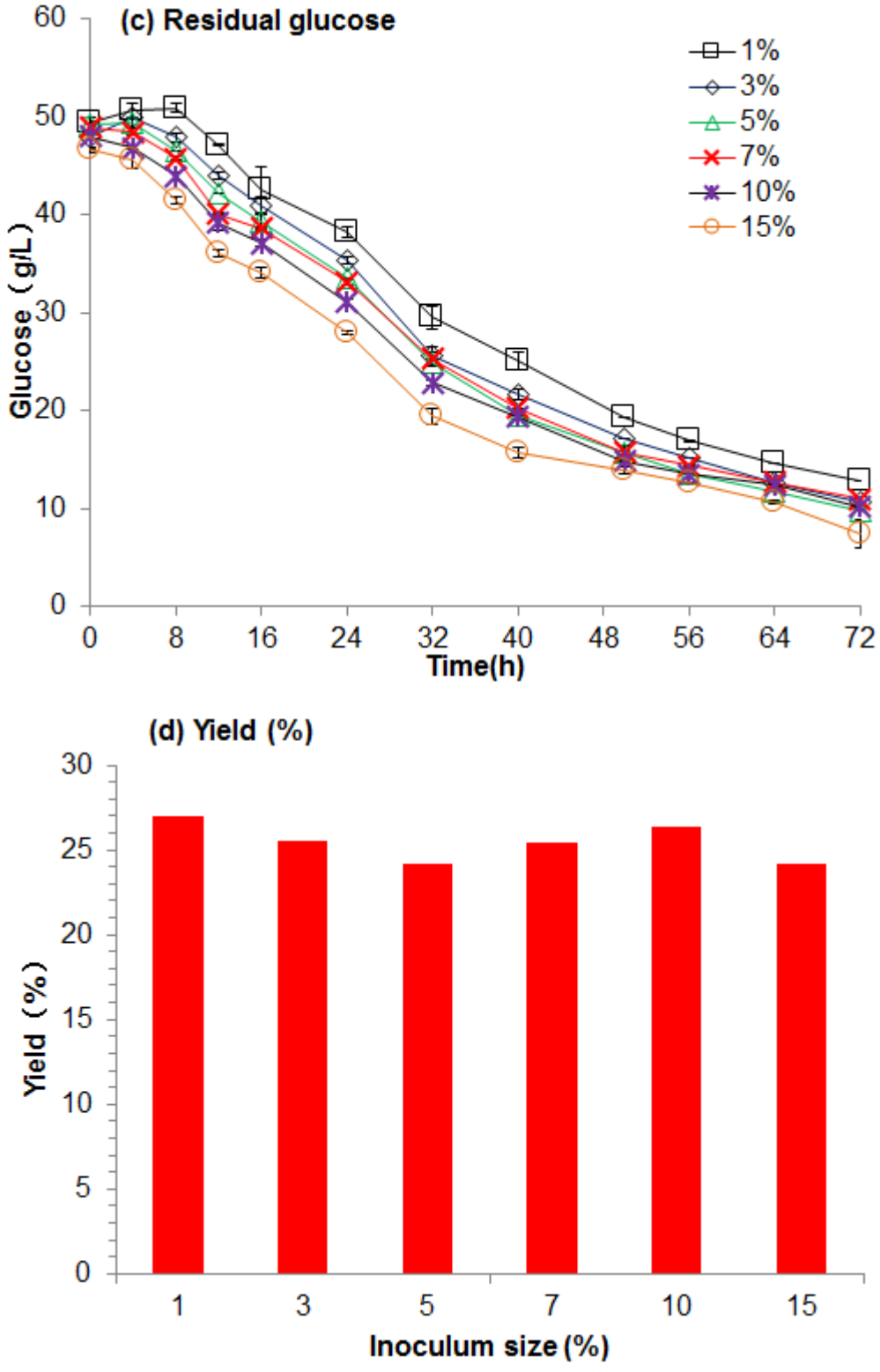
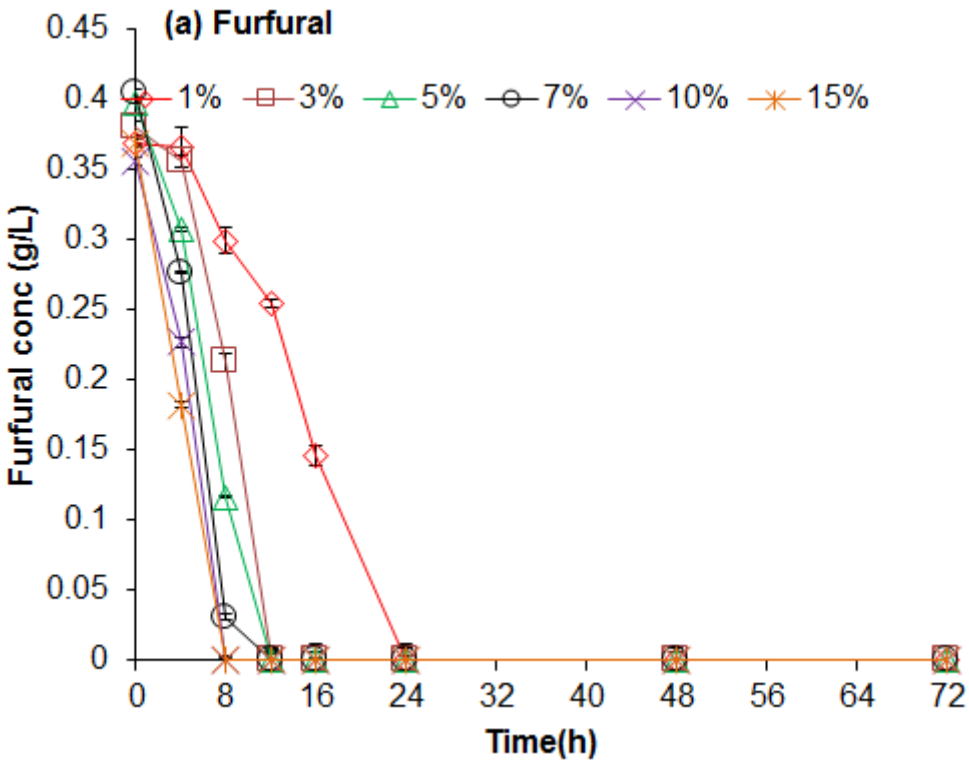
