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Kinetic Study and Modelling of Ethanol Production from Glucose/Xylose Mixtures Using the Genetically-Engineered Strain *Escherichia coli* KO11

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Kinetic Study and Modelling of Ethanol Production from Glucose/Xylose Mixtures Using the Genetically-Engineered Strain *Escherichia coli* KO11

By

Xin Li

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ABSTRACT

Kinetics of ethanol fermentation on different synthetic glucose/xylose mixtures using *Escherichia coli* KO11 was studied. No substrate inhibition was observed in both glucose-only and xylose-only experiments. In single substrate fermentations, results show that glucose was the preferred substrate and its ethanol conversion rate was 1.5 times higher than the one obtained with xylose. In mixed-sugar fermentations, glucose was fully consumed significantly earlier than xylose, indicating an initial inhibition on xylose fermentation. The xylose consumption rate after the depletion of glucose significantly decreased with an increase in the initial glucose content.

An unstructured kinetic model for ethanol production from glucose, xylose, and their mixtures using *Escherichia coli* KO11 was developed based on metabolic analysis. Ethanol inhibition and effect of pH were taken into account in the proposed model. Terms related to the inhibition on xylose metabolism by glucose were also included. Good agreement between model predictions and experimental data was obtained.

RÉSUMÉ

La cinétique de la production d'éthanol pour différents mélanges glucose/xylose utilisant la bactérie *Escherichia coli* KO11 a été étudiée. Aucune inhibition de la production d'éthanol n'a été observée lors de la fermentation du glucose ou de la xylose. Lors des fermentations à substrat unique, les résultats démontrent que le glucose est le substrat de préférence vu son taux de conversion en éthanol 1.5 fois supérieur à celui qui a été observé pour la xylose. Lors de la fermentation des mélanges glucose/xylose, la consommation complète du glucose s'est faite bien avant celle de la xylose. L'augmentation de la concentration initiale de glucose lors de la fermentation du mélange glucose/xylose, a eu pour effet de diminuer le taux de consommation de la xylose.

Un modèle cinétique non structuré pour représenter la production d'éthanol pour des solutions de glucose, xylose ainsi que pour un mélange des deux a été développé en utilisant l'information d'une analyse métabolique et les résultats expérimentaux. L'inhibition par l'éthanol et les effets du pH ont été pris en compte dans le modèle proposé. Des paramètres représentant la répression du métabolisme relié à la consommation de la xylose par le glucose ont été inclus dans les équations décrivant la croissance cellulaire et la production d'éthanol. Les prédictions du modèle représentent bien les données expérimentales.

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NOMENCLATURE

A_1, A_2, A_3	Coefficient in Arrhenius equation (Dimensionless)
C	Conversion efficiency (%)
E_{max}	Maximum ethanol concentration (g/L)
E_1, E_2, E_3	Activation energy (kJ/mol)
k	Kinetic rate constant (g/L)
k_i	Energy-related constant (h^{-1})
K_i	Ionization constant ($M \cdot L^{-3}$)
K_m	Michaelis constant (g/L)
K_{mg}	Monod constant for glucose fermentation (g/L)
K_{ms}	Monod constant for pentose fermentation (g/L)
K_S	Monod coefficient, also called the saturation constant (g/L)
$K_{S,G}$	Saturation constant for glucose fermentation (g/L)
$K_{S,X}$	Saturation constant for xylose fermentation (g/L)
K_{SX}	The saturation constant in Contois equation(g/L)
K_I	Substrate inhibition constant (g/L)
K_I	Glucose inhibition constant (Dimensionless)
$K_{I,0}$	Glucose metabolite inhibition constant (Dimensionless)
K_{ig}	Inhibition constant for glucose in glucose fermentation (g/L)
K_{igs}	Inhibition constant for pentoses in glucose fermentation (g/L)
K_{is}	Inhibition constant for pentoses in pentose fermentation (g/L)
K_{isg}	Inhibition constant for glucose in pentose fermentation (g/L)
K_I	Inhibition constant for xylose fermentation in mixed-substrate fermentations (Dimensionless)
m_G	Maintenance coefficient for glucose fermentation (h^{-1})
m_X	Maintenance coefficient for xylose fermentation (h^{-1})
m	Maintenance coefficient (h^{-1})
M	The total initial sugar concentration (g/L)
n	Constant in product inhibition model (Dimensionless)
P	Ethanol concentration (g/L)

P_m	Product concentration above which cells do not grow (g/L)
Q_E	Volumetric ethanol productivity ($g \cdot L^{-1} \cdot h^{-1}$)
Q_G	Glucose utilization rate ($g \cdot L^{-1} \cdot h^{-1}$)
Q_X	Xylose utilization rate ($g \cdot L^{-1} \cdot h^{-1}$)
R	Ideal gas constant ($kJ \cdot mol^{-1} \cdot K^{-1}$)
S	Substrate concentration (g/L)
S_G	Glucose concentration (g/L)
$S_{G,0}$	Initial glucose concentration (g/L)
S_X	Xylose concentration (g/L)
t	Fermentation time (h)
T	Temperature (^oC or K)
X	Cell mass concentration (g/L)
X_0	Initial cell mass concentration (g/L)
X_m	Carrying capacity (g/L)
$Y_{E/S}$	Ethanol yield coefficient (g ethanol/g substrate)
$Y_{E/S, G}$	Ethanol yield coefficient for glucose fermentation (g ethanol/g substrate)
$Y_{E/S, X}$	Ethanol yield coefficient for xylose fermentation (g ethanol/g substrate)
$Y_{X/S}$	Cell mass yield coefficient for glucose fermentation (g cells /g substrate)
$Y_{X/S, G}$	Cell mass yield coefficient for xylose fermentation (g cells /g substrate)
$Y_{X/S, X}$	Cell mass yield coefficient (g cells /g substrate)
$Y'_{E/S}$	Ethanol yield (g ethanol/g substrate)
$Y'_{X/S}$	Cell mass yield (g cells /g substrate)
V_m	Maximum ethanol production rate (h^{-1})
V_{mg}	Maximum ethanol production rate in glucose fermentation (h^{-1})
V_{ms}	Maximum ethanol production rate in pentose fermentation (h^{-1})

Greek Letters

γ	Constant in growth limits model (Dimensionless)
θ	The critical initial glucose concentration below which there is no apparent inhibition of glucose on xylose.

α	Growth related product formation coefficient (g product/g cells)
α_G	Growth related product formation coefficient for glucose fermentation (g product/g cells)
α_X	Growth related product formation coefficient for xylose fermentation (g product/g cells)
B	Non-growth related product formation coefficient ((g product) · (g cells)$^{-1}$ · h^{-1})
β_G	Non-growth related product formation coefficient for glucose fermentation ((g product) · (g cells)$^{-1}$ · h^{-1})
β_X	Non-growth related product formation coefficient for xylose fermentation ((g product) · (g cells)$^{-1}$ · h^{-1})
M	Specific growth rate (h^{-1})
μ_m	Maximum specific growth rate in Monod equation (h^{-1})
υ	Enzyme-catalyzed reaction rate (g · L^{-1} · h^{-1})
υ_m	The maximum rate of enzyme reaction (h^{-1})
ξ	Catabolic inhibition coefficient (Dimensionless)

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CHAPTER 1

INTRODUCTION

1.1 Development of the Bioethanol Industry

Since the crude oil price has broken $140 per barrel in the early July of 2008 (Figure 1.1), and it was feared that it could reach $170 in the coming months (recently predicted by the President of OPEC, Chakib Khelil), the development of renewable energy resources such as biofuels is receiving tremendous attention. There exist many types of biofuels: bioethanol, biodiesel, biogas, straight vegetable oil, and many others. Among these, much attention has been placed on bioethanol because of its use as a gasoline substitute.

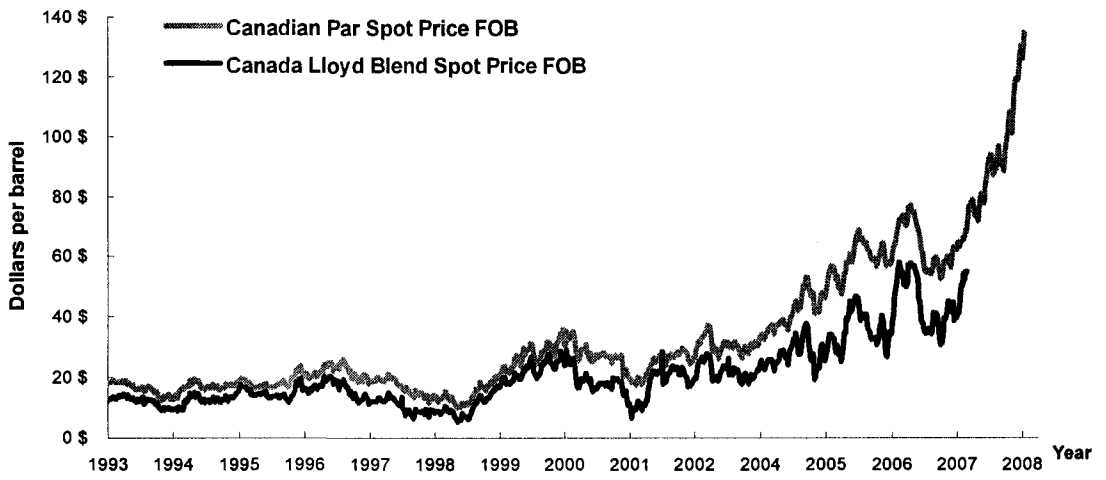

Figure 1.1. Canada oil price statistics since 1993 based on data derived from EIA (Official Energy Statistics from the U.S. Government).

Ethanol, also called grain alcohol or ethyl alcohol, is a good replacement for gasoline and has been used in gasohol (mixture of gasoline and ethanol) or as a fuel oxygenate since the 1980s (Sun and Cheng, 2002). Ethanol is produced mainly by two methods. One method uses chemical synthesis via a high-temperature catalytic reaction from ethylene or ethylene

sulfite. The other method is through fermentation of sugars obtained from biomass. The three most common types of biomass from which sugars are extracted are sugarcane, corn and lignocellulosic materials. Bioethanol production in the United States in 2007, according to the statistics of RFA (Renewable Fuel Association), was 25 000 million liters (6500 millions gallons), which is 7 times larger than the 1990 production, and is still increasing according to the 2008 monthly statistics of U.S. bioethanol production reported by RFA (http://www.ethanolrfa.org/). The International Energy Agency (IEA) predicts that 10 percents of the world's gasoline can be replaced by ethanol by 2025 and 30 percents by 2050 (Whittington, 2006). Furthermore, market analysis shows a faster increase in global bioethanol demand (http://www.eia.doe.gov/). Although utilizing ethanol as an automotive fuel still releases a significant amount of greenhouse gas, this can be considered partly "recycled CO_2" since it can be absorbed by plants when they grow and these plants can be once again fermented to ethanol. From this point of view, it can be argued that ethanol burning adds no net CO_2 to the atmosphere (Costello and Chum, 1998). This statement is however the subject of many interpretations.

There are no technology barriers to expanding the level of ethanol in automotive fuel. The largest bioethanol producer in the world is Brazil with 40% of the world market. Ethanol from Brazil is obtained by fermentation of sugars extracted from sugarcane. Most ethanol is used domestically substituting 40% of the total automotive fuel consumption. Brazil is undoubtedly the pioneer in this field with 77% of light vehicles that were sold in 2006 using flexible-fuel (Greenergy Ltd., 2007). Bioethanol is used either in blending the ethanol with gasoline or as a complete substitute to gasoline. In Brazil, ethanol-powered and flexible-fuel vehicles have indeed already been developed to be capable of using ethanol ranging from 20% to 100%. Worldwide automotive ethanol capabilities vary widely and most spark-ignited gasoline style engines have the capability of using mixtures of 10% ethanol and 90% gasoline. In addition, the higher blend ethanol fuel E85 (about 85% ethanol with 15% gasoline) was designed to be used in flexible fuel vehicles with about 36% lower selling price per liter than gasoline (Goettemoeller and Goettemoeller, 2007). Bioethanol offers significant potential as a fossil fuel substitute.

1.2 Ethanol from Lignocellulosic Biomass

Apart from Brazil, the traditional ethanol process is starch-rich substrate based, usually obtained from corn. Some claim that it is uneconomical and the increase in bioethanol demand has given rise to an ethical conflict between food use and industrial use of crops (Maynard, 2007). Recently, a sudden and rapid increase in food prices around the world occurred and the increasing biofuel production was identified as one of the contributors for this increase although it is highly suspected that market speculation was the main cause. Consequently, to partly alleviate this problem, it is preferable to find an alternative source of fermentable sugars for ethanol production that is inexpensive and abundant and at the same time not contributing to food market competition. Lignocellulosic materials, a lower-value substrate and in particular agricultural and forest residues, could offer this alternative biomass source, and has been the subject of significant research in recent years. Lignocellulosic materials offer a significant potential for reducing the bioethanol production cost.

Sources of lignocellulosic materials currently targeted for ethanol production include sugarcane bagasse, switch grass, corn stovers, cottonwoods, paper, wood, corn stalks, soybean residues, wheat straw, as well as many types of municipal solid waste (Wiselogel et al., 1996). This natural and abundant polymer is estimated to account for about 50% of the biomass in the world (Zaldivar et al., 2001). The lack of indigenous microorganisms which can rapidly and efficiently convert completely the hydrolysates of lignocellulose to ethanol is the main barrier for bioethanol production. Thus, a large number of engineered or genetically modified microorganisms, including yeasts, bacteria and fungi, for a more efficient production of ethanol were developed and studied in last two decades.

1.3 Project Objectives

Ethanol production from lignocellulosic hydrolysates using a variety of microorganisms has many advantages compared to other biological ethanol production processes because it uses renewable resources and can be integrated within another process to use the residues of that process (ex.: agricultural wastes and forest residues) to produce ethanol. The main

fermentable sugars obtained from lignocellulosic hydrolysates are glucose and xylose. Microorganisms that use glucose to produce ethanol exist naturally and have been used for centuries. On the other hand, xylose accounting for approximately one third of the fermentable sugars cannot be used by these microorganisms and one must resort to genetically engineered microorganisms in order to use both sugars. The current investigation focuses on ethanol production using genetically engineered *Escherichia coli* KO11 on complex media containing glucose, xylose, or their mixtures.

The main objectives of this research may be summarized as follows:

1. To determine the growth kinetics of *Escherichia coli* KO11 on single-sugar substrates and mixed-sugar substrates under anaerobic conditions.
2. To investigate the effects of different initial sugar concentrations, different initial ethanol concentrations, and different pH on the ethanol formation under anaerobic conditions.
3. To construct an unstructured mathematical kinetic model to represent the rates of cell growth, ethanol production and substrate consumption, and to estimate model parameters to describe these rates while potentially including substrate and product inhibition. To generalize even further the model, the impact of pH variation on cell growth and ethanol production will be taken into account in the kinetic model by allowing the model parameters to vary with pH.

1.4 Structure of the Thesis

The main body of this thesis consists of two papers that are presented in Chapters 3 and 4. Both of these papers will be submitted for publication shortly after the thesis defence.

Chapter 2 presents a literature survey on the work carried out on the conversion of lignocellulosic hydrolysates to ethanol using a variety of microorganisms, and recent studies performed on the microorganism that is of interest for this project, the engineered strain *Escherichia coli* KO11. Chapter 2 also reviews a variety of fermentation kinetic models that could be used to represent the results obtained in this study.

Chapter 3 presents the experimental results obtained for ethanol production, using genetically engineered strain *Escherichia coli* KO11, from glucose, xylose and their mixtures under anaerobic conditions. A description of fermentation kinetics on single-sugar substrates and mixed-sugar substrates at various sugar ratios is also presented and provides valuable information on the use of these sugars.

In Chapter 4, kinetic studies of ethanol fermentation from glucose and xylose mixtures using *Escherichia coli* KO11 were carried out to develop an unstructured dynamic mathematical model incorporating the effects of ambient pH, substrate inhibition, product inhibition, and mutual influence of the two sugars. Finally, Chapter 5 presents the conclusions and recommendations for future work

CHAPTER 2

LITERATURE SURVEY

2.1 Hydrolysates of Lignocellulosic Biomass

Lignocellulosic biomass is primarily composed of hemicellulose, cellulose and lignin. Lignin is a rigid aromatic polymer found in plant cell walls and binds the biopolymers together to give the rigid structure characteristic of lignocellulosic material (Thomas et al., 2007). Cellulose is the predominant component, contributing 38-50% of total dry weight, formed as a linear homopolymer of anhydrous D-glucose. Hemicellulose can account for approximately 23-32% and is mainly composed of xylose units (Johnson, 2006). Bioconversion of lignocellulose to ethanol mainly involves two processes: pretreatment and hydrolysis of lignocellulose to monomeric sugars; and fermentation of the sugars to ethanol (van Maris et al., 2006; Foyle et al., 2007).

Different pretreatment processes based on physical, physico-chemical, chemical, or biological techniques have been developed in the last two decades (Table 2.1.). These processes are most often used in combination. During the pretreatment process, most of the hemicellulose and lignin are readily degraded and dissolved. Cellulose is separated as the solid fraction and further hydrolyzed using cellulase enzyme to produce usable carbon (mainly glucose) for ethanol fermentation (Garde et al., 2002). Hemicellulose can also be hydrolyzed chemically or enzymatically to release galactose, mannose, rhamnose, fucose, arabinose, uronic acids, and xylose, the predominant component (Zaldivar et al., 2001). Because of its distinctive polymeric structure and chemically distinct subunits, lignin is hard to degrade and not was considered previously as fermentable carbon sources (van Maris et al., 2006). Previous attempts to reduce the lignin content with a concomitant increase in cellulose of genetically modified plants have been successful and show a potential of more effective biorefinery operation (Ragauskas et al., 2006; Chen and Dixon, 2007). Additional

research was also done to explore lignin degradation enzymes from some organisms, such as white rot fungi and termite, and offered a novel insight into biomass delignification (Weng et al., 2008).

Table 2.1. Current pretreatment processes of lignocellulosic biomass (Sun and Cheng, 2002).

Physical pretreatment	**Physico-chemical pretreatment**	**Chemical pretreatment**	**Biological pretreatment**
Mechanical comminution	Steam explosion	Ozonolysis	Fungal degradation
Pyrolysis	Ammonia fiber explosion	Acid hydrolysis	Enzymatic hydrolysis
	CO_2 explosion	Alkaline hydrolysis	
		Oxidative delignification	
		Organosolv process	

Even though the proportion of monomeric sugars varies depending on the species of lignocellulosic feedstock, the main two constituents of lignocellulosic hydrolysates are glucose and xylose (Table 2.2). One important factor in sustaining an economically viable bioconversion of lignocellulosic biomass to ethanol is the interaction between released monomeric sugars and microorganisms. The microbe must be able to rapidly and efficiently convert all of the available carbohydrates, including both pentoses and hexoses, present in lignocellulosic hydrolysates, to ethanol at near theoretical yield with high tolerance to ethanol and process hardiness. Resulting from the lack of natural microorganisms able to perform this complex task, worldwide interests in bioethanol research are focused on obtaining efficient fermentative organisms through metabolic engineering technology and optimizing the fermentation process integration for high ethanol productivity.

Table 2.2. Major monomeric sugar components (%) of common agricultural lignocellulosic hydrolysates (van Maris et al., 2006).

	Corn stover	Wheat straw	Bagasse	Cotton gin	Sugar beet pulp	Switch grass
Glucose	34.6	32.6	39.0	37.1	24.1	31.0
Mannose	0.4	0.3	0.4	1.1	4.6	0.2
Galactose	1.0	0.8	0.5	2.4	0.9	0.9
Xylose	19.3	19.2	22.1	9.4	18.2	0.4
Arabinose	2.5	2.4	2.1	2.3	1.5	2.8

2.2 Microorganisms Used in Ethanol Production

Genetic engineering, through the modification of a specific bioreaction, addition of a new metabolic pathway, or depletion of genes encoding byproduct formation enzymes, was performed on a large number of bacteria and yeasts in an attempt to construct the ideal strain for ethanol fermentation. In the last two decades, numerous microorganisms were used, including *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *Pichia stipitis*, *Pachysolen tannophilus* and many others. Those approaches are summarized in Table 2.3 with a particular attention to ethanol yield and productivity, ethanol tolerance, culture characteristics, and different substrate feedstocks.

Table 2.3. Comparison of wild-type and genetically engineered ethanologenic microorganisms.

Microorganism	Description	Substrates	Culture conditions	Ethanol yield	Overall ethanol productivity	Ethanol tolerance	References
Pichia stipitis (ATCC58785)	Wild type	Glucose and xylose	30℃, pH=5 oxygen-limited	0.45 g/g	0.24 g/(L·h)	64 g/L	Agbogbo and Wenger, 2006; Agbogbo et al., 2007
Pichia stipitis (FPL-Shi21)	cyc1-Δmutant strain	Glucose and xylose	25℃, anaerobic	0.46 g/g	0.43 g/(g cells·h)	N/A	Shi et al., 1999
Pachysolen tannophilus （CBS 4044 ）	Wild type	Pentose, hexose, and cellobiose	30℃, pH=4.5, aerobic	0.37 g/g	0.62 g/(g cells·h)	50 g/L	Barbosa et al., 1990; Sánchez et al., 2002
Candida shehatae (CSIR-Y492)	Wild type	Glucose and xylose	30℃, pH=4, aerobic	0.37 g/g	0.9 g/(L·h)	48 g/L	du Preez et al., 1986; du Preez et al., 1987
Klebsiella oxytoca (P2)	Containing *pdc*, *adhB* genes from *Z. mobilis*	Pentose, hexose, and cellobiose	32℃-35℃, pH=5.0-5.2, anaerobic	0.46 g/g	1.6 g/(L·h)	36 g/L	Wood and Ingram, 1992; Golias et al., 2002
Fusarium oxysporum (VTT-D-80134)	Wild type	Glucose and xylose	30℃, pH=4.5, oxygen-limited	0.38 g/g	0.15 g/(g cells·h)	50-60 g/L	Suihko and Enari.,1981; Ruiz et al., 2007
Saccharomyces cerevisiae（BO 15）	Wild type	Sucrose	25℃, anaerobic	0.91 g/g	1.0 g/(L·h)	18.7 vol% (147.5 g/L)	Esser and Stahl, 1982
Saccharomyces cerevisiae（TMB3001）	Carrying *XYL1*, *XYL2* genes from *P. stipitis*	Glucose and xylose	30℃, pH=5.5 anaerobic	0.35-0.38 g/g	0.24-0.30 g/(g cells·h)	N/A	Eliasson et al., 2000

Table 2.3 (cont.) Comparison of wild-type and genetically engineered ethanologenic microorganisms.

