Co-fermentation of hexose and pentose by *Zymomonas mobilis* for enhanced ethanol production

A Thesis

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By

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STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India, under the supervision of Prof. Debasish Das.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Co-fermentation of hexose and pentose by *Zymomonas mobilis* for enhanced ethanol production**" by Mrs. Payel Sarkar for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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*"**At times, our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us**."*

—Albert Schweitzer

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Abstract

Rapid expansion and development of industries along with exponential growth in world population have led to a surge in the global energy demand in recent times. An escalating increase in dependence on conventional non-replenishing fossil-based fuels have triggered concerns on the reduction in their reserves and subsequent increase in global carbon footprint with its detrimental effects on climate change. To that end, extensive efforts and culmination of ideas prompted development of sustainable and renewable fuels. Biofuels, especially bioethanol and biodiesel, have emerged as prospective alternative renewable energy source with the potential to significantly reduce the thrust on the depleting fossil fuel reserve as well as carbon dioxide emissions. Bioethanol through microbial fermentation of organic matter or wastes has been considered as a redoubtable competitor of the existing petroleum centric transportation fuels, owing to its desirable properties such as easy blendability with gasoline, reduced greenhouse gas emission, carbon neutral etc., to mention a few. Conventional use of food crops, as substrates, for bioethanol production rendered assailable socio-economic conditions, which led to the exploitation of inexpensive, sustainable and abundant alternative substrate e.g. lignocellulose, for bioethanol production through microbial fermentation. Lignocellulose is majorly composed of cellulose, hemi-cellulose and lignin, which upon hydrolysis yields a mixture of sugars comprising majorly of hexose (D-glucose) and pentoses (D-xylose and L-arabinose). The typical composition of lignocellulosic hydrolysates necessitates the development an ethanologenic microbial platform with the ability to effectively utilize both hexose as well as pentose sugars, which would also mark its significance in commercial bioethanol fermentation process. *Zymomonas mobilis*, owing to its higher ethanol yield, productivity, and ethanol-tolerance, proved to be an exceptional candidate bioethanol producer, when compared to commercially acknowledged ethanol producing *S. cerevisiae*.

However, owing to lack of a complete pentose phosphate pathway, wild type *Z. mobilis* cannot metabolize xylose or arabinose.

The present study has been designed and implemented with the primary rationale to overcome the challenges existing towards efficacious pentose fermentation, by development of engineered *Z. mobilis* strains adept in utilizing hexose and pentose sugars with comparable efficiencies, for commercially realizable production of bioethanol. To that end, two *Z. mobilis* strains, i) *Zymomonas mobilis* ATCC 31821 or ZM4 (a wild type *Z. mobilis* strain) and ii) *Zymomonas mobilis* ATCC ZW658 (a recombinant *Z. mobilis* strain), were characterized in terms of growth, substrate utilization, and product formation. Based on their performance, *Z. mobilis* ATCC 31821 or ZM4 was initially selected for genetic manipulation towards simultaneous utilization of glucose and xylose. Directed metabolic engineering was employed to modify ZM4 with heterologous xylose utilizing genes unresponsive to glucose inhibition (Carbon catabolite repression). However, the engineered ZM4 strain did not manifest the desired traits as expected. Thereafter, *Zymomonas mobilis* ATCC ZW658 (ZW658) endowed with heterologous xylose metabolizing genes integrated in its genome was selected for genetic engineering using a systematic Adaptive Laboratory Evolution (ALE) strategy. ZW658 was subjected to extended ALE involving 50 transfers, carried out over a period of 200 days. The strain was grown under strict selection pressure of increasing xylose concentration from 30 g L^{-1} to 100 g L^{-1} to obtain an adapted strain designated as AD50. Process engineering strategies were designed to enhance the phenotypic response of AD50 in terms of xylose utilization and ethanol formation. However, since the primary objective of this study was to develop a *Z. mobilis* strain that could simultaneously utilize glucose & xylose and produce high amounts of ethanol, AD50 was further subjected to adaptation in presence of glucose under two different strategies. In the first strategy undertaken, AD50 was alternatively subcultured in media

containing 10% (w/v) xylose or 2% (w/v) glucose. In the second strategy, AD50 was serially subcultured in media containing 8% (w/v) xylose in the initial 48 h of growth followed by the addition of 2% (w/v) glucose in the same cultivation media and the culture was allowed to grow further for 24 h. After 72 h of cultivation, the serial subculture steps were repeated. After 6 serial transfers, single isolated colony was obtained, one each from the first and second strategy of ALE in glucose and xylose based media. The adapted strains were designated as AS1-6 and AS2-6, respectively. The adapted strains AD50, AS1-6 and AS2-6 were further characterized in media containing 5% (w/v) glucose and 5% (w/v) xylose. It was observed that AD50 outperformed AS1-6 and AS2-6 in terms of specific xylose uptake rate in presence of glucose. The evolved strain (AD50) exhibited 1.65 times increase in the overall specific xylose utilization rate when compared with the parent strain (ZW658) and other developed strains reported till date. AD50 also displayed enhanced performance in terms of co-fermentation of xylose in presence of glucose with specific xylose utilization rate of 1.34 g g^{-1} h^{-1}, as compared to that of the parent strain ZW658 (0.13 g g^{-1} h^{-1}). High throughput (Next-gen) sequencing revealed novel mutations in xylose assimilating, metabolizing, and crucial regulatory pathway genes, which substantiate the improved phenotypic response of AD50 in terms of co-utilization of glucose and xylose, higher ethanol and reduced xylitol production. Enzyme activity assays were carried out to validate the performance of the strain with high confidence.

Although, directed ALE proved to be an efficient strategy for development of a *Z. mobilis* strain, which can co-utilize glucose and xylose at comparable rate, however, there was scope for improvement in terms of simultaneous utilization of xylose in presence of glucose by AD50. Previous research endeavours have highlighted bottlenecks associated with xylose metabolism in *Z. mobilis*. Hence, our second objective was to investigate 'transport' as a possible obstacle for simultaneous of glucose and xylose. In particular, we hypothesized that the slow uptake of xylose through the promiscuous Glf transporter may limit the efficiency of

xylose metabolism in *Z. mobilis*. To test this hypothesis, an array of constructs with xylose specific transporters were developed using AD50 as the host organism. XylE, the low-affinity xylose transporter from *Escherichia coli*, XylE* (a mutant variant of XylE), XylFGH, the ABC type transporter from *E. coli*, was expressed in AD50. Introduction of the xylose transporters, especially an ABC type transporter system, manifested into increased rate of xylose utilization by 48.9%, in presence of glucose by the novel engineered *Z. mobilis* strain, as compared to that of the host strain (AD50), leading to notably reduced fermentation time and co-utilization of glucose and xylose coupled with comparable ethanol production to that of AD50. The specific utilization rate of xylose in presence glucose was observed to be 2.04 g g^{-1} h^{-1}, which is comparable to that of increased glucose (2.49 g g^{-1} h^{-1}). Thus, in the ensued study an improvement of 14.7-fold was observed in term of specific utilization rate of xylose in presence glucose as compared to that of the parent strain ZW658, used as the initial platform strain for genetic manipulation. The ethanol titer was observed to be 47.4 g L^{-1}, with productivity and yield of 1.97 g L^{-1} h^{-1} and 0.472 g g^{-1}, respectively. This study confers an apprehension of the effect of different xylose transporters in *Z. mobilis*, which have not been explored till date. The phenotypic response of the best performing strain, Zm-Ppdc-XFGH was observed to be consistent under scale up conditions in a bioreactor.

Hence, the ensued study successfully demonstrates development of an efficient bioethanol producing *Z. mobilis* strain with a potential to co-utilize glucose and xylose, which might aid towards commercial realization of ethanol biosynthesis.

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INTRODUCTION

"The beginning is the promise of the end."

Henry Ward Beecher, *American Congregationalist*

CHAPTER 1

Introduction

1.1 Background and motivation

"We shall need a new way of thinking if humanity is to survive", by - Albert Einstein, gives a felicitous justification to the motivation behind this study. Energy crisis is a global concern that is aggravating since the last few decades (Yang et al., 2016). The exponential rise in world's population and the subsequent increase in energy demand, has led to this predicament which has severe environmental, economic and sociopolitical impacts (Peralta-Yahya et al., 2012). Depleting fossil fuel reserves has highlighted the exigency to produce sustainable and renewable fuels (Peralta-Yahya et al., 2012). "Bio-fuels" ~ liquid fuels derived from renewable plant biomass and animal wastes, have emerged as an alternative to fossil fuels. Biofuels *e.g.* bio-ethanol and bio-diesel have drawn global attention owing to their environmental merits over fossil fuels (Nieves et al., 2015; Peralta-Yahya et al., 2012).

Bio-ethanol has emerged as the most promising bio-fuel at commercial scale (Yang et al., 2016). Easy blending ability with gasoline and reduced greenhouse gas emissions are the unique traits which have rendered bioethanol as the most desirable bio-fuel (Pal, 2011). Conventionally, the first-generation bio-fuel was being produced from food crops, eventually triggering economic vulnerability (Strogen, Horvath, & McKone, 2012). This has led to the utilization of non-food feedstock *e.g.*, lignocellulose, in recent times, typically referred to as second generation feedstock for bioethanol production (Nieves et al., 2015). Lignocellulose, an inexpensive and eco-friendly alternative, is principally composed of cellulose, hemi-

cellulose and lignin. The hydrolysis of lignocellulose yields a sugar mixture consisting majorly of hexoses (glucose) and pentoses (xylose and arabinose) (Nieves et al., 2015).

Model organisms namely *Escherichia coli, Zymomonas mobilis* and *Saccharomyces cerevisiae* have been exploited for the production of bioethanol by reconstituting certain metabolic pathways. *E. coli* has been reported to utilize multiple substrates for ethanol production, yet has low ethanol tolerance, narrow pH range for growth and yields low ethanol productivity (Dien, Cotta, & Jeffries, 2003). Although, *S. cerevisiae* is a robust organism with high ethanol productivity, it is constrained by the production of by-products and its susceptibility to contamination. On the other hand, *Z. mobilis* shows resistance to contaminations, higher specific rate of ethanol production and high ethanol tolerance compared to other organisms. These properties exhibited by *Z .mobilis* make it a potential candidate for ethanol production (He et al., 2014).

Zymomonas mobilis, a gram-negative bacterium was isolated from plaque and tainted cider in the late 1970's (Swings & De Ley, 1977). Unlike other ethanol producing model organisms, *Zymomonas,* a facultative anerobic bacterium utilizes hexoses like glucose, sucrose and fructose by the Entner- Doudoroff (ED) pathway (Nieves et al., 2015), a characteristic of strictly aerobic organisms. ED pathway enzymes are very stable and do not show any inhibitory effect even at high glucose concentration (Kim, Shoemaker, & Mills, 2009). *Z. mobilis* exhibits a unique behaviour known as "uncoupled growth phenomenon" where sugars are fermented to ethanol after being transported into the cell, regardless of the growth requirement by the organism (He et al., 2014). As a result, *Zymomonas* produces less cell biomass and the carbon flux is majorly channeled to the production of the desired fermentation product (He et al., 2014). This eventually leads to reduced cell biomass production coupled with high ethanol titer. In addition to the complete ED pathway, *Z. mobilis* also contains most of the enzymes involved in Embden Meyerhof Parnas (EMP) pathway and Tricarboxylic Acid (TCA) cycle

but lacks most of the enzymes involved in Pentose Phosphate Pathway (PPP) (He et al., 2014). Thus, native *Z. mobilis* strains cannot ferment pentose sugars like xylose.

Studies on metabolic engineering of *Z. mobilis* for co-utilization of multiple substrates and enhanced ethanol production has mainly focused on: a) incorporating metabolic genes for pentose utilization from other organisms such as *E. coli* into *Z. mobilis*, b) adaptive laboratory evolution (ALE) techniques to induce adaptation based mutations in the genome of the organism, c) incorporating transporter genes for arabinose and xylose into *Z. mobilis* and d) Over expression or knock down of genes against inhibitors and high ethanol concentration (Agrawal, Mao, & Chen, 2011; Dunn & Rao, 2014; Zhang et al., 1995). National Renewable Energy Laboratory (NREL), USA was the first to develop a *Z. mobilis* strain, which could consumed xylose as its sole carbon source, by heterologous expression of transaldolase, xylose isomerase, xylulokinase, and transketolase genes from *E. coli* (Zhang et al., 1995). However, this strain could not efficiently simultaneously utilize pentose (xylose) and hexose (glucose) sugars. This might be due to some intrinsic regulatory mechanisms similar to carbon catabolite repression (CCR) (Agrawal et al., 2011; Dunn & Rao, 2014, 2015; Gao, Zhang, Mcmillan, & Kompala, 2002; Mohagheghi, Evans, Chou, & Zhang, 2002; Zhang et al., 1995).

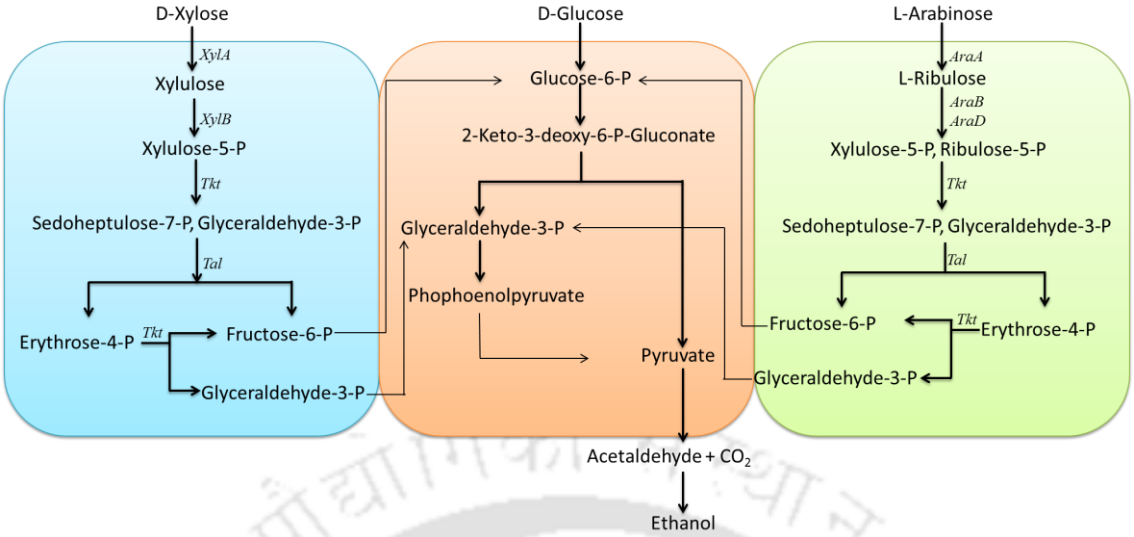

Fig. 1.1 Simplified schema of the hexose (glucose), and pentose (xylose and arabinose) metabolism in recombinant *Z. mobilis*.

Unlike directed metabolic engineering strategies, Adaptive Laboratory Evolution (ALE) is an organic process of achieving beneficial mutations in multiple genes and regulatory regions of microorganisms in an unbiased fashion under specific selection pressure (Portnoy, Bezdan, & Zengler, 2011). Agrawal et. al. (2011) reported the modification of a xylose utilizing *Z. mobilis* strain for efficient multi-substrate utilization through ALE. A mutation in the promoter region of the cloned xylose isomerase gene was identified which resulted in fivefold enhanced expression and subsequent increased enzyme activity, revealing the importance of this xylose assimilating enzyme in xylose metabolism (Agrawal et al., 2011). The ethanol yield obtained was 92% of the maximum theoretical yield though there was a lag of 12 h in complete xylose utilization compared to glucose.

In further research endeavours, other bottlenecks associated with pentose metabolism in *Z. mobilis* were also investigated. Dunn and Rao (2015) found that expression of mutated glucose facilitated diffusion protein obtained through ALE increased the rate of xylose fermentation in adapted strain, suggesting that inefficient transport is a key bottleneck in pentose utilization (Dunn & Rao, 2014). In a separate study, they also expressed XylE, a xylose

specific transporter of *E. coli*, in *Z. mobilis*. In the resulting strain, the rate of xylose metabolism was enhanced, but only at high-xylose concentration. Although, these studies could highlight the potential targets for future metabolic engineering towards efficient xylose metabolism in the evolved *Z. mobilis*, the strains could not exhibit the phenotypic traits of simultaneous utilization of glucose and xylose in the true sense. These identified challenges, in terms of simultaneous utilization of glucose and xylose by *Z. mobilis*, forms the rationale behind framing the objectives of the ensued study.

1.2 Objectives of the study

The investigations carried out in the thesis titled "**Co-fermentation of hexose and pentose by *Zymomonas mobilis* for enhanced ethanol production**" had the following objectives formulated based upon the current bottlenecks in terms of substrate utilization for bioethanol production by *Z. mobilis*:

- *Adaptive Laboratory Evolution (ALE) of Z. mobilis directed towards simultaneous utilization of glucose and xylose*
- *Construction of an array of plasmids containing xylose transporter genes and expression of transporters in the adapted strains of Z. mobilis (AD50) for efficient glucose & xylose uptake by the organism.*
- *Performance evaluation of the adapted Z. mobilis strain under scale-up condition in a bioreactor.*

1.3 Approach of thesis

Present study is modelled around addressing key bottlenecks towards dual substrate (hexose and pentose sugars) utilization by *Z. mobilis* for bioethanol production, namely (i) inefficient xylose utilization, (ii) unknown intrinsic metabolic regulations leading to preferential utilization of glucose over xylose and (iii) absence of xylose specific transporter

leading to inept xylose uptake. The bottlenecks along with their possible remedial strategies have been systematically enumerated in the forthcoming thesis chapters.

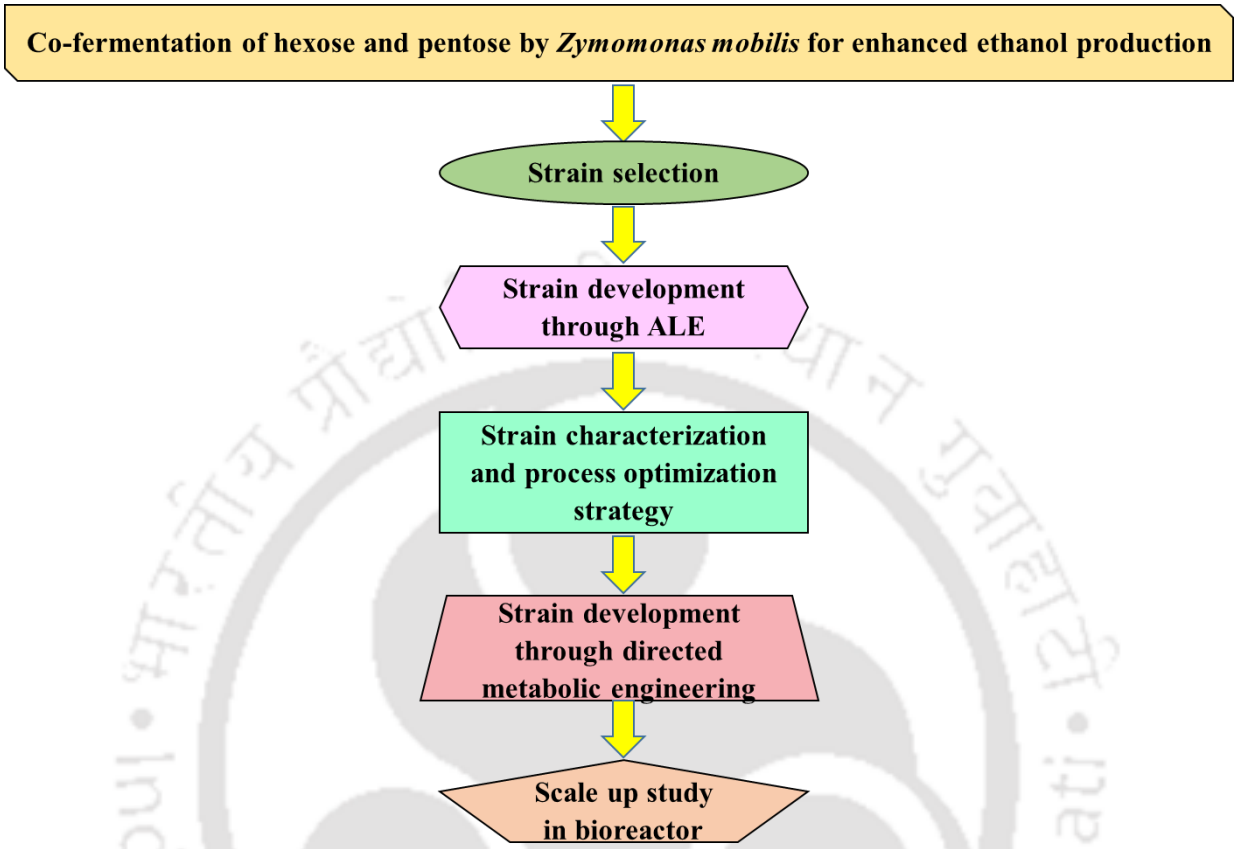

Fig. 1.2 Organization of the thesis enumerating the methodologies employed for undertaking the proposed strategy in turn addressing the key limitations in hexose and pentose utilization by *Z. mobilis* for ethanol biosynthesis.

In the first step, two *Z. mobilis* strains were characterized for possible use as a potential platform for genetic manipulation. The strain with the best characteristic traits towards substrate utilization was selected for further strain development studies. In the next step, ALE was used as a tool to ensure that the evolved strain imbibes the desired traits of simultaneous utilization of glucose and xylose. The evolved strain was characterized at genome level as well as in terms of biochemical attributes. Further, directed metabolic engineering was employed to express heterologous specific sugar transporters in the evolved *Z. mobilis* strain to further

enhance simultaneous uptake of dual substrate and subsequent utilization. Extensive characterization of the mutant strains were carried out under optimized growth conditions. In an effort to reduce the titer of xylitol (obtained as a by-product), the process was aimed to be improvised by the addition of divalent metal ions at different concentrations. Finally, scale up studies were conducted to evaluate the performance of the engineered *Z. mobilis* strain in bioreactor.

1.4 Thesis organization

Chapter 1 establishes the background and motivation for the current study. This chapter highlights the existing gaps towards development of an efficient *Z. mobilis* strain capable of simultaneously utilizing hexose and pentose sugars. It also elaborates the challenges to bridge the gaps and the potential hypotheses, to resolve the obstacles in the current state-of-the-art technology. It also outlines the strategies employed in the present study to counter the major bottlenecks assessed from the existing literatures towards successful development of a *Z. mobilis* strain with broadened substrate utilizing and enhanced product forming capabilities.

Chapter 2 presents a detailed literature survey on the current scenario of diminishing fossil reserves that is predicted to trigger a global energy crisis with a special emphasis of biofuels particularly bioethanol as a potential and sustainable alternative to combat the exponential energy demand for liquid transportation fuels. The chapter delves in establishing the importance of *Zymomonas* sp. as the suitable microbial platform for bioethanol synthesis with in-depth study of its biology, physiology, biochemical and metabolic pathways. Further, the chapter highlights the current state-of-the-art in terms of various strategies employed towards genetic manipulation of *Zymomonas* strains for broadening its substrate utilization capability and efficiency leading to improved product formation. It also reports the different strain improvement rationales directed towards maximization of product titer, productivity, and yield.

Chapter 3 encompasses the strategies designed in this study to develop *Z. mobilis* strains, capable of efficiently co-utilizing glucose and xylose. It illustrates the rationale behind selection of a suitable platform for genetic manipulation. It presents a systematic Adaptive Laboratory evolution (ALE) strategy undertaken to let the selected *Z. mobilis* strain organically evolve, for efficient xylose utilization, especially in presence of glucose. Process engineering strategies were designed to enhance the phenotypic response of the adapted strain in terms of glucose and xylose co-utilization and ethanol formation. High throughput (Next-gen) sequencing revealed novel mutations in xylose assimilating, metabolizing, and crucial regulatory pathway genes, which substantiate the improved phenotypic response of AD50 in terms of co-utilization of glucose and xylose. Enzyme activity assays were carried out to validate the performance of the strain with high confidence.

Chapter 4 enumerates the effect of different heterologous xylose specific transporters, expressed in the evolved *Z. mobilis* strain, towards simultaneous utilization of glucose and xylose. Directed ALE proved to be an efficient strategy for development of a *Z. mobilis* strain, which could co-utilize glucose and xylose at comparable rates. However, the xylose consumption rate of the evolved strain still lagged marginally behind glucose consumption rate, which in turn extends the fermentation time. Hence, this chapter entails the rationale behind selection of the xylose specific transporter genes leading to construction of a transporter library and subsequent expression in the adapted strain. It also presents a detailed characterization of all the recombinant strains developed through directed metabolic engineering strategy. Further, various divalent metal ions were screened to reduce by-product formation by the engineered strain, with best phenotypic response, in terms of simultaneous utilization of glucose and xylose. It also provides a comprehensive performance evaluation of the developed *Z. mobilis* strain, under scale-up conditions. It highlights the feasibility of commercial application of the recombinant strain.

Chapter 5 summarizes the key research highlights obtained from the present study and elucidates the future prospects stemming out of the key outcomes of the thesis.

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Chapter 2

REVIEW OF LITERATURE

"Research is formalized curiosity. It is poking and prying with a purpose."

Zora Neale Hurston, American author

CHAPTER 2

Review of Literature

2.1 Reflections on global energy crisis and rise of renewable fuels

Energy is the core driving force for everything thriving on planet earth (Coyle D. & Simmons A., 2014). The incessant quest for energy was never as huge as it does today. This has led to a perturbed situation of global energy-crisis (Coyle D. & Simmons A., 2014). There has been a direct synergy between the exponential rise in population associated with major demographic disruptions which led to an escalation in global energy demand thus, adversely affecting the global climate (BP p.l.c., 2019). It has been appraised that the global GDP will be doubled by 2040 owing to the increasing opulence of the rapidly developing nations. This apparent prediction coupled with anticipated population increase, which is expected to be around 9.2 billion by 2040, would result in an eventual exponential increment in energy demand, where, India and China would account for two-third of the increase (BP p.l.c., 2019). Annual statistical report of British petroleum accounts China's energy utilization to be maximum, closely followed by United States of America and India (BP p.l.c., 2019). Continued dependence on fossil-based fuels for energy generation and transportation have been witnessed in the present day world. Globally, crude oil (31%), coal (28%) and natural gas (25%) have been the major contributors towards energy generation, whereas, India's energy production is majorly dependent on coal (56%) and oil (30%) (BPSTATS, 2019). Extensive utilization of these naturally borne, non-renewable resources has resulted in their rapid depletion, where, oil is predicted to be completely exhausted in the next 50 years (Barreto, 2018). Furthermore, extensive utilization of these resources has led to over-accumulation of greenhouse gases

(GHG) such as carbon dioxide, methane, etc. in the atmosphere resulting in disruption of global climatic condition. Global temperature was reported to increase at a rate of 0.07 °C per decade, on average, between 1880 to 1980 (National Centers for Environmental Information, 2019). Shockingly, in 2019, the average global temperature elevated at an alarming rate of 0.95°C as compared to that of average of 20th century (National Centers for Environmental Information, 2019). This flustered condition can be associated with carbon dioxide incrementing at a rate of 3 ppm annually, currently highest at 410 ppm (Fig 2.1) (Friedlingstein et al., 2019).

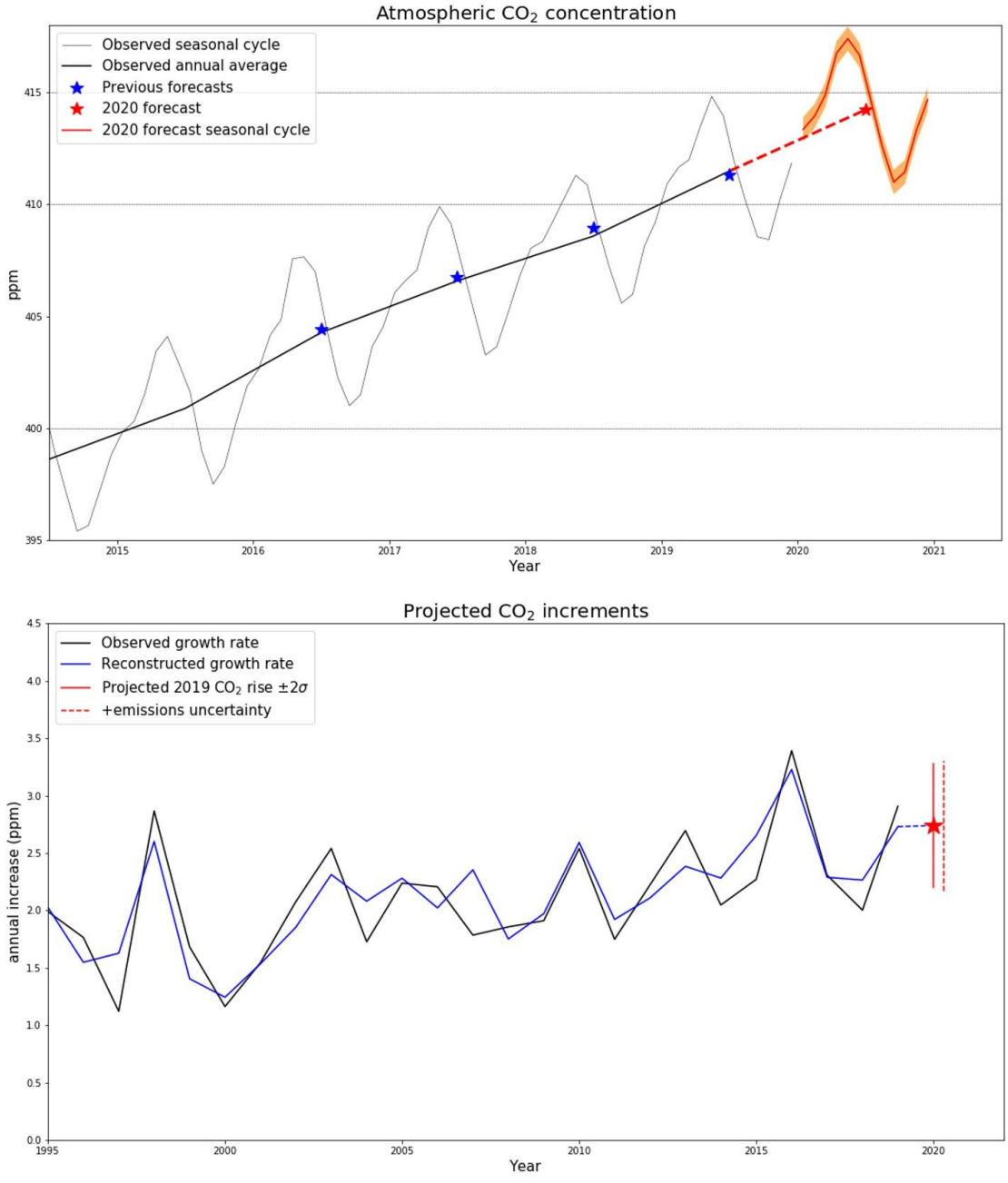

Fig. 2.1 Graphical representation of **A)** forecast of global CO_2 concentration and **B)** projected annual increment in CO_2 concentration. Sourced from (Friedlingstein et al., 2019)

An estimation of the contribution of different sectors towards global greenhouse gas emission has been depicted in figure 2.2**.** There is a collegial effect of increasing carbon dioxide emission to deteriorating climatic condition, pollution and health conditions. It is evident that conventional fossil-based liquid transportation fuels and coal dependent electricity generation contribute a maximum of 28% each towards global carbon footprint, followed by 22% contribution by industrial emissions (Hockstad L. & Hanel L., 2018). Fossil fuel based energy generation is still prevalent, however, due to rapid depletion of fossil reserves along with emission of harmful greenhouse gas (GHG), has triggered a keen interest amongst researchers, worldwide, to identify and devise a better and sustainable alternative of the same. Renewable sources, which can provide nonperishable energy, include solar, wind, hydro, geothermal, and biomass. In terms of recognizing a replacement for non-renewable transportation fuels, electric vehicles are under research trials but have technical issues that hinder widespread usage. Considering the socio-economic indicators of the world, biofuels through blending, would serve as a competent and potential substitute to fossil-based transportation fuels.

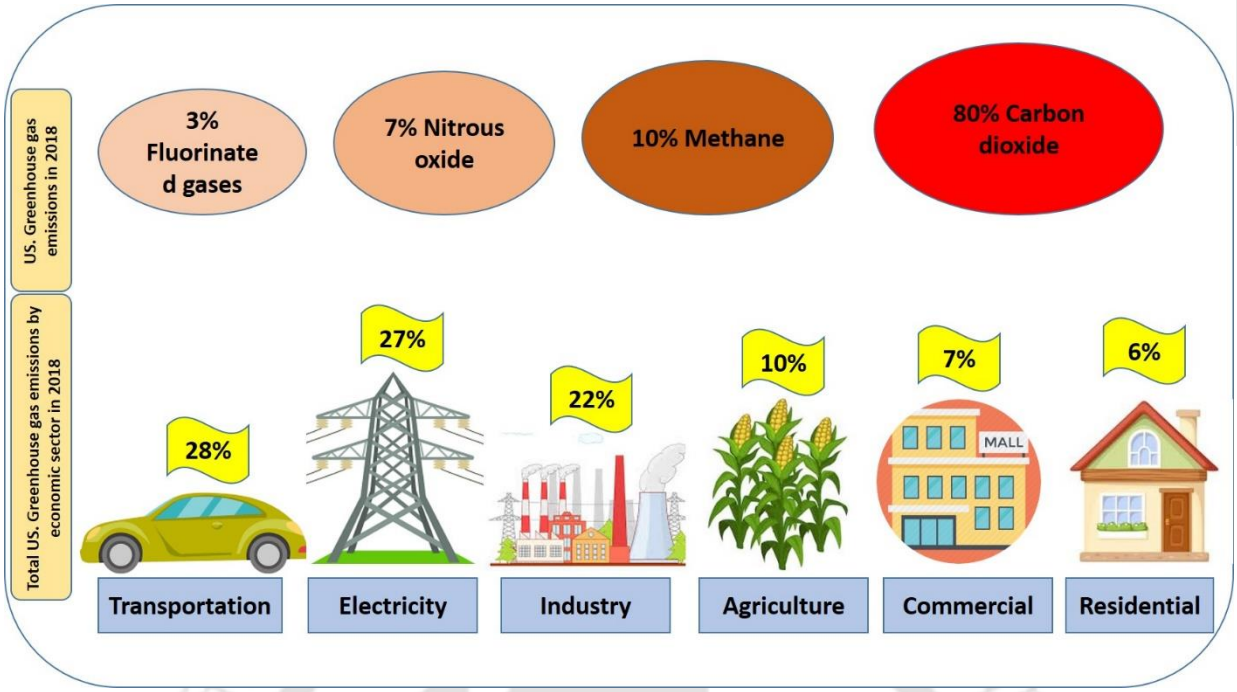

Fig. 2.2 Schematic representation of contribution of different sectors towards global greenhouse gas emissions. Sourced from (Hockstad L. & Hanel L., 2018)

The significance of biomass based biofuels have been recognized over the last few decades by many academicians and industrialists. Current trend, in the biomass based biofuel research, is majorly inclined towards micro-algal biodiesel production, lignocellulosic-based bio-alcohol and higher alcohol production, towards possible blending and partial substitution of the non-renewable fuels in use at present. The importance and use of biofuels have been growing at a positive rate and is expected to increase manifolds by the year 2050 where they will dominate 73% of the total market share (International Energy Agency, 2018) (Fig 2.3).

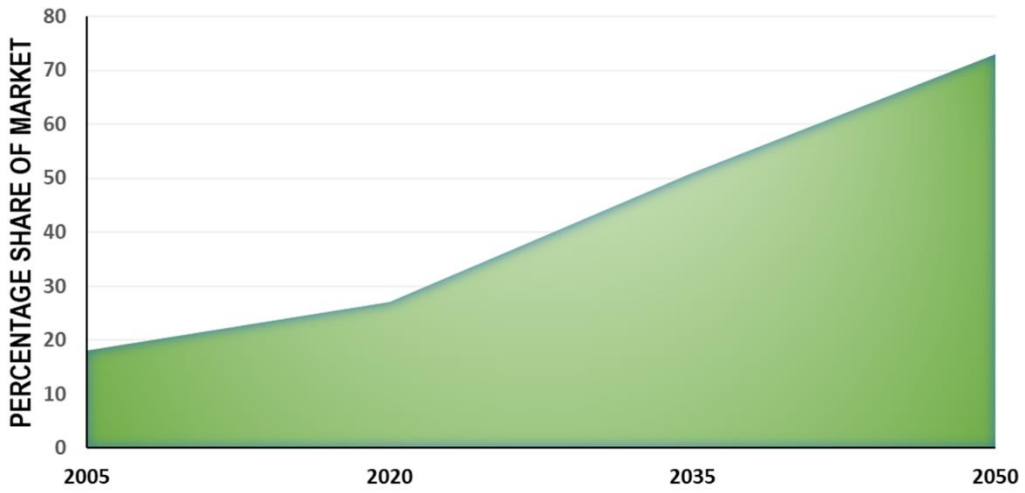

Fig. 2.3 Representation of the total market size of biofuels ranging from 2005 to 2050 (International Energy Agency, 2018)

BP Sustainability report (2018) prognosticates a 46 % reduction in global carbon footprint by the year 2040 as a consequence of synergistic rise and dependence on renewable fuel production accompanied by substantial decrease in coal and fossil based oil consumption (Fig 2.4). Consumption of renewable energy is necessary step, as envisaged, towards eradicating the repercussions of global warming for a healthier environment and sustenance.

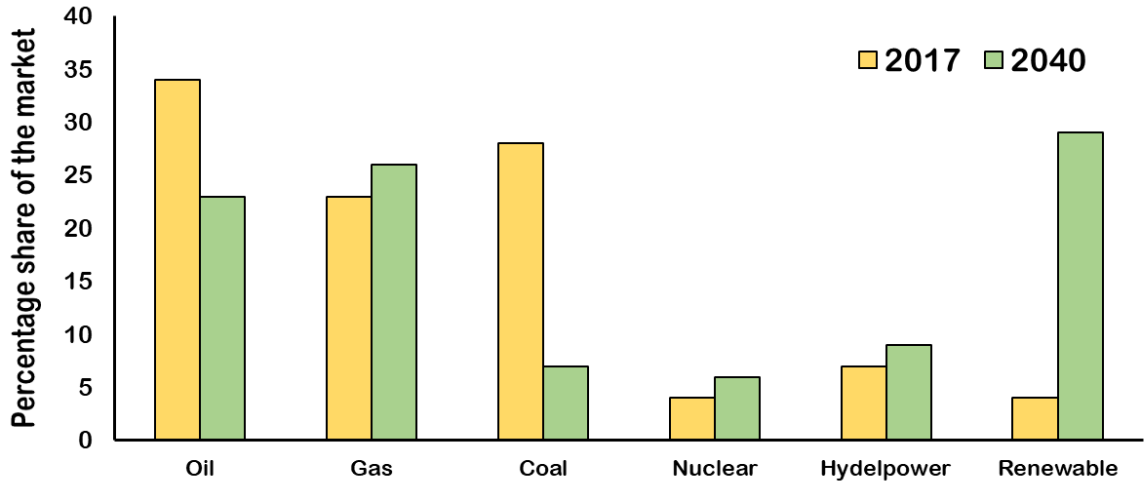

Fig. 2.4 Representation of the change in different energy producers on the total market size (BP Sustainability report (2018))

The USA is the leading country, in the world, in terms of biofuel production (Köhler, Walz, Marscheder-Weidemann, & Thedieck, 2014), with a massive production volume of 1,190.2 thousand barrels/day (43.9% of the global share), while China and India ranked very low constituting 2.6% and 0.5%, respectively of the global share (Fig 2.5).