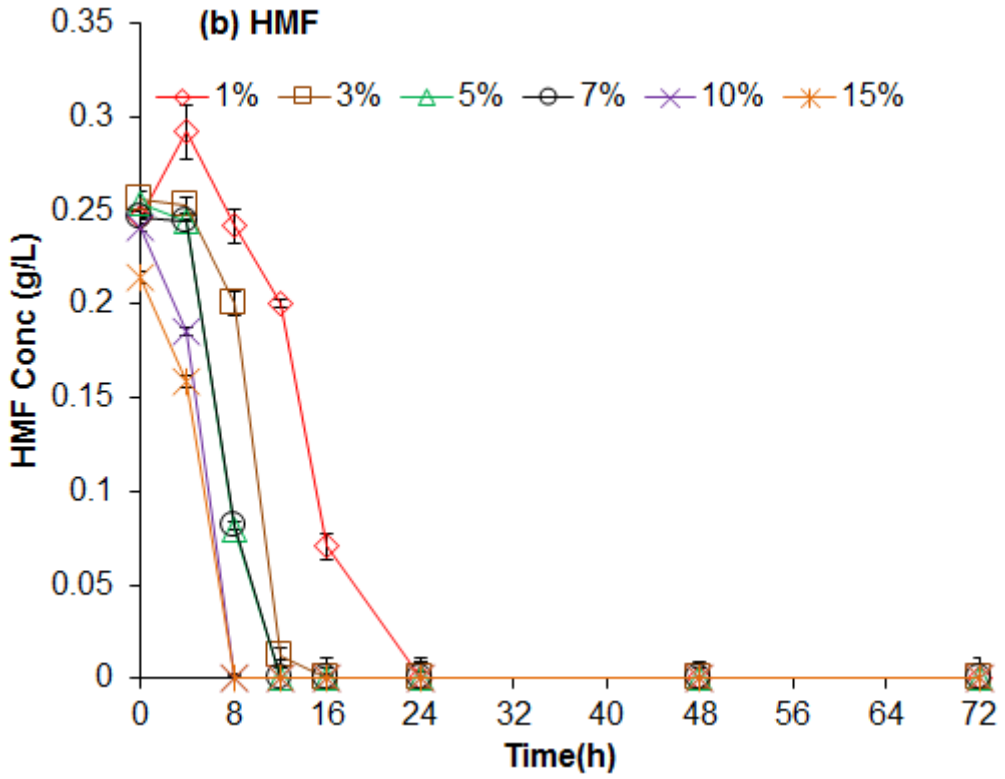