Microorganism	Description	Substrates	Culture conditions	Ethanol yield	Overall ethanol productivity	Ethanol tolerance	References
Saccharomyces cerevisiae 1400 (pLNH32)	Carrying *XR*, *XDH* genes from *P. stipitis* and over-expressing *XK* gene	Pentose and hexose	30°C, pH=5.5 anaerobic	0.46 g/g	1.6 g/(L·h)	N/A	Moniruzzaman et al., 1997; Ho et al., 1998
Zymomonas mobilis ZM4	Wild type	Glucose	30°C, pH=5, anaerobic	0.50 g/g	3.9 g/(L·h)	16 vol% (126 g/L)	Davis et al., 2006
Zymomonas mobilis ZM4 (pZB5)	Carrying two operons: P_{gap}-*xylA/xylB*, P_{eno}-*tal/tktA*	Glucose, xylose, and arabinose	30°C, pH=5, anaerobic	0.46 g/g	1.29 g/(L·h)	63 g/L	Joachimsthal et al., 1999
Zymomonas mobilis AX101	Insertion of 7 genes into the genome.	Glucose, xylose, arabinose,and fructose	30°C, pH=5.5, anaerobic	0.45 g/g	3.3 g/(L·h)	50 g/L	Mohagheghi et al., 2002; Lawford and Rousseau, 2003
Escherichia coli （FBR5）	Carrying the plasmid pLOI297	Pentose and hexose	35°C, pH=6.5, anaerobic	0.46 - 0.51 g/g	0.9 g/(L·h)	50 g/L	Zhang et al., 1995; Qureshi et al., 2006
Escherichia coli KO11	Containing *pdc*, *adhB* genes from *Z. mobilis*	Pentose and hexose	30°C, pH=6.0-6.5, anaerobic	0.53 g/g	1.3 g/(L·h)	50 g/L	Ohta et al., 1991
Clostridium thermocellum SS2	Wild type	Cellulose	60°C, pH=7.5, anaerobic	0.33 g/g	0.021 g/(L·h)	8 vol% (63 g/L)	Sudha Rani et al., 1996; Sudha Rani and Seenayya,, 1999
Geobacillus thermoglucosidasius (M10EXG)	Wild type	Pentose and hexose	50-80°C, pH=6-8, anaerobic	0.082 g/g	0.0083 g/(L·h)	10 vol% (79 g/L)	Fong et al., 2006; Tang et al., 2007

Saccharomyces cerevisiae, the top-fermenting yeast in the brewing process and bread making, was proved as the safest and most effective microorganism in crop-based ethanol fermentation process on an industrial scale. However, it is unable to grow and produce ethanol on xylose and other pentoses because it lacks the necessary pentose metabolic pathway. A good number of genetically engineered *Saccharomyces cerevisiae* strains were constructed by inserting the *Pichia stipitis* genes encoding xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) which add a xylose utilization pathway for ethanol production. The resulting strains were reported to have higher ethanol yield (Table 2.3) under aerobic conditions. But the xylitol yield was still high and the arabinose present in the substrates was not metabolized (Moniruzzaman et al., 1997; Ho et al., 1998). Additionally, *Saccharomyces cerevisiae* can produce high-yield ethanol only when exact small amounts of oxygen are provided to balance growth requirements and over-respiration. In an industrial-scale process, such accurate addition of oxygen to the viscous solid-liquid feedstocks is not only uneconomical, but also virtually impossible. Another approach in *Saccharomyces cerevisiae* studies has been successfully investigated to transform the xylose isomerase gene from the obligate anaerobic fungus *Piromyces* sp.E2 to *Saccharomyces cerevisiae* with high expression level. Due to the very slow xylose consumption rate that was observed in mixed-sugar fermentation, the constructed anaerobic strain RWB217 was further genetically modified to improve ethanol production rate when glucose was present with xylose as carbon sources. A final ethanol yield of 0.41 g/g and higher production rate of 0.49 g/(g·h) under strictly anaerobic conditions were obtained (Kuyper et al., 2005). It is an important breakthrough in the development of *Saccharomyces cerevisiae* strains for ethanol fermentation from lignocellulosic biomass.

With its unique anaerobic glucose metabolic pathway, the Entner–Doudoroff (ED) pathway, the gram-negative bacterium *Zymomonas mobilis* has emerged as a potential biocatalyst for ethanol production. Contrary to yeasts, which produce two moles adenosine triphosphate (ATP) per mole of glucose through the Embden-Meyerhof-Parnas (EMP) pathway, *Zymomonas mobilis* only has a net yield of one mole ATP from each mole of glucose through the ED pathway. This low ATP yield leads to a lower biomass production and, as a result, more carbon is utilized for the production of ethanol (Zhang et al., 1995).

Furthermore, *Zymomonas mobilis* has a very high ethanol tolerance estimated at 16 vol% (Table 2.3) and was considered as the ideal biocatalyst for fuel ethanol production. However, the lack of a complete pentose metabolic pathway is the main technical roadblock of *Zymomonas mobilis* fermenting the mixture of sugars obtained from lignocellulosic biomass to effectively produce ethanol. In 1995, these researchers have successfully introduced and expressed four *Escherichia coli* genes encoding xylose assimilation and pentose phosphate pathway enzymes, including xylose isomerase, xylulose kinase, transketolase, and transaldolase in *Zymomonas mobilis*. The same engineering strategy was also used to construct the arabinose-utilizing strain for ethanol production. Further development led to *Zymomonas mobilis* strain AX101 which can metabolize both xylose and arabinose. This strain was constructed by inserting seven necessary recombinant genes into the chromosomal DNA, and has given rise to a very high ethanol productivity of 3.3 g/(L·h) and ethanol yield of 0.45 g/g (Table 2.3). However, the ethanol tolerance of this engineered strain was decreased by one half (Table 2.3). Furthermore, the efficiency of this bacterium decreased due to its very high sensitivity to acetic acid present in typical industrial substrates. Acetic acid is formed during the de-acetylation of hemicellulose. Moreover, the sensitivity of *Zymomonas mobilis* to acetic acid was found to be exacerbated in the presence of ethanol (Lawford and Rousseau, 2002). The reason was probably related to membrane potential which is unknown. Improving acetic acid tolerance of *Zymomonas mobilis* was found to be very challenging and has hampered the development of this bacterium for ethanol production at commercial scale from lignocellulosic biomass.

Escherichia coli, the most ubiquitous and well known gram-negative microorganism, plays an important role in modern biological engineering and is frequently altered to serve as the "factory" or the "workhorse" in molecular biology. This microorganism is very well known and has a long history of laboratory culture and manipulation. *Escherichia coli* can metabolize a wide spectrum of sugars, including glucose, xylose, and arabinose and was one of the first successful metabolic engineering strain to selectively produce ethanol (Ingram et al., 1987). *Escherichia coli* has been primarily used to produce recombinant protein in food factories and was considered as safe. It is considered to be an excellent microorganism to be used as a biocatalyst for ethanol production. Low ethanol yield of *Escherichia coli* was due

to the unbalanced protons in the sugar metabolic pathway, in which one NADH is generated for each pyruvate made from sugars, coupled with two NADH required for converting pyruvate into ethanol. The construction of an ethanologenic *Escherichia coli* strain was performed by introducing a pyruvate-to-ethanol pathway from *Zymomonas mobilis* into the *Escherichia coli* genome. The resulting strain *Escherichia coli* KO11 displayed a very high ethanol yield up to 0.53 g/g and ethanol productivity of 1.3 g/(L·h) when cultivated on xylose-only media (Table 2.3). This highly promising ethanologenic strain will be introduced in the next section.

Other naturally ethanologenic microbial biocatalysts, such as *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae*, *Klebsiella oxytoca,* and *Fusarium oxysporum* were also investigated. Among these, a group of thermophilic microorganisms have attracted more attention recently. Cultivating microorganisms under higher temperatures can lower the risks of contamination and facilitate the end-product recovery. Some thermophiles, such as *Clostridium thermosaccharolyticum*, *Geobacillus thermoglucosidasius, Clostridium thermohydrosulfuricum*, and *Thermoanaerobacter ethanolicus*, have been studied and they can metabolize a broad range of sugars to produce ethanol. However, they are strict anaerobes and can only yield a small amount of ethanol as products. Further progress is also expected in engineering these thermophilic microorganisms for ethanol production by metabolic engineering techniques to improve sugar transport.

2.3 Engineered *Escherichia coli* KO11

Escherichia coli KO11, which was constructed by Professor Ingram and his colleagues in 1998 (US patent 5821093), is one of the best recombined ethanologenic bacterial strains and has a high ethanol productivity and high tolerance to hemicellulose hydrolysates (Rodney et al., 1999). It was constructed by introducing two genes (*pdc* and *adhB*), which encode pyruvate decarboxylase and alcohol dehydrogenase, from the ethanol fermentation pathway of *Zymomonas mobilis* into the chromosome of *Escherichia coli*. Further genetic modification was performed to disrupt the fumarate reductase gene in order to eliminate succinate production. For screening, a chloramphenicol resistance marker (*cat*) was also chromosomally integrated into *Escherichia coli*. This final strain, designated KO11, compared to the wide type in Figure 2., can be used to metabolize both pentoses and hexoses to ethanol with yields approaching more than 100% of the theoretical sugar conversion in laboratory media containing excess complex nutrients (Ohta et al., 1991).

Escherichia coli KO11 strains have been successfully developed for the fermentation of pine waste (Barbosa et al., 1992), willow (von Sivers et al., 1994), rice hull (Moniruzzaman and Ingram., 1998), sugar cane bagasse (Takahashi et al., 2000), corn cob (de Carvalho Lima et al., 2002), cotton gin residues (Agblevor et al., 2003), corn stovers (Kim et al., 2006), and brewery wastewater (Rao et al., 2007) to produce ethanol. In spite of some reports about the phenotypic instability of *Escherichia coli* KO11 in batch or continuous culture (Lawford and Rousseau, 1995; Lawford and Rousseau, 1996), experiments performed by Dumsday et al. (1997, 1999) showed that *Escherichia coli* KO11 gave a stable ethanol production on glucose, mannose, xylose and galactose substrates in repeat batch culture and on glucose substrates in continuous culture without antibiotics. Furthermore, it has been reported to appear relatively resistant to potential process errors. Without antibiotics, even after deliberate contamination with up to 10% soil, the dominance of *Escherichia coli* KO11 still prevailed. After being exposed to extreme temperatures (2 h at 5°C or 50°C) or pH (2 h at pH 3 or pH 10), *Escherichia coli* KO11 has recovered after the fermentation broth was returned to the optimal fermentation conditions, although longer times were required in most cases. Ethanol yields were not altered by exposure to extreme temperatures, but were reduced by exposure to extreme pH (Moniruzzaman et al., 1998). Consequently, *Escherichia*

coli KO11 was considered to be the most promising strain for commercial, industrial-scale ethanol production.

However, high levels of complex nutrients (tryptone and yeast extract) are required for the rapid ethanol production by *Escherichia coli* KO11. The high cost of these substrates hampers the low production cost of ethanol. Further investigation was performed and it was found that the growth of *Escherichia coli* KO11 during xylose fermentation was limited by flux through citrate synthase, and was not limited by nutrients, by the lack of biosynthetic enzymes, or insufficient ATP from xylose metabolism. This hypothesis was tested by cloning the *Bacillus subtilis citZ* gene encoding NADH-insensitive citrate synthase in *Escherichia coli* KO11, resulting in an increase in cell growth and ethanol production, which substantially reduced the need for complex nutrients (Underwood et al., 2002).

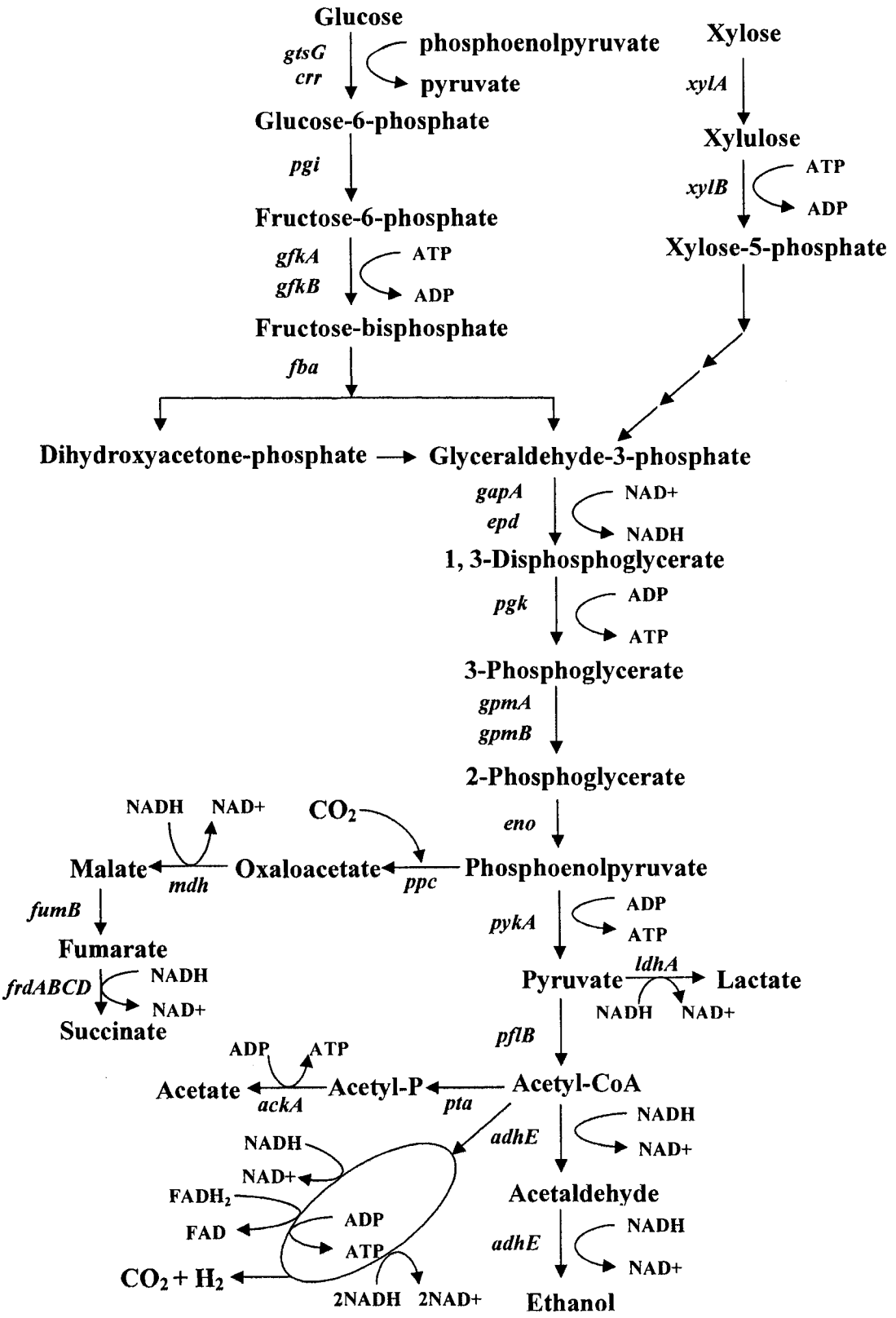

Figure 2.1 A. Carbon flow through central metabolism in wild-type *Escherichia coli*.

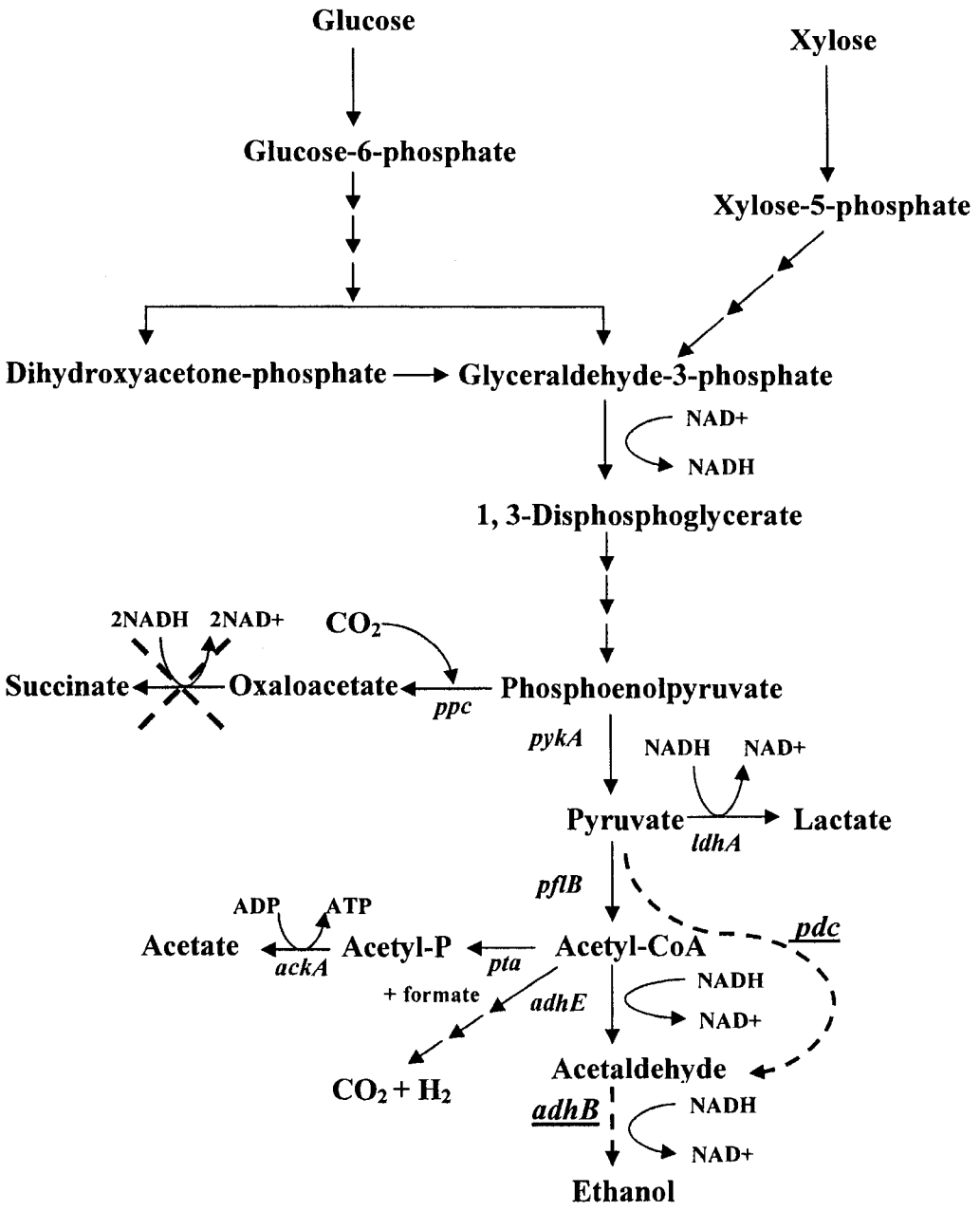

Figure 2.1 B. Carbon flow through central metabolism in the genetically engineered strain *Escherichia coli* KO11.

2.4 Batch Fermentation Kinetics

To help understand and predict microbial behaviour, optimize the fermentation process, determine suitable control strategies, and designing an adequate bioreactor for a given fermentation, a mathematical kinetic model of the process is often required. Fermentation is a complex process where numerous interactions between microbes and culture environment can occur and affect cellular physiological activities and, as a result, the bioprocess performance. A large number of mechanisms, such as the inhibitory effect of substrates, products, and byproducts on cellular growth and product formation, and the response of cellular biological function to the variation of environmental pH, temperature, shear stress, and other fermentation conditions, could all be taken into account in the development of a kinetic model depending on the level of complexity of the models to be developed. Batch fermentation is undoubtedly the most common mode of operation and it has been widely studied and a variety of mathematical models have been proposed. Two kinds of models are available. Considering an individual cell as a multicomponent system, grouping some of cellular constituents into a small number of general classes, and investigating part of the intracellular metabolic way as a result of individual action of enzymes, was designated as a structured model. Considering that all cells are homogeneous, assuming the biomass composition is constant for all operating conditions and treating cells as a single reacting species, was designated as an unstructured model (Sinclair and Kristiansem, 1987; Vázquez and Murado, 2008). Even though in the case of a growing culture the cell population may be heterogeneous, an unstructured model was widely accepted as the simplest and proved to provide a very useful description of experimental reality of the cells. However, special care is needed because the parameters appearing in unstructured kinetic models seldom have a real physical meaning and vary with cellular species or even for the same cells exposed to different experimental conditions.

2.4.1 General unstructured kinetic model

An unstructured model is usually constructed based on fundamental concepts. In a fermentation system, the rate of cell mass production is proportional to biomass

concentration, there usually exists a saturation limit for growth rate on each substrate, and cells need a substrate and may synthesize products even when they do not grow (Nielsen and Villadsen, 1994). Many approaches have been used to develop unstructured kinetic models. The simplest expression relating the kinetic properties to the enzyme reactions is the Michaelis-Menten equation, which is giving by the following expression:

$$\upsilon = \frac{\upsilon_m S}{K_m + S} \tag{2.1}$$

This equation was derived from the assumed irreversible enzyme-catalyzed reaction of a product (P) from a substrate (S). The enzyme (E) must first come into physical contact with the substrate to form an intermediate enzyme-substrate complex (ES). This reaction scheme can be represented as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \tag{2.2}$$

In the Michaelis-Menten equation, the maximum rate (υ_m) would be achieved when all the enzyme molecules form an enzyme-substrate complex (ES) under the conditions that [S] is much greater than [E]. K_m is a combined constant term which is defined as $K_m = \frac{k_{-1} + k_2}{k_1}$ and represents the substrate concentration at which the reaction occurs at half of the maximum rate (υ_m). If it is assumed that the maximum reaction rate (υ_m) is controlled by the concentration of enzymes, which is proportional to the cell concentration, the Michaelis-Menten equation can be written using an analogous expression, the Monod kinetic equation:

$$\frac{1}{X}\frac{dX}{dt} = \mu = \frac{\mu_m S}{K_S + S} \tag{2.3}$$

μ_m (h^{-1}) is the maximum specific growth rate and can be considered as proportional to the concentration of the controlling enzyme; X (g/L) represents the cell concentration; K_S (g/L) is the Monod coefficient, also called the saturation constant because it corresponds to the substrate concentration at which μ is one-half of its maximum.

Monod equation has been found to successfully represent the exponential growth phase followed by the decelerating growth phase during batch growth when a single limiting substrate is used. For the case where there are two substrates, both required for cell growth,

the modified Monod growth expression can be derived (Sinclair and Kristiansen, 1987):

$$\mu = \mu_m \left\{ \frac{S_1}{K_1 + S_1} \right\} \left\{ \frac{S_2}{K_2 + S_2} \right\} \tag{2.4}$$

This expression can only be applied when two required substrates S_1 and S_2 are both present at low concentrations such that the cell growth rate is limited by both. However, Eq. (2.4) does not apply in the case of ethanol production from mixed sugar substrates by yeast or bacteria, because the microorganism can use each sugar independently.

In fact in the case of ethanol production, when cells are cultivated in the presence of glucose combined with another carbon source, glucose is usually consumed first and at a much faster growth rate than the other substrate. The synthesis of the necessary enzymes for the utilization of another substrate is inhibited until glucose is virtually exhausted (Lee and Huang, 2000; Krishnan et al., 1999; Lee et al., 1995; Leksawasdi, 2001; Olsson and Hahn-Hägerdal, 1995). Considering the additive behaviour with respect to the two substrates, the growth rate can be described by adding a catabolite inhibition coefficient as shown in the Eq. (2.5) (Lee et al., 1995; Lee and Huang, 2000; Olsson and Hahn-Hägerdal, 1995):

$$\mu = \frac{\mu_{m1} S_1}{K_1 + S_1} + \frac{\mu_{m2} S_2}{K_2 + S_2} \cdot \xi \tag{2.5}$$

Although the modified Monod equation (2.5) can reasonably express substrate-limited growth in most instances, it is only valid when the growth is slow and cell population density is low.

Many other equations for the specific growth rate have also been proposed and widely used (Shuler and Kargi, 1992).

Logistic Model: $$\mu = \mu_m \left[1 - \frac{X}{X_m}\right] \quad (2.6)$$

Tessier Model: $$\mu = \mu_m \left[1 - e^{-KS}\right] \quad (2.7)$$

Moser Model: $$\mu = \frac{\mu_m S^n}{K_S + S^n} = \mu_m (1 + K_S S^{-n})^{-1} \quad (2.8)$$

Contois Model: $$\mu = \frac{\mu_m S}{K_{SX} X + S} \quad (2.9)$$

The logistic model, proposed by Pierre Verhulst (1838), assumes that the rate of population increase is limited by the maximum biomass concentration that can be achieved and the shape of the growth curve can be represented by a sigmoid shape. Recently, the logistic model has been widely used due to its "goodness to fit" (Speers et al., 2003), but it is only useful when the inhibitory effects of substrates and products are negligible and independent of the substrate concentration. The Moser equation is the most general form of all the substrate-limited growth equations and reduces to the Monod equation for a value of n of unity. Given that the saturation constant of the Contois model is proportional to the cell concentration, its expression is appropriate at describing substrate-limited growth at high cell densities. Even though the classic semi-empirical Monod equation is widely used in describing batch fermentation, in some cases, these other proposed models may give better fits of experimental data than Monod equation (Wang et al., 2004).

2.4.2 Models with growth inhibition factors

The models described in the previous section imply that the growth rate usually increases with the substrate concentration but the rate of increase may be dependent on the substrate concentration. However, in some fermentations, the growth rate starts to decline above a specific value of the substrate concentration and the metabolic activities of cells are reduced due to an inhibition caused by the high substrate concentration. Some of the possible reasons for this phenomenon have been proposed, such as the modification of the chemical potential

of substrates, the alteration of cell membrane permeability, the variation of enzyme activity or the limited osmotolerance of cells (Jones et al., 1981). The noncompetitive substrate inhibition effect, generally observed in ethanol fermentation, is often modelled using the following equation:

$$\mu = \frac{\mu_m S}{K_S + S + S^2 / K_I} \tag{2.10}$$

Ethanol was also reported as an inhibitor of cell growth and activity due to its possible effect on key glycolytic enzymes, cell membrane and constituents (Jones et al., 1981). The noncompetitive ethanol inhibition can be expressed as follows:

$$\mu = \frac{\mu_m S}{K_S + S} \left(1 - \frac{P}{P_m}\right)^n \tag{2.11}$$

Where P_m is the product concentration above which cell growth rate is reduced to zero.

