Isolation, Characterization and Kinetic Modeling of Carboxymethyl Cellulose Degrading Ethanologenic Bacterial Strains

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MS THESIS WORK

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We hereby recommend that the dissertation prepared under our supervision by: HAMZA IHSAN Reg No. NUST201260336MASAB91012 **Titled**: "Isolation, Characterization, and Kinetic Modeling of Carboxymethyl Cellulose degrading Ethanologenic Bacteria Strains ", be accepted in partial fulfillment of the requirements for the award of MS Industrial Biotechnology degree with (A grade).

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No portion of the work presented in this report has been submitted in support of any other degree of this or any other university or institute of learning.

Date _____ Hamza Ihsan

I dedicate this dissertation to my parents and grandparents whose unending love, support and encouragement has never let me down.

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Abstract

Ethanol has become a major target as an alternate fuel in the wake of a fossil fuel crisis. Cellulose provides a cheap biomass for the production of ethanol. In the present study, bacterial strains were isolated from different environmental sources and screened for their cellulolytic activity upon Carboxymethyl Cellulose (CMC). Ethanol production by the bacterial isolates was tested using the potassium dichromate test and subsequently confirmed through High Performance Liquid Chromatography. Two of the bacterial isolates were identified as Lactococcus lactis (HI-W) and Lactobacillus pentosus (HI-Lb2), which showed highest cellulose degrading activity at 0.5% CMC concentration, temperature 37ºC, pH 6.0, under oxygen limiting conditions, producing a reducing sugar concentration of 2.3mg/ml and 2.1mg/ml respectively and an ethanol concentration of 1.764mg/ml and 1.684mg/ml respectively in batch fermentation. A third unidentified bacterial strain produced 1.8mg/m of reducing sugars and 1.544mg/ml ethanol at 37ºC and pH 7.0 under the same conditions. Furthermore the reducing sugar and ethanol production by the strains was modeled through Hybrid Petri Nets using kinetic data obtained in the wet lab. The computational model results showed good correlation with the wet lab results with respect to reducing sugar concentration and ethanol production. The current study can be can be taken forward through a better understanding of media requirements and using advanced molecular techniques such as metabolic and enzyme engineering for enhancing ethanol production with the help of improvements in the kinetic model.

Chapter One

Introduction

Introduction

The increased dependence on fossil fuels has brought the world to the verge of a global energy crisis leading to an exponential rise in the demand of these fuels, creating a supply demand-situation, resulting in increased fuel prices (IAE, 2008). Furthermore the excessive use of the fossil fuels is leading to adverse environmental effects along with their depletion becoming a serious threat to the global economy (Goldemberg, 2008). These fuels also have the drawbacks of not being renewable and sustainable in addition to the immense greenhouse gas emissions (He *et al.*, 2010). All these problems have led to the appreciation of biofuels as a realistic option as alternate fuels, ready to replace the current but aging trend of fossil fuel usage (Lynd & Wang, 2003). The word biofuels is self-explanatory of the fact that the fuels are derived directly or indirectly from living organisms. Under the umbrella of biofuels, fall a number of fuels such as biogas, biohydrogen, bioethanol, biodiesel, and fischer-tropsch gasoline (Jaecker-Voirol *et al.*, 2008).

Biofuels are roughly classified as primary and secondary biofuels. Primary biofuels involving the use of biomass in its natural form, which includes fire wood, manure and crop residue for burning, along with landfill gas. These fuels do not need human intervention for being generated. In contrast, secondary biofuels are those that require certain transformation processes, and it is the secondary biofuels that are set to be the potential successors of the conventional fuels (Nigam & Singh, 2011). These secondary biofuels have been recently classified further as first, second, third and fourth generation biofuels based on the processes involved (Larson, 2008). The first generation biofuels require simple processes to be synthesized, their derivation being essentially from the food part of the crops, making use of the sugar content and grains. Bioethanol from the fermented starch from wheat, maize, potato, and barley and biodiesel by trans-esterification of plant oils such as rapeseed, sunflower, palm and coconut are the most common of the first generation biofuels (Love *et al.*, 1998). Utilization of the non-food component of the crop, and processes involved in their synthesis, sets apart the second generation biofuels from their predecessors. These fuels make use of the lingo-cellulosic and cellulosic parts of the plant, hence rendering crops such as grasses available to be utilized (Stevens *et al.*, 2004). Certain processes are required to be carried out so that these tough materials can be used for biofuel production. Biological (enzymatic) and thermo-chemical methods of processing are employed in the generation of the second generation biofuels.

Bioethanol and biomethane from fermentation and fischer-tropsch gasoline and biodiesel by thermo-chemical processing are major representatives of these fuels (Larson, 2008). The third generation biofuels focus on a different substrate altogether, causing a shift from the usage of plant-based substrates to algal-based substrates (Patil *et al.*, 2008). Algae and sea weeds are being employed for the production of biodiesel, hydrogen and bioethanol, either as substrates or as the producing organisms in the case of algae. The latest in the line of biofuels are the fourth generation biofuels, which involve the metabolic engineering of algae for the production of various fuels, targeting the photosynthetic capabilities of the algae, with the basic aim of enhancing the production of the fuels and the substrate utilization of the organism itself (Lü *et al.*, 2011).

Bioethanol has found a special importance in the midst of all. The long prospected potential of bioethanol and the recent realization of its application in industry and transport is becoming fast a reality (Lynd *et al.*, 1991). Earlier production of bioethanol made use of the extracted sugars, from sugarcane and sugar beet, and molasses from the sugarcane industry (Aggarwal *et al.*, 2001), while corn, cassava, shorghum along with grains such as wheat, rye, barley and triticale were exploited for their starchy content (Singh *et al.*, 1995). Sugars such as sucrose, hexoses and pentoses are easily utilized by micro-organisms such as yeast and bacteria and need no pre-treatment (Badger, 2002).

However recently, all the focus has shifted towards the use of cellulosic feed stock which is non-food, the "waste" part of the plant as it may be called (Lynd *et al.*, 1991). Around 50% of the biomass produced annually is cellulosic biomass and is estimated to be around 10-50 billion tones. Many forms of cellulosic feed stocks exist which can roughly be classified as crop residue which includes crops after food processing, hardwood which includes wood from trees such as poplar and aspen, cellulose waste which comprises processed wood in the form of paper and newspapers, softwood consisting of conifers, municipal solid wastes and last but not the least herbaceous plants that comprise of the grasses (Hess *et al.*, 2007)

Pretreatment of the lignocelluosic biomass is done to separate cellulose, hemicellulose and lignin contents. Physical methods (communition, pyrolysis) (Sun & Cheng, 2002) (Kilzer & Broido, 1965), physico-chemical pretreatment (steam explosion, liquid hot water, ammonia fiber, and CO_2 explosion) (McMillan, 1994)

(Taherzadeh & Karimi, 2008) (Abril & Abril, 2009), chemical pretreatments (acid, alkaline, ozonolysis, organosolv and lime) (Mosier *et al.*, 2005). Biological pretreatment method includes treating the lignocelluosic biomass with fungi such as white and brown rot fungi (Taherzadeh & Karimi, 2008). The cellulose component of the biomass is further processed for ethanol production.

Hydrolysis of cellulose releases glucose which is used for ethanol production. Chemical and biological hydrolysis are the two major techniques. Chemical hydrolysis involves the use of both concentrated and dilute acid (Taherzadeh & Karimi, 2007). The biological method involves the use of microorganisms capable of cellulolytic activity through enzymes known as cellulases (BEguin & Aubert, 1994). The cellulases are specific in their action and produce reducing sugars as hydrolysis products including glucose. Both bacteria and fungi are employed for carrying out biological hydrolysis. Commonly used bacteria include *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus* and others (Sun & Cheng, 2002). Fungi include *Sclerotium rolfsii, Phenarochete chrysosporium, Trichoderma, Schizophylum, Aspergillus* and *Penicillium*. Among the fungi, *Trichoderma reesei* is the best known producer of cellulases (Sternberg, 1976).

In recent times, synthetic substrates, such as cellulose derivatives have been employed in ethanol production. Carboxymethyl cellulose (CMC) is one of the most widely used among these derivatives. This cellulose ether has been used in detergents, textile industry, oil exploration and food (Horner *et al.*, 1999). Having a structure similar to cellulose, CMC has been used to isolate cellulolytic microorganisms, study cellulase production and simulate production of ethanol directly from cellulose without the use of cellulose. CMC is essentially a cellulose polymer chain, with each glucose molecule having an ether linkage with carboxymethyl functional group (-CH_2-COOH). Mostly the sodium salt of the derivative i.e. Sodium Carboxymethyl Cellulose is used (Bogati, 2011).

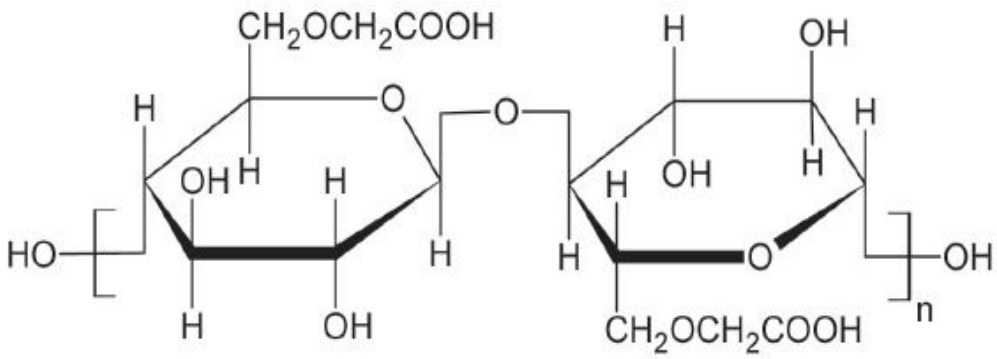


Figure 1: Structure of Carboxymethyl celulose (Horner *et al.*, 1999)

Although theses synthetic derivatives have been used for various ethanol studies, the use of these derivatives is limited to research based experimental approaches and are not used at the industrial production of bioethanol (Sadhu *et al.*, 2013).

Bioethanol is produced by a number of yeasts and some bacteria. The best known producer in the yeasts is the Baker's yeast (Herrero, 1983), Saccharomyces cerevisiae. Among bacteria, *Zymomonas mobilis* is the chief producer (Rogers *et al.*, 2007). In contrast to *S. cerevisiae, Z. mobilis* gives 5% higher ethanol yields and almost 5-fold higher volumetric production and is now the becoming the preferred organism for the job. *Z. mobilis* can ferment glucose, fructose and starch efficiently giving up to a 97% theoretical yield (Weuster-Botz *et al.*, 1993). Both of these microbes do not have the ability to degrade cellulose, hence cannot be employed directly in fermentations, which is fast becoming the target of many bioethanol producing groups, which is being termed as Consolidated Bioprocessing (CBP) or Direct Microbial Conversion (DMC), surpassing the older strategies such as Simultaneous Saccharification and Fermentation (SSF), and Separate Hydrolysis and Fermentation (SHF) (Lynd *et al.*, 1991).

A number of other yeasts such as *Pichia stiplis, Candida shehatae,* and *Kluyveromyces marxianus,* and bacteria such as *Escherichia coli, Thermoanaerobaacterium saccharolyticum, Thermoanaerobacter ethanolicus,* and *Clostridium thermocellum* have the ability to produce ethanol directly from cellulose (Limayem & Ricke, 2012).

Pakistan is an agriculture based economy and one of the biggest crops in the country is sugar cane. The sugar industry is the second largest after the textile

industry more than 70 operational sugar mills each having a capacity to process around 300,000 tons of sugar cane per day. Ethanol is produced using the molasses obtained from the sugar cane industry with 21 distilleries having the capacity of processing 2 million tons of molasses to produce 400,000 tons of ethanol (Malik, 2008).

Aims and Objectives

- Isolation of carboxymethyl cellulose degrading bacteria and their screening for ethanol production
- Optimization of culture conditions including pH, temperature, substrate concentration and agitation to obtain increased ethanol yields
- Kinetic modeling of the wet lab results using Hybrid Petri Nets

Chapter Two

Literature Review

2.1 The Need for Biofuels

Increasing economic and environmental problems rising due to the elevated use of fossil fuels is turning the focus of the world to the development of biofuels. Fossil fuels have disadvantages of being non-renewable, production of environmentally harmful byproducts and dependence on countries with high fossil fuel reserves (Naik *et al.*, 2010). Biofuels present various advantages over conventional fuels in terms of economic impacts being derived from cheap renewable resources, environmental benefits of being degradable and recyclable and energy security as they ward off dependence on oil and gas (Balat, 2011). Among a number of biofuels such as biogas, biohydrogen, biodiesel, and Fischer-Tropsh oil, bioethanol has found a unique place as a replacement for traditional fossil fuels (Otero *et al.*, 2007).

2.2 Cellulosic Biomass Resources

Cellulose makes up almost 50% of the lignocellulosic biomass (Gírio *et al.*, 2010) and is the most abundant biomass resource. Cellulose is a long chain of B-1,4-glycosidic linkages containing solely glucose residues in its structure (Ebringerova *et al.*, 2005). Cellulose makes up to 30% of a plant by weight and is the most abundant polymer in the natural world, cotton being one of the purest forms of cellulose with (80-95%) cellulose composition. Cyclic carbon chains in its structure and rigidity due to covalent bond formation with hemicellulose makes lignin a tough target for any pre-treatment method (Mielenz, 2001). Hardwoods (45%), softwoods (42%), cornstalk and wheat straw (37-47%) And chemical pulp (60-80%), are sources containing the highest amount of cellulose (Balat, 2011).

2.3 Biological Pretreatment of Cellulose

In the process of conversion of cellulosic biomass to ethanol, rate limitations are presented by the pretreatment method being adopted due to cost inputs. Biological pretreatment strategy is employed over physical, physic-chemical and chemical methods due to low costs and their environmental friendly nature (Wan & Li, 2012). As implied, the biological pretreatment makes use of cellulolytic fungi and bacteria for the process. Filamentous fungi are generally employed and are considered efficient for biomass such as wood shavings, wheat straw and softwood (Akin *et al.*, 1995). Brown rot fungi are another group of fungi that are commonly used to

selectively degrade cellulose and hemicellulose. Lignin is not affected during the process (Rasmussen *et al.*, 2010). Fungi degrade the lignocelulosic biomass using a combination of two mechanisms 1) oxidative and 2) hydrolytic. The oxidative mechanism is carried out through the production of ROS (reactive oxygen species) which generally comprise of hydroxyl radicals (Hammel *et al.*, 2002). Hydrolytic enzymes such as endo-(1,4)-β-glucanase (endocellulase), cellobiohydrolase (exocellulase), and β-glucosidase (Baldrian & Valaskova, 2008) are key to the hydrolytic mechanism of cellulose degradation. The glycosidic linkages in the polymer carbohydrate are broken to release monomeric gucose residues (Feijoo *et al.*, 2008). The major disadvantages of the biological pretreatment are the slow reaction rate and the resulting long residence times needed for the process (Taherzadeh & Karimi, 2008). *Phanerochete chrysosporium* gave a 44.7% reducing sugar yield after 18 days using rice husk (Potumarthi *et al.*, 2013). White rot fungi *Ceriporia lacerata*, *Stereum hirsutum* and *Polyporus brumalis* were used for pretreatmet of Japanese Red Pine (Lee *et al.*, 2007).

2.4 Enzymatic Hydrolysis

The enzyme based hydrolysis of the cellulosic biomass components is carried out by hydrolases known as the glycosylhyrolases (Henrissat, 1991) as shown by the Figure 2.5. Carboxymethyl cellulose is essentially a derivative of cellulose and has almost identical structure, thus the hydrolysis of CMC also follows the same pathway for the production of glucose as depicted in Figure 2.5. The hydrolysis process involves three main glycosyl hydrolases namely endo-glucanse (E.C. 3.2.1.4), cellobiohydrolase (E.C. 3.2.1.91) and β-glucosidase (E.C. 3.2.1.21). These enzymes hydrolyze cellulose through a general process that includes enzyme adsorption to cellulose, biodegradation of cellulose and desorption of the cellulolytic enzymes (Wyman, 1996). The endo-glucanases act on the cellulose chain at low crystalline points, cleaving the β-1,4-glycoside linkages, forming shorter cellulose chains. Cellobiohydrolases act on the short chains to produce cello-oligomers and celobiose. β-glucosidase cleaves the cellobiose units into glucose monomers, ready for conversion into ethanol by microorganisms (Taherzadeh & Karimi, 2007). Feedback inhibition of the enzyme cellobihydrolase by cellobiose and β-glucosidase by glucose presents some difficulty in total hydrolysis of the cellulose biomass (Gusakov & Sinitsyn, 1992). Enzymatic hydrolysis possesses certain advantages over chemical dilute acid hydrolysis which are; 1) mild conditions for hydrolysis, 2) high hydrolysis

yields, 3) no inhibitory product formation during enzymatic hydrolysis (Lee *et al.*, 1999). On the other hand, enzymatic hydrolysis used expensive enzymes which add to the process cost, as well as longer residence times are also a nuisance for industrial upgradation (Tengborg *et al.*, 2001).

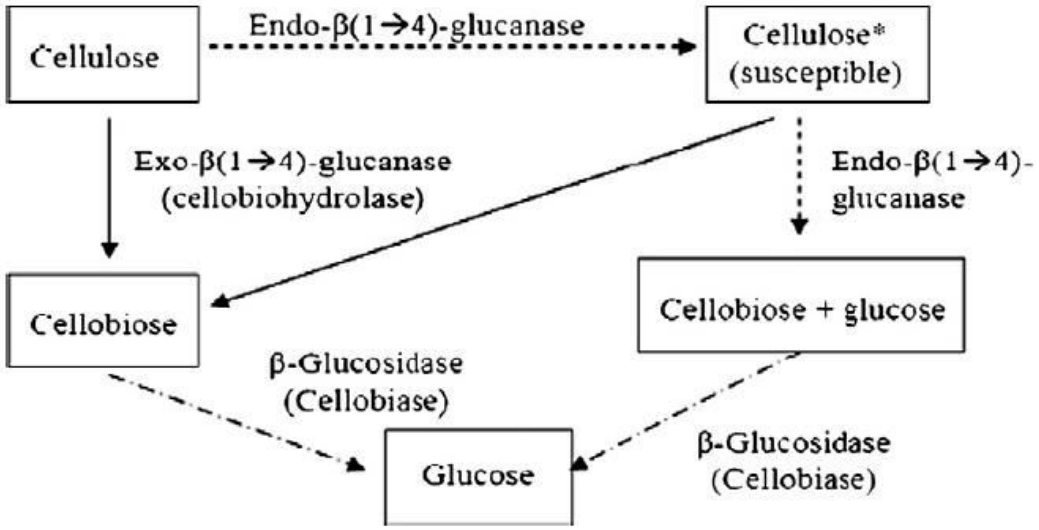


Figure 2.5: The enzymatic hydrolysis of cellulose to glucose (Kim & Holtzapple, 2005)

Among the microorganisms, top cellullase producing bacterial species include *Clostridium, Cellulomonas, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Streptomyces, Bacteriodes, Acetovibrio,* and *Microbispora (Sun & Cheng, 2002).* Lactic acid bacteria such as *Lactobacillus* and *Lactococcus* spp. have also been reported to produce cellulases. The well-known fungal species enlist *Penicillium, Fusarium, Trichoderma, Phanerchete, Humicola* and *Schizophillum* (Rabinovich *et al.*, 2002).

2.5 Isolation of Cellulolytic Bacteria using CMC

Carboxymethyl cellulose has been employed for the isolation of cellulose/CMC degrading bacteria as alternative to naturally available cellulose. A number of studies regarding cellulose/CMC degrading bacteria have used this cellulose derivative.

Ariffin (Ariffin *et al.*, 2006), isolated CMCase producing *Bacillus pumilis EB3* using CMC as a sole carbon source in the media. The bacteria showed good results of CMCase production on the substrate with the cellulase having a wide temperature (30-70ºC) and pH (5.0-9.0) range.

Ekperigin (Ekperigin, 2007) isolated two bacterial species *Acinetobacter anitratus* and *Branhamela sp*. from the snail *Archachatina marginata* using CMC as

the sole carbon source in the media. Best cellulase activity was found to be during the logarithmic phase.

Huang (Huang *et al.*, 2012) studied the diversity of cellulolytic bacteria residing in the hind gut of the scareb beatle *Holotrichia parallela*. The isolations were performed on carboxymethyl cellulose medium and it was found that many cellulolytic bacteria including *Pseudomonas, Orchobactrum, Rhizobium* and others can be isolated on CMC agar.

Mazzucotelli *et al.* (2013) isolated bacterial strains including *Lactococcus, Enterococcus, Bacillus, Stenotrophomonas,* and *Klebsiella sp*. using 1% carboxymethyl cellulose. Subsequent staining with congo red confirmed CMCase production.

Vinotha and Maheshwari (2014) isolated cellulolytic bacterial strains from cellulosic wastes such as rotting leaves and bagasse. The isolated bacteria contained *Bacillus sp., Pseudomonas sp., Micrococcus sp., and Lactobacillus sp.* The highest cellulase activity was showed by Bacillus sp. which reached 7.8 IU/ml using 1% CMC and measuring the reducing sugar production using the DNS method. The Lactobacillus sp. was observed to have an optimal temperature of 30ºC and a pH of 5.7-6.0.

2.6 Microorganisms for Ethanol Production

Ethanol production from lignocellulosic and cellulosic substrates has been of major importance in recent times. Restrictions in the efficient conversion of the cellulosic biomass in to ethanol, limited substrate utilization ability and the economic feasibility of the process present limitations in the up staging of the process. None of the existing organisms has the ability to covert cellulose into ethanol at an appreciable rate (Hahn-Hägerdal *et al.*, 2006). Hence the first step towards the whole ethanol production starts with strain development (Bothast *et al.*, 1999). The ideal microbe should have a broad substrate range, high ethanol tolerance, higher conversion rates and a good substrate to ethanol ratio (Zaldivar *et al.*, 2001). High stress tolerance and tolerance to product and substrate inhibition are also some of the desired attributes.