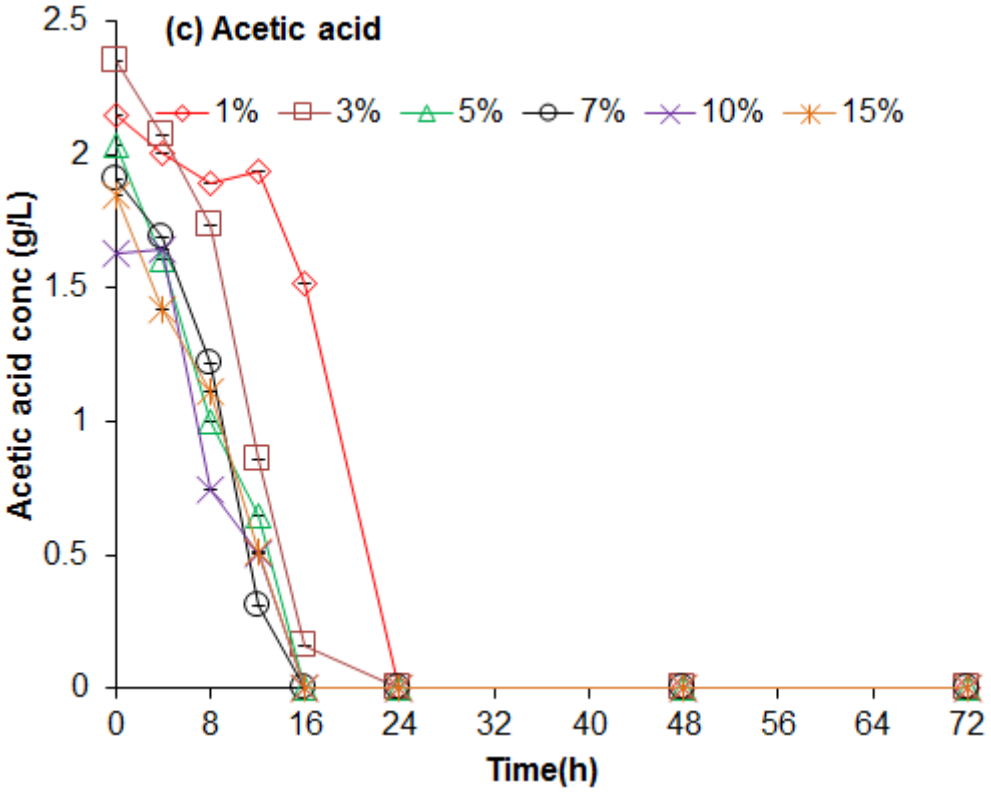
Fig. 3.4. Effect of different inoculum ratios on lysine fermentation by *Corynebacterium glutamicum* SIIM B253 using non-biodetoxified hydrolysate. Fermentation conditions: 30 ℃, 200 rpm for 72 hours in flasks. pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval. (a) 1%, (b) 3%, (c) 5%, (d) 7%, (e) 10%, (f) 15%. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