A unique equation describing ethanol fermentation that accounts for the growth inhibition due to substrate and ethanol does not exist because cell growth varies with the type of strain, chemical and physical conditions and the physiological state of the cells. Therefore, the choice between these expressions is partially a matter of convenience as long as the model can effectively represent the experimental results.

2.4.3 Environmental effects

Temperature and pH are two important factors that affect cellular behaviour. Since cell growth and metabolism are truly achieved through a series of chemical reactions, the effect of temperature on the cellular behaviour should be related to an Arrhenius-type equation. For example, the Monod equation could be expressed as follows:

$$\mu = \frac{\mu_m(T)\, S}{K_S(T) + S} \tag{2.12}$$

Where both μ_m and K_S are functions of the temperature and can usually be expressed using the following forms:

$$\mu_m(T) = A_1 \exp\left(-\frac{E_1}{RT}\right) - A_2 \exp\left(-\frac{E_2}{RT}\right) \tag{2.13}$$

and

$$K_S(T) = A_3 \exp\left(-\frac{E_3}{RT}\right) \tag{2.14}$$

The first term in the expression of $\mu_m(T)$ accounts for the general increase in the growth rate with temperature, while the second term is associated with a rapid reduction in the activity of cells when the temperature rises above a certain point (Sinclair and Kristiansen, 1987).

Even though cells are able to adjust their internal hydrogen ion concentration when a change in environmental pH occurs, the maintenance energy required is obviously affected. It is believed that the external pH has an important effect on cell metabolism and cell membrane permeability (Sinclair and Kristiansen, 1987). A general approach using a statistical thermodynamic method to account for the influence of pH on the kinetics of microbial growth was proposed by Tan et al. (1998). Their equation is based on the enzyme kinetics and on the number of functional ionizable groups and binding energy levels as follows:

$$\mu_m = \frac{\frac{k_t}{K_1K_2\cdots K_t}[H]^t + \frac{k_{t+1}}{K_1K_2\cdots K_tK_{t+1}}[H]^{t+1} + \cdots + \frac{k_{m-r}}{K_1K_2\cdots K_{m-r}}[H]^{m-r}}{1 + \frac{1}{K_1}[H] + \frac{1}{K_1K_2}[H]^2 + \cdots + \frac{1}{K_1K_2\cdots K_m}[H]^m} \tag{2.15}$$

Where m is the number of functional ionizable groups in a basic unit, in which t of the m functional ionizable groups must be in a protonated state and r of the m functional ionizable groups in a deprotonated state. The empirical parameters of k_i and K_i are functions of the binding energy levels and/or the reaction rate constants.

Another approach was reported where the pH variation was taken into account in the Monod equation by allowing the model parameters, such as the maximum specific growth rate (μ_m), the cell yield coefficient ($Y_{P/S}$), and the saturation constant (K_S), to vary as a function of pH. The optimal parameter sets are correlated with pH by empirical equations. This method was used successfully to simulate lactic acid production by *Lactobacillus plantarum* (Fu and Mathews, 1999).

2.4.4 Substrate utilization and product formation

Like many other extracellular products released by cells, ethanol formation can be simply represented using the Luedeking-Piret equation:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad (2.16)$$

This expression was originally suggested by Luedeking and Piret (1959) as an empirical model and successfully described the kinetics of lactic acid fermentation. This equation implies that the rate of formation of the product varies linearly with both the instantaneous cell mass concentration (X) and growth rate ($\frac{dX}{dt}$). It also assumes that a sufficient substrate concentration prevails.

In general, the substrate is used to form cell material and metabolic products of which the formation rate is stoichiometrically related to the substrate utilization rate. In most cases, it is regulated by yield coefficients ($Y_{X/S}$ and $Y_{P/S}$). Cells may require substrate even in the absence of growth to provide energy for maintenance. Thus, the substrate utilization kinetics can be modified by introducing a maintenance term, which is usually assumed to be first order with respect to cell concentration (Eq. 2.17).

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + mX \qquad (2.17)$$

2.4.5 Kinetic model of ethanol production by engineered *Escherichia coli* KO11.

The kinetics of ethanol production by *Escherichia coli* KO11 were studied both in a laboratory medium and in a condensate of steam-pretreated willow by Olsson and Hahn-Hägerdal (1995). An empirical model describing the ethanol formation and sugar utilization in the investigated experimental domain was developed and expressed as the modified Monod kinetics including substrate and product inhibition as follows:

$$\frac{dP}{dt} = \frac{X V_m S}{K_m + S\left[1 + S/K_I\right]} \left[1 - \frac{P}{P_m}\right]^n \tag{2.18}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}} \frac{dP}{dt} \tag{2.19}$$

The values of parameters V_m, K_S, K_I, $Y_{P/S}$, P_m and n in Eqs. (2.18-2.19) were determined in single sugar fermentations of glucose or xylose, respectively. The model was then extended to take into account mixed glucose and xylose fermentation using a combination of each equation and adding a catabolic inhibition coefficient (K_I) as follows:

$$\frac{dP}{dt} = \frac{X V_{mg} G}{K_{mg} + G\left[1 + G/K_{ig} + S/K_{igs}\right]} \left[1 - \frac{P}{P_m}\right]^n + \frac{X V_{ms} S}{K_{ms} + S\left[1 + S/K_{is} + G/K_{isg}\right]} \left[1 - \frac{P}{P_m}\right]^n \cdot K_I \tag{2.20}$$

Where G represents the glucose concentration and S the xylose concentration. The mutual inhibition on the sugar utilization rate by glucose and xylose is reflected by the inhibition constants K_{igs} and K_{isg}, respectively. They did not estimate the cell growth kinetics and a constant cell mass concentration determined using an empirical equation (2.21) was applied in the ethanol formation kinetic equations.

$$\Delta X = \frac{4.30\, M}{37.6 + M} + \frac{0.77\, X_0}{X_0 - 0.11} \tag{2.21}$$

Where ΔX gives the growth, M represents the total initial sugar concentration, and X_0 is the initial cell mass concentration.

This model could accurately describe the fermentation performance within the experimental domain with a fixed environmental pH. However, only one experimental result of mixed sugar fermentation with a higher amount of xylose than glucose was simulated using the proposed model. It can hardly be expanded to other ethanol fermentation from lignocellulosic hydrolysates of different biomass sources. In addition, environmental effects were not included in this model. As an attractive and promising industrial ethanol production strain, *Escherichia coli* KO11 needs to be studied more comprehensively to derive a generalized kinetic model that could be used to represent the fermentation kinetic behaviour for different lignocellulosic substrates.

CHAPTER 3

Ethanol Production from Glucose/Xylose Mixtures Using Genetically-Engineered Strain *Escherichia coli* KO11

Abstract

Escherichia coli KO11 is currently one of the most promising strains for industrial ethanol production from lignocellulosic material. In this study, the two main sugars obtained from lignocellulosic materials, glucose and xylose, were fermented separately and simultaneously at various ratios using *Escherichia coli* KO11. In single substrate fermentations, results show that glucose was the preferred substrate and its ethanol conversion rate was 1.5 times higher than the one obtained with xylose. The highest final ethanol yield of 0.56 g/g was produced in 30 g/L xylose media and the highest ethanol productivity of 0.87 g/(L h) was observed when *Escherichia coli* KO11 was cultured on 33 g/L glucose. In mixed-sugar fermentations, a strong interaction occurred where glucose was first consumed followed by the consumption of xylose. The xylose consumption rate after the depletion of glucose significantly decreased with an increase in the initial glucose content. This decreased consumption rate was probably due to the inhibition on xylose metabolic enzymes caused by glucose metabolites.

Keywords: *Escherichia coli* KO11, ethanol, glucose, xylose, fermentation.

3.1 Introduction

The world is confronted with a second wave of energy crisis caused by the expected rapid depletion of nonrenewable fossil fuel, which invariably drives the price of petroleum-derived products to new heights. Indeed, the price of crude oil was in the vicinity of $140 per barrel in July 2008 (EIA, 2008). On the transportation side, the price of gasoline has more than doubled in one decade (EIA, 2008). Using an increasing proportion of renewable and cleaner energy resources must therefore be encouraged to reduce the dependency on oil and mitigate the net addition of greenhouse gases to the atmosphere. Ethanol-blend fuel is one of the possible actors in the overall strategy required to meet those needs. Recently, ethanol production by fermentation of biomass, such as starch and cellulose, has received renewed interest. Considerable development is currently taking place in order to decrease the production cost and at the same time to reduce the net CO_2 emission. Lignocellulosic material, often available as a lower-value substrate, is undoubtedly a viable option for reducing the ethanol production cost, especially if agricultural and forest wastes are used as the primary substrates. This natural and abundant polymer is estimated to account for about 50% of the biomass in the world (Zaldivar et al., 2001).

Unlike starch, lignocellulose is a more complex substrate composed of a mixture of polymers: cellulose (35%~50%), hemicellulose (20%~35%), and lignin (Saha, 2004). Except for lignin, which is a rigid aromatic polymer and not a carbohydrate, cellulose and hemicellulose can be completely hydrolyzed into fermentable sugars which can subsequently be partly converted to ethanol. Unlike corn processing which only gives glucose, lignocellulosic hydrolysates include small amounts of arabinose, galactose and mannose, and more significant amounts of glucose and xylose (typically comprising roughly 90% of released sugars) along with some cellobiose. To achieve the best possible conversion to ethanol and to ensure economic feasibility, it is required that the selected microorganisms be able to fully utilize all sugar units and more particularly both glucose and xylose.

Escherichia coli is the most ubiquitous and well known gram-negative microorganism. It can

metabolize a wide spectrum of sugars, including glucose, xylose, and arabinose. It was one of the first successful metabolic engineering strains to selectively produce ethanol (Ingram et al., 1987). *Escherichia coli* KO11, which was patented by Ingram and his colleagues in 1998 (US patent 5821093), is currently one of the best recombinant ethanologenic bacterial strains. It has high ethanol productivity and high tolerance to hemicellulosic hydrolysates (Bothast et al., 1999). It has been successfully developed for the fermentation of hydrolysates of pine waste (Barbosa et al., 1992), willow (von Sivers et al., 1994), rice hull (Moniruzzaman and Ingraml., 1998), sugar cane bagasse (Takahashi et al., 2000), corn cob (de Carvalho Lima et al., 2002), cotton gin residues (Agblevor et al., 2003), corn stovers (Kim et al., 2006), and brewery wastewater (Rao et al., 2007) to ethanol. In spite of some reports about the phenotypic instability of *Escherichia coli* KO11 in batch or continuous cultures (Lawford and Rousseau, 1995; Lawford and Rousseau, 1996), experiments performed by Dumsday et al. (1997, 1999) showed that *Escherichia coli* KO11 gave a stable ethanol production on glucose, mannose, xylose and galactose substrates in repeated batch culture and on glucose substrates in continuous culture without antibiotics. Furthermore, it has been reported to be relatively resistant to potential process errors, including deliberate contamination, temperature swing, and pH swing during ethanol fermentation (Moniruzzaman et al., 1998). Consequently, *Escherichia coli* KO11 was considered to be currently the most promising strain for the industrial scale ethanol production.

The hydrolysates from different lignocellulosic biomass result in different ratios of glucose to xylose. In this study, it desired to investigate the kinetics of cell growth, substrate utilization and ethanol production on glucose, xylose and their mixtures throughout the fermentation using *Escherichia coli* KO11.

3.2 Materials and Methods

3.2.1 Microorganism

Escherichia coli KO11 was obtained from the American Type Culture Collection (ATCC) as ATCC 55124. It is a mutant ethanol producing strain in which the recombinant genes *pdc* and *adh*B, introduced from *Zymomonas mobilis*, for the production of ethanol as well as the *cat* gene conferring resistance to chloramphenicol (Cm) were integrated into the

chromosome. For short term storage, all cultures were stored at 4°C after incubation on solid Luria Bertani (LB) medium (composition described below) supplemented with 20 g/L glucose and 40 mg/L chloramphenicol at 37°C for 24 h. For long term storage, a medium of similar composition without agar was prepared for stock cultures and cultivated at 37°C with 100 rpm orbital rotation. After 9 hours, the cultures were diluted in sterile 20% glycerol (v/v) and then stored in 1 mL volumes at -80°C.

3.2.2 Culture medium and conditions

The solid LB medium contains per liter: 10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 15 g agar. For the liquid LB medium, agar was not included. Cultures were grown in a complex medium containing liquid LB medium supplemented with 20 g/L of glucose or xylose, unless otherwise specified. Except for the stock culture and the solution used for inoculation, aqueous LB media and sugar solution were sterilized separately by autoclaving at 121°C for 15 min. Filter-sterilized chloramphenicol was only added to the autoclaved stock culture after cooling. Inoculum was prepared by transferring 2~3 large single isolated colonies from agar plates to 100 mL culture medium, which contains 20 g/L glucose as carbon source for glucose-only fermentation or 20 g/L xylose for xylose-only and mixed-sugar fermentation, after shaking at 30°C for nearly 8~9 h on a rotary shaker at 100 rpm to ensure the cells were in the late exponential phase based on a predetermined growth curve (Appendix A).

3.2.3 Fermentation conditions

Fermentations were conducted with approximately 10% (v/v) inoculum in order to get an initial optical density of cells at 550 nm of 0.5 with an approximate cell concentration of 0.12 g/L. This relatively high level of inoculation was used to reduce the effect of lag phase and shorten the log phase time to reach the maximum cell mass concentration. Experiments were all performed in 300 mL Erlenmeyer flasks containing 100 mL medium at 30°C and placed in a rotary shaker (MaxQ 5000, Geneq INC.) with 100 rpm orbital rotation, which was determined by previous studies of the reported optimal fermentation conditions (Appendix B). The flask was sealed with a rubber stopper in which was inserted a sterilized wine air-lock partly filled with distilled water to maintain anaerobic conditions. No pH control was

performed during cultivation. The initial pH ranged between 6.7 and 5.3 which was adjusted by the addition of 2M NaOH solution. The sugar mixtures were prepared separately with different glucose to xylose ratios (1:0, 1:1, 1:2, 1:3, 3:1, 2:1, 0:1). All experiments were carried out in duplicate parallel flasks.

3.2.4 Analytical methods

Cell concentration was estimated using a pre-determined calibration curve (Appendix C) between the cell dry weight and the optical density at a wavelength of 550 nm measured using a spectrophotometer (Perkin Elmer Lambda 25 UV/Vis). For preparing the calibration curve, the cell dry weight was measured using pre-weighed alumina weighing dishes, which were dried in an oven at 100°C for approximately 24 h and then weighed. Cell pellets were obtained from the centrifuged fermentation broth including duplicate washing to eliminate residual elements of the fermentation broth before drying. The calibration curve showed a linear relationship of one unit of turbidity at 550 nm corresponding to 0.2484 g dry cells/L at absorbance range of 0.1-0.4.

Fermentations were monitored by taking 2 mL samples at appropriate time intervals for analysis of cell mass by measuring the optical density. Before testing, samples needed to be diluted to insure that the optical density was in the range of 0.1 to 0.4. The pH of the broth was measured with a Barnant 30 pH meter, which was calibrated with pH reference buffer solutions of 4.0 and 7.0 prior to each measurement. Glucose, xylose, and ethanol concentrations in the broth were determined by HPLC using a Sugar-Pak[I] cation-exchange column (6.5mm×300mm) obtained from Waters Ltd., On, CA, a Waters Breeze system and a Waters 2414 refractive index (RI) detector. The separation was performed at 90°C using distilled de-ionized water as the mobile phase at a flow rate of 0.5 mL/min. To maintain the equilibrium and prevent inversion, 50 mg/L calcium EDTA was added in the mobile phase. Samples taken for HPLC analysis were centrifuged at 12000 g for 6 min to remove cells and then, the supernatant was filtered with a 0.22 μm Millipore membrane filter. The resulting solution was diluted with distilled de-ionized water to give a final concentration in the range of 0.01 g/L to 3 g/L of sample. Calibration curves and chromatograms for sugar and ethanol

are shown in Appendix D. Standard sugar and ethanol solutions were run periodically to ascertain the accuracy of the calibration curves.

For sugar analysis, the recorded chromatograms showed a concentration profile with two overlapping peaks because of similar retention times of glucose and xylose at 9.871 min and 10.745 min, respectively. To estimate the individual peak of each sugar, the chromatograms were analyzed by peak-deconvolution software, Cutter version 5.0, created by J. G. Shackman, using the ETG (Empirically Transformed Gaussian) function (Shackman et al., 2004). The accuracy of the deconvoluted results was evaluated by testing single sugar solutions of known concentration separately and mixtures of them at different ratios. Area measurements of deconvoluted peaks are shown in Appendix E and the estimated error was within 4% of the true value.

3.2.5 Calculation of fermentation parameters

The ethanol yield ($Y'_{E/S}$) and the cell mass yield ($Y'_{X/S}$) were assumed constant for a given fermentation and were calculated as the maximum amounts of ethanol and cell mass produced divided by total amount of substrates consumed. The specific growth rate (μ) that was reported here by calculating $\Delta \ln(X)$ divided by Δt, where X was the cell mass concentration at tangent points, varied in the exponential phase of cell growth which is around the first 6 hours of cultivation. The volumetric ethanol productivity (Q_E), glucose utilization rate (Q_G), and xylose utilization rate (Q_X) were estimated from the linear slope of the concentration versus time plot after the initial delay. In mixed-sugar fermentations, Q_X was calculated after glucose exhaustion. A maximum theoretical ethanol yield of 0.51 g ethanol/g sugar from glucose or xylose was used as a basis for the calculation of sugar conversion efficiency (C), defined as the ratio of the ethanol yield and the theoretical yield.

3.3 Results and Discussion

3.3.1 Fermentation with individual sugars

The following section discusses the results of growing *Escherichia coli* KO11 on single-sugar substrates for various initial glucose or xylose concentrations in batch culture without pH control. The objective of these experiments was to study the effect of various initial sugar concentrations on cell growth, substrate utilization, ethanol production, and duration of fermentation in order to gain a better understanding of the fermentation of *Escherichia coli* KO11 when a single substrate is used and to be able to compare the results with those obtained with more than one substrate.

3.1.1.1 Cell growth

Figures 3.1 and 3.2 present the cell density profiles as a function of time during batch fermentations with an initial glucose concentration in the range of 18 g/L to 65 g/L and with an initial xylose concentration in the range of 19 g/L to 73 g/L, respectively. Cultivated on glucose-only and xylose-only media, the microorganism showed a rapid growth within the first 12 and 20 h, respectively. There was no significant period of adaptation because of the high initial cell density and the exponential growth phase started immediately at the beginning of incubation in all experiments. For each substrate, similar growth rates were observed at all concentrations except for the highest initial xylose concentration (73 g/L) where a substrate inhibition started to prevail. The duration of the exponential growth phase and the final cell mass concentration increased with the initial sugar concentration. However, it appears that there exists a maximum cell mass concentration in the range of 1.8 to 2.0 g/L for both substrates. For experiments performed at higher sugar concentrations, following the exponential growth phase up to the maximum cell mass concentration, a stationary phase was observed for a considerable time period until all the substrate had been consumed.

Only one experiment behaved differently to the behaviour discussed above, the one performed with an initial xylose concentration of 73 g/L (Figure 3.2). The growth rate was smaller than for lower concentrations and a maximum cell concentration of 2.52 g/L was

obtained followed by a slow decrease toward the maximum cell mass concentration observed for all the other fermentations. It may be attributed to the different strain of *Escherichia coli* KO11 which had to be purchased because the first one appeared to have lost its ability to use xylose.

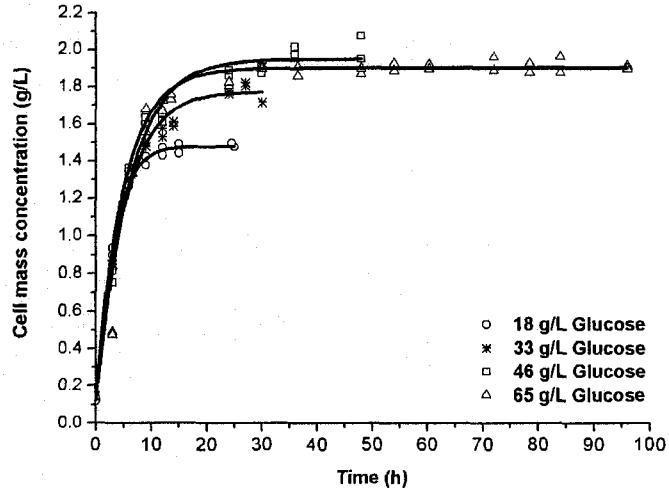

Figure 3.1. Growth of *Escherichia coli* KO11 on pure glucose substrate with an initial concentration ranging from 18 g/L to 65 g/L under anaerobic conditions and an initial cell mass concentration of 0.12 g/L. Solid lines are the sigmoidal curve fit using data of the two replicates.

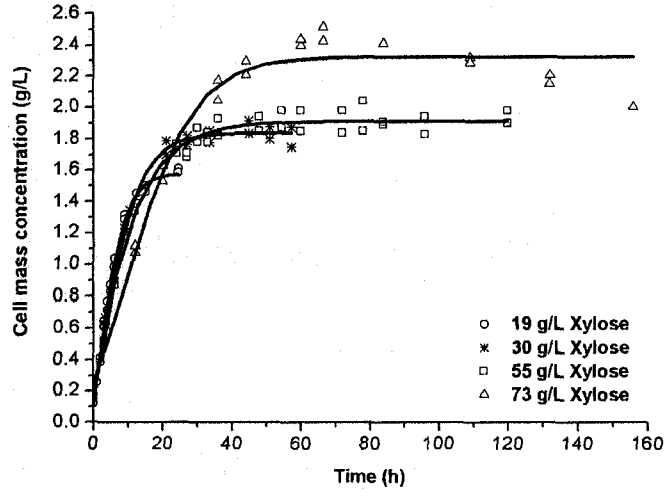

Figure 3.2. Growth of Escherichia coli KO11 on pure xylose substrate with an initial concentration ranging from 19 g/L to 73 g/L under anaerobic conditions and an initial cell mass concentration of 0.12 g/L. Solid lines are sigmoidal curve fits using data of the two replicates.

3.1.1.2 Sugar consumption and ethanol production

Figures 3.3 and 3.4 present the substrate and ethanol concentrations for fermentations performed with glucose and xylose, respectively. For the set of glucose-only experiments, no lag phase was present and nearly identical rates of sugar consumption and ethanol production were observed even though the initial glucose concentration increased from 18 g/L to 65 g/L. It can be hypothesized that there is no substrate inhibition within the observed concentration range. This finding differs from the observations of Olsson. (1995). In their experiments, *Escherichia coli* KO11 was grown under a controlled pH of 6.0 on the glucose-only medium with an initial cell mass concentration of 1.1 g/L. They observed an increase in ethanol production rate for initial glucose concentrations up to 30 g/L and then the rate decreased showing a significant inhibition. This may be due to the higher sodium chloride concentration (10 g/L) used in this investigation in comparison with the 5 g/L they used. In addition, in our investigation no base was added to the broth for pH control.

For experiments performed with xylose as a single substrate (Figure 3.4), with an increase in the initial xylose concentration up to 73 g/L, a slightly increased sugar consumption rates and ethanol production rates were observed. These rates are however smaller than the rates observed for glucose. Xylose was completely consumed at the end of the experiments except for the experiment performed at 73 g/L and the concentration of ethanol continuously increased throughout the fermentation. In the experiment with an initial xylose concentration of 73 g/L, xylose was not completely consumed and a residual xylose concentration of 4 g/L was observed. This result indicates that there was no apparent substrate inhibition because the initial rate of xylose consumption was not affected but the null rate of consumption at the end of fermentation is likely due to the combined effects of reduced pH, by-product accumulation and ethanol inhibition on *Escherichia coli* KO11.

Extended tails of sugar consumption were observed at the end of both glucose-only and xylose-only experiments and were longer with an increase in the initial sugar concentration (Figures 3.3 and 3.4). These tails at the end of fermentation are undoubtedly caused by accumulated ethanol and other metabolites.