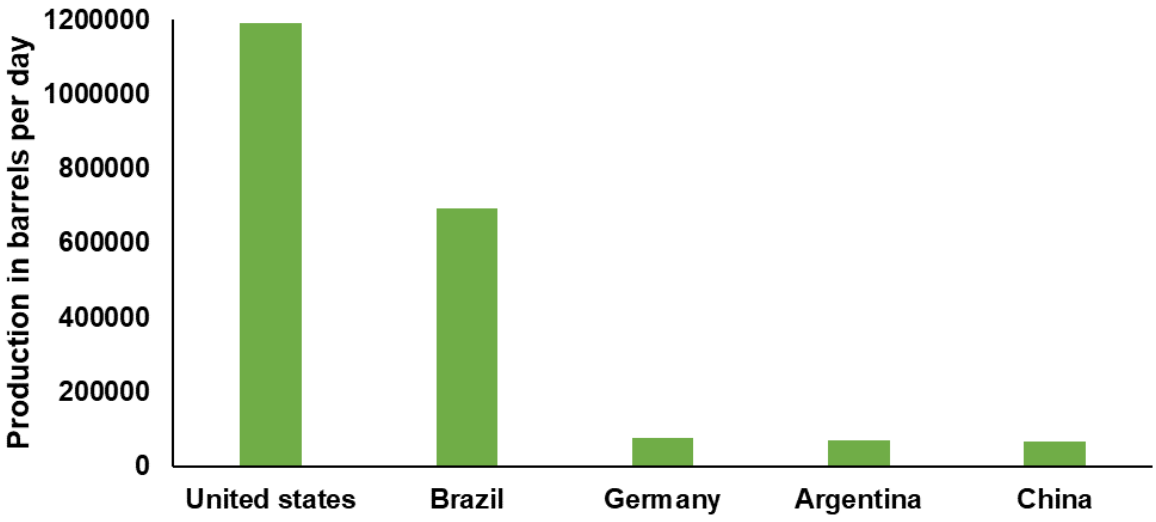

Fig. 2.5 Graphical representation of the leading countries based on biofuel production (Source: NS Energy)

Biofuels are primarily classified based upon the biomass, used as substrate during production process as shown in (Dutta, Daverey, & Lin, 2014; Nigam & Singh, 2011). Biofuels, sourced from crop plants such as soybean, sunflower, corn, rapeseed, barley, wheat, palm, sugarcane, etc. are termed as first generation (1G) biofuels. 1G biodiesel are produced via esterification and transesterification of oils, whereas, fermentation and thermochemical processes are employed for the production of 1G bioethanol and biobutanol. Although, implementation of 1G biofuel led to moderate reduction in GHG emissions, however, they are land intensive and give rise to food versus fuel debate. To that end, efforts have been made towards generation of advanced biofuels using non-food feedstocks, which are designated as second generation (2G) biofuels. 2G biofuels are mainly produced from non-edible oil seeds, agricultural and plant residue, grass, weeds, municipal waste, etc., which are collectively coined as lignocellulosic biomass. They serve as a solution of mitigating food versus fuel crisis coupled with utilization of waste for energy

generation. A major impediment in recognizing 2G biofuel at commercial scale is the costly pretreatment process involved in the conversion of biomass into readily utilizable sugars. Third and fourth generation biofuels involve use of algae and genetically modified algae / microbes respectively. Although, algae is an exceptional feedstock for bio-refinery, in terms of reducing the competition for utilization of food crops, and use of wastewater for its growth, however, use of algae pose obvious challenges in terms of high energy requirement for its cultivation (for mixing, filtration, centrifugation, etc.), low lipid content, chances of biomass contamination in open cultivation systems, and high cost of photo-bioreactor operation. In recent years, bioethanol has emerged as one of the potential alternative liquid fuel (Yang et al., 2016)and has drawn sedulous interest of researchers and industries, globally, towards the production of ethanol and its impact on environment.

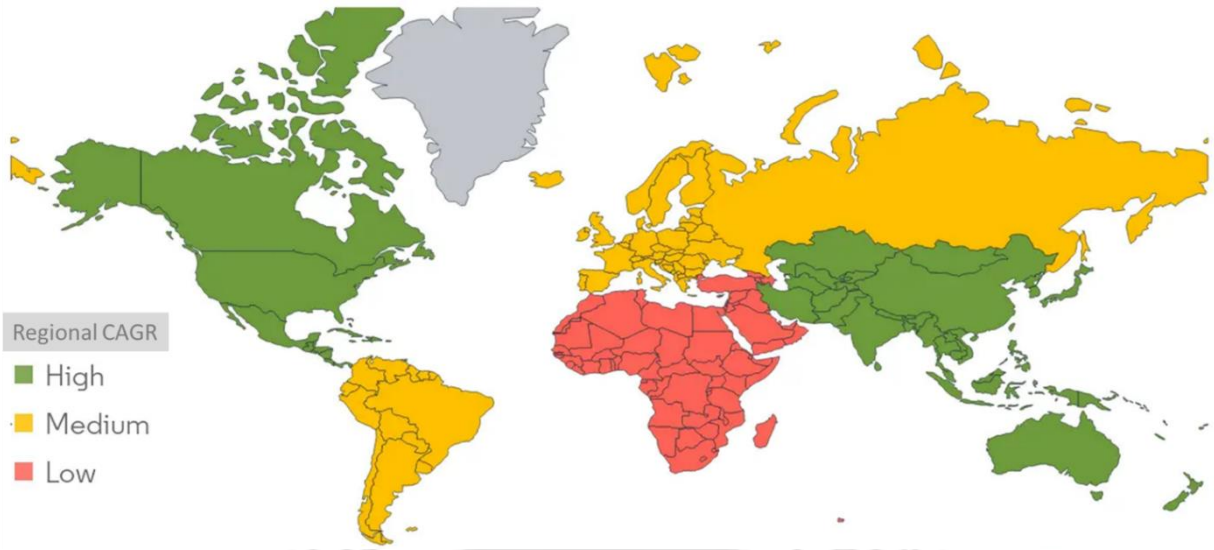

Fig. 2.6 Projected annual growth rate of market demand of bioethanol by the year 2025 (Source: Mordor intelligence)

2.2 History of Ethanol

With a history, dated as far back as 12th century, when production of pure ethanol began. However, imposition of tariff on ethanol production, as a consequence of providing financial assistance in the civil war and availability of inexpensive kerosene, ethanol was promptly replaced by kerosene (Morris, 1993). It was 19th century that this trade set-off as an industry with massive

production figures due to the economic development of the distilling process. It was only in the 20th century; ethanol was realized as a competent alternative fuel for various combustion engines, especially for automobiles. The interest in application of fuel ethanol was reinvigorated in the 1970's due to the oil crisis. The National Alcohol Fuels Commission was established to undertake detailed study on the prospective for alcohol based fuels (Lansing, 1983). In 1980's, the significance of ethanol, as a transport fuel was conceded, when Chrysler, Ford and General Motors released statements that ethanol with blends of up to 10% would be covered in their vehicle warranties (Sharma & Aggarwal, 2020). Now, as we look into the growth of ethanol production, it encountered from less than a billion litres in 1975 to 15,800 billion gallons by the US (only) in 2019 (Licht, 2006) (Fig 2.6).

2.3 Bioethanol and its properties

Unlike petro-chemically-derived alcohols, bioethanol, chemically known as ethyl alcohol, is produced by microbial fermentation from fermentable sugars, derived from plant sources (Chin & H'ng, 2013). Bioethanol possesses specific characteristic traits that makes it one of the most desirable among the known biofuels produced in recent times. Bioethanol is a colourless liquid, low in toxicity, biodegradable and environmentally sustainable (Chin & H'ng, 2013). With a high octane number, bioethanol, when blended with gasoline at varying concentrations, can serve a renewable alternative to petroleum-centric fuels. Bioethanol exhibits high heat of evaporation and high flammability temperature, which has a positive influence on the engine performance along with increased compression ratio. The amount of carbon dioxide absorbed by the plant sources, during growth, is volumetrically similar to the amount of carbon dioxide produced during combustion of bioethanol, making it carbon neutral in nature. Thus, application of bioethanol certainly have advantages over fossil-based fuels in terms of reducing air pollution (Wang, Saricks, & Santini, 1999). It also undergoes complete combustion, thereby, shows reduction in green house (GHG) emissions. On a life cycle basis, ethanol

produced from corn results in a reduction of 20 percent in GHG emissions relative to gasoline. It was anticipated that, future endeavors towards cellulosic bioethanol production has the potential to reduce GHG emissions by 86 percent as relative to gasoline (Office of Transportation and Air Qulaity, 2007). A detailed list of properties of bioethanol has been tabulated below (table 2. 1).

Table 2.1 Properties of bioethanol

Parameters	**Ethanol properties**
Molecular formula	C2H5OH
Molecular mass	46.07 g/mol
Water solubility	(between –117°C and 78°C)
Density	0.789 kg/l
Boiling temperature	78.5°C
Freezing point	–117°C
Flash point	12.8°C
Ignition temperature	425°C
Explosion limits	Lower 3.5% (v/v) Upper 19%(v/v)
Vapour pressure at 38°C	50 mm Hg
Higher heating value (at 20°C)	29,800 KJ/kg
Lower heating value (at 20°C)	21,090 KJ/kg
Specific heat	Kcal/Kg 60°C
Acidity (p*Ka*)	15.9
Viscosity	1.200 mPa.s (20°C)
Refractive index (*n*D)	1.36 (25°C)
Octane number	99

Sourced from (Walker, 2011)

2.4 Bioethanol feedstocks

For years, intensive research endeavors are focused on discovering cheap and viable carbohydrate sources for production of bioethanol (Mohanty, Behera, Swain, & Ray, 2009). Bona fide assessment of feedstocks for bioethanol production is an imperative step towards its

sustainable commercial application (Gnansounou & Dauriat, 2005). The important factors that defines the usability of the feedstocks include (1) chemical composition of the biomass, (2) emission of greenhouse gases, (3) energy balance, (4) absorption of minerals to soil and water, (5) cultivation practices, (6) soil erosion, (7) availability of land, 8) injection of pesticides, 9) contribution to biodiversity and landscape, 10) raw cost of the biomass, 11) logistic cost, 12) the economic value of feedstocks based on the co-products, 13) appointment of employment, and 14) water availability and requirements etc. Bioethanol feedstocks can be categorized into three major groups namely, 1) First generation, 2) Second generation, and 3) Third generation feedstocks.

2.4.1 First generation feedstock

First generation bioethanol was produced from first generation feedstocks that comprises of agricultural cereal and sugar crops, which also served as a source of human food. The sugar sources that have been exploited for first generation bioethanol production are sorghum (Mamma et al., 1995), sugarcane (Office of Transportation and Air Qulaity, 2007), sugar beet (Içöz, Mehmet Tuğrul, Saral, & Içöz, 2009; Ogbonna, Mashima, & Tanaka, 2001), and molasses (Roukas, 1996). Some studies also reported use of starchy grains like maize (Gaspar et al., 2007) and root crops like cassava (Amutha & Gunasekaran, 2001) for first generation bioethanol production. Sugar extraction process from sugar crops require a milling process, which is rather simpler unlike other multi-step sugar extraction processes. Ethanol can be directly fermented from beet juice or sugar cane juice etc., as a by-product, after the sugar extraction (Içöz et al., 2009). On the other hand, sugar extraction process from starchy grains involve saccharification before fermentation. The steps involved in saccharification include gelatinization of starch followed by enzymatic hydrolysis, to generate glucose monomers, which can be further fermented by microorganisms to ethanol (Mussatto et al., 2010). First generation bioethanol acted as a stepping-stone towards establishing the necessary

infrastructure and policy drivers, to support renewable transport fuels in the international market (U.S. EIA, 2012). However, bulk production of bioethanol from first generation feedstocks, met with several limitations. Exploitation of food crops for future biofuel production was highly unsustainable due to its negative impact on food security, considerable land requirement, high water and fertilizer requirements etc. Thus, as a sustainable alternative approach, second generation bioethanol production was initiated.

2.4.2 Second generation feedstock

Second-generation bioethanol production using non-food feedstock, such as lignocellulose, emerged as a feasible alternative to the impractical first generation approach. Lignocellulose is the most abundant form of carbon present on earth in the form of waste biomass (Xu, Li, & Mu, 2016). Lignocellulose is the most abundant, renewable organic component of the biosphere, which accounts for 50% of world biomass with an approximate annual production of 10 to 50 billion tons (Claassen et al., 1999). Lignocellulosic biomass obtained in the form of agricultural residues is hypothetically inexhaustible due to its photosynthetic based production. Additionally, they are abundantly available and annually renewable. Majorly lignocellulosic feedstocks can be grouped under six categories as represented in table 2.2.

Table 2.2 Six categories of lignocellulosic feedstock

Biomass Category	**Examples**
Agricultural residues	Corn Stover, Sugarcane bagasse etc.
Industrial cellulosic waste	Paper mill and saw mill waste etc.
Municipal solid waste	Newsprint papers, office waste papers etc.
Dedicated herbaceous	Switch grass, Biomass Alfalfa hays etc.
Softwoods	Pine, Spruce etc.

Hardwoods	Aspen, Poplar etc.

Sourced from (Lin & Tanaka, 2006; Sánchez & Cardona, 2008; Sun & Cheng, 2002)

Other advantages of using lignocellulose for production of ethanol includes low net carbon emission, low energy dependency, high energy efficiency, positive impact on food security and diversification of rural economies (Antizar-Ladislao & Turrion-Gomez, 2008). Lignocellulose is majorly composed of cellulose, hemi cellulose and lignin (fig 2.7).

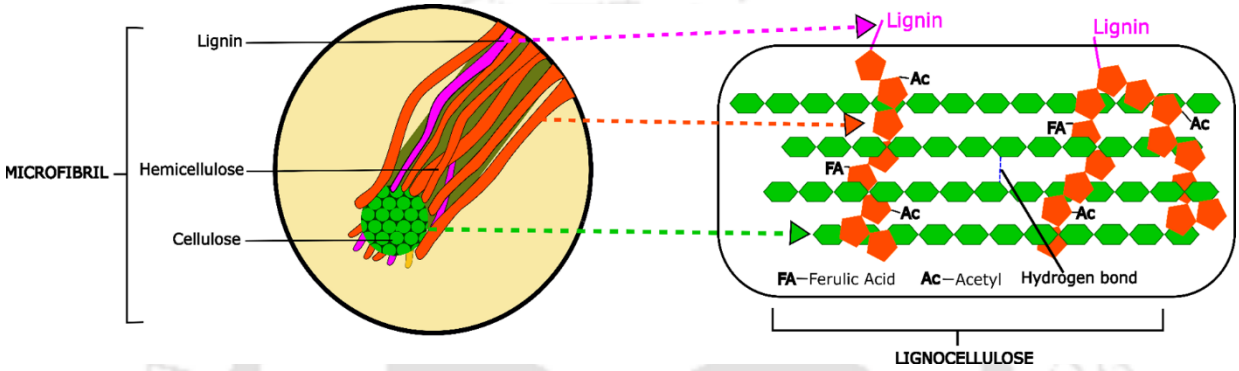

Fig. 2.7 Schematic representation of the composition of lignocellulosic biomass

Cellulose and hemi cellulose are considered to be of great importance as feedstocks for second-generation bioethanol production. Cellulose is a primary building material of plant's cell wall and is a homopolymer composed of β linked D-glucose units (O'Sullivan, 1997). Hemi-cellulose is a heteropolymer consisting of C5 and C6 sugar units, namely D-glucose, D-xylose, D-galactose, D-mannose, L-rhamnose and L-arabinose (Gírio et al., 2010). Unlike cellulose and hemi-cellulose, lignin is a complex biopolymer with heterogeneity in its structure (Vanholme, Demedts, Morreel, Ralph, & Boerjan, 2010). Lignocellulose are being processed to extract the cellulose and hemi-cellulose from the biomass separating the lignin from the usable feedstock (fig 2.8). Cellulose and hemicellulose are further enzymatically hydrolyzed into its monomeric units i.e. D-glucose from cellulose and D-xylose and L-arabinose from hemicellulose.

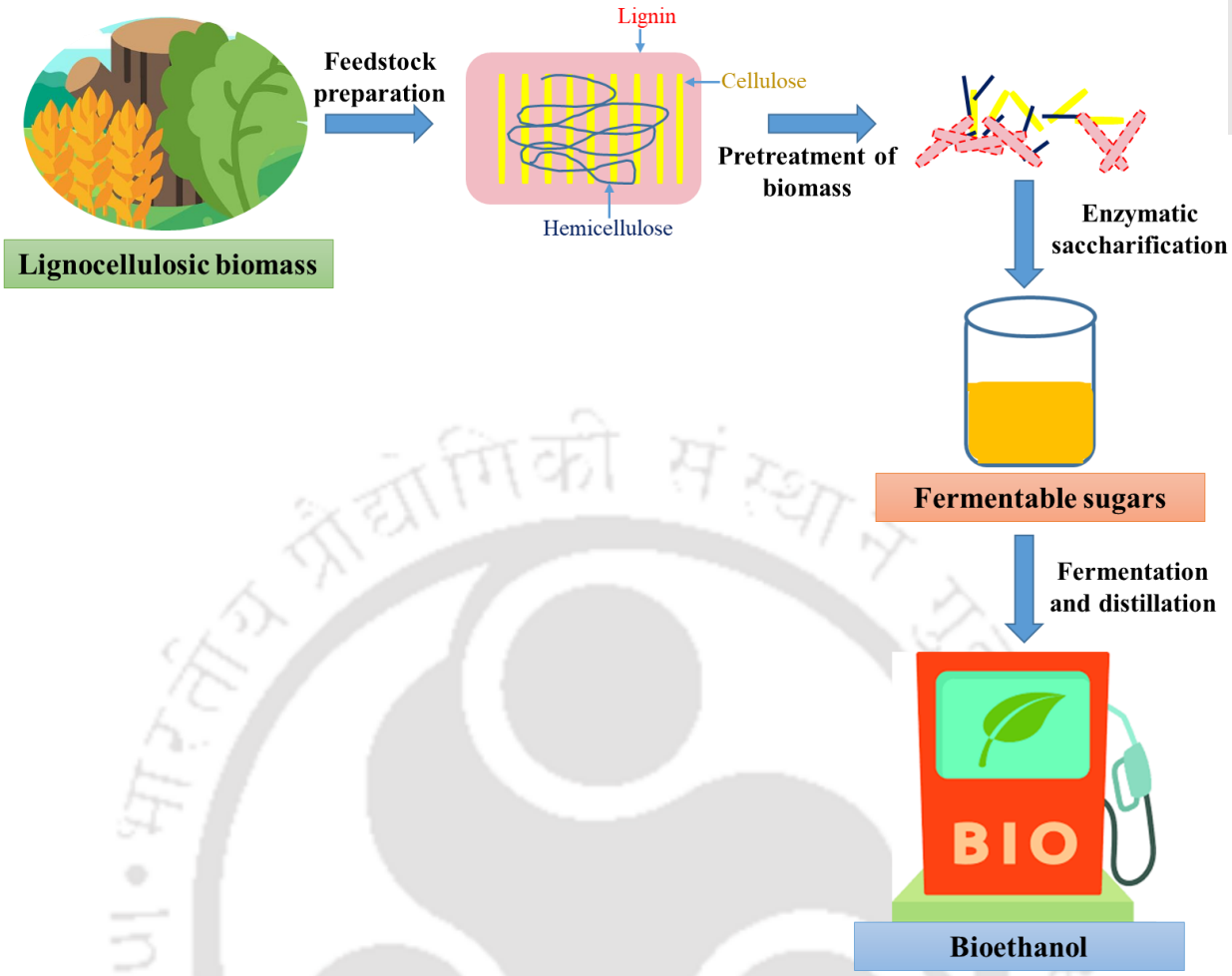

Fig. 2.8 Schematic representation of lignocellulosic biomass conversion for bioethanol production

2.4.3 Third generation feedstock

Biofuels produced from algal biomass represents the third generation feedstock based biofuels (Brennan & Owende, 2010). Microalgae with their exceptional features of high surface productivity, requirement of simple cultivation condition, and power to alleviate the concern of greenhouse gas emission; have made it a potential substrate for biofuel production (Brennan & Owende, 2010; Li-Beisson & Peltier, 2013). However, its large scale exploitation is limited by its high cost of cultivation, biomass harvest and process for extraction of oil. Moreover, certain microalgal species are not expedient for industrial application due to the fatty acid composition of their lipids, which may not be optimum for use as biofuel. Stress conditions

required to maximize microalgal lipid content mostly results in inhibition of cell growth and division, leading to a hindered biomass productivity (Li-Beisson & Peltier, 2013). Thus, microalgae based biofuel is not yet sustainable.

2.5 Microbial fermentation of lignocellulosic hydrolysate

Efficient microbial fermentation of lignocellulosic feedstock is a prerequisite to utilize all forms of carbon present in the waste biomass towards improved ethanol production. Various potential biocatalysts have been reported to utilize hexose sugar from the lignocellulosic biomass and convert it to ethanol. Most promising microbes identified for bioethanol production include yeasts like *Saccharomyces cerevisiae* (*S. cerevisiae*), *Candida shehatae* etc. (Hickert, Da Cunha-Pereira, De Souza-Cruz, Rosa, & Ayub, 2013), and bacteria like *Zymomonas mobilis* (*Z. mobilis*) and *Escherichia coli* (*E. coli*) (Ohta, Beall, Mejia, Shanmugam, & Ingram, 1991). *S. cerevisiae* is the most exploited and widely used biocatalyst for industrial bioethanol production, owing to its tolerance to wide range of pH, with acidic pH being optimum for cultivation (Tesfaw & Assefa, 2014). This robust nature of *S. cerevisiae* makes it less susceptible to contamination during fermentation, which is a desirable trait for realizing a microbe at commercial scale towards ethanol production. However, native *S. cerevisiae* is limited by low ethanol yield, ethanol tolerance and inadequacy of efficient genetic manipulative methods (Karsch, Stahl, & Esser, 1983). *Z. mobilis* offers potential advantages over *S. cerevisiae* in terms high ethanol yield & productivity and high alcohol tolerance (Yang et al., 2016). *Z. mobilis* can grow under a broad range of pH ranging from 3.5 to 7.5 and possess generally regarded as safe status. While *S. cerevisiae* uses the Embden-Meyerhof-Parnas (EMP) pathway for glycolysis, *Z. mobilis* operates on the Entner-Doudoroff (ED) pathway, which is a typical characteristic trait of strict aerobic microorganisms (Yang et al., 2016). Thus, ED pathway based fermentation leads to 50% lower ATP production relative to the EMP pathway, leading to higher ethanol yield (Dawes, Ribbons, & Large, 1966; He et al., 2014). A

higher specific cell surface area of *Z. mobilis* aids faster consumption of glucose leading to higher ethanol productivity as compared to that of *S. cerevisiae*. Several desirable characteristics exhibited by *Z. mobilis* makes it a potential candidate, to be exploited for bioethanol production.

2.6 *Zymomonas mobilis*: a natural ethanologen

Zymomonas mobilis, a natural ethanol producer (Panesar, Marwaha, & Kennedy, 2006), has gained considerable attention of researchers worldwide, owing to its unique characteristics. *Z. mobilis* naturally thrives in high sugar containing solutions *e.g.* cell sap, cider etc., thereby, fermenting sugars to ethanol with high yield and productivity. *Z. mobilis* ferments glucose or fructose to ethanol with a high yield of 97 % of the maximum theoretical yield, while, utilizing sucrose it can produce ethanol with a yield of 90 % due to the formation of additional by-products like levan and sorbitol. A probable reason for the high ethanol yield showcased by *Z. mobilis* is its limited use of substrate (2 to 2.6 % of the total sugar substrate utilized) for biomass formation. *Z. mobilis* also displays high ethanol productivity, which is 2.5 times higher as compared to that of yeast (Rogers, Lee, Skotnicki, & Tribe, 1982). It is exceptional in terms of ethanol tolerance (up to 16% (v/v)), glucose tolerance (40% (v/v)) (Swings & De Ley, 1977) and tolerance towards acetic acid (0.8% (w/v)) (In S. Kim, Barrow, & Rogers, 2000). It is a robust microbe, which can sustain a wide range of pH, making its cultivation less susceptible to contamination and rendering the fermentation process more economically feasible.

In spite of harboring such fascinating traits, certain crucial factors prevent the use of *Z. mobilis* as a commercial biocatalyst for ethanol production. The prime facet is its limited substrate range, which is restricted to glucose, sucrose and fructose (Goldman et al., 2008; Gunasekaran et al., 1990; Song, Joo, & Rhee, 1993). Wild type *Z. mobilis* lacks the ability to ferment pentose sugars like xylose and arabinose (Yang et al., 2016), which are the second and third most abundant form of sugars, respectively, present in lignocellulosic hydrolysates. *Z.*

mobilis has slow specific growth rate and cannot tolerate toxic inhibitors such as acetic acid, phenolic compounds etc., present in lignocellulosic hydrolysates.

2.7 *Zymomonas mobilis*: History and Physiology

Z. mobilis is gram negative, rod-shaped, non-sporulating, facultative anaerobic and polarly- flagellated bacterium (Swings & De Ley, 1977). Bacteria, which belong to the genus of *Zymomonas*, were traditionally used in tropical areas of Asia, Africa and America, to prepare local alcoholic beverages by fermenting plant saps. The bacteria was also found to be present in palm juice, ripening honey and sugarcane juice and as a contaminant in spoiled beer, Perry and cider (Swings & De Ley, 1977). In the early 20th century, the demand for clear and sweet cider vanquished the rough and dry farm made cider. The production of sweet cider encountered tremendous difficulties owing to the secondary fermentation of the sweet cider, known as "cider sickness". Barker and Hillier (1912) extensively studied the phenomenon of cider sickness and described the bacterium responsible for the distinctive aroma and flavor of the cider. The growth of the bacteria was marked to commence with frothing and gas formation followed by transforming the cider turbid, which was observed to get clear later with formation of deposits. Barker and Hillier successfully isolated a bacterial strain from the fermented cider in 1911 (Barker & Hillier, 1912). Upon cultivation on sterile cider, the bacteria continued to produce the typical aroma and flavor. The culture appeared creamy white and slimy with a moderate growth in solid medium and was able to ferment glucose and fructose to ethanol and carbon dioxide. Thus, the first strain of *Zymomonas* came into being.

It was in the early 1950's, Gibbs and DeMoss discovered the anaerobic catabolism of glucose follows the Entner-Doudoroff (ED) pathway in *Zymomonas*, which intrigued biochemists, all over the world, to extensively investigate the metabolic pathways of this organism (GIBBS & DEMOSS, 1951; GIBBS & DEMOSS, 1954). This unprecedented discovery marked the beginning of *Zymomonas* era, since it stood as an exception, to follow

ED pathway for glucose metabolism, which is a characteristic of strictly aerobic bacteria (Kersters & De Ley, 1968). The three identified subspecies of *Z. mobilis* include *Z. mobilis* subsp. *mobilis*, *Z. mobilis* subsp. *pomaceae* and *Z. mobilis* subsp. *francensis*. *Z. mobilis* strains namely ATCC 31821 (ZM4), CP4, ATCC29191 (ZM6), ATCC 10988 (ZM1), and and NCIMB 11163 from *Z. mobilis* subsp *mobilis* & ATCC 29192 from *Z. mobilis* subsp. *pomaceae* are well characterized and considered as the model organism in *Z. mobilis* research (He et al., 2014). The first whole genome sequence of *Z. mobilis* ZM4 was reported in 2005. The complete genome of ZM4 consists of a circular chromosome of 2,056,416-bp and five circular plasmids (Seo et al., 2005). Complete genome sequence of nine *Zymomonas* strains have been reported till date, which provides an opportunity for in-depth study on fundamental insights and enable strain development (Chacon-Vargas et al., 2017; Desiniotis et al., 2012; V. N. Kouvelis et al., 2014; Vassili N. Kouvelis et al., 2011, 2009; Katherine M. Pappas et al., 2011; Seo et al., 2005; Zhao, Bai, Zhao, Yang, & Bai, 2012; Zhao, Pan, Liu, & Cheng, 2016).

2.8 *Zymomonas mobilis*: central metabolic pathways and uncoupled growth

Z. mobilis, the obligately fermentative bacterium, produces equimolar, near-theoretical yields of ethanol and carbon dioxide utilizing sugars like, fructose and sucrose through ED pathway. The ED pathway, along with pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) enzymes, is known as the 2-keto-3-deoxy-6-phosphogluconate (KDPG) pathway (Dawes et al., 1966; GIBBS & DEMOSS, 1954). Unlike the EMP pathway, which produces 2 moles of ATP, ED pathway produces one mole of ATP per mole of glucose (Tyrrell Conway, 1992; Kalnenieks, 2006). The ED pathway is the primary sugar metabolism pathway in *Z. mobilis* because its genome does not have the genes that code for the 6-phosphofructokinase enzyme of the EMP pathway (RAPS & DEMOSS, 1962). It also does not contain the gene sequence for the malate dehydrogenase and 2-oxoglutarate dehydrogenase complex of the tricarboxylic acid (TCA) cycle (Seo et al., 2005). *Z. mobilis* lacks most of the

genes that code for the pentose phosphate pathway (PPP) enzymes (Seo et al., 2005). The PPP and TCA cycle enzymes that are present, along with the malic enzyme and phosphoenolpyruvate carboxylase are utilized to drive anaplerotic reactions (Bringer-Meyer & Sahm, 1989; Sprenger, 1996). Sucrose metabolism in *Z. mobilis* initiates by the degradation of sucrose, by extracellular levansucrases, into glucose and fructose monomers coupled with the formation of levan as a by-product (Goldman et al., 2008; Gunasekaran et al., 1990; Kannan, Mukundan, et al., 1995; Kannan, Pitchaimani, Gunasekaran, Aït-abdelkader, & Baratti, 1995; Senthilkumar, Rajendhran, Busby, & Gunasekaran, 2009; Silbir, Dagbagli, Yegin, Baysal, & Goksungur, 2014; Song et al., 1993; Song, Lee, Joo, & Rhee, 1994). About 2 to 2.6% of the total consumed carbon source gets converted its biomass thus, making it an ideal platform for product synthesis (BELAUICH & SENEZ, 1965; McGill & Dawes, 1971). *Z. mobilis* demonstrates an idiosyncratic phenomenon known as "uncoupled growth" (BELAUICH & SENEZ, 1965). This phenomenon describes the rapid consumption of sugar by the bacteria regardless of its growth requirement. The catabolic rates in *Zymomonas* are five times faster as compared to that of yeast. Most of the ED pathway proteins are constitutively expressed, with all the enzymes exhibiting high-level of expression. ED pathway proteins constitute half of the total protein content of the cell (Algar & Scopes, 1985; An, Scopes, Rodriguez, Keshav, & Ingram, 1991). The high catabolic rate of *Z. mobilis* compensates the ATP requirement of the cell, in spite of the low ATP yield of the ED pathway (Kalnenieks, 2006). *Zymomonas* efficiently maintains an equilibrium between the energy consumption reactions for utilizing the ATP and the energy production reactions during the log phase (LAZDUNSKI & BELAICH, 1972). Apart from *Z. mobilis*, *Streptococcus bovis* also demonstrates the phenomenon of uncoupled growth (Forrest, 1967). *Streptococcus bovis* performs ATP spilling by the ATPase, which is coupled to a cycling of protons across the membrane (Cook & Russell, 1994). Apparently, *Z. mobilis* was predicted to have similar conformation, where its ATPase does not

dissipate energy, rather converts it into proton motive force (PMF) (Kalnenieks, 2006). Although a substantial pH gradient has been observed across the membrane of *Z. mobilis* (BARROW, COLLINS, ROGERS, & SMITH, 1984; Osman, Conway, Bonetti, & Ingram, 1987), however, accurate determination of the transmembrane electric potential has not been made to verify this hypothesis (Ruhrmann & Kramer, 1992). It was proposed that the export of bicarbonate ions across the membrane is responsible for the dissipation of PMF (Kalnenieks, 2006). Later this hypothesis was endorsed by the presence of carbonic anhydrase gene, in the genome of *Z. mobilis*, which catalyzes the conversion of carbon dioxide into bicarbonate ions (Seo et al., 2005).

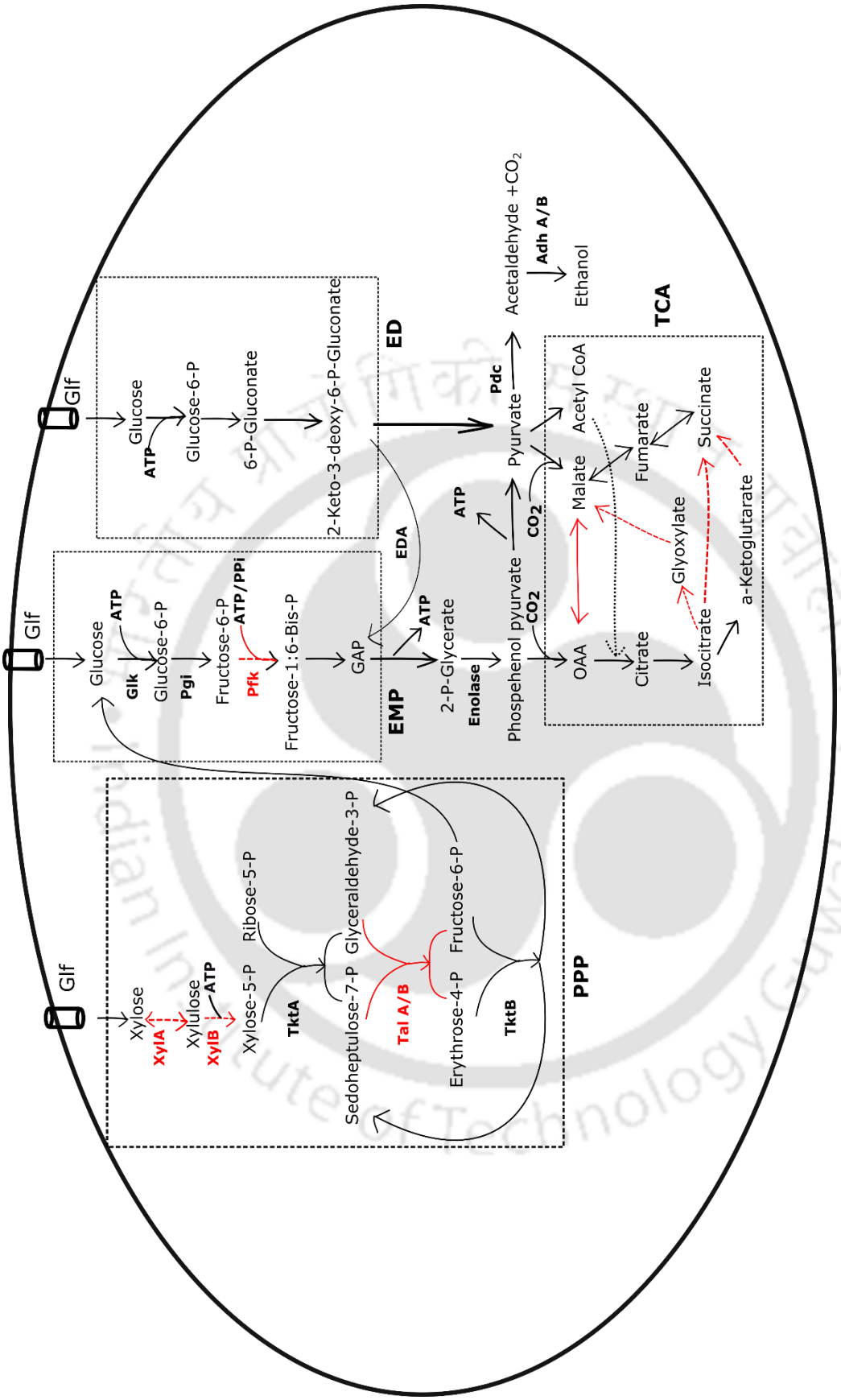

Fig. 2.9 Central metabolic pathways in *Z. mobilis*. Biochemical pathway reactions and genes which are absent in *Z. mobilis* are represented in red. Only relevant & significant enzymes and metabolites have been depicted

2.9 Nutrition and environmental effects on *Z. mobilis*

Z. mobilis can promptly utilize sugars such as glucose, fructose and sucrose with great efficiency. It can tolerate high concentrations of sugar of up to 400 g L^{-1} (Swings & De Ley, 1977). Sucrose is metabolized to produce sorbitol by *Z. mobilis* using glucose-fructose oxidoreductase (GFOR) enzyme, which facilitates the organism to overcome the osmotic stress in high sugar concentrations. Addition of sorbitol in the culture medium, containing sugars other than sucrose, improves growth of *Z. mobilis*, under high sugar, salt and ethanol (up to 160 g L^{-1}) concentrations (Loos, Kramer, Sahm, & Sprenger, 1994; Sootsuwan et al., 2013). It can tolerate up to 20 g L^{-1} of sodium chloride, above which the growth of the organism is impeded (Swings & De Ley, 1977). It can sustain at a wide temperature range of 28°C to 37°C, with optimum temperature for the cultivation being 30°C (Fieschko & Humphrey, 1983; Swings & De Ley, 1977). Temperature above 37°C causes a decline in the lipid to protein ratio in the membrane, inducing leakage of the soluble proteins outside the cell (Benschoter & Ingram, 1986). *Z. mobilis* can survive in a wide range of pH (3.5 to 7.5) (Swings & De Ley, 1977), which was hypothesized to a result of the expression of a proton-buffering peptide (Baumler et al., 2006). When cultivated in minimal medium, *Z. mobilis* require pantothenate, a precursor of coenzyme A, for its growth (BELAUICH & SENEZ, 1965; Swings & De Ley, 1977). Rich medium (RM) was designed to be the optimal medium for *Z. mobilis* cultivation, which comprises of yeast extract, a fermentable sugar and buffering salt (Swings & De Ley, 1977). Ammonium salts, peptides and amino acids act as the nitrogen source for the bacteria (BELAUICH & SENEZ, 1965). Sulfate and methionine are responsible for sulfur assimilation inside the cell (Rogers et al., 1982). Addition of certain metal ions and inorganic phosphates have also been reported enhance *Z. mobilis* growth (BELAUICH & SENEZ, 1965; Dawes & Large, 1970).

2.10 Genetic engineering of *Z. mobilis*: Traditional methods

In 1979, P. L Rogers brought the potentiality of *Z. mobilis* towards ethanol production under lime light, which inveigled several attempts to modify the strain for industrial use (Rogers, Lee, & Tribe, 1979). In 1980, Skotnicki and coworkers established conjugation from *E. coli* and *Pseudomonas aeruginosa* as a prospective method to transfer plasmids of the IncP and IncFII incompatibility groups (Skotnicki, Tribe, & Rogers, 1980). Carey and coworkers employed a conjugable plasmid to express a lactose operon in the *Z. mobilis* strain, in order to use it as a platform for genetic modification, towards broadening its substrate range (Carey, Walia, & Ingram, 1983). A hybrid vector was developed by fusing a native vector from *Z. mobilis* and an *E. coli vector*, which was used to demonstrate chemical transformation in *Z. mobilis* (Browne, Skotnicki, Goodman, & Rogers, 1984). Consequently, native vectors from *Z. mobilis* have been extensively characterized and used to design shuttle vectors (Amalia S. Afendra, Vartholomatos, Arvanitis, & Drainas, 1999; Arvanitis et al., 2000; Dally, Stokes, & Eveleigh, 1982; Misawa & Nakamura, 1989; Reynen, Reipen, Sahm, & Sprenger, 1990; Scordaki & Drainas, 1990; SCORDAKI & DRAINAS, 1987; Strzelecki, Goodman, & Rogers, 1987). In 1987, Conway and coworkers constructed the first vector, specifically designed for protein expression in *Z. mobilis* (T. Conway, Byun, & Ingram, 1987). The vector constituted a broad-host-range origin of replication, a chloramphenicol acyltransferase gene as an antibiotic resistance marker, and a native *Z. mobilis* promoter, upstream of the restriction site designed for gene cloning. The promoter region of the pyruvate decarboxylase was also sequenced, as it is one of the most abundant protein identified in *Z. mobilis* (T. Conway, Osman, Konnan, Hoffmann, & Ingram, 1987). This is one of most widely used strong promoter that have been used for expressing heterologous proteins in *Z. mobilis*, since then. Further, Conway and coworkers using lacZ as a reporter deduced the structure of consensus promoter sequence in *Z. mobilis* (T. Conway, Osman, & Ingram, 1987). The comparative analysis study identified

several similar features between the promoter sequences of *Z. mobilis* and *E. coli*. The -10 region contains similar consensus sequence while -35 region had a partial sequence homology (T. Conway, Osman, & Ingram, 1987). The possible ribosome-binding site, with the sequence GGA, appeared 8 to 12 bp upstream of the start codon. For the first time in 1997, transposable elements, present on suicide vectors, were promulgated to mutagenize the *Z. mobilis* chromosome (K. M. Pappas, Galani, & Typas, 1997). The authors successfully established the use of mini Mu transposon to develop stable *Z. mobilis* auxotrophs, thereby manifesting the feasibility of this method for chromosomal integrations.

2.11 Limitations associated with traditional genetic modification techniques and recent advancements

While early attempts, made towards genetic modification of *Z. mobilis* strains proved to be promising, however, several drawbacks associated with those traditional methods, were later realized. Major efforts were towards expanding the substrate range of *Z. mobilis* using metabolic engineering. However, attempts were met with difficulties leading to limited or no success (Byun, Kaper, & Ingram, 1986; Carey et al., 1983; Feldmann, Sahm, & Sprenger, 1992; Liu, Goodman, & Dunn, 1988; Yanase, Kurii, & Tonomura, 1988). Earlier, conjugation was considered as a reliable method for plasmid insertion in *Z. mobilis* above chemical transformation and electroporation (Buchholz & Eveleigh, 1986; Sprenger, 1993). Post transformation in *Z. mobilis*, plasmid stability was recognized as a major challenge, especially when the plasmid was devoid of origin of replication from native *Z. mobilis* vectors (A. S. Afendra & Drainas, 1987; Brestic-Goachet, Gunasekaran, Cami, & Baratti, 1990; Dong, Bao, Ryu, & Zhong, 2011; K. M. Pappas et al., 1997; Scordaki & Drainas, 1990; Strzelecki, Goodman, Cail, & Rogers, 1990). It was hypothesized that the presence of intrinsic type I and type IV restriction modification system in *Z. mobilis* is the primary reason for degradation of the heterologous plasmids (Kerr, Jeon, Svenson, Rogers, & Neilan, 2011; Typas & Galani,

1992). Methods dedicated towards chromosomal integration and/or knock-out of genes have not advanced much, since the origination of suicide vector technique for *Z. mobilis* (K. M. Pappas et al., 1997). Targeted metabolic engineering using suicide vector technique requires several hundreds of base pairs of homologous sequence, however, yield very few transformants (Delgado, Martínez, Abate, & Siñeriz, 2002; Kerr et al., 2011; Senthilkumar, Rameshkumar, Busby, & Gunasekaran, 2004). Further, a consensus promoter sequence and ribosome binding site (RBS) was not identified, that is reliable and could be accepted for precise regulation of gene expression in *Z. mobilis*. Till date, expression of heterologous genes in *Z. mobilis*, was performed using constitutive promoters leading to uncontrolled gene expression, further complicating optimization of gene expressions in the strain (Jeon, Svenson, & Rogers, 2005; M. Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995) . The defiance of *Z. mobilis* towards genetic modification has led to the development of few recombinant strain (Kerr et al., 2011). However, recent advancements have been made towards optimization of plasmid insertion and subsequent expression of heterologous genes in *Z. mobilis*. Although chemical transformation still could not yield recombinant *Z. mobilis* strains, however, electroporation has been proved beneficial (Dunn & Rao, 2014; Gliessman, Kremer, Sangani, Jones-Burrage, & McKinlay, 2017; Jeon et al., 2005; Lam, O'Mullan, & Eveleigh, 1993; Zou et al., 2012). With veritable modification of parameters, plasmids of various sizes have been successfully transformed in *Z. mobilis*. To address the difficulty in terms of instability of heterologous plasmids in *Z. mobilis*, a specific protein preparation known as TypeOne restriction inhibitor was developed, using phage protein called overcome classical restriction (ocr) (Zou et al., 2012). The use of this protein during plasmid transformation, increases the transformation efficiency of the unmodified plasmid DNA, by blocking the DNA binding site of type I restriction modification enzymes, thereby inhibiting DNA cleavage (Zavilgelsky & Rastorguev, 2009). In other studies, use of unmethylated plasmid DNA, generated using modified *E. coli* strains like *E. coli* JM110,

E. coli GM119 etc., have been reported to yield more transformants as compared to that of methylated plasmid DNA (Agrawal, Mao, & Chen, 2011; Dunn & Rao, 2014; Zou et al., 2012). Further, the λ Red-mediated knockout protocol have been successfully implemented to generate modified *Z. mobilis* strains with gene knock-outs (Lal et al., 2019). Although advancements have been made towards improvising genetic modification techniques, however, many challenges associated with the developed methods, still need to be addressed.