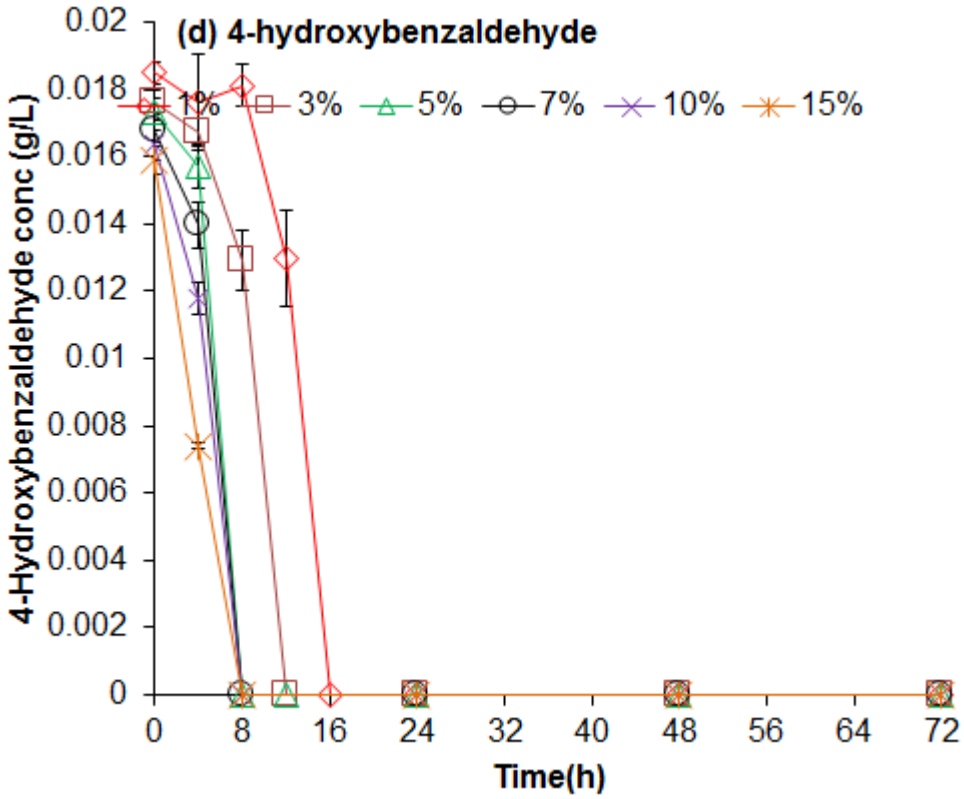
3.3.9. Effect of different inoculum ratios on inhibitors degradation by *C. glutamicum* in non-biodetoxified corn stover hydrolysate