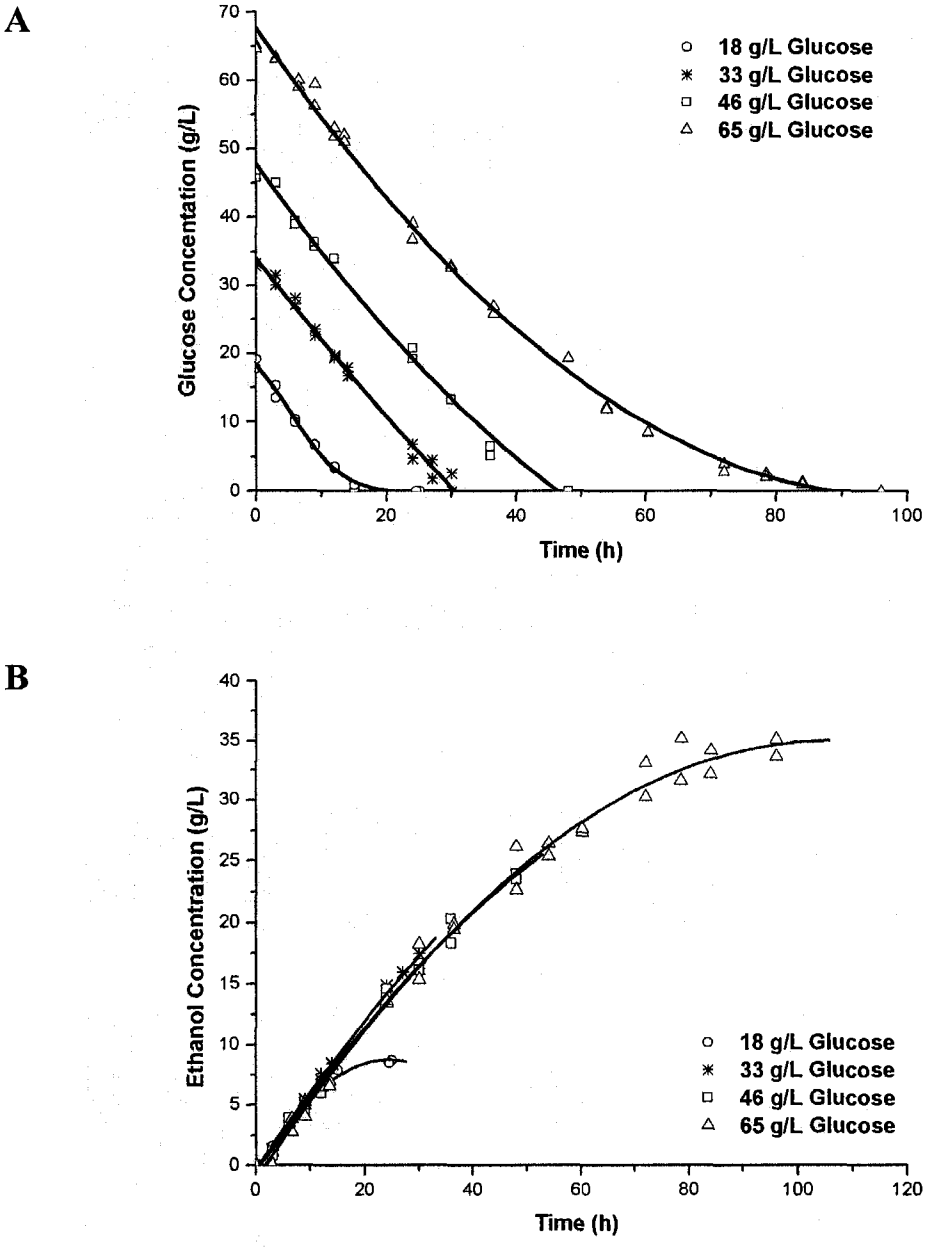

Figure 3.3. Substrate consumption (A) and ethanol production (B) by *Escherichia coli* KO11 with pure glucose having an initial concentration ranging from 18 g/L to 65 g/L under anaerobic conditions and with an initial cell mass concentration of 0.12 g/L. Solid lines are sigmoidal curve fits based on duplicate experimental data.

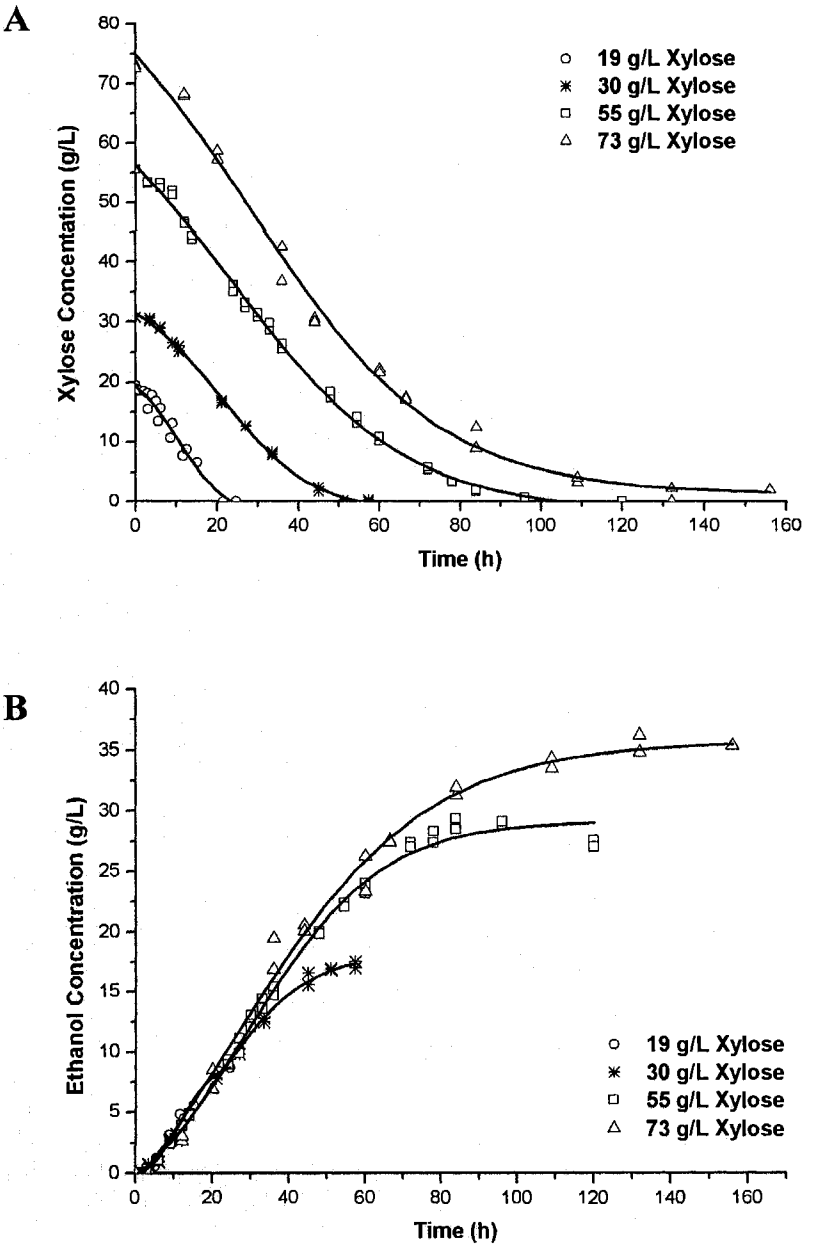

Figure 3.4. Substrate consumption (A) and ethanol production (B) by *Escherichia coli* KO11 with pure xylose with an initial concentration ranging from 19 g/L to 73 g/L under anaerobic conditions and an initial cell mass concentration of 0.12 g/L. Solid lines are sigmoidal curve fits based on duplicate experimental data.

The fermentation parameters of single-sugar experiments are summarized in Table 3.1. A maximum ethanol concentration of 35.79 g/L was obtained when *Escherichia coli* KO11 was cultivated in a medium containing xylose at a concentration of 73 g/L with a resulting ethanol yield of 0.50 g/g. The calculated ethanol productivity of *Escherichia coli* KO11 cultivated on single substrates was around 0.53 $g \cdot L^{-1} \cdot h^{-1}$ for glucose-only experiments and 0.47 $g \cdot L^{-1} \cdot h^{-1}$ for xylose-only experiments, respectively. These results indicate that there was no apparent substrate inhibition. A higher value of ethanol productivity of 1.3 $g \cdot L^{-1} \cdot h^{-1}$ was obtained by Ohta et al. (1991) for an 80 g/L xylose fermentation under pH control. This higher value is probably due to initial biomass concentration (0.33 g/L) that was thrice the initial cell mass concentration was used in this investigation. Sugar consumption rate varied from 1.06 $g \cdot L^{-1} \cdot h^{-1}$ to 1.15 $g \cdot L^{-1} \cdot h^{-1}$ for glucose-only experiments and from 0.82 $g \cdot L^{-1} \cdot h^{-1}$ to 0.97 $g \cdot L^{-1} \cdot h^{-1}$ for xylose-only experiments. The lower sugar consumption rate of xylose compared to that of glucose can be explained by the adenosine triphosphate (ATP) yield from xylose metabolism (0.67 ATP from per xylose) that is one third of the yield from glucose (2 ATP per glucose) on a molar basis. This is due to the separate energy requirement of xylose fermentation for substrate uptake (ATP-dependent transporter or proton symport) which prior to sugar phosphorylation (xylulokinase) (Underwood et al., 2002).

The overall ethanol yield ($Y'_{E/S}$) varied from 0.44 g/g to 0.56 g/g and was found to be relatively independent of the initial sugar concentration. Ethanol yields, often exceeding the theoretical yield of 0.51, were achieved in both glucose-only and xylose-only fermentations and have reached values up to 0.56 g/g. The higher yield is undoubtedly due to the carbon present in the LB broth and which led to ethanol (Ohta et al., 1991). The lower specific cell growth rate (μ) in xylose-only media compared to that in glucose-only media (Table 3.1) has been attributed to slower xylose metabolism, resulting in a lower ATP formation, which is necessary to maintain growth (Underwood et al., 2002). Since there is a maximum achievable biomass concentration, the biomass yield has no significance for the entire fermentation. Comparing ethanol productivity, sugar consumption rate, specific growth rate, and total fermentation time of *Escherichia coli* KO11 reveals a higher fermentation rate for glucose than for xylose, but the final ethanol and biomass yields are independent on the type

of sugar.

Table 3.1. Parameters for single-sugar fermentation by *Escherichia coli* KO11 with different initial sugar concentrations.

Initial sugar concentration (g/L)	E_{max} (g/L)	Q_E ($g \cdot L^{-1} \cdot h^{-1}$)	Q_G or Q_X ($g \cdot L^{-1} \cdot h^{-1}$)	$Y'_{E/S}$ (g/g)	$Y'_{X/S}$ (g/g)	μ (h^{-1})	C (%)	t[a] (h)
Glucose-only								
18	8.74	0.52	1.11	0.47	0.081	0.39	92	15
33	17.47	0.57	1.15	0.51	0.056	0.37	100	30
46	23.93	0.53	1.07	0.51	0.044	0.37	100	48
65	35.12	0.53	1.06	0.53	0.030	0.34	104	85
Xylose-only								
19	8.70	0.50	0.97	0.44	0.085	0.35	86	21
30	17.51	0.45	0.82	0.56	0.061	0.29	110	51
55	27.61	0.47	0.86	0.53	0.036	0.27	104	97
73	35.79	0.52	0.90	0.50	0.034	0.13	99	132

[a] Time required for the maximum ethanol concentration to be reached, or until termination of the cultivation.

3.3.2 Fermentation with mixed sugars

Xylose and glucose are the main contributors to the total reducing sugar present in lignocellulosic hydrolysates. This section presents the results obtained with *Escherichia coli* KO11 for fermentations where both glucose and xylose were present. Various ratios of the two sugars were used while keeping the total sugar concentration in the range of 20 g/L to 40 g/L. The biomass, sugar and ethanol concentrations were measured as a function of time for all fermentations. The plots of these variables as a function of time are presented in Figures 3.5-3.8. Table 3.2 summarizes the calculated fermentation parameters with *Escherichia coli* KO11 for mixed-sugar experiments. Except for the set of experiments performed at a 40 g/L total sugar concentration, *Escherichia coli* KO11 was able to use effectively these

combinations of sugars. For a total sugar concentration of 40 g/L, the rate of consumption of xylose was very much lower than the rate obtained at 20 and 30 g/L of total sugar. Nevertheless, for all three total sugar concentrations, the overall ethanol production yield was approximately 91% of the theoretical yield.

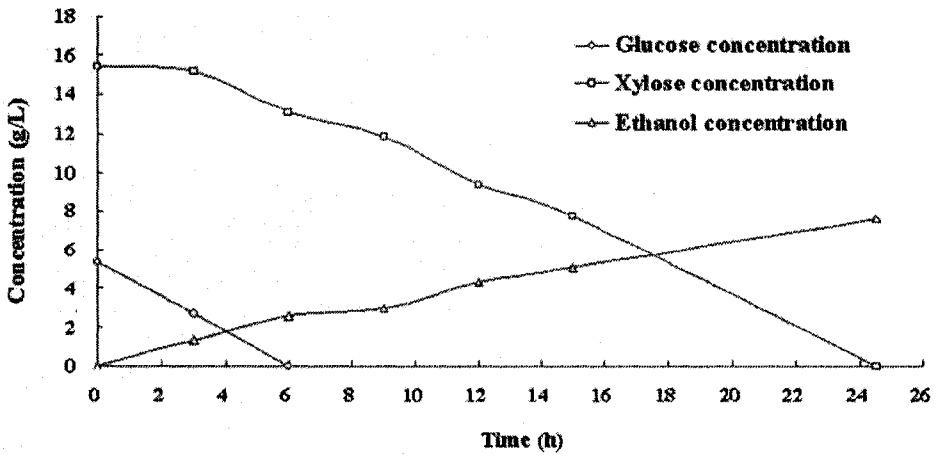

Figure 3.5. Ethanol production from mixed sugars (Glucose: Xylose = 1:3) of 20 g/L total concentration in anaerobic fermentations with *Escherichia coli* KO11.

In mixed-sugar media, the exponential growth phase terminated when cell growth reached the maximum cell concentration ranged from 1.4 g/L to 2.0 g/L. The cell mass yield ($Y'_{X/S}$) decreased with an increase in the total sugar concentration. The average values of the biomass yield were 0.087 g/g, 0.068 g/g, and 0.055 g/g based on initial sugar concentrations of 20, 30 and 40 g/L, respectively. Because of the observed maximum cell population, these yields simply reflect that the substrate is used to achieve this maximum and most of the remaining sugar is used for ethanol production. For all experiments, because of the large amount of inoculum used, there was no lag phase and the exponential growth phase started immediately. The specific growth rate in the exponential phase for all experiments was similar and ranged from 0.27 to 0.39 h^{-1} and was independent of the glucose to xylose ratio.

Table 3.2. Parameters for mixed-sugar fermentation by *Escherichia coli* KO11 with initial sugar concentrations of 20g/L, 30 g/L, and 40 g/L.

Initial sugar concentration (g/L)	Ratio of glucose to xylose	$Y'_{X/S}$ (g/g)	$Y'_{E/S}$ (g/g)	C (%)	μ (h^{-1})	Q_G ($g \cdot L^{-1} \cdot h^{-1}$)	Q_X[b] ($g \cdot L^{-1} \cdot h^{-1}$)	Q_E ($g \cdot L^{-1} \cdot h^{-1}$)	Q_E[b] ($g \cdot L^{-1} \cdot h^{-1}$)	t[a] (h)
20	0:1	0.085	0.44	86	0.35	-	0.97	0.50	0.50	21
	1:3	0.089	0.47	93	0.35	0.93	0.78	0.49	0.44	24
	1:2	0.098	0.49	97	0.35	1.36	0.70	0.54	0.39	30
	1:1	0.093	0.40	78	0.37	1.53	0.34	0.53	0.12	36
	2:1	0.086	0.42	82	0.36	1.46	0.23	0.50	0.10	48
	3:1	0.078	0.42	82	0.37	1.20	0.21	0.59	0.10	48
	1:0	0.081	0.47	92	0.39	1.11	-	0.52	-	15
30	0:1	0.061	0.56	110	0.29	-	0.82	0.45	0.45	51
	1:3	0.078	0.45	88	0.35	0.62	1.02	0.43	0.48	23
	1:2	0.070	0.47	93	0.34	1.38	0.99	0.45	0.25	35
	1:1	0.068	0.46	91	0.37	1.48	0.35	0.48	0.16	47
	2:1	0.070	0.44	87	0.38	1.79	0.08	0.76	0.04	95
	3:1	0.074	0.46	77	0.38	1.77	0.10	0.86	0.03	95
	1:0	0.056	0.51	100	0.37	1.15	-	0.57	-	30
40	0:1	0.036	0.53	104	0.27	-	0.860	0.47	0.47	97
	1:3	0.069	0.46	91	0.35	0.61	0.082	0.76	0.03	>300
	1:2	0.070	0.45	87	0.36	0.71	0.077	0.68	0.03	>300
	1:1	0.062	0.47	84	0.31	1.23	0.089	0.87	0.03	>300
	2:1	0.054	0.45	77	0.34	1.30	0.042	0.87	0.02	>300
	3:1	0.053	0.45	75	0.34	1.35	0.028	0.84	0.01	>300
	1:0	0.044	0.51	100	0.37	1.07	-	0.53	-	48

[a] Time required for the maximum ethanol concentration to be reached, or until termination of the cultivation.

[b] Calculated after glucose exhausted.

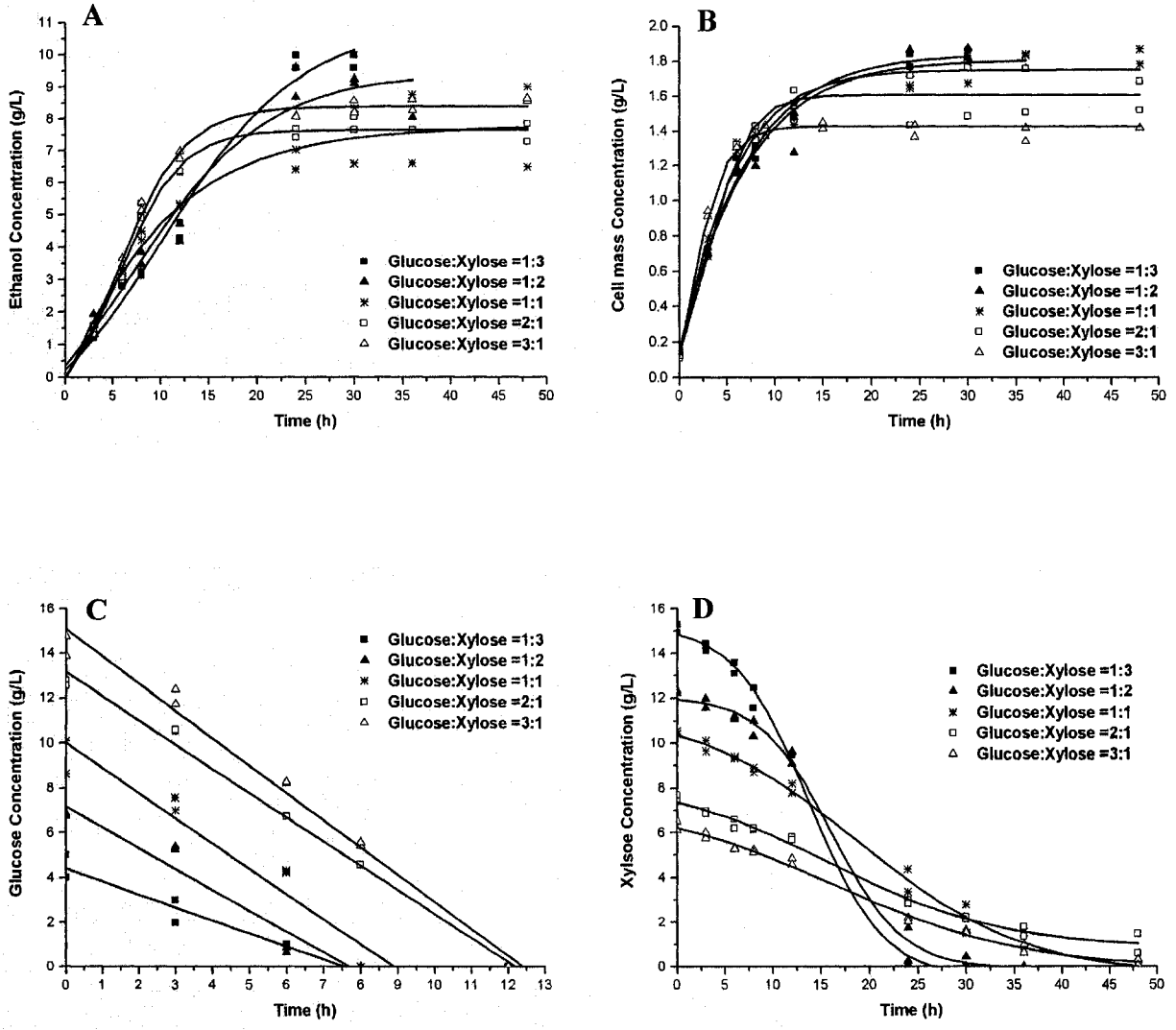

Figure 3.6. Ethanol production (A), cell growth (B), glucose consumption (C), and xylose consumption (D) by *Escherichia coli* KO11 in mixed 20 g/L total sugar media with glucose to xylose ratios of 1:3, 1:2, 1:1, 2:1, and 3:1 and with an initial cell mass concentration of 0.12 g/L. Solid lines are the sigmoidal curve fits of duplicate experimental data.

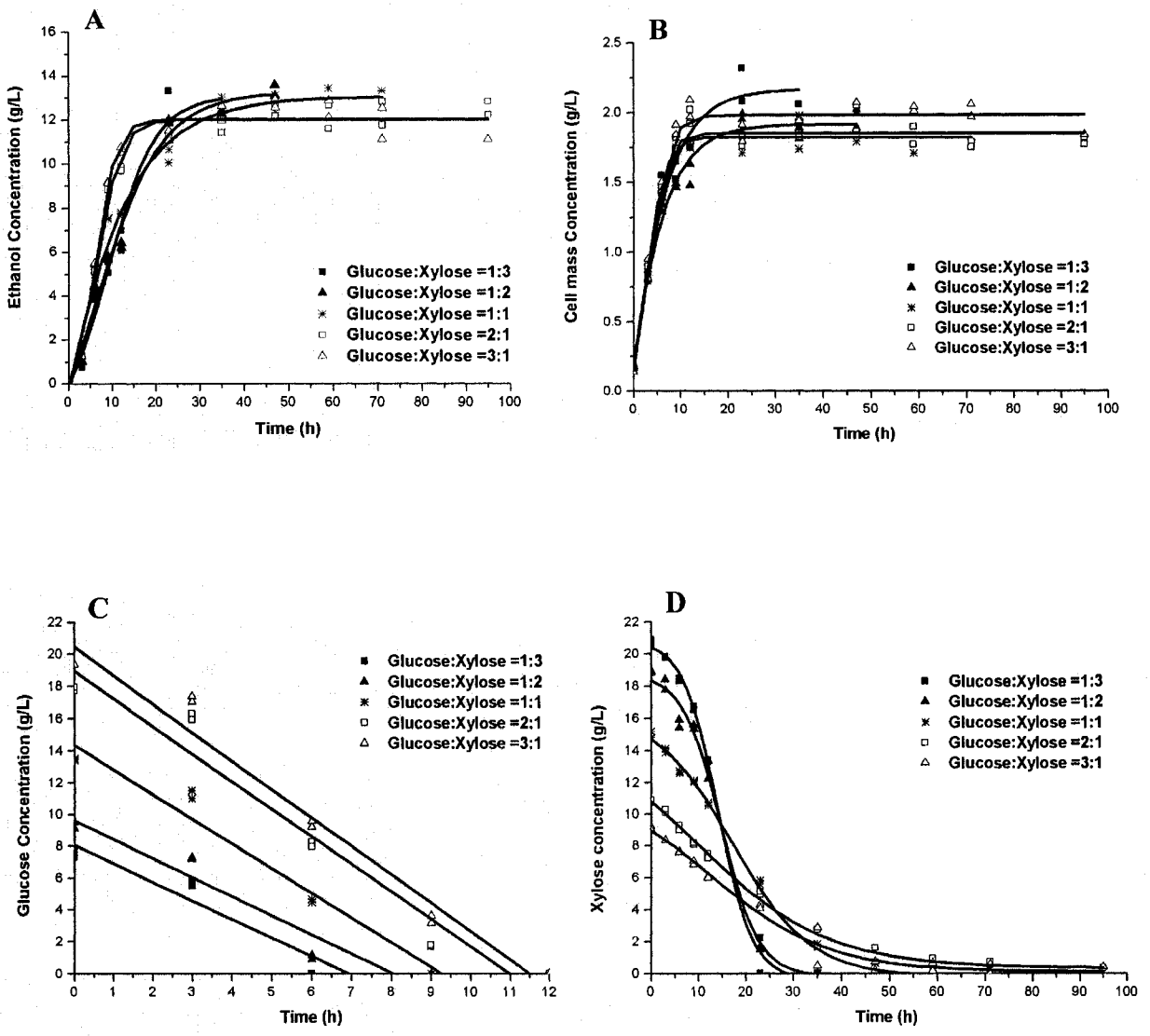

Figure 3.7. Ethanol production (A), cell growth (B), glucose consumption (C), and xylose consumption (D) by *Escherichia coli* KO11 in mixed 30 g/L total sugar media with glucose to xylose ratios of 1:3, 1:2, 1:1, 2:1, and 3:1 and with an initial cell mass concentration of 0.12 g/L. Solid lines are the sigmoidal curve fits of duplicate experimental data.