2.12 *Z. mobilis* for cellulosic ethanol production: Development of engineered strains

Z. mobilis is a prospective contender for an industrial cellulosic ethanologen, owing to its high ethanol productivity, yield and tolerance (Rogers et al., 1979; Swings & De Ley, 1977). *Z. mobilis* also has an extremely high tolerance to the sugars present in lignocellulosic hydrolysates (Rogers et al., 1979). Till date, no viral invader has been reported to contaminate *Z. mobilis* culture, making it a robust organism (Sahm, Bringer-Meyer, & Sprenger, 2006). Since, *Z. mobilis* is aero-tolerant, it does not require oxygen for growth, thus, simplifying the process design requirements leading to reduction in production cost (Swings & De Ley, 1977). In spite of having such interesting characteristics, *Z. mobilis* has not been accepted for industrial cellulosic ethanol production. The primary reason that has prevented *Z. mobilis* to be industrially recognized for cellulosic ethanol production, is its narrow substrate range. It can only ferment hexose sugars, while it can utilize pentose sugars like D-xylose and D-arabinose, present in the lignocellulosic hydrolysate (Swings & De Ley, 1977). Additionally, *Z. mobilis* is highly sensitive to acetic acids and has very low tolerance to other potential inhibitors, present in the hydrolysate (Jeon, Svenson, Joachimsthal, & Rogers, 2002; In S. Kim et al., 2000; Ranatunga, Jervis, Helm, Mcmillan, & Hatzis, 1997). To overcome these drawbacks, several genetic engineering strategies have been undertaken on *Z. mobilis*, in order to develop a potential platform cellulosic ethanol.

2.13 *Z. mobilis* towards improved cellulosic ethanol production: Genetic engineering approaches

The preliminary approach, taken towards engineering *Z. mobilis* for cellulosic ethanol production, focused on broadening the organism's substrate range (Pan et al., 2014; Rogers, Jeon, Lee, & Lawford, 2007). In 1987, with an aim of modifying *Z. mobilis* for xylose utilization, Liu and coworkers expressed a xylose permease, xylose isomerase and xylulokinase from *Xanthomonas* XA1-1, however, the strain was unable to grow on medium with xylose as sole carbon source (Liu et al., 1988). Feldmann and coworkers also adopted a similar approach by heterologously expressing xylose isomerase and xylulokinase, from *E. coli*, in *Z. mobilis* (Feldmann et al., 1992). The developed strain was used as a platform to isolate a mutant strain that would produce less xylitol phosphate, which is a potential inhibitor of xylose metabolic pathway. Additionally, the mutant strain was exploited as a host to express *E. coli* transketolase, an important enzyme for xylose assimilation that catalyzes the conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate. The activities for all the heterologously expressed enzymes were confirmed, however, the engineered strain could not grow on xylose. A detailed investigation on the failed attempt led to the hypothesis that absence of detectable transaldolase activity in *Z. mobilis*, could be the reason behind the xylose unresponsive mutant, however, the author did not venture it further (Feldmann et al., 1992). In 1995, a breakthrough endeavor by the researchers at the National Renewable Energy Laboratory, led to the construction of a recombinant *Z. mobilis* strain CP4 (pZB5) that could grow on xylose as its sole carbon source (M. Zhang et al., 1995). They heterologously expressed four xylose metabolizing enzymes namely, xylose isomerase, xylulokinase, transketolase and transaldolase, from *E. coli*, in a wild type *Z. mobilis* strain i.e. *Z. mobilis* CP4. Further, the same group of researchers successfully engineered a wild type *Z. mobilis* strain i.e. *Z. mobilis* ATCC 39676 to develop *Z. mobilis*

(pZB206) that could utilize arabinose as its sole carbon source, by expressing the arabinose isomerase, ribulokinase, ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase genes from *E. coli* (Deanda, Zhang, Eddy, & Picataggio, 1996). In 1999, Joachimsthal et al., developed a xylose fermenting strain, using the same strategy as employed by NREL research group, however, he used *Z. mobilis* (ZM4/ATCC 31821) as a platform to transform pZB5 plasmid with integrated xylose assimilating (xylose isomerase and xylulokinase) and pentose phosphate pathway (transketolase and transaldolase) genes (E. Joachimsthal, Haggett, & Rogers, 1999). *Z. mobilis* ZM4 was realized to have enhanced characteristics in terms of ethanol productivity and tolerance as compared to that of *Z. mobilis* CP4. Antibiotic resistance markers bearing pentose-utilizing *Z. mobilis* strains were not desirable for industrial application. To enhance the stability of the recombinant *Z. mobilis* strains, the same group of scientists from NREL, developed an engineered strain (AX101) by integrating all seven genes necessary for xylose and arabinose utilization into the genome of wild type *Z. mobilis* (Mohagheghi, Evans, Chou, & Zhang, 2002). Continuing with the aim of modifying *Z. mobilis* for efficient pentose utilization, Gao et. al. reported two xylose metabolizing strains namely C25 and 39676/pZB4L, using *Z. mobilis* ATCC 39676 as the host strain (Gao, Zhang, Mcmillan, & Kompala, 2002). C25 was engineered by integration of the four essential xylose-metabolizing genes, where *xylA/xylB* and *talB/tktA* genes were present as two distinct synthetic operons, in the genome of *Z. mobilis* ATCC 39676. 39676/pZB4L was constructed by transforming plasmid pZB4L that contains the *xylA/xylB* and *talB/tktA* genes in separate synthetic operons, and serial adaptation on hardwood hemicellulose hydrolysate. Although several successful attempts have been made towards expanding the substrate range of *Z. mobilis*, however, efficient pentose metabolism continued to be a pre-eminent area of research. The primary reason being inefficacious xylose and arabinose metabolism, demonstrated by the modified *Z. mobilis* strains, when compared to that of glucose (Gao et al., 2002; Mohagheghi

et al., 2002; M. Zhang et al., 1995). Even though the ATP yields for xylose and glucose are same on molar basis, the rate of xylose and arabinose utilization was highly compromised (when supplemented as sole carbon source) as compared to that of glucose (Agrawal et al., 2011; De Graaf et al., 1999; Deanda et al., 1996; E. L. Joachimsthal & Rogers, 2000; Lawford & Rousseau, 2000, 2002; Mohagheghi et al., 2002; M. Zhang et al., 1995). Consequently, the volumetric ethanol productivity was also noted to be 4-fold lower on xylose compared to glucose. Xylose, being the second major sugar component of lignocellulosic hydrolysate led to further research endeavors, which were majorly dedicated towards enhancing the rate of xylose metabolism by modified *Z. mobilis* strains.

Firstly, efforts were made towards investigating the probable bottlenecks, present in xylose metabolism in *Z. mobilis*. Kinetic and nuclear magnetic resonance (NMR) studies identified number of factors, which could be responsible for the reduced rates of xylose metabolism in *Z. mobilis*, even when xylose was supplemented as sole carbon source in the fermentation medium. The significant factors that were addressed further are: (i) the increased metabolic burden for plasmid maintenance, (ii) low level of expression of xylose assimilating genes, (iii) the production of by-products such as xylitol, acetate, lactate etc., (iv) growth inhibition by the putative inhibitory compound xylitol phosphate (synthesized from xylitol) (Akinterinwa & Cirino, 2009; Feldmann et al., 1992; In Seop Kim, Barrow, & Rogers, 2000a), and (v) the less energized state of the *Z. mobilis* cells during xylose metabolism (De Graaf et al., 1999; In Seop Kim et al., 2000a). To alleviate the metabolic for plasmid maintenance, xylose-metabolizing genes were integrated into the genome of *Z. mobilis*, thus enhancing the stability of the modified strain. The less-energized state of *Z. mobilis* during xylose fermentation could possibly be due to low level of expression and subsequent weak activities of the heterologously expressed xylose isomerase and xylulokinase enzymes (In Seop Kim, Barrow, & Rogers, 2000b). To mitigate this problem of subdued expression of xylose

assimilating genes, Jeon and coworkers successfully overexpressed the xylulokinase enzyme in *Z. mobilis* strain, which was hitherto endowed with xylose metabolizing genes (Jeon et al., 2005). However, increased expression of xylulokinase enzyme did not transpire into increased rate of xylose metabolism, in the recombinant *Z. mobilis* strain. Rather, it negatively affected the rate, perhaps due to production of xylitol (a by-product along produced along with ethanol during xylose fermentation in *Z. mobilis*). In a separate study, a xylose metabolic gene bearing was adapted under high xylose concentration for extended period (Agrawal et al., 2011). Interestingly, a point mutation in the promoter region of the xylose isomerase gene, presumably led to a five-fold higher expression of xylose isomerase with its augmented activity, subsequently enhancing xylose utilization by the evolved strain (Agrawal et al., 2011). This observation was in accordance with a finding reported by Gao et al., in 2002, which identified xylose isomerase as a probable bottleneck and rate-limiting enzyme in xylose metabolism, due to its lowest activity detected among the four foreign xylose-metabolizing enzymes, expressed in two modified *Z. mobilis* strains (Gao et al., 2002). Another significant obstruction that hindered efficient xylose metabolism in engineered *Z. mobilis* strains is production of xylitol and its counterpart xylitol phosphate during xylose fermentation. Two pathways were identified that contribute to xylitol formation, i) involving an NADPH-dependent xylose reductase, and ii) involving the glucose-fructose oxidoreductase (GFOR) of central metabolism (Feldmann et al., 1992; X. Zhang, Chen, & Liu, 2009). A study reported, an evolved *Z. mobilis* strain through extended adaptive laboratory evolution (ALE) in xylose as sole carbon source, acquired a mutation in the NADPH-dependent xylose reductase during the course of evolution (Agrawal et al., 2011). The mutation greatly reduced the activity of the enzyme, further, upon subsequent deletion of the gene encoding the NADPH-dependent xylose reductase from the genome of the evolved *Z. mobilis* strain, led to decrease in xylitol production with a concomitant increase in rate of xylose fermentation by the strain (Agrawal & Chen, 2011). In

a separate study, Viitanen et al. deleted the gene encoding GFOR enzyme, from a xylose fermenting *Z. mobilis* strain, which subsequently transpired into increase in the rate of xylose utilization by the engineered strain (Viitanen et al., 2008). Apart from xylitol, acetate or acetic acid, as determined by Ranatunga and coworkers, exerts maximum detrimental effect on xylose fermentation by the recombinant *Z. mobilis* strains (Ranatunga et al., 1997). Kim and coworkers, using NMR studies, deduced the process in which acetic acid inhibits the xylose-fermenting *Z. mobilis* strain by acidifying the cytoplasm and subsequently decreasing the concentrations of sugar phosphates and nucleotide triphosphates present in the cell (In S. Kim et al., 2000). To that end, acetic acid tolerant mutant strain was isolated using chemical mutagenesis and adaptation on increasing the concentration of sodium acetate (Yang et al., 2010). The mutated *Z. mobilis* strain was discovered to have increased expression of the sodium-proton antiporter gene *nhaA* that perhaps can be correlated with the increased tolerance of the strain (Yang et al., 2010). Chen and coworkers exploited ALE to isolate an acetic acid tolerant *Z. mobilis* strain (Chen et al. 2009), which was later used by Agrawal et al., as a platform to incorporate mutations in xylose reductase and xylose isomerase gene that resulted in decrease and increase of the their corresponding activity, respectively (Agrawal, Wang, & Chen, 2012). Thus, emanating into an enhanced *Z. mobilis* strain that efficiently fermented xylose in presence of acetic acid. Significant progress was evidenced in the development of modified *Z. mobilis* strains with enhanced xylose utilizing capability, in presence of xylose as sole carbon source. Although, these studies could highlight the potential targets for future metabolic engineering towards efficient xylose metabolism in the evolved *Z. mobilis* (in presence of xylose as sole carbon source), however, these strains exhibited selective utilization of the preferred substrate glucose over xylose in medium supplemented with glucose and xylose mixture (Agrawal et al., 2011; Gao et al., 2002; Jeon et al., 2005; Mohagheghi et al., 2002, 2015; Viitanen et al., 2008). Thus, development of an efficient glucose and xylose co-

utilizing *Z. mobilis* strain is a pre-requisite, before lignocellulosic biomass could be realized at commercial scale.

It would be noteworthy to mention that the coherent application of targeted metabolic engineering along with ALE led to the development of efficient *Z. mobilis* strains, which could utilize xylose faster as compared to the mutant *Z. mobilis* strains, modified using directed metabolic engineering strategies only, in binary sugar mixture (glucose and xylose). A3, an adapted *Z. mobilis* strain, developed by Agrawal et al. (Agrawal et al., 2011) has been contemplated as one of the promising *Z. mobilis* strain for mixed sugar fermentation. When cultivated in medium supplemented with 5% (w/v) each of glucose and xylose, A3, exhibited a maximum xylose uptake rate of 1.8 g g^{-1} h^{-1} along with an ethanol titer 49.9 g L^{-1}, which corresponds to 96.6% of the maximum theoretical yield. A3 displayed a remarkable improvement in xylose metabolism, yet it could not efficiently utilize xylose, in presence of glucose, present in the fermentation media. Viitanen et al. had used a similar approach previously, where directed metabolic engineering coupled with ALE led to the development of a xylose-fermenting *Z. mobilis* strain, *Z. mobilis* ATCC ZW658 (ZW658) (Viitanen et al., 2008). Although, ZW658 could utilize xylose in presence of glucose, the rate of xylose uptake appeared to be significantly low as compared to that of glucose. Additionally, the same group investigated the effect of *himA* gene inactivation on the performance of a xylose-fermenting *Z. mobilis* strain (Viitanen et al., 2009). The mutant strain could convert higher concentrations of xylose to ethanol, but only after complete utilization of glucose, present as a mixture in the cultivation medium. Another major aspect that might vindicate the preferential utilization of glucose over xylose is lack of xylose specific transporter protein in *Z. mobilis*. *Z. mobilis* has been reported to transport xylose through Glf (Glucose facilitated diffusion protein), which is an innate glucose transporter (Dunn & Rao, 2014). However, higher affinity of Glf towards glucose hinders simultaneous xylose uptake, in dual sugar supplemented medium. Considering

transport as a potential bottleneck in xylose utilization by the recombinant *Z. mobilis* strains, heterologous expression of low-affinity xylose specific transporter in *Z. mobilis* led to the development of strain 31821 (pKLD4) (Dunn & Rao, 2014). The strain could successfully enhance the xylose uptake rate as compared to its host strain, however, it did not match efficiency of glucose utilization demonstrated by 31821 (pKLD4). The modified *Z. mobilis* strains reported till date, manifested a typical diauxic behavior in mixed sugar fermentation, which often resulted in prolonged fermentation time along with incomplete xylose utilization.

2.14 Definition of the problem

The ethanologenic potential of *Z. mobilis* distinguishes it from other ethanol-producing organisms however, before recognizing its capability at industrial cellulosic ethanol production, the challenge that still prevails is its inefficacious xylose utilization in presence of glucose. Rationally designed strategy to induce effective genetic modifications with the synergistic use of directed metabolic engineering, appeared to be a prerequisite step towards development of an efficient glucose and xylose co-utilizing strain. *Z. mobilis* strains have been engineered to utilize xylose efficiently, when cultivated under xylose as single carbon source. Preferential utilization of glucose over xylose by engineered *Z. mobilis* strains might be attributed to a phenomenon called carbon catabolite repression (CCR) or glucose effect, where glucose exerts repressive pressure on utilization of other sugars e.g., xylose. Although, such phenomenon has not been identified and elucidated in *Z. mobilis*. Unlike directed metabolic engineering, it would be cogent to employ ALE on a xylose-fermenting *Z. mobilis* strain under stringent selection pressure, to organically induce modifications necessary to bypass any intrinsic regulatory mechanism (such as CCR), present in the engineered strain with augmented xylose and glucose co-utilizing capacity. Apart from the metabolic aspect of xylose utilization, xylose transport was also conceded as a major bottleneck hindering co-utilization of glucose and xylose by the engineered strains of *Z. mobilis*. Limited studies have been undertaken to

unravel the effect of various xylose specific transporters on simultaneous uptake of xylose in presence of glucose. The ensued study ventured different possibilities to address the potential bottlenecks persisting in efficient co-utilization of glucose and xylose by a modified *Z. mobilis* strain, leading to the development of a prospective, commercially viable biocatalyst for enhance ethanol production.

2.15 References

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Chapter 3

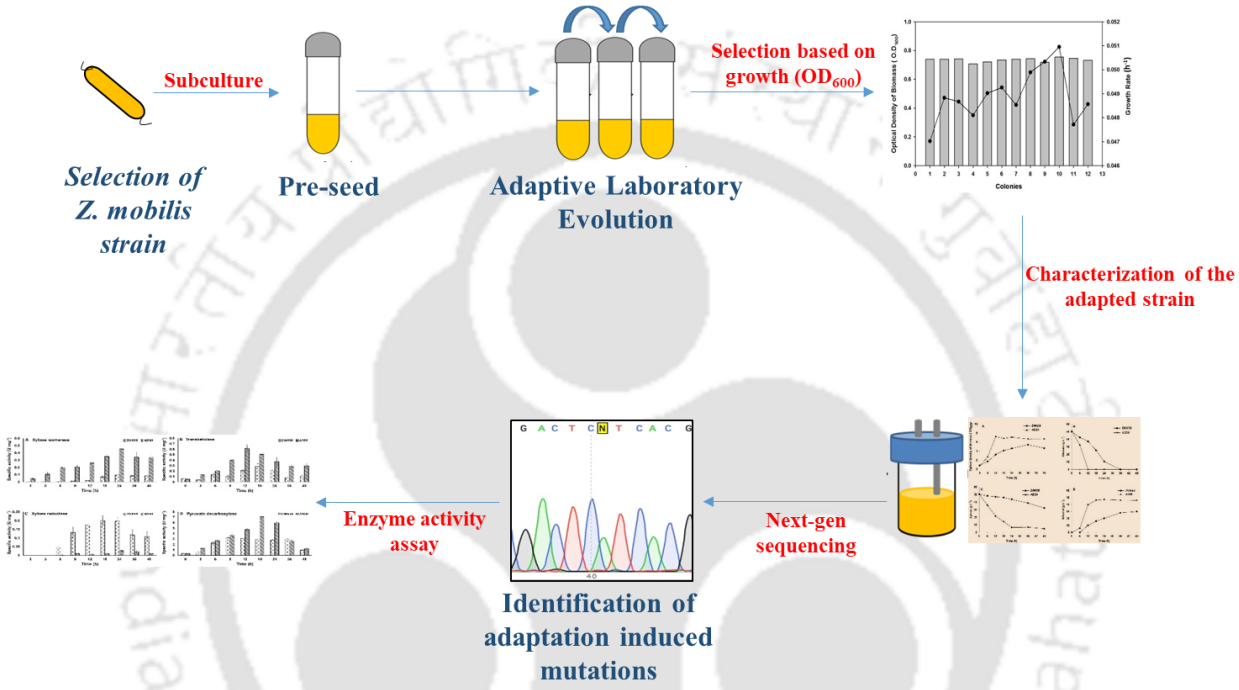
LABORATORY EVOLUTION OF *Z. MOBILIS* DIRECTED TOWARDS SIMULTANEOUS UTILIZATION

"Adapt or perish, now as ever, is nature's inexorable imperative."

HG Wells, *English writer*

CHAPTER 3

Adaptive Laboratory Evolution of *Z. mobilis* directed towards simultaneous utilization of glucose and xylose



Schematic representation of the summary of developmental process of a potential *Z. mobilis* strain directed towards co-utilization of glucose and xylose

3.1 Background and motivation

The conjugal effect of population rise with exponentially declining fossil fuel reserve led to adverse climatic changes that has kindled studies towards development of alternate and sustainable form of energy (Peralta-Yahya, Zhang, Del Cardayre, & Keasling, 2012). Bioethanol has been established as a feasible alternative; with a prospective commercial aspect to replace the inexhaustible insistence on petroleum centric fuels (Yang et al., 2016). However, consumption of food crops for bioethanol production questioned the economic and sociopolitical stability (Tenenbaum, 2008). Lignocellulosic biomass was appraised as one of the most abundant renewable feedstock, available on earth. Thus, exploitation of lignocellulosic biomass for production of bioethanol, while bypassing the concerns related to utilization of food sources, was rendered as a sustainable solution (Nieves, Panyon, & Wang, 2015). However, its compositional complexity and heterogeneity impedes its commercial application (J. H. Kim, Block, & Mills, 2010). Hydrolysis of lignocellulosic biomass yields hexose and pentose sugars (Nieves et al., 2015). Nature inherent ethanologenic microorganisms that can utilize both hexose (glucose) and pentose (xylose and arabinose) sugars to produce ethanol have not been unearthed till date. Thus, development of a microbial strain that could efficiently utilize both the sugars, yielding high ethanol, is imperative to recognize lignocellulosic biomass as an industrially viable alternative carbon source for bioethanol production. Several rationally designed genetic engineering strategies have been employed to develop microbial strains that could utilize hexose and pentose sugars. Engineered *Saccharomyces cerevisiae* and *Zymomonas mobilis* deserves special mention in lieu of their ability to produce ethanol using hexose (D-glucose) and pentose (D-xylose) sugars. Although, *S. cerevisiae* has been industrially exploited for ethanol production, however, its efficiency was realized only in presence of glucose as its sole carbon source. To that end, *Z. mobilis*, owing to its exceptional characteristics, has the potentiality to be acknowledged as an alternative to *S.*

cerevisiae, at industrial level. *Z. mobilis* is a promising natural ethanologen with higher ethanol yield, productivity, and tolerance in comparison to *S. cerevisiae*. It can also tolerate high concentrations of sugar and has a broad range of pH (3.5-7.5) for production, making it an ideal biocatalyst for commercial scale ethanol fermentation (Browne, Skotnicki, Goodman, & Rogers, 1984; Gunasekaran & Chandra Raj, 1999; Panesar, Marwaha, & Kennedy, 2006; Swings & De Ley, 1977). While native *Z. mobilis* strains can efficiently utilize glucose, they have an incomplete pentose phosphate pathway (PPP). Thus, absence of the crucial PPP enzymes hinders *Z. mobilis* to metabolize xylose (Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995). To converse this obstacle, *Z. mobilis* strains have been endowed with crucial xylose metabolizing genes either through genome integration or by heterologous expression using plasmids (Mohagheghi, Evans, Chou, & Zhang, 2002; Viitanen et al., 2008; Zhang et al., 1995). However, one of the major challenges that persisted in lieu of exploiting lignocellulosic biomass at commercial scale, is selective utilization of the preferred substrate glucose over xylose, even by the mutant variants of native *Z. mobilis* strains leading to prolonged fermentation time with incomplete xylose utilization at higher xylose concentrations (Dunn & Rao, 2015; Liu, Goodman, & Dunn, 1988; Mohagheghi et al., 2002; Viitanen et al., 2008; Zhang et al., 1995).

Preferential utilization of glucose over xylose by *Z. mobilis* might be attributed to a phenomenon called carbon catabolite repression where glucose exerts repressive pressure on utilization of other sugars e.g., xylose (Ren, Chen, Zhang, Liang, & Lin, 2009). This mechanism of glucose mediated repression is not well elucidated in *Z. mobilis* and may involve multi-level regulations e.g., inhibition of alternate sugar transporter; through cyclic AMP; or through Catabolite Repressor/Activator (Cra), a dual transcriptional regulator (J. H. Kim et al., 2010). Various genetic engineering strategies have been adopted to improve co-utilization of sugars in microorganisms including *Z. mobilis* such as engineering strains with relaxed glucose

repression (Agrawal, Mao, & Chen, 2011; Dunn & Rao, 2015), overexpression of heterologous xylose transporters (Dunn & Rao, 2014) or metabolizing genes (Jeon, Svenson, & Rogers, 2005), and reprogramming hexose transporters to achieve altered affinities for xylose (Nijland et al., 2014; Ren et al., 2009; Young, Tong, Bui, Spofford, & Alper, 2014). While very limited success has been achieved in terms of xylose metabolism through overexpression of xylose transporters, genetic alterations of the glucose transporters has resulted in reduced efficiency in glucose transport (Young et al., 2014).

Unlike directed metabolic engineering strategies, Adaptive Laboratory Evolution (ALE) is an organic process of achieving beneficial mutations in multiple genes and regulatory regions of microorganisms in an unbiased fashion under specific selection pressure (Portnoy, Bezdan, & Zengler, 2011). In order to achieve co-fermentation of glucose and xylose by *Z. mobilis*, research endeavors over the years have combined ALE strategies with directed metabolic engineering (Agrawal et al., 2011; Dunn & Rao, 2015; Mohagheghi et al., 2015). An ALE strategy was demonstrated to isolate a mutant strain of *Z. mobilis* that outperformed the engineered parent strain by three folds in terms of xylose utilization with reduction in xylitol production (Agrawal et al., 2011). Dunn and Rao have followed a similar approach, where high-throughput sequencing was used to identify the potential genetic alterations responsible for improved pentose utilization by an adapted *Z. mobilis* strain (Dunn & Rao, 2015). ALE has also been exploited to develop robust *Z. mobilis* strain that efficiently utilized xylose in presence of glucose and other model inhibitory compounds from pre-treated corn stover (Mohagheghi et al., 2015). Although, these studies could highlight the potential targets for future metabolic engineering towards efficient xylose metabolism in the evolved *Z. mobilis*, the strains could not exhibit the phenotypic traits of simultaneous utilization of glucose and xylose in the true sense.

This chapter encompasses the strategies designed with the aim to develop a *Z. mobilis* strains, capable of efficiently co-utilizing glucose and xylose. To that end, selection of a suitable platform for genetic manipulation was a pre-requisite step. Two *Z. mobilis* strains, i) *Zymomonas mobilis* ATCC 31821 or ZM4 (a wild type *Z. mobilis* strain) and ii) *Zymomonas mobilis* ATCC ZW658 (a recombinant *Z. mobilis* strain), were characterized in terms of growth, substrate utilization, and product formation. Based on their performance, *Z. mobilis* ATCC 31821 or ZM4, was initially selected for genetic manipulation towards simultaneous utilization of glucose and xylose. Directed metabolic engineering was employed to engineer ZM4 with heterologous xylose utilizing gene, unresponsive to glucose inhibition (Carbon catabolite repression). However, the recombinant *Z. mobilis* strain did not manifest the desired traits as expected. Thereafter, *Zymomonas mobilis* ATCC ZW658 (ZW658) endowed with heterologous xylose metabolizing genes, integrated in its genome, was selected for genetic modification using a systematic Adaptive Laboratory Evolution (ALE) strategy. We have explored ALE as a platform to develop a potential *Z. mobilis* strain with highly beneficial traits directed towards efficient co-utilization of glucose and xylose. ZW658 was subjected to extended ALE involving 50 transfers, carried out over a period of 200 days. The strain was grown under strict selection pressure of increasing xylose concentration from 30 g L^{-1} to 100 g L^{-1}. The selected evolved strain (designated as AD50) showed several fold increase in xylose utilization rate when compared with the parent strain and other reported strains till date. Further, the strain displayed enhanced performance in terms of co-fermentation of xylose in presence of glucose. High throughput (Next-gen) sequencing revealed novel mutations in xylose assimilating, metabolizing, and crucial regulatory pathway genes, which substantiate the improved phenotypic response of AD50 in terms of co-utilization of glucose and xylose. Enzyme activity assays have been carried out to validate the performance of the strain with high confidence.

3.2 Materials and methods

3.2.1 Microorganisms, plasmids and media composition

Zymomonas mobilis ATCC 31821 (ZM4), a wild type strain, was received as a gift from Dr. J. McKinlay, Indiana University Bloomington. *Zymomonas mobilis* ATCC ZW658 (ZW658), a mutant strain with xylose metabolizing genes, integrated in its genome, was procured from American Type Culture Collection (ATCC). ZM4 and ZW658 were revived in Rich Medium containing glucose (RMG) composed of 1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, and 5% (w/v) D-glucose (until specified otherwise), at 30 °C under shaking at 150 rpm. Agar plates were prepared using the same media composition with an additional 1.5% (w/v) agar. All *Escherichia coli* strains namely *E. coli* TOP10 and *E. coli* JM110, were procured from Coli Genetics Stock Center and were cultivated in Luria Bertani (LB) medium at 37 °C under shaking at 180 rpm. Plasmid pZ7-184 was received as a kind gift from Dr. Rory Watt, University of Hong Kong. Plasmid pBBR1MCS-2 was purchased from Addgene. Antibiotics such as chloramphenicol and kanamycin were used at a concentration of 100 µg/mL and 200 µg/mL, respectively, for recombinant *Z. mobilis* cells. Similarly, for transformed *E. coli* strains chloramphenicol and kanamycin were used at a concentration of 25 µg/mL and 50 µg/mL, respectively.

3.2.2 Characterization of ZM4 and ZW658 on mono sugar glucose/xylose and binary sugar mixture of glucose-xylose directed towards selection of *Z. mobilis* strain for metabolic engineering

For selection of a potential platform for incorporation of xylose metabolizing genes, performance of ZM4 and ZW658 was assessed using batch fermentations in medium supplemented with glucose or xylose or mixture of glucose and xylose. Pre-seed cultures were prepared by inoculating glycerol stock of ZM4 and ZW658, in liquid RM supplemented with glucose (RMG) and grown till stationary phase. Seed cultures were prepared by harvesting and

inoculating 20% (v/v) of the stationary phase pre-seed culture to obtain an optical density (OD_{600}) of ~ 0.7 to 0.8. Both pre-seed cultures and seed cultures were grown in 2% (w/v) glucose or xylose at 30°C under shaking at 150 rpm. For batch fermentations, 20% (v/v) seed cultures of ZM4 and ZW658, were harvested and centrifuged at 7000 g for 2 min. The supernatant was discarded and cell pellets were resuspended in 1 mL of fresh medium and subsequently inoculated in 250 mL flasks with a working volume of 100 mL of medium. For characterization on mono-sugar, the RM was supplemented with 2% (w/v), 4% (w/v), 6% (w/v), 8% (w/v), 10% (w/v) and 15% (w/v) of glucose or xylose. For characterization on binary sugar mixture, the RM was supplemented with 2% (w/v), 4% (w/v) and 6% (w/v) of glucose along with 2% (w/v) of xylose (based on the performance of the *Z. mobilis* strain). After inoculation, the set up was kept under shaking (150 rpm) at 30 °C. Samples were withdrawn at regular intervals to obtain dynamic profiles of growth, glucose and/or xylose utilization and ethanol production. The experiments were performed in duplicates and the data have been expressed as mean ± standard error. The best performing *Z. mobilis* strain was selected as the host for metabolic engineering.

3.2.3 Construction of plasmids with xylose metabolizing genes

Two plasmids namely pZ7-184 (Fig. 3.1A) and pBBR1MCS-2 (Fig. 3.1B), with distinct features were screened for expressing the xylose metabolizing genes in ZM4. All plasmids used in the present study are listed in table 3.1. Xylose assimilating genes namely *xylA* encoding xylose isomerase (from *Lactobacillus brevis*) and *xylB* encoding xylulokinase (from *E. coli*) & the necessary PPP genes namely *talB* encoding transaldolase and *tktA* encoding transketolase (both from *E. coli*) were synthesized by PCR amplification. The *xylA* gene from *L. brevis* was codon optimized for *Z. mobilis* ATCC 31821 (ZM4) and synthesized (Genscript, USA) fused to the native ZM4 constitutive promoter of glyceraldehyde-3-phosphate gene (P_{gap}). Further, *xylB*, *talB* and *tktA* was PCR amplified using *E. coli* K-12 genomic DNA as template. All the

primers used in this study are listed in table 3.2. While the expression of *xylA* and *xylB* was under the influence of 310 bp promoter P_{gap}, expression of *talB* and *tktA* was designed to be controlled under 308 bp promoter of native enolase gene (P_{eno}) of *Z. mobilis* strain ZM4. Promoter P_{eno} was PCR amplified using ZM4 genomic DNA as template. Overlap extension PCR was performed to generate the two operons i.e. P_{gap}-*xylAB* and P_{eno}-*talBtktA*. P_{gap}-*xylAB* was constructed by fusing synthesized P_{gap}-*xylA* with amplified *xylB* (along with its ribosome binding site and terminator), having complementary overlapping sequence to the 3' end of *xylA* gene. Similarly, P_{eno}-*talBtktA* was constructed by fusing P_{eno} with *talB* followed by *tktA* (along with its ribosome binding site and terminator), having complementary overlapping sequence to the 3' end of *talB* gene. The two operon P_{gap}-*xylAB* (3.3 kb) and P_{eno}-*talBtktA* (3.35 kb) were designed such a way that the 3' end of *xylB* terminator of P_{gap}-*xylAB* operon has a complementary overlapping sequence to the 5' end of P_{eno} of the P_{eno}-*talBtktA* operon. The overlap extension PCR reactions were performed in 80 µL volume, containing equal molar concentrations of DNA encoding the xylose metabolizing genes, and promoters. The PCR conditions for the amplification of promoters and xylose metabolizing genes are listed in table 3.3. The forward primers for the promoter P_{gap} and the reverse primers of the ultimate gene of the full length construct i.e. *tktA* had complementary overlapping sequences with 5` and 3` end of the plasmids (pZ7-184 and pBBR1MCS-2), respectively. Plasmid pZ7-184 and pBBR1MCS-2 was linearized using BamHI and BamHI-SacI, respectively, to accommodate the operons P_{gap}-*xylAB* followed by P_{eno}-*talBtktA*, using Gibson assembly reaction. The reaction mix was transformed into chemically competent *E. coli* TOP10 cells. The transformed cells were plated on LB agar medium with 25 µg mL^{-1} of chloramphenicol and 50 µg mL^{-1} of kanamycin for pZ7-184 and pBBR1MCS-2 based constructs, respectively. After 12 h, colonies appeared on the plate. The colonies were tested for the presence of the cloned genes using colony PCR. All PCR reactions were performed using *pfu* polymerase enzyme in Veriti™ 96-

Well Fast Thermal Cycler (Thermo Fisher Scientific, USA). The initial denaturation temperature used was 98°C for 5 min followed by 25 DNA amplification cycles with denaturation temperature of 98°C for 30 s, template and primer specific annealing temperature (table 3.3), extension temperature was set at 72°C for 2 min. All the PCR were concluded after a final extension at 72°C for 7 min followed by cooling at 4°C.

Table 3.1 List of plasmids used and constructed in this study

Plasmid name	Genotype	Reference
pZ7-184	Cm^r, rep (pZMO7), ori (pZMO7), ori (p15A)	(So, Chen, Lacap-Bugler, Seemann, & Watt, 2014)
pBBR1MCS-2	Km^r, pBBR1-lacZα	(Kovach et al., 1995)
pZ7-184-Construct 1	pZ7-184-P_{gap} *xylAxylB*-P_{eno} *talBtktA*	This study
pBBR1MCS-2-Construct 1	pBBR1MCS-2- P_{gap} *xylAxylB*-P_{eno} *talBtktA*	This study

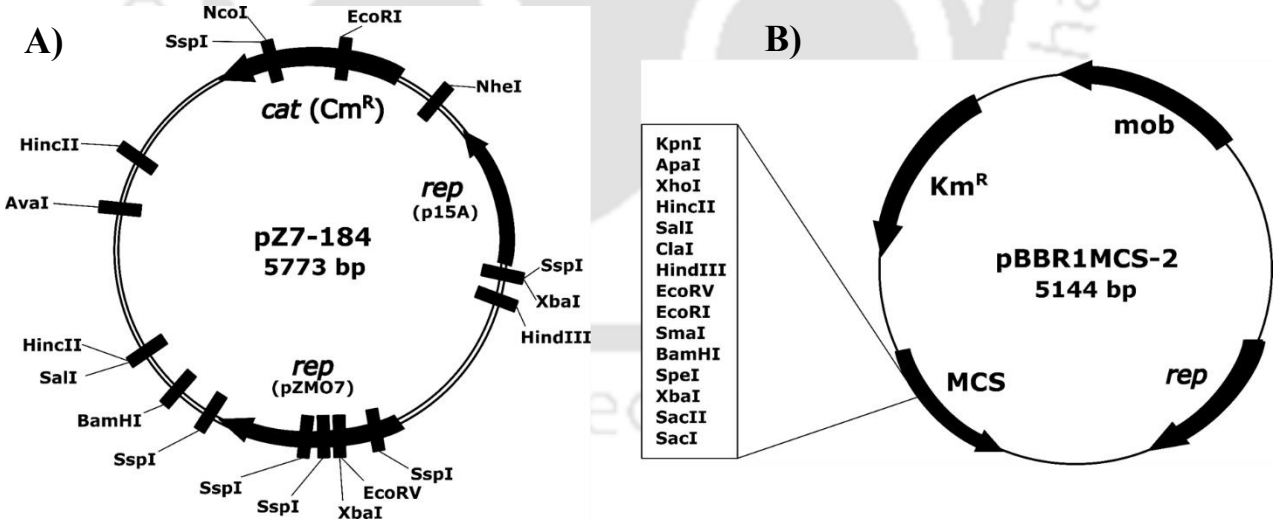


Fig. 3.1 Schematic representation of plasmid **A)** pZ7-184 and **B)** pBBR1MCS-2

Table 3.2 List of primers in this study

Primer name	Primer application	Primer sequence
pZ7- *P_{gap}*-FP	Forward primer of *P_{gap}* for *xylA* & *xylB* gene, sequence overlap with pZ7-184	TAAAAGATCAATACGGATCCCATATGACTTTGTTCGATCAACAACCC
pZ7- *tktA*-RP	Reverse primer of *tktA* gene, overlap with pZ7-184	CACGATGCGTCCGGCGTAGAGGATCCCCGCAAACGGACATATCAAGGTA
pBBR- *P_{gap}*-FP	Forward primer of *P_{gap}* for *xylA* & *xylB* genes, sequence overlap with pBBRMCS-2	GATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTCATATGACTTTGTTCGATCA ACAACCCGAA
***xylA*-RP**	Reverse primer of *xylA* gene, overlap with *xylB* gene	GCCAAGATCTATCCCGATATACATATCGATCGTTCCTTAAAAAAATGCCC
***xylB*-FP**	Forward primer of *xylB* gene, overlap with *xylA* gene	GGGCATTTTTTTAAGGAACGATCGATATGTATATCGGGATAGATCTTGGC
***xylB*-RP**	Reverse primer of *xylB* gene, overlap with *P_{eno}*	ACTCATGAAGCAAAACATGAAGGAACGATCTCCATATCTACCAGC

***P_{eno}*-FP**	Forward primer of *P_{eno}* for *talB* & *tktA* genes, overlap with *xylB* gene	GCTGGTAGATATGGAGATCGTTCCTTCATGTTTTGCTTCATGAGT
***P_{eno}*-RP**	Reverse primer of *P_{eno}*, overlap with *talB* gene	GAAGGGAGGTCAATTTGTCCGTCATATCGAAACCTTTCTTAAAATCTTTT
***talB*-FP**	Forward primer of *talB* gene, overlap with *P_{eno}*	AAAAGATTTTAAGAAAGGTTTCGATATGACGGACAAATTGACCTCCCTTC
***talB*-RP**	Reverse primer of *talB* gene, overlap with *tktA* gene	GACATTTTGACTCCAGATCGGATGATTACAGCAGATCGCCGATCATTTTT
***tktA*-FP**	Forward primer of *tktA* gene, overlap with *talB* gene	AAAAATGATCGGCGATCTGCTGTAATCATCCGATCTGGAGTCAAAATGTC
pBBR-*tktA-RP*	Reverse primer of *tktA* gene, overlap with pBBRMCS-2	TACGACTCACTATAGGGCGAATTGGAGCTCCCGCAAACGGACATATCAAGGTAATAAAAAAGGTCGCC

Table 3.3 Optimized annealing conditions for PCR amplification of xylose metabolizing genes and native ZM4 promoters

Amplicon name	Primers used	Optimized annealing temperature and time
pZ7-*P_{gap}*-*xylA*	pZ7- *P_{gap}*-FP, *xylA*-RP	62°C, 45 s
pBBR -*P_{gap}*-*xylA*	pBBR- *P_{gap}*-FP, *xylA*-RP	65°C, 45 s
xylB	*xylB*-FP, *xylB*-RP	58°C, 45 s
P_{eno}	*P_{eno}*-FP, *P_{eno}*-RP	58°C, 45 s
talB	*talB*-FP, *talB*-FR	62°C, 45 s
***tktA*- pZ7**	*tktA*-FP , pZ7- *tktA*-RP	65°C, 45 s
***tktA*- pBBR**	*tktA*-FP, pBBR-*tktA-RP*	69°C, 45 s

3.2.4 Optimization of electroporation conditions and plasmid transformation into ZM4 using electroporation

Efficiency of transformation of plasmid DNA into *Z. mobilis* is extremely low and plasmid stability was reported to be a challenge (Zou et al., 2012). To enhance the probability and efficiency of plasmid transformation in ZM4 cells, optimization of transformation parameter at every step was imperative. Since, electro-transformation was widely accepted as a satisfactory method for plasmid transformation in *Z. mobilis* (Agrawal et al., 2011; Dunn & Rao, 2014; Gliessman, Kremer, Sangani, Jones-Burrage, & McKinlay, 2017; Jeon et al., 2005), parameters involved in electroporation protocol for the organism was optimized. The details of optimization is tabulated in table 3.4.