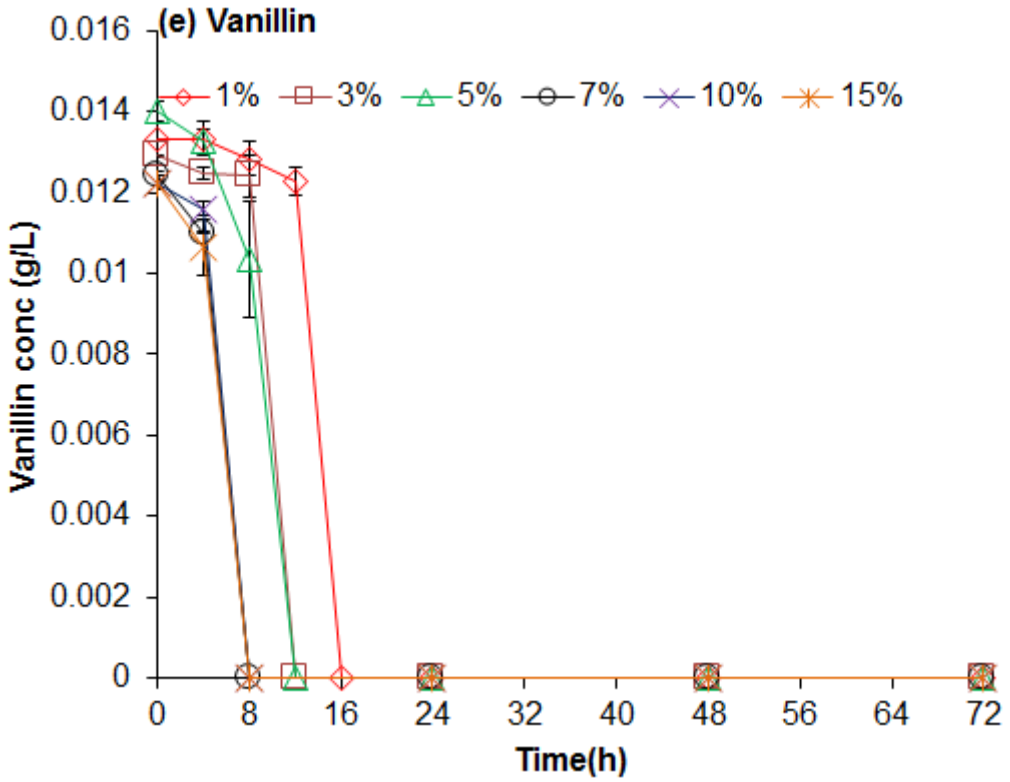
Increased inoculum size is an important conventional method that hasbeen used to overcome the inhibitory effects of furfural and HMF, since no strain has been available to grow in the presence of these inhibitors [177, 242, 247, 248]. *Corynebacterium glutamicum* was grown in non-biodetoxified hydrolysate with different inoculum ratios. Strain demonstrated excellent capability to tolerate and degrade the inhibitors. Increased inoculum ration decreased the degradation time for all inhibitors, while minimum inoculum ratio of 1% also degraded the inhibitors in short time. No difference was observed for inhibitors degradation time between 10% and 15% inoculum ratios. Furfural was degraded within 24 h with 1% inoculum and within 8 h with 10 and 15% inoculum (Fig. 3.5a). HMF was all degraded within 24 to 8 h with inoculum size of 1 to 15% (Fig. 3.5b). Fig. 3.5(c) demonstrates acetic acid degradation within 24 and 16 h with 1% to 15% inoculum ratios. Strain demonstrated quick degradation of 4-Hydroxybenzaldehyde within 8 h with inoculum size 5 to 15%, while 1% inoculum degraded 4-Hydroxybenzaldehyde within 16 has shown in Fig. 3.5 (d). Vanillin was also degraded by using 1% inoculum within 16 h, while 7 to 15% inoculum ratios degraded vanillin within 8 h (Fig. 3.5e). Strain could not degrade syringaldehyde within 72 h (Fig. 3.5f).