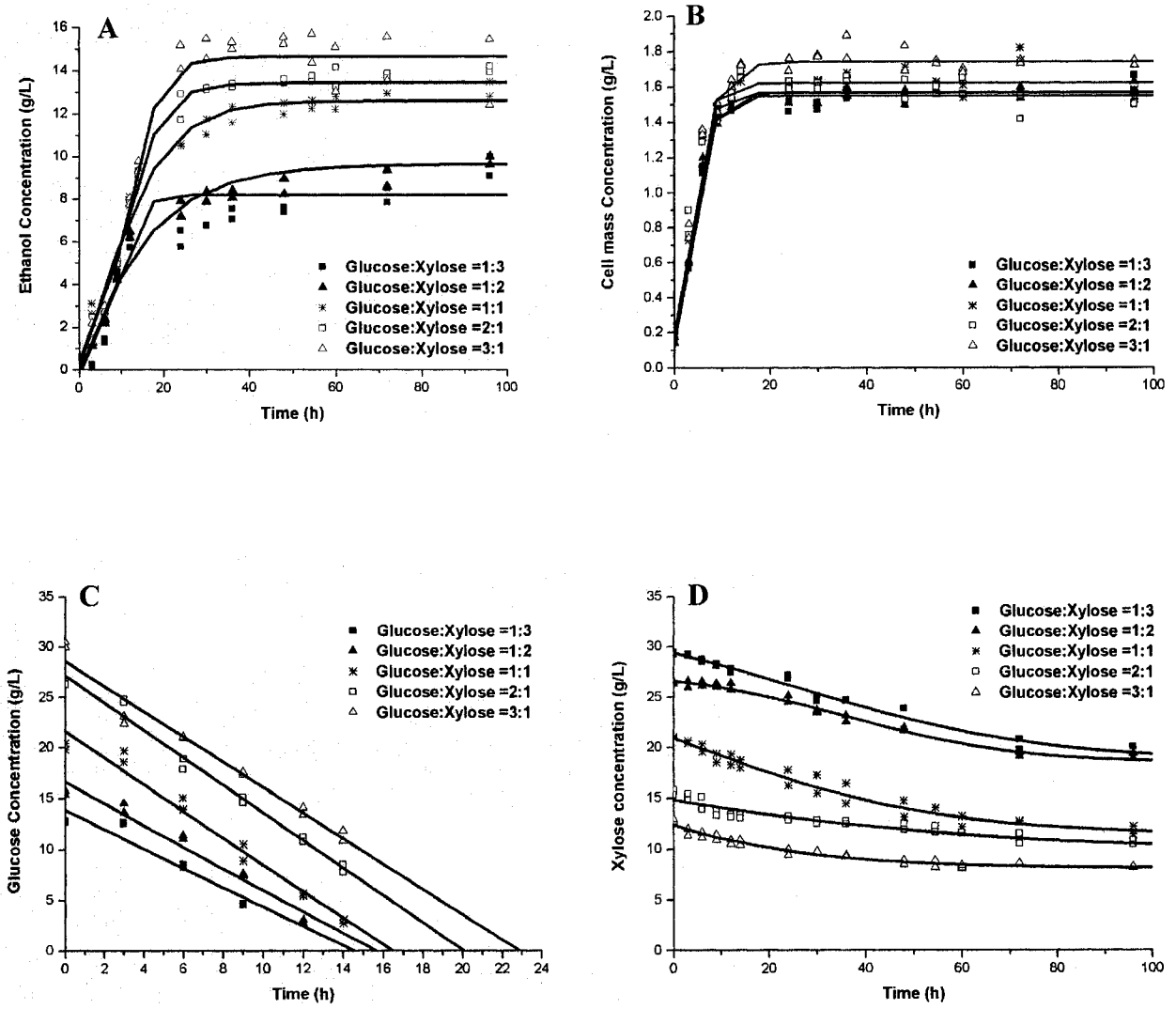

Figure 3.8. Ethanol production (A), cell growth (B), glucose consumption (C), and xylose consumption (D) by *Escherichia coli* KO11 in mixed 40 g/L total sugar media with glucose to xylose ratios of 1:3, 1:2, 1:1, 2:1, and 3:1 and with an initial cell mass concentration of 0.12 g/L. Solid lines are the sigmoidal curve fits of duplicate experimental data.

The consumption of glucose and xylose started in the first hours of fermentation but the xylose consumption rate was much slower as compared to that of glucose. This observation indicates that *Escherichia coli* KO11 preferred glucose. The presence of glucose appeared to slow down the fermentation of xylose with a severe lag and xylose metabolism was only dominant after glucose was depleted (Figure 3.5). When the cells were grown on xylose-only media with the same sugar concentration, there was no lag in xylose metabolism and a higher consumption rate was obtained compared to that of the mixed sugar fermentation even after glucose was exhausted. The reason for this behaviour is the inhibition on xylose utilization caused by glucose (Cirino et al., 2006).

In all sets of experiments at different total initial sugar concentrations, *Escherichia coli* KO11 showed similar results where a mutual effect of glucose and xylose was observed. Glucose was depleted in 12 h in both 20g/L and 30 g/L mixed-sugar fermentations, (Figures 3.6 and 3.7). That period was extended to 24 h when *Escherichia coli* KO11 was cultivated in the media containing 40 g/L total sugar (Figure 3.8). For the sets of experiments performed with 20 g/L and 30 g/L sugar concentration, all sugars were consumed and the final ethanol yield ranged from 0.42 g/g to 0.49 g/g which was lower than the yield obtained from the single substrates experiments. However, the rate of ethanol formation strongly depended on the concentration ratio of glucose to xylose.

For a constant total sugar concentration, the rate of glucose consumption was relatively independent of the proportion of glucose such that the time required to consume glucose was proportional to the initial glucose concentration. When glucose was depleted, the rate of consumption of xylose increased significantly. It is interesting to note that the rate of xylose consumption is much greater for a low proportion of initial glucose. As Figures 3.6 D and 3.7 D show, the slopes of all glucose concentration versus time plots are approximately identical and the slopes of xylose plots decreased with a increase in initial glucose concentration. This particular behaviour indicates that there are very strong mutual effects of glucose and xylose (Figures 3.6 and 3.7), which is partly in agreement with the findings of Olsson and Hahn-Hägerdal (1995). However, the final ethanol yield was independent of the glucose to xylose ratio.

Similar results were observed for the set of experiments performed at 40 g/L total sugar concentration even if the rate of consumption of xylose became very small at the end of fermentation and a large residual xylose concentration remained in the broth. The acceleration of the xylose consumption rate with a decrease in glucose concentration for 20 and 30 g/L was not reported by other researchers. Further experiments are required to elucidate this observed phenomenon from a physiological point of view and an enzyme activity study.

Even though the initial ethanol volumetric productivity using *Escherichia coli* KO11 cultivated in 40 g/L total sugars was considerably higher (Table 3.2), no ethanol production and sugar consumption were detected after approximately 160 h fermentation. This is probably the integrated effect of low pH stress (below 4.9) and metabolite inhibition. Much higher mixed sugar concentration (60g/L glucose and 30 g/L xylose) has been effectively fermented by Lindsay et al. (1995) and almost 38 g/L ethanol was obtained. The possible reason for the high inhibition happening in this study is the low ambient pH which leads to more protons being transported out of the cell by an ATP-consuming transport system to maintain the intracellular pH (Olsson and Hahn-Hägerdal, 1995), which together with the lower energy yield from xylose negatively influenced the fermentation rate. If pH were controlled in the optimal range, unmetabolized residual xylose might be avoided.

3.4 Conclusions

The ability of *Escherichia coli* KO11 to ferment glucose and xylose separately and simultaneously at various ratios was investigated using the same initial cell concentration of 0.12 g/L under anaerobic conditions without pH control. In single-sugar experiments, ethanol yields are similar in both glucose-only and xylose-only media, but sugar consumption rate and ethanol production rate with glucose were almost 1.5 times higher than those with xylose. There is no substrate inhibition observed in both sugar consumption and ethanol production for all glucose-only and xylose-only experiments. When the initial xylose concentration in the medium was 73 g/L, a strong combined inhibition was observed after 120 h with an incomplete utilization of xylose. It is hypothesized that metabolite inhibition and lower pH (< 5.0) contributed to the

incomplete fermentation.

In mixed-sugar fermentations with no pH control, glucose and xylose were utilized simultaneously but the consumption rate of xylose was much slower and a severe lag was observed even after glucose was exhausted. Xylose metabolism was only dominant after glucose was consumed. With a high percentage of glucose, sugar depletion time increased leading to a low ethanol production rate. However, the final ethanol yield was not affected. These results show that *Escherichia coli* KO11 has a higher ethanol production rate when growing on high xylose fractions. It has the potential of fermenting pretreated biomass substrates with a high xylose fraction into ethanol in a relatively short time.

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CHAPTER 4

Kinetic Studies of Ethanol Production by *Escherichia coli* KO11 in Batch Culture

Abstract

An unstructured kinetic model for ethanol production from glucose, xylose, and their mixtures using *Escherichia coli* KO11 is proposed, based on the Monod equation. This model was developed on the basis of metabolic analysis and many experimental findings. Ethanol inhibition and pH effect on cell growth, sugar consumption, and ethanol production were investigated and were taken into account in the proposed model. Previous kinetic studies of *Escherichia coli* KO11 on mixed sugar substrates indicate that glucose strongly inhibits xylose metabolism. An inhibition term related to the initial glucose concentration present in the medium was included in both cell growth and ethanol production equations. Good agreement was found between model predictions and experimental data for *Escherichia coli* KO11.

Keywords: *Escherichia coli* KO11; Ethanol; Glucose; Xylose; Fermentation; Kinetic model.

4.1 Introduction

To reduce the production cost of ethanol and greenhouse gas emissions, lignocellulosic material, mainly providing glucose and xylose upon hydrolysis, has been recognized as one of the most promising feedstocks. The efficient fermentation of lignocellulosic hydrolysates is paramount for the development of an economically viable bioethanol process. *Escherichia coli* is a gram-negative bacterium capable of producing ethanol from pyruvate using pyruvate formate lyase (PFL) under anaerobic conditions on a wide variety of substrates. However, this fermentation pathway is unbalanced due to a shortage of protons which is made up by producing acetic and succinic acids simultaneously, resulting in a low ethanol yield (Dien et al., 2003). The replacement of the native fermentation pathway in *Escherichia coli* with a homo-ethanol pathway from *Zymomonas mobilis* has been performed and resulted in a much more effective strain for industrial ethanol production from lignocellulosic materials. This strain, referred to as *Escherichia coli* KO11, carries the recombinant genes *pdc* and *adh*B for ethanol production and the *cat* gene conferring resistance to chloramphenicol (Cm) which were integrated into the chromosome (Ohta et al., 1991). It has been shown to have a very high ethanol yield (up to 0.53 g ethanol/g sugar) by fermenting both glucose and xylose.

Preliminary research performed in our laboratory has shown that the fermentation performance of *Escherichia coli* KO11 is closely related to changes in environmental conditions, especially variations in pH. To gain a better understanding of the kinetics of growth, substrate consumption and ethanol production when *Escherichia coli* KO11 is used to produce ethanol from glucose, xylose or a mixture of the two, a mathematical model can be developed. Kinetic models of fermentation can be divided into two types: unstructured models and structured models. Compared with structured models, which are based on both intracellular metabolic pathways and the biochemical structure of the microorganisms, unstructured models are simple and equally useful, where biomass is seen as a holistic entity and represented using a single variable in the equation (Vázquez and Murado, 2008). Previous kinetic models for *Escherichia coli* KO11 fermenting both glucose and xylose have been proposed in the literature (Olsson and Hahn-

Hägerdal, 1995) and included substrate inhibition, product inhibition, and mutual inhibition of glucose and xylose. But no pH effect was considered and the kinetics of the cell growth was not reported.

In this paper, experimental data of glucose and xylose fermentation using *Escherichia coli* KO11 in batch cultivation were used in order to develop an unstructured kinetic model to represent the kinetics of growth, substrate consumption, and ethanol production. In this study, the influence of pH and the composition of the culture medium (concentration of glucose, xylose, and ethanol) on the fermentation performance has been analyzed and taken into account in developing the kinetic model.

4.2 Materials and Methods

4.2.1 Microorganism

Escherichia coli KO11, obtained from the American Type Culture Collection (ATCC) as ATCC 55124, has been described in the introduction. Cultures were maintained as frozen stocks in 20% (v/v) glycerol at -80°C and in a complex medium containing LB medium and 2% (w/v) glucose with addition of 40 mg/L chloramphenicol.

4.2.2 Culture media and inoculum preparation

Cultures were grown in Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) and 20 g/L of glucose or xylose, unless otherwise specified. Except for the stock culture and the solution used for inoculation, aqueous LB media and sugar solution were sterilized separately by autoclaving at 121°C for 15 min. Filter-sterilized chloramphenicol was only added to the autoclaved stock culture after cooling.

Inoculum was prepared by transferring 2~3 large single isolated colonies from agar plates to 100 mL culture medium, which contains 20 g/L glucose as carbon source for glucose-only

fermentation or 20 g/L xylose for xylose-only and mixed-sugar fermentation, after shaking at 30°C for nearly 8~9 h on a rotary shaker at 100 rpm to ensure the cells were in the late exponential phase based on a predetermined growth curve (Appendix A).

4.2.3 Fermentation conditions

Fermentations were conducted with almost 10% (v/v) inoculum in order to get an initial optical density of cells at 550 nm of 0.5 with an approximate cell concentration of 0.12 g/L. This relatively high level of inoculation was used to reduce the effect of lag growth phase and shorten the time to reach the maximum cell mass concentration.

Three-hundred-millilitre erlenmeyer flasks containing 100 mL of substrate solution were used as fermentation vessels. The flasks were sealed with a rubber stopper in which was inserted a sterilized wine air-lock partly filled with distilled water to maintain anaerobic conditions. Experiments were all performed at 30°C in a rotary shaker (MaxQ 5000, Geneq INC.) with 100 rpm orbital rotation, which was determined by previous studies of the reported optimal fermentation conditions (Appendix B). No pH control was performed during cultivation except where pH effects on ethanol production were studied. The initial pH, adjusted by the slow addition of 2M NaOH, ranged between 6.7 and 5.3. All experiments were carried out in duplicates.

For the set of experiments where the pH effect on fermentation performance of *Escherichia coli* KO11 was evaluated, the pH was maintained within the desired range by directly mixing 10 mL of a phosphate buffer solution with the fermentation medium to get a final volume of 100 mL. The buffer solution was composed of sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4). In order to regulate pH throughout the fermentation within a specific range, various buffer solutions were prepared as described in Table 4.1. However, when the desired pH was below 5.5, 10 mL of a buffer solution containing only potassium phosphate monobasic (KH_2PO_4) (Table 4.1) was added to the fermentation medium followed by pH adjustment with concentrated HCl.

Table 4.1. Composition of the phosphate buffers.

Desired pH Range	Phosphate Buffer Components
7.0-6.5	55.2 g/L NaH_2PO_4 and 218.7 g/L Na_2HPO_4
6.5-6.0	74.9g/L NaH_2PO_4 and 138.6 g/L Na_2HPO_4
6.0-5.5	240 g/L NaH_2PO_4 with the slow addition of 2M NaOH
Below 5.5	100 g/L KH_2PO_4 with the slow addition of concentrated HCL.

4.2.4 Analytical methods

Cell concentration was estimated using a pre-determined calibration curve (Appendix C) between the cell dry weight and the optical density at a wavelength of 550 nm measured using a spectrophotometer (Perkin Elmer Lambda 25 UV/Vis). For preparing the calibration curve, the cell dry weight was measured using pre-weighed alumina weighing dishes, which were dried in an oven at 100°C for approximately 24 h and then weighed. Cell pellets were obtained from the centrifuged fermentation broth including duplicate washing to eliminate residual elements of the fermentation broth before drying. The calibration curve showed a linear relationship of one unit of turbidity at 550 nm corresponding to 0.2484 g dry cells/L over an absorbance range of 0.1 to 0.4.

Fermentations were monitored by taking 2 mL samples at appropriate time intervals for analysis of cell mass concentration by measuring the optical density. Before testing, samples needed to be diluted to insure that the absorbance was in the range of 0.1-0.4. The pH of the broth was measured with a Barnant 30 pH meter, which was calibrated with pH reference buffer solutions of 4.0 and 7.0 prior to each measurement. Glucose, xylose, and ethanol concentrations in the broth were determined by HPLC using a Sugar-Pak^{I} cation-exchange column (6.5mm×300mm) obtained from Waters Ltd., On, CA, a Waters Breeze system and a Waters 2414 refractive index (RI) detector. The separation was performed at 90°C using distilled de-ionized water as the mobile phase at a flow rate of 0.5 mL/min. However, to maintain the equilibrium and prevent

inversion, 50 mg/L calcium EDTA was added in the mobile phase. Samples taken for HPLC analysis were centrifuged at 12000 g for 6 min to remove cells and then, the supernatant was filtered with a 0.22 μm Millipore membrane filter. The resulting solution was diluted with distilled de-ionized water to give a final concentration in the range of 0.01 g/L to 3 g/L of sample. Calibration curves and chromatograms for sugar and ethanol are shown in Appendix D. Standard sugar and ethanol solutions were run periodically to ascertain the accuracy of the calibration curves.

For sugar analysis, the recorded chromatograms showed a concentration profile with two overlapping peaks because of similar retention times of glucose and xylose at 9.871 min and 10.745 min, respectively. To estimate the individual peak of each sugar, the chromatograms were analyzed by peak-deconvolution software, Cutter version 5.0, created by J. G. Shackman, using the ETG (Empirically Transformed Gaussian) function (Shackman et al., 2004). The accuracy of the deconvoluted results was evaluated by testing single sugar solutions of known concentration separately and mixtures of them in different ratios. Area measurements of deconvoluted peaks are shown in Appendix E and the estimated error was within 4% of the true value.

4.3 Unstructured Mathematical Modeling

4.3.1 Theoretical background

Ethanol production by yeast and bacteria under anaerobic conditions can be effectively described by the biochemical knowledge based models (Olsson and Hahn-Hägerdal, 1995; Wang et al., 2004; Krishnan et al., 1999; Leksawasdi et al., 2001). Most of these models are known as unstructured models. In an unstructured kinetic model, it is desired to simultaneously relate biomass growth, substrate consumption and ethanol production throughout the fermentation. Previous studies (see Chapter 3) indicated that the kinetics of cell growth and ethanol production by *Escherichia coli* KO11 was highly affected by the environmental pH and the composition of culture medium. In this investigation, fermentations were performed in Erlenmeyer flasks. In this environment, fine control of pH was not achievable even using buffer solutions. Nevertheless, the

pH range throughout the fermentation with buffer solutions was relatively small to allow studying with confidence the effect of pH on the parameters of the kinetic models.

Microbial growth is most commonly limited by one of three factors: (a) exhaustion of available nutrients; (b) accumulation of toxic metabolic products; and (c) change in ion equilibrium, especially pH (Monod, 1949). The first factor is obviously the most common and the Monod equation is used to relate the limiting nutrient concentration to biomass growth rate:

$$\frac{1}{X}\frac{dX}{dt} = \mu = \frac{\mu_m S}{K_S + S} \tag{4.1}$$

The inhibition of the metabolic products can be expressed as a product inhibition term $\left(1 - \frac{P}{P_m}\right)^n$ which was added to the Monod equation as follows:

$$\mu = \frac{\mu_m S}{K_S + S}\left(1 - \frac{P}{P_m}\right)^n \tag{4.2}$$

The effect of pH variation on cell growth can be taken into account in the Monod equation by allowing the model parameters to vary with pH as shown in Eq. (4.3).

$$\mu = \frac{\mu_m(pH)\, S}{K_S(pH) + S}\left(1 - \frac{P}{P_m}\right)^n \tag{4.3}$$

Preliminary experimental results showed that the anaerobic batch culture of *Escherichia coli* KO11 with high initial cell mass concentration led to a fairly short exponential growth phase, during which a relatively small amount of the initial nutrient stock in the medium was consumed. The short growth phase was followed by a long stationary phase, where most of the ethanol was produced. The stationary phase prevailed until the substrate was completely consumed. With analysis of the growth of *Escherichia coli* KO11 at various compositions of the culture medium (glucose, xylose, and ethanol), it became obvious that the growth of *Escherichia coli* KO11 was not only limited by the above three factors.

There was always a maximum population density, which is identical to the ecological concept of carrying capacity, was detected under the specific culture environmental conditions. There have

been numerous hypotheses proposed to explain this cell growth limitation. It can be explained as the exhaustion of biological space and therefore the rate of limiting nutrients diffusing to the cells is felt to such value that cells can not generate or process energy rapidly enough to meet the demands for growth (Ruchanan et al., 1997). On the other hand, the optimal growth pattern of *Escherichia coli* was investigated by Varma and Pallson (1993). They reported that ATP is very important for cell maintenance and it has a significant influence on the maximum biomass yields. The upper limit of cell mass concentration was similar in all experiments performed by using *Escherichia coli* KO11 under anaerobic conditions, which was around 1.4-1.8 g/L. From the point of view of the intracellular metabolism of *Escherichia coli* KO11, all living organisms generate the needed energy and carbon skeletons for biosynthesis by catalyzing a set of coupled oxidation-reduction reactions (Figure 2.1 A). Under anaerobic conditions, without external input of the terminal electron acceptors for respiration, higher levels of NADH are accumulated and results in the formation of other metabolic products (acetate and ethanol) to maintain the overall redox balance (Hasona et al., 2004). Compared with the complete oxidation of the carbon source to CO_2 and H_2O, less ATP and carbon skeletons are generated during anaerobic growth and, as a result, cell growth is limited and a lower level of the stationary phase is obtained. Additionally, expression of *Zymomonas mobilis pdc* (pyruvate decarboxylase) in *Escherichia coli* KO11 reduces the flow of pyruvate carbon into native fermentation pathways as desired, but it also restricts the flow of carbon skeletons into the tricarboxylic acid pathway (TCA) for biosynthesis. This hypothesis was validated by the addition of pyruvate or 2-ketoglutarate into the cultivating medium which led to an increase of cell growth and ethanol production (Underwood et al., 2002).

Consequently, a maximum cell population term $\left(1-\frac{X}{X_m}\right)^{\gamma}$, similar to the product inhibition term, was added to the cell growth rate expression. It is assumed that this term includes all the effects of limiting biological spaces, ATP constraint and deficient carbon skeletons for biosynthesis.

As a result, the final expression of cell growth model was derived as follows:

$$\mu = \frac{\mu_m(pH)\, S}{K_S(pH) + S} \left(1 - \frac{P}{P_m}\right)^n \left(1 - \frac{X}{X_m}\right)^\gamma \qquad (4.4)$$

where X_m and P_m are the maximum levels of bacterial population and ethanol concentrations that completely inhibit the cellular activities.

In Chapter 3, the influence of varying the initial sugar concentration on single-sugar fermentation performances of *Escherichia coli* KO11 was investigated. No substrate inhibition was observed over a range of initial glucose concentration of 18 g/L to 65 g/L and xylose concentration of 19 g/L to 73 g/L. A substrate inhibition term will therefore not be included in the single-substrate model.