To prepare electro-competent cells, ZM4 cells were grown in Rich Medium (RMG) containing 5% (w/v) glucose to an optical density (OD_{600}) of 0.4. The cells were harvested and

first washed with an equal volume of 10 % (v/v) glycerol and then with half volume of 10 % (v/v) glycerol. Finally, cells were resuspended in 10 % (v/v) glycerol to an OD_{600} of 20. 60 µL of electro-competent ZM4 cells were transformed with a mix containing TypeOne Restriction Inhibitor (Lucigen Corporation, USA) added at a concentration of 2.5 µg to 1.5 µg of unmethylated plasmid DNA isolated from *E. coli* strain JM110. The reaction mixture was added to pre-chilled cuvettes with 0.1 cm gap, containing the competent cells. The cuvettes were placed in a Bio-Rad Xcell gene pulser (Bio-Rad Laboratories, USA) set at 18 kV, 200 Ω, and 25 µF. Immediately after the pulse was applied to the cuvettes containing the transformation mixture, cells were resuspended in mating media containing glucose (MMG) composed of 1% (w/v) yeast extract, 0.5% (w/v) tryptone, 0.2% (w/v) KH_2PO_4, 0.25% (w/v) $(NH_4)_2SO_4$, 0.025% (w/v) $MgSO_4$ and 5% (w/v) D-Glucose and allowed to recover without shaking at 30°C for 3 h. The transformed cells were plated on to MMG agar plates containing 5% (w/v) glucose, supplemented with 25 µg mL^{-1} of chloramphenicol and 50 µg mL^{-1} of kanamycin for pZ7-184 and pBBR1MCS-2 based constructs, respectively. The plates were incubated at 30°C for 3-4 days. The transformed colonies were tested for the presence of the cloned genes using colony PCR.

Table 3.4 Optimization of parameters for electro-transformation of *Z. mobilis*

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Culturing media	RMG	RMG	RMG	RMG*	RMG*	RMG**	RMG	RMG
OD_{600}	0.6	0.8	1	0.3	0.4	0.8	1	0.4
Restriction inhibitor (µl)	0.3	0.5	0.5	1	0.5	0.5	0.3	1.5
Cuvette(mm)	2	2	2	1	1	1	1	1
Pulse (kV)	18	16	16	16	18	16	18	18

Recovery media	RMG	RMG	RMG	RMG*	RMG**	RMG**	RMG	MMG
Recovery time (h)	1.5	3	22	1.5	3	18	24	3
Shaking while incubatio (rpm)	Yes	yes	yes	no	no	No	no	No
Plating media (agar)	RMG	RMG	RMG	RMG*	RMG**	RMG**	RMG	MMG

Where, RM is Rich Media containing 20 g L^{-1} glucose, 10 g L^{-1} yeast extract and 2 g L^{-1} KH_2PO_4

RM is 20 g L^{-1} glucose, 10 g L^{-1} yeast extract and 2 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4$, 1 g L^{-1} $(NH_4)_2SO_4$,*

*RM** is 20 g L^{-1} glucose, 10 g L^{-1} yeast extract and 2 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$, 2.5 g L^{-1} $(NH_4)_2SO_4$*

MMG is 50 g L^{-1} glucose, 10 g L^{-1} yeast extract and 2 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$, 2.5 g L^{-1} $(NH_4)_2SO_4$

3.2.5 Adaptive Laboratory Evolution of ZW658

ZW658 was revived from glycerol stock in 10 mL liquid RM containing 3% (w/v) xylose as sole carbon source till attainment of stationary phase (OD_{600} of 0.5). The stationary phase cells were then harvested by centrifugation at 10,000 g at 4 °C for 2 min and resuspended in fresh liquid RM containing 3% (w/v) xylose. In the first step, the cells were subjected to serial subculture into identical cultivation condition for 10 transfers (stage I of adaptation) (Fig. 3.2). Each transfer was performed after 96 h from the time of inoculation, when the cells reached stationary phase. After stage I, cells were subcultured in liquid RM containing higher xylose concentration of 5% (w/v) for successive 20 transfers (stage II of adaptation) (Fig. 3.2). Finally, in stage III of ALE, the concentration of xylose was further increased to 10% (w/v) in liquid RM for additional 20 transfers to maintain the stringent selection pressure on the evolving *Z. mobilis* strain as shown in figure 3.2. Adaptation of the cells beyond 50 transfers did not result in any further improvement in growth. Therefore, the adapted cells, post 50 transfers, were considered for screening, isolation, and selection of the best performing colony. In order to screen single isolated colonies, based on their growth performance, the cells evolved after 50 transfers were plated on to RM agar with 10%

(w/v) xylose. After 2 to 3 days of incubation at 30 °C, 40 single isolated colonies appeared on RM agar plate. A two-stage screening was carried out to confirm and select the colony with the best growth response under xylose as sole carbon source. In the first stage of screening, 40 single isolated colonies were inoculated in 200 µL of RM supplemented with 10% (w/v) xylose in a 96-well microtiter plate and incubated at 30 °C without shaking for 40 h till attainment of stationary phase. OD_{600} for each well, inoculated with single isolated colony of the adapted ZW658 cells, was obtained using micro-plate reader (Infinite M200PRO, Tecan, Switzerland). 12 colonies out of 40 were selected based on their growth (OD_{600}) for further screening in the second stage. The screening was carried out in a 96-well plate under similar cultivation conditions as above and the OD_{600} for each inoculated well was monitored at an interval of 6 h. The second stage of screening was performed to select the strain with dual attributes of maximum growth and highest specific growth rate in xylose supplemented medium. The selected strain was designated as AD50. Further, to exploit the efficiency of ALE, we serially subcultured AD50 until 60^{th} transfer in presence of 10% (w/v) of xylose, and conducted similar experiments to select an isolated colony of adapted strain designated as AD60. The efficiency of AD50 and AD60 was examined by characterizing the two strains under different cultivation conditions. The growth assays were performed in triplicate and the data has been expressed as mean ± standard error.

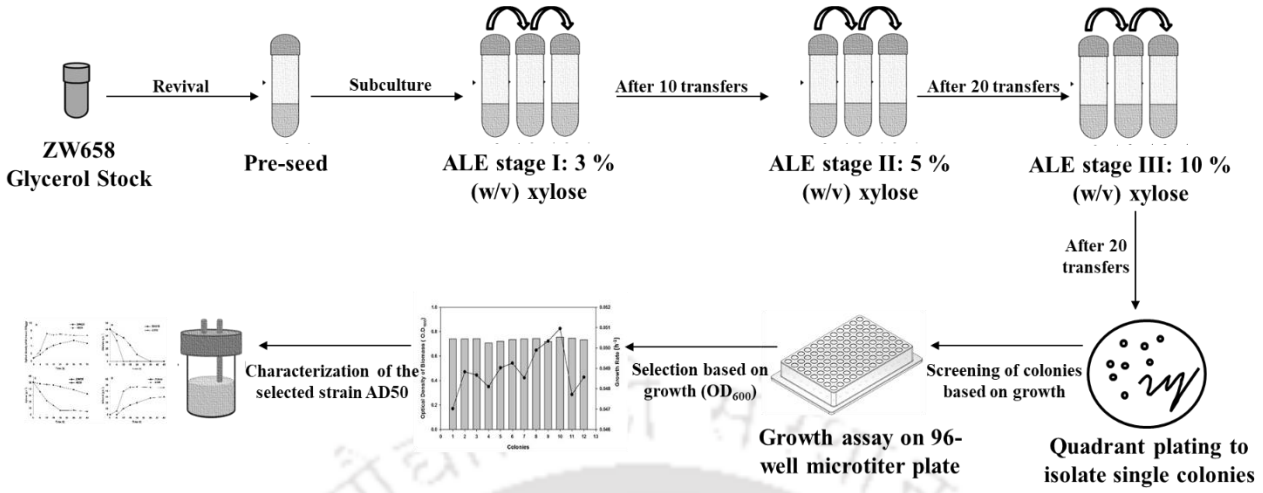


Fig. 3.2 Schematic representation of the systematic ALE strategy implemented to evolve ZW658 towards enhanced xylose utilization capability

3.2.6 Adaptive Laboratory Evolution of AD50

Since the primary objective of this study was to develop a *Z. mobilis* strain that could simultaneously utilize glucose & xylose and produce high amounts of ethanol, AD50 was further subjected to adaptation in presence of glucose under two different strategies. AD50 was revived in 10 mL liquid RM containing 10% (w/v) xylose as sole carbon source till attainment of stationary phase. The stationary phase cells were then harvested by centrifugation at 10,000 g at 4 °C for 2 min and resuspended in fresh liquid RM containing 2% (w/v) glucose or 8% (w/v) xylose as specified in two different ALE strategies. In the first strategy undertaken, AD50 was alternatively subcultured in media containing 10% (w/v) xylose or 2% (w/v) glucose (Fig. 3.3). In the second strategy, AD50 was serially subcultured in media containing 8% (w/v) xylose in the initial 48 h of growth followed by the addition of 2% (w/v) glucose in the same cultivation media and the culture was allowed to grow further for 24 h. After 72 h of cultivation, the serial subculture steps were repeated (Fig. 3.3). The adapted cells, obtained post 6 serial transfers in two strategies, were considered for screening, isolation, and selection of the best performing colony. In order to screen

single isolated colonies, based on their growth performance, the cells evolved after 6 transfers were plated on to RM agar with 5% (w/v) glucose and 5% (w/v) xylose. After 2 to 3 days of incubation at 30 °C, 10 single isolated colonies were selected for further screening. 10 single isolated colonies were inoculated in 200 µL of RM supplemented with 5% (w/v) glucose and 5% (w/v) xylose in a 96-well microtiter plate and incubated at 30 °C without shaking for 48 h till attainment of stationary phase. The OD_{600} for each inoculated well was monitored at an interval of 6 h using micro-plate reader (Infinite M200PRO, Tecan, Switzerland). Single isolated colony was obtained, one each from the first and second strategy of ALE in glucose and xylose based media. The adapted strains were designated as AS1-6 and AS2-6, respectively.

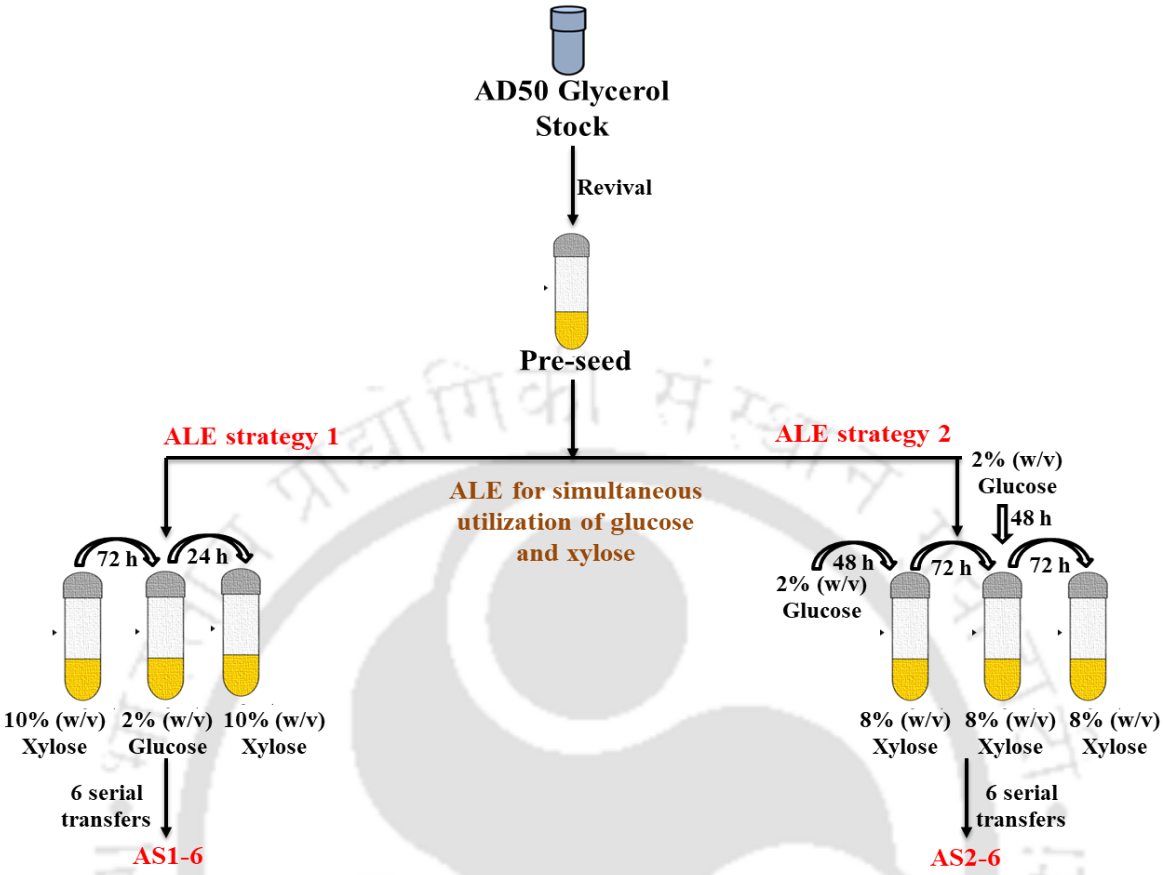


Fig. 3.3 Schematic representation of the dual substrate based ALE strategy implemented to evolve AD50 towards enhanced glucose & xylose co-utilization capability

3.2.7 Optimization of cultivation conditions of AD50

With the aim of enhancing the rate of xylose utilization and subsequent improvement in ethanol productivity, AD50 was subjected to different process engineering strategies. To that end, four cultivation conditions were screened (Fig. 3.4). For the designed experiment, pre-seed cultures were prepared by inoculating glycerol stocks of AD50 in liquid RM supplemented with xylose (RMX) and grown till stationary phase. Seed cultures were prepared by harvesting and inoculating 20% (v/v) of the stationary phase pre-seed culture at an optical density (OD_{600}) of ~ 0.7 to 0.8. Both pre-seed cultures and seed cultures were prepared in 10% (v/v) of xylose as present

in the production media. 20% (v/v) seed culture was harvested and centrifuged at 7000 g for 2 min. The supernatant was discarded and cell pellets were resuspended in 1 mL of fresh (RM) medium supplemented with 10% (v/v) of xylose and subsequently inoculated in 250 mL airtight customized cultivation bottles with a working volume of 100 mL of medium. In the first strategy, designated as N_2NS, the culture was purged with 99.9% pure gaseous nitrogen (N_2) for 10 min and kept without shaking at 30°C. In the second strategy, designated as N_2WS, the culture was purged with 99.9% pure gaseous nitrogen (N_2) for 10 min and kept under shaking at 150 rpm at 30°C. Third strategy, designated as AE, involved cultivation of the AD50 culture under shaking at 150 rpm at 30°C. Finally in the fourth strategy, designated as AEN_2, the culture was kept at 30°C with shaking at 150 rpm, after 6 h, the culture was purged with 99.9% pure gaseous nitrogen (N_2) and kept under same condition. Samples were withdrawn at regular intervals to obtain dynamic profiles of growth, xylose utilization and ethanol production. The experiments have been performed in duplicates and the data have been expressed as mean ± standard error.

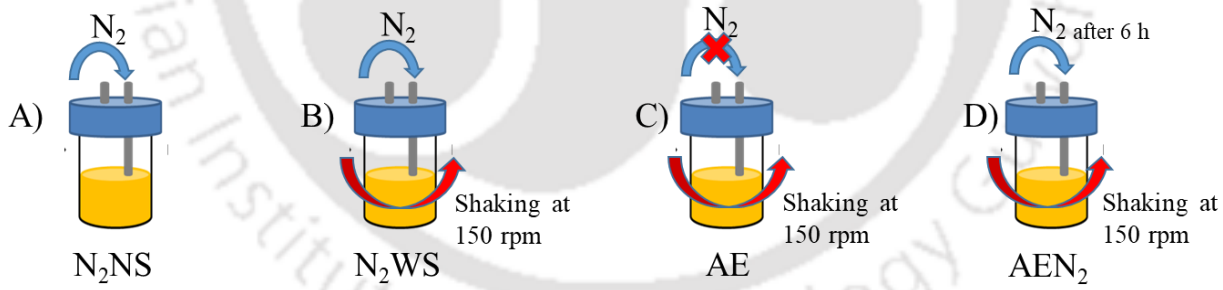


Fig. 3.4 Schematic representation of four distinct process engineering strategies screened, where, **A)** is the culture was purged with pure N_2 gas and kept without shaking at 30°C, **B)** is the culture was purged with pure N_2 gas and kept with shaking at 150 rpm at 30°C, **C)** is the culture was kept at 30°C with shaking at 150 rpm, and **D)** is the culture was kept at 30°C with shaking at 150 rpm, after 6 h N_2 gas was purged and kept under same condition.

3.2.8 Optimization of substrate concentrations for AD50 in binary sugar mixtures

To understand and obtain the best concentrations of glucose and xylose, in mixture, required for optimum growth and comparable binary sugar utilization rate by AD50, pre-seed cultures were prepared by inoculating glycerol stocks of AD50 in liquid RM supplemented with xylose (RMX) and grown till stationary phase. 20% (v/v) of pre-seed cultures were harvested at stationary phase and inoculated to prepare seed cultures such that the initial optical density obtained was (OD_{600}) 0.7 to 0.8. Seed cultures were prepared in same amount of sugar (glucose and xylose) as present in the production media at 30 °C under shaking at 150 rpm in a shaker incubator (Orbitek, Scigenics biotech, India). For all the nine combinations of glucose and xylose concentrations tested, 20% (v/v) seed culture was harvested and centrifuged at 7000 g for 2 min. The supernatant was discarded and cell pellets were resuspended in 1 mL of fresh (RM) medium and subsequently inoculated in 250 mL airtight customized cultivation bottles with a working volume of 100 mL of medium. The concentrations of both glucose and xylose was varied from 1 % (w/v) to 9 % (w/v) such that a high overall sugar concentration of 10 % (w/v) was maintained in the fermentation media. After inoculation, 100 mL RM medium was purged with 99.9% pure gaseous nitrogen for 10 min and the set up was kept under shaking (150 rpm) at 30 °C. Samples were withdrawn at regular intervals to obtain the kinetic parameters depicting the rate of glucose and xylose utilization and amount of ethanol formation by AD50 under investigated combinations of binary sugars.

3.2.9 Characterization of ZW658, AD50, AS1-6 and AS2-6 on mono sugar xylose and binary sugar mixture of glucose-xylose

For batch fermentation experiments with ZW658, AD50, AS1-6 and AS2-6, pre-seed cultures were prepared by inoculating glycerol stocks of *Z. mobilis* strains in liquid RM

supplemented with xylose (RMX) and grown till stationary phase. Seed cultures were prepared by harvesting and inoculating 20% (v/v) of the stationary phase pre-seed culture at an optical density (OD_{600}) of ~ 0.7 to 0.8. Both pre-seed cultures and seed cultures were prepared in same amount of sugar (glucose and/or xylose) as present in the production media at 30 °C under shaking at 150 rpm in a shaker incubator (Orbitek, Scigenics biotech, India).

For all batch fermentations, 20% (v/v) seed culture was harvested and centrifuged at 7000 g for 2 min. The supernatant was discarded and cell pellets were resuspended in 1 mL of fresh (RM) medium and subsequently inoculated in 250 mL airtight customized cultivation bottles with a working volume of 100 mL of medium. For characterization on mono-sugar, the RM was supplemented with 10% (w/v) xylose and for characterization on binary sugar mixture, the RM was supplemented with 5% (w/v) of glucose and xylose each. After inoculation, 100 mL RM medium was purged with 99.9% pure gaseous nitrogen for 10 min and the set up was kept under shaking (150 rpm) at 30 °C. Samples were withdrawn at regular intervals to obtain dynamic profiles of growth, glucose, xylose, xylitol, and ethanol. The specific sugar uptake rate (q_s, g g^{-1} h^{-1}) was determined using equation (1);

$$q_s = \frac{1}{x}\frac{ds}{dt} \qquad (1)$$

Where, s is the concentration of substrate in the broth (g L^{-1}) and x is the dry cell weight (g)

The experiments have been performed in duplicates and the data have been expressed as mean ± standard error.

3.2.10 Genomic DNA extraction and whole genome sequencing of AD50 and AS1-6

The mid-log phase AD50 and AS1-6 cells, grown in liquid RM with 10% (w/v) xylose, were used for chromosomal DNA extraction using genomic DNA extraction kit (GeneJET Genomic DNA purification kit, Thermo Fisher Scientific). The concentration of the genomic DNA

was quantified at 260 nm using nanodrop UV-Vis spectrophotometer (NanoPhotometer, PLEN NP80). Whole Genome Sequencing was performed using Illumina HiSeq platform (Sandor Lifesciences, India), generating pair end reads for the sample. 8.2 million reads were generated using the forward and reverse reactions for the genomic DNA sample. The mutations identified in the genomic regions have been validated by analyzing their corresponding de novo sequences using nucleotide blast (blastn).

3.2.11 Enzyme assays to understand the adaptation induced modulation in metabolic pathway of xylose metabolism and ethanol biosynthesis in *Zymomonas mobilis*

Temporal expression profile of the following four enzymes, intrinsic to xylose metabolism and ethanol biosynthesis, were obtained for both AD50 and ZW658: Xylose isomerase (XylA), Transketolase (TklB), Xylose reductase (XyrA), and Pyruvate decarboxylase (Pdc). ZW658 and AD50 cells were cultivated in 200 mL of liquid RM containing 5% (w/v) xylose and 5% (w/v) glucose for 48 h at 30 °C under shaking at 150 rpm. In order to obtain temporal profile of the enzymes, samples were collected at specific intervals starting from 3 h till the end of the fermentation.

For cell lysate preparation, 10 mL of cultures were collected in 50 mL centrifuge tubes under aseptic conditions and harvested by centrifugation at 3,000 g at 4 °C for 15 min. Harvested cells were washed once with freshly prepared lysis buffer composed of 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithioerythritol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Washed cell pellets were resuspended in the lysis buffer in order to obtain a final OD_{600} of 50. The resuspended cells were sonicated for 8 cycles of 10 s pulse and 30 s of cooling period. Sonicated cells were centrifuged at 17,000 g at 4 °C for 40 min and the cell debris was discarded. The supernatant was collected and re-centrifuged at 17,000 g at 4 °C for 40 min. The supernatant was

used as cell free extract for performing enzyme activity assays. Bradford assay was used for estimation of the total protein concentration in the cell free extract using Bovine Serum Albumin (BSA) as standard.

All enzyme activity assay reactions were carried out in a 96-well microtiter plate with a final volume of 200 µL. A reaction mixture containing 1 mM triethanolamine (TEA) pH 7.5, 0.3 mM NADH, 100 mM xylose, 10 mM $MgSO_4$, 4 U/mL sorbitol dehydrogenase, and 10 µL of crude cell extract was used for xylose isomerase assay at 30 °C (Agrawal et al., 2011). Transketolase activity assay was performed at 30 °C in a solution containing 42 mM Tris–HCl pH 8.5, 5 mM $MgCl_2$, 1 mM thiamine pyrophosphate (TPP), 0.5 mM ribose-5-phosphate, 0.2 mM NADH, 2.5 U/mL glycerol-3-phosphate dehydrogenase, 10 U/mL triose phosphate isomerase (TPI), and 10 µL crude cell extract (Agrawal et al., 2011). The reaction was initiated by adding 5mM xylulose-5-phosphate in the solution. In order to assess the performance of xylose reductase, a reaction mixture composed of McIlvaine buffer pH 7.2, 0.35 mM NADPH, 200 mM xylose, and 10 µL crude cell extract was used (Agrawal et al., 2011). McIlvaine buffer was prepared by adding 16.5 mL of 0.2 M sodium phosphate dibasic to 3.5 mL of 0.1 M citric acid. The assay reaction was monitored at 30 °C. Pyruvate decarboxylase was assayed at 30 °C, in a reaction mixture containing 100 mM citrate buffer pH 6.0, 20 mM $MgSO_4$, 18 mM sodium pyruvate, 0.15 mM NADH, 10 U alcohol dehydrogenase, 1.5 mM TPP, and cell free extract (Bringer-Meyer, Schimz, & Sahm, 1986). The enzyme activities for XyrA and for XylA, TklB, & Pdc were measured as the change in NAD(P)H and NADH absorbance respectively, monitored at 340 nm using spectrophotometer (Infinite M200PRO, Tecan, Switzerland).

3.2.12 Analytical methods

For all characterization experiments, samples were collected at regular intervals and centrifuged at 13,000 g at 4 °C for 10 min (Multifuge X3R, Thermofisher Scientific, USA). The pellets were resuspended in MilliQ water and cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}), using a UV-Vis spectrophotometer (Cary 100, Varian, Australia). The dry cell weight (DCW) was determined and an OD_{600} of 1 corresponded to 0.47 mg of DCW per mL of culture broth. The supernatant was collected and analyzed for the estimation of glucose, xylose, ethanol, and xylitol, using high performance liquid chromatography (Ultimate 3000, Dionex, Thermofisher Scientific, USA) equipped with Rezex-ROA column (300 × 7.8 mm, Phenomenex, USA). A solution of 0.005 N H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL min-1. Glucose, xylose, ethanol, and xylitol were detected with Refractive Index Detector (RID). The temperature of the column oven was maintained at ambient temperature and that of RID at 37°C.

3.3 Results and discussion

3.3.1 Selection of a potential *Z. mobilis* strain as a platform for metabolic engineering directed towards efficient utilization of xylose

Batch fermentations were carried out to characterize and compare the performance of ZM4 and ZW658 in terms of growth, glucose and/or xylose utilization, and ethanol production. A superior growth performance of ZM4 was evident from a 2.5-fold higher biomass titer as compared to ZW658 under the different concentrations of glucose screened (Fig. 3.5A). A maximum OD_{600} of 13.8 was obtained for ZM4 cultivated in media supplemented with 160 g L^{-1} of glucose, while maximum OD_{600} of 5.5 was recoded for ZW658, in media supplemented with 80 g L^{-1} of glucose. It was observed that ZM4 could utilize glucose within 10 h and maximum of 20 h, when subjected

to 20 g L^{-1} and 160 g L^{-1} of glucose, respectively. However, ZW658 demonstrated a significantly slower glucose utilization within 20 h under 20 g L^{-1} of glucose and within 45 h when grown in 80 g L^{-1} of glucose (Fig. 3.5B). Additionally, ZW658 exhibited incomplete glucose utilization at a concentration beyond 80 g L^{-1}. ZM4 proved to be superior in terms of substrate utilization i.e. glucose, with a maximum specific glucose uptake rate of 1.5 g g^{-1} h^{-1} as compared to that of 1 g g^{-1} h^{-1} in case of ZW658, when cultivated in 80 g L^{-1} of glucose. Consistently, ZM4 remarkably exceeded in terms of ethanol production with a maximum ethanol titer of 80 g L^{-1}, observed in presence of 160 g L^{-1} of glucose, as opposed to 32.5 g L^{-1} in case of ZW58, in presence of 80 g L^{-1} of glucose (Fig. 3.5C). When compared, the maximum ethanol yield was observed to be in the range of 0.49 to 0.5 g g-1 in case of ZM4, under all glucose concentrations screened, which corresponds to 98% of the maximum theoretical yield. While the maximum ethanol yield marked for ZW658 was 0.36 g g^{-1}, in presence of 80 g L^{-1} of glucose, which corresponds to 71.1% of the maximum theoretical yield. Similarly, the maximum ethanol productivity exhibited by ZM4 was 4.05 g $L^{-1}h^{-1}$ as compared to that of 0.95 g $L^{-1}h^{-1}$ for ZW658.

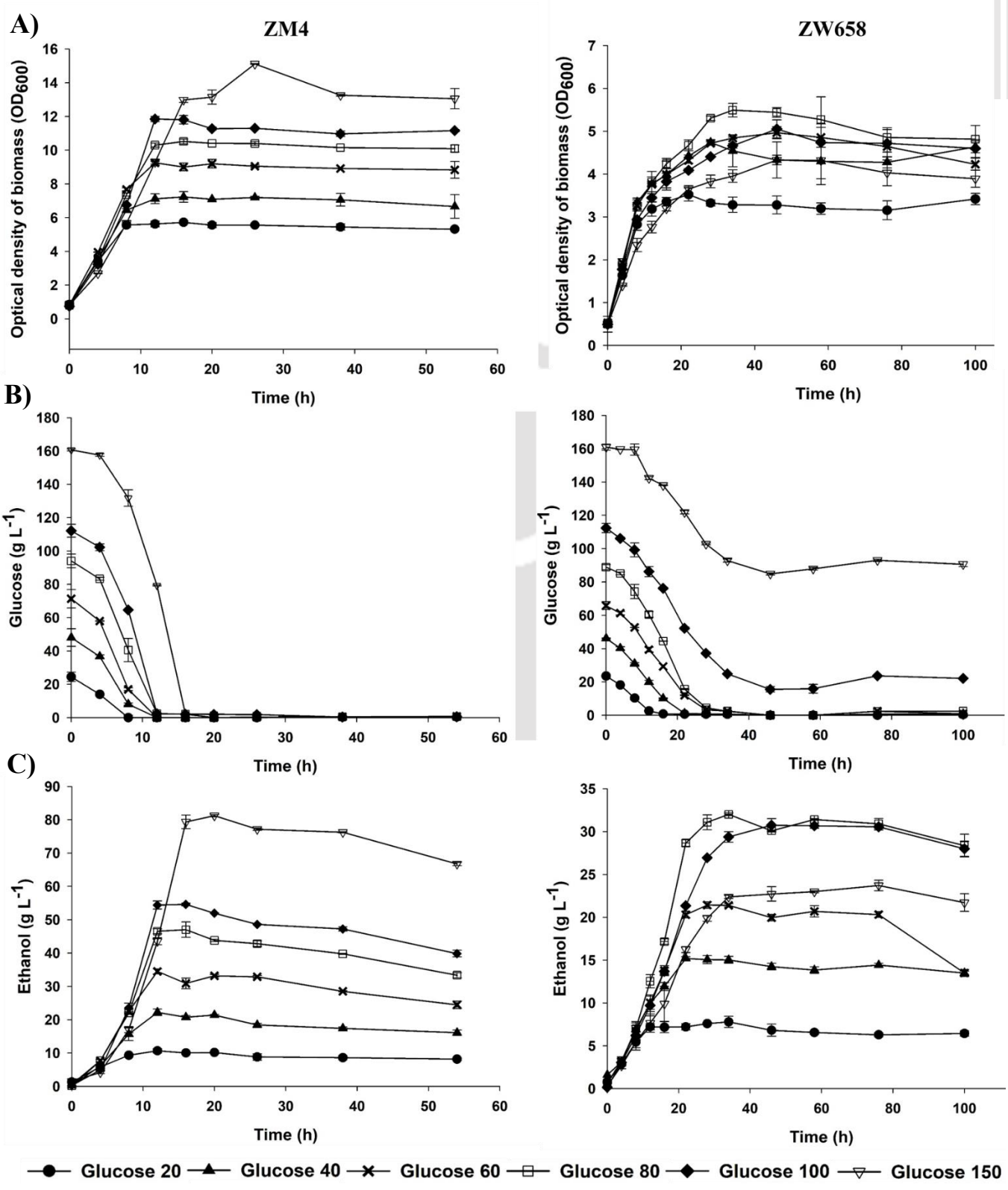


Fig. 3.5 Dynamic profile of **A)** growth, **B)** glucose utilization, and **C)** ethanol production by ZM4 and ZW658, respectively, when cultivated under various concentrations of glucose.

However, when both *Z. mobilis* strains were characterized under different concentration of xylose, ZM4 could not demonstrate any growth, substrate utilization and subsequent ethanol formation, which was in lieu of the absence of xylose metabolizing enzymes in wild type *Zymomonas* strains (Zhang et al., 1995). When the performance of ZW658 was investigated under different concentrations of xylose as sole carbon source, surprisingly, instead of being engineered with xylose metabolizing genes, the strain could not utilize xylose as it did in case of glucose. It showed maximum growth, OD_{600} of 2.5, when cultivated in 80 g L^{-1} of xylose (Fig. 3.6A). Presence of significant amounts of residual xylose was consistent for all the conditions tested (Fig. 3.6B). Relatively, maximum xylose utilization was observed in presence of 20 g L^{-1} of xylose and consequently this led to the highest ethanol yield of 0.31 g g^{-1}, which corresponds to 60.8% of the maximum theoretical yield. Maximum ethanol titer obtained was 4.5 g L^{-1} with an overall ethanol productivity of 0.08 g L^{-1} h^{-1}, when ZW658 was grown in presence of 80 g L^{-1} of xylose (Fig. 3.6C).

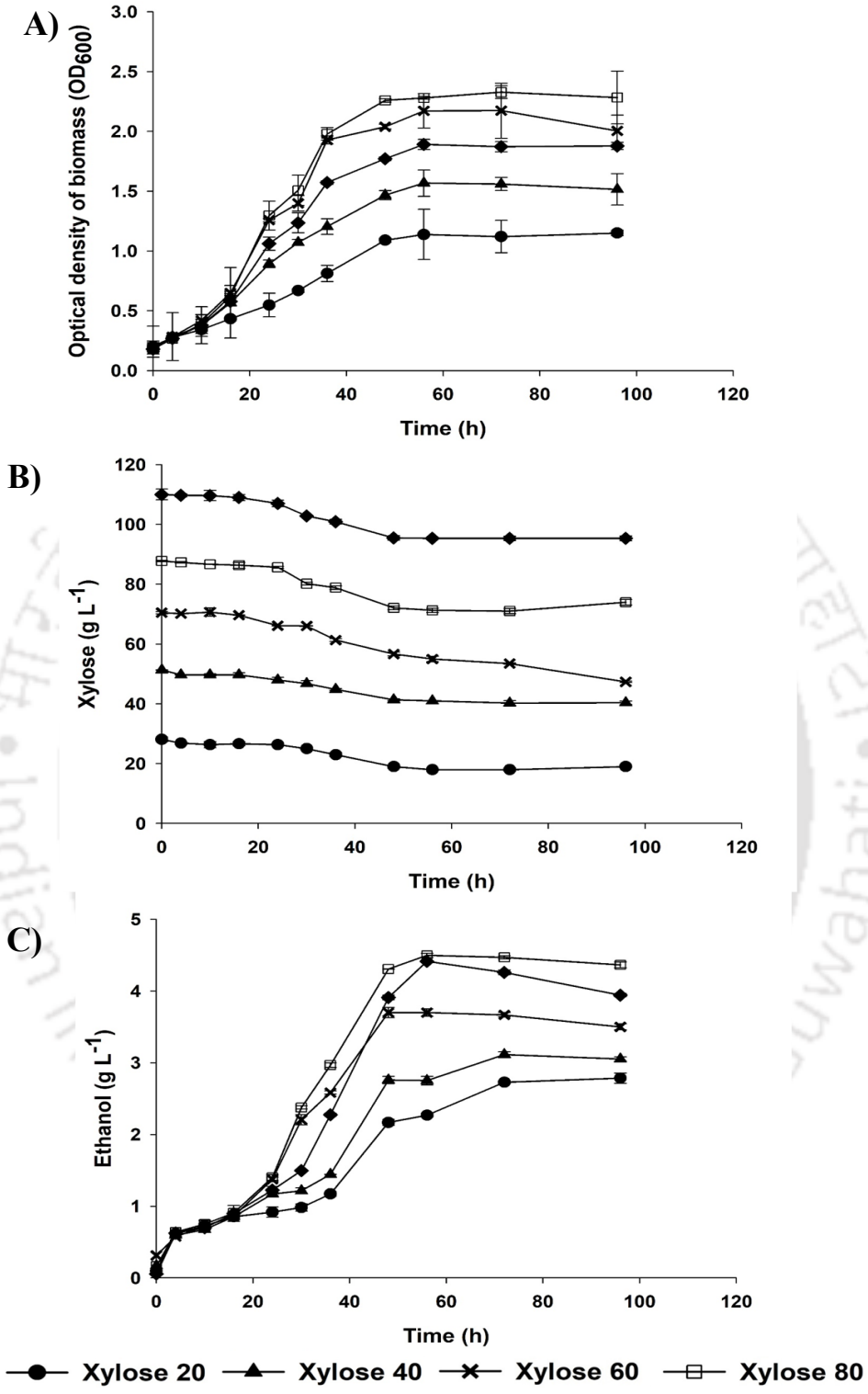


Fig. 3.6 Dynamic profile of **A)** growth, **B)** xylose utilization, and **C)** ethanol production by ZW658, when cultivated under various concentrations of xylose.

Based on the performance of the *Z. mobilis* strains under mono sugar substrates, ZW658 was in further characterized under binary sugar mixture i.e. in presence of glucose and xylose (Fig. 3.7). Since, ZW658 exhibited incomplete glucose utilization above 80 g L^{-1} and in presence of xylose highest ethanol yield was observed for 20 g L^{-1} of xylose, the glucose and xylose concentrations selected for ZW658 characterization were 20 g L^{-1} glucose, 40 g L^{-1} and 60 g L^{-1} of glucose along with 20 g L^{-1} of xylose. Among the three different xylose concentrations tested, the highest OD_{600} of 2.8 was observed when ZW658 was cultivated in presence of 20g/l of xylose and 40 g/l of glucose (Fig. 3.7A). All the conditions showed incomplete xylose consumption (Fig. 3.7B). Interestingly, xylose utilization by the ZW658 was commenced only after complete consumption of glucose. In terms of glucose consumption, 20 g/l of glucose was completely consumed while higher concentrations led to incomplete glucose utilization (Fig. 3.7C). In terms of ethanol yield, out of all the conditions tested, 20 g/l of glucose with 20 g/l of xylose showed highest yield of 0.2 mol/mol of mixed sugar, which is 39% of the maximum theoretical yield with an ethanol titer of 6 g L^{-1} (Fig. 3.7D).

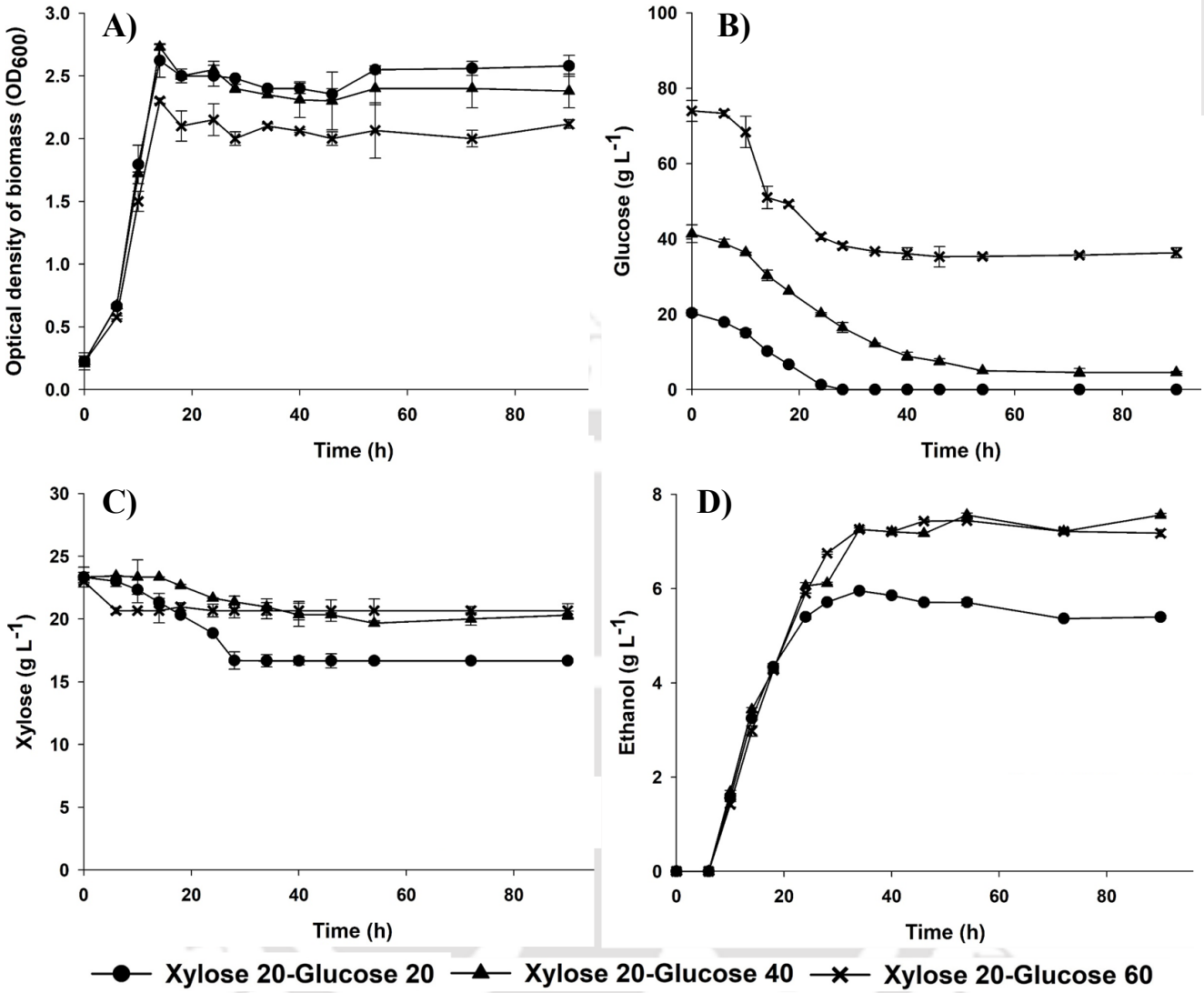


Fig. 3.7 Dynamic profile of **A)** growth, **B)** glucose utilization, **C)** xylose utilization, and **D)** ethanol production by ZW658, when cultivated under various concentrations of binary sugar mixture (glucose and xylose).