Products of cellulosic biomass pretreatment and hydrolysis are subjected to fermentation for the production of ethanol using microorganisms which include both yeast and bacteria. Traditionally, the most abundantly used microbe for ethanol production is the yeast *Saccharomyces cerevesiae*. This wide usage is owing to the yeast's capability of high ethanol production, up to 18% (v/v), high ethanol tolerance,

and robustness (Lin & Tanaka, 2006). In similarity to most microorganisms, the yeast can metabolize a range of simple sugars except for pentoses. The pathway for fermentation follows the Embden-Meyerhoff-Parnas (glycolysis) pathway for ethanol production. Among bacteria, *Zymomonas mobilis* has gained importance in terms of an ethanol producer (Hayes, 2009). *Z. mobilis* gives a higher ethanol yield on glucose than *S. cerevesiae*. A 5% higher ethanol yield and 5 times volumetric production as compared to yeast has been reported (Sáez‐Miranda *et al.*, 2006). *Z. mobilis* uses the Entner-Doudoroff pathway for the anaerobic metabolism of glucose which results in lower biomass and higher ethanol yields and is a homo-ethanol fermentative organism (Matthew *et al.*, 2005). *Zymomonas mobilis* ethanol production is limited by its utilization exclusively of sucrose, glucose and fructose (Alterthum & Ingram, 1989). Some yeast such as *Pichia stipilis* (Slininger *et al.*, 1991), *Candida shehatae* (Prior *et al.*, 1989) and *Pachysolen tannophilus* (Slininger *et al.*, 1982) have the ability to ferment xylose into ethanol, giving considerable yields.

Inherent ability of thermophilic bacteria such as members of the genus *Clostridium* (*C. thermocellum, C. thermohydrosufuricum, C. thermosaccharolyticum*), *Thermoanaerobacter mathranii, Thermoanaerobium brockii* to degrade cellulose directly into ethanol has been explored (Balat, 2011). On the other hand, some filamentous fungi also can ferment cellulose to ethanol. These include *Monilla, Neurospora crassa, Zygosaccharomyces rouxii, Aspergillus spp. Trichoderma viride and Paecilomyces spp.* (Lin & Tanaka, 2006). However, these microbes show little ethanol production and the process time is high (3-12 days) for efficient conversion of biomass to ethanol. The low production is attributed to the low ethanol tolerance of the microbes to ethanol and to the formation of byproducts such as lactic and acetic acid which make fermentation conditions unfavorable (Sarkar *et al.*, 2012).

Recent trends in metabolic engineering have enabled advances in the production of bioethanol from cellulosic feedstocks, turning wild type, non-cellulose degrading bacteria and fungi in to cellulose degraders for the cellulosic ethanol production. Bacteria that have been engineered for the purpose include, *E. coli* and *K. oxytoca* for producing ethanol as a primary product (Ingram *et al.*, 1987) (Wood & Ingram, 1992). *Erwinia chrysanthemi* has also been targeted for metabolic engineering (Zhou *et al.*, 2001). E. coli has been incorporated with the pyruvate decarboxylase and alcohol dehydrogenase of *Z. mobilis* for better production of ethanol (Ingram *et al.*, 1987). Wood and Ingram (1992) engineered *K. oxytoca* strain

with the *Z. mobilis* ethanol producing genes for ethanol production from amorphous cellulose. In addition to these bacterial species, metabolic engineering of cellulolytic bacterium *Clostridium cellulolyticum* has been done to produce ethanol (Guedon *et al.*, 2002), and the ethanol producing bacterium *Lactobacillus casei* has been engineered with cellulases from *Bacillus spp.* and ethanol producing genes from *Z. mobilis* (Gold *et al.*, 1996). Promising prospects have been observed using the techniques of metabolic engineering in the field of direct ethanol production from cellulosic feed stocks but still further work needs to be done before the efficient ethanol production can be achieved from these resources (Senthilkumar & Gunasekaran, 2005).

2.7 Ethanol Production using Cellulose and Synthetic Cellulose Derivatives

Cellulose has been used as a resource for ethanol production. To ease the cellulose hydrolysis at the laboratory scale, substitutes instead of cellulose are used. These include CMC, Avicel, MN300, and Solka Flok (Ng *et al.*, 1981).

Groposo *et al.,* (2013), reported ethanol production by *Clostridium thermocellum ATCC 27405* using sugar cane bagasse cellulose component after pretreatment. A maximum of 21.9mM ethanol concentration was achieved under static conditions after 72 hours of incubation.

Gupta (Gupta *et al.*, 2012) isolated cellulose degrading bacteria from termites, snail caterpillar and bookworm, using basal salt medium and filter paper as the cellulose source. The bacterial isolates were confirmed for cellulase production using the congo red staining. The cellulose hydrolysis products were tested for ethanol production using yeast *Saccharomyces cerevisiae* using simultaneous saccharification and fermentation. Ethanol production was achieved after five days of incubation. Ethanol production was confirmed through Jones reagent (potassium dichromate).

Ng (Ng *et al.*, 1981) have reported ethanol production from synthetic cellulosic substrates including Solka Floc, MN 300, and Avicel using co-cultures of thermophilic bacterium Clostridium thermocellum and Clostridium thermohydrosulfuricum. Highest ethanol yield of 1.8mol/mol of glucose unit was achieved using MN 300.

Deshpande (Deshpande *et al.*, 1986) optimized fermentation conditions for Neurospora crassa for the production of ethanol. The mold grown on 1% Avicel and 2% Alkali treated cellulose powder yielded 5.5g/l and 10g/l ethanol after 4 days of incubation.

Rao (Rao *et al.*, 1983) have also reported two Neurospora crassa strains capable of producing ethanol directly from cellulose with the ethanol production starting from an early incubation period of 48 hours. The cellulose to ethanol conversion was reported at > 60% making N. crassa a potential producer for ethanol.

Zertuche et al. (1984) studied the direct conversion of cellulose into ethanol using thermophilic bacterium *Clostirdium thermocellum*. The continuous process was carried out under anaerobic conditions. It was also observed that the temperature had a positive effect on growth rate of the bacterium. Agitation seemed to negatively influence the ethanol production by the bacterium. At 1.5% cellulose concentration, maximum ethanol production of 0.75g/g cellulose consumed. The optimum temperature for ethanologenesis was found to be 60ºC at pH 7.0.

2.8 Fermentation Strategies

The steps involved in the conversion of cellulosic biomass comprise of pretreatment of the biomass, hydrolysis and the conversion of hydrolyzed biomass into fuels ethanol (Taherzadeh & Karimi, 2007). Certain strategies have been developed over time, which are being used to carry out this task. Stages of the conversion of biomass into ethanol have been described which include 1) production of cellulases, 2) hydrolysis of the biomass (cellulolse), and 3) fermentation of cellulose hydrolysis products that are soluble. Four strategies have been developed to address these processes; 1)separate hydrolysis and fermentation (SHF), 2) simultaneous saccharification and fermentation (SSF), 3) simultaneous saccharifcation and co-fermentation (SSCF) and 4) consolidated bioprocessing (CBP) (Lynd *et al.*, 2002).

Cellulase enzymes act at their best at temperatures ranging from 45-50ºC depending up on the microbe involved, whereas ethanol fermentation by most ethanologenic microorganisms is from 30-37ºC (Saha *et al.*, 2005). It has been observed that even a 6g/l cellobiose concentration inhibits enzyme activity by 60% (Philippidis & Smith, 1995). On the other hand, glucose inhibits β-glucosidase and a glucose concentration of 3g/l lowers β-glucosidase activity by 75% (Philippidis *et al.*, 1993).

2.8.1 Simultaneous Saccharification and Fermentation (SSF)

One of the better methods for ethanol production from cellulosic biomass is the simultaneous saccharification and fermentation (SSF), which combines the

hydrolysis of the biomass and its fermentation to ethanol in one single step (Eklund & Zacchi, 1995). The process bypasses the inhibition of cellulses by the cellobiose and glucose formed as the products are immediately consumed/fermented by the ethanol producing microbes (Sun *et al.*, 1995). Another advantage of the process is the low risk of contamination of the process as the ethanol present in the system restricts microbial invasion from outside. Furthermore, the number of chambers/reactors used in the process, are less than those used in SHF so there is less investment to run the process (Limayem & Ricke, 2012). The process integrates hydrolysis and ethanol fermentation and thus requires that the conditions be close to optimal for both processes. Obviously the task of optimizing both processes simultaneously is near to impossible but the best strategy is to run SSF at conditions which suit both the combined processes (Karimi *et al.*, 2006). Co-cultures of *T. reesi* and *S. cerevisiae* for ethanol production while using ceulosic biomass have been reported to perform best at 38ºC which is a median range between the optimal conditions for both organisms (Tengborg, 2000). Use of thermophilic yeasts such as *Candida acidothermophilum* and *Kluyveromyces marxianus* is being explored to raise the ethanol fermentation temperature to match hydrolysis (Kadam & Schmidt, 1997) (Ballesteros *et al.*, 2004). Ethanol accumulation during the process is also considered as a drawback in SSF. A 30g/l ethanol concentration inhibits enzyme activity by 25% (Wyman, 1996).

SSF was employed using Fusarium oysporum and S. cerevisiae for the ethanol production from cellulose (Lezinou *et al.*, 1994). An ethanol production of 29.7g per 100g of substrate was obtained in mixed culture through fed-batch process. Mohan (Mohan *et al.*, 2012) reported an ethanol production of 17.03% (w/v) using S. cerevesiae with bagasse.

2.8.2 Consolidated Bioprocessing

Consolidated bioprocessing (CBP) also known as direct microbial conversion (DMC) is based on the strategy that all of the involved processes during the fermentation of cellulose or hemicellulose to ethanol are carried out in a single bioreactor. This implies that unlike previous strategies, CBP integrates both the hydrolysis and the fermentation steps into one single process. During the process, the biomass is hydrolyzed and ethanol is produced by a single microbial colony or a co-culture employing a mixed microbial community. CBP is considered to be the ultimate goal in strategies for ethanol fermentation which cuts costs on external

enzyme requirements as well as multiple bioreactors (Lynd *et al.*, 2005) (Hamelinck *et al.*, 2005). The main hurdle in the upgradation of the process is the microbes that have the capability of both cellulose hydrolysis and ethanol production. Development of microorganisms for the process is a two pronged strategy, one aiming at microbes that are ethanol producers and lack cellulase activity, while the second strategy works on cellulase producers that do not produce ethanol (Hogsett *et al.*, 1992). Some bacteria such as *Clotridium thermocellum, Thermoanaerobacter mathranii, and Thermoanaerobium brockii* produce cellulase as well as ethanol but ethanol concentration is reported to be not more than 4% which is not economically viable. Fungi that have the same ability face the same problem. The overall process also takes normally 3-12 days which adds to the cost (Lynd *et al.*, 2002). Attributes that are desirable for microbes to be used in direct microbial conversion include high ethanol tolerance, resistance to substrate and product inhibition, ethanol production as a primary metabolite and low production of harmful byproducts such as lactic and acetic acid (Zaldivar *et al.*, 2001). A comparison of SSF and CBP was done by South et al. (South *et al.*, 1993) using *T. reesei*, and *S. cerevisiae* and *C. thermocellum* for the processes respectively. It was observed that the substrate conversion in CBP which was 77% was much higher than what was achieved in SSF which was 31%.

Tiwari (Tiwari *et al.*, 2011) isolated cellulose degrading bacteria from rotten fruits and reported ethanol production from cellulosic sources such as maize, barley, oat and sugar beet. The bacterial strains which were named A, B, X, and Y gave an ethanol production of 3.0ml, 3.3ml, 1.5ml and 3.4ml per 100ml of fermentation media.

2.9 Kinetic Modeling of Ethanol Production

Capital costs of carrying out a full process from start to end, from the pretreatment o hydrolysis and from hydrolysis to ethanol fermentation are much too high for a hit and trial approach towards efficient process development. Therefore kinetic models of the process have taken the pace to pre-operation settings such that the model defines what parameters to set and predicts what results can be expected, saving large investments which could easily go down the drain without any fruitful outcomes (Lin & Tanaka, 2006).

Conventional modeling generally focuses on the process design, kinetics and general parameters in microbial growth, whereas recent developments in kinetic modeling take into account the metabolic pathway kinetics, rate of change of substrate

and product and the effects of various culture conditions (Marwan & Blätke, 2012). Petri nets, mathematical modeling, Langmuir type models are some examples of the modeling techniques that are being used today (Heiner & Koch, 2004). Petri nets have gained importance recently since their emergence and have been used for modeling metabolic pathways such as glycolysis, carbon metabolism in potato tubers and apoptosis (Hengartner, 2000, Matsuno *et al.*, 2003).

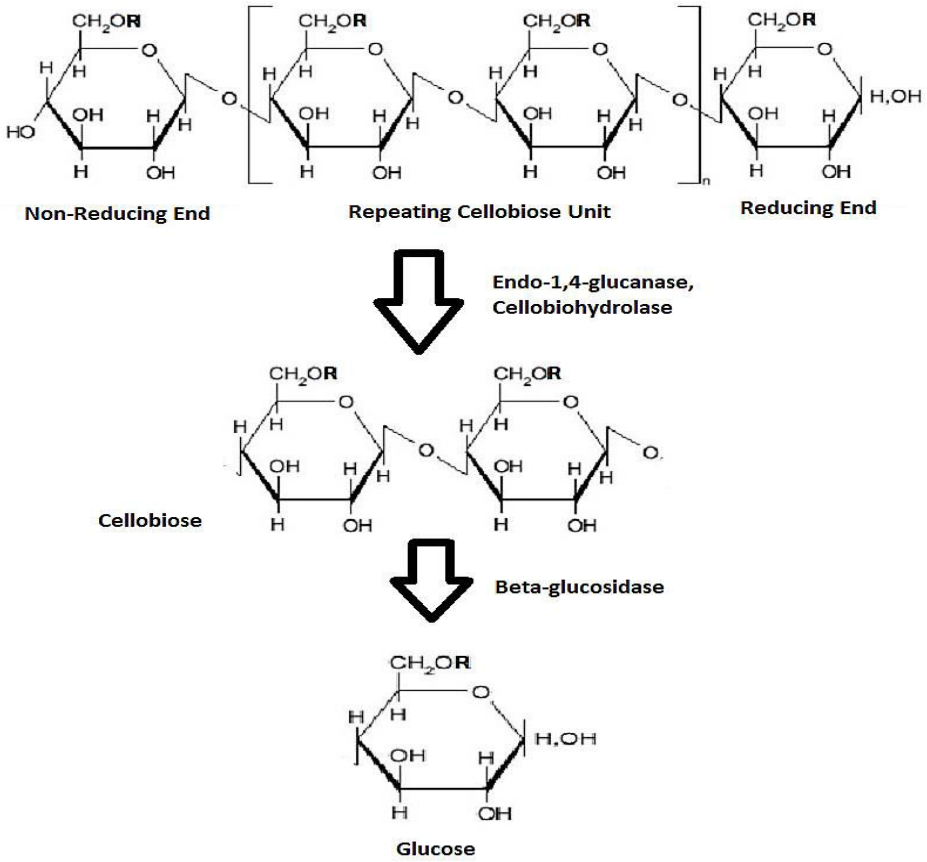
A number of softwares for modeling metabolic pathways are available which include Snoopy (Heiner *et al.*, 2012), Cell Illustrator (Nagasaki *et al.*, 2010), COPASI (Mendes *et al.*, 2009). These computational modeling tools provide features that can be used for modeling the metabolic pathways according to the wet lab conditions to predict reslts as close to real lab conditions as possible.

Further understanding of the metabolic pathway kinetics can be obtained with further increasing the set of parameters that control the model and then application of these mode results into wet lab experiments and the integration of both for efficient ethanol production by the desired microorganism.

2.9.1 Metabolic Pathway used for Kinetic Modeling

The phosphoketoase pathway is the main metabolic pathway used by the hetrofermentative lactic acid bacteria for ethanol production from simple sugars. The pathway was proposed by DeMoss (1951), and is found in lactic acid bacterial genera such as *Streptococcus, Lactococcus, Pediococcus, Microbacterium, and Lactobacillus spp*. the pathway leads to the formation of lactic acid, ethanol and CO_2 and sometimes acetate. The pathway is named after the key enzyme phosphoketolase enzyme (E.C 4.1.2.9) which cleaves xylulose-5-phosphate to acetate and glyceraldehyde-3-phosphate. It has been shown that the pathway dominates in hetrofermentative acetic acid bacteria (Årsköld *et al.*, 2008). The glucose to the pathway is fed by the hydrolysis of carboxymethyl cellulose as shown in figure 2.5 and the resulting glucose is fermented to ethanol by the bacteria. Carboxymethyl cellulose is degraded by CMCases (endo-1,4,glucanase) and cellobiohydrolases which act on the β-1,4-glycosidic linkage in CMC and cellobiose is produced. The cellobiose is acted upon by β-glucosidase which cleaves it to produce monomer glucose units. This released glucose is used by the bacteria to produce ethanol through the phosphoketolase pathway illustrated in Figure 2.5 (b). The functional group (R= CH_2COOH) in CMC and (R= -CH_2COONa) in the sodium salt of carboxymethyl cellulose which is used generally.

(a)



(b)

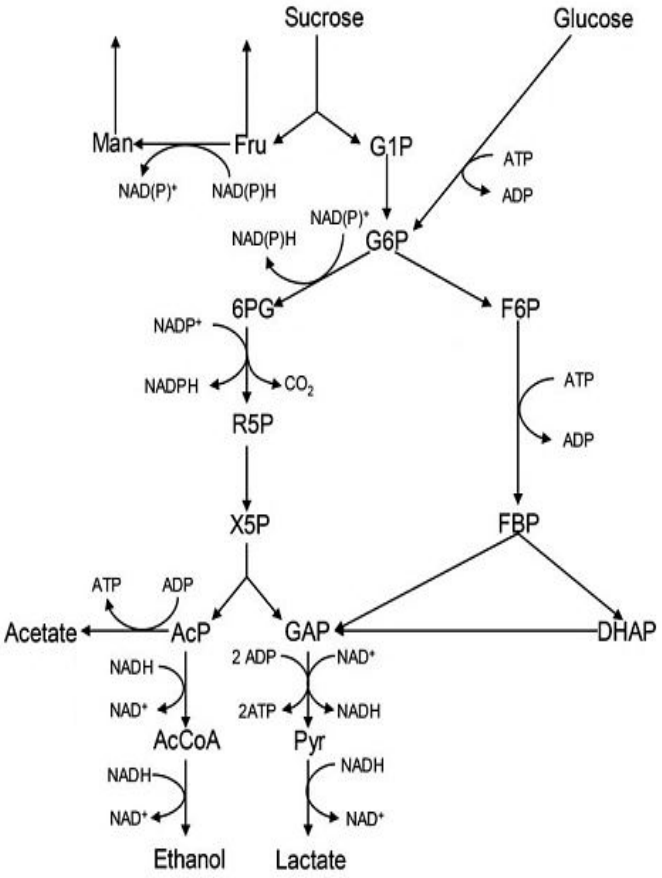


Figure 2.6.1: CMC degradation to ethanol (a) CMC degradation to glucose by cellulase enzymes (b) the phosphoketolase pathway for ethanol fermentation in hetrolactic bacteria as adapted from (Årsköld *et al.*, 2008)

2.10 Bioethanol in Pakistan

Being the 5th largest sugar cane cultivator in the world and ranked in the top 10 sugar cane producing countries and the 3rd largest molasses exporter for the last 2 decades, Pakistan has a lot of potential in bioethanol production. Investment in the ethanol production sector has increased ethanol exports in recent years from 100,000 tons in 2004 to 225,000 tons in 2010. A highest ethanol export was reached in 2008 when 350,000 tons of ethanol was exported. At present number of distilleries in operation, the ethanol production can easily go up to 400,000 tons.

A number of distilleries are producing ethanol from sugar cane molasses using the yeast *Saccharomyces cerevisiae*. Premier group with Premier sugar mill and distillery (Mardan, KPK)(1958), and Frontier sugar mill and distiellry (Takht-e-bhai, KPK)(1987), Unicol distillery (Mirpurkhas, Sindh)(2006). Colony sugar mills, have sugar mills at Phalia and Mian Chunu and a distillery at Phalia (2007) with 125,000L/day of ethanol production capacity. The plant is also equipped with a CO_2 recovery unit with a recovery capacity of 48 metric tons/day (Arshad, 2011).

It has been reported that exporting raw molasses, a maximum of $100 million are earned while the value added product of molasses which is ethanol can earn the country in excess of $600 million (Sibtain, 2009). Recently, Pakistan State Oil (McFeeters *et al.*) introduced E-10 in three major cities of Pakistan, Islamabad, Karachi and Lahore, with the collaboration of Hydrocarbon Development Institute of Pakistan. 25 cars were monitored for their E-10 fuel usage, mileage and performance patterns. After 6 months, the pilot project showed promising results and the project is now planned to spread to the entire country. Sufficient ethanol can be produced (400,000 tons) by the distilleries working at the moment in Pakistan to cut down fuels prices and fuel import prices (Umar *et al.*, 2008).

Chapter Three

Materials and Methods

3.1 Chemicals

All the chemicals used in the experiments were analytical grade. Carboxymethyl cellulose was purchased from Fluka. The salts for media preparation were of either Merck, Germany or Sigma Aldrich, USA. Sulfuric acid used for the potassium dichromate test was purchased from Scharlau, Spain. HPLC grade ethanol was purchased from Reanal Chemicals, Hungry while Acetonitrile, n-Hexane and HPLC grade Water were purchased from Sigma. 3,5-dinitrosalicylic acid was purchased from Unichem, China, while all the other ingredients used for the DNS reagent test were purchased from Merck, Germany except for phenol which was purchased from Carl Roth Laboratories, GmbH.

3.2 Collection of Samples

Isolation of cellulose degrading bacterial strains was performed from samples collected from various environmental sources including soils of different localities of Islamabad, Rawalpindi, Lahore, and Gujranwala, waste water from different sites in Islamabad and Rawalpindi, and food sources such as rotting Tomatoes. Soil samples were collected in ziplock bags, while the water samples were collected in sterile jars.

3.3 Enrichment of Cellulolytic Bacteria in Samples

The collected soil and waste water samples were diluted in autoclaved distilled water, up 1.0ml of 10^5 times dilutions were used to inoculate Nutrient broth containing 5.0g/l carboxymethyl cellulose in a 250ml Erlenmeyer flask and incubated at 37ºC for 24-48 hrs. The media was sterilized by autoclaving at 121ºC and 15psi for 20 minutes. Medium pH was adjusted before autoclaving with 1N HCl or 1N NaOH.