Ethanol production from glucose or xylose by *Escherichia coli* KO11 is accompanied with lactate and acetate formation (Figure 2.1 B). When fermentation acid accumulates in vessels, pH decreases and affects cell growth and ethanol formation (Beall et al., 1991). Ambient pH shift could lead to slight but significant changes of intracellular pH. In general, microbial cell growth and metabolite formation are considered to be the result of a sequence of enzymatic reactions (Tan et al., 1998). The ionic forms of metabolic enzymes vary as pH changes and the active centers in enzymes could be inhibited if conditions move away from the optimal pH, thereby leading to reduced performance.

Very few studies have been conducted to quantify and to incorporate the effects of pH into kinetic fermentation models. A general approach using a statistical thermodynamic method to simulate the kinetics of microbial growth influenced by pH was proposed by Tan et al. (1998). They conducted their experiments to relate enzyme kinetics to the number of functional ionizable groups and binding energy levels. Another approach was reported where the pH variation was taken into account in the Monod equation by allowing the model parameters, such as the maximum specific growth rate (μ_m) and the saturation constant (K_S), to vary as a function of pH. The cell yield coefficient ($Y_{P/S}$) can also be made a function of pH. The modified kinetic model proved effective and useful in simulating lactic acid production by *Lactobacillus plantarum* (Fu and Mathews, 1999).

In this study, an unstructured kinetic model, based on the modified Monod equation and with growth-associated ethanol production, to simulate product formation and substrate consumption in single sugar and mixed-sugar fermentations using *Escherichia coli* KO11. The pH effect on the kinetic parameters of ethanol production was also quantified.

4.3.2 Model development

The kinetic model proposed in this study for glucose-only and xylose-only fermentation by *Escherichia coli* KO11 is presented by Eqs. (4.5) through (4.7). Eq. (4.5) describes the dry weight biomass growth rate using a pH-dependent Monod model to which was added two inhibition terms, one for product inhibition and one to account for maximum cell population. Eq. (4.6) describes the ethanol production rate as the summation of two contributions. The first term accounts for the production of ethanol associated with growth and the second term associated with the cell mass population. Eq. (4.7) states that the rate of substrate consumption is a function of the biomass and ethanol production as well as what is necessary to maintain metabolic activities.

$$\frac{dX}{dt} = \frac{X\,\mu_m(pH)\,S}{K_S(pH) + S}\left(1-\frac{P}{P_m}\right)^n\left(1-\frac{X}{X_m}\right)^\gamma \tag{4.5}$$

$$\frac{dP}{dt} = \alpha(pH)\,\frac{dX}{dt} + \beta(pH)\,X \tag{4.6}$$

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}(pH)}\frac{dX}{dt} + \frac{1}{Y_{P/S}(pH)}\frac{dP}{dt} + m(pH)\,X \tag{4.7}$$

Eqs. (4.8) through (4.15) present the kinetic model that is proposed to describe the kinetics for the simultaneous fermentation of glucose and xylose using *Escherichia coli* KO11. Eqs. (4.8-4.9) are nearly identical to Eq. (4.5) and account for the biomass growth rates due to glucose and xylose consumption, respectively. To make both equations as general as possible, two key parameters of the Monod equation were made to vary with pH. In Eqs. (4.8-4.9), terms for product inhibition and maximum cell population were included as for single substrate fermentation. Because of the observed inhibition on xylose consumption and ethanol production

caused by glucose, an additional term related to the glucose concentration (S_G/K_I) was added to the denominator of Eq. (4.9) to account for this inhibition. Eq. (4.10) is simply the summation of the biomass growth rates of glucose and xylose, respectively. Eqs. (4.11-4.13) gives the rate of ethanol production due to glucose and xylose separately. It is similar to Eq. (4.6) for single substrate. Again, because of the inhibition on xylose metabolism by glucose even after glucose was exhausted, an inhibition constant $K_{I,0}$ was added in Eq. (4.11) to take this phenomenon into account. This inhibition was hypothesized as the cellular regulation of xylose metabolism by glucose metabolites. Therefore this inhibition was related to the initial glucose concentration ($S_{G,0}$) in the medium. Parameter θ is the critical initial glucose concentration below which there is no apparent inhibition of glucose on xylose. Finally, Eqs. (4.14-4.15) gives the rates of glucose and xylose consumption akin to Eq. (4.7) for single substrate and account for the use of substrate for biomass growth, ethanol production and maintenance.

$$\left[\frac{dX}{dt}\right]_G = \frac{X_n\,\mu_{m,G}(pH)\,S_G}{K_{S,G}(pH)+S_G}\left(1-\frac{P}{P_m}\right)^n\left(1-\frac{X}{X_m}\right)^\gamma \tag{4.8}$$

$$\left[\frac{dX}{dt}\right]_X = \frac{X_n\,\mu_{m,X}(pH)\,S_X}{K_{S,X}(pH)+S_X+S_G/K_I}\left(1-\frac{P}{P_m}\right)^n\left(1-\frac{X}{X_m}\right)^\gamma \tag{4.9}$$

$$\frac{dX}{dt} = \left[\frac{dX}{dt}\right]_G + \left[\frac{dX}{dt}\right]_X \tag{4.10}$$

$$\left[\frac{dP}{dt}\right]_G = \alpha_G(pH)\left[\frac{dX}{dt}\right]_G + \frac{\left[\frac{dX}{dt}\right]_G}{\left[\frac{dX}{dt}\right]_G+\left[\frac{dX}{dt}\right]_X}\beta_G(pH)\,X_n \tag{4.11}$$

$$\left[\frac{dP}{dt}\right]_X = \alpha_X(pH)\left[\frac{dX}{dt}\right]_X + \frac{\left[\frac{dX}{dt}\right]_X}{\left[\frac{dX}{dt}\right]_G+\left[\frac{dX}{dt}\right]_X}\beta_X(pH)\,X_n\,K_{I,0}^{(S_{G,0}-\theta)} \tag{4.12}$$

$$\frac{dP}{dt} = \left[\frac{dP}{dt}\right]_G + \left[\frac{dP}{dt}\right]_X \tag{4.13}$$

$$-\left[\frac{dS}{dt}\right]_G = \frac{1}{Y_{X/S,G}(pH)}\left[\frac{dX}{dt}\right]_G + \frac{1}{Y_{P/S,G}(pH)}\left[\frac{dP}{dt}\right]_G + m_G(pH)\,X_n \tag{4.14}$$

$$-\left[\frac{dS}{dt}\right]_X = \frac{1}{Y_{X/S,X}(pH)}\left[\frac{dX}{dt}\right]_X + \frac{1}{Y_{P/S,X}(pH)}\left[\frac{dP}{dt}\right]_X + m_X(pH)\,X_n \quad (4.15)$$

4.3.3 Parameter estimation

The parameters in the above model were determined by minimizing the sum of squares of the differences between the predicted values of the biomass, ethanol and substrate concentrations and their respective experimental values:

$$J = \sum(X_p - X_e)^2 + \sum(S_p - S_e)^2 + \sum(P_p - P_e)^2 \quad (4.16)$$

4.4 Results and Discussion

4.4.1 Kinetics of glucose-only and xylose-only fermentation

4.4.1.1 Influence of initial ethanol concentration

To determine the effect of the ethanol concentration on the fermentation performance of *Escherichia coli* KO11, experiments with varying initial ethanol concentration in the glucose-only and xylose-only media were performed. For all experiments, an initial sugar concentration of approximately 20 g/L was selected in order to separate ethanol inhibition effects from those of substrate limitation. For fermentations having an initial ethanol concentration ranging from 1.25% to 5% (v/v), the specific growth rate (μ) was calculated. Table 4.2 gives this performance parameter as a function of the initial ethanol concentration for glucose and xylose fermentations.

Table 4.2. Effect of the initial ethanol concentration for the glucose and xylose fermentation of *Escherichia coli* KO11.

Initial Ethanol Concentration (v/v)		0%	1.25%	2.5%	5%
μ (h^{-1})	Glucose-only	0.39	0.30	0.29	0.26
	Xylose-only	0.35	0.21	0.20	0.20

Results of Table 4.2 show that there was an apparent decrease in the growth rate of *Escherichia*

coli KO11 when the initial ethanol concentration was higher. However, the inhibition information that can be obtained from experimental data cannot be used directly to determine the parameters P_m and n of the product inhibition term $\left(1 - \frac{P}{P_m}\right)^n$ in the modified Monod equation because this term does not only include the contribution of ethanol but also other metabolites that are produced simultaneously with ethanol by *Escherichia coli* KO11, such as acetate, succinate and lactate. In addition, it has been argued that exogenous ethanol existing in the fermentation broth is less toxic than endogenous ethanol produced by the microorganisms (Dasari et al., 1990). The substrate, product, and cell mass profiles during fermentation for various initial ethanol concentrations are plotted in Appendix F.

4.4.1.2 Influence of environmental pH

The impact of pH on ethanol production by *Escherichia coli* KO11 was studied with regard to the kinetic parameters related to biomass and ethanol production as well as sugar consumption. Kinetic parameters were estimated from the experimental data of anaerobic fermentations which all started at the same initial sugar concentration of approximately 20 g/L and different buffer solutions were used to control pH in a range that varied between 4.5 and 7.0.

The values of kinetic parameters obtained from experiments performed at different pH values are summarized in Tables 4.3 and 4.4 for glucose-only and xylose-only fermentations, respectively. The highest values of the maximum specific growth rate (μ_m) were obtained in the range of pH between 6.5 and 7.0, which is close to the reported optimal pH for *Escherichia coli* growth (Singleton and Sainsbury, 1978). Even though ethanol production by Escherichia coli KO11 is partly growth-associated, the ethanol yield ($Y_{E/S}$) increased with a decrease in pH. This trend can be explained due to a decline in organic acid formation with a decrease in environmental pH which was previously reported for similar *Escherichia coli* strains, ATCC 11303 (PLOI297) (Beall et al., 1991) and FBR5 (Qureshi et al., 2006), both carrying the plasmid pLOI297 containing the same *Zymomonas mobilis* genes *pdc* and *adh*B as *Escherichia coli* KO11. More pyruvate conversion to acetate in place of ethanol will generate more ATP (Figure 2.1) for cell growth. That is in agreement with the increased cell mass yield coefficient ($Y_{X/S}$) observed with

an increase in pH.

The optimal pH for ethanol production by *Escherichia coli* KO11 was observed at pH 6.0-6.5 based on experimental data. This is consistent with earlier studies using this strain (Barbosa et al., 1992; von Sivers et al., 1994; Moniruzzaman et al., 1998; Takahashi et al., 2000; Lima et al., 2002; Agblevor et al., 2003; Kim et al., 2006; Rao et al., 2007; Lawford et al., 1995, 1996). The corresponding parameter α was found to have the highest value at this pH range. The maximum specific growth rate (μ_m) was much lower for pH below 5.0 and the associated ethanol production also declined because low-pH stress offers a higher consumption of energy for cell maintenance.

The parameters accounting for ethanol inhibition (P_m, n) and cell population inhibition (X_m, γ) were assumed to be identical for all experiments and independent of pH (Tables 4.3 and 4.4) in order to simplify the model. During the fitting procedure, it was clear that these four parameters were correlated. It should be noted that the value of P_m was set at 68 g/L which corresponds to the highest concentration of ethanol produced by *Escherichia coli* KO11 (Guimaraes et al., 1992). The value of parameter X_m was set between 1.49 g/L to 2.52 g/L which were the highest achieved cell mass concentration in different experiments. During the fitting procedure, the values of X_m and γ were found to be 2.29 g/L and 3.86, respectively. The higher value of γ, compared with a value of n of 0.146, indicates that biomass production from glucose or xylose was more sensitive to ATP constraint as well as limited carbon skeletons for biosynthesis and limited biological space than to ethanol inhibition. It is consistent with the experimental results of *Escherichia coli* KO11 fermentation on various mediums with the addition of an initial amount of ethanol. The value of the cell mass yield coefficient ($Y_{X/S}$) was obtained after simulating both glucose-only and xylose-only fermentation results at different pH. The values ranged from 0.28 g/g to 0.42 g/g, which are reasonable considering that the maximum cell mass yield value of 0.588 g/(g glucose) in the absence of maintenance requirement was previously reported (Varma and Pallson, 1993). The prediction of the cell mass growth, ethanol production and substrate consumption throughout the fermentation performed at different pH is in good agreement with the experimental data (Figures 4.1 and 4.2).

Table 4.3. Estimated values of model parameters for glucose-only fermentation with *Escherichia coli* KO11 at different pH.

pH	μ_m	K_S	P_m	α	β	$Y_{X/S}$	$Y_{E/S}$	m	n	X_m	γ
pH 7.0-6.3	2.23	0.17	68	0.90	0.29	0.41	0.39	1.91E-08	0.146	2.29	3.86
pH 6.5-6.0	1.67	0.17	68	1.87	0.29	0.40	0.50	1.91E-08	0.146	2.29	3.86
pH 6.0-5.4	1.53	0.17	68	1.23	0.29	0.35	0.51	1.91E-08	0.146	2.29	3.86
pH 5.4-5.0	1.51	0.17	68	1.07	0.29	0.31	0.53	1.91E-08	0.146	2.29	3.86
pH 5.2-4.8	1.04	0.17	68	1.03	0.29	0.30	0.56	1.91E-08	0.146	2.29	3.86

Table 4.4. Estimated values of model parameters for xylose-only fermentation with *Escherichia coli* KO11 at different pH.

pH	μ_m	K_S	P_m	α	β	$Y_{X/S}$	$Y_{E/S}$	m	n	X_m	γ
pH 7.0-6.3	1.83	2.01	68	0.30	0.222	0.42	0.39	1.91E-08	0.146	2.29	3.86
pH 6.3-6.0	1.30	2.01	68	1.12	0.222	0.41	0.47	1.91E-08	0.146	2.29	3.86
pH 6.0-5.5	0.90	2.01	68	1.05	0.222	0.37	0.48	1.91E-08	0.146	2.29	3.86
pH 5.5-5.0	0.71	2.01	68	1.03	0.222	0.28	0.51	1.91E-08	0.146	2.29	3.86
pH 5.0-4.5	0.45	2.01	68	1.03	0.222	0.28	0.56	1.91E-08	0.146	2.29	3.86

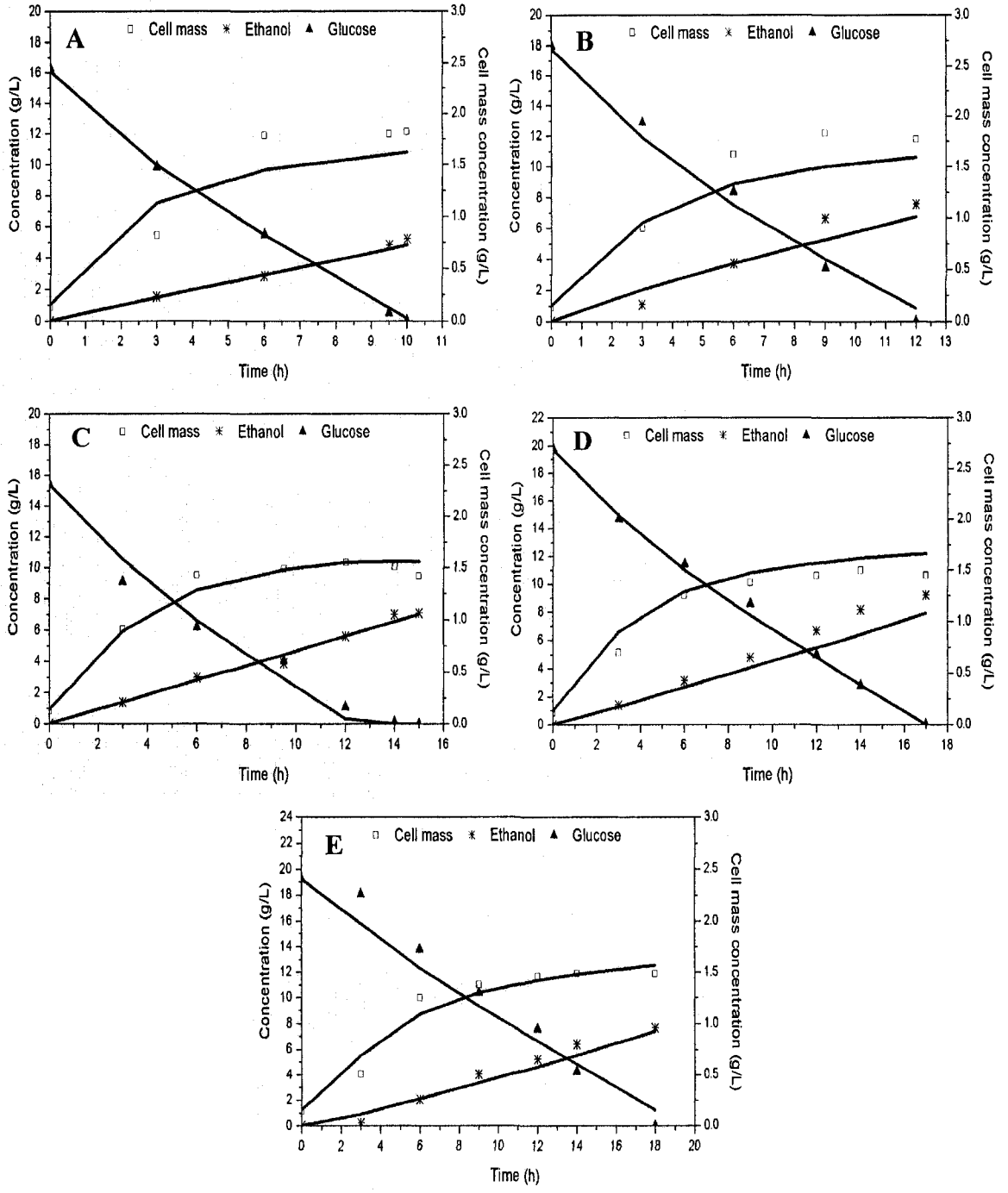


Figure 4.1. Experimental and predicted values at pH 7.0-6.3 (A), pH 6.5-6.0 (B), pH 6.0-5.4 (C), pH 5.4-5.0 (D), and pH 5.2-4.8 (E) of *Escherichia coli* KO11 fermentation on glucose-only media with an initial cell mass concentration of 0.12 g/L under anaerobic conditions. Solid lines are the predictions and symbols represent experimental data.

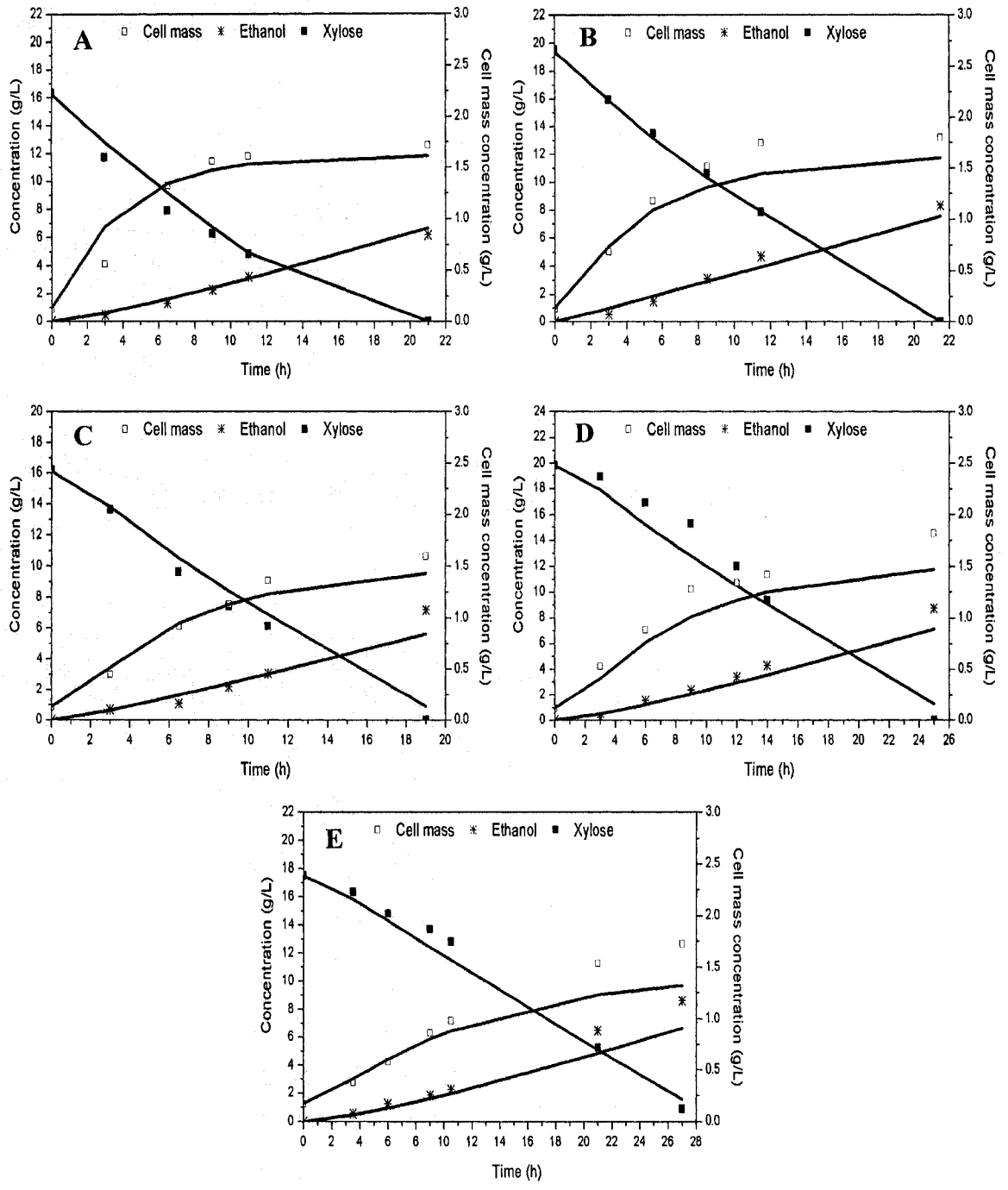


Figure 4.2. Experimental and predicted values at pH 7.0-6.3 (A), pH 6.3-6.0 (B), pH 6.0-5.5 (C), pH 5.5-5.0 (D), and pH 5.0-4.5 (E) of *Escherichia coli* KO11 fermentation on xylose-only media with an initial cell mass concentration of 0.12 g/L under anaerobic conditions. Solid lines are the predictions and symbols represent experimental data.

4.4.1.3 Kinetics of fermentation at different initial sugar concentrations.

The experimental results of glucose-only and xylose-only fermentation with different initial sugar concentration have been reported in Chapter 3. These data were used to test the validity of the kinetic model. Figures 4.3 and 4.4 compared the predicted substrate, product, and cell concentration profiles with experimental data. The predicted rates of ethanol formation and substrate consumption fit the experimental data well. The profile of the biomass concentration is also very well predicted except that the predicted maximum cell population for the fermentation performed with a 73 g/L initial xylose concentration is much lower than the experimental data. This difference may be attributed to the different strain of *Escherichia coli* KO11 which had to be purchased because the first one appeared to have lost its ability to use xylose.