The observations noted from the characterization of ZM4 and ZW658 based on batch fermentations, led to the conclusion that wild type strain ZM4 is highly efficient in terms of glucose utilization and ethanol production compared to the engineered strain *Z. mobilis* ATCC ZW658. Additionally, it can also be observed that it can tolerate higher sugar concentrations and utilize it

within 20h. Moreover, ZW658 could not demonstrate efficient xylose utilization. Thus, ZM4 was selected as a platform for directed metabolic engineering towards efficient xylose utilization.

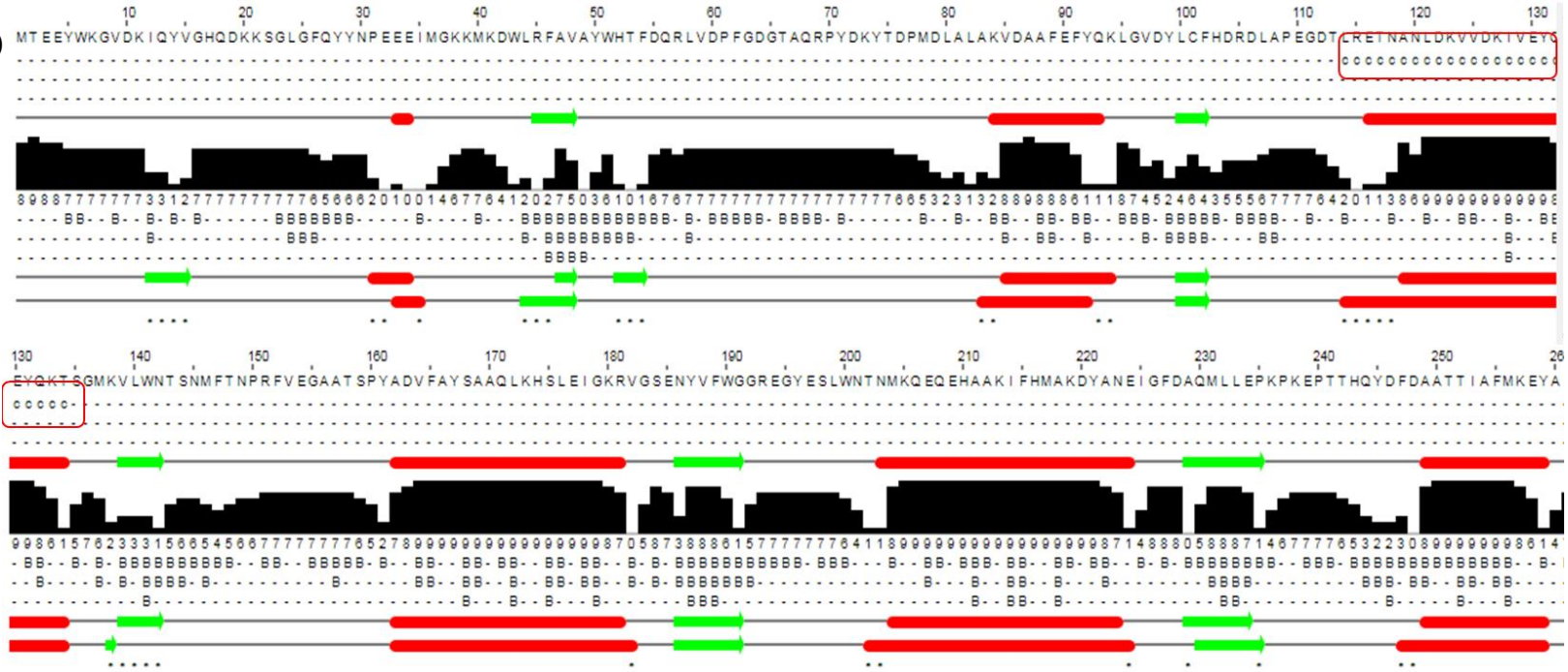
3.3.2 Construction of plasmids carrying xylose-metabolizing genes

In order to construct plasmids with genes encoding xylose assimilating enzymes i.e. XylA & XylB and PPP enzymes i.e. TalB & TktA, two plasmids namely pZ7-184 and pBBR1MCS-2, with distinct features have been selected. While genes encoding XylB, TalB and TktA were chosen from *E. coli*, gene encoding XylA was selected from *Lactobacillus brevis*. *L. brevis* was reported to be unresponsive towards CCR or glucose effect, thus exhibiting simultaneous utilization of glucose and xylose (J. H. Kim et al., 2010). Additionally, XylA was found to be a rate-limiting enzyme in xylose metabolic pathway (Gao, Zhang, Mcmillan, & Kompala, 2002) and indeed, higher expression of XylA was reported to improve xylose utilization in engineered *Z. mobilis* (Agrawal et al., 2011). State-of-the-art investigations were performed using *xylA* sourced from *E. coli*, which is a CCR positive organism (Yang et al., 2016). Thus, in this study we sought to test the effect of *xylA*, sourced from a CCR negative organism i.e. *L. brevis*, towards enhanced xylose utilization in the *Z. mobilis* strain, even in presence of glucose.

Xylose isomerase (XylA) catalyzes the conversion of xylose to xylulose, which is the first step in conversion of xylose towards ethanol. In terms of specific activity, XylA from *L. brevis* showed higher efficiency than XylA from other organisms. The specific activity of XylA from *L. brevis* obtained was 6.5 µmol min^{-1} mg^{-1} (Yamanaka, 1975) as compared to that from *E. coli* that corresponds to 2 µmol min^{-1} mg^{-1} (Schellenberg et al., 1984). Preference of XylA, towards divalent metal ions, also varied amongst the two organism. While XylA from *L. brevis* prefer Mn^{2+} for its efficient functioning, XylA from *E. coli* prefers Mg^{2+} for its activity (Schellenberg et al., 1984; Yamanaka, 1975). Moreover, sequence analysis of XylA from *L. brevis* and *E. coli* was performed

using blastp showed 45% identity for 98% query coverage with an e-value of $5e^{-147}$. The substrate and divalent metal binding residues were also found to be highly conserved with minor change in the positions of these residues in XylA from *E. coli*. Further, sequence based structure analysis was performed for XylA from both of the organisms. The XylA sequence from *L. brevis* and *E. coli* were submitted in Jpred for the prediction of the secondary structures (Drozdetskiy, Cole, Procter, & Barton, 2015) and solvent accessibility for the two XylA proteins (Fig. 3.8). Chou and Fasman Secondary Structure Prediction Server (CFSSP) had been used to predict the percentage distribution of helix, sheets and turns present in both the enzymes (Ashok Kumar, 2013). Structural analysis of XylA from both the organisms highlighted marked variations in distribution of secondary structural components. While *L. brevis* XylA was found to have stretches of α-helix and β-sheets, spanning amino acid position 1 to 150, *E. coli* XylA was observed to have β-sheets, dominantly present within the same stretch of amino acid residues as that of the enzyme from *L. brevis*. One striking difference perceived between the two protein structures is presence of a coiled coil region in *L. brevis* XylA, positioned from amino acid residues 115 to 135, which was missing from the structure of XylA from *E. coli*. The prediction using CFSSP program was also in alignment with Jpred results where *L. brevis* XylA had 68.2 % of α-helix, 26.9 % of β-sheet and 13.4 % of turns as opposed to *E. coli* XylA, which had 75 % of α-helix, β-27 % of sheet and 8 % of turns, present in its structure. Thus, it can be inferred that there is a significant variation in the secondary structural component distribution among the same protein from two different organisms. This preliminary information proffered more confidence to select XylA from *L. brevis*, to be incorporated in *Z. mobilis* along with XylB, TalB and TktA from *E. coli*, to achieve simultaneous utilization of xylose along with glucose.

A)



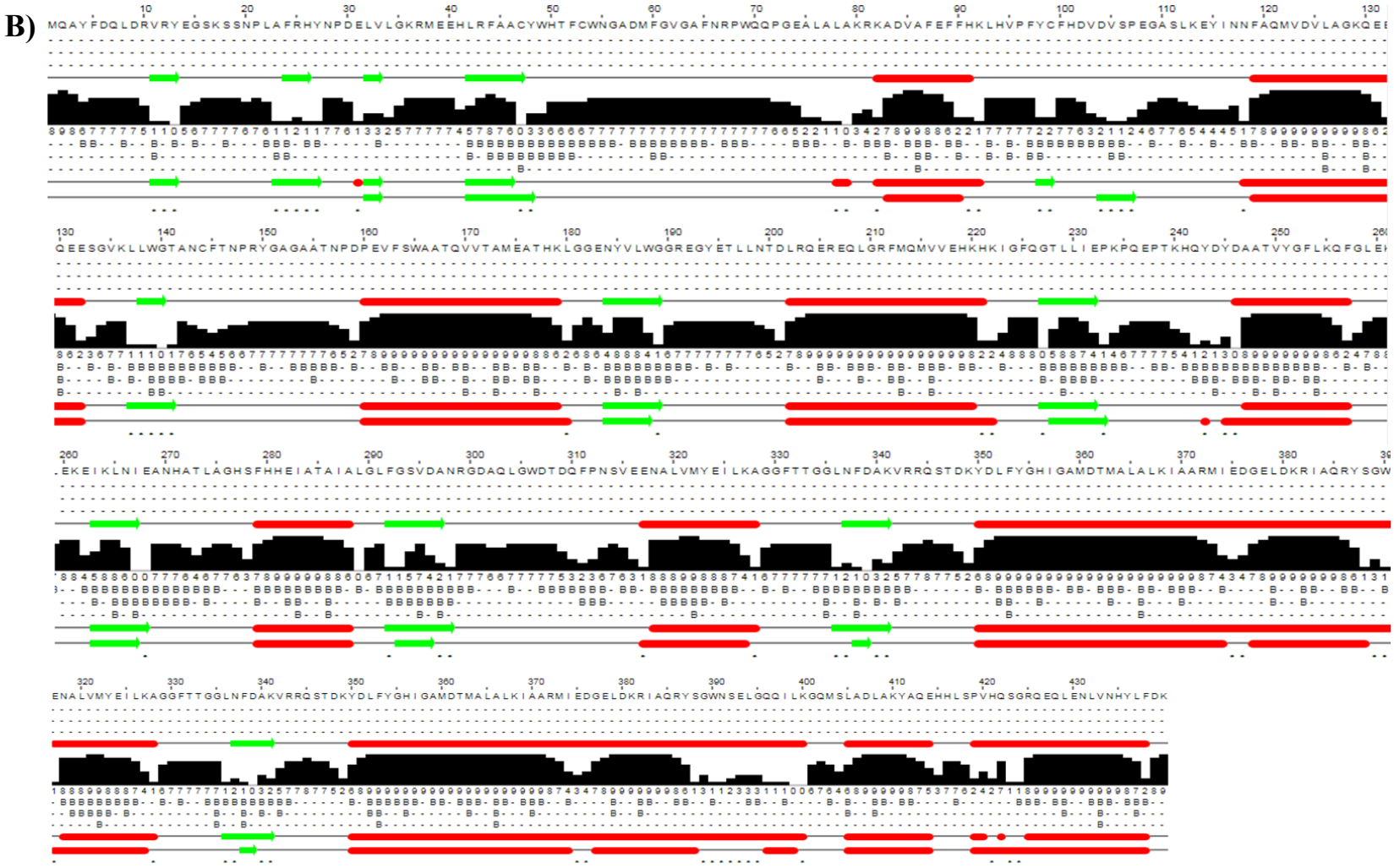


Fig. 3.8 JPred analysis of XylA protein sequence from **A)** *L. brevis*, and **B)** *E. coli*, where red tubes denote helices and green tube denotes sheets

The performance of *xylA* & *xylB* and that of *talB* & *tktA* was investigated when expressed under the control of P_{gap} (promoter of native glyceraldehyde-3-phosphate dehydrogenase gene) and P_{eno} (promoter of native enolase gene), using pZ7-184 or pBBR1MCS-2 as the vector for xylose-metabolizing gene integration. The designs of the engineered constructs are shown in Fig. 3.9.

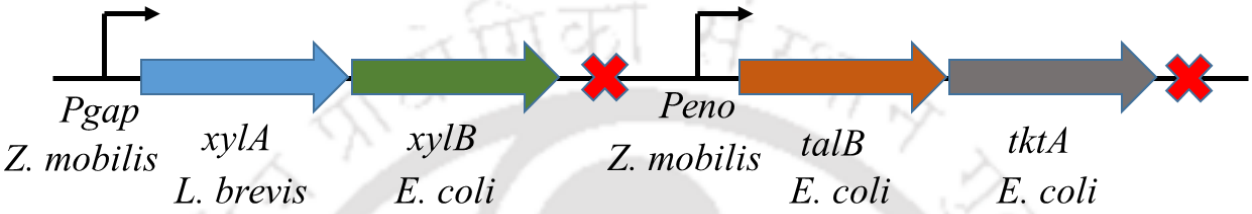
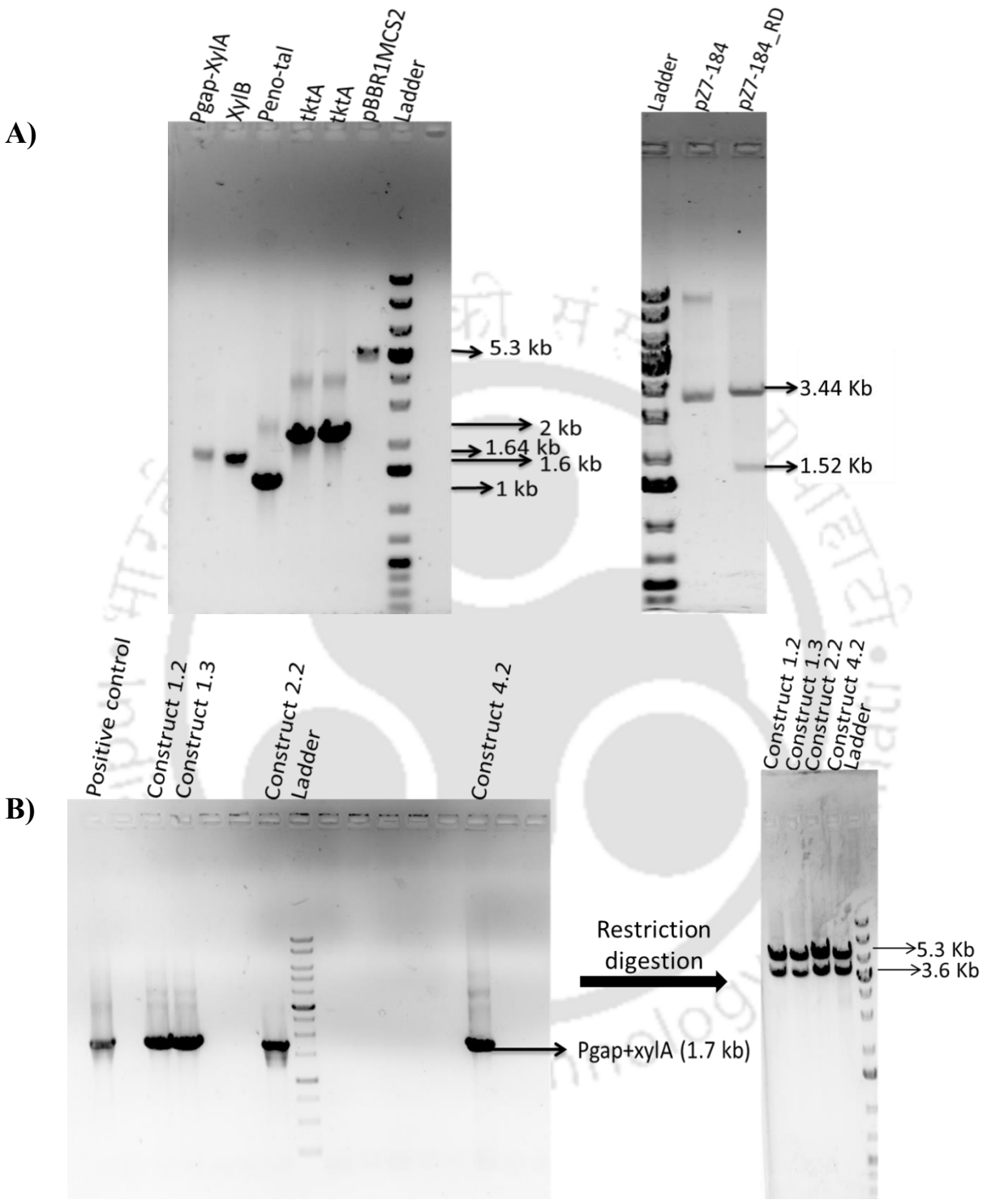


Fig. 3.9 Design of the construct to introduce xylose assimilating and PPP genes, under the influence of strong native promoters, in ZM4, where red coloured cross denotes the terminator sequence

PCR amplification was performed to obtain the *Pgap-xylA*, *xylB*, *Peno-talB* and *tktA* amplicons (3.10A). Restriction digested pZ7-184 or pBBR1MCS-2 were used as backbone in Gibson assembly reaction with the amplified DNA fragments. Thus, two constructs were developed namely, pZ7-184-Construct 1 (Fig. 3.10B), and pBBR1MCS-2-Construct 1, which were further confirmed using colony PCR and restriction digestion (Fig. 3.10C).



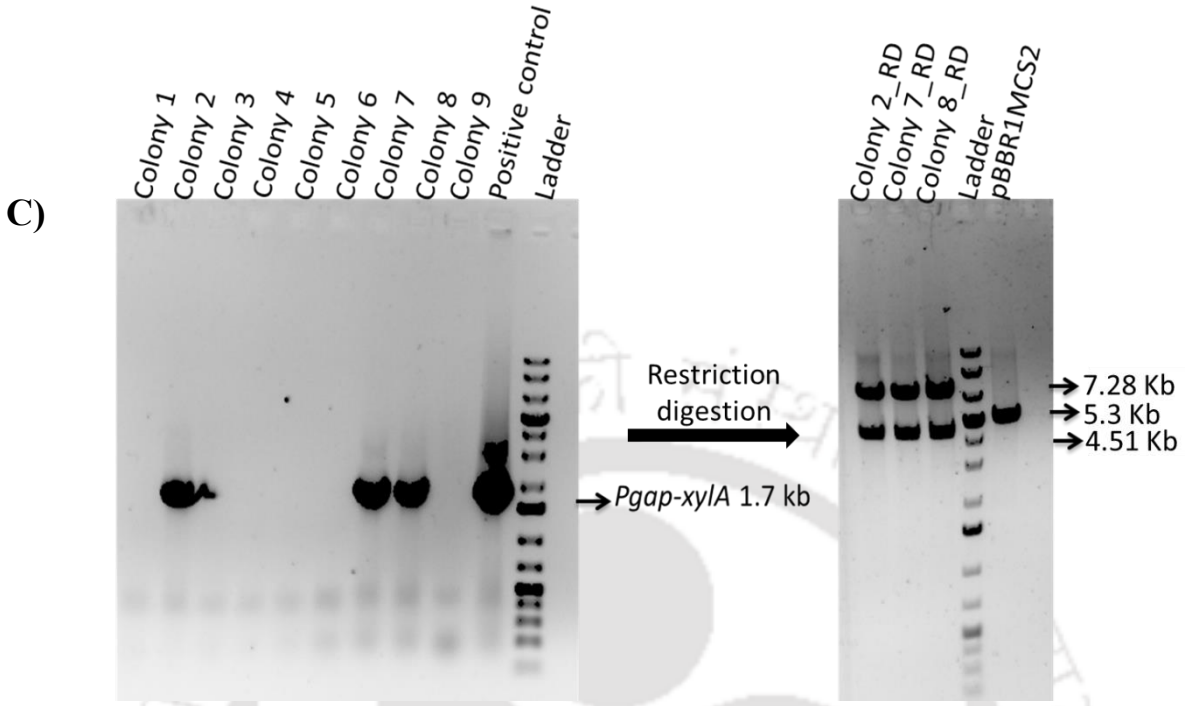


Fig. 3.10 Agarose gel analysis shows **A)** PCR amplified promoters, xylose metabolizing genes and restriction digested vectors, **B)** Clone confirmation through colony PCR and subsequent restriction digestion of clone plasmid, for construct pZ7-184-Construct 1, and **C)** Clone confirmation through colony PCR and subsequent restriction digestion of clone plasmid, for construct pBBR1MCS-2-Construct 1

Transfecting these constructs in ZM4 evinced to be challenging due to poor plasmid stability inside the cells. Thus, optimization of electro-transformation parameters resulted in successful transfection of pBBR1MCS-2-Construct 1 in ZM4 cells (Fig. 3.11). Although, repeated attempts have been made to transform pZ7-184-Construct 1 in ZM4, however, due to possible incompatibility of the plasmid, pZ7-184-Construct 1 carrying ZM4 mutant could not be developed.

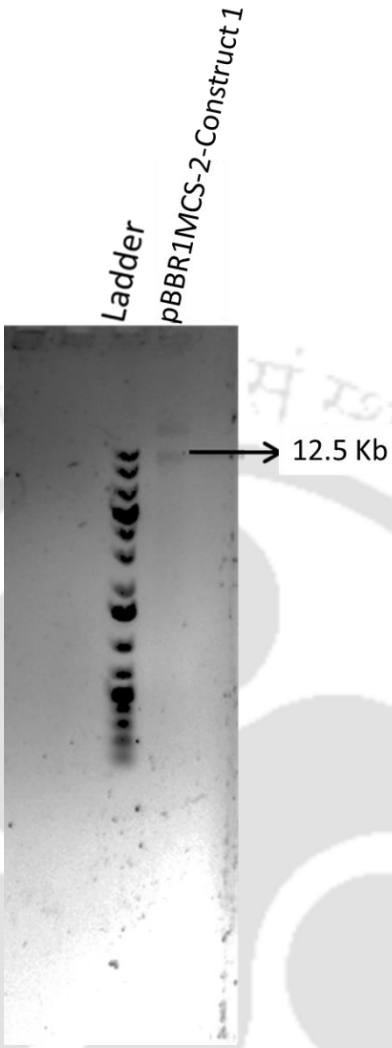


Fig. 3.11 Agarose gel analysis shows a band corresponding to construct pBBR1MCS-2-Construct 1 (12.5 kb) cloned in pBBR1MCS-2 vector, extracted from transformed ZM4 cells

Initially, the engineered ZM4 cells carrying pBBR1MCS-2-Construct 1 was inoculated in 2% (w/v) of RMG (Rich Media supplemented with Glucose) and subsequently subcultured in 2% RMX (Rich Media supplemented with Xylose) for 5 serial transfers. The growth of the engineered strain was monitored by observing the OD_{600} of the culture using UV-Vis spectrophotometer. Even after the initial short period of adaptation, in RMX media, the mutant was unable to grow beyond

an OD_{600} of 0.2-0.3. This observation called for an alternative strategy to develop an engineered *Z. mobilis* strain, which can demonstrate an efficient utilization of xylose.

3.3.3 Adaptive Laboratory Evolution of ZW658 leads to development of AD50

ALE harnesses the power of natural selection of beneficial genetic variations under the influence of strict selection pressure (Portnoy et al., 2011). ALE is a potent and effective strategy for development of microbial strains with exceptional phenotypic traits (Agrawal et al., 2011; LaCroix et al., 2015; Sandberg, Lloyd, Palsson, & Feist, 2017). In the present study, ALE has been implemented to develop a *Z. mobilis* strain with dual capabilities of: efficient xylose fermentation and co-utilization of glucose & xylose. To that end, ZW658, an engineered *Z. mobilis* strain endowed with four *E. coli* xylose metabolizing genes (Viitanen et al., 2008) was used as a platform for employing a rational ALE strategy. Although, ZW658 was reported to successfully express the heterologous genes namely *xylA*, *xylB*, *talB*, and *tktA*, which encode xylose metabolism enzymes. However, in the present study it has been observed that the strain was unable to efficiently utilize xylose even as a sole carbon source. The need for an initial adaptation of metabolically engineered *Z. mobilis* with xylose metabolizing genes, in xylose containing medium was previously reported (Agrawal et al., 2011). ZW658 was systematically subjected to an increasing concentration of xylose starting from 3% (w/v) for 10 transfers (stage I) through 5% (w/v) for 20 transfers (stage II) to 10% (w/v) for 20 transfers (stage III) in order to maintain a constant stringent selection pressure. The initial adaptation in stage I resulted in an improvement in growth, which was evident from increase in OD_{600} from 0.5 to 1.5. With the extended adaptation process in stage II, time taken by the culture to reach stationary phase significantly reduced to 80 h from 96 h taken in stage I. This result point towards increased growth rate of the culture and in turn, augmented xylose utilization rate. At the end of the stage II of adaptation, growth of the culture further increased to

an OD_{600} of 3.2. In the final stage, the OD_{600} of the adapted strain reached a maximum of 5.9 within 48 h, which was 10.8 folds higher than the unadapted strain. This highlights a significant improvement in xylose metabolism and its positive influence on growth of the organism. ALE of ZW658 was terminated after 50 transfers conducted over a period of 200 days, since no further improvement in growth was observed after 50^{th} transfer. After the completion of adaptation, in order to select the best evolved cells a two-stage screening was performed. At the end of two-stage screening, the single isolated colony (numbered as colony 10), exhibiting maximum growth with highest growth rate, was selected for further study (Fig. 3.12). The selected evolved strain was designated as AD50.

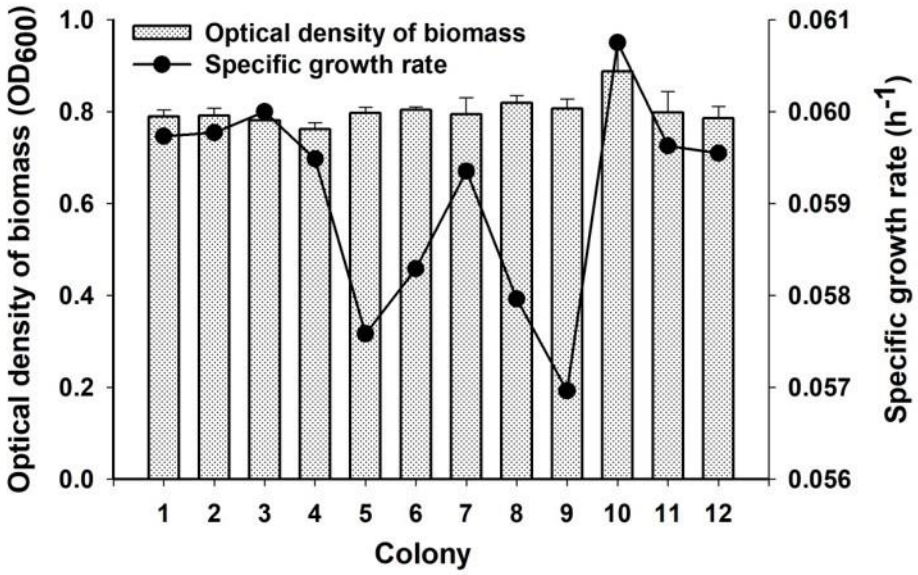


Fig. 3.12 Screening and selection of best-adapted colony in terms of growth and specific growth rate obtained from three-stage ALE strategy. The data have been expressed as mean ± standard error

3.3.4 Characterization of AD50 in presence of xylose

Batch fermentations were carried out to characterize and compare the performance of ZW658 and AD50 in terms of growth, xylose utilization, and ethanol production. AD50 was cultivated in media supplemented with different concentrations of xylose, to assess the extent of improvement in terms of xylose utilization, achieved by the evolved strain in lieu of the extended adaptation process. An improved growth performance was observed in case of AD50 (Fig. 3.13A), with the maximum biomass titer of OD_{600} 4.5 in presence of 10 % (w/v) of xylose, as compared to that of ZW658 (OD_{600} of 2.5) under similar cultivation conditions. This enhanced growth of AD50 may be attributed to the increase in extent and rate of xylose utilization in comparison to ZW658. AD50 could utilize a maximum of 78.6% of xylose i.e. 15.72 g L^{-1} in 2 % (w/v) RMX media compared to 32.38% i.e. 9.1 g L^{-1} of xylose in case of ZW658. A coherent pattern of enhancement in xylose utilization by AD50 was marked under all the screened xylose concentrations (Fig. 3.13B). At the highest xylose concentration tested, 10% (w/v) of RMX, AD50 utilized 42.73% of the supplemented xylose that corresponded to 42.73 g L^{-1} of xylose as compared to that of 14.66% i.e. 14.66 g L^{-1} of xylose in case of ZW658. However, there was incomplete substrate utilization in all the conditions tested. In terms of ethanol production, AD50 proved to be superior over ZW658. AD50 could produce a maximum of 11.7 g L^{-1} of ethanol utilizing 42.73 g L^{-1} of xylose in media containing 100 g L^{-1} of xylose, as compared to 4.49 g L^{-1} in case of ZW658 in presence of 80 g L^{-1} of xylose (Fig. 3.13C). Although, AD50 showcased considerable improvement in terms of xylose utilization and subsequent ethanol production when compared to that of ZW658, however, its behaviour was not encountered to be exceptional unlike the seed culture of the AD50 prepared as an inoculum of the batch fermentation. In the seed culture, AD50 was observed to metabolize 68.774 g L^{-1} of xylose in 10 % (w/v) of RMX media producing 18 g L^{-1} of ethanol. A

comparative analysis of the performance of AD50 against ZW658 have been depicted in Fig. 3.13D. This led us to further investigate the rationale behind this feature of AD50.

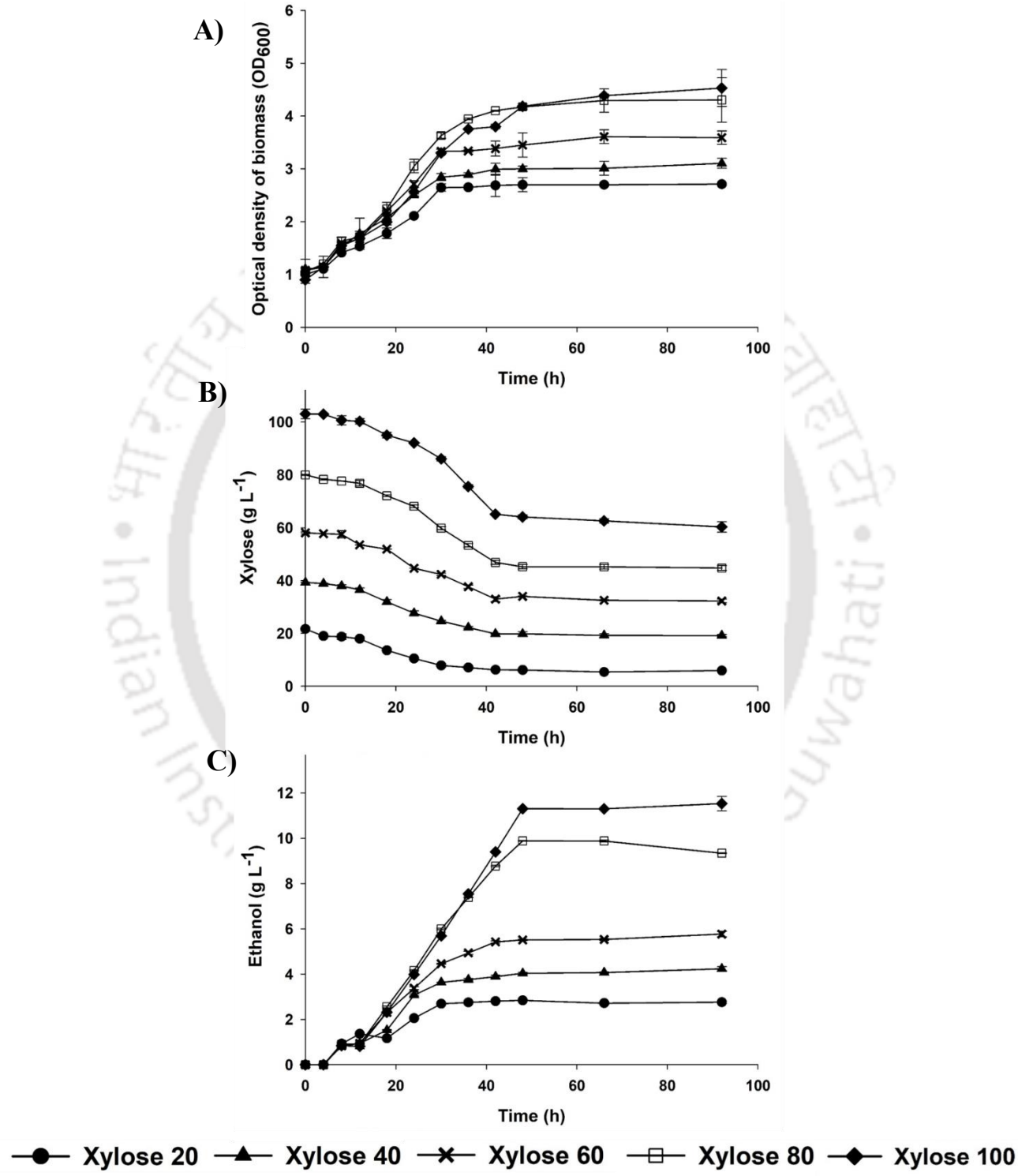


Fig. 3.13 Dynamic profile of **A)** growth, **B)** xylose utilization, and **C)** ethanol production by AD50, when cultivated under various concentrations of xylose.

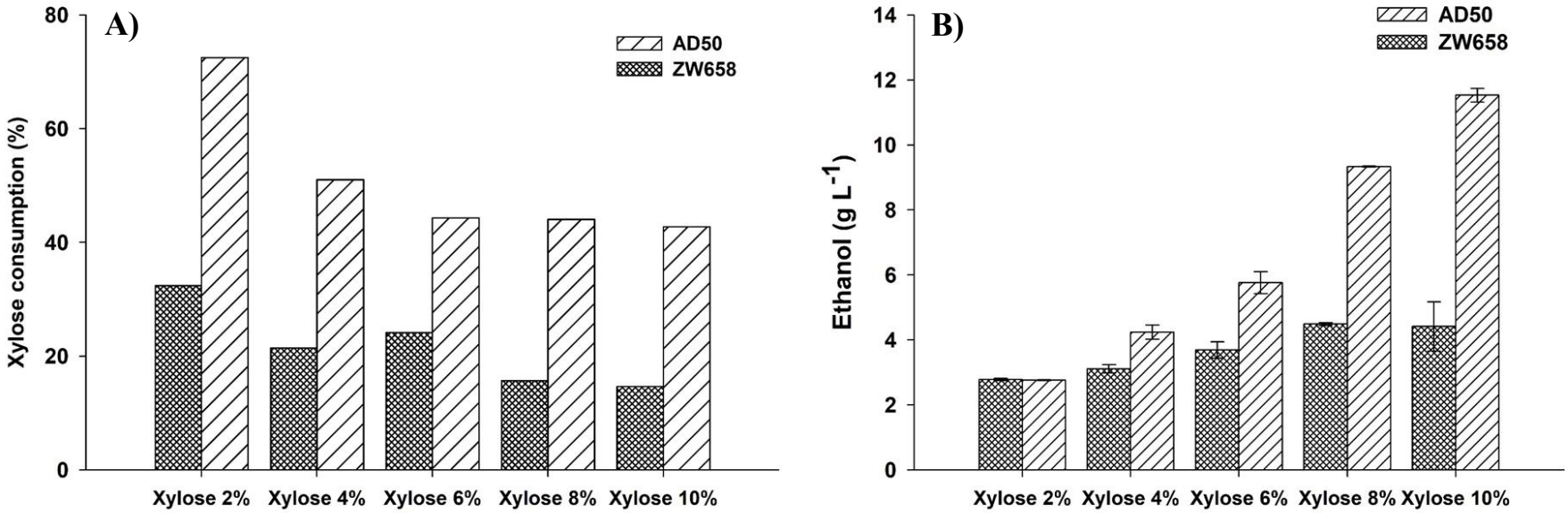


Fig. 3.14 Comparative performance analysis of **A)** xylose consumption, and **B)** Ethanol production by ZW658 and AD50. Concentrations are expressed in terms of (% (w/v))

3.3.5 Process engineering strategies screened towards improved xylose utilization by AD50

As per the reports from significant studies, aerobic environment was perceived to be unsuitable for efficient utilization of xylose by *Z. mobilis* mutant strains. Aerobic culture condition induces the production of acetic acid, which in turn activates xylitol synthesis pathway. In turn, xylitol was reported to inhibit xylose isomerase, thus, the flux shifts from ethanol to xylitol. Based on these studies and considering the distinct behaviour of AD50 under two different conditions, rational process-engineering strategies were designed and screened, to evaluate the performance of AD50 under different cultivation conditions. Out of the four conditions screened, N_2WS in which the AD50 culture was purged with pure N_2 gas and kept with shaking at 150 rpm at 30°C, showed drastic increase in rate of xylose utilization by the strain and increased production of ethanol with 98.6% of the maximum theoretical yield. AD50 demonstrated remarkably rapid utilization of 97.8% of the 100 g L^{-1} of xylose present in the media within 48 h (Fig. 3.15A), with a concomitant increase in the ethanol titer (49.1 g L^{-1}) (Fig. 3.15B). The maximum ethanol productivity for AD50 was also observed to be superior in N_2WS condition, which corresponded to 1.22 g L^{-1} h^{-1} (Fig. 3.15C). Although, N_2WS emerged as the best condition for cultivation of the evolved strain, however, the other three tested conditions also proved to be efficacious towards improving the xylose utilizing capability and subsequently enhanced ethanol production by AD50. While under all four conditions, AD50 could utilize maximum amount of xylose within 48 h, however, N_2WS excelled in terms of maximum xylose uptake rate of 3.9 g g^{-1} h^{-1} as compared to that observed under N_2NS (2.1 g g^{-1} h^{-1}), AE (3.7 g g^{-1} h^{-1}) and AEN_2 (1.9 g g^{-1} h^{-1}). These observations further emphasized the fact that aerobic condition was not suitable for production of ethanol from xylose by *Z. mobilis*. A significant study also reported N_2 gas as an effective fertilizer for ethanol production by *Z. mobilis* (Kremer, LaSarre, Posto, McKinlay, & Ingram, 2015).

Notably, purging N_2 gas in the cultivation media of AD50 along with optimal mixing of nutrients evinced to be an effective strategy towards enhancing the xylose rate and extent of xylose utilization by the evolved strain. A comparative analysis of the various cultivation conditions used to test the most effective condition for AD50 growth, substrate utilization and product formation (Fig.3.16).