3.4 Isolation of Cellulolytic Bacteria

After incubation the culture broth from enrichment culture medium was spread on CMC agar plates containing CMC as the sole carbon source. This was done to isolate cellulose degrading bacteria which could use CMC as the sole carbon source. CMC medium after Ariffin et al, (2006) was used which contained 10.0g/l CMC, 10.0g/l NaCl, 4.0g/l KH2PO4, 4.0/l K2HPO4, 1.4g/l MgSO4 and 3.0g/l NH4Cl. The plates were incubated at 37ºC for 24 to 48 hours, under microaerophilic conditions in an anaerobic jar candle jar which was purchased from Oxoid.

Pure cultures were isolated from the colonies growing on the selective medium by streaking further on to new plates. Pure culture was maintained done by refreshing the cultures every 2 weeks on new cellulose agar plates. All cultures were cultivated under oxygen limited conditions using anaerobic jar.

3.5 Congo Red Test

The confirmation of cellulose degradation from isolated bacterial strains was performed by the congo red test for cellulose (Teather & Wood, 1982). The plates were stained with 0.01% of congo red and allowed to stand for 30 minutes at room temperature. After decanting, the plates of congo red were flooded with a solution of 1.0N NaCl and further allowed to stand for 15 minutes at room temperature. Flooding with NaCl was done to wash the excess stain on the CMC agar plate. The CMC agar was stained red and the zone of hydrolysis of CMC appeared yellow on a red background.

3.6 Batch Fermentation

3.6.1 Inoculum Preparation

Inoculum was prepared for the fermentation by preparing 100 mL of 0.5% cellulose media as described earlier (section 3.4) in a 250mL Erlenmeyer flask and inoculating the media with a single bacterial colony picked from the CMC agar plate. The inoculum was prepared aerobically. An overnight incubated inoculum was used.

3.6.2 Batch Fermentation

The fermentation experiments were carried out in 250mL Erlenmeyer flasks containing 230mL of cellulose medium whose composition was 5.0g/l CMC, 10.0g/l NaCl, 4.0g/l KH_2PO_4, 4.0/l K_2HPO_4, 1.4g/l $MgSO_4$ and 3.0g/l NH_4Cl. The medium was inoculated with 1% (v/v) inoculum in a pre-warmed media and rubber stopper were used to close the flasks. Anaerobic conditions were achieved in a short while as the inoculated bacteria consumed the residual oxygen in the headspace of the flasks (Ramos *et al.*, 2000). The flasks were placed in the anaerobic jar for the conditions to be microaerophilic. The flasks were incubated at 37ºC under anaerobic conditions for an incubated for a period of 120 hours. Samples were drawn aerobically after every 24 hours. The isolated strains were checked for ethanol production, reducing sugar production and cell growth

Samples for the investigation of reducing sugars and ethanol production were centrifuged at 5000Xg for 10 minutes in Sigma 90616 microcentrifuge to remove cells and the supernatant was used to analyze the target products. Samples for investigating cell growth were used as such.

3.7 Analysis of the Fermentation Samples

3.7.1 Cell Growth

Cell growth was monitored by collecting 1.0mL of broth sample and taking absorbance OD_{600}. Absorbance studies were done using an Optima (SP-300) spectrophotometer.

3.7.2 Ethanol Production

For qualitative analysis of ethanol production through batch fermentation. potassium dichromate test and HPLC analysis was performed.

3.7.2.1 The Potassium Dichromate Test

The potassium dichromate test was and was used for the initial detection of ethanol (Bennett, 1971). Acidified potassium dichromate reagent was prepared according to by dissolving 7.5g of $K_2Cr_2O_7$ in dilute (6N) H_2SO_4 and the volume was raised to 250mL with distilled water. The reaction mixture consisted of 1mL of sample and 2mL of potassium dichromate reagent. The reaction mixture was boiled in a water bath for 10 minutes and allowed to cool at room temperature followed by taking absorbance at 590nm using an Optima (SP-300) spectrophotometer.

The dichromate test was used because of easily detectable color change for alcohol detection. Potassium dichromate reacts with alcohols during which Cr(VI) is reduced to Cr(III) (Rasmussen *et al.*) producing a sea-green to blue coloration. The alcohol in the reaction is oxidized to the corresponding aldehyde (Zimmermann, 1963).

$$K_2Cr_2O_7 + H_2SO_4 + R_2C\text{-}OH \longrightarrow R\text{-}COH + K_2SO_4 + Cr_2(SO_4)_3 + H_2O$$

3.7.2.2 HPLC Analysis

The broth samples were subjected to HPLC analysis for qualitative confirmation of ethanol production. The broth samples were taken after confirmation of product synthesis by the potassium dichromate test. A 1.0mL sample was extracted in a 1.5mL eppendorff tube and centrifuged at 5000Xg for 10 minutes. The supernatant was filtered using a 0.20µm syringe filter and used as the test sample for HPLC analysis. The HPLC equipment used was the Agilent 1260 equipped with a C18 reversed phase column. The mobile phase used was 100% acetonitrile for the entire run time. The run time was 30 minutes, with an injection volume of 20uL and a flow rate of 0.5ml/min (McFeeters *et al.*, 1984).

3.7.2.3 Quantitative Test for Ethanol Production

A standard curve of ethanol concentration was prepared using $K_2Cr_2O_7$, with different ethanol concentrations. Ethanol dilutions of 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55 and 0.6mg/ml, were made. The method employed was the same as described earlier. 1ml of ethanol standard dilution was added to 2ml of potassium dichromate reagent. The reaction mixture was boiled in a water bath for 10 minutes and allowed to cool at room temperature. After this, absorbance was noted at 590nm after setting the blank with distilled water.

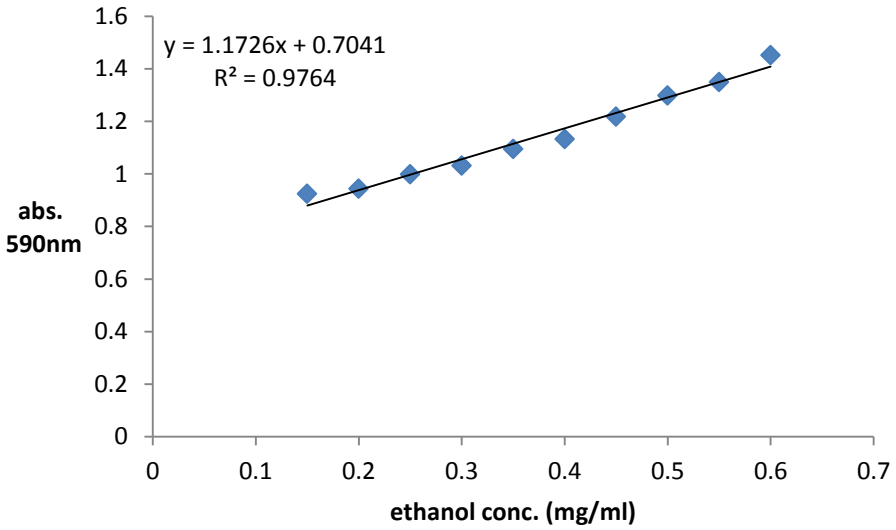


Figure 3.7.2.2: Standard curve for Ethanol using potassium dichromate

3.7.3 Estimation of Reducing Sugars

The reducing sugars were estimated using the DNS reagent (Miller, 1959), for the evaluation of cellulose utilization rate by the isolated bacterial strains. The reducing sugar concentrations are also used to model the metabolic pathway in section (3.11)

3.7.3.1 Dinitrosalicylic Acid Reagent Preparation

DNS reagent test was used to measure the reducing sugar quantity in the fermentation broth after Miller (1959).

The DNS reagent was prepared by dissolving 20.0g NaOH in 200 mL of distilled water. After dissolution, 10.0g of 3,5-dinitrosalicylic acid was added to the solution and stirred on hot plate equipped with a magnetic stirrer. Also added 2.0g phenol and 0.5g sodium sulfite into this solution. After the dissolution of all the

components, the final volume was raised to 1.0L with distilled water. The reagent was stored in an amber colored bottle in a dark place.

A 40% solution of sodium-potassium tartrate was also prepared by dissolving 40.0g of the salt in 80.0mL of distilled water and the final volume was raised to 100.0mL. The Na-K-Tartrate solution is added to the DNS reagent for the color stabilization after color has developed after heating as described in the method (section 3.7.3.2).

3.7.3.2 Measurement of Reducing Sugars

The amount of reducing sugars released during the fermentation process was measured using DNS reagent after Miller (1959). The reducing sugars were measured using a glucose standard curve. Dilution of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9mg/mL were made. Each reaction mixture consisted of 0.1ml glucose standard and 0.4mL DNS reagent. The reaction mixture was heated in a boiling water bath at 90.0ºC for 15 minutes for color development. After this, immediately 1.0mL of Na-K-Tartrate solution was added to the reaction mixture before the cooling of the tubes. After cooling each sample at room temperature, the absorbance was measured at 550nm.

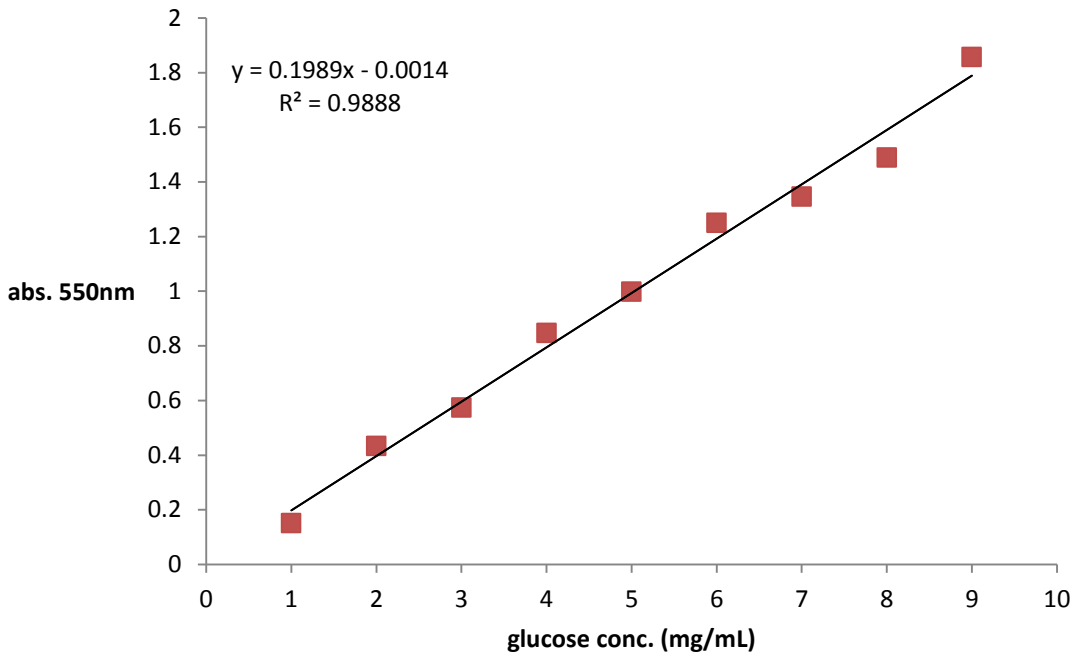


Figure 3.7.3.2: Standard curve for Glucose concentration using DNS reagent

3.8 Optimization of Physical Parameters for Ethanol Production

Physical characterization of the strains HI-W, HI-Lb1 and HI-Lb2 for ethanol production and reducing sugar synthesis was done by optimizing the cultural conditions at different initial substrate concentration, agitation, pH and temperature along with monitoring ethanol production in both static and agitated culture. For optimization studies, batch fermentation experiments were setup in 250ml Erlenmeyer flasks with cellulose medium as previously mentioned (section 3.6.2).

3.8.1 Effect of Substrate Concentration

The isolated strains were cultivated on carboxymethyl cellulose as a sole carbon source as described earlier. To find out the most suitable concentration of CMC, strains were grown on 0.5, 1.0, 1.5 and 2.0% CMC in batch fermentations. The samples were collected after every 24 hours to monitor of the progress of the strains HI-W, HI-Lb1 and HI-Lb2 on different substrate concentrations. The total incubation time was 120 hours, with temperature at 37ºC and pH at 7.0. The batch fermentations were carried out in static conditions the bacterial strains were isolated from mesophilic environments. Methods for estimation of reducing sugars, and cell growth measurement have been discussed earlier (section 3.7.1 and 3.7.3).

3.8.2 Effect of Agitation

The agitated cultures were fermented at 145rpm in a shaking incubator (Heidolph). Samples were drawn after every 24 hours to monitor ethanol production and reducing sugar estimation by the strains HI-W, HI-Lb1 and HI-Lb2 according to the methods described previously (section 3.7.2 and 3.7.3). An incubation temperature of 37ºC and an initial medium pH of 7.0 were used. The incubation time for the batch fermentation was 120 hours. Optimized conditions were used in subsequent experiments to further confirm the optimum temperature and pH.

3.8.3 Effect of Temperature

Effect of temperature was confirmed on the ethanol production by the strains HI-W, HI-Lb1 and HI-Lb2 by incubating the flasks at different temperatures. The temperatures selected were 30, 37 and 45ºC again keeping in mind the possible mesophilic nature of the isolated strains. The fermentations were continued for 120

hour incubation period. Samples were drawn from the fermentation broth after every 24 hours to inquire the ethanol production, cell growth and production of reducing sugars by methods already described. Once optimized, further fermentations were carried out at the determined optimized temperature.

3.8.4 Effect of pH

In order to investigate the effect of initial pH changes on cell growth, and ethanol production, the strains HI-W, HI-Lb1 and HI-Lb2 were incubated at varying initial pH ranging from 5.0-7.0. The temperature was set at 37ºC and the fermentation was performed in static condition. Changes in the cell growth and ethanol production were monitored after every 24 hours up to a 120 hours. Medium pH was adjusted using 1.0N NaOH or 1.0N HCl, depending on the pH of the medium.

3.9 Identification of Isolated Bacterial Strains

The 16s rDNA sequencing was performed by Macrogen Inc. Seoul, Korea. Isolated single bacterial colonies were sent to Macrogen on agar plate. The sequencing was performed following isolation of bacterial gDNA.

16S rRNA gene sequencing was performed for the identification of isolates. The full length gene was amplified from DNA using 27F' (5-AGAGTTTGATCCTGGCTCAG-3') and 1494R' (5'-CTACGGCTACCTTGTTACGA-3') bacterial primers. The 20 µl reaction mixture for PCR consisted of template DNA 1 µl, PCR buffer 2 µl, deoxynucleotide triphosphate (dNTP) mix 2 µl, forward and reverse primer 2 µl each, ex-Taq DNA polymerase 0.5µl and nuclease free water 10.5 µl. At first, the reaction mixture was incubated at 96 ºC for 4 min. Then performed 35 amplification cycles at 94ºC for 45s, 55ºC for 60s, and 72ºC for 60s. Reaction was further incubated for 7 min at 72ºC. DNA fragments are amplified about 1,400bp in the case of bacteria. A positive control (Escherichia coli genomic DNA) and a negative control were included in the PCR. The PCR product was purified using Montage PCR Clean up kit (Millipore) in order to remove unincorporated PCR primers and dNTPs from PCR products. The purified PCR products of approximately 1,498bp were sequenced by using primers, 518F' (5'-CCAGCAGCCGCGGTAATACG-3') and 800R' (5'-TACCAGGGTATCTAATCC-3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied Bio-Systems, USA). Sequencing products were resolved on an Applied

Bio-Systems model 3100 automated DNA sequencing system (Applied Bio-Systems, USA) at the Macrogen, Inc., Seoul, Korea. BLASTn (Zhang *et al.*, 2000) report was provided by Macrogen Inc. Seoul, Korea.

3.10 Computational Modeling

Kinetic modeling of the metabolic pathway for the conversion of cellulose to ethanol was done using the petri net editor Snoopy 2.0. The metabolic pathway was modeled with the help of hybrid petri nets using kinetic rate data and rate constants from literature and then correlating the data previously reported, with the wet lab experiments carried out. Rate constants were determined using rate of substrate utilization and the subsequent production of reducing sugars and the eventual production of ethanol.

3.10.1 Modeling the Cellulose hydrolysis to ethanol pathway

Ethanol production from cellulose proceeds from cellulose hydrolysis which in usual conditions is the production of glucose monomers. These glucose monomeers are released in the medium and are further metabolized through the glycolytic pathway for energy production. Under aerobic conditions, in most bacteria, glucose is metabolized to pyruvate which is further processed into energy yielding molecules. However under anaerobic conditions, the glucose metabolism yields different products which include acids, alcohols and gases. Ethanol is one of the major well known anaerobic fermentation products which is produced during alcoholic fermentation in most microbes, and in hetro-lactic acid fermentation in lactic acid bacteria.

Modeling any metabolic pathway requires for each reaction to be investigated independently. A metabolic reaction is similar to a chemical reaction in many ways. It has a reactant (substrate), a catalyst (enzyme) and a product (Heiner & Koch, 2004). The conversion of any metabolite into a product and the reaction that represents it can be written as an individual reaction. The modeling of the ethanol production from cellulose also comprises of similar individual reactions which can be modeled using petri nets. A single reaction is treated as one complete chemical reaction. Consider the first step of the pathway under consideration which is the ethanol production from cellulose,

Cellulose ⟶ Cello-oligomers

The reaction can be divided into the substrates (left hand side) and the products (right hand side). In the reaction, cellulose is being hydrolyzed by the enzyme endoglucanase (EG) into cello-oligomers. All the substrates and products are represented by places in the petri nets. Places contain values which are known as markings called tokens which represent the amount/concentration of substrate or product. The enzyme participating in the reaction is represented by a two way edge which shows that the enzyme is involved in the reaction but it is not being used up or consumed and regenerates after catalyzing the reaction. This is a static state of the model and the reaction will only take place when the transition is fired or the system is executed. After the execution has been initiated, the model will show the reaction as proceeding and the changes in concentration of the substrates and products appear in the form of markings or in the case of hybrid petri nets, a plotted graph.

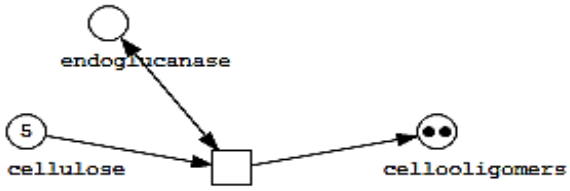


Figure 3.10.1: Representation of a metabolic reaction in petri nets.

3.10.2 Michaelis-Menten Kinetics of Enzyme-Substrate Reaction

Any metabolic reaction which is to take place has all the basic components, which are substrate, enzyme and product. The generalized proceeding of an enzyme catalyzed reaction is represented as,

Enzyme + Substrate ⟶ Enzyme-Substrate Complex ⟶ Product + Enzyme

which implies that before a substrate can be converted into a product, an enzyme substrate complex is formed and the enzymes interacts with its substrate in a way which is necessary for product formation. This reaction mechanism can be represented by a petri net as follows,

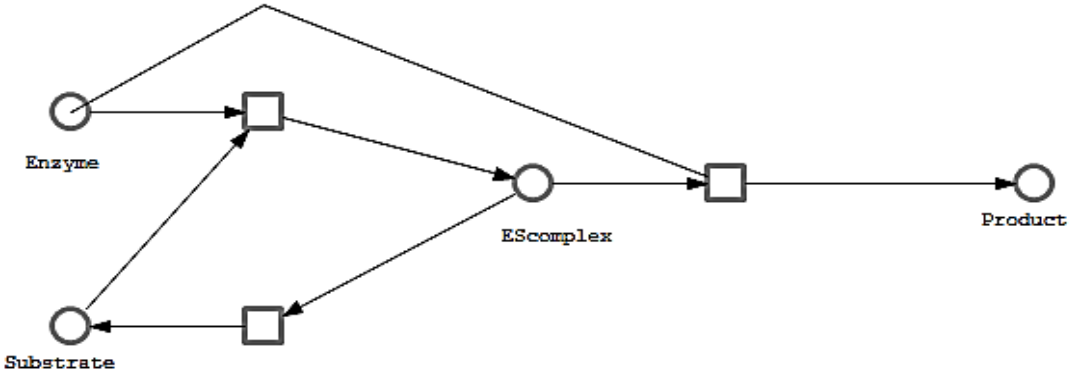


Figure 3.10.2.1: A typical enzyme catalyzed reaction as depicted in petri net

The figure shows the possible interactions between enzyme, substrate, transition states, and the enzyme-substrate complex to form the product.

This complex interaction can be simplified if we use the Michaelis-Menten kinetics according to which if the substrate concentration is appropriate, then the then the conversion of a substrate to product can be written as,

$$V = V_{max}[S]/[S] + K_m$$

The equation can be further simplified if we assume that the substrate concentration and the enzyme concentration are the same, so it can be said that the amount of substrate equals the amount of product. This equation of a chemical reaction will have the same representation as shown in figure 1, with places representing substrate, enzyme and product, and the transition representing the velocity with which the reaction is taking place (Matsuno *et al.*, 2003).

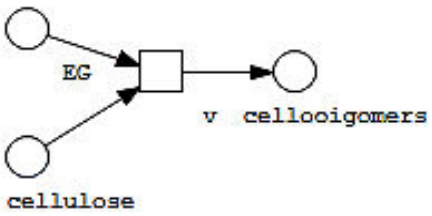


Figure 3.10.2.2: Simplified form of enzyme catalyzed reaction as represented by Michaelis-Menten kinetics.

3.10.3 Modeled Pathway for Ethanol Production in *Lactobacillus sp.*

The pathway for ethanol production in *Lactobacilus sp.* as modeled in petri net editor Snoopy 2.0 is shown in Figure 3.10.3 and was modeled based on the phosphoketolase pathway proposed by DeMoss (DeMoss *et al.*, 1951) which is shown literature review (section 2.6.1). The CMC degradation depiction is according to Kim

and Dale (2004). The phosphosketolase pathway is known for the enzyme Phosphoketolase that brings about the splitting of pentose phosphate in to glyceraldehyde-3-phosphate (G3P) and acetylphosphate. G3P moves through the glycolytic pathway to produce lactic acid through pyruvate. On the other hand acetylphosphate is converted into acetyl-CoA producing acetate and ethanol at the end of the metabolic pathway. All the substrates and enzymes in the pathway were represented by continuous places (circles), transitions (squares) and were inked by arcs (arrows).