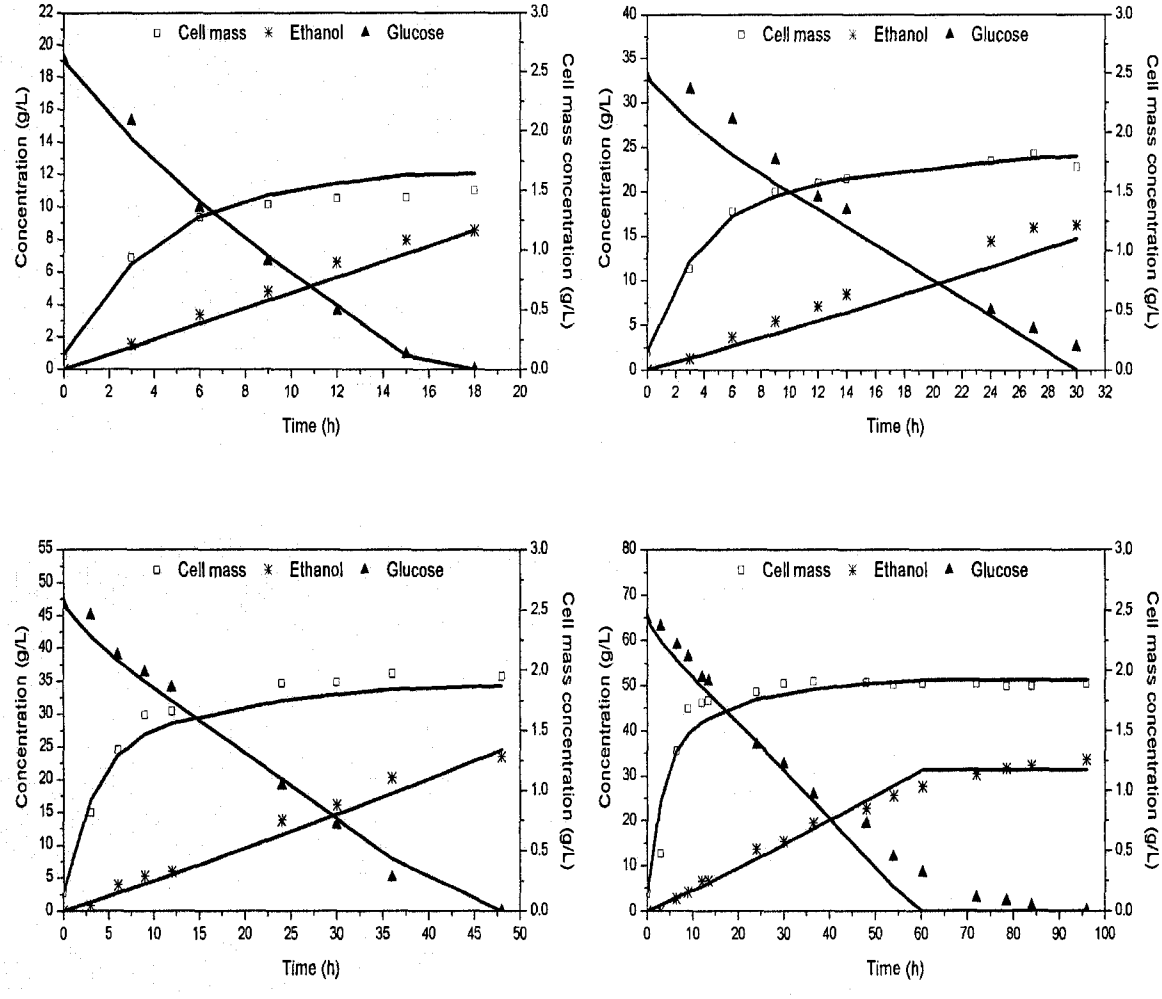


Figure 4.3. Experimental and predicted profiles of cell growth, glucose consumption, and ethanol production at various initial glucose concentrations using *Escherichia coli* KO11 and with an initial cell mass concentration of 0.12 g/L under anaerobic conditions. Solid lines are the predictions and symbols represent experimental data.

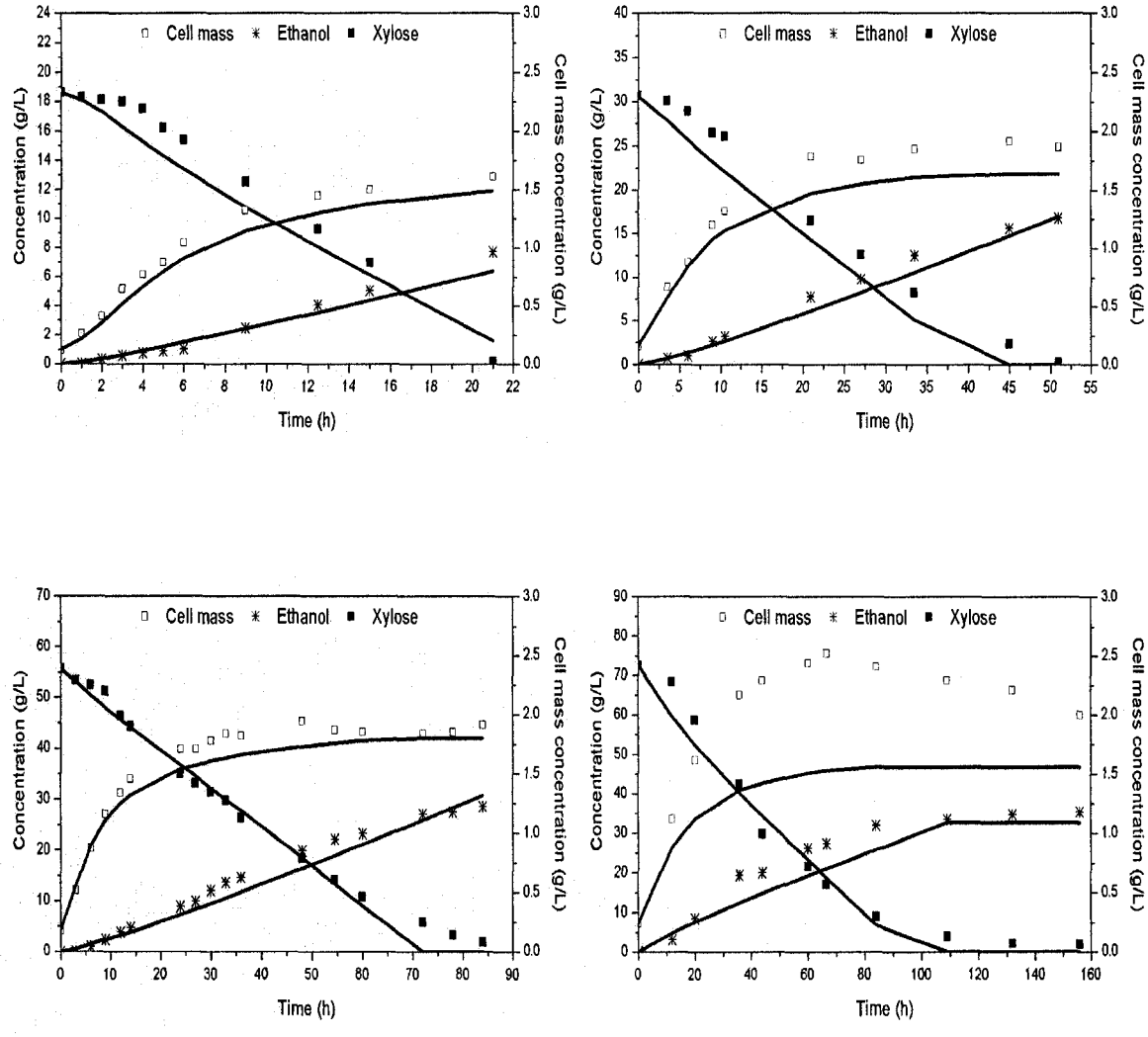


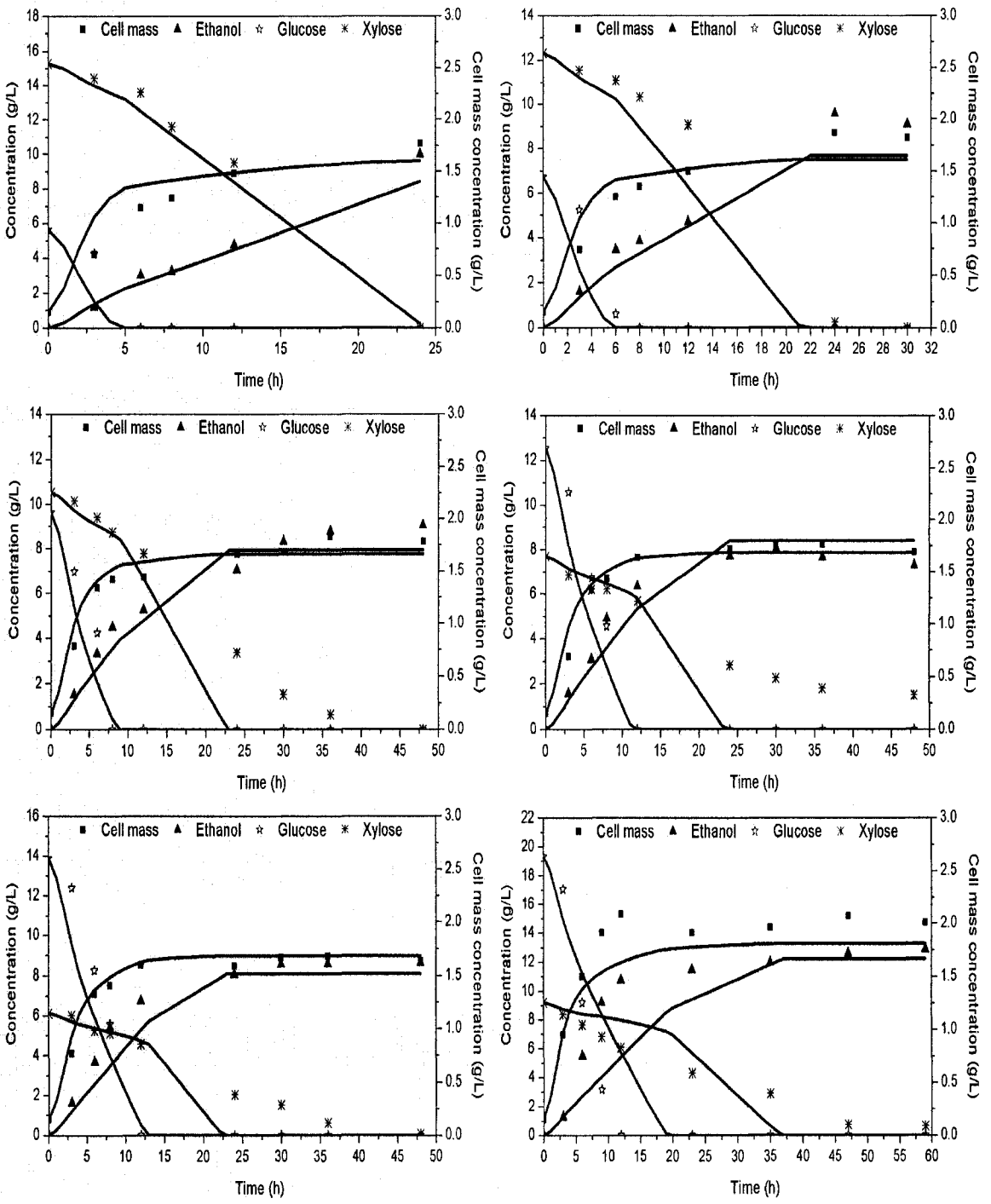
Figure 4.4. Experimental and predicted profiles of cell growth, glucose consumption, and ethanol production at various initial xylose concentrations using *Escherichia coli* KO11 and with an initial cell mass concentration of 0.12 g/L under anaerobic conditions. Solid lines are the predictions and symbols represent experimental data.

4.4.2 Kinetics of mixed sugar fermentation

The ability of *Escherichia coli* KO11 to ferment glucose-xylose mixtures to grow and produce ethanol was studied for the series of sugar mixtures presented in Table 4.5. Previous experimental results revealed that glucose and xylose were utilized by *Escherichia coli* KO11 simultaneously but a very strong mutual effect of glucose and xylose was detected. With glucose present, the uptake rate of xylose was much slower with a severe lag even after glucose was exhausted. Comparing the experimental results of different sugar compositionst, a decrease in the rate of xylose consumption was observed when the initial glucose content increased. This particular behaviour of *Escherichia coli* KO11 was taken into account in the modelling and has been expressed using the parameters $K_{I,0}$, K_I, and θ. The values of other parameters estimated from single sugar fermentation were kept constant in the mixed sugar kinetic model and were set equal to their previously obtained values (Tables 4.3 and 4.4). The parameter fitting resulted in values of $K_I = 0.53$ and $K_{I,0} = 0.95$, which shows that even after glucose was depleted, the xylose fermentation rate was slower when compared with xylose-only fermentation. The parameter θ was determined to be 3.98 g/L and indicates that there is no inhibition of glucose metabolites on xylose metabolism when the initial glucose concentration is below this value. As Figure 4.5 shows, the predicted results were in relatively good agreement with the experimental data. It is obviously difficult to fit all experimental data simultaneously given the wide range of sugar concentration and glucose-xylose ratio but it is believed that the model is able to capture the essential of the kinetics of fermentation.

Table 4.5. Composition of mixed sugar used in fermentation with *Escherichia coli* KO11

Mixture	Glucose (g/L)	Xylose (g/L)	Mixture	Glucose (g/L)	Xylose (g/L)
1	5.62	15.28	6	7.32	20.59
2	6.75	12.31	7	9.11	18.83
3	9.62	10.52	8	13.52	15.19
4	12.53	7.68	9	17.72	10.76
5	13.87	6.11	10	19.34	9.20



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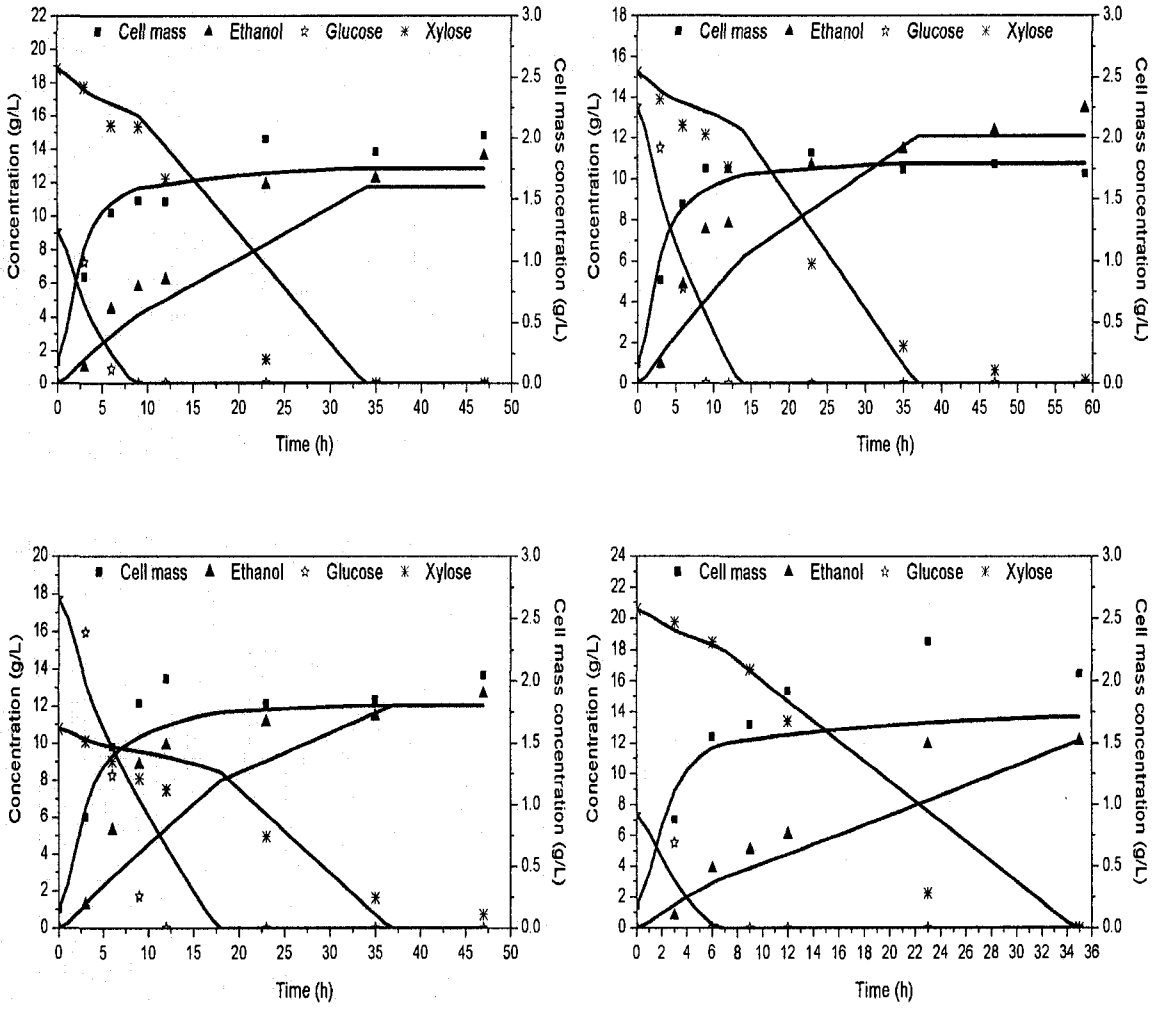

Figure 4.5. Experimental and predicted profiles of cell growth, glucose consumption, xylose consumption, and ethanol production of *Escherichia coli* KO11 fermented with an initial cell mass concentration of 0.12 g/L on media containing glucose and xylose with various initial concentrations under anaerobic conditions. Solid lines are the predictions and symbols represent experimental data.

4.5 Conclusions

An unstructured kinetic model for ethanol production from glucose, xylose, and their mixtures using *Escherichia coli* KO11 was proposed based on metabolic analysis and various experimental findings. This model could be used to describe the culture of *Escherichia coli* KO11 in the pH-uncontrolled batch mode. It is evident that the model can provide increased insight into the various factors influencing the fermentation kinetics of *Escherichia coli* KO11 on glucose/xylose media. In the area of model development, the inclusion of the pH effect on the parameters, the maximum cell population limit on cell growth, as well as the inhibition of glucose and glucose metabolites on xylose metabolism, enabled a generalized model to be constructed. Previously reported experimental data of single sugar fermentation was used to test the validity of the proposed model. The good agreement of the predicted model results with the fermentation performance of *Escherichia coli* KO11 on glucose/xylose media indicate that this model can provide a basis for future fermentation optimization studies.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Experiments were performed with *Escherichia coli* KO11 fermenting glucose and xylose separately and simultaneously at various ratios using the same initial cell concentration of 0.12 g/L under anaerobic conditions in order to study the fermentation kinetics under various experimental conditions. The conclusions that were reached in this project concerning the production of ethanol are listed below.

In single-sugar experiments with no pH control, ethanol yields were similar in both glucose-only and xylose-only media, but sugar consumption and ethanol production rates for glucose were almost 1.5 times higher than those with xylose. The maximum ethanol concentrations obtained in this study were 35.12 g/L and 35.79 g/L in 65 g/L glucose medium and 73 g/L xylose medium. When the initial xylose concentration in the medium was 73 g/L, a strong combined inhibition (low pH stress and metabolites) was observed after 120 h with an incomplete utilization of xylose. There was no substrate inhibition observed in both sugar consumption and ethanol production for all glucose-only and xylose-only experiments.

In mixed-sugar fermentations with no pH control, glucose and xylose were utilized simultaneously but the consumption rate of xylose was much slower with the presence of a severe lag even after glucose was exhausted. Xylose metabolism was only dominant after glucose was consumed. With a high percentage of glucose, sugar depletion time increased for the same total sugar concentration, thereby leading to a lower ethanol production rate. However, the final ethanol yield was not affected.

An unstructured kinetic model for ethanol production from glucose, xylose, and their mixtures using *Escherichia coli* KO11 was proposed, based on the Monod equation. The possible

inhibition factors were investigated by challenging cultures with the addition of different amounts of ethanol. The same initial sugar concentration of approximately 20 g/L was selected to better separate ethanol inhibition effects from those of substrate limitation. There is an obvious decrease in the growth rate of *Escherichia coli* KO11 when cultivated on media with increased initial ethanol concentration. Consequently, product inhibition was included in the proposed kinetic model. The effects of ATP constraint, limited carbon skeletons for biosynthesis, and limited biological space were also considered and expressed by adding a term into the model to account for the maximum cell population.

The effect of pH variation on the fermentation kinetics of *Escherichia coli* KO11 was studied by maintaining the pH relatively constant throughout the fermentation using phosphate buffer. The proposed model also took into account the pH variation by allowing the model parameters to vary as a function of pH.

A mathematical expression of the inhibition on xylose metabolism caused by glucose was developed and included in the kinetic model. Three parameters $K_{I,0}$, K_I, and θ were introduced into the kinetic equations to better describe the cell growth and xylose consumption rate for *Escherichia coli* KO11.

The kinetic parameters in the model were evaluated by minimizing the sum of squares of the errors between the predicted values of the biomass, substrates and ethanol and their experimental values. Previously reported experimental data for the fermentation of glucose, xylose, and their mixtures were used to test the validity of the proposed model. Predicted values of cell growth, ethanol production and substrate consumption matched relatively well with the experimental data.

Recommendations

Based on the results obtained in this investigation, the following recommendations are made:

Ethanol yield and productivity of *Escherichia coli* KO11 for glucose and xylose mixtures are much lower than those observed from single sugar substrates fermentation. A much higher inhibition on xylose metabolism caused by glucose was also observed. A high glucose:xylose ratio of 3:1 in the medium was shown to extend the fermentation time to twice the time observed with xylose-only experiments. Separation of glucose and xylose prior to the fermentation would give higher productivity at the expense, however, of an increase in the complexity of the process.

A very strong decline in fermentation performance was observed when the pH exceeded the optimal pH range of 6.0 to 6.5. Lower ethanol productivity and cell growth were also observed below pH 5.0. Results indicated that pH control strategy is very important to efficiently produce ethanol using *Escherichia coli* KO11. Beall et al. (1991) performed fermentations under controlled pH using both buffer solutions and continuous control using a base with a similar microorganism, *Escherichia coli* B (pLOI297), as the one used in this investigation. *Escherichia coli* B (pLOI297) was constructed using the same parent strain *Escherichia coli* B and carries a plasmid pLOI297 containing the same *Zymomonas mobilis* genes *pdc* and *adh*B as KO11. They reported that in buffered fermentations with high salt concentration levels, the time required to consume 80 g/L xylose was doubled compared to the same fermentation where pH control was performed continually using a base. However, the final yield was similar. Additionally, the use of a buffer is not practical for commercial fermentations. Online monitoring of pH is necessary to improve the efficiency of ethanol production by *Escherichia coli* KO11. However, to determine rapidly the optimal operating conditions, flask fermentations still remain the best alternative unless one has a large number of small fermenters that can all be controlled independently.

Since a buffer cannot perfectly control pH at a fixed value and a slight decline in pH was observed during the fermentation, pH effect was expressed into the proposed model using different values of parameters for different ranges of pH. An alternative is to perform more experiments at rigorously controlled pH to determine more accurately the effect of pH and then derive equations to capture the variation of each parameter with pH.