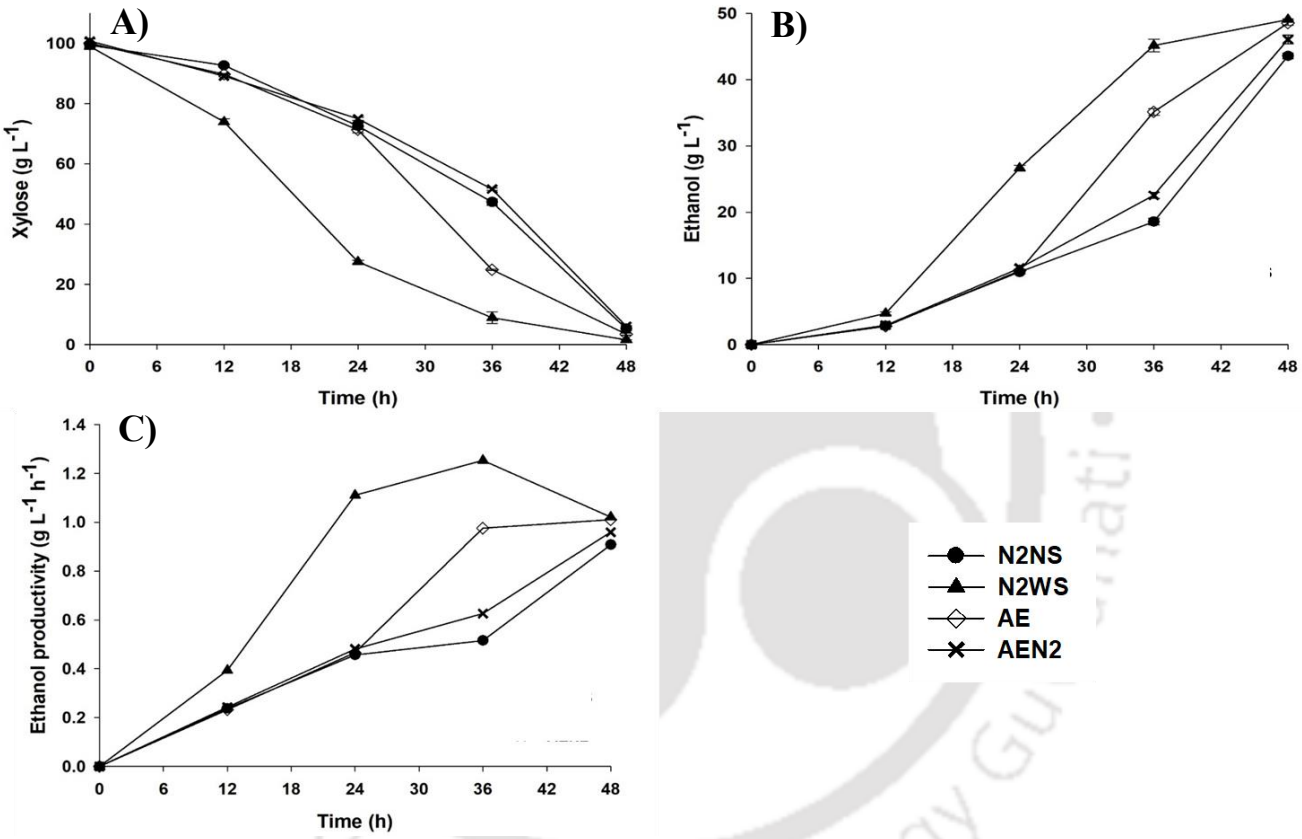


Fig. 3.15 Dynamic profile of AD50 under various cultivation conditions screened, wherein **A)** xylose utilization, **B)** ethanol titer, and **C)** ethanol productivity of AD50 under different cultivation conditions.

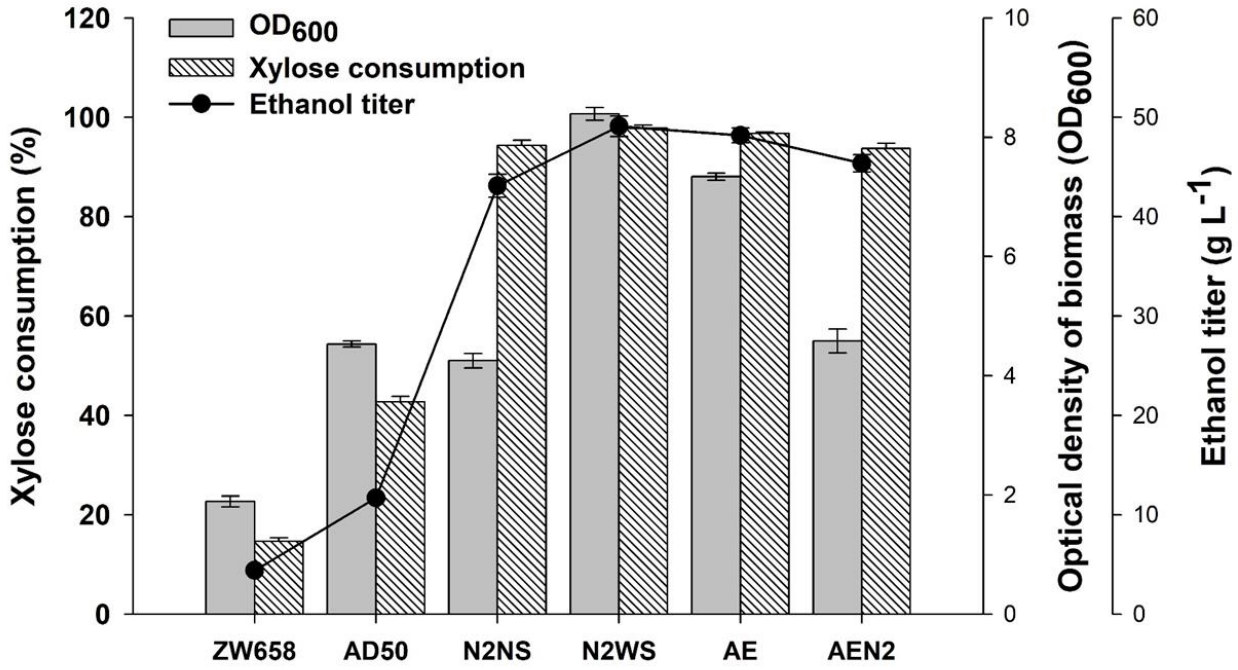


Fig. 3.16 Comparative performance analysis of growth, xylose consumption, and ethanol production by un-adapted ZW658 and AD50 under different cultivation condition

Similarly, the performance of AD60 was also investigated under similar cultivation conditions (Fig. 3.17). Although, the maximum ethanol productivity of AD60 (1.51 g L^{-1} h^{-1}) was marked to be higher as compared to that of AD50, however, AD50 surpassed AD60 in terms of extent of xylose utilization and ethanol titer (Fig. 3.18). Thus, AD50 was selected for further improvisation. ALE coupled with process engineering manifested into an efficient xylose metabolizing strain, AD50, however, the primary objective of this study is to develop a strain of *Z. mobilis* that could co-utilize glucose and xylose and produce high amounts of ethanol. To that end, strategies have been designed and implemented to augment the characteristic features of AD50.

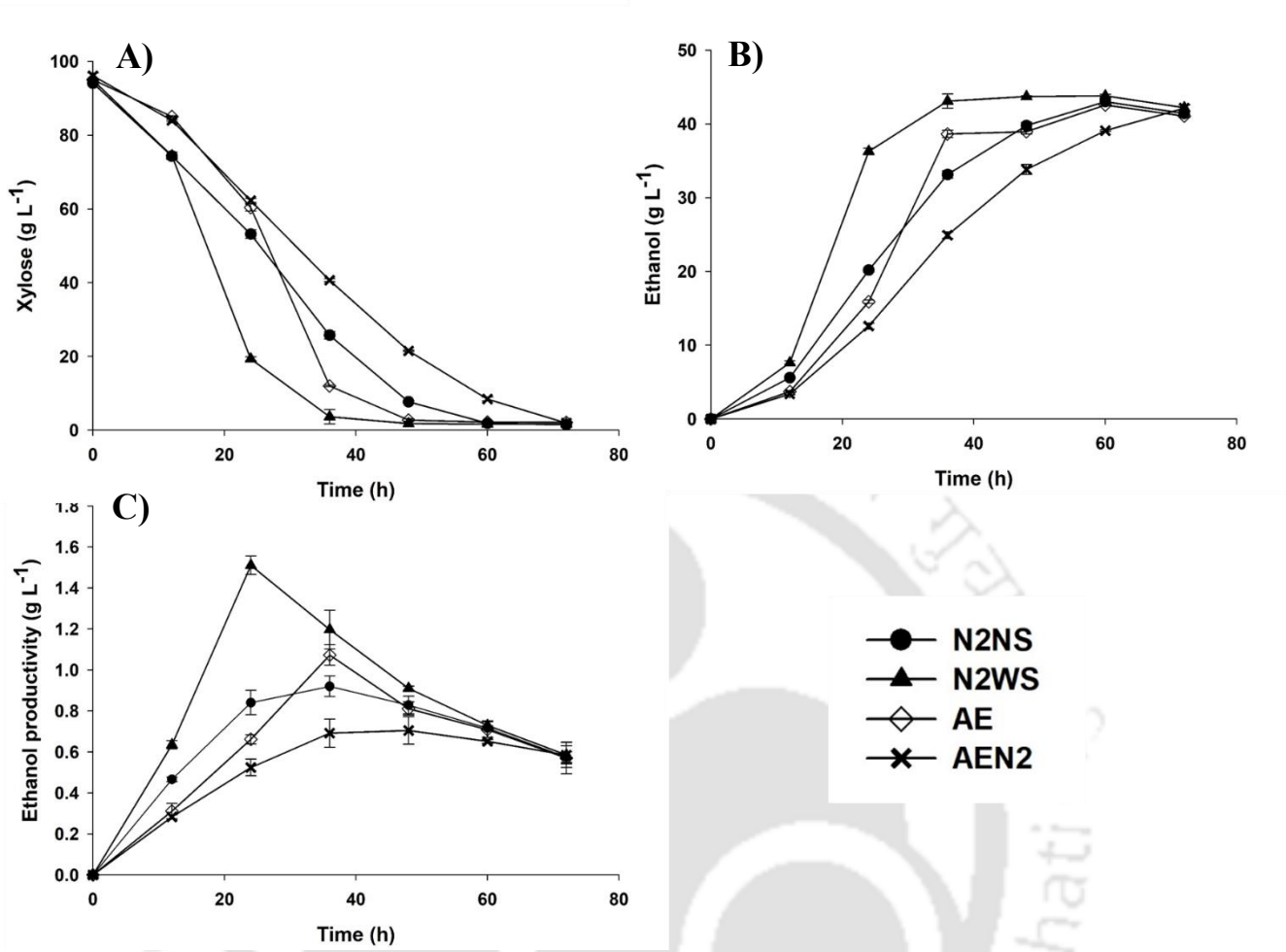


Fig. 3.17 Dynamic profile of AD60 under various cultivation conditions screened, wherein **A)** xylose utilization, **B)** ethanol titer, and **C)** ethanol productivity of AD50 under different cultivation conditions.

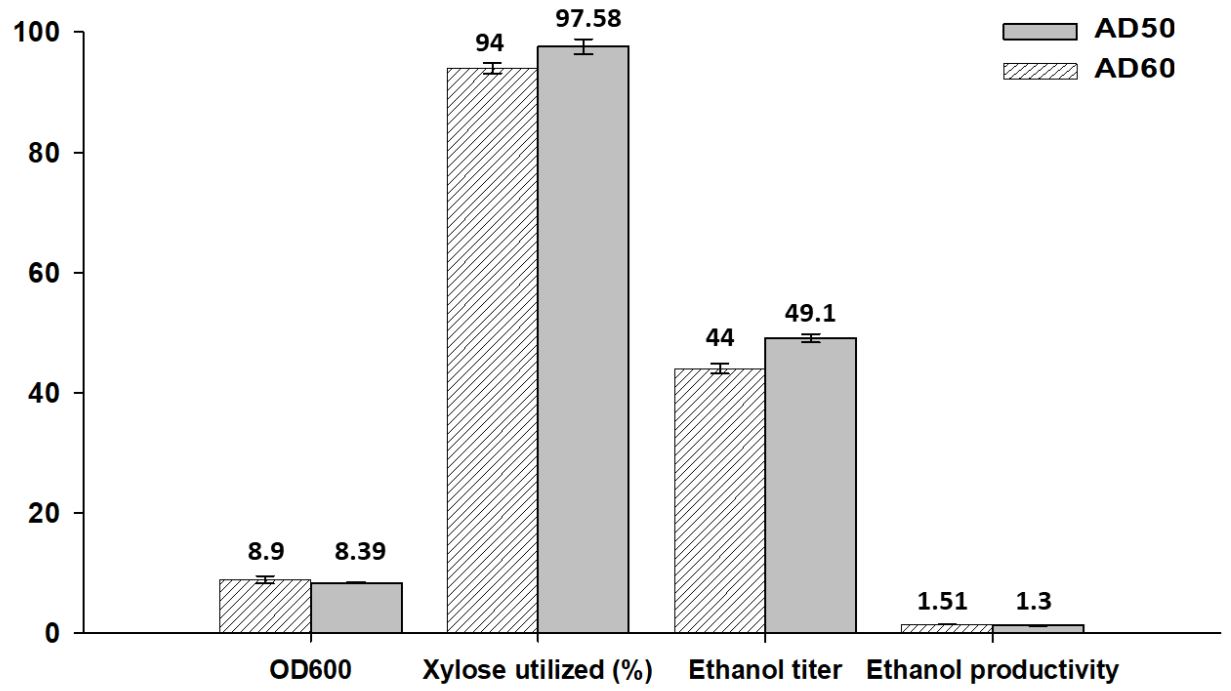


Fig. 3.18 Comparative performance analysis of growth, xylose consumption, ethanol titer and ethanol productivity by AD50 and AD60 under same cultivation condition (N_2WS)

3.3.6 Selection of the best binary sugar concentration for optimum performance of AD50 towards substrate utilization and product formation

In order to identify the ideal concentration of glucose and xylose mixture towards optimum performance of AD50, 9 different combinations of the hexose and pentose sugars were tested. In accordance with the objective of efficacious xylose utilization, AD50 exhibited maximum percentage xylose utilization of 97.6% in medium containing 8% (w/v) of xylose and 2 % (w/v) of glucose (Fig 3.19A). In terms of ethanol formation, AD50 marked highest ethanol titer of 47.1 g L^{-1} under equal glucose and xylose supplementation (3.19B). Although, a highest ethanol yield of 95.5 % of the maximum theoretical yield was obtained, it could be rationalized as an outcome of the dominant glucose concentration i.e. 9% (w/v) of glucose. Equal concentrations of glucose

and xylose too presented a remarkable ethanol yield of 94.6 % of the maximum theoretical yield by AD50.

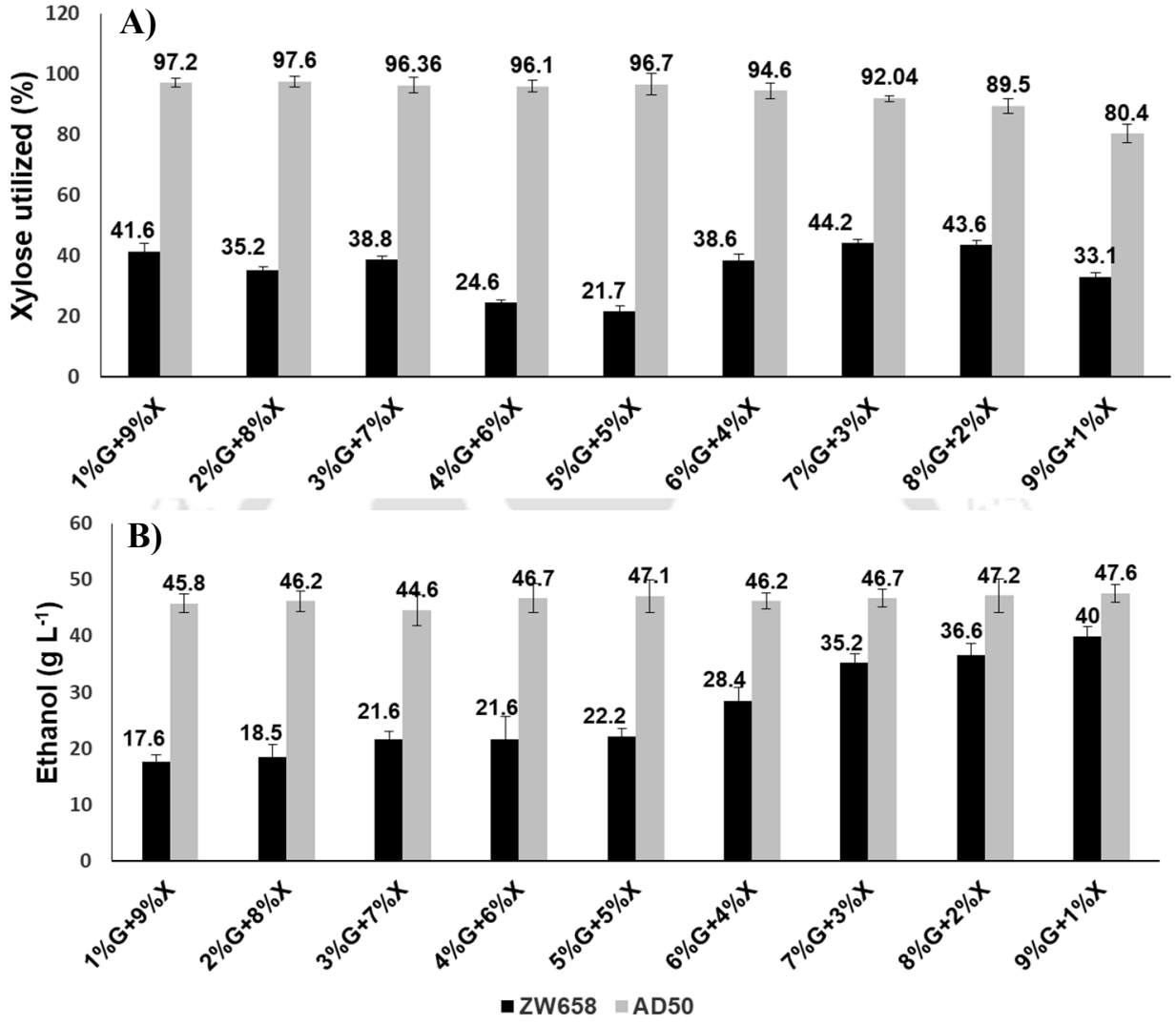


Fig. 3.19 Comparative performance analysis of **A)** xylose consumption, and **B)** ethanol titer by AD50 under various combinations of glucose and xylose concentrations

While, maximum specific xylose uptake rate of 0.8 g g^{-1} h^{-1} was obtained at highest xylose concentration supplemented condition of 9 % (w/v) of xylose and 1 % (w/v) of glucose (3.20A), maximum specific glucose uptake rate of 1.69 g g^{-1} h^{-1} was obtained in medium containing 2 % (w/v) of xylose and 8% (w/v) of glucose (3.20B). It was evident from the dynamic substrate uptake

rate of AD50 that the uptake rate for both the sugars attained its peak at their respective highest concentrations. The comparable and optimal glucose and xylose utilization rate was depicted by AD50 in medium supplemented with 5 % (w/v) of both glucose and xylose. In lieu of selecting an optimum substrate concentration in terms of binary sugar mixture towards enhanced performance of AD50, 5 % (w/v) of both glucose and xylose supplemented medium converged into an ideal condition for further experiments using AD50.

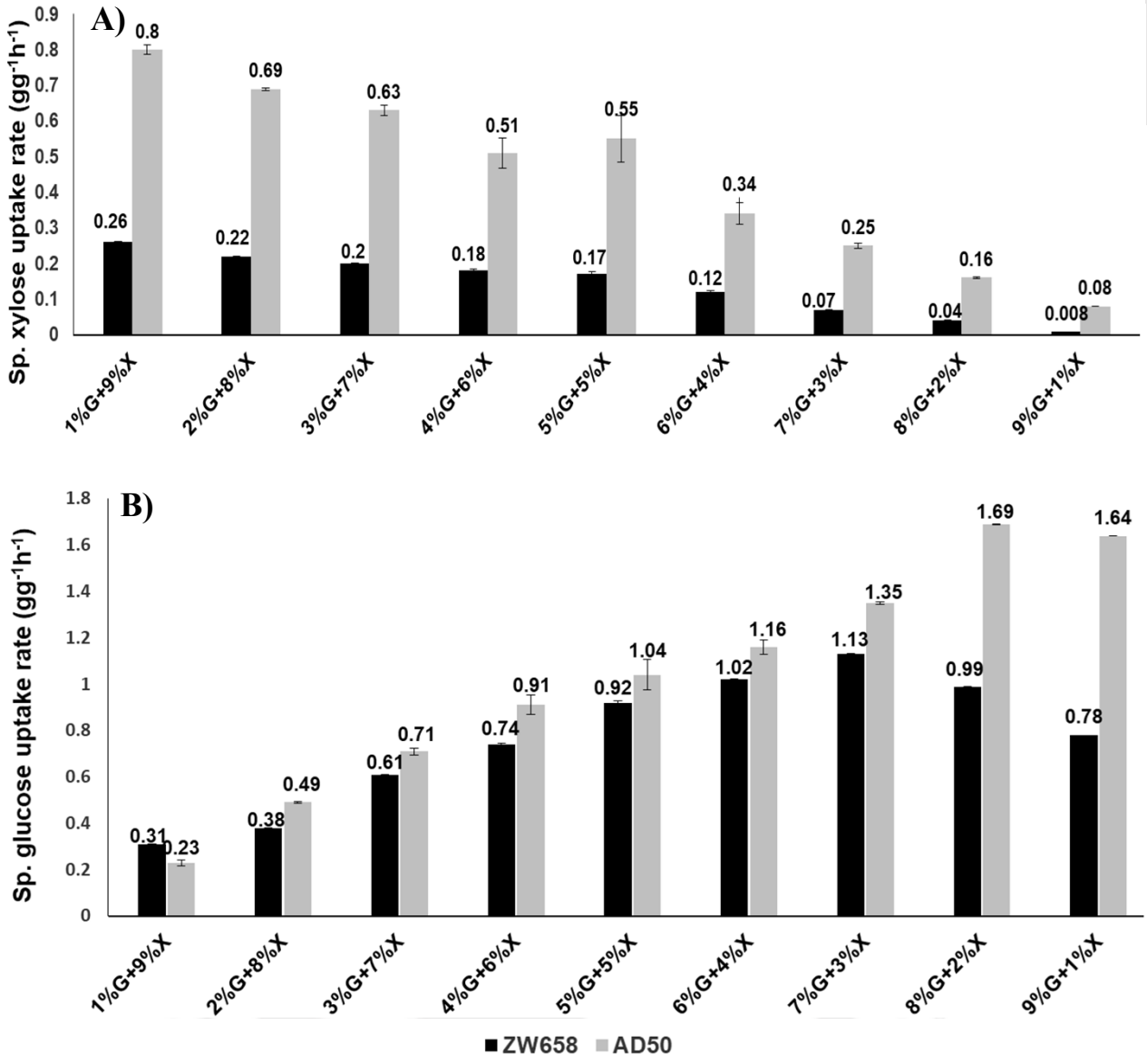


Fig. 3.20 Comparative performance analysis in terms of **A)** Specific xylose uptake rate, and **B)** Specific glucose uptake rate by AD50 under various combinations of glucose and xylose concentrations

3.3.7 Adaptation of AD50 in presence of glucose and xylose directed towards efficient co-utilization of both the sugars by the evolved strains

With the prime objective to develop an engineered *Z. mobilis* strain with exceptional glucose and xylose co-utilizing capability, AD50 was further subjected to two rationally designed

dual substrate based ALE strategies. The adapted strains obtained from the two strategies were designated as AS1-6 and AS2-6. The phenotypic response of the two evolved strains was characterized in terms of their ability to co-utilize glucose & xylose, and compared to that of AD50, in media containing 5% (w/v) glucose and 5% (w/v) xylose. The dynamic profile of AS1-6 and AS2-6 unveiled interesting behavioural pattern of the strains (Fig. 3.21). While both AS1-6 and AS2-6 exhibited complete consumption of glucose by 12h of fermentation (3.21A), however, AS1-6 showed a lag in xylose utilization compared to glucose at the beginning of the fermentation *i.e.* till 6th hour, a lag in xylose utilization was seen from 8 h to 12 h in case of AS2-6 (Fig. 3.21B). The maximum ethanol titer of 48.2 g L^{-1} was obtained in case of AD50 (Fig. 3.21C).

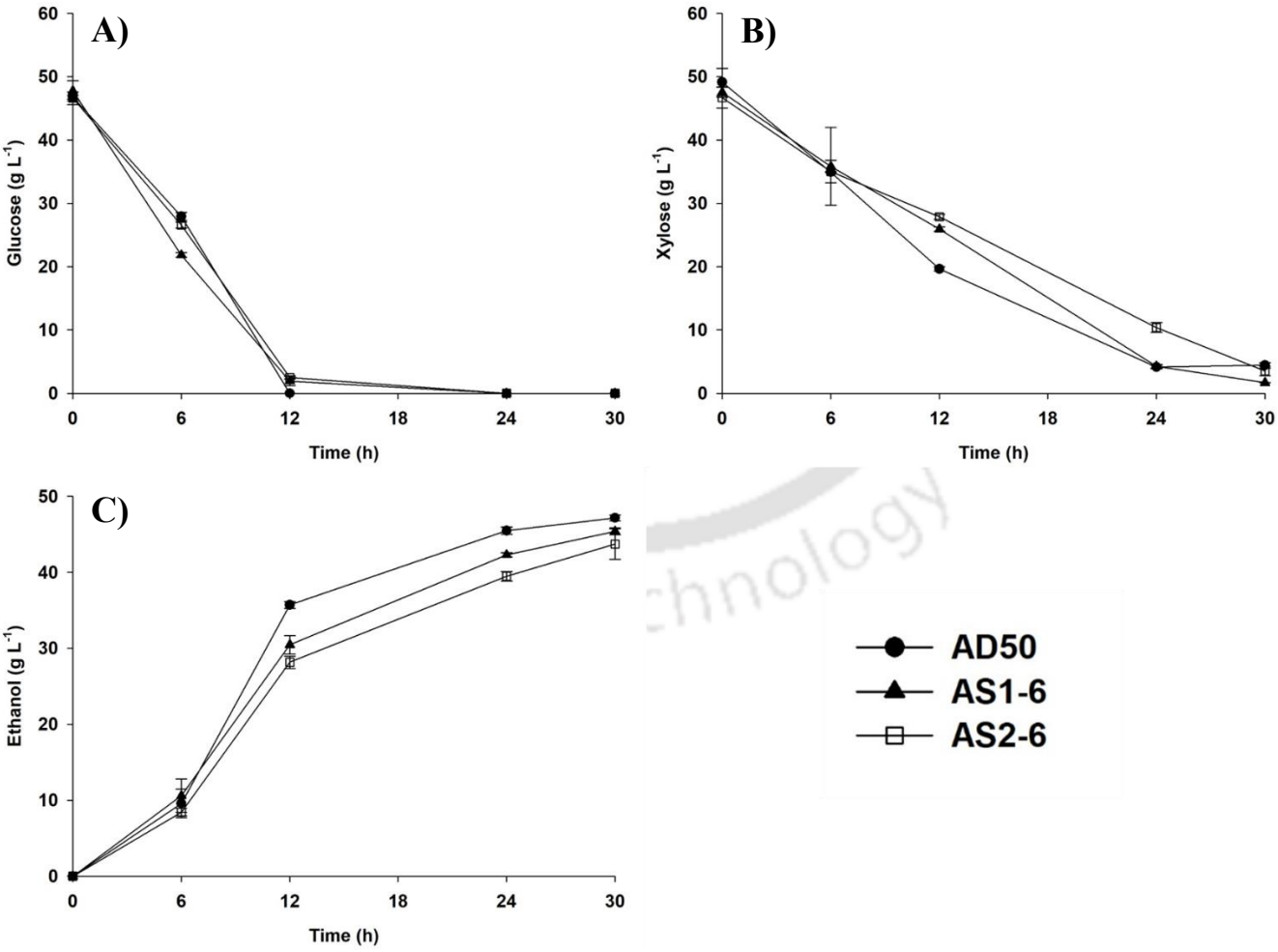


Fig. 3.21 Comparative dynamic profiles of **A)** glucose consumption, **B)** xylose consumption, and **C)** ethanol titer by AD50, AS1-6 and AS2-6 under 5 %(w/v) each of glucose and xylose

A comparative performance analysis showed the percentage of xylose utilized by AS1-6 was 96.7%, which is comparable to AS2-6 *i.e.* 96.2% (Fig. 3.22). The ethanol yield obtained in both cases were found to be same *i.e.* 94.5% of the maximum theoretical yield (Fig. 3.22).

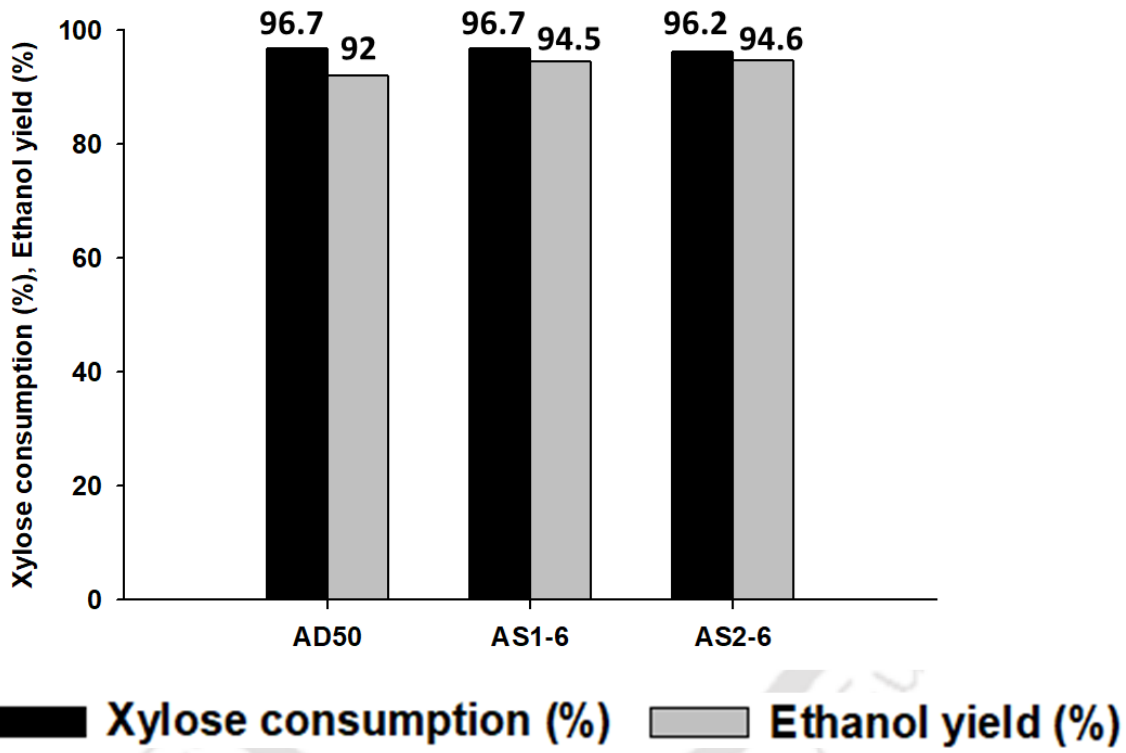


Fig. 3.22 Comparative performance analysis of xylose consumption (%), and ethanol yield (%) by AD50, AS1-6 and AS2-6 under similar cultivation conditions

Amongst the adapted strains, AD50, AS1-6 and AS2-6 the overall specific xylose uptake rate was observed to be marginally higher for AS1-6 (0.64 g g^{-1} h^{-1}) when grown in an equimolar glucose-xylose mixture. However, the aim of this study is to develop an efficient *Z. mobilis* strain that could simultaneously utilize glucose and xylose. To that end, it was necessary to evaluate the

specific xylose uptake rate of the adapted strains for the duration of fermentation during which glucose was still present in the medium. AD50 was quantified to exhibit higher specific xylose uptake rate in presence of glucose i.e. when glucose was present in the media AD50 could utilize xylose faster as compared to AS1-6 and AS2-6 (Table 3.5). This led us to select AD50 for further analyses to achieve our aim of selecting most efficient glucose-xylose co-utilizing *Z. mobilis* strain. A detailed quantitative analysis of the performance of evolved strain AD50 was undertaken.

Table 3.5 Specific xylose uptake rate of adapted *Z. mobilis* strains

strains	**specific xylose uptake rate (g g^{-1} h^{-1})***
AD50	**1.3**± 0.04
AS1-6	0.93± 0.02
AS2-6	0.66± 0.05

***** *Until complete consumption of glucose*

3.3.8 Characterization and comparative performance evaluation of ZW658 and AD50 when grown on glucose or xylose as sole carbon source under optimized cultivation condition

Batch fermentations were carried out to characterize and compare the performance of ZW658 and AD50 in terms of growth, xylose utilization, and ethanol production. An improved growth performance of AD50 was evident from a 2-fold higher biomass titer (OD_{600} of 8) as compared to ZW658 (Fig. 3.23A). This improved growth performance of AD50 may be attributed to the increase in both, extent and rate of xylose utilization in comparison to ZW658. While, AD50 utilized 98% of total xylose present in the fermentation medium, unadapted ZW658 consumed only 32% of the available xylose (Fig. 3.23B). In contrary to the present study, ZW658 developed by Viitanen et. al., (2008) showed complete utilization of xylose within 24 h of cultivation, when

grown in RM medium containing 8% (w/v) xylose (Viitanen et al., 2008). However, in the present study, despite of several trials, the performance of ZW658 as observed by Viitanen et. al., (2008) could not be reproduced. This could be attributed to the stability and viability of the recombinant strain. Further, an enhanced specific xylose uptake rate of 0.53 g g^{-1} h^{-1} was recorded for AD50 as compared to ZW658, which utilized xylose at a rate of 0.32 g g^{-1} h^{-1} (Table 3.6). These parameters point towards a successful outcome of the ALE strategy, where one of the objectives was to develop a *Z. mobilis* strain with key phenotypic trait of improved xylose utilization. Ethanol production ability of AD50 (49 g L^{-1}) remarkably surpassed the performance of ZW658 (12.65 g L^{-1}) when grown on xylose as sole carbon source (Fig. 3.23C). It is important to note that, AD50 exhibited an ethanol yield ($Y_{P/S}$) of 0.5 g of ethanol. g of xylose^{-1} with an overall productivity of 1.02 g L^{-1} h^{-1} (Table 3.6). The ethanol yield of AD50 is comparable to the theoretical yield of ethanol from xylose (0.51 g of ethanol. g of xylose^{-1}). Similar to growth, xylose utilization, and ethanol production, ZW658 exhibited a lower ethanol yield and productivity in comparison to AD50.

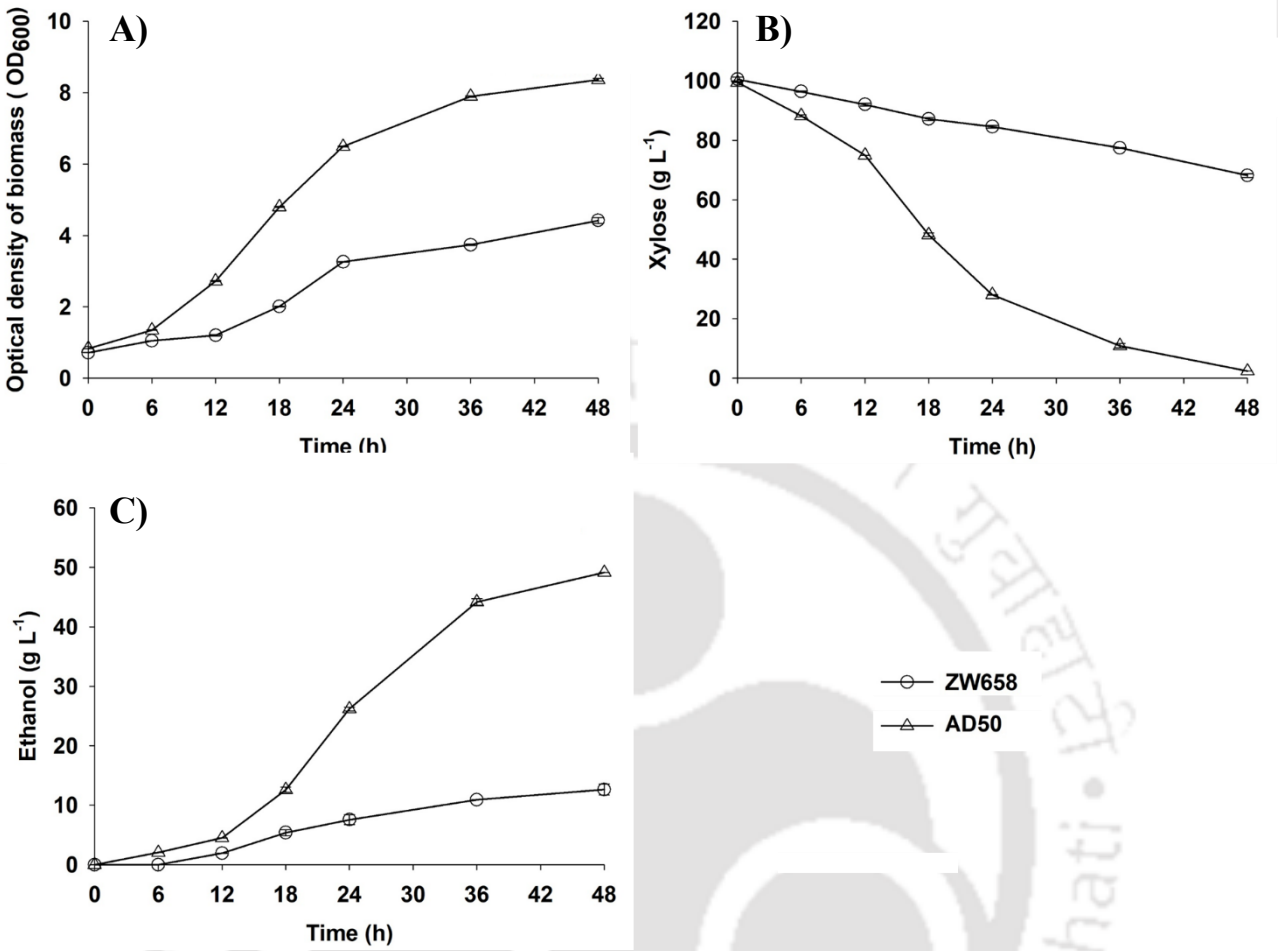


Fig. 3.23 Dynamic profile of **A)** growth**, B)** xylose utilization, and **C)** ethanol production by AD50, when cultivated in medium supplanted with 10 %(w/v) of xylose

Similarly, AD50 exhibited superior performance, in terms of glucose utilization, as compared to ZW658 (Fig. 3.24). Although the biomass titer was similar and both the strains utilized glucose completely, the specific glucose uptake rate of AD50 was 2.57 g g^{-1} h^{-1} as compared to 1.1 g g^{-1} h^{-1} for ZW658. This enhancement in glucose utilization by the adapted stain could be rationalized as a positive impact of the ALE strategy.

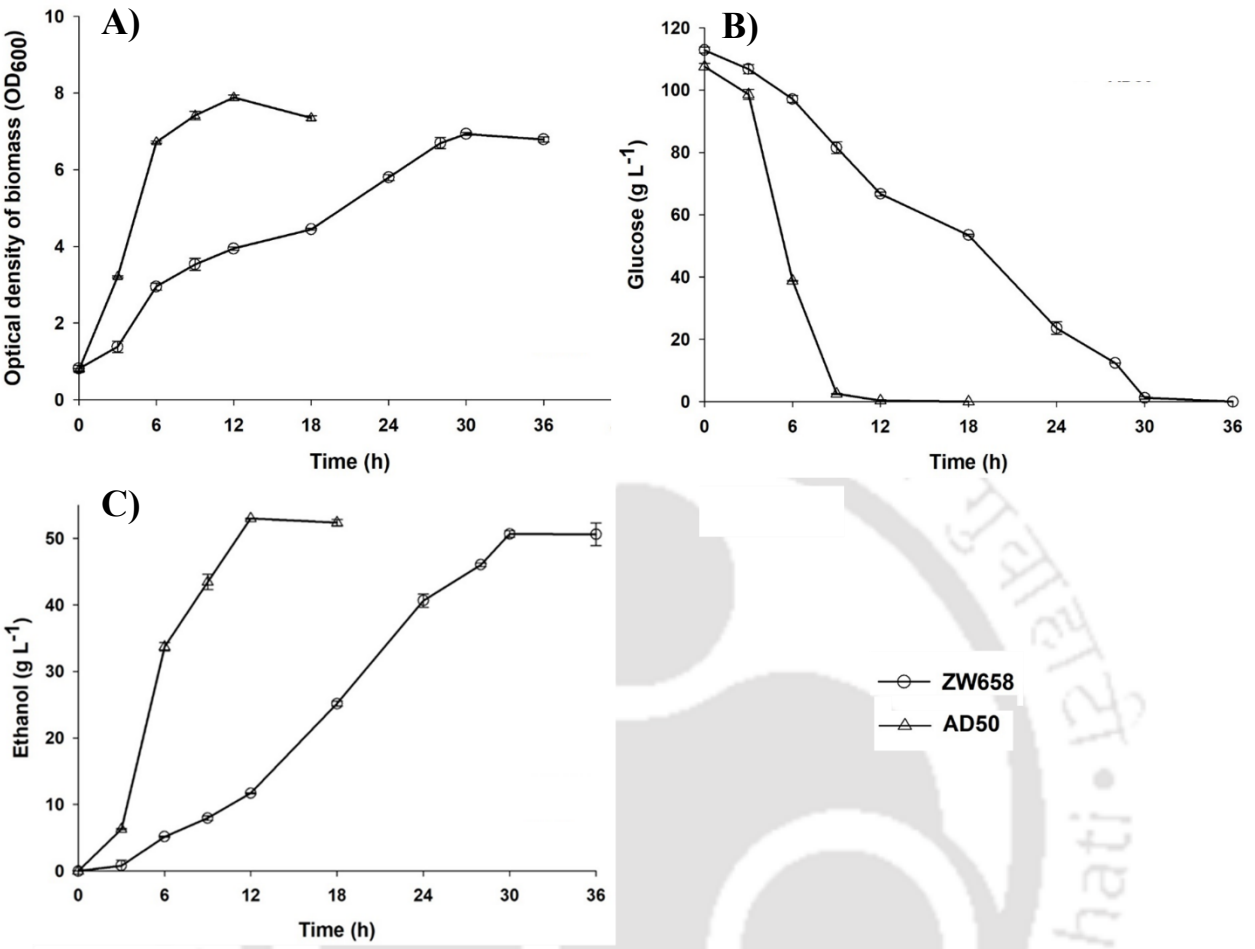


Fig. 3.24 Dynamic profile of **A)** growth, **B)** glucose utilization, and **C)** ethanol production by AD50, when cultivated in medium supplanted with 10 %(w/v) of glucose

In the present study, AD50 developed through a rational ALE strategy, has proved to be an exceptionally efficient biocatalyst with unprecedented performance in terms of xylose utilization and subsequent ethanol production. Previous metabolic engineering attempts have faced challenges to develop efficient xylose fermenting *Z. mobilis* strains that would help in designing a cost effective technology to produce lignocellulosic ethanol, due to several unknown metabolic regulations and bottlenecks in terms of xylose utilization (Dunn & Rao, 2014; Gao et al., 2002; Mohagheghi et al., 2002; Zhang et al., 1995). The restrained performance of the strains were even

more prominent at higher concentrations of xylose where sluggish utilization or incomplete consumption of sugar was marked (Dunn & Rao, 2014, 2015). AD50 not only outperformed its parental strain ZW658, under similar cultivation conditions, but also other metabolically engineered and/or adapted *Z. mobilis* strains reported till date (Table). AD50 has been the best so far in terms of extent and rate of xylose utilization when compared with previously developed notable *Z. mobilis* strains such as CP4(pZB5), AX101, C25, 39676/pZB4L, A3, ATCC 31821 (pKLD3), and ATCC 31821 (pKLD3, pKLD4) (Agrawal et al., 2011; Dunn & Rao, 2014, 2015; Gao et al., 2002; Mohagheghi et al., 2002; Zhang et al., 1995). Unlike directed metabolic engineering, *Z. mobilis* strains developed using ALE have proved to be efficacious in terms of broadening their substrate utilizing capabilities (Agrawal et al., 2011; Dunn & Rao, 2015; Gao et al., 2002; Supple, Joachimsthal, Dunn, & Rogers, 2000). A3, a notable *Z. mobilis* strain developed using ALE has demonstrated pronounced xylose utilizing efficiency over other xylose fermenting *Z. mobilis* strains (Agrawal et al., 2011). Intrigued by this study, Dunn and Rao implemented ALE for isolating pentose (xylose and arabinose) fermenting strains with rapid growth rate, pentose consumption and ethanol production rates (Dunn & Rao, 2015). However, the overall performance of the resultant adapted *Z. mobilis* strain (KLD1) was unsatisfactory as it metabolized 10% (w/v) of xylose over a duration of 125 h, which expectedly lowered its maximum xylose uptake rate with a lower rate of ethanol formation (Dunn & Rao, 2015). In this study, AD50 showed a striking maximum specific xylose uptake rate of 6.9 g g^{-1} h^{-1} as compared to A3 (3.44 g g^{-1} h^{-1}) which has been reported to exhibit one of the highest xylose utilization rate till date (Table 3.6). Besides superior xylose fermenting ability, the strain also registered an improvement in the ethanol titer and ethanol yield as compared to the previously demonstrated highest ethanol titer of 43.1 g L^{-1} and yield of 0.44 g g^{-1} (Agrawal et al., 2011). Improved xylose utilization efficiency and higher

ethanol production capability of AD50, as compared to other distinct xylose utilizing strains, highlights an altered xylose metabolism and product formation potential of the strain because of the extended and coherent ALE strategy.