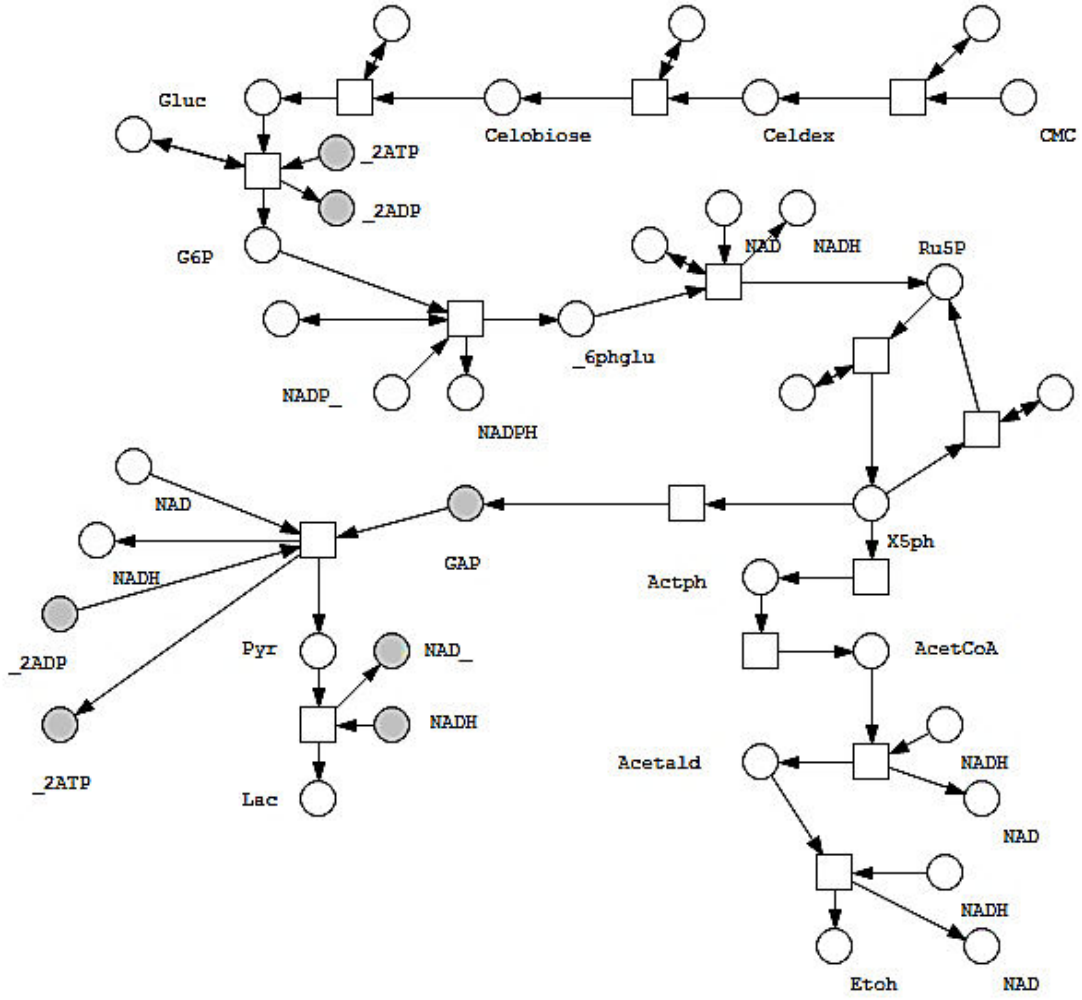


Figure 3.10.3.2: The phosphoketolase pathway, as modeled in petri net editor Snoopy 2.0

Chapter Four

Results

4.1 Isolation of Bacterial Strains

Cellulose degrading bacteria were isolated using culture enrichment and serial dilution technique. The collected samples of soil, food and water were suspended in cellulose medium as described in materials and methods. Serially diluted samples were spread on agar plates containing 1.0g/l carboxymethyl cellulose as a sole carbon source. The growing strains were subjected to the congo red test as mentioned materials and methods (section 3.4). All isolated strains produced a yellow clearing zone on CMC agar plates as a result of congo red staining and subsequent washing with NaCl. The congo red test was performed to confirm the cellulose utilization ability of the isolated strains. As congo red binds to cellulose in the agar plate, it stains the agar red, except for the parts where the cellulose has been degraded by the bacterial cellulase production, which remains yellow (Teather and Wood, 1982). The congo red test was used as a confirmation test as to determine the cellulase production ability of the isolated strains. Congo red test was used to select the isolates that showed a high cellulase activity on CMC agar.

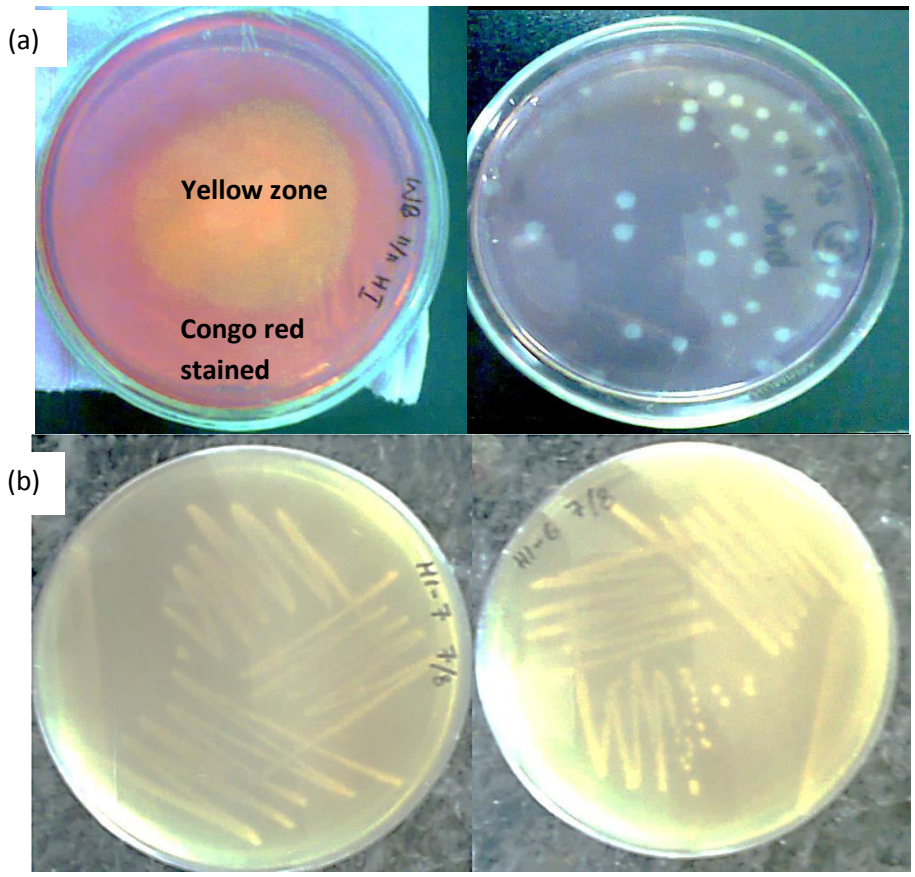


Figure 4.1: Isolated bacterial strains on CMC agar (a) isolated bacterial strains showing positive congo red test for the production cellulase on CMC agar plate. (b) streaked plates for obtaining pure colonies of the isolated strains.

4.2 Batch Fermentation

Batch fermentations were set up for the investigation of the isolated strains for ethanol production. Fermentations were setup using the cellulose medium under anaerobic conditions in 250 ml Erlenmeyer flasks as described in materials and methods (section 3.6.2). Anaerobic conditions were maintained (Ramos *et al.*, 2000). Fermentations were carried out under both static and agitated conditions. The strains were characterized for ethanol production using potassium dichromate test and High Performance Liquid Chromatography (HPLC) analysis, which were both used as qualitative tests. Furthermore, the potassium dichromate test was also used for the quantitative analysis of ethanol production (Zimmermann, 1963).

4.3 Potassium Dichromate as a preliminary test for ethanol production

The samples were drawn from the broth cultures periodically at 24 hour intervals up to a time of 120 hours. The broth samples were centrifuged at 5000Xg for 5 minutes to remove the cells and subjected to the potassium dichromate test. For color development and stabilization, the tubes containing the reaction mixture were incubated in a boiling water bath for 10 minutes, allowed to cool at room temperature and the absorbance was measured at 590nm with an Optima (SP-300) spectrophotometer. The test was performed according to Bennett (1971) as discussed in the materials and methods (section 3.7.2.1.1).

Supernatants from the bacterial cultures that produced a sea-green to blue coloration were taken to produce alcohol, which was an indication towards possible ethanol production. This result was in agreement with the potassium dichromate test performed for the production of ethanol by *Zymomonas mobilis (Deka et al., 2013).*

Out of the eight strains isolated for cellulase production HI-1, HI-2, HI-3, HI-4, HI-5, HI-W, HI-Lb1 and HI-Lb2 on CMC agar medium, three strains HI-W, HI-Lb1, and HI-Lb2 tested positive for ethanol production and were further characterized regarding ethanol production. Optimum, substrate concentration (0.5%, 1.0%, 1.5%, 2.0%), culture conditions agitation and static, temperature (30ºC, 37ºC, 45ºC), and pH (5.0-7.0).

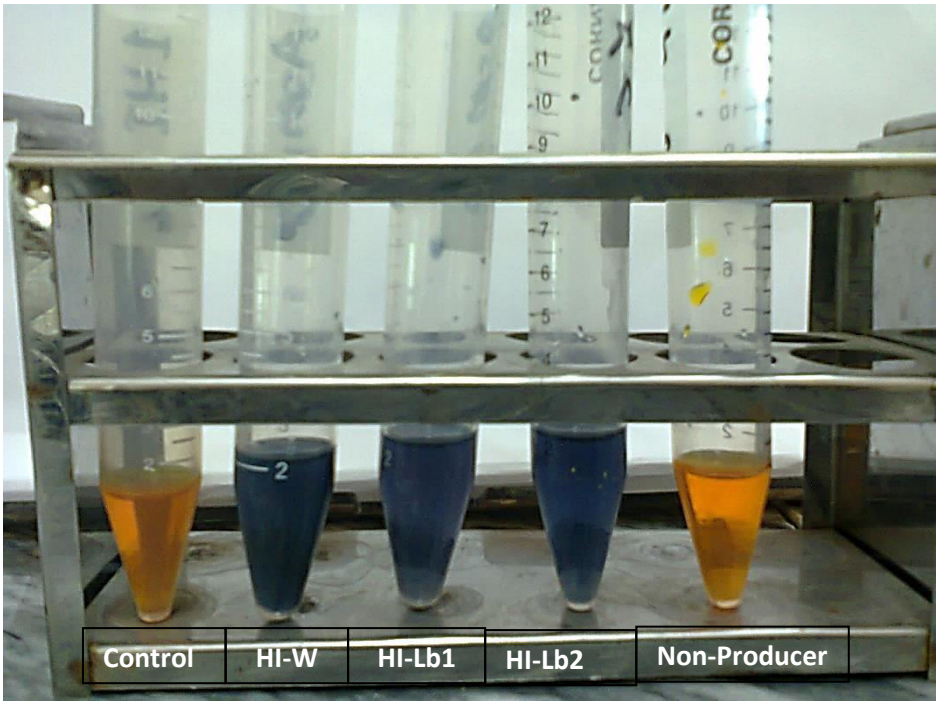


Figure 4.3: Color change with potassium dichromate test by isolated ethanologenic strains

4.4 HPLC determination

HPLC determination of the produced ethanol by the strains HI-W, HI-Lb1 and HI-Lb2 was performed using the Agilent 1260 Infinity series HPLC equipment which was equipped with a C18 column, UV detector, and an auto sampler. Figure 4.4 (a) shows the ethanol standard which was analyzed through High Performance Liquid Chromatography. HPLC grade ethanol was used which was purchased from Reanal Chemicals, Hungry. The standard ethanol showed a retention time of 0.605 min on the RP-C18 column. A low retention time is due to the fact that polar compounds such as ethanol are not retained by the hydrophobic C18 column which was used (Restek Technical Solutions). A mobile phase of 100% acetonitrile was used for a run time of 30 mins with a flow rate of 0.5ml/min. The absorbance of the UV detector was set at 210nm which is the λ_{max} for ethanol. Figure 4.4 (b) and (c) show the subsequent analysis of the sample broth through HPLC using the conditions as described above. For HPLC analysis, the samples were drawn after every 24 hours from the batch fermentations, the cells were centrifuged at 5000Xg using a microcentrifuge (Sigma 96061) for 10 minutes. The samples show a retention time of 0.618min and 0.610min which is slightly more than the standard as shown in Figures 4.4(b) and (c). This can be due to the fact that the fermentation broth contains multiple metabolites which can interact with the analysis of the ethanol content in the sample. Ethanol can interact with organic acids in the broth sample which can extend the retention time (McFeeters *et al.*, 1984).

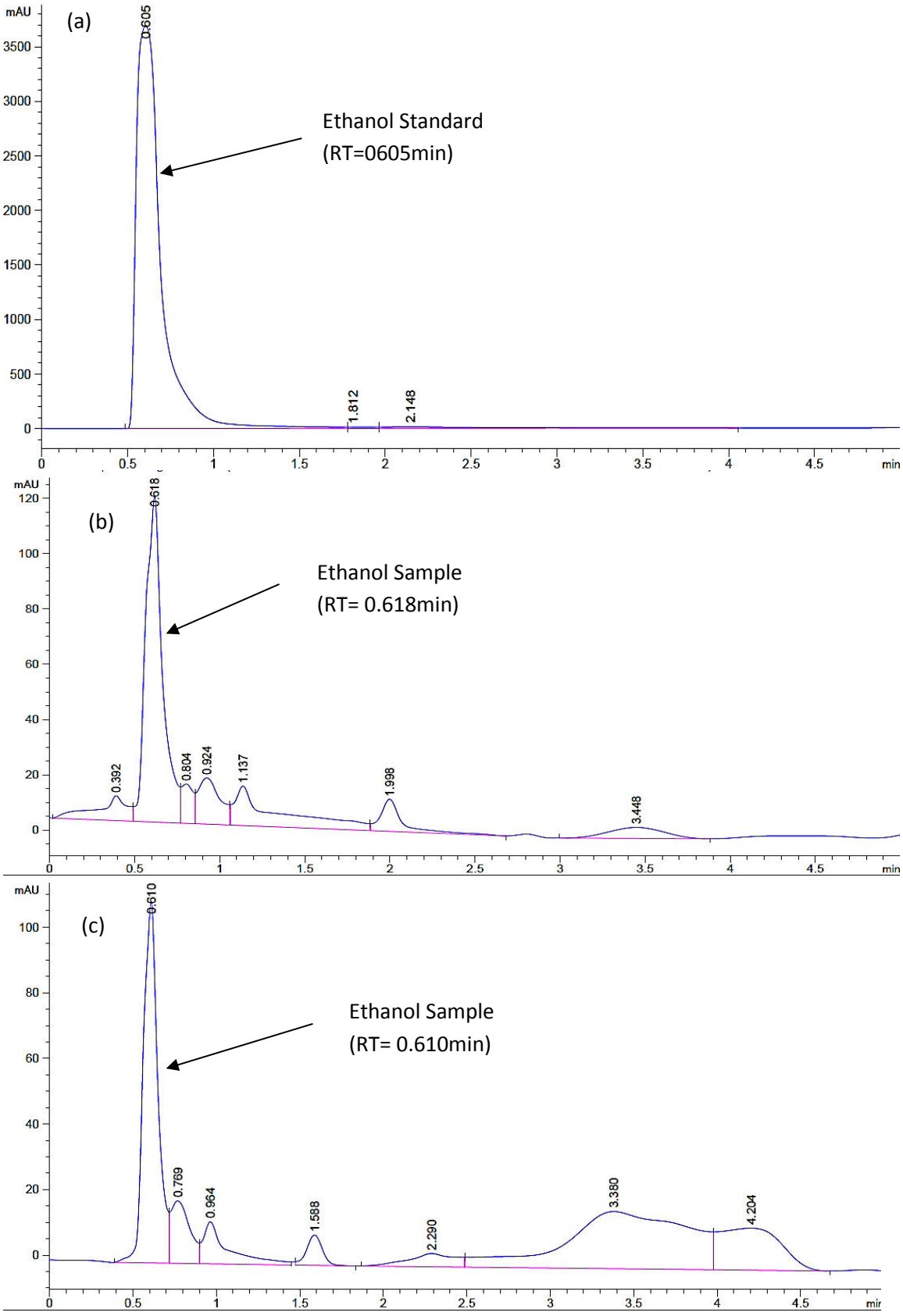


Figure 4.4: Chromatographic analysis of Ethanol (a) standard; retention time (RT) 0.605min(b) sample HI-W; RT 0.618 min (c) sample HI-Lb2; RT 0.610 min. Column C18 Sample size 10µl, UV detection 210nm, Flow rate 0.5ml.min, Run time 30 mins. Mobile Phase 100% Acetonitrile

4.5 16S Identification of the Isolated Bacterial Strains

4.5.1 Sequencing of the 16S rDNA

The bacterial strains HI-W, HI-Lb1 and HI-Lb2 were identified using 16S rDNA universal primers using 27F' (5-AGAGTTTGATCCTGGCTCAG-3') and 1494R' (5'-CTACGGCTACCTTGTTACGA-3') for 16S rRNA gene amplification and subsequent sequencing using universal primers 518F' (5'-CCAGCAGCCGCGGTAATACG-3') and 800R' (5'-TACCAGGGTATCTAATCC-3') at Macrogen Inc. Seoul, Korea as described in materials and methods (section 3.9).

The PCR products for the strains HI-2, HI-3 and HI-Lb2, are shown in Figure 4.5 (a) in lane 1, 2 and 3 respectively. The PCR products are run along with a 3Kb DNA ladder in lane 6. It can be seen that the bands of the PCR products lie in the range of approximately 1400bp which is the length of the 16S rRNA gene.

Similar results can be observed in Figure 4.5 (b) where the amplified 16S rRNA gene from the strain HI-W has been amplified and run on gel along with the 3Kb DNA ladder at Macrogen Inc. Seoul, Korea. The band for HI-W also lies in the 1400bp region.

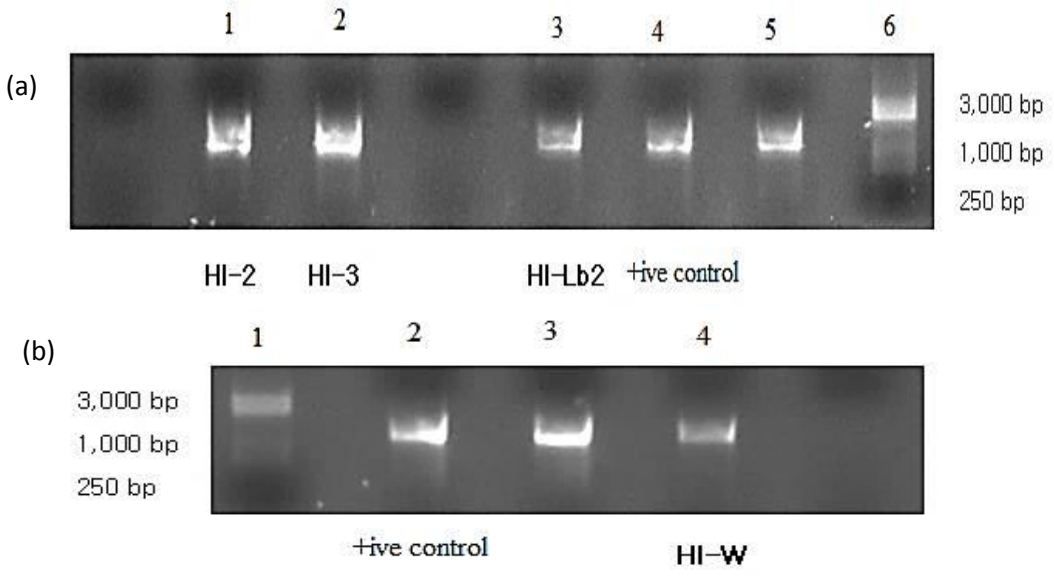


Figure 4.5: Amplification of 16S rDNA. (a) and (b) Gel showing the bands for of 16S rDNA; bands for HI-W and HI-Lb2 are at approximately 1400bp mark (Source: Macrogen Inc. Korea). A positive control, (*Escherichia coli*) run along with the samples.

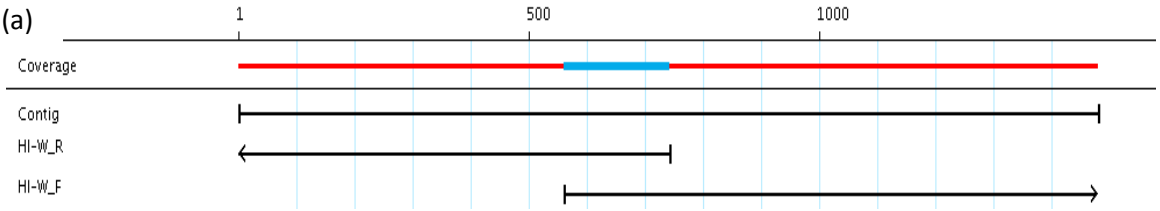
4.5.2 Blast n

Nucleotide BLAST (BLASTn) report was provided by Macrogen Inc. Seoul, Korea according to Zhang et al. (2000).

4.5.2.1 HI-W

Strain HI-W showed the highest hit with *Lactococcus lactis subsp. lactis* (gb#CP006766.1) with a bit score of 2713 and an E value of 0.0 showing a very high level of similarity to the strain. The contig align is shown in Figure 4.5.2.1 (a) which shows the overlapping region of the extension resulting from the reverse and forward primer extension. Figure 4.5.2.1(b) shows the read length and the quality of the read length, Q20 indicating the number of bases having a 99% accuracy and shows the GC content of the individual primer extended sequences as well as for the aligned contig which has a GC content of 51.68% in case of HI-W. Figure 4.5.2.1 (c) shows the top ten BLASTn results. Strain HI-W shows the most hits with *Lactococcus lactis subsp. lactis*. Thus it can be concluded that strain HI-W is *Lactococcus lactis* as it shows 99% similarity.