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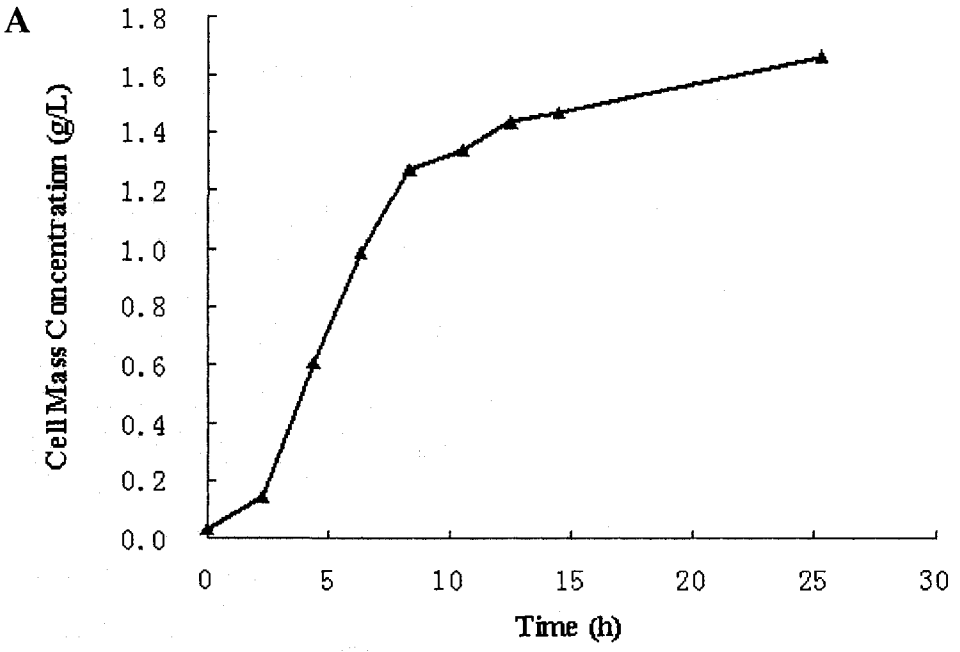
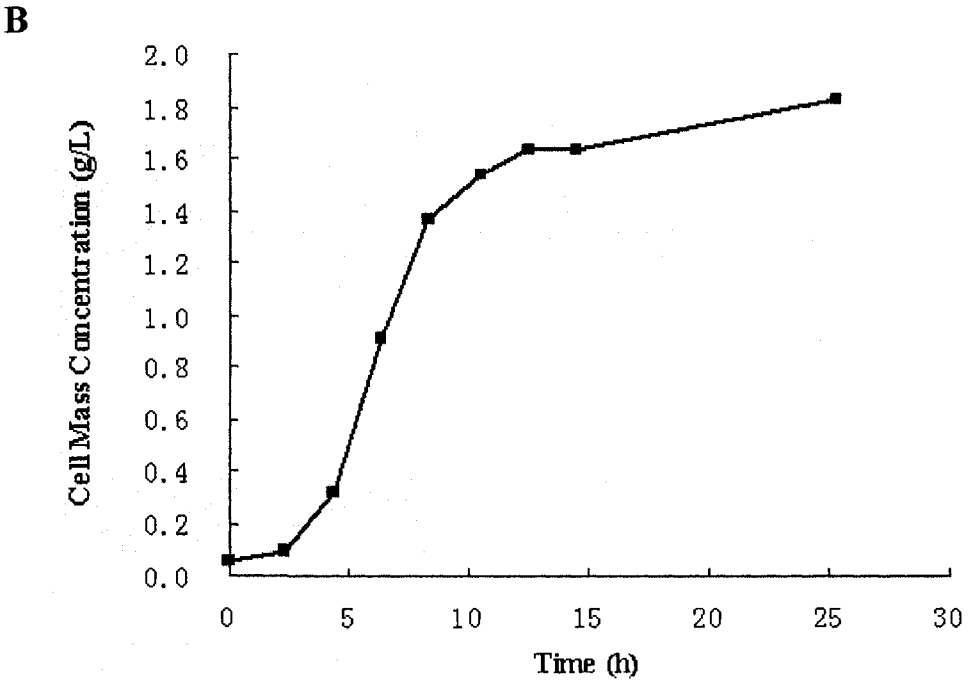
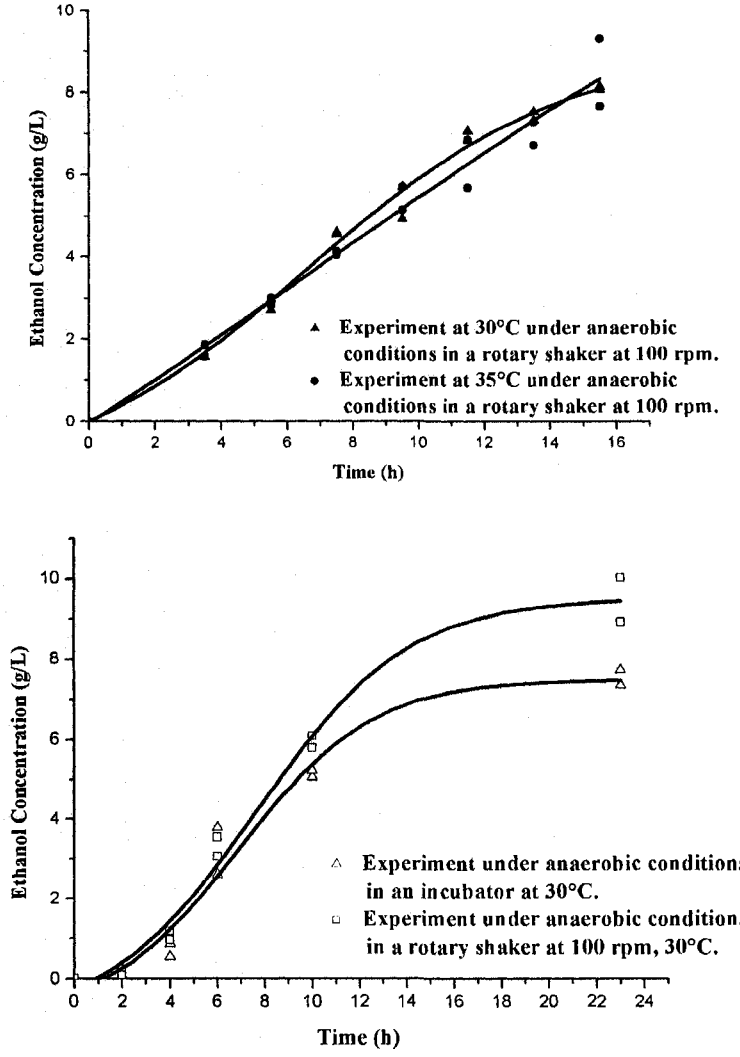
APPENDICES

Appendix A. Growth curves of *Escherichia coli* KO11 when cultivated in shake flasks using glucose (A) or xylose (B) media under aerobic conditions in a rotary shaker at 100 rpm, 30°C.





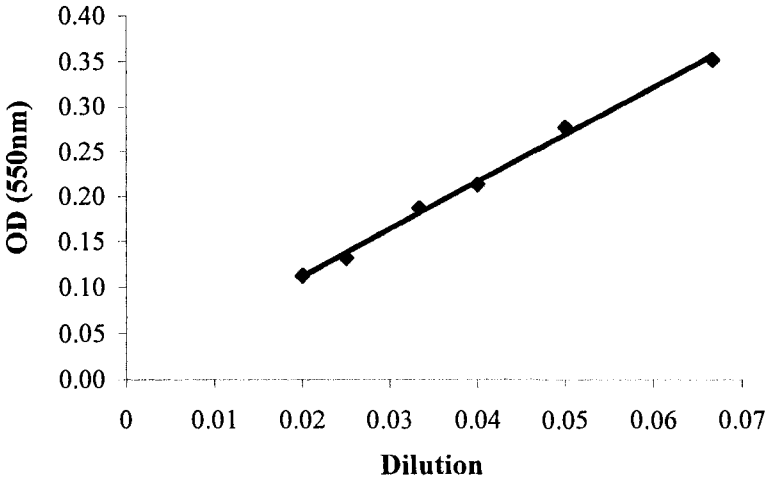
Appendix B. Comparison of ethanol production by *Escherichia coli* KO11 under different operating conditions.



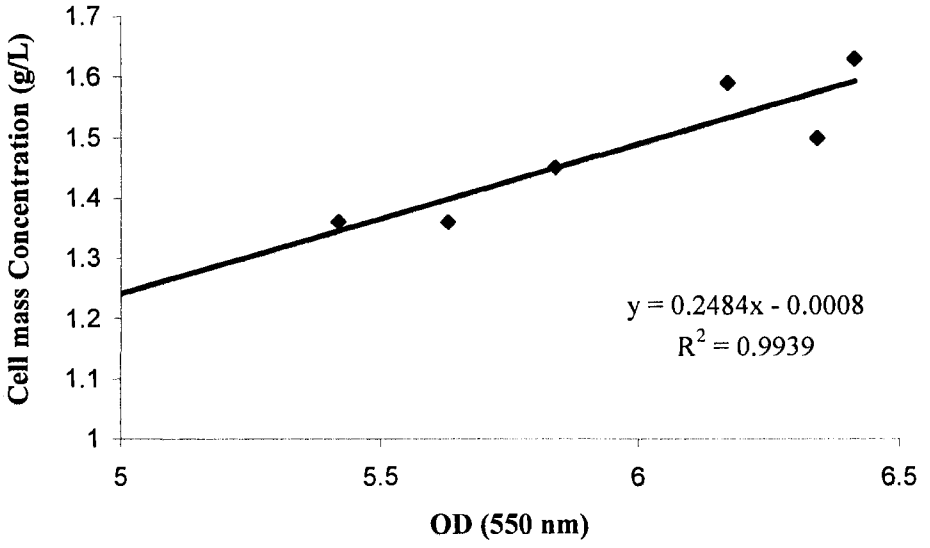
No apparent difference of ethanol production rate was observed between experiments performed at 30°C and 35°C. For energy-saving and ethanol evaporation reduction, 30°C was chosen in this study. Ethanol production by *Escherichia coli* KO11 was enhanced when cultivated in a rotary shaker. After comparison, experiments in this study were all performed at 30°C and placed in a rotary shaker with 100 rpm orbital rotation.

Appendix C.

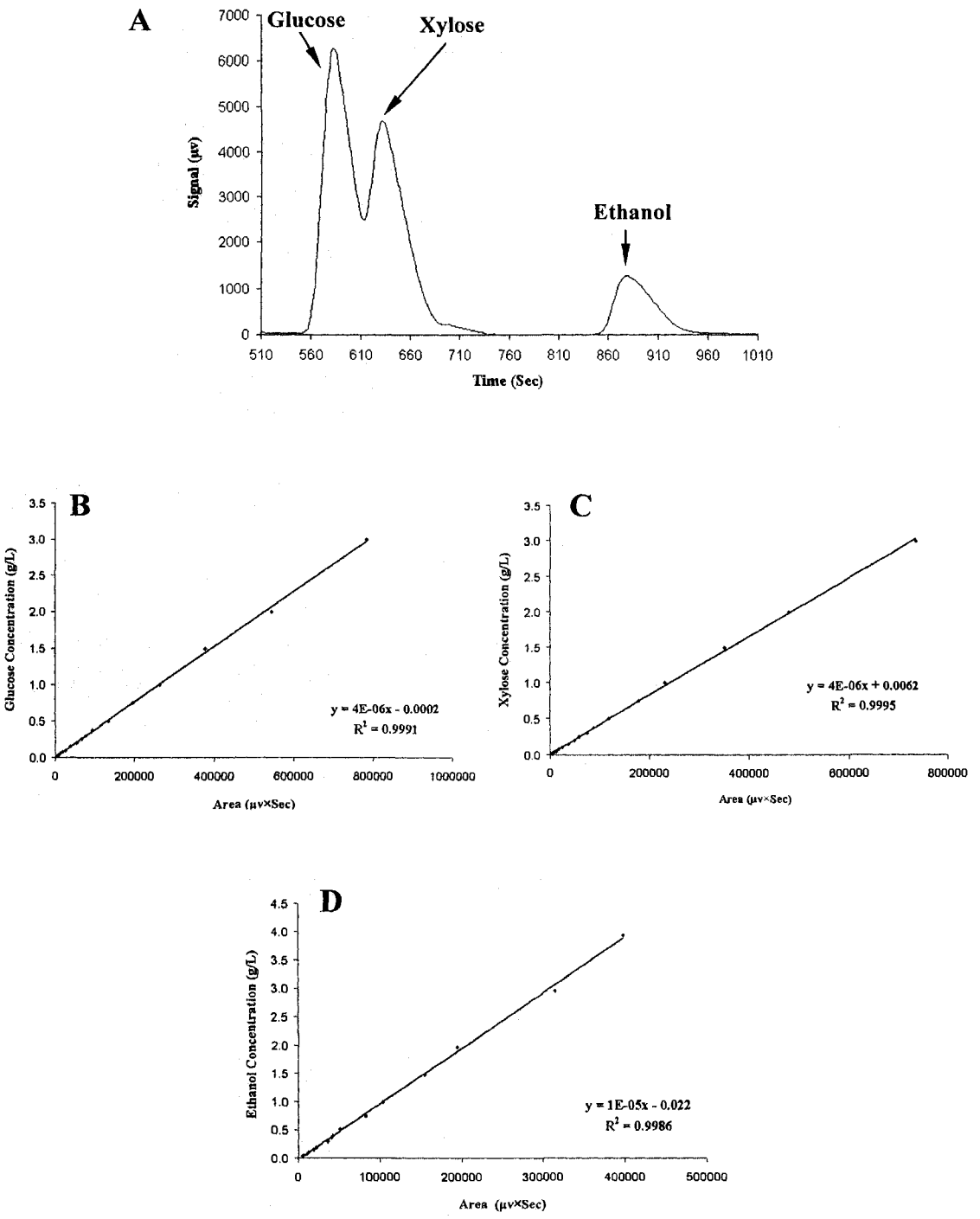
Optical density at 550 nm of series diluted fermentation broths which were obtained after 6 h cultivation of *Escherichia coli* KO11 on a complex medium containing liquid LB medium and 20 g/L glucose.



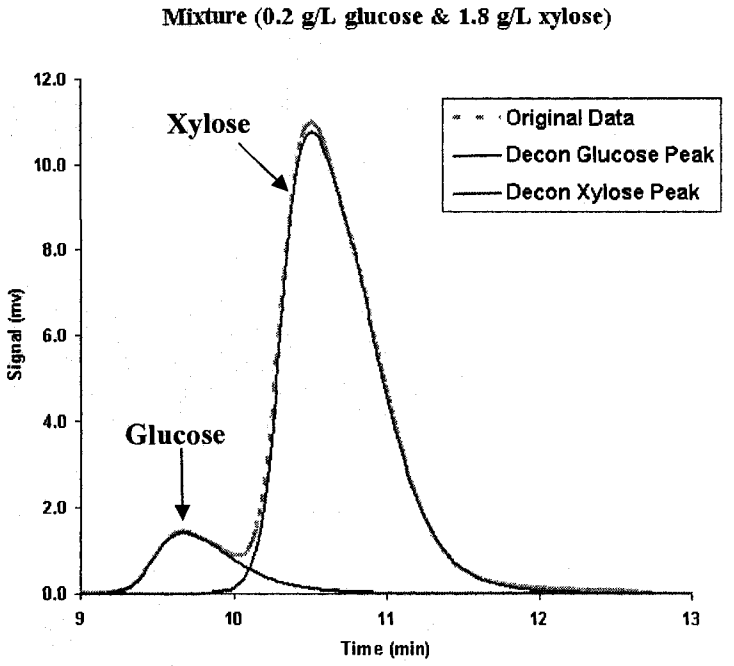
Calibration curve between the cell mass concentration and the optical density at a wavelength of 550 nm measured using a spectrophotometer.

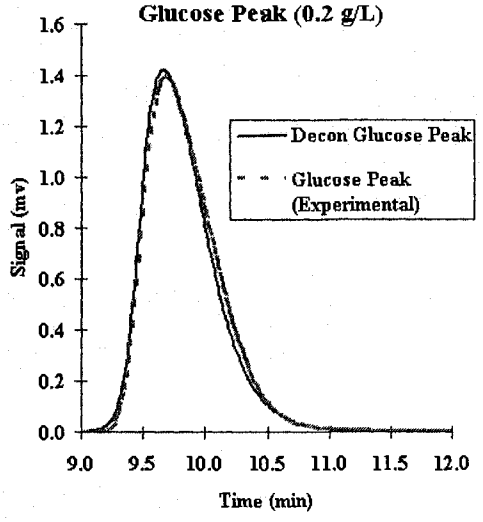


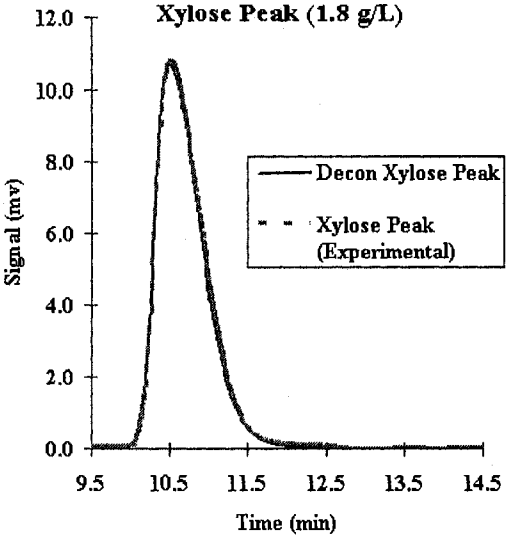
Appendix D. Chromatograms of fermentation broth (A) and calibration curves of glucose (B), xylose (C), and ethanol (D).

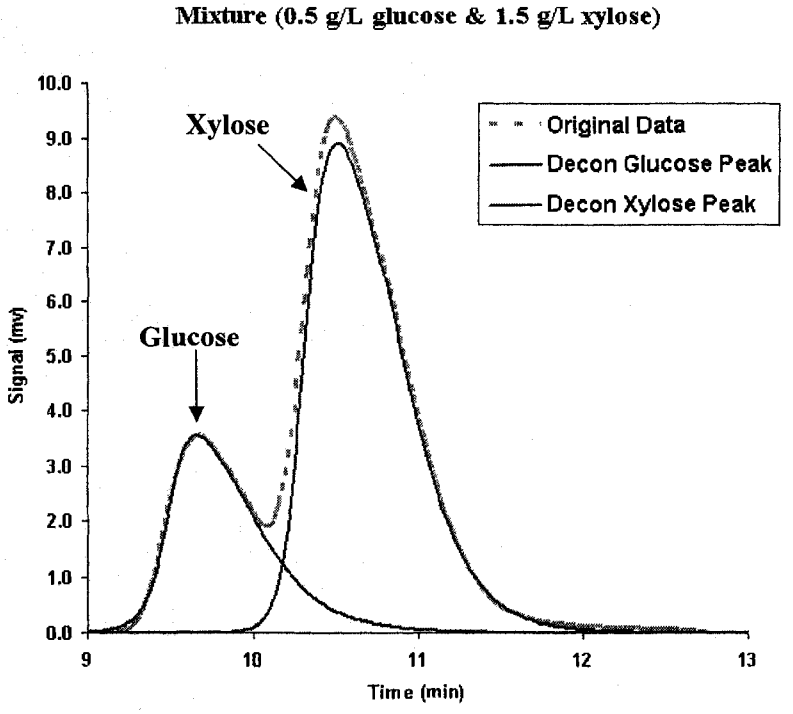


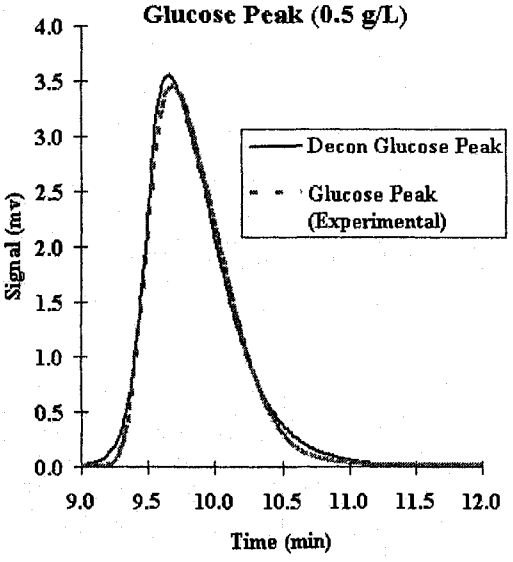
Appendix E. Comparison of the results of deconvoluted peaks of sugar mixtures with peaks generated from single sugar solutions at the same concentrations.

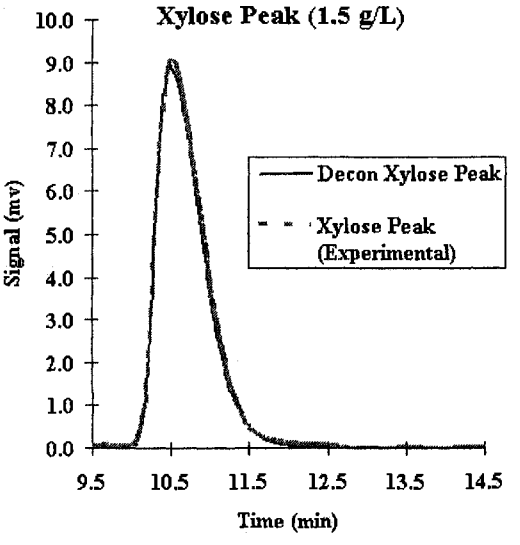


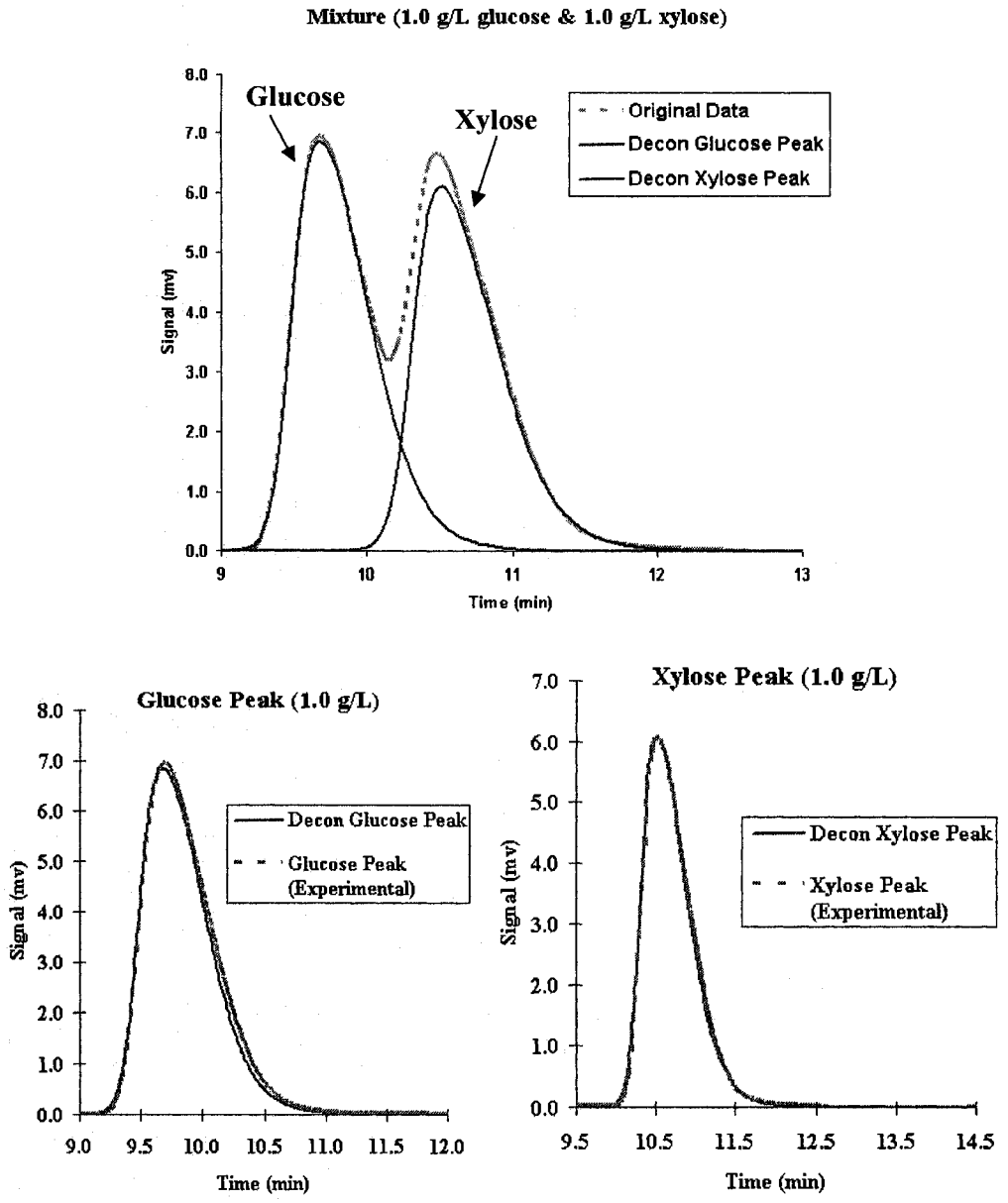





Mixture (0.5 g/L glucose & 1.5 g/L xylose)
Xylose
Glucose
Original Data
Decon Glucose Peak
Decon Xylose Peak
Signal (mv)
Time (min)


Glucose Peak (0.5 g/L)
Decon Glucose Peak
Glucose Peak (Experimental)
Signal (mv)
Time (min)


Xylose Peak (1.5 g/L)
Decon Xylose Peak
Xylose Peak (Experimental)
Signal (mv)
Time (min)



Comparison of calculated areas of deconvoluted peaks with data generated from single sugar solutions.

		0.2 g/L Glucose	1.8 g/L Xylose	0.5 g/L Glucose	1.5 g/L Xylose	1.0 g/L Glucose	1.0 g/L Xylose
Peak Area (mv · min)	Deconvoluted Peaks	0.91357	7.59547	2.38453	6.26347	4.45013	4.28831
	Peaks (single sugar solution)	0.82080	7.57579	2.43662	6.35375	4.53378	4.14275

Appendix F. The substrate (a), product (b), and cell mass (c) profiles of *Escherichia coli* KO11 when cultivated on glucose (A) or xylose (B) media with various initial ethanol concentrations under anaerobic conditions in a rotary shaker at 100 rpm, 30°C.