(b) HMF
1%
3%
5%
7%
10%
15%
HMF Conc (g/L)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
8
16
24
32
40
48
56
64
72
Time(h)


(c) Acetic acid
1%
3%
5%
7%
10%
15%
Acetic acid conc (g/L)
2.5
2
1.5
1
0.5
0
0
8
16
24
32
40
48
56
64
72
Time(h)


(d) 4-hydroxybenzaldehyde
1%
3%
5%
7%
10%
15%
4-Hydroxybenzaldehyde conc (g/L)
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
8
16
24
32
40
48
56
64
72
Time(h)


(e) Vanillin
1%
3%
5%
7%
10%
15%
Vanillin conc (g/L)
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
8
16
24
32
40
48
56
64
72
Time(h)

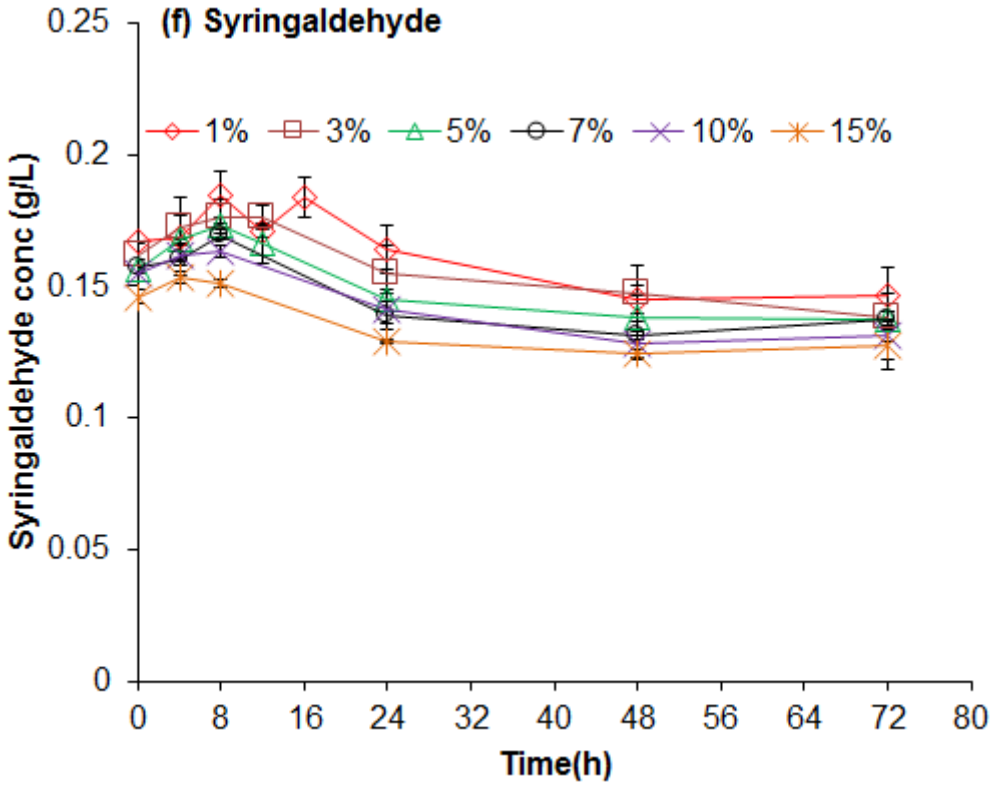


Fig. 3.5. Effect of different inoculum ratios on inhibitor degradation by *Corynebacterium glutamicum* SIIM B253 using non-biodetoxified hydrolysate. Fermentation conditions: 30 ℃, 200 rpm for 72 hours in flasks. pH was adjusted to value of 7.0 with addition of 5M NaOH at regular interval. (a) Furfural, (b) HMF (c) Acetic acid, (d) 4-Hydroxybenzaldehyde (e) Vanillin, (f) Syringaldehyde. The experiments were performed in duplication and data presented in Figures are average of two parallel experiments. Error bars are shown for standard deviation.

3.4. Conclusion

Inhibitor tolerance and biodegradation capability of *C. glutamicum* SIIM B253 was investigated and experimentally analyzed. *C. glutamicum* showed strong tolerance for almost all of the pretreatment inhibitors present in typical hydrolysate and pretreated material. *C. glutamicum* exhibited excellent capability to degrade furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid in very short time when added in the synthetic medium in isolation. No significant synergistic effects were observed as cell growth and lysine production were not affected by the use of non-biodetoxified hydrolysate. *C. glutamicum* also degraded furfural, HMF, acetic acid, vanillin and 4-hydroxybenzaldehyde in non-biodetoxified hydrolysate. This study provides attractive means to improve the performance of amino acid producing strains from agro-industrial residues.