(a)



(b)

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
HI-W_contig_1	1480	1396	1394	51.689189189189186
HI-W_F	919	901	886	52.01305767138194
HI-W_R	742	732	732	50.673854447439346

(c)

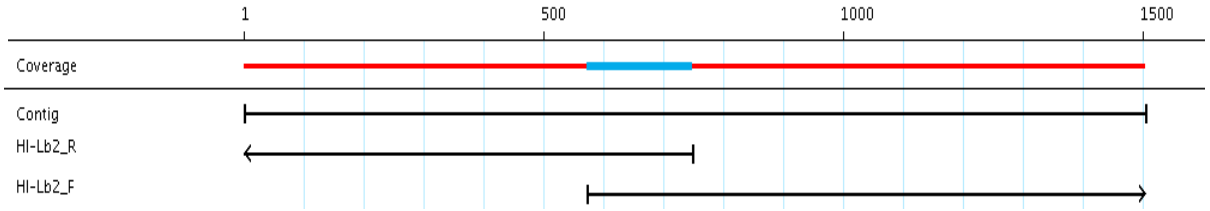
Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
5	1480	Lactococcus lactis subsp. lactis KLDS 4.0325, complete genome	CP006766.1	2589250	559142	560615	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis strain KG-8 16S ribosomal RNA gene, partial sequence	KF263161.1	1483	1	1474	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis Il1403 strain IL1403 16S ribosomal RNA, complete sequence	NR_103918.1	1548	25	1498	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Uncultured bacterium clone MY 88 16S ribosomal RNA gene, partial sequence	JN245705.1	1511	17	1490	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus sp. Cd31 gene for 16S rRNA, partial sequence	AB673464.1	1483	3	1476	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis strain YF11 16S ribosomal RNA gene, partial sequence	JQ364952.1	1536	17	1490	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis CV56, complete genome	CP002365.1	2399458	1963730	1962257	2713	1469	0.0	1474	1476	99	Plus/Minus
5	1480	Lactococcus lactis subsp. lactis strain NM53-4 16S ribosomal RNA gene, partial sequence	HM218236.1	1489	5	1478	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis KF147, complete genome	CP001834.1	2598144	529845	531318	2713	1469	0.0	1474	1476	99	Plus/Plus
5	1480	Lactococcus lactis subsp. lactis Il1403, complete genome	AE005176.1	2365589	537585	539058	2713	1469	0.0	1474	1476	99	Plus/Plus

Figure 4.5.2.1: BLASTn results for HI-W (a) Contig alignment according to the forward and reverse primer extensions of the 16S rDNA (b) Contig properties as compared to the forward and reverse primers (c) Top ten comparabe sequences obtained through BLASTn Source: Macrogen Inc., Seoul, Korea)

4.5.2.2 HI-Lb2

Strain HI-Lb2 showed the highest hit with *Lactobacillus pentosus* strain *IHB 6854* (gb#KF668473.1) with a bit score of 2747 and E value 0.0 showing a high degree of similarity. The contig align is shown in Figure 4.5.2.2 (a) which shows the overlapping region of the extension resulting from the reverse and forward primer extension. Figure 4.5.2.2(b) shows the read length and the quality of the read length, Q20 indicating the number of bases having a 99% accuracy and shows the GC content of the individual primer extended sequences as well as for the aligned contig which has a GC content of 51.26% in case of HI-W. Figure 4.5.2.1 (c) shows the top ten BLASTn results. Strain HI-W shows the most hits with *Lactococcus lactis subsp. lactis*. Thus it can be concluded that strain HI-W is *Lactococcus lactis* as it also shows 99% similarity.

(a)



(b)

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
HI-Lb2_contig_1	1504	1421	1419	51.26329787234043
HI-Lb2_F	931	924	920	51.66487647690655
HI-Lb2_R	748	744	741	50.668449197860966

(c)

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
11	1504	Lactobacillus pentosus strain IHB B 6854 16S ribosomal RNA gene, partial sequence	KF668473.1	1534	16	1507	2747	1487	0.0	1492	1494	99	Plus/Plus
12	1504	Lactobacillus plantarum strain H11 16S ribosomal RNA gene, partial sequence	KC550299.1	1519	1	1493	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus sp. T3R2C13 16S ribosomal RNA gene, partial sequence	JX193626.1	1531	16	1507	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum subsp. plantarum gene for 16S rRNA, partial sequence, strain: Ni729	AB601179.1	1525	16	1507	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum subsp. plantarum gene for 16S rRNA, partial sequence, strain: Ni1325	AB598974.1	1529	17	1508	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum WCFS1 complete genome	AL935263.2	3308273	1169186	1170677	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: NRIC 1954	AB362768.1	1550	18	1509	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: NRIC 1838	AB362759.1	1561	18	1509	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: NRIC 1832	AB362753.1	1554	11	1502	2747	1487	0.0	1492	1494	99	Plus/Plus
11	1504	Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: NRIC 1737	AB362738.1	1538	18	1509	2747	1487	0.0	1492	1494	99	Plus/Plus

Figure 4.5.2.2: BLASTn results for HI-Lb2 (a) Contig alignment according to the forward and reverse primer extensions of the 16s rDNA (b) Contig properties as compared to the forward and reverse primers (c) Top ten comparabe sequences obtained through BLASTn (Source Macrogen Inc. Seoul, Korea)

4.6 Optimization of Physical Parameters for Ethanol Production

Characterization of the isolated ethanol producing strains was done using batch fermentations on different initial substrate concentrations (0.5%, 1.0%, 1.5%. and 2.0%), culture conditions regarding agitation and static fermentations, effect of medium pH (5.0-7.0), and incubation temperature (30º, 37º and 45ºC). The above mention parameters were selected to optimize condition for increasing the bacterial ethanol yield

4.6.1 Effect of Initial Substrate Concentration

The inoculum for each isolated strain was prepared in 100.0mL of cellulose medium as described in materials and methods (section 3.6.1). The media for batch fermentation for all three strains were inoculated with 1% (v/v) of one day old inoculum (OD_{600} 0.5). The batch fermentations were monitored for cell growth at and reducing sugar production for the determination of optimized initial substrate concentration for the isolated strains HI-W, HI-LB1 and HI-LB2 grown on 0.5, 1.0, 1.5 and 2.0% CMC. Samples were drawn at 24 hours up to a time of 120 hours and the samples analyzed for production of reducing sugars and cell growth by methods described in materials and methods (section 3.6.2).

For analysis of reducing sugars, samples were drawn from the broth cultures at 24 hour intervals, cells centrifuged at 5000xg for 10 minutes and the supernatant was subjected to the dinitrosaicylic acid reagent test (Miller, 1959) for the estimation of reducing sugars produced over the five day incubation period as discussed in materials and methods (section 3.7.3).

The trends in cell growth and reducing sugar production among the strains HI-W, HI-Lb1, and HI-Lb2 are shown in Figure 4.6.1(a). At a substrate concentration of 0.5%, strain HI-W (*Lactococcus lactis*) showed the highest growth rate along with the highest amount of reducing sugars (2.1mg/ml) at 48hours of incubation time. Strains HI-Lb1 and HI-Lb2 (*Lactobacillus pentosus*) showed the same pattern i.e. an increase in growth rate along with an increase in production of reducing sugar of 1.8mg/ml for 1.9mg/ml respectively during incubation of 48hrs. After 48 hours, the amount of measurable reducing sugars in the supernatant started declining for strains HI-W and HI-Lb2 however strain HI-Lb1 continued releasing reducing sugars into the media even after 48hrs of incubation. The decline in reducing sugar production by strains

HI-W and HI-Lb2 is attributed to the fast cell growth rate and the production of other metabolites in the media (Ortega *et al.*, 2001). Strain HI-Lb1 showed an increase in reducing sugar production (1.8mg/ml) until 72hr incubation. Cell growth for strains HI-W, HI-Lb1 and HI-Lb2 reached a maximum absorbance of 1.157, 0.951 and 1.014 respectively after 96 hours of incubation. After this time, the cell growth declined which may be due to the harmful byproducts accumulation of cellulose degradation such as cellobiose, which is an inhibitor of endoglucanase, responsible for cellulose hydrolysis (Gilkes *et al.*, 1991).

The trends on decrease in sugar production along with cell growth were observed at 1.0% CMC concentration. The observed growth was highest for strain HI-W (OD_{600} 0.964) followed by HI-LB2 (OD_{600} 0.869) and the least was observed for HI-Lb1 (OD_{600} 0.784) as shown in Figure 4.6.1(b). Similarly the highest yield in reducing sugar was observed for HI-W reaching a maximum of 1.7mg/ml during the initial 72 hours whereas train HI-Lb1 showed slow growth on 1.0% CMC, producing a maximum of reducing sugars of 1.3mg/ml after 96 hours. Strain HI-Lb2 showed maximum yield of reducing sugars (1.6mg/ml) after 72 hours of incubation. The yield of reducing sugars produced by all three strains at CMC concentration of 1.0% is not much different from that on a 0.5% CMC concentration, however slow bacterial growth was observed on 1.0% CMC as shown in Figure 4.5.1(b). This shows that the activity of the cellulases produced is better on a lower initial substrate concentration which is in accordance to Liu and Yang (2007), at a higher substrate concentration, the reaction rate of cellulases is decreased due to substrate inhibition.

At a 1.5% substrate concentration, the growth rate for the strains HI-W, HI-Lb1 and HI-Lb2 was even slower than that observed on 1.0% concentration which concludes that an increase in substrate concentration effects bacterial growth rate [Figure 4.6.1(c)]. All three bacterial strains showed less production of reducing sugars comparing to 0.5 and 1.0% CMC concentration [Figure 4.6.1(c)] HI-W, HI-Lb1 and HI-Lb2 showed a maximum production of 1.3mg/ml, 1.1mg/m and 1.2mg/ml of reducing sugars during 72 hour for HI-W and HI-Lb2 and after 96 hours for the strain HI-Lb1 during the batch fermentation. Maximum OD_{600} for strains HI-W, HI-Lb2 and HI-Lb1 observed was 0.814, 0.648 and 0.821 respectively. After 72 hours of incubation, cell growth retarded for the strains HI-W and HI-Lb2. Strain HI-Lb1 showed a decrease in cell growth after 96 hours of incubation. Optimal substrate

concentration is characteristic of any bacterial strain and a higher concentration compared to optimized one decreases the production of cellulases along with the decrease in production of reducing sugars (Singhania *et al.*, 2007).

At 2.0% substrate concentration [Figure 4.6.1 (d)] the lowest cell growth along with the lowest quantity of measurable reducing sugars which may be due to the fact that a higher cellulose concentration results in an increased amount of unfermented cellulose hydrolysis products such as cellobiose, which inhibit the further hydrolysis of cellulose by inhibiting the endoglucanases activity produced by the bacterial strains. In addition to cellobiose, accumulation of glucose in the media inhibits cellobiohydrolase which is responsible for the cleavage of cellobiose into glucose (George *et al.*, 2001).

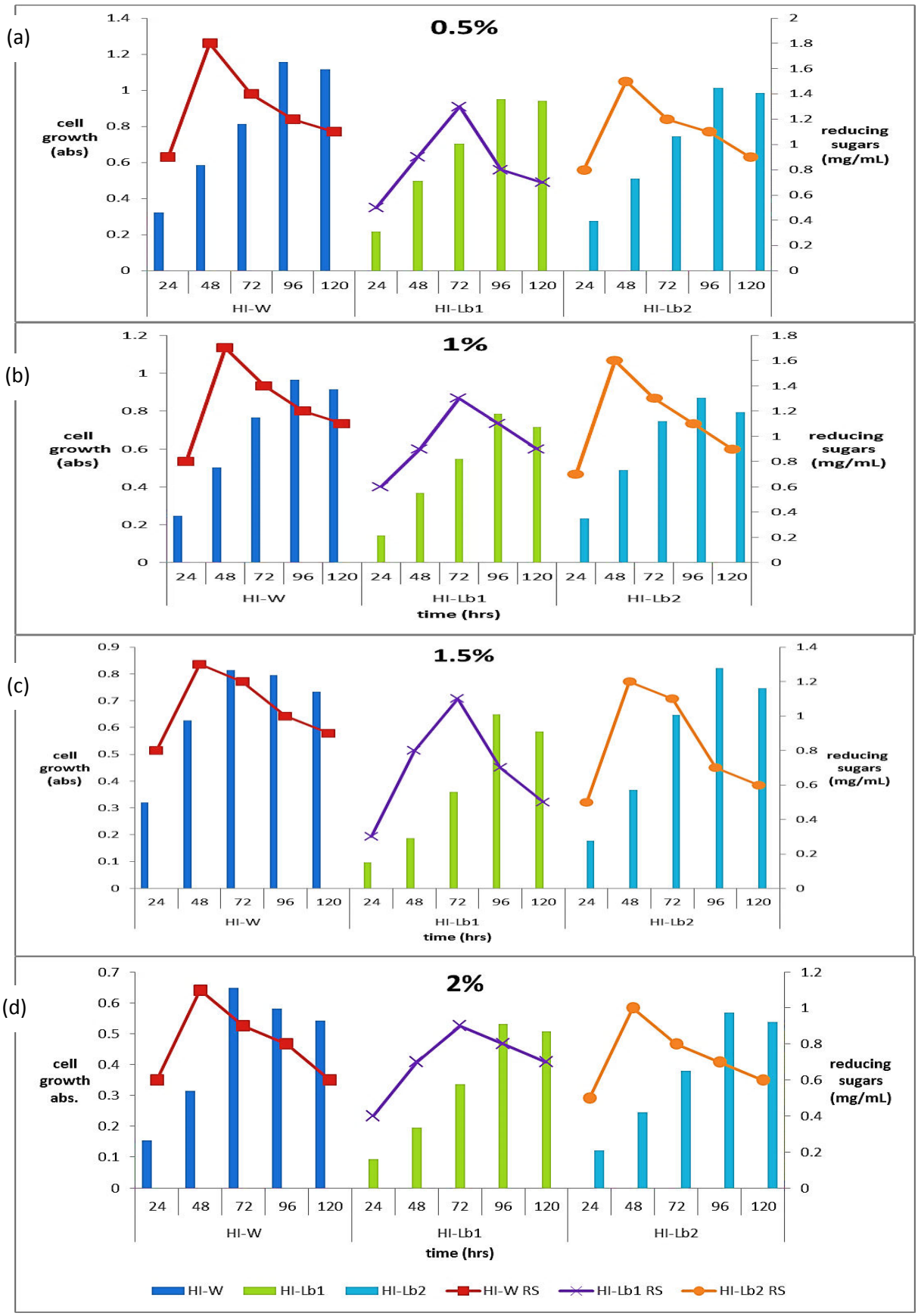


Figure 4.6.1: The optimization of substrate concentration for HI-W, HI-Lb1 and HI-Lb2, (a) 0.5%, (b) 1.0%, (c) 1.5% and (d) 2.0%. The cells were grown at 37ºC, with pH 7.0.

4.6.2 Effect of Agitation

The effect of agitation was assessed by carrying out fermentations at both static and agitated conditions. The static cultures were incubated in a static incubator (Memmert: Incubator I) while the agitated cultures were incubated in a shaking incubator (Heidolph: Incubator 1000) at 145 rpm. The cultures were incubated for 120 hours and samples were drawn to confirm ethanol production through the potassium dichromate method and the concentration of reducing sugars produced using the DNS method as described in the materials and methods (section 3.8.2).

The effect of agitation on the production of reducing sugars is shown by Figure 4.6.2(a). All three strains produce higher amount of reducing sugars in agitated flasks than the static cultures. Strain HI-W showed a production of 2.4mg/ml of reducing sugars as compared to 2.1mg/ml in static cultures whereas strain HI-Lb1 produced 2.1mg/ml and HI-Lb2 produced 2.3mg/ml reducing sugars respectively in agitated cultures as compared to 1.5mg/ml and 1.6mg/ml at static conditions. This may be explained that agitation increases the distribution of medium components and makes available the dissolve oxygen to the bacterial strains (Rajagopalan & Krishnan, 2008). The provision of oxygen and, the distribution of dissolved oxygen is also greater under agitated conditions which increase cellulase production and overall bacterial activity (Singh *et al.*, 2000).

Agitation is an important factor that can affect the ethanol production during the fermentation process. In the current study, agitation at 145 rpm was studied for its effects on bacterial ethanol production and as shown in Figure 4.5.2(b), ethanol production is much higher in static cultures as compared to the agitated ones. Strain HI-W showed the highest ethanol yield of 1.51 mg/mL as compared to ethanol yield during agitated conditions (0.73mg/ml). An increase in ethanol production was observed for strains HI-Lb1 (1.544mg/ml) and HI-Lb2 (1.684mg/ml) in static cultures as compared to agitated cultures (0.549mg/ml and 0.711mg/ml respectively). The diminishing effects on ethanol production inflicted by agitation may be due to the higher oxygen transfer rates to the strains (Cazetta *et al.*, 2007). Agitation may also lead the metabolic pathway away from ethanol production due to oxygenation (Ishikawa *et al.*, 1990).

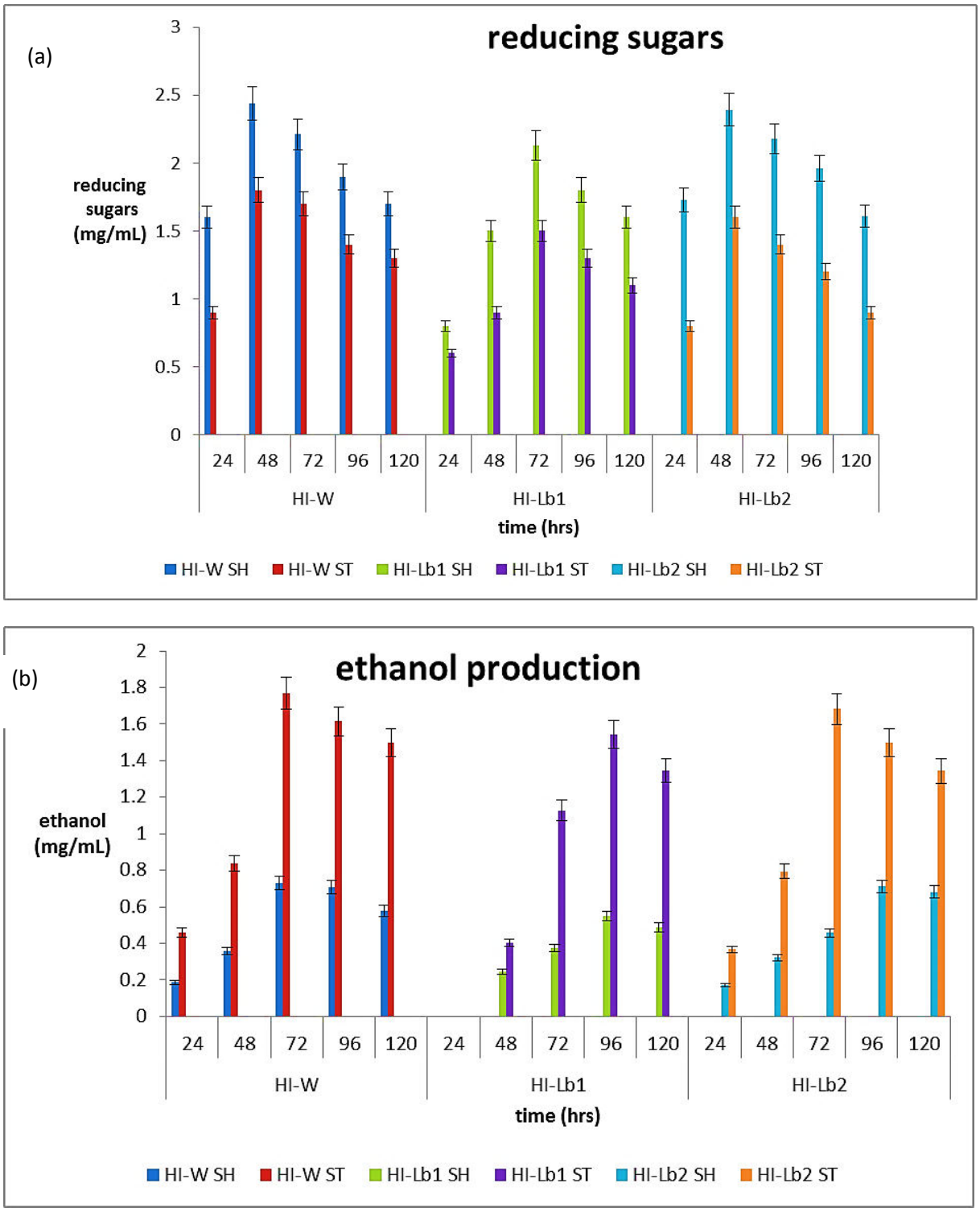


Figure 4.6.2: The comparison of agitated vs static cultures for ethanol production by strains HI-W, HI-Lb1 and HI-Lb2. (a) The comparison of production of reducing sugars (b) The comparison of production of ethanol. Cells were grown at 37ºC, at pH 7.0, 5.0mg/ml CMC.

4.6.3 Effect of different Temperatures

The fermentation for ethanol production was carried out at 30ºC, 37ºC and 45ºC to determine the optimal temperature for cell growth and ethanol production for the strains HI-W, HI-LB1, and HI-LB2. All experiments were carried out at pH 7.0 and the fermentations were setup according to conditions described in materials and methods (section 3.8.3).

At 30ºC, as shown in Figure 4.6.3(a), a considerable cell growth was observed for HI-W and HI-LB2 along with ethanol production of 0.73mg/ml and 0.71mg/ml respectively after 72 hours. The third strain HI-LB1 showed relatively slow growth along with 0.39mg/ml of ethanol production. Cell growth was observed to be increase up to 72 hours for strains HI-W and HI-LB2 and up to 96 hours for HI-Lb-2. This proves that ethanol production is growth associated.