Table. 3.6 Kinetic parameters for utilization of sugars and ethanol production

		Specific sugar uptake rate (g g^{-1} h^{-1})		
Strain	**Initial sugar concentration (%, w/v)**	**Glucose**	**Xylose**	
			Overall	**In presence of glucose***
ZW658	5% Glucose + 5% Xylose	0.57± 0.01	0.17± 0.04	0.13± 0.02
ZW658	10% Xylose	NA	0.32± 0.05	NA
AD50	5% Glucose + 5% Xylose	1.24± 0.03	0.59± 0.02	1.34± 0.05
AD50	10% Xylose	NA	0.53± 0.2	NA
		Ethanol		
		Yield (g g^{-1})	**Titer (g L^{-1})**	**Overall Productivity (g L^{-1} h^{-1})**
ZW658	5% Glucose + 5% Xylose	0.42± 0.002	29.7± 0.09	0.62± 0.002
ZW658	10% Xylose	0.39± 0.03	12.65± 0.91	0.26± 0.019
AD50	5% Glucose + 5% Xylose	0.49± 0.002	47± 0.12	1.96± 0.005
AD50	10% Xylose	0.5± 0.005	49± 0.08	1.02± 0.002

** Until complete consumption of glucose*

3.3.9 Evaluating the performance of AD50 in a dual substrate mixture of glucose and xylose

Based on the promising phenotypic response of AD50 in presence of xylose as the sole carbon source, its performance was examined in dual substrate mixture containing 5% (w/v) of glucose and 5% (w/v) xylose. AD50 and ZW658 were compared in terms of growth, glucose utilization, xylose utilization, ethanol production, and xylitol production (Fig. 3.25). Improved

growth performance of AD50 (Fig. 3.25A) was marked by higher optical density (OD_{600} of 7.2) and higher maximum specific growth rate (μ^{max}= 0.155 h^{-1}) in comparison to ZW658 (OD_{600} of 5.4 and μ^{max} = 0.123 h^{-1}). This noticeable improvement in the growth of AD50 may be attributed to greater extent and higher rate of xylose utilization in presence of glucose (Fig. 3.25B). While AD50 consumed 90.3% of the total xylose present in the sugar mixture within 24 h, ZW658 utilized only 36% in 48 h (Fig. 3.25B). Further, the overall specific xylose uptake rate for AD50 was 0.59 g g^{-1} h^{-1}, substantially higher as compared to 0.17 g g^{-1} h^{-1} in case of ZW658 (Table 3.6). Interestingly, even though both AD50 and ZW658 completely utilized glucose present in the fermentation medium, AD50 registered a higher specific glucose uptake rate (Fig. 3.25C) of 1.24 g g^{-1} h^{-1} in comparison to ZW658 (0.57 g g^{-1} h^{-1}). It is important to note that, when rate of xylose utilization was analysed for the duration of fermentation in which glucose was present in the culture medium, AD50 showed a specific rate of 1.34 g g^{-1} h^{-1}, which was 9.4 folds higher than the rate exhibited by ZW658 (0.13 g g^{-1} h^{-1}) (Table 3.6). Unlike ZW658, ability of AD50 to co-utilize both the sugars was evident from comparable utilization rate of 1.34 g g^{-1} h^{-1} and 1.24 g g^{-1} h^{-1} for xylose (until glucose was present in the fermentation medium) and glucose (overall), respectively (Table 3.6). In case of ZW658, it was observed that the xylose utilization started once 50% of the glucose, present in the fermentation medium, was exhausted. In media containing both glucose and xylose, AD50 produced an ethanol titer of 47 g L^{-1} with an overall productivity of 1.96 g L^{-1} h^{-1} while, ZW658 produced only 29.7 g L^{-1} of ethanol with a productivity of merely 0.62 g L^{-1} h^{-1} (Fig. 3.25D). However, ethanol yield of both AD50 and ZW658 was estimated to be comparable (Table 3.6). The comparative dynamic profile of xylitol production revealed significantly different rate and amount of xylitol produced by AD50 and ZW658 under mixed sugar fermentation. ZW658 produced 3 times higher xylitol as compared to AD50 with final xylitol

titer of 0.7 g L^{-1} for AD50 as compared to 2.1 g L^{-1} for ZW658 (Fig 3.25E). This behavioural pattern of AD50 confirms a shift in the xylose flux towards ethanol over xylitol.

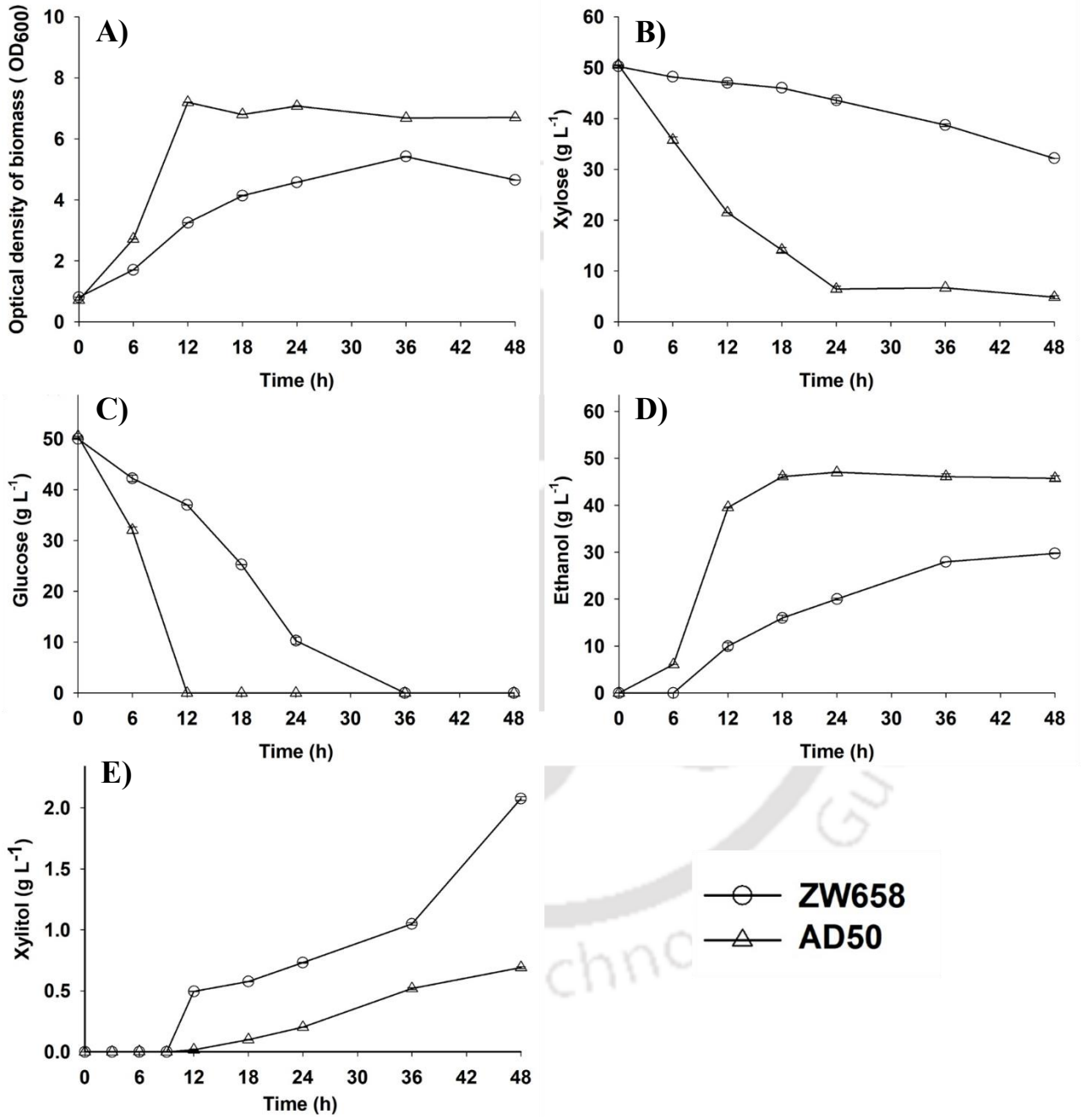


Fig 3.25 Dynamic profiles of **A)** growth, **B)** xylose utilization, **C)** glucose utilization, **D)** ethanol production, and **E)** xylitol production by ZW658 and AD50 when grown on dual carbon source of 5% (w/v) glucose and 5% (w/v) xylose

Although synergistic use of targeted metabolic engineering and ALE has led to the construction of *Z. mobilis* strains, which could utilize glucose and xylose in sequential manner, co-utilization of these sugars remains a challenge (Agrawal et al., 2011; Dunn & Rao, 2015; Gao et al., 2002; Supple et al., 2000). Such diauxic behaviour in mixed sugar fermentation often results in prolonged fermentation time. In a previous study, when ZW658 was cultivated in mixed sugar (10%, w/v glucose and 8%, w/v xylose) as opposed to only glucose, xylose utilization was found to be initiated only after 10 h of cultivation followed by an incomplete utilization (only 57% of its initial concentration was used) (Viitanen et al., 2008). Although xylose was utilized in presence of glucose, the rate of xylose uptake appeared to be significantly lower as compared to that of glucose. These results point towards sequential utilization of glucose and xylose by ZW658 instead of simultaneous utilization. Directed metabolic engineering strategy was employed to construct *Z. mobilis* strain CP4 (pZB5) which could utilize glucose and xylose within 30 h of cultivation with an ethanol yield of 95% of the maximum theoretical yield (Zhang et al., 1995). However, dynamic profile of sugar utilization by this revealed that xylose utilization was initiated only after complete utilization of glucose, depicting a sequential utilization of the sugars. Although *Z. mobilis* strain A3 developed by Agrawal et al. (Agrawal et al., 2011) has been highlighted as one of the most promising *Z. mobilis* strain for mixed sugar fermentation, yet it failed to utilize xylose in presence of glucose in the fermentation media. AD50 exhibited the higher specific xylose uptake rate of 1.34 g g^{-1} h^{-1} in presence of glucose in the dual substrate fermentation medium as compared to the adapted 39676/pZB4L with a specific rate of xylose uptake rate of 1.2 g g^{-1} h^{-1}, which is amongst

the fastest observed till date (Gao et al., 2002). The ALE strategy employed in this study proved to be distinctly beneficial to overcome the previously reported challenges such as sequential sugar utilization, incomplete xylose utilization, and prolonged fermentation time. The potential of AD50 towards co-utilization of glucose and xylose is found to be the best reported till date (Table 3.7). Hence, in the present study, we have successfully demonstrated the development of an improved biocatalyst with the potential to efficiently co-utilize glucose and xylose.

Table. 3.7 Comparison of maximum specific xylose uptake rate, ethanol titer, ethanol yield and maximum ethanol productivity of different mutants of *Z. mobilis* when grown on xylose only or dual substrate mixture of glucose and xylose

***Z. mobilis* strains**	**Initial sugar concentration (%, w/v)**	**Maximum specific xylose uptake rate ($g\ g^{-1}\ h^{-1}$)**	**Ethanol titer ($g\ L^{-1}$)**	**Ethanol yield (%)**	**Maximum ethanol productivity ($g\ L^{-1}\ h^{-1}$)**	**References**
AD50	**10% Xylose**	**6.9**	**49**	**98**	**1.23**	**This study**
ZW658*	8% Xylose	-	40	98	-	(Viitanen et al., 2008)
A3	10% Xylose	3.44	43.1	88.2	-	(Agrawal et al., 2011)
31821 (pKLD4)	10% Xylose	0.7	21.8	78.4	0.18	(Dunn & Rao, 2014)
KLD1	10% Xylose	-	30	58.8	-	(Dunn & Rao, 2015)
AD50	**5 % Glucose - 5% Xylose**	**3.94**	**47**	**96**	**3.3**	**This study**
CP4 (pZB5)	2.5 % Glucose- 2.5 % Xylose	-	24.22	95	1.21	(Zhang et al., 1995)
AX101	4 % Glucose - 4 % Xylose - 2 % Arabinose	-	42.8	84	1.8	(Mohagheghi et al., 2002)
39676/pZB4L	4% Glucose – 4% Xylose	2.9	37.09	85	3.9	(Gao et al., 2002)
C25	4% Glucose – 4% Xylose	1.6	38.9	96.5	4.03	(Gao et al., 2002)
ZW658*	10% Glucose – 8% Xylose	-	66	71.9	-	(Viitanen et al., 2008)
A3	5 % Glucose - 5% Xylose	1.8	49.9	96.6	-	(Agrawal et al., 2011)
31821 (pKLD4)	5 % Glucose - 5% Xylose	0.82	39.6	90.1	1.06	(Dunn & Rao, 2014)

** Ethanol titer and yield was calculated based on the graphs shown in (Viitanen et al., 2008)*

3.3.10 Adaptive Laboratory Evolution induced genetic variations in AD50

High throughput sequencing was performed to unravel possible genetic mutations that might have occurred in the genome of AD50 during the course of ALE. These mutations may explain the altered phenotypic response of AD50 with respect to ZW658 in terms of improved xylose utilization, efficient co-utilization of mixed sugars, enhanced growth, and ethanol production. In view of the unavailability of the whole genome sequence for ZW658, the genome sequence of AD50 was compared against the updated genome sequence of wild type *Z. mobilis* ATCC 31821 (GenBank: CP023715.1) and the gene sequences of *xylA*, *xylB*, *talB* and *tktA* from *E. coli* str. K-12 substr. MG1655 (GenBank: CP032667.1). The genetic variations observed after analysing and comparing the whole genome sequence of AD50 against the wild type *Z. mobilis* ATCC 31821 genome sequence and the *E. coli* str. K-12 substr. MG1655 genes have been tabulated in Table 3.8. The mutations were distributed among various metabolic pathways, transporters, transposases, and intergenic regions of the adapted strains (Fig. 3.26A).

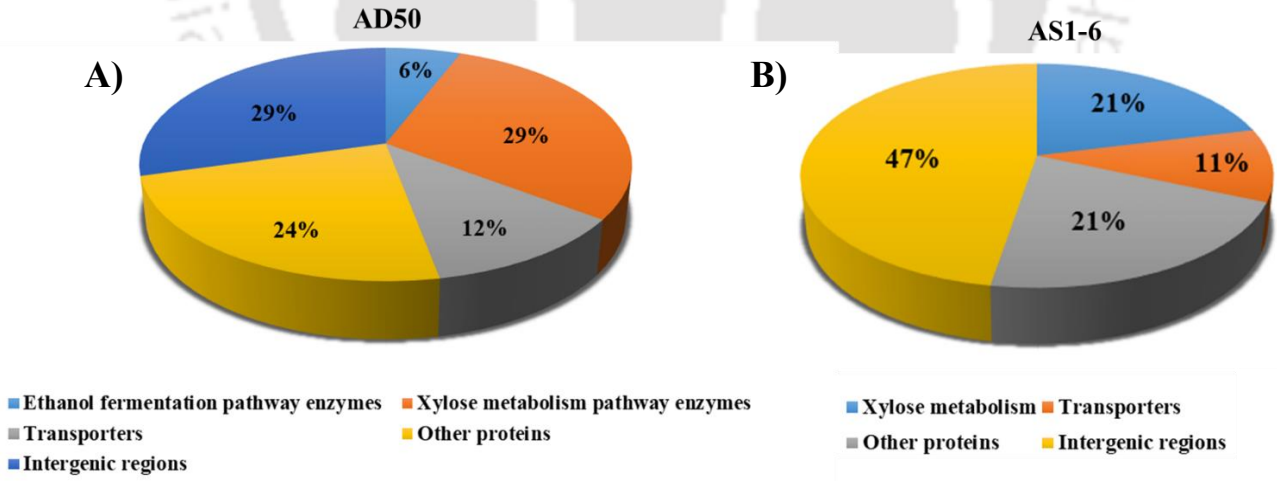


Fig. 3.26 Statistical representation of the ALE induced mutations distributed among various metabolic pathways, transporters, transposases, and intergenic regions of the adapted strain **A)** AD50, and **B)** AS1-6

The most intriguing mutations were observed in heterologous genes integrated in the genome of ZW658 for xylose metabolism and a gene involved in ethanol fermentative pathway. When compared with the native *xylA* gene sequence from *E. coli*, heterologous *xylA* gene in AD50 was observed to carry mutations in its coding region. Interestingly, *xylA* was also found to carry a novel mutation in the promoter region (promoter of glyceraldehyde-3-phosphate dehydrogenase gene from *Z. mobilis, Pgap*) at position -152 upstream of the start codon. Previously, improved xylose utilization by an engineered *Z. mobilis* strain A3 in presence of glucose was attributed to the mutation in the *Pgap* promoter region of *xylA* at position -23 upstream of the start codon, which, in turn resulted in higher *xylA* activity (Agrawal et al., 2011). The *Pgap* promoter has been reported to carry two mutations at position -89 and -190 upstream of the start codon of *xylA* gene in ZW658, which in turn, resulted in, increased expression of *xylA* (Viitanen et al., 2008). Both synonymous and non-synonymous mutations were identified in heterologously expressed *xylB* and *talB* in AD50 as compared to the corresponding native gene sequences from *E. coli*. The *tklB* gene, which encodes for the native transketolase in *Z. mobilis* also acquired an adaptation mediated novel mutation in its coding region (Table 3.8). Transketolase is an important enzyme for xylose assimilation that catalyzes the conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate (Feng, Liu, Weber, & Li, 2018). Therefore, the distinctly enhanced xylose fermentation and efficient co-utilization of glucose and xylose can be speculated to be an outcome of the observed mutations in the xylose assimilating and metabolizing genes. Xylose is converted to xylitol by xylose reductase or to xylulose by xylose isomerase (Agrawal et al., 2011). The xylose isomerase pathway is the preferred pathway for subsequent production of ethanol. A reduced xylitol production had been proved as one of the major reasons for enhanced ethanol production from xylose fermentation (Agrawal et al., 2011).

In this study, we identified novel mutations in the xylose reductase gene of the parent strain, ZW658, which hints towards a shift of the xylose flux towards enhanced ethanol production in AD50. The *pdc* gene encoding pyruvate decarboxylase enzyme, which is involved in the downstream pathway for ethanol production, also exhibited a synonymous nucleotide change (A to C) in the coding region of AD50 as compared to the wild type *Z. mobilis* ATCC 31821 (Table 3.8). Although the single nucleotide polymorphism (SNP) in the coding region of pdc in AD50 ultimately coded for the same amino acid (Tyrosine, Y) but these silent mutations have been shown to play significant functional role in mRNA folding and translational efficiency of the subsequent protein (Hunt, Simhadri, Iandoli, Sauna, & Kimchi-Sarfaty, 2014). It would be noteworthy to highlight the nucleotide (T) insertion in the +1028 position from the start codon of *glf* gene, which codes for glucose facilitated diffusion protein, which is native to *Z. mobilis* and is considered as the only low affinity xylose transporter present in the organism. Although the affinity of *glf* for glucose is significantly higher than xylose (Dunn & Rao, 2014), the enhanced rate of xylose utilization by AD50 might indicate a positive change in the protein in terms of xylose transport due to the genetic variation. Adaptation induced *glf* mutations (A18T) have also been identified in a recombinant *Z. mobilis* strain that exhibited enhanced xylose fermentation and ethanol production (Dunn & Rao, 2015). Other important SNPs observed in the genome of AD50 were present in genes *amtB*, *gcrA*, *gyrB2*, and *kdsB*, which code for an ammonium transporter, cell cycle regulator (a σ70 cofactor that regulates gene expression from a subset of methylated promoters), DNA gyrase subunit B, and 3-deoxy-manno-octulosonate cytidylyl-transferase, respectively. Apart from the coding region, mutations in intergenic regions also hold significance in terms of the expression of their downstream genes and activity of their encoded proteins (Hossein Khademi, Sazinas, & Jelsbak, 2019). We also observed a few interesting SNPs in the upstream region of

genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gapd*), ribose-5-phosphate isomerase B, and ubiquinol-cytochrome c reductase iron-sulfur subunit genes of AD50 (Table 3.8). The point mutation (G to A) observed at the genomic location, 163848, in the upstream region of *gapd*, might alter the expression level of gapd gene or activity of the encoded protein. A positive outcome of this mutation could be considered as a possible rationale behind the stimulated glucose consumption by AD50 as compared to ZW658 (Jojima, Fujii, Mori, Inui, & Yukawa, 2010). It seemed be interesting to probe the function of these mutant proteins to understand their effect on the phenotypic response of the evolved strain AD50.

Further, to identify and understand the crucial mutation that in effect induced the desirable phenotypic changes, the genome of AS1-6, an evolved *Z. mobilis* strain obtained through dual substrate ALE strategy of AD50 with enhanced glucose and xylose co-utilization capability as compared to that of ZW658, was sequenced. The observed mutations rationally hinted and explicated the superior performance of AD50 over AS1-6 (Fig. 3.26B) towards efficient dual substrate co-utilization. The identified mutations in AS1-6 genome is tabulated in table 3.9.

Table. 3.8 ALE induced mutations identified in AD50 as compared to wild type *Z. mobilis* ATCC 31821 (GenBank: CP023715.1) or *E. coli* str. K-12 substr. MG1655 genes (GenBank: CP032667.1)

Reference	Position	Reference nucleotide	Changed nucleotide	Mutation type	Gene/ Intergenic Region (IR)	Locus tag	Description
CP032667.1	651	T	C	SNP, Synonymous	*xylA*	-	Xylose isomerase
CP032667.1	1263	T	G	SNP, Non-synonymous	*xylB*	-	Xylulokinase
CP032667.1	300	T	C	SNP, Synonymous	*talB*	-	Transaldolase
	863	A	G	SNP, Non-synonymous			
CP023715.1	163688	G	T	SNP, Stop gained	*tklB*	ZMO1_ZMO0176	Transketolase
	163725	G	T	SNP, Non-synonymous			
CP023715.1	995008	A	C	SNP, Non-synonymous	*xyrA*	ZMO1_ZMO0976	Xylose reductase
	995013	T	A	SNP, Non-synonymous			
	995021	G	A	SNP, Non-synonymous			
	995084	G	A	SNP, Non-synonymous			
	995041	G	N**	SNP			
CP023715.1	342180	C	G	SNP, Non-synonymous	*amtB*	ZMO1_ZMO0346	Ammonium transporter
CP023715.1	372109	NA	+T	Insertion	*Glf*	ZMO1_ZMO0366	Glucose facilitated diffusion protein
CP023715.1	411206	T	A	SNP, Non-synonymous	*gcrA*	ZMO1_ZMO0407	Cell cycle regulator
CP023715.1	1623266	G	A	SNP, Non-synonymous	*gyrB2*	ZMO1_ZMO1583	DNA gyrase subunit B
CP023715.1	1516533	T	G	SNP, Synonymous	*kdsB*	ZMO1_ZMO1489	3-deoxy-manno-octulosonate cytidylyltransferase
CP023715.1	1377054	A	C	SNP, Non-synonymous	*Pdc*	ZMO1_ZMO1360	Pyruvate decarboxylase
CP023715.1	973383	T	C	SNP, Non-synonymous	Transposase	ZMO1_ZMO0955	Transposase
	973389	C	T	Synonymous	DDE domain containing Non-protein		
	973424	G	A	SNP, Synonymous			
	973460	A	T	Stop lost			
	973680	A	G	SNP, Synonymous			

	973709	A	G	Stop lost			
	973713	C	T	SNP, Synonymous			
	973722	C	A	SNP, Synonymous			
	973733	T	C	SNP, Non-synonymous			
	973845	C	T	SNP, Synonymous			
	973893	T	C	SNP, Synonymous			
CP023715.1	163848	G	A	SNP	promoter *gapd*	IR	Promoter region of Glyceraldehyde-3-phosphate dehydrogenase
CP023715.1	973927	G	A	SNP	Promoter ubiquinol-cytochrome c reductase iron-sulfur subunit	IR	Promoter of ubiquinol-cytochrome c reductase iron-sulfur subunit
CP023715.1	-189*	G	T	SNP	Promoter *xylA* (*Pgap*)	IR	Upstream region of Xylose isomerase
	-152*	G	T	SNP		IR	
	-29*	G	A	SNP		IR	
	-22*	T	-	Deletion		IR	
CP023715.1	1227078	G	A	SNP	Promoter *rpiB*	IR	Promoter region of Ribose-5-phosphate isomerase B
CP023715.1	1614974	A	G	SNP	ZMO1_ZMOt037	IR	tRNA-Met

* Positions are mentioned with respect to the start codon of integrated heterologous *xylA* gene

** "N" any nucleotide base except the reference nucleotide

Table. 3.9 ALE induced mutations identified in AS1-6 as compared to wild type *Z. mobilis* ATCC 31821 (GenBank: CP023715.1) or *E. coli* str. K-12 substr. MG1655 genes (GenBank: CP032667.1)

Reference	Position	Reference nucleotide	Changed nucleotide	Mutation type	Gene/ Intergenic Region (IR)	Locus tag	Description
CP032667.1	651	T	C	SNP, Synonymous	*xylA*	-	Xylose isomerase
CP032667.1	300	T	C	SNP, Synonymous	*talB*	-	Transaldolase
	863	A	G	SNP, Non-synonymous			
CP023715.1	163725	G	T	SNP, Non-synonymous	*tklB*	ZMO1_ZMO0176	Transketolase
CP023715.1	995008	A	C	SNP, Non-synonymous	*xyrA*	ZMO1_ZMO0976	Xylose reductase
	995013	T	A	SNP, Non-synonymous			
	995040	G	N**	SNP			
	995041	G	N**	SNP			
	995059	C	T	SNP, Synonymous			
	995084	G	A	SNP, Non-synonymous			
CP023715.1	342180	C	G	SNP, Non-synonymous	*amtB*	ZMO1_ZMO0346	Ammonium transporter
CP023715.1	372109	NA	+T	Insertion	*Glf*	ZMO1_ZMO0366	Glucose facilitated diffusion protein
CP023715.1	411206	T	A	SNP, Non-synonymous	*gcrA*	ZMO1_ZMO0407	Cell cycle regulator
CP023715.1	973680	A	G	SNP, Synonymous	Transposase DDE domain containing protein	ZMO1_ZMO0955	Transposase
	973709	A	G	Stop lost			
	973722	C	A	SNP, Synonymous			
	973733	T	C	SNP, Non-synonymous			
	973845	C	T	SNP, Synonymous			
	973893	T	C	SNP, Synonymous			
	973713	C	T	SNP, Synonymous			
CP023715.1	166614	G	C	SNP	Hypothetical protein	ZMO1_ZMO2045	Unknown
	166628	G	C	SNP			

	166629	C	G	SNP			
CP023715.1	163848	G	A	SNP	promoter *gapd*	IR	Promoter region of Glyceraldehyde-3-phosphate dehydrogenase
CP023715.1	-152*	G	T	SNP	Promoter *xylA* (*Pgap*)	IR	Upstream region of Xylose isomerase
	-222*	T	C	SNP		IR	
CP023715.1	1227078	G	A	SNP	Promoter *rpiB*	IR	Promoter region of Ribose-5-phosphate isomerase B
CP023715.1	1614974	A	G	SNP	ZMO1_ZMOt037	IR	tRNA-Met
	1615142	A	G	SNP			
	1615144	G	T	SNP			
CP023715.1	1913717	T	C	SNP	ZMO1_ZMOt046	IR	tRNA-Met

* Positions are mentioned with respect to the start codon of integrated heterologous *xylA* gene

** "N" any nucleotide base except the reference nucleotide

3.3.11 Adaptive Laboratory Evolution induced modulation in the activity of key metabolic enzymes

Genetic mutations resulting in an alteration of protein expression levels or post-transcriptional modification of proteins can cause resulting phenotypic changes (Agrawal et al., 2011; Bhattacharya, Rose, Burley, & Prlić, 2017). While, polymorphism in the promoter region can alter the rate of transcription, variations in the coding sequence can affect RNA stability, protein stability or translation rate (Foss et al., 2011). Mutations that lead to amino acid variations may influence the structure, function or binding properties of the corresponding protein (Bhattacharya et al., 2017; Yasukawa & Inouye, 2007). In order to decipher the rationale behind the altered phenotypic response of the adapted strain AD50 and corroborate it with the observed mutations, temporal activity profiles of the key metabolic enzymes, involved in xylose metabolism and ethanol fermentation were obtained for both AD50 and ZW658. As explained in previous section, several crucial genes of AD50 involved in xylose metabolism and ethanol fermentation had acquired genetic variations under the strong selection pressure of high xylose concentration. Co-utilization of xylose and glucose along with enhanced ethanol biosynthesis by AD50 prompted us to probe the activity of specific enzymes: (i) *xylA* and *xyrA*, enzymes involved in the initial steps of xylose metabolism; (ii) *tklB*, one of the enzymes that plays a major role in linking xylose metabolic pathway to glycolysis; and (iii) *pdc*, which associates glycolysis with ethanol biosynthesis (Fig. 3.27)

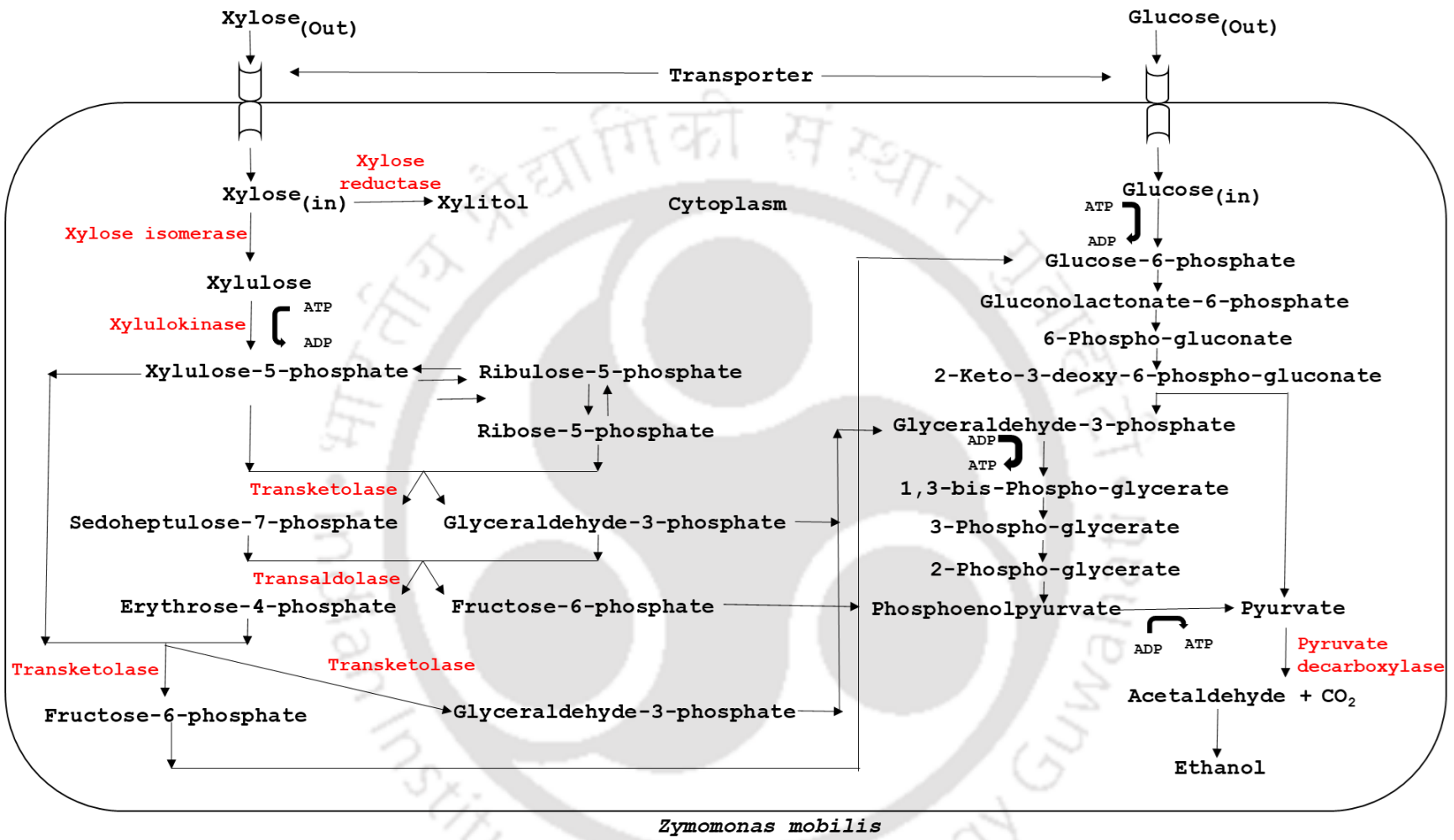


Fig. 3.27 Schematic representation of xylose metabolism and Entner–Doudoroff pathway in ZW658. Enzymes highlighted in red represent the heterologously expressed xylose metabolizing genes and one of the genes involved in ethanol biosynthesis pathway

In case of both AD50 and ZW658, activity of xylose isomerase was found to increase linearly with the progression of cultivation (Fig. 3.28A). However, throughout the entire course of fermentation, activity of XylA in case of ZW658 remained significantly lower as compared to AD50. The maximum specific activity of xylose isomerase from AD50 was 0.46 U mg^{-1} at 24 h of fermentation, which was 4.6 times higher as compared to the same enzyme from ZW658 (Fig. 3.28A). In earlier studies, activity of XylA was observed to be low as compared to the other heterologously expressed proteins (Agrawal et al., 2011; Gao et al., 2002). The low XylA activity was considered as one of the primary reasons for unsatisfactory xylose fermentation by engineered *Z. mobilis* (Agrawal et al., 2011; Gao et al., 2002). However, in the present study activity of XylA in AD50 was found to be comparable to the activity of transketolase enzyme. The enhanced activity of XylA can be attributed to the extended adaptation acquired mutation in the promoter and coding regions of *xylA* in AD50. A similar observation was also reported previously where a point mutation in the promoter region (*Pgap*) of *xylA* resulted in an enhanced activity of the enzyme (Agrawal et al., 2011). The activity of transketolase from AD50 was moderately upregulated (maximum of 0.61 U mg^{-1} at 12 h) throughout the course of fermentation when compared with the activity of transketolase from ZW658 (maximum of 0.28 U mg^{-1} at 18 h) (Fig. 3.28B). Incidentally, overexpression of transaldolase and transketolase was reported to be responsible for improved cell growth and xylose consumption in organisms like yeast (Feng et al., 2018). An elevated transketolase expression was observed to improve xylose fermentation in recombinant yeast (Salusjärvi et al., 2003). The accelerated xylose utilization and subsequent higher ethanol titer of AD50 was apparently prompted by a shift in xylose flux towards xylulose by xylose isomerase instead of xylitol by xylose reductase. Xylose reductase converts xylose to xylitol, which has been reported to be toxic and a primary bottleneck in xylose fermentation to

ethanol (Agrawal et al., 2011; Jeon et al., 2005; I. S. Kim, Barrow, & Rogers, 2000). The improvement in xylose metabolism of AD50 was validated by investigating the activity of xylose reductase. In the present study, negligible activity of the xylose reductase might be correlated to ALE induced SNPs in the gene encoding xylose reductase (Fig. 3.28C). The maximum specific activity of xylose reductase from AD50 was observed to be only 0.023 U mg^{-1}, which is 7.7 folds lower as compared to the activity of xylose reductase from ZW658 (0.201 U mg^{-1}). The xylose reductase activity of ZW658 and AD50 does endorse the dynamic profile obtained for xylitol production by the two strains (Fig. 3.28C). Similar results were outlined for previously reported A1 and A3 mutants where xylose reductase activity was barely detectable resulting in reduced xylitol synthesis. This downregulated xylose reductase was partially responsible for the improved xylose metabolism by these strains (Agrawal et al., 2011). The conversion of xylose to xylitol is also carried out by glucose fructose oxidoreductase (GFOR). A knock out of this gene has also been reported to trigger an accelerated xylose metabolism towards ethanol formation in recombinant *Z. mobilis* (Viitanen et al., 2008). Further, to understand the enhanced ethanol production by AD50, activity of pyruvate decarboxylase was assayed. Pyruvate decarboxylase encoded by *pdc* plays a key role in ethanol biosynthesis in *Z. mobilis* where it catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. The synonymous mutation in *pdc* of AD50 was expected to alter either its expression or activity of its encoded protein and in turn, extent of ethanol fermentation. The pyruvate decarboxylase activity for both AD50 and ZW658 was observed to increase steadily with the progression of fermentation (Fig. 3.28D). The maximum specific activity displayed by Pdc from AD50 was 7 U mg^{-1} at 18 h as compared to 3.8 U mg^{-1} at 24 h for ZW658. This elevated activity can be corroborated with an increased ethanol titer by AD50. Previously, a *Clostridium thermocellum* DSM1313 strain engineered with *Z.*

mobilis pdc gene was shown to have increased Pdc activity resulting in an enhanced ethanol production by the strain (Kannuchamy, Mukund, & Saleena, 2016). Taken together the results of the present study, point towards an outcome of collective and multilevel modifications in metabolic pathways that enabled rapid and enhanced xylose flux towards higher ethanol biosynthesis by the adapted *Z. mobilis* strain AD50.