Chapter 4

General conclusions and future perspectives

The objectives of the present study were to alleviate the high lignocellulosic processing and operational cost to produce glutamic acid and lysine, and to make the lignocellulosic biorefinery economically feasible and environmentally sustainable. In this study, several strategies were evaluated to improve the lignocellulosic bioconversion to glutamic acid and lysine for reducing the lignocellulosic processing cost. Various conclusions can be drawn from the results and many suggestions can be made for future studies.

Corynebacterium glutamicum SIIM B460 was evaluated for degradation capability of inhibitors present in typical hydrolysate and pretreated material. *C. glutamicum* degrades furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid in very short time. To increase the tolerance and degradation capability of the strain, and to achieve higher glutamic acid titer, long term evolutionary adaptation was performed using corn stover hydrolysate for 128 days. Cell growth and glutamic acid production were increased by the evolutionary adaptation of *C. glutamicum*. Tolerance of the strain increased and inhibitor degradation time shortened after evolutionary adaptation. These results clearly indicate that *C. glutamicum* B460 is potential strain and can be used to produce glutamic acid using non-biodetoxified corn stover hydrolysate as cheap energy source. This strain shows strong tolerance and degradation capability towards pretreatment inhibitors. The results provide important information for process intensifications of inhibitors removal from the pretreated materials and strain modifications for enhancing the inhibitor tolerance and degradation capacity.

This study also reports the inhibitor tolerance and biodegradation capability of *Corynebacterium glutamicum* SIIM B253 in lysine fermentation using pretreated non-biodetoxified corn stover feedstock. *C. glutamicum* demonstrated strong tolerance against almost all tested inhibitors, furans (furfural, 5-hydroxymethylfurfural (HMF)), various organic acids (acetic acid, formic acid, levulinic acid), and phenolic compounds (4-hydroxybenzaldehyde, vanillin, syringaldehyde and coniferyl aldehyde). Biodegradation of inhibitors by *Corynebacterium glutamicum* SIIM B253 was experimentally performed using each inhibitor in synthetic media. *C. glutamicum* SIIM B253 showed excellent degradation capability and degraded furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid in very short time. Cell growth and lysine production by *C. glutamicum* SIIM B253 was better in non-biodetoxified corn stover hydrolysate in comparison to synthetic media and biodetoxified corn stover hydrolysate, which again reflects the strong tolerance of the strain against pretreatment inhibitors. Strain degraded toxic inhibitors, furfural, HMF, acetic acid, 4-hydroxybenzaldehyde and vanillin using corn stover hydrolysate. Different parameters were optimized for higher lysine titer. The results suggest the efficiency of *C.*

glutamicum for the production of lysine using corn stover hydrolysate without prior biodetoxification.

Research presented in this thesis shows great potential for using *Corynebacterium glutamicum* strains for the glutamic acid and lysine production using corn stover hydrolysate as cheap energy source and cost effective process. Due to its excellent tolerance and degradation capability, *Corynebacterium glutamicum* can be used in non-biodetoxified hydrolysate, reducing the biodetoxification step, saving time and cost.

This study represents an attractive means to improve the economics of corynebacterial processes for production of amino acids and other valuable products.

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List of Publications

- Evolutionary adaptation of *Corynebacterium glutamicum* for enhanced inhibitor tolerance and degradation to hydrolysate of lignocellulose biomass. In submission.

- Evaluation of tolerance and degradation of lysine producing *Corynebacterium glutamicum* to lignocellulose-derived inhibitors. In submission.

Poster presentation

- **Imrana Khushk**, Yanqiu Xiao, Qiuqiang Gao, Jie Bao. Inhibitor Tolerance and biodegradation of *Corynebacterium glutamicum* in glutamic acid fermentation from lignocellulose feed stock in International Symposium on Biosystem and Biodesign Engineering (2015): Microbial Cell Signaling and Cell Factories (ISBBE 2015).

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