At 37ºC, the cell growth of all the strains HI-W, HI-LB1 and HI-LB2 was associated with ethanol production of 1.51mg/ml, 1.54mg/ml and 1.34mg/ml respectively as shown in Figure 4.5.3 (b). The results are in agreement with those reported by Cazetta et al (2007), as increased cell growth is associated with ethanol production. Ethanol production rate observed for HI-W was (0.02mg/ml/h) and for HI-Lb2 (0.018mg/l/h) for 96 hour incubation. Strain HI-LB1 showed an ethanol production rate of 0.016mg/ml/h. Both ethanol production and cell growth show a rapid decrease as the cells enter the stationary phase which again confirms that ethanol production is growth associated.

Figure 4.5.3 (c) shows the fermentation analysis at 45ºC. A further increase in temperature reduced the cell growth and ethanol production drastically. In the Figure it can be seen that at 45ºC, none of the three strains, HI-W, HI-Lb1, and HI-Lb2 showed a significant growth rate or ethanol production. The ethanol production at the 45ºC reaches 0.31 mg/mL (0.0043mg/ml/h), for HI-W, 0.22 (0.0022mg/ml/h) for HI-LB1 and 0.28 g/L (0.0038mg/ml/h) for HI-LB2. This trend can be attributed to the fact that bacterial ethanol production is greatly affected at temperatures higher than 37ºC (Lee *et al.*, 1981). At temperatures higher than the optimum temperatures, enzymes can be affected adversely and hence the cellulase producing ability of the isolated bacterial strains is also retarded evident by the low growth levels at high temperature (Siripornadulsil *et al.*, 2014).

Significant level of ethanol production was observed at 30ºC, 37ºC, and 45ºC with all three strains. Strains HI-W and HI-Lb2 however showed a high cell growth and ethanol production but at 37ºC, HI-Lb1 shows the highest ethanol production as can be seen by Figure 4.5.3 (b).

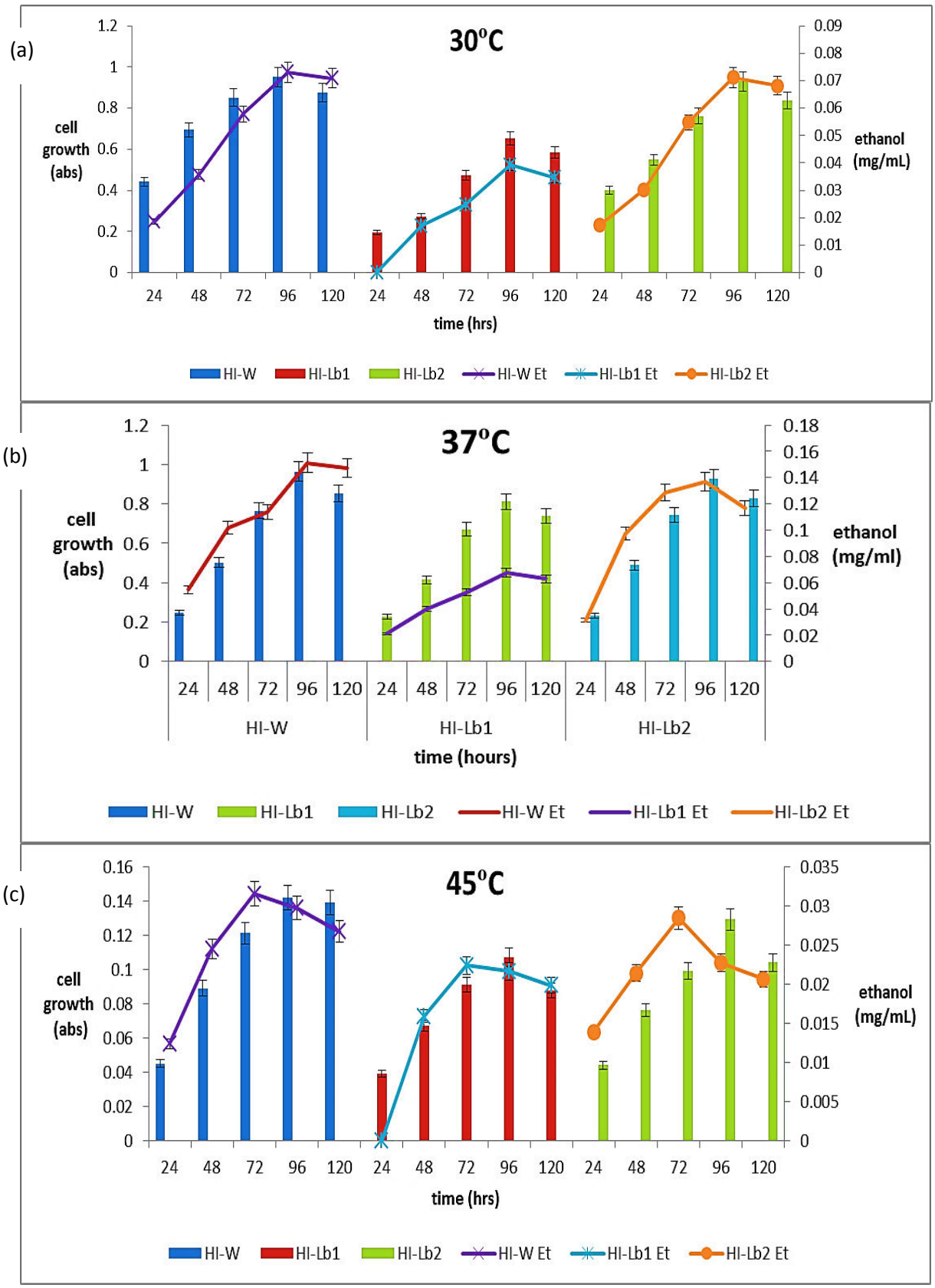


Figure 4.6.3: Ethanol production by HI-W, HI-Lb1 and HI-Lb2 at different temperatures, (a) 30ºC (b) 37ºC and (c) 45ºC. The bar graph shows the cell growth whereas the line graph shows the ethanol production by the strains.

4.6.4 Effect of Different Initial pH

Ethanol fermentation was evaluated at various pH (5.0-7.0) to determine the optimal conditions for ethanol production and cell growth according to methods as described in materials and methods (section 3.8.4). All batch fermentations were carried out at 37ºC at which maximum cell growth and ethanol production were observed.

The batch fermentation carried out at pH 5.0, and temperature 37ºC showed considerable cell growth by the strains HI-W and HI-Lb2 (OD_{600}: 1.015 and 0.874 respectively). Ethanol production was observed with 0.986 and 0.784 for HI-W and HI-Lb2 respectively. The strain HI-Lb1 showed restricted growth with maximum OD_{600} of 0.241 and an ethanol production of 0.63mg/ml as seen from the Figure 4.6.4(a). Strains HI-W and HI-Lb2 show as faster growth rate as compared to HI-LB1. The pH of the medium effected the growth of the HI-Lb1 adversely. Changes in pH of the fermentation medium can affect the production of cellulases and their respective efficiencies (Angsana et al, 2009). Changes in pH also disturb the cellular integrity of the bacteria especially when a lower medium pH is used for fermentations, affecting cell growth (Kashket, 1987).

At pH 6.0 strains HI-W, HI-Lb1 and HI-Lb2 showed better growth as compared to that on pH 5.0 producing ethanol at a concentration of 1.76mg/l, 0.67mg/l and 1.68mg/ respectively with an ethanol production rate of 0.024mg/ml/h, 0.0069mg/ml/h and 0.0233mg/ml/h respectively [4.5.4(b)]. Highest ethanol yield was observed 72 hours of incubation by strains HI-W and HI-Lb2, however in the case of HI-Lb1, highest ethanol yield was observed after 96 hours. Changing pH can have adverse effects on the growth of bacterial cells as well as enzymes produced by these bacteria (Ariffin *et al.*, 2006) (Hu *et al.*, 2004). A higher than optimal pH however affects the 3-D structure of the enzymes due to the charge imbalance created in the medium (Nelson & Cox, 2004).

The experimental setup does not include the effect of pH 7.0 in this section as cell growth, reducing sugar production and ethanol production from all three strains HI-W, HI-Lb1 and HI-Lb2 has already been studied at pH 7.0 in previous sections.

The optimum pH for ethanol production and cell growth was observed at pH 7.0 for HI-Lb1 and pH 6.0 for strains HI-W and HI-Lb2 (Figure 4.6.3).

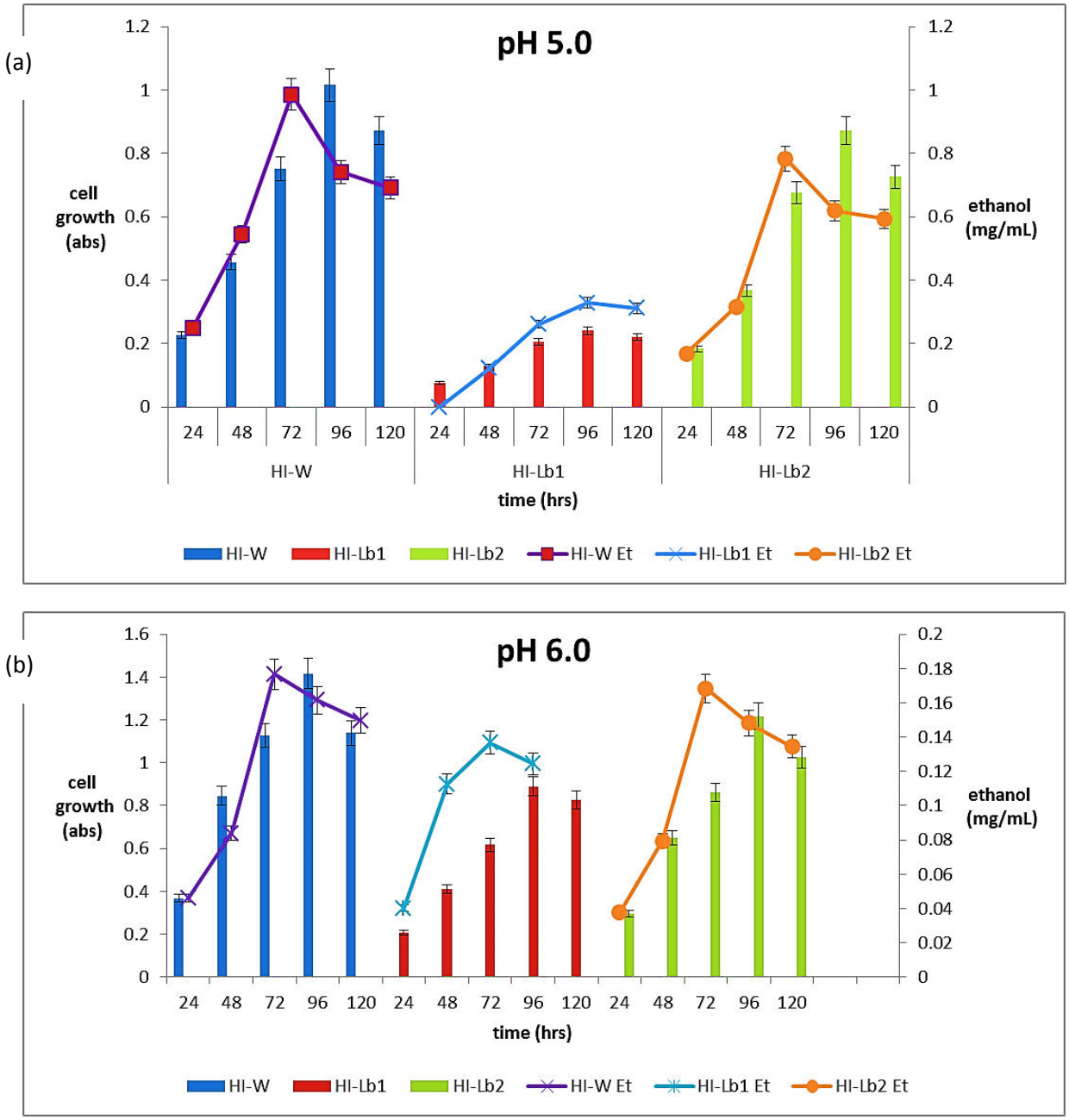


Figure 4.6.4: Strain characterization at different pH (a) 5.0 (b) 6.0 and (c) 7.0. The bar graph shows cell growth while line graph shows the ethanol produced at 37ºC, 0.5% CMC

4.7 Validation through Computational Modeling

The data obtained from the batch fermentations carried out in the wet lab was validated through modeling the pathway using petri nets. The modeling results showed some degree of similarity with the wet lab batch fermentation results for the strains HI-W, HI-Lb1, and HI-Lb2.

4.7.1 At Shaking conditions

The comparative results obtained from the wet lab experiments and the predicted results which were obtained through computational modeling using petri nets are given in Table 4.7.1. The comparative results are discussed for ethanol production and reducing sugar formation which includes both cellobiose and glucose produced as a result of cellulose degradation. Reducing sugars were measured as a corresponding concentration of glucose, and are considered a sum of cellobiose and glucose produced.

As observed from the Table 4.7.1 that the values obtained through the model show a slight increase in ethanol production while a slight decrease in reducing sugar production can be seen for all three strains HI-W, HI-Lb1 and HI-Lb2. Strains HI-W and HI-Lb2 show a similar amount of ethanol and reducing sugars in both the wet lab and the model. The slightly lower ethanol value obtained in the wet lab might be due to the metabolic by products that are produced in the wet lab. The prepared model does not take into consideration the production of side products. In contrast to strains HI-W and HI-Lb2, the strain HI-Lb1 which shows an ethanol production lower than the other two strains, has a higher value of both ethanol and reducing sugars in the predicted model. This is possible due the agitation rate affecting the growth rate and product production adversely in the case of strain HI-Lb1 as has been discussed earlier in section (4.6.2). The predicted values are shown in Table 4.6.1 and the predicted trends for the production of reducing sugars glucose, cellobiose, cellodextrins and ethanol along with the consumption of CMC (cellulose) are shown in Figure (4.6.1). According to both, the wet lab results and the predicted results, strain HI-w shows the highest amount of reducing sugars and ethanol production (4.7.1).

Table 4.7.1: Produced and predicted concentration of reducing sugars and ethanol, under agitation conditions**.**

	Agitation (145rpm)					
	Wet lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugars (mg/ml)	2.4	2.13	2.3	2.06	1.85	2.05
Ethanol (mg/ml)	0.731	0.549	0.711	0.946	1.86	0.929

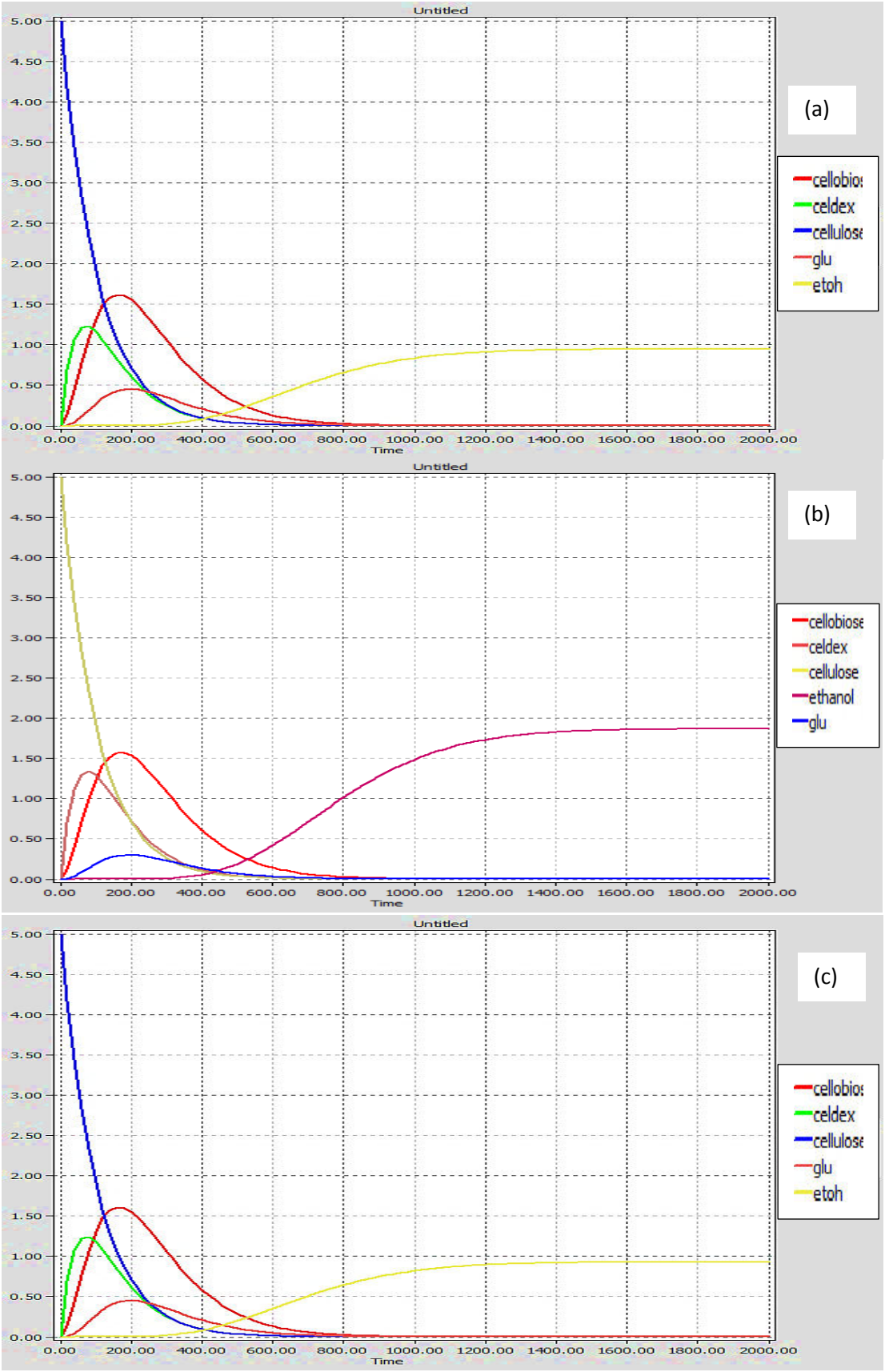


Figure 4.7.1: Petri net computational model showing trends of ethanol and reducing sugar production under agitated conditions for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2.

4.7.2 At Static Conditions

The data obtained from the batch fermentation of ethanol production from cellulose at static conditions was validated using petri net model that was constructed based on the collected data. Table 4.7.2 shows the amount of reducing sugars and ethanol produced during the batch fermentations carried out under static conditions for strains HI-W, HI-Lb1 and HI-Lb2. As observed in the case of agitation conditions, strains HI-W and HI-Lb2 show a correlation between the results obtained in the wet lab and the data predicted using the petri net model. A slight difference is observed in the predicted values and the experimentally obtained data for the strains HI-W and HI-Lb2. Nevertheless, both strains show ethanol production concentrations corresponding to the wet lab data. Table shows the ethanol concentration along with the reducing sugars produced by all strains HI-W, HI-Lb1 and HI-Lb2. Strain HI-Lb1 shows deviation from the predicted results, showing a marked decreased ethanol (1.544 mg/ml) production as compared to the predicted values (3.12 mg/ml). Figure 4.7.2 shows the trends in change in ethanol production and reducing sugar concentration with time.

Table 4.7.2: Produced and predicted concentration of reducing sugars and ethanol, under static conditions.

	Static					
Strain	**Wet lab**			**Model**		
	HI-W	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugars (mg/ml)	1.8	1.5	1.6	1.93	1.71	1.87
Ethanol (mg/ml)	1.514	1.544	1.368	1.487	3.12	1.451

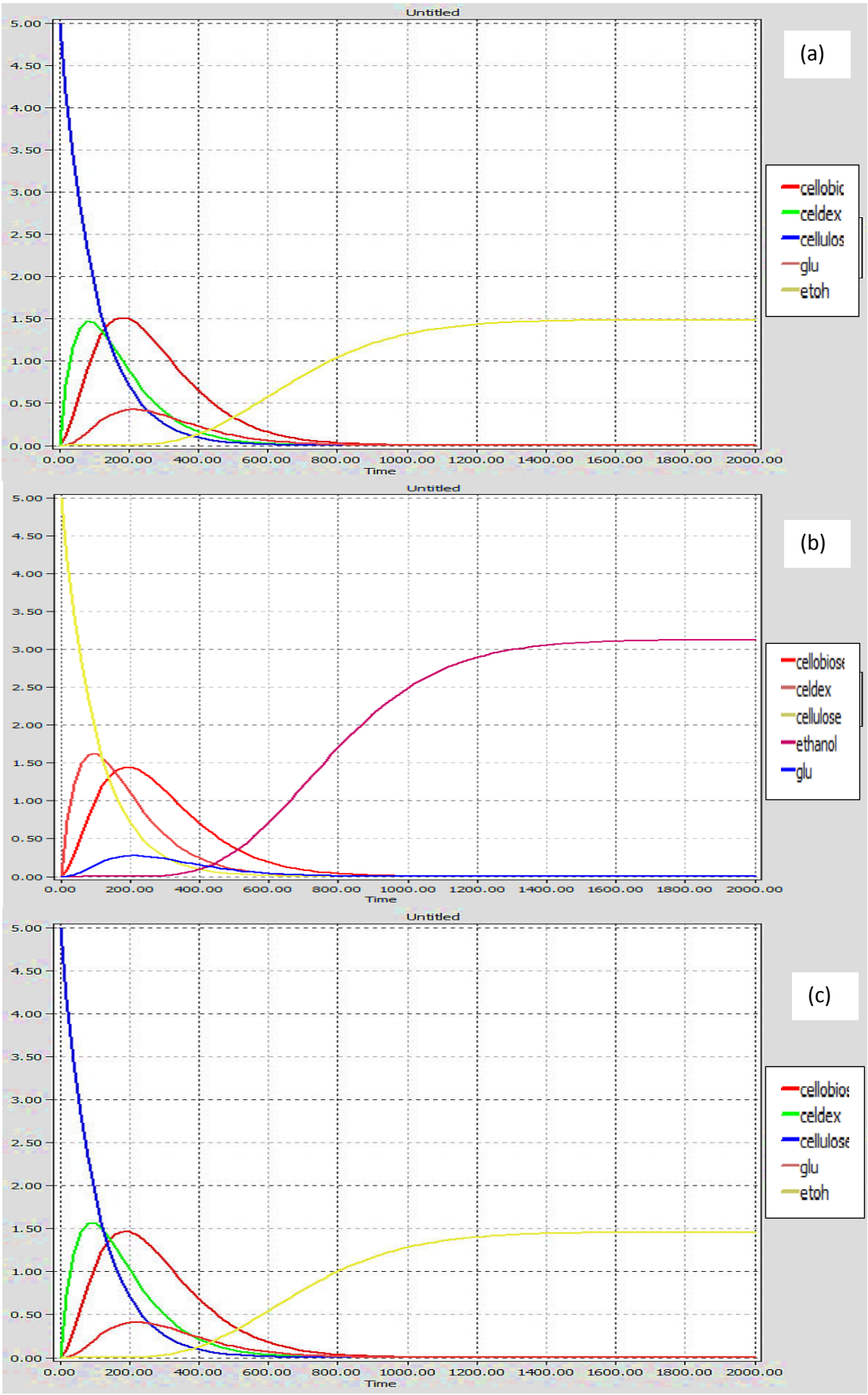


Figure 4.7.2: Trends of ethanol production and reducing sugars concentration under agitated conditions for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2.

4.7.3 At Different Temperatures

At different temperatures i.e. 30, 37, and 45ºC, the production of ethanol and reducing sugars as validated by the model using petri nets is more or less the same as the data obtained in the wet lab batch fermentations.

4.7.3.1 At 30ºC

At 30ºC, strains HI-W and HI-Lb2 show increased ethanol production in the results predicted by the model as compared to the wet lab data as seen in Table 4.7.3.1. As for the reducing sugar production, the values from both sources, the wet lab and model, correlate which validates the experimental procedures performed in the lab. In the wet lab, strain HI-W produced 1.9 mg/ml of reducing sugars as compared to the model prediction of 1.95 mg/m. similarly strain HI-Lb2 produced 1.8 mg/ml in comparison to 1.93 mg/ml predicted by the model. On the other hand, strain HI-Lb1 showed a lower ethanol production of 0.392 mg/ml as compared to the model prediction of 1.49 mg/ml. The reducing sugar production by the strain HI-Lb1 correlated with the predicted values which were 1.7 mg/ml from the wet lab and 1.77 mg/ml from the petri net prediction. Figure 4.6.3.1 shows the trends in the changing concentration over time in the reducing sugar and the ethanol concentration.

Table 4.7.3.1: Produced and predicted concentration of reducing sugars and ethanol, at 30ºC.

Temp. (ºC)	**30ºC**					
	Wet Lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugars (mg/ml)	1.9	1.7	1.8	1.95	1.77	1.93
Ethanol (mg/ml)	0.731	0.392	0.711	0.946	1.49	0.929

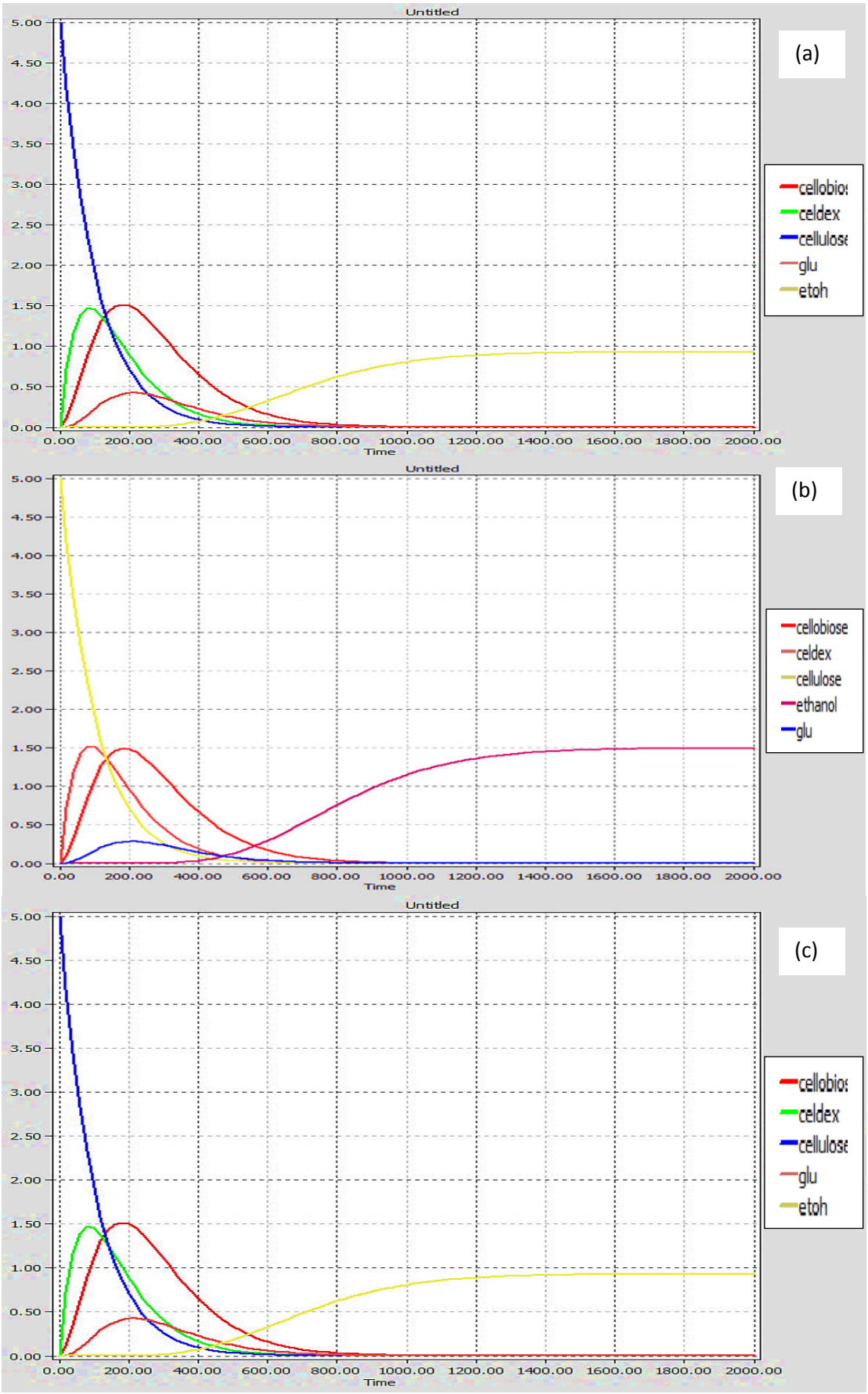


Figure 4.7.3.1: Trends of ethanol production and reducing sugars concentration at 30ºC for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2.

4.7.3.2 At 37ºC

At 37ºC, as shown in Table 4.6.3.2, the strains HI-W, HI-Lb1 and HI-Lb2 showed considerable correlation with the predicted results obtained from the model constructed using petri net editor Snoopy 2.0. Strain HI-W and HI-Lb2 showed a predicted ethanol production of 1.393 mg/ml and 1.33 mg/ml respectively in contrast to 1.514 mg/ml and 1.364 mg/ml by strain HI-W and HI-Lb2. This is in close proximity to the results obtained using the model. The production of reducing sugars also showed a correlation between the predicted results and the actual results obtained in the wet lab. Strain HI-W showed a production of 2.1 mg/ml of reducing sugars while strain HI-Lb2 showed 1.96 mg/ml in comparison to the predicted values of 2.0 mg/ml and 1.96 mg/ml respectively for strain HI-W and HI-Lb2. Strain HI-Lb1 again showed a decreased ethanol production of 1.544 mg/ml as compared to the predicted value of 3.16 mg/ml. however, the reducing sugar concentration measured was in correlation with the predicted value of 1.79 mg/ml against a wet lab value of 1.8 mg/ml. Figure 4.7.3.2 shows the trends in the changing concentration of ethanol and reducing sugars during the 120 hour incubation period.

Table 4.7.3.2: Produced and predicted concentration of reducing sugars and ethanol, at 37ºC.

Temp. (ºC)	**37ºC**					
	Wet Lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugar (mg/ml)	2.1	1.8	1.9	2.0	1.79	1.96
Ethanol (mg/ml)	1.514	1.544	1.368	1.393	3.16	1.33

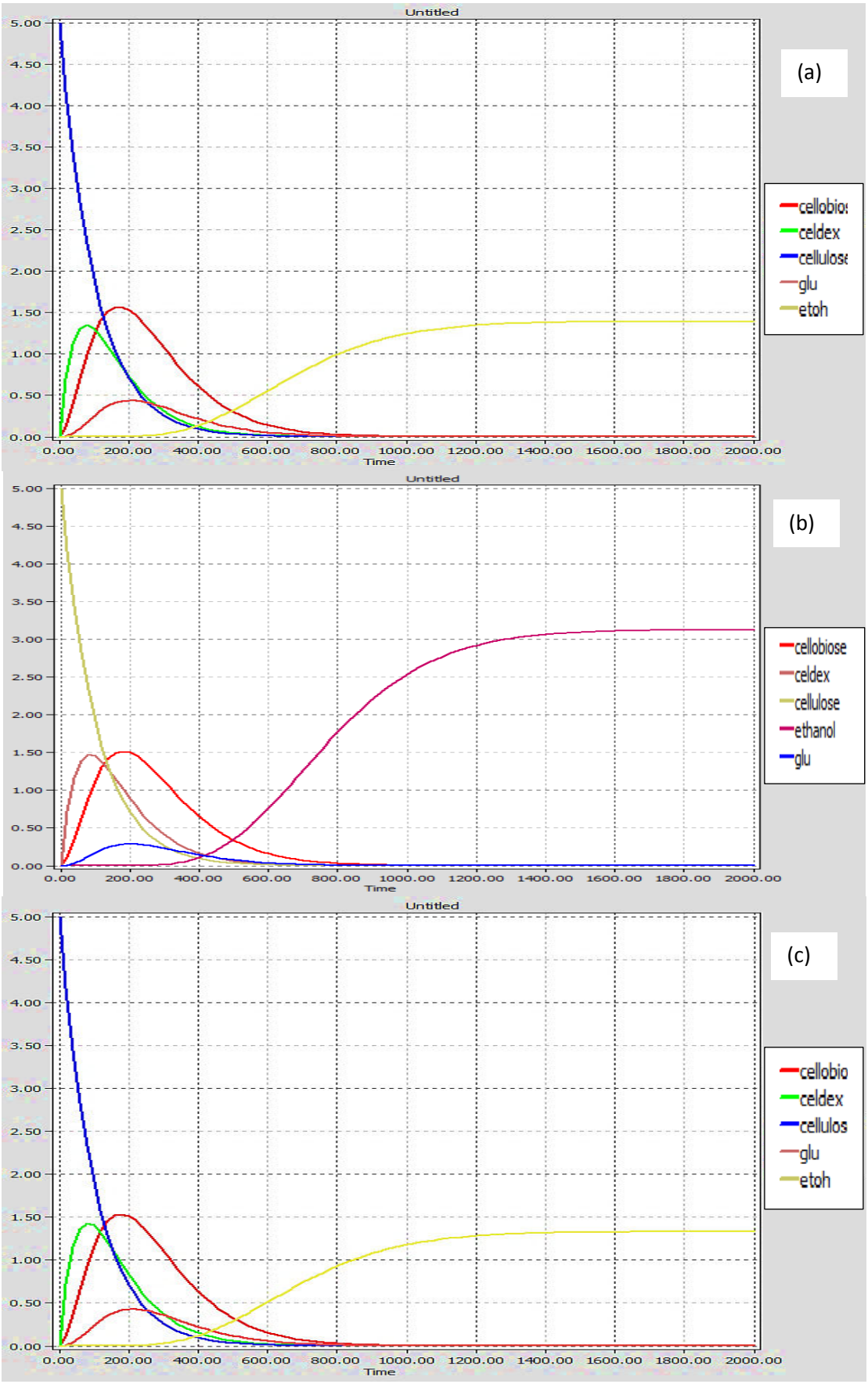


Figure 4.7.3.2: Trends of ethanol production and reducing sugars concentration at 37ºC for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2

4.7.3.3 At 45ºC

Table 4.7.3.3 shows the data obtained from the wet lab and the kinetic model for ethanol and reducing sugar concentration produced by the strains HI-W, HI-Lb1 and HI-Lb2. Regarding the reducing sugar production, all three strains showed comparable results to the values obtained from the model. Strain HI-W produced 1.4 mg/ml to a 1.8 mg/ml predicted value. Strain HI-Lb1 and HI-Lb2 showed a production of 1.2 and 1.3 mg/ml as compared to a predicted value of 1.66 and 1.48 mg/ml respectively. Ethanol production in all the strains was found to be less than the predicted values. The predicted values of 0.518, 0.97 and 0.477 mg/ml for the strains respectively showed slight deviation from the predicted values for strains HI-W and HI-Lb2 which showed 0.315 and 0.284 mg/ml of ethanol in the wet lab. However, the ethanol production by strain HI-Lb1 was far off from the predicted value of 0.97 mg/ml. Figure 4.7.3.3 shows the changing trends with time in the ethanol and reducing sugar concentrations. Reducing sugars are represented by cellobiose and glucose.

Table 4.7.3.3: Produced and predicted concentration of reducing sugars and ethanol, at 45ºC**.**

Temp. (ºC)	**45ºC**					
	Wet Lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugar (mg/ml)	1.4	1.2	1.3	1.80	1.66	1.48
Ethanol (mg/ml)	0.315	0.224	0.284	0.518	0.97	0.477

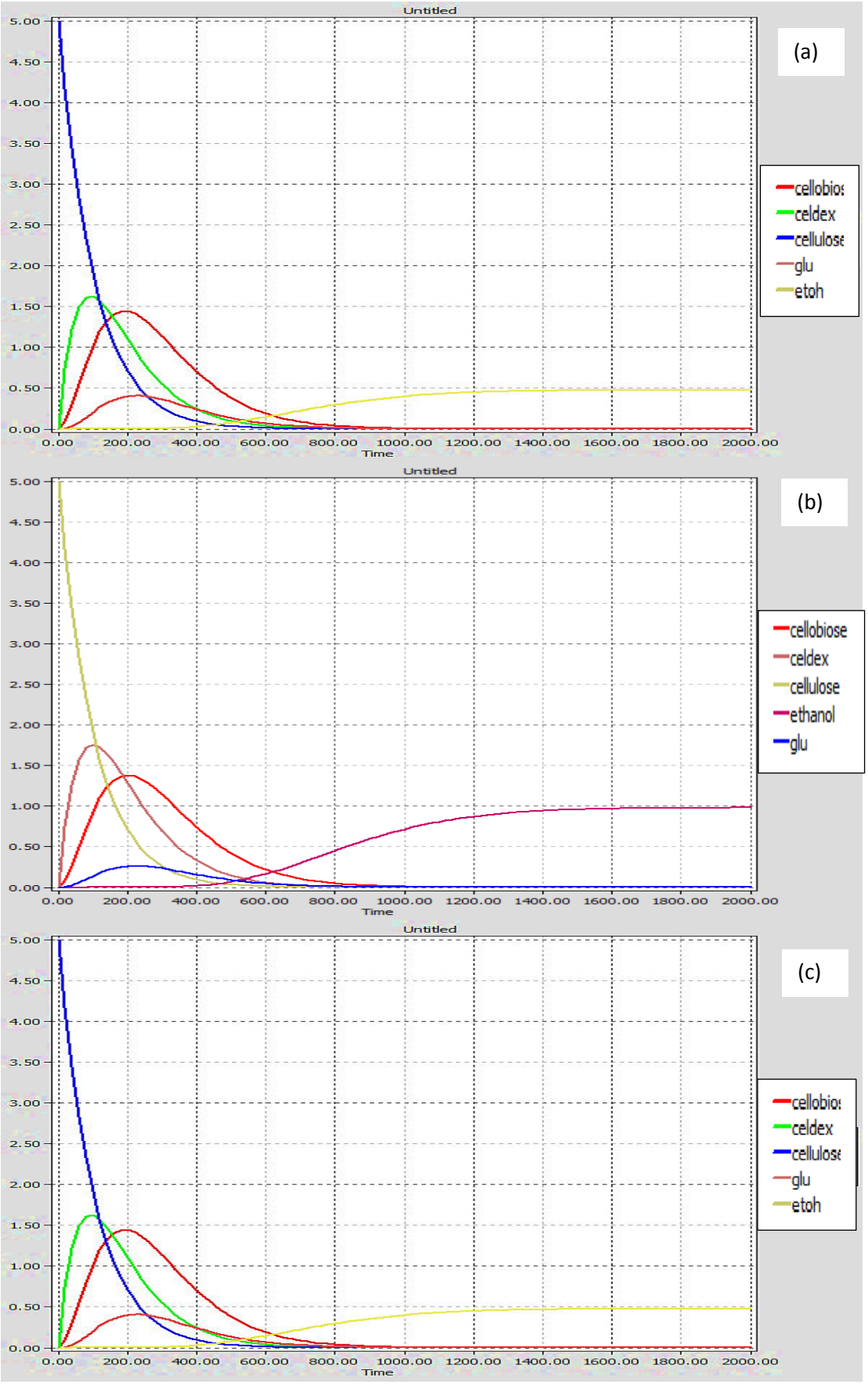


Figure 4.7.3.3: Trends of ethanol production and reducing sugars concentration at 45ºC for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2

4.7.4 At different pH

Batch fermentations were carried out at different pH (5.0-7.0), temperature (30ºC, 37ºC, and 45ºC) agitated (145 rpm) and static conditions. The incubation time, for each batch fermentation, was 120 hours and the substrate concentration used was 5mg/ ml carboxymethyl cellulose. The data obtained in the wet lab batch fermentations was validated using the petri nets in the petri net editor Snoopy 2.0.

4.7.4.1 At pH 5.0

The results obtained at pH 5.0 for the strains for ethanol concentration and reducing sugar production are mentioned in Table 4.7.4.1. The values from the model reflect the wet lab values for both the parameters. As observed for various temperatures, the actual ethanol production and reducing sugar values were less than those predicted by the model. Strain HI-W produced 1.9 mg/ml of reducing sugars as opposed to 1.96 mg/ml of predicted value. Strain HI-Lb1 showed a reduced production of reducing sugar producing 1.3 mg/ml as compared to a predicted value of 1.66 mg/ml. Similarly strain HI-Lb2 produced 1.4 mg/ml in comparison to a predicted value of 1.93 mg/ml of reducing sugars. Figure 4.6.4.1 shows the changing trends in ethanol production and reducing sugar concentration with time at pH 5.0 as predicted by the kinetic model.

Table 4.7.4.1: Produced and predicted concentration of reducing sugars and ethanol, at pH 5.0

pH	**5.0**					
	Wet Lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugar (mg/ml)	1.9	1.3	1.4	1.96	1.66	1.93
Ethanol (mg/ml)	0.986	0.637	0.684	1.262	2.04	0.907

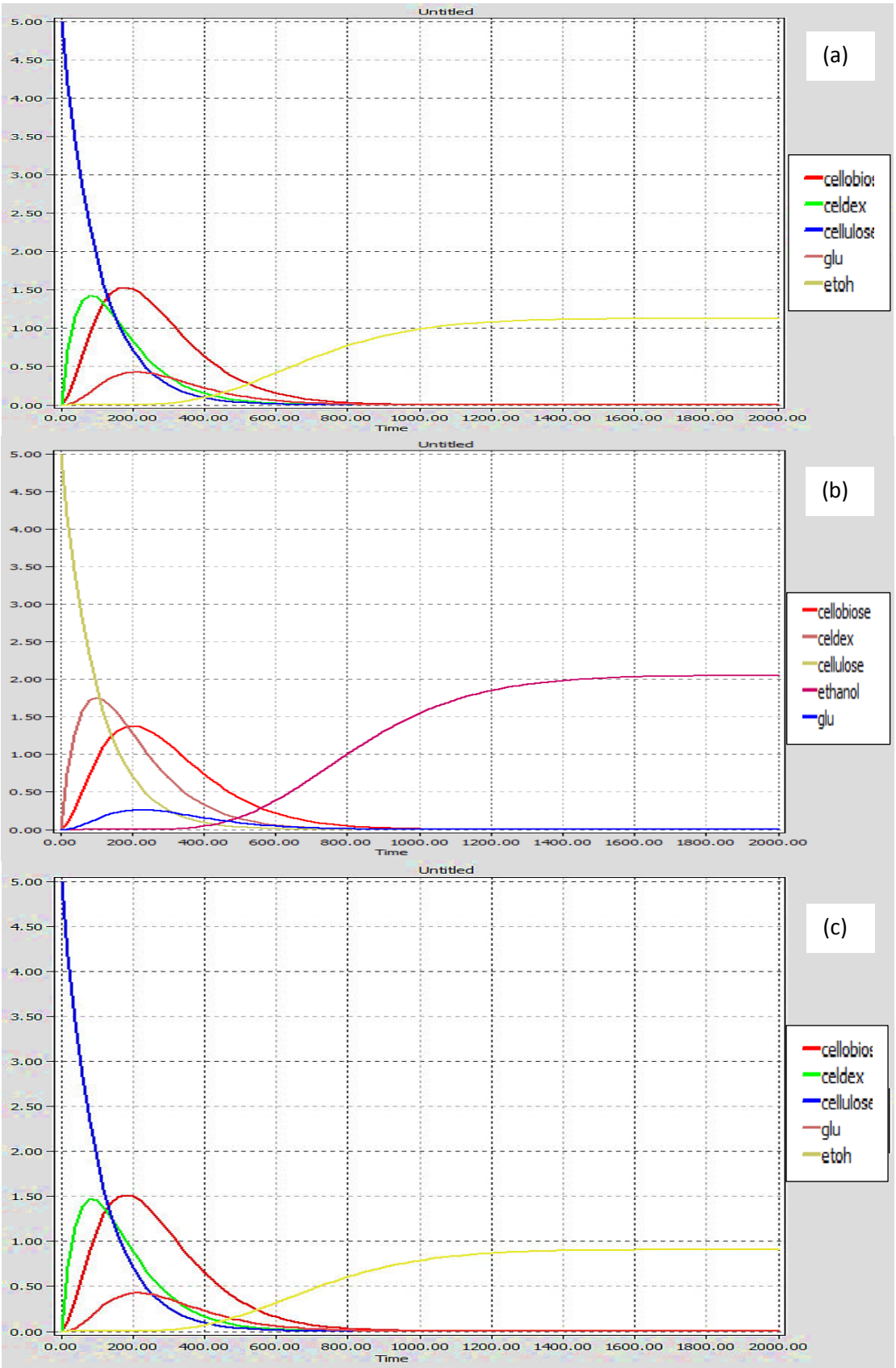


Figure 4.7.4.1: Trends of ethanol production and reducing sugars concentration at pH 5.0 for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2

4.7.4.2 At pH 6.0

Strains showed significant correlation with the predicted values at pH 6.0 under batch fermentation as seen from the Table 4.6.4.2. Strain HI-W produced 2.3 mg/ml of reducing sugars under wet lab conditions which was close to the predicted value of 2.03 mg/ml. Ethanol production shown by strain HI-W 1.768 mg/ml was more than the predicted value which was 1.467 mg/ml. the predicted values for reducing sugar production for strains HI-Lb1 and HI-Lb2 were 1.79 and 2.0 mg/ml respectively as compared to the 1.8 and 2.1 mg/ml calculated from the wet lab experiments. Ethanol production varied for strains HI-Lb1 and HI-Lb2 from the predicted values. Strain HI-Lb1 showed a lower 0.673 mg/ml ethanol production than the predicted value of 2.1 mg/ml. On the hand strain HI-Lb2 showed a more than predicted ethanol concentration of 1.684 as compared to 1.456 mg/ml. Figure 4.6.4.2 shows the trends in the concentration of ethanol and reducing sugars with respect to time.

Table 4.7.4.1: Produced and predicted concentration of reducing sugars and ethanol, at pH 6.0

pH	**6.0**					
	Wet Lab			**Model**		
Strain	**HI-W**	**HI-Lb1**	**HI-Lb2**	**HI-W**	**HI-Lb1**	**HI-Lb2**
Reducing Sugar (mg/ml)	2.3	1.8	2.1	2.03	1.79	2.0
Ethanol (mg/ml)	1.768	0.673	1.684	1.467	2.1	1.456

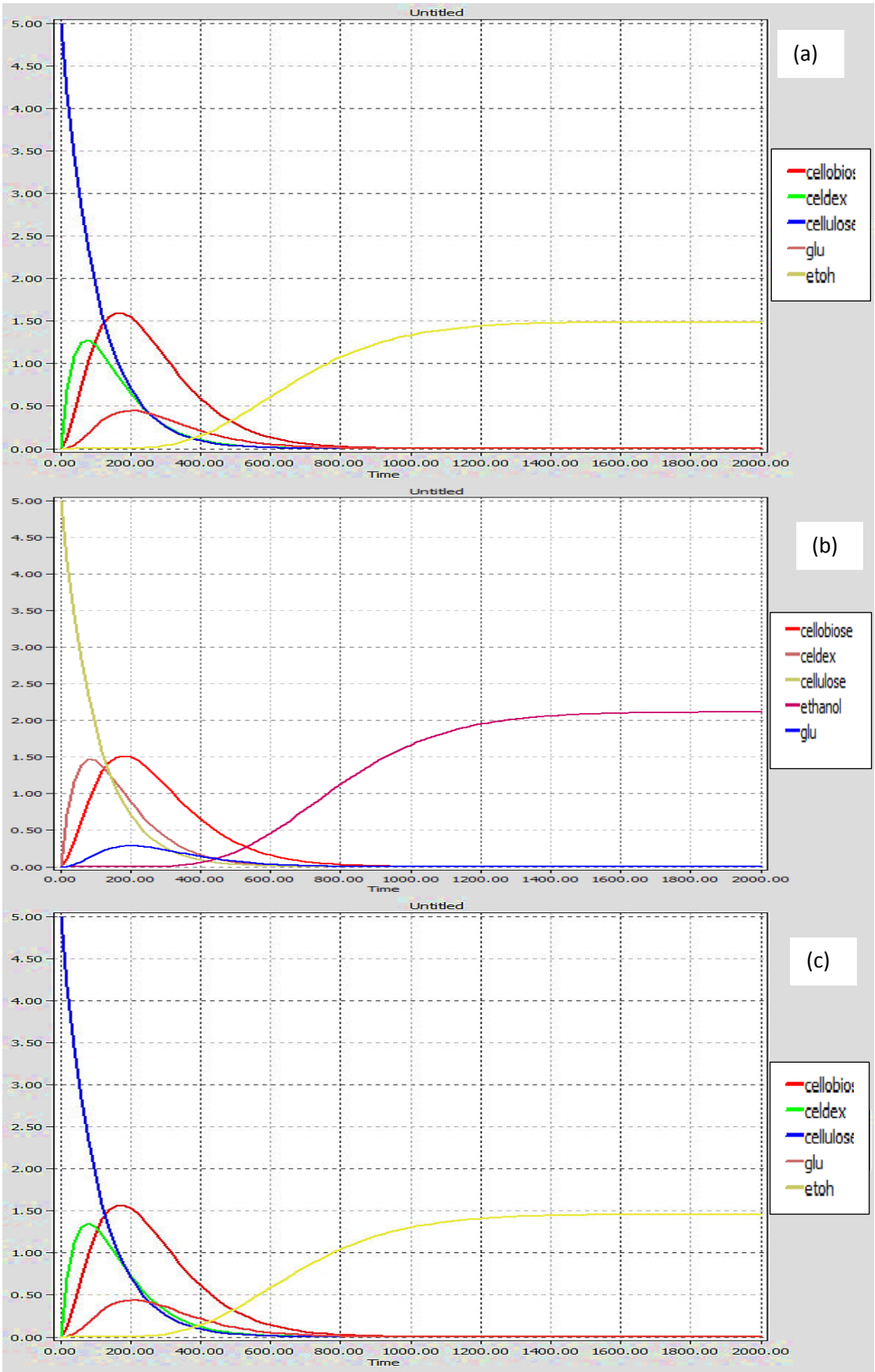


Figure 4.7.4.2: Trends of ethanol production and reducing sugars concentration under agitated conditions for strains (a) HI-W, (b) HI-Lb1 and (c) HI-Lb2

Chapter Five

Discussion

Carboxymethyl cellulose was used in the present study for the isolation of bacterial isolates capable of producing ethanol using CMC as the sole carbon source. A total of 8 CMC degrading bacteria were screened for their cellulase activity. These strains were further confirmed for ethanol production. Cellulase production was confirmed through Congo red staining of the CMC agar plate according to the protocol published by Teather and Wood (1982). 3 of the 8 bacterial isolates were confirmed for ethanol production using the potassium dichromate test and High Performance Liquid Chromatography. The 3 bacterial isolates were named HI-W, HI-Lb1 and HI-Lb2 and two of these isolated strains HI-W and HI-Lb2 were identified as *Lactococcus lactis* and *Lactobacillus pentosus* respectively. The lactic acid bacteria are not famous for their cellulase and ethanol production but this is not an un-noted observation. El-Sheikh (El-Sheikh, 2013) isolated cellulose degrading *L. lactis* from different environmental sources. Meilenz (2001) has deemed the *Lactobacilli* as worthy candidate for direct microbial conversion of cellulose to ethanol after further improvements. Singhvi *et al*., (Singhvi *et al.*, 2010) have used mutant *Lactobacillus lactis* for lactic acid production. Nordkvist *et al*., (Nordkvist *et al.*, 2003) observed the ethanol production by *L. lactis* under microaerobic conditions. Mazzucotelli *et al*., (Mazzucotelli *et al.*, 2013) also reported a cellulose degrading *Lactococcus garvieae* while using carboxymethy cellulose as a substrate.

A carboxymethyl cellulose concentration of 0.5%, 1.0%, 1.5%, and 2.0% was employed in the current study to determine the optimal growth of the isolated strains HI-W, HI-Lb1 and HI-Lb2. Optimal reducing sugar production was shown by strains HI-W (*Lactococcus lactis*), HI-Lb1 and HI-Lb2 (*Lactobacillus pentosus*) at an initial substrate concentration of 0.5% producing a reducing sugar concentration of 1.8mg/ml, 1.5mg/ml and 1.6mg/ml respectively after 48 hours under static batch fermentation conditions. It was observed that the reducing sugars reached maximum concentration at 0.5% CMC and the measurable reducing sugar concentration decreased at concentrations above 0.5%. As observed in the present study and previously reported research, the initial substrate concentration effects both reducing sugar yield and the rate of hydrolysis (Iqbal *et al.*, 2010). A lower substrate concentration increases the hydrolysis rate by increasing the enzyme to substrate ratio (Singhania *et al.*, 2007). As in any enzyme catalyzed reaction, the rate of reaction only increases up to a certain level of increase in substrate concentration, therefore a high substrate concentration may cause low hydrolysis rates in addition to substrate

inhibition of the cellulase enzymes (Jørgensen *et al.*, 2007) (Cheung & Anderson, 1997). Ortega et al. (2001) found that the optimal CMC concentration for cellulase production after 48 hours was 0.5% while working with *Trichoderma reesei*. Verma (Verma *et al.*, 2012) reported similar results with *Bacillus subtilis* with a reducing sugar production of 1.25mg/ml at 1.5% CMC. The maximum rate of reducing sugar production at 0.5% CMC reached 0.0375mg/ml/h for strain HI-W (*Lactococcus* lactis) while the same strain showed a rate of 0.0351mg/ml/h 1.0% CMC. A further increase in substrate concentration further decreases the rate of reducing sugar production dropping to 0.0229mg/ml/h on 2.0% CMC concentration by the strain HI-W. Shaikh *et al.,* (2013) also observed the same results at 0.5% CMC concentration with *Bacillus sp*. Further increase in substrate concentration resulted in decrease in cellulase activity by the bacterium. However after reaching the optimum level, the substrate loading inculcates inhibitory effects on cellulose hydrolysis. A high substrate loading greatly diminishes the enzyme activity and decreases the hydrolysis rate. The substrate inhibition depends on the enzyme loading and the substrate concentration ratio (Penner & Liaw, 1994). The isolated bacterial strains HI-W, HI-Lb1 and HI-Lb2 showed similar trends in reducing sugar production at higher CMC concentration as shown in Results (section 4.5.1). All three strains show a high reducing sugar production (1.8mg/ml, 1.3mg/ml and 1.5mg/ml respectively) at 0.5% CMC concentration but show a decreased reducing sugar production at higher substrate concentrations (1.1mg/ml, 0.9mg/ml and 1.0mg/ml respectively at 2.0% CMC). A higher substrate concentration lowers the product yield with respect to substrate concentration and an excess substrate is left unfermented in the form of inhibitory agents such as cellobiose (*Fenske et al.,* 1999).

Batch fermentations under agitation (145rpm) and static conditions were performed for the determination and comparison of reducing sugars released and ethanol production. Agitation was found to positively reinforce cellulase activity and the production of reducing sugars by the isolates HI-W (*L. lactis*), HI-Lb1 and HI-Lb2 (*L. pentosus*) but showed a negative effect on the ethanol production by all three strains. Reducing sugar production was found to be the highest under agitation conditions by HI-W (*L. lactis*) (2.4mg/ml) as compared to static conditions (1.8mg/ml). Deka *et al*, (2013) reported an optimal cellulase activity at 121rpm using *Bacillus subtilis*, but reported a decrease in cellulase activity on further increase in agitation rate due to possible cell shearing. Similar results were obtained by Sreedevi

et al, (2013) and (Bairagi *et al.*) at 150rpm for cellulase production using *Bacillus sp*. Agitation promotes an increase in aeration of the medium and a better distribution of the media components thus enhancing enzyme activity. In contrast to reducing sugar production, ethanol production showed marked increase in non-stirred batch fermentations as compared to agitated cultures. Strain HI-W (*L. lactis*) showed the highest ethanol production in static culture (0.176mg/ml) in comparison with agitated conditions (0.0731mg/ml). Groposo *et al*, 2013 observed similar diminishing effect of agitation on ethanol production from bagasse, observing a two time increase in ethanol production by *Clostridium thermocellum* in non-stirred fermentation as compared to agitation. Aeration is increased as a result of formation of smaller oxygen bubbles which increase the rate of oxygen mass transfer to the bacteria (Singh *et al*, 2000). The strains HI-W (*L. lactis*), HI-Lb1 and HI-Lb2 (*L. pentosus*) are facultative anaerobes which can be concluded by observing that under high oygen transfer rates, all 3 strains are able to grow (Holt *et al.*, 1984). Smetankova (Smetanková *et al.*, 2012) observed a slightly higher ethanol production during aerobic conditions by *Lactobacillus plantarum*. An increase in H_2 production in the fermentation medium causes a shift towards ethanol or lactate production (Lamed *et al*, 1988). A low ethanol yield during agitation causes higher oxygen transfer rates to cells which promote production of other metabolites along with a higher cell growth, compromising ethanol production (Sharma *et al.*, 2007). This observation correlates with one in present study where a lower amount of ethanol production by all isolated strains HI-W, HI-Lb1 and HI-Lb2 was seen when agitated conditions were used whereas the reducing sugar production increased.

In the current study, the effect of different temperature (30ºC, 37ºC, 45ºC) on the production of reducing sugars and the ethanol production by the strains *L. lactis* (HI-W), HI-Lb1 and HI-Lb2 (*L. pentosus*) was investigated. The highest reducing sugar production was observed with the strain HI-W which produced 1.8mg/ml reducing sugars and 0.176mg/ml of ethanol during 120 hour incubation period with the substrate concentration of 0.5% at pH 6.0 at 37ºC. Strains HI-Lb1 (1.5mg/ml and 0.1544mg/ml) and HI-Lb2 (*L. pentosus*) (1.6g/ml and 0.1684mg/ml) produced reducing sugars and ethanol respectively at 37ºC, pH 7.0 and 6.0 respectively. The yield of ethanol and reducing sugars increased with the increase in temperature from 30ºC to 37ºC but dropped at 45ºC. This shows that the ethanol production by bacterial strains is temperature dependent as reported by Cazetta *et al*, (2007). The rate of

enzyme catalyzed reaction increases with increasing temperatures up to a certain level, after this the rate of reaction drops significantly due to increase in internal kinetic energy of the enzyme which effects enzyme stability (Tipton & Dixon, 1979) (Nelson & Cox, 2004). The cell growth was also found to be maximum at this temperature (37ºC) which proves that ethanol is a growth associated. Temperature effects the cell growth and hence ethanol production (Lee *et al.*, 1981). At high temperatures, the intracellular concentration of ethanol rises (Navarro and Durand, 1978) which inhibits the fermentative pathway that ultimately effects ethanol production. Owing to this accumulation of ethanol inside the cell also effects the cell growth (Laudrin & Goma, 1982). In yeast, reported by Lee *et al*, (1981), intracellular ethanol accumulation effects the yeast cell growth adversely also in *Zymomonas mobilis*, the ethanol production is more severely affected (Laudrin & Goma, 1982). Cell maintenance is defined as the energy spent by the bacteria in processes other than growth related functions (Van Bodegom, 2007). At increased temperatures, no new cells are produced but only the existing cells are maintained which causes which causes shifts in metabolic pathways, resulting in lower product yields (Fieschko & Humphrey, 1983). An increase in susceptibility to ethanol inhibition at high temperatures has also been proposed (Lee *et al*., 1980). During the current study, an increase in incubation temperature from 37ºC to 45ºC, effects the cell growth, reducing sugar production and ethanol yield decreased significantly.

In the current study the optimum pH for the strains HI-W (*L. lactis*), HI-Lb1 and HI-Lb2 was in the pH range of 5.0-7.0. The optimum pH for the isolated bacterial strains HI-W (*L. lactis*) and HI-Lb2 (*L. pentosus*) was observed at pH 6.0 where highest reducing sugars (2.1mg/m and 1.9mg/ml respectively) and highest ethanol production (1.786mg/ml and 1.684mg/ml respectively) was observed. However, pH 7.0 was observed to be optimum for cell growth, reducing sugar release (18mg/ml) and ethanol production (1.544mg/ml) for strain HI-Lb1. Changes in pH of the growth medium can effect bacterial cell growth and the rate of fermentation along with a possible metabolic shift (Zhu & Yang, 2004). The effect of changes in pH on CMCase activity has been canvassed and is attributed to the change in the ionic balance of the enzymes involved in cellulose degradation and also ethanol production (Rice & Stephen, 2002). Changes in the ionic balance results in disrupted net charge on the enzyme, causing destabilization of the enzyme 3D structure and, causes denaturation under strong pH shifts (Hu *et al.*, 2004). The pH variation also causes slower cell

growth rate and lower overall cell growth which contributes to low cellulase activity and ethanol yields (Ariffin *et al.*, 2006).

The wet lab results for all the three strains HI-W (*L. lactis*), HI-Lb1 and HI-Lb2 (*L. pentosus*) were modeled using hybrid petri nets using the petri net editor Snoopy 2.0. The modeling of the metabolic pathway was performed using the phosphoketolase pathway which is used by the Lactic acid bacteria for ethanol production described by DeMoss (1951). The pathway along with CMC as the substrate is shown in Literature review section 2.6.1 [Figure (2.6.1)]. Modeling was done according to Matsuno *et al*., (2013). Petri nets and other modeling techniques employ differential equations to represent complex metabolic networks, having no definite solutions. These metabolic networks are solved one reaction at a time by the software, investigating the effect of the concentrations on individual reactions. Studying the effect of all the individual reactions provides an overall continuous view of the metabolic pathway (Marwan & Blätke, 2012). All individual reactions in the modeled pathway are in coherence with each other so as to portray the combined effect of the reactions and the model as a whole (Heiner & Koch, 2004).

Previous models developed for cellulose degradation have focused on the cellulose hydrolysis mechanism, cellulase adsorptions, and the end product inhibition of β-glucosidase and cellobiohydrolase by glucose and cellobiose respectively (Wald *et al.*, 1984), (Gusakov *et al.*, 1985), (South *et al.*, 1995) and (Philippidis & Hatzis, 1997). Kadam *et al*, 2004 developed a Langmuir type kinetic model for the enzymatic hydrolysis of lignocellulosis. The model focused on three hydrolysis reactions covering the breakdown of cellulose to cellobiose and glucose and the third depicting the cellobiose to glucose breakdown. The model parameters were estimated by wet lab results model showed good correlation with the wet lab results.

The current model was aimed to study the rate of cellulose hydrolysis and ethanol production from the subsequently released reducing sugars. This was done by caluculating the rates of reaction for the reducing sugar and ethanol production according to the wet lab results using the equation given in materials and methods (section 3.10.2). Furthermore the currently developed model considers the effect of changes in the initial substrate concentration, pH, temperature and fermentation conditions (agtitation and static) using Hybrid Petri Nets. The model replicated the effect of various physical conditions (pH temperature and agitation) on the production of the reducing sugars and ethanol production according to the results that are

observed in the wet lab. Highest ethanol production, and reducing sugar content was shown by the model at the conditions observed in the batch fermentations at 37ºC temperature, pH 6.0 for HI-W and HI-Lb2 and pH 7.0 for HI-Lb1 with a substrate concentration of 0.5%.

Conclusions

It can be concluded from the current study that direct microbial conversion of carboxymethyl cellulose in to ethanol is possible using mesophilic bacterial strains such as *Lactococuss lactis* and *Lactobacillus pentosus* which grow well at 37ºC and a pH of 6.0. Though the isolated bacterial strains converted carboxymethyl cellulose over an incubation period of 5 days, the strains are nominal producers of CMCase enzyme, being able to utilize a meager 0.5% CMC concentration efficiently. Ethanol production by the bacterial strains is affected by changes in temperature, pH, and culture condition such as agitation during batch fermentation. The ethanol production efficiency might be increased in fed batch fermentation as substrate inhibition can be curbed. Kinetic modeling results achieved good correlation with the wet lab results so a further optimization of the physical parameters in the wet lab and some fine tuning of the computational model can be done to study the ethanol yield by the isolated bacterial strains *L. lactis* and *L. pentosus*. Also helpful in the development of the kinetic model would be a more insightful knowledge of the intermediate reaction rates and mechanisms.

Future Prospects

Work on mesophiic bacteria for their potential for ethanol production from ceulose has been limited and scarce. Mostly thermophilic bacteria such as members of the genera *Clostridium, Thermoanaerobacter,* and *Thermoanaerobium*, have been explored for this capability. Lactic acid bacteria such as *Lacctobacilli* and *Lactococcus* have been reported for cellulase production. Though the production is low in terms of cellulase enzyme activity, the bacteria have the ability to produce ethanol naturally along with their primary metabolic product which is lactic acid. Work can be done to improve production of ethanol and decrease the proction of side products, mainly lactic acid through metabolic engineering and medium optimization studies. Computational modeling can also pay major role in obtaining better yields by narrowing down the possible optimization studies and by increasing the understanding of the metabolic pathways involved in the ethanol production by these bacteria.

Employment of mesophilic bacteria in the industrial ethanol production can decrease the energy costs for maintaining a high temperature for thermophilic bacteria. Furthermore, LAB are known to have a high ethanol as well as organic acid tolerance that can be present during the fermentation process, which can result in possible high yields of ethanol and lower inhibition by these substances.

Chapter Six

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Appendix-I

List of Abbreviations

^{o}C	Degree centigrade (Celsius)
ml	Milliliter
pH	Power of hydrogen ion
Conc.	Concentration
Fig.	Figure
g	Gram
h	Hour(s)
mg/ml	Milligram per milliliter
O.D	Optical Density
U	Enzyme Unit
U/ml	Units per milliliter
CH_3COOH	Acetic Acid
T	Absolute temperature
mM	milli molar
nm	Nano-meter
µm	Micro-meter
min(s)	Minute(s)
S	Shaking
St	Static
rpm	Revolutions per minute
K_m	Michaelis-Menten constant
V_{max}	Maximum enzyme velocity
λ_{max}	Maximum wavelength
[S]	Substrate concentration
bp	base pairs
CBP	Consolidated Bioprocessing
SSF	Simultaneous Saccharification and Fermentation
RT	Retention time

HPLC	High Performance Liquid Chromatography
Etoh	Ethanol
Glu	Glucose
Celdex	Celodextrins
PCR	Polymerase Chain Reaction
DNS	Dinitrosalicylic acid
HI	Hamza Ihsan
ASAB	Atta ur Rahman School of Applied Biosciences
NUST	National University of Sciences and Technology
KPK	Khayber Pakhtun Khwah