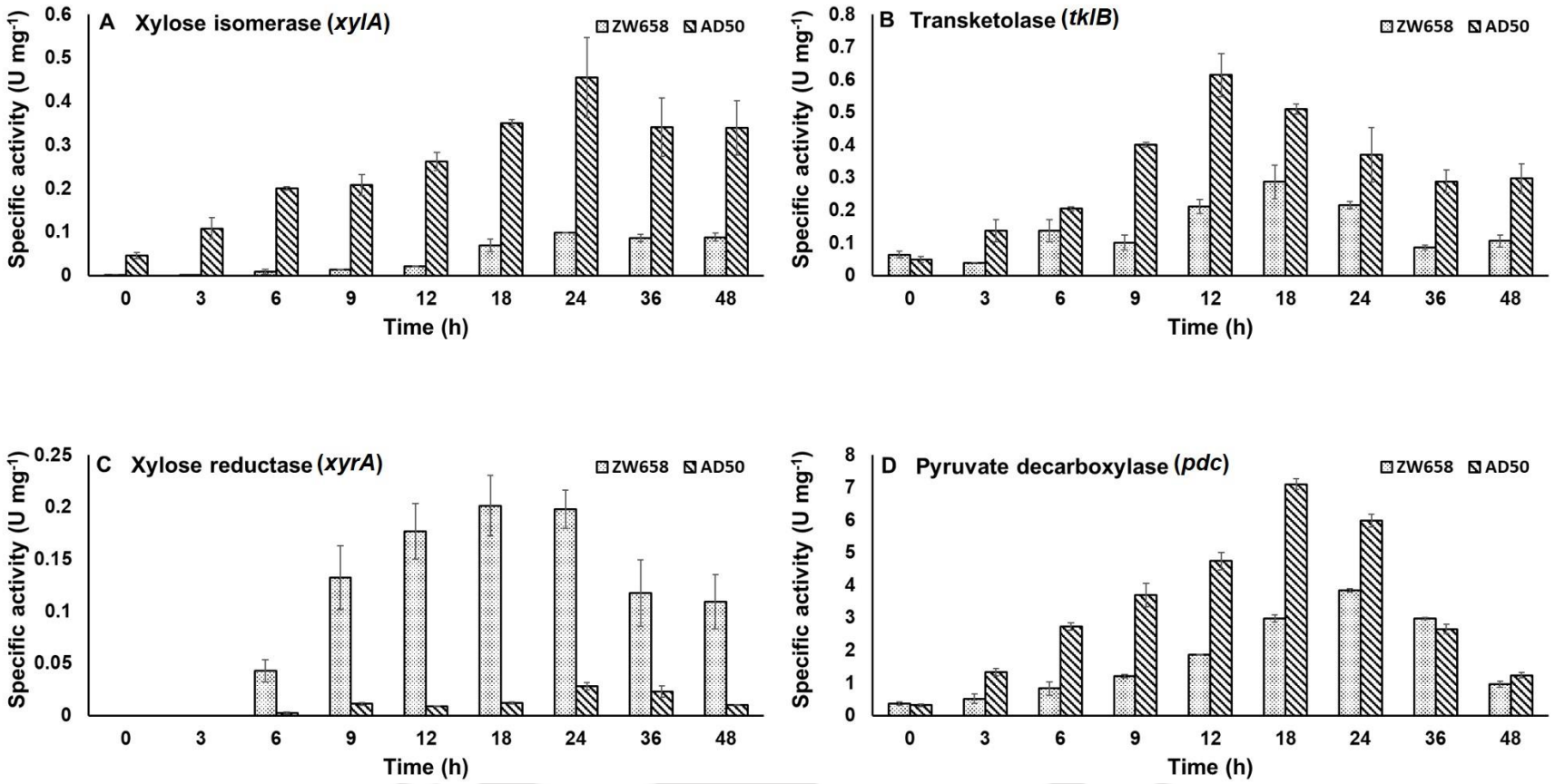


Fig. 3.28 Dynamic profiles of specific activities (U mg^{-1}) of **A)** Xylose isomerase, **B)** Transketolase, **C)** Xylose reductase, and **D)** Pyruvate decarboxylase. The data have been expressed as mean ± standard error

3.4 Conclusions

- ✓ *Z. mobilis* ATCC ZW658, which is a recombinant xylose fermenting strain was subjected to extended Adaptive Laboratory Evolution involving 50 transfers, carried out over a period of 200 days under increasing concentrations of xylose starting from 30 g L^{-1} to 100 g L^{-1}.
- ✓ The evolved strain (designated as AD50) showed 1.65 times increase in the overall specific xylose utilization rate when compared with the parent strain.
- ✓ AS1-6 and AS2-6 developed through extended ALE of AD50 in presence of glucose & xylose based under two distinct strategies also exhibited superior xylose utilization compared to that of ZW658.
- ✓ AD50 showed highest maximum specific xylose uptake rate of 6.9 g g^{-1} h^{-1}, when compared to other reported strains till date.
- ✓ AD50 displayed exceptional performance in terms of co-fermentation of xylose in presence of glucose with specific xylose and glucose utilization rate of 1.34 g g^{-1} h^{-1} and 1.24 g g^{-1} h^{-1}. AS1-6 and AS2-6 also exhibited higher specific xylose utilization rate of 0.93 g g^{-1} h^{-1} and 0.66 g g^{-1} h^{-1}, respectively, in presence of glucose, as compared to that of ZW658.
- ✓ High throughput (Next-gen) sequencing revealed novel mutations in xylose assimilating, metabolizing, and crucial regulatory pathway genes, which substantiate the exceptional phenotypic response of AD50 in terms of co-utilization of glucose and xylose.
- ✓ Comparative analysis of the distinct adaptation strategy induced genomic mutations of AD50 and AS1-6 highlighted the essential mutations for enhance co-utilization of glucose and xylose.
- ✓ Enzyme activity assays were carried out to validate the performance of the strain AD50 with high confidence.

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Chapter 4

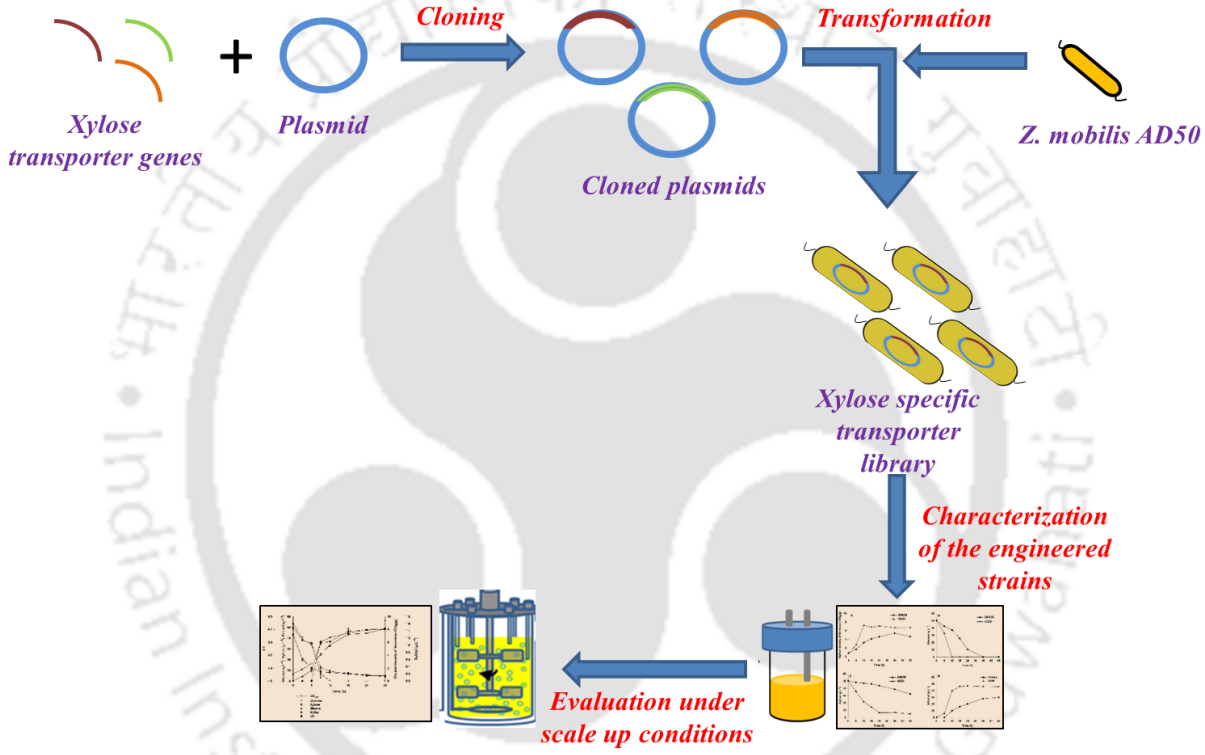
CONSTRUCTION AND PERFORMANCE EVALUATION OF *Z. MOBILIS* STRAINS ENGINEERED WITH VARIOUS XYLOSE SPECIFIC TRANSPORTER GENES TOWARDS EFFICIENT XYLOSE & GLUCOSE CO-UTILIZATION

"Perfection is achieved, not when there is nothing more to add, but when there is nothing left to take away."

Antoine de Saint-Exupery, *Writer*

CHAPTER 4

Construction and performance evaluation of *Z. mobilis* strains engineered with various xylose specific transporter genes towards efficient xylose & glucose co-utilization



Schematic representation of summary of the developmental process of an efficient *Z. mobilis* strain through directed metabolic engineering of *Z. mobilis* strain AD50 towards simultaneous utilization of glucose and xylose and performance evaluation under scale up conditions

4.1 Background and motivation

Bioethanol is widely recognized as a potential and competent alternative to fossil fuels to alleviate the current global concern about energy crisis (S. Yang et al., 2016). Lignocellulose has proven to be an eco-friendly and inexpensive alternative feedstock to conventionally exploited substrates i.e. food crops, for the production of bioethanol (Nieves et al., 2015). Lignocellulosic biomass, upon hydrolysis, produces a sugar mixture, majorly comprising of hexose sugar (majorly D-glucose) and pentose sugars (majorly D-xylose & L-arabinose) (Nieves et al., 2015). However, microbial conversion of both hexose (D-glucose) and pentose (D-xylose and D-arabinose) sugars of lignocellulosic hydrolysate into ethanol remains a challenge due to lack of ethanol producing organism with inherent pentose metabolizing capability. This chemical composition of lignocellulosic hydrolysate drives a prerequisite to develop an ethanologenic microbial platform with the ability to efficiently utilize both hexose as well as pentose sugars. This is even more relevant with respect to achieving improved ethanol yield and productivity in an industrially viable fermentation process. *Zymomonas mobilis* offers various advantages of higher ethanol yield, productivity, and ethanol-tolerance, when compared to commercially acknowledged ethanol producing *S. cerevisiae* (He et al., 2014). However, wild type *Z. mobilis* cannot metabolize xylose or arabinose owing to lack of innate pentose phosphate pathway enzymes (PPP) (M. Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995). Therefore, there is an avenue of research to be explored towards development of modified *Z. mobilis* strains proficient in consuming hexose and pentose sugars with comparable efficiencies, for commercially viable production of bioethanol.

Over the years, several rationally designed metabolic engineering approaches have been employed to enable and augment pentose utilization ability of *Z. mobilis* (Dunn & Rao, 2014, 2015; Jeon, Svenson, & Rogers, 2005; Mohagheghi, Evans, Chou, & Zhang, 2002;

Viitanen et al., 2008; M. Zhang et al., 1995). Xylose, being more abundant than other pentose sugars present in lignocellulosic feedstocks (Nieves et al., 2015), has attracted maximum scientific attention on its metabolism by *Z. mobilis*. The initial hurdle of introducing heterologous xylose metabolic pathway genes was successfully overcome, leading to development of a xylose fermenting *Z. mobilis* strain. However, the strain displayed typical diauxic behavior when grown in a dual sugar mix of glucose and xylose (M. Zhang et al., 1995). This phenotypic response of diauxic growth by the engineered *Z. mobilis* strain hinted towards presence of a well-known global regulatory phenomenon, carbon catabolite repression (CCR) where sugar metabolism (except glucose) is repressed by glucose. However, such intrinsic mechanism has not been well elucidated in *Z. mobilis* (Kim, Block, & Mills, 2010; Ren, Chen, Zhang, Liang, & Lin, 2009). Ever since, several significant research endeavors have been made towards engineering *Z. mobilis* strains to aid improved dual substrate utilizing capabilities towards enhanced ethanol production, encompassing overexpression or knock-down of crucial xylose metabolizing genes (Dunn & Rao, 2014; Jeon et al., 2005; Viitanen et al., 2008). Directed metabolic engineering has also been coupled with adaptive laboratory evolution (ALE) for isolation of mutant strains that could co-utilize glucose and xylose with comparable efficiencies (Agrawal et al., 2011; Sarkar et al., 2020). Although, coherent use of ALE in combination with directed metabolic engineering manifested into development of efficient xylose utilizing *Z. mobilis* strain, a major challenge that has persisted, is the lower rate of xylose utilization as compared to glucose present in a dual sugar mixture, leading to extended fermentation time and in turn, lower productivity (Agrawal et al., 2011; Dunn & Rao, 2015; Sarkar et al., 2020).

Apart from metabolic aspect of xylose utilization, xylose transport has also been considered as a major bottleneck hindering efficient xylose utilization by the engineered strains

of *Z. mobilis* (Dunn and Rao, 2014). Wild type *Z. mobilis* has been reported to transport xylose through Glf, (Glucose facilitated diffusion protein) which is an innate glucose transporter (Dunn & Rao, 2014; Supple, Joachimsthal, Dunn, & Rogers, 2000). However, higher affinity of Glf towards glucose results in competitive inhibition of xylose uptake in dual sugar supplemented medium (Weisser, Krämer, & Sprenger, 1996). Introduction of a low affinity xylose transporter i.e. XylE in a *Z. mobilis* strain has been demonstrated to escalate its xylose uptake rate. However, the fermentation time for xylose was significantly higher as compared to that of glucose (Dunn & Rao, 2014). This hinted that uptake of xylose may also be a bottleneck towards efficacious co-consumption of xylose and glucose by *Z. mobilis*. Concurrently, inefficient xylose transport by *Z. mobilis* has not been explored and addressed much in relevant scientific literature. Therefore, we hypothesized that a prospective approach should be to introduce well-characterized xylose-specific transporters into an efficient xylose utilizing *Z. mobilis* strain, to further enhance xylose uptake rate and in turn, augment co-fermentation of xylose and glucose.

This chapter entails the development of an efficient *Z. mobilis* strain, which can metabolize glucose and xylose with equal efficacy towards production of ethanol with improved productivity. To that end, we constructed an array of xylose specific transporter genes from *E. coli*, under the influence of two different native *Z. mobilis* promoters. The xylose transporter genes included an H^+ symporter (XylE), a mutant form of the H+ symporter developed through site-directed mutagenesis (XylE*) and a xylose specific ATP Binding Cassette (ABC) type transporter system (XylFGH). Interestingly, the ensued study is the first to engineer and report the effect of a mutant form of XylE and ABC-type xylose transporter XylFGH in *Z. mobilis,* under the control of different native *Z. mobilis* promoters of variable strengths. Previously, we reported an efficient xylose metabolizing *Z. mobilis* strain using adaptive laboratory evolution strategy, designated as AD50 (Sarkar et al., 2020). In the ensued

study, AD50 was used as a host for introducing the xylose specific transporter genes to accomplish co-utilization of xylose and glucose as a desired phenotypic outcome. We screened the efficacy of the xylose specific transporters based on the rate of xylose uptake and extent of xylose consumption by the recombinant *Z. mobilis* strains, specifically in presence of glucose in dual sugar mixture.

4.2 Materials and methods

4.2.1 Bacterial strains, media and growth condition

Zymomonas mobilis ATCC ZW658 (ZW658), a recombinant strain engineered with xylose assimilating and metabolizing genes, incorporated in its genome, was purchased from American Type Culture Collection (ATCC). AD50, an evolved *Z. mobilis* strain with an efficient xylose metabolizing capability was developed using adaptive laboratory evolution of ZW658 (Sarkar et al., 2020). In the current study, AD50 was used as the host for developing mutant strains of *Z. mobilis* with xylose specific transporters. ZW658 and AD50 were initially recovered in Rich Medium containing xylose (RMX) composed of 1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, and 5% (w/v) D-xylose. Agar plates were prepared by using the same media composition supplemented with 1.5% (w/v) agar. Pre-seed cultures, for all experiments, were prepared by inoculating glycerol stock of ZW658, AD50 and single isolated colonies of all the xylose-specific transporter mutant *Z. mobilis* strains, in liquid RMX and grown till stationary phase. 20% (v/v) of the stationary phase pre-seed cultures were harvested and inoculated in fresh media to an optical density (OD_{600}) of ~ 0.7 to 0.8, to prepare seed cultures. The pre-seed and seed cultures were cultivated in 10% (w/v) xylose at 30 °C under shaking at 150 rpm. All *Escherichia coli* strains namely *E. coli* DH5α and *E. coli* JM110, were procured from Coli Genetics Stock Center and were cultivated in liquid Luria Bertani (LB) medium at

37 °C under shaking at 180 rpm. Tetracycline was supplemented at a working concentration of 10 µg mL^{-1}, when specified.

4.2.2 Construction of plasmids for expressing xylose specific transporters

Plasmids containing xylose specific transporters i.e., XylE (H^+ symporter) and XylFGH (ABC type transporter complex) were constructed by PCR amplification of the genes from *E. coli* K-12 genomic DNA using primers listed in table 4.1. In order to express *xylE* and the operon of *xylFGH* in AD50, the genes were fused to a constitutive promoter native to *Z. mobilis*. Two promoters, *Ppdc* and *Pglf* that control the genes encoding pyruvate decarboxylase and glucose facilitated diffusion transporter, respectively, were screened for optimal expression of the transporter genes. Nucleotides encoding *Ppdc* and *Pglf* promoters were PCR amplified from *Z. mobilis* genomic DNA using primers listed in table 4.1. The amplified transporter genes were fused to the promoters using overlap extension PCR. The overlap extension PCR reactions were performed in 80 µL volume, containing equal molar concentrations of DNA encoding the transporters, and promoters. The DNA fragments were annealed and extended using *pfu* polymerase at an annealing temperature of 58°C for pdc promoter & the downstream gene and 62°C for *glf* promoter & the downstream gene. The forward primer for the promoters and the reverse primers of the transporter genes had overlapping sequences with 5` and 3` end of the plasmid, pSRKTc, respectively. The amplified and gel purified 1867 bp Pglf-xylE, 1864 bp Ppdc-xylE, 4170 bp Pglf-xylFGH and 4167 bp Ppdc-xylFGH were inserted into a, Nde I and Xho I digested, plasmid, pSRKTc, using Gibson assembly reaction. The reaction mix was transformed into chemically competent *E. coli* DH5α cells. The transformed cells were plated on LB agar medium with 10 µg mL^{-1} of tetracycline. After 12 h, colonies appeared on the plate. The colonies were tested for the presence of the cloned genes using colony PCR. The plasmids carrying the *xylE* and *xylFGH* gene under the influence of *Ppdc* or *Pglf* was designated as

pSRKTc-Ppdc-XE, pSRKTc-Pglf-XE and pSRKTc-Ppdc-XFGH, pSRKTc-Pglf-XFGH, respectively (Table 4.2).

Table 4.1 List of primers used for PCR amplification of promoters and genes in this study

Names of primers	Primer application	Primer sequence
P-Ppdc-XE-FP	Forward primer of *Ppdc* for *xylE* gene, sequence overlap with pSRKTc	GAGCGGATAACAATTTCACACAGGAAAC AGCATATGACCTTTTTCAAGGTGTCCCGT TCCTTTTTC
P-Ppdc-XE-RP	Reverse primer of *Ppdc* for *xylE* gene sequence overlap with *xylE* gene	TATATAACTGGAATTATACTGGGTATTCA TTGCTTACTCCATATATTCAAAACACTAT GT
XE-FP	Forward primer for *xylE* gene	ATGAATACCCAGTATAATTCCAGTTATAT ATTTTCGA
XE-His-RP	Reverse primer for *xylE* gene with His-tag in C-terminal	CGAATTGGGTACCGGGCCCCCCCTCGAG TTAATGGTGGTGGTGATGATGCAGCGTA GCAGTTTGTTGTGTTTTCTT
XE*-RP¥	Reverse primer for inducing mutation in *xylE* gene	TAATTGCCAGAACGGTGAAGGTGAG**GAA** GATAACTCCGACAATAATGGTCTGC
P-Pglf-XE-FP	Forward primer of *Pglf* for *xylE*, sequence overlap with pSRKTc	AGCGGATAACAATTTCACACAGGAAACA GCATATGTTTTTTAAAAAGAAAACTGTTT TTTTAAACACTTATGTTGCCT
P-Pglf-XE-RP	Reverse primer of *Pglf* for *xylE* with overlap sequence against *xylE* gene	TATAACTGGAATTATACTGGGTATTCATG GCGATTCCTCTCCCGCCTTAATCTG

P-Ppdc-XF-RP	Reverse primer of *Ppdc* for *xylFGH* operon with overlap sequence against *xyl*F gene	AAAGGGTGAGTAGAATGTTCTTTATTTTC ATTGCTTACTCCATATATTCAAAACACTA TGTC
P-Pglf-XF-RP	Reverse primer of *Pglf* for *xylFGH* operon with overlap sequence against *xyl*F gene	AAGGGTGAGTAGAATGTTCTTTATTTTCA TGGCGATTCCTCTCCCGCCTTAATCTGTA AA
XF-FP	Forward primer for *xyl*F gene	ATGAAAATAAAGAACATTCTACTCACCC TT
XF-RP	Reverse primer for *xyl*F gene	AATGTTCTTCATTTCAAGTAGATAAGGCA T
XG-FP	Forward primer for *xyl*G gene	ATGCCTTATCTACTTGAAATGAAGAACA TT
XG-RP	Reverse primer for *xyl*G gene	TTTCACTTCAGACGGATTGCTTTTCGACA T
XH-FP	Forward primer for *xylH* gene	ATGTCGAAAAGCAATCCGTCTGAAGTGA A
XH-His-RP	Reverse primer for *xylH* gene with His-tag in C-terminal	CTATAGGGCGAATTGGGTACCGGGCCCC CCCTCGAGTCAATGGTGGTGGTGATGATG AGAACGGCGTTTGGTTGCGGAGTC

¥The underlined nucleotide codon in XE-RP represents mutated codon*

4.2.3 Site-directed mutagenesis of *xylE* transporter

Site-directed mutagenesis was performed by introducing the desired mutation (N325F) (Farwick, Bruder, Schadeweg, Oreb, & Boles, 2014) in the oligonucleotide primers. The oligonucleotide carrying the mutated nucleotides (XE*-RP with GAA in place of GTT) was used as a reverse primer along with forward primer, XE-FP to amplify a 1000 bp region of *xylE* gene, starting from the start codon (Table 4.1). The conditions used for PCR reactions included initial denaturation at followed by 25 cycles of denaturation, annealing and extension at 95°C for 30 s, at 65°C for 45 s and 72°C for 2 min, respectively, and after 25 cycles the reaction was incubated at 72°C for 10 min for final extension. The amplified fragment of *xylE* gene carrying the mutated nucleotides was used as forward primer to amplify the full-length *xylE* gene with a reverse primer encoding a 3` His-tag (Table 4.1). The PCR amplification of *xylE* was performed at an annealing temperature of 62°C. Post amplification, *xylE* with the mutated nucleotide was gel purified and analyzed on 0.8% (w/v) agarose gel. Subsequently, the mutant *xylE* gene (*xylE**) was fused to *Ppdc* promoter generating an 1864 bp fragment, Ppdc-xylE* and to *Pglf* promoter generating an 1867 bp fragment, Pglf-xylE*, using overlap extension PCR. The amplified fragments were inserted into the Nde I and Xho I digested pSRKTc plasmid using Gibson assembly reaction. The Gibson assembly reaction mix was transfected into chemically derived competent *E. coli* DH5α cells. Further, the transformed cells were plated onto LB agar medium with 10 µg mL^{-1} of tetracycline. After 12 h, colonies that appeared on the plate were tested for presence of the cloned genes using colony PCR. The plasmids carrying the *xylE** gene under the influence of *Ppdc* and *Pglf* were designated as pSRKTc-Ppdc-XE* and pSRKTc-Pglf-XE*, respectively (Table 4.2).

Table 4.2 List of plasmids used and constructed in this study

Names of plasmids	Genotype	Reference
pSRKTc	Tet^r, pBBR1MCS-3- lacZα	Gliessman, Kremer, Sangani, Jones-Burrage, & McKinlay, 2017; Khan, Gaines, Roop, & Farrand, 2008)
pSRKTc-Ppdc-XE	pSRKTc-P_{pdc} *xylE*	This study
pSRKTc-Ppdc-XE*	pSRKTc-P_{pdc} *xylE**	This study
pSRKTc-Ppdc-XFGH	pSRKTc-P_{pdc} *xylFGH*	This study
pSRKTc-Pglf-XE	pSRKTc-P_{glf} *xylE*	This study
pSRKTc-Pglf-XE*	pSRKTc-P_{glf} *xylE**	This study
pSRKTc-Pglf-XFGH	pSRKTc-P_{glf} *xylFGH*	This study

4.2.4 Plasmid transformation into AD50 using electroporation

To prepare electro-competent cells, AD50 cells were grown in Rich Medium (RMG) containing 5% (w/v) glucose to an OD_{600} of 0.4. The cells were harvested and first washed with 10 % (v/v) glycerol, of equal volume, and then with half volume of 10 % (v/v) glycerol. Finally, cells were resuspended in 10 % (v/v) glycerol to an OD_{600} of 20. 60 µL of electro-competent AD50 cells were transformed with a mix containing TypeOne Restriction Inhibitor (Lucigen Corporation,

USA) added at a concentration of 2.5 µg to 1.5 µg of unmethylated plasmid DNA, which were isolated from *E. coli* strain JM110. The reaction mixture was added to pre-chilled cuvettes with 0.1 cm gap, containing the competent cells. The cuvettes were placed in a Bio-Rad Xcell gene pulser (Bio-Rad Laboratories, USA) set at 18 kV, 200 Ω, and 25 µF. Immediately after the pulse was applied to the cuvettes containing the transformation mixture, cells were resuspended in mating media (MMG) composed of 1% (w/v) yeast extract,, 0.5% (w/v) tryptone, 0.2% (w/v) KH_2PO_4, 0.25% (w/v) $(NH4)_2SO_4$, 0.02% (w/v) $MgSO_4$ and 5% (w/v) D-Glucose and allowed to recover without shaking at 30°C for 3 h. The transformed cells were plated on to MMG agar plates comprising of 5% (w/v) glucose, supplemented with 10 µg mL^{-1} of tetracycline. Further, the plates were incubated at 30°C for 3-4 days. The transformed colonies were investigated for the presence of the cloned genes using colony PCR. The six *Z. mobilis* strains carrying xylose specific transporter genes under the influence of native promoters from *Z. mobilis* were designated as Zm-Ppdc-XE, Zm-Pglf-XE, Zm-Ppdc-XE*, Zm-Pglf-XE*, Zm-Pglf-XFGH and Zm-Ppdc-XFGH (Fig. 4.1).

4.2.5 Expression analysis of recombinant *Z. mobilis* strains using western blot

For cell lysate preparation, 10 mL of cultures were collected in 50 mL centrifuge tubes under aseptic conditions and harvested by centrifugation at 3,000 g at 4 °C for 15 min. Harvested cells were washed once with freshly prepared lysis buffer composed of 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithioerythritol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Washed cell pellets were resuspended in the lysis buffer in order to obtain a final OD_{600} of 50. The resuspended cells were sonicated for 8 cycles of 10 s pulse and 30 s of cooling period. Sonicated cells were centrifuged at 17,000 g at 4 °C for 40 min and the cell debris was discarded. The supernatant was collected and re-centrifuged at 17,000 g at 4 °C for 40 min. The supernatant was

used as cell free extract for performing western blot analysis. Bradford assay was used for estimation of the total protein concentration in the cell free extract using Bovine Serum Albumin (BSA) as standard.

Equal amounts of cell lysate from all six recombinant *Z. mobilis* cells were used for western blot analysis. The cell lysates were resolved on an SDS-PAGE gel and blotting was performed onto a Polyvinylidene Difluoride (PVDF) membrane. Upon transfer, the membrane was blocked using 5% (w/v) non-fat milk in TBST (10 mM Tris, pH 8.0, 130 mM, NaCl, 0.1% v/v Tween 20) followed by an incubation with primary rabbit-anti-His antibody (BioBharati LifeScience Pvt. Ltd., India). The membrane was further washed three times with TBST, followed by incubation with a 1:10,000 dilution of horseradish peroxidase conjugated antibody, Goat anti-rabbit antibody (BioBharati LifeScience Pvt. Ltd., India) for 1 hour at room temperature. Subsequently, the membrane was subsequently washed and developed with the Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, USA).

4.2.6 Characterization of mutant *Z. mobilis* strains on binary sugar mixture

For all batch fermentations experiments performed, 20% (v/v) seed cultures of ZW658, AD50 and the six recombinant *Z. mobilis* strains containing xylose specific transporters (Zm-Ppdc-XE, Zm-Pglf-XE, Zm-Ppdc-XE*, Zm-Pglf-XE*, Zm-Ppdc-XFGH, and Zm-Pglf-XFGH), were harvested by centrifuging at 7000 g for 2 min. The supernatant were discarded and cell pellets were resuspended in 1 mL of fresh medium and inoculated in 100 mL of fresh medium, sterilized in 250 mL customized airtight cultivation bottles. Characterization of the recombinant *Z. mobilis* strains, AD50 and ZW658 was carried out in a medium, supplemented with binary sugar mixture comprising of 5% (w/v) of glucose and 5% (w/v) of xylose (RMGX). Further, the inoculated medium was flushed with 99.9% pure N_2 gas for 10 min and the culture bottles were kept under

shaking at 150 rpm at 30 °C. Samples were collected at specific time intervals to generate dynamic profiles of growth, glucose, xylose, ethanol, and xylitol. Specific uptake rate of sugars (q_s, g g^{-1} h^{-1}) was quantified using equation (1) and ethanol productivity (Q, g L^{-1} h^{-1}) was calculated using equation (2);

$$q_s = \frac{1}{x}\frac{ds}{dt} \quad (1)$$

$$Q = \frac{(Et - E0)}{t} \quad (2)$$

Where, *s* is the substrate concentration present in the broth (g L^{-1}) and *x* is the dry cell weight (g) in equation 1

In addition, $E0$ (g L^{-1}) is the initial concentration of ethanol present in the broth & Et (g L^{-1}) is the concentration of ethanol present in the broth at specific time point *t* (h) in equation 2

The experiments were executed in duplicates and the data is presented as mean ± standard error. The best performing mutant *Z. mobilis* strain was selected for further characterization.

4.2.7 Characterization of the selected xylose specific transporter mutant strain in presence of two divalent metal ions Cu^{2+} and Zn^{2+}

Seed culture (20% (v/v)) of the selected xylose specific transporter mutant strain was harvested by centrifuging at 7000 g for 2 min. The supernatant was discarded and cell pellet was resuspended in 1 mL of fresh medium and inoculated in 100 mL of fresh medium, sterilized in 250 mL customized airtight cultivation bottles. Characterization of the selected xylose specific transporter mutant *Z. mobilis* strain was carried out in a medium, supplemented with binary sugar mixture comprising of 5% (w/v) of glucose and 5% (w/v) of xylose (RMGX). Firstly, to assess the effect of Zn^{2+} on the performance of recombinant *Z. mobilis* strain, $ZnSO_4.7H_2O$ was supplemented as exogenous source of Zn^{2+} in the medium at three different concentrations of 0.5 mM, 1 mM and

2 mM in the fermentation medium. Similarly, to evaluate the efficacy of Cu^{2+} supplementation on the performance of mutant *Z. mobilis* strain, five different concentrations of $CuSO_4.5\ H_2O$ i.e., 0.05 Mm, 0.075 mM, 0.1 mM, 0.25 mM and 0.5 mM, was added, , as exogenous source of Cu^{2+}, in the fermentation medium. Further, the inoculated medium was flushed with 99.9% pure N_2 gas for 10 min and the culture bottles were kept under shaking at 150 rpm at 30 °C. Samples were collected at specific time intervals to generate dynamic profiles of growth, glucose, xylose, ethanol, and xylitol.

4.2.8 Characterization of the selected xylose specific transporter mutant strain (Zm-Ppdc-XFGH) in bioreactor

For characterization of the selected recombinant *Z. mobilis* strain, Zm-Ppdc-XFGH, 200 mL of pre-seed culture was prepared in a 500 ml airtight customized culture bottle, kept at 30°C under shaking at 150 rpm. Further, 20% (v/v) of stationary phase pre-seed culture was harvested and inoculated in 600 mL of fresh RMX containing 10% (w/v) of xylose, to prepare the seed culture. At late exponential phase of growth, the seed culture was harvested by centrifugation at 7000 g for 5 min in 300 mL sterilized centrifuge tubes. The supernatant was discarded and the cell pellet, after resuspension, was inoculated in 3 L of sterile RMGX supplemented with 5% (w/v) each of glucose and xylose in a bioreactor to achieve a starting OD_{600} of 0.7. The fermenter used (New Brunswick, Eppendorf, Germany) for the experiment had a capacity of 7.5 L, with working volume of 3 L. Fermentation was conducted for 36 h at 30°C at a constant agitator speed of 100 rpm and initial pH of 5.8. Anaerobic condition was maintained throughout the course of the batch fermentation by flushing N_2 for 2h, after inoculation. The exhaust was occasionally opened to release the gas formed during the course of fermentation. Samples were collected routinely at specific time points to generate dynamic profiles of growth, glucose utilization, xylose utilization,

and ethanol & xylitol formation. The specific sugar uptake rate and ethanol productivity was determined using equation (1) & (2), respectively.

4.2.9 Analytical methods

For all characterization experiments, samples were routinely collected at specific time intervals and centrifuged at 13,000 *g* for 10 min at 4°C (Multifuge X3R, Thermofisher Scientific, USA). The pellets were resuspended in MilliQ water and cell growth was estimated by measuring the absorbance of the biomass at 600 nm (OD_{600}), using UV-Vis spectrophotometer (Cary 100, Varian, Australia). Quantification of the dry cell weight (DCW) was carried out, wherein an OD_{600} of 1 corresponded to 0.47 mg of DCW per mL of liquid culture. Subsequently, the collected supernatant was analyzed to estimate the concentration of glucose, xylose, ethanol, and xylitol, using HPLC (Ultimate 3000, Dionex, Thermofisher Scientific, USA) fortified with Rezex-ROA column (300 × 7.8 mm, Phenomenex, USA). A solution of 0.005 N H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. Glucose, xylose, ethanol, and xylitol were detected with Refractive Index Detector (RID). The temperature of the column oven was maintained at ambient temperature and that of RID at 37°C.

4.3 Results and discussions

4.3.1 Construction of an array of xylose-specific transporter engineered plasmids

In order to construct the plasmids carrying three different xylose transporters XylE, a xylose specific transporter from *E.coli* (Dunn & Rao, 2014); XylE*, a mutant form of XylE and XylFGH, a high affinity xylose uptake system that belongs to the ATP Binding Cassette (ABC) superfamily of transporters from *E. coli* (Zhao, Xian, Liu, & Zhao, 2020), were selected. XylE has been reported to bind glucose with an affinity similar to xylose (K_{dxyl}= 0.35 mM vs. K_{dglc} = 0.77 mM) (Henderson & Maiden, 1990; Lam, Daruwalla, Henderson, & Jones-Mortimer, 1980; Sun et

al., 2012). A close homologue of XylE is a yeast hexose transporter, Gal2, which has lower affinity towards xylose as compared to glucose (Farwick et al., 2014). However, a mutation of Asn376 to Phe in Gal2 increased its affinity for xylose as evident from reduction in K_m value from 200 mM for wild type to 91mM for mutant, while the capacity to transport glucose was abrogated (Farwick et al., 2014). This alteration in functionality of the transporter was attributed to the steric hindrance offered by the large side chain of Phe residue to glucose residues and not to xylose residues. With the aim of constructing a glucose unresponsive mutant of XylE, sequence alignment was used to identify the position of the residue in XylE that corresponds to the Asn376 residue of Gal2. Based on the sequence alignment, a point mutation of N325F was introduced in XylE through site directed mutagenesis to obtain XylE*. The other candidate xylose transporter, selected for expression in AD50, was XylFGH. XylFGH is a high affinity xylose binding ABC-type transporter complex, and sequence homology analysis proposed XylF to be the periplasmic xylose binding protein, while XylG was predicted to be the ATP-binding protein, XylH was imitative to a membrane component of the ABC transporter system (Ahlem, Huisman, Neslund, & Dahms, 1982; Sofia, Burland, Daniels, Plunkett, & Blattner, 1994). XylFGH displays a high affinity towards xylose with K_m of 0.2 to 4 µM (Sumiya, Davis, Packman, McDonald, & Henderson, 1995). The performance of these transporters was compared when expressed under the control of *Ppdc* (promoter of native pyruvate decarboxylase gene) and *Pglf* (promoter of native glucose facilitated diffusion protein), using pSRKTc as the vector for transporter gene integration. The designs of the engineered constructs and plasmid are shown in fig. 4.1.

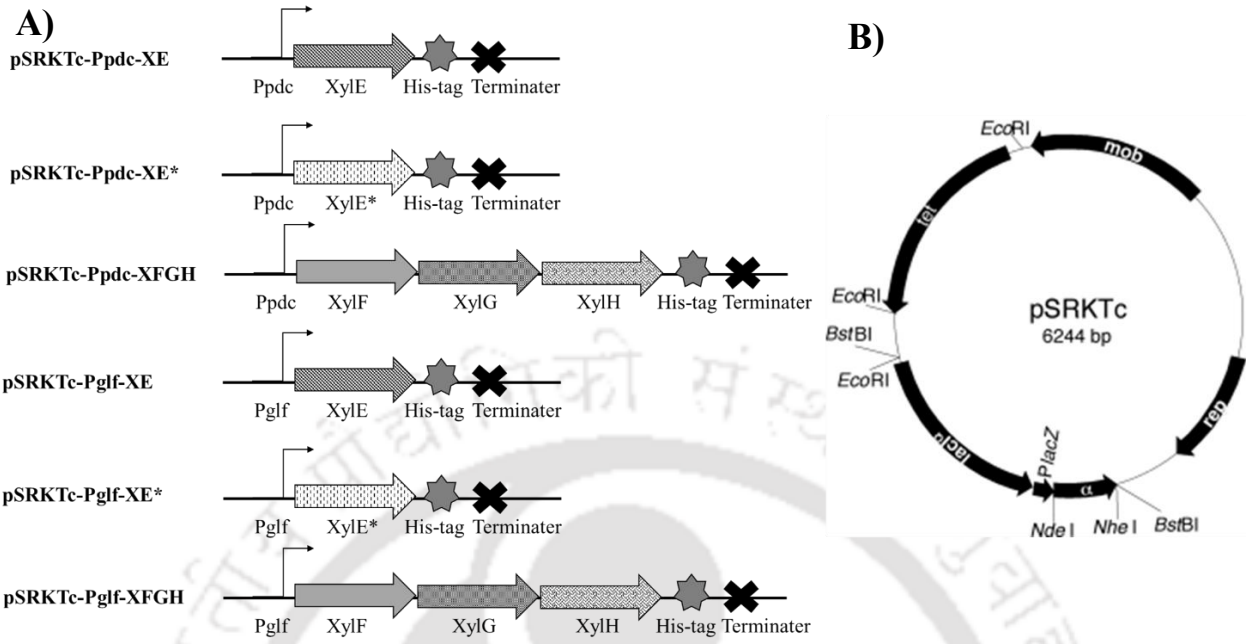


Fig. 4.1. Schematic representation of **A)** design of the constructs, where, *xylE*, *xylE**, and *xylFGH* have been cloned in pSRKTc under the influence of *Ppdc* and *Pglf* promoter; and **B)** pSRKTc plasmid

The PCR amplified DNA fragments were purified and analyzed by agarose gel electrophoresis as depicted in fig 4.2.

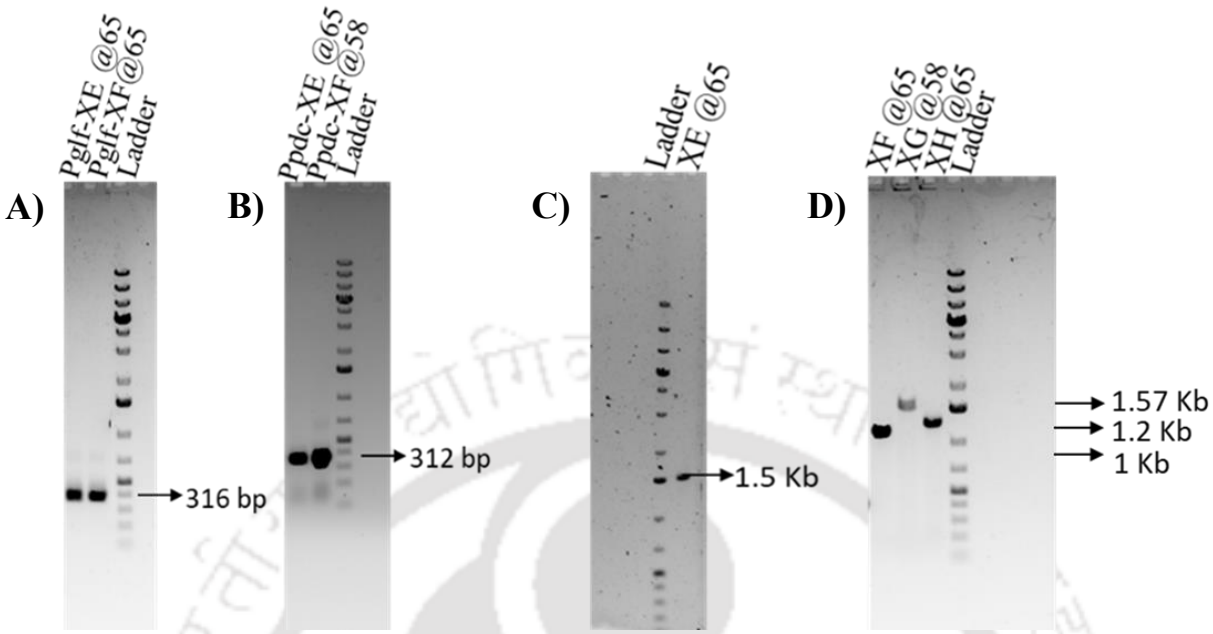


Fig. 4.2. Agarose gel electrophoresis of **A)** PCR amplified and purified P_{glf} promoter for *xylE* and *xylF* at 65°C, **B)** PCR amplified and purified P_{pdc} promoter for *xylE* and *xylF* at 65°C & 58°C respectively, **C)** PCR amplified and purified *xylE* gene at 65°C, and **D)** PCR amplified and purified *xylF*, *xylG*, *xylH* genes at 65°C, 58°C & 65°C respectively

The steps involved and resultant DNA fragments in site directed mutagenesis of *xylE* has been depicted in fig 4.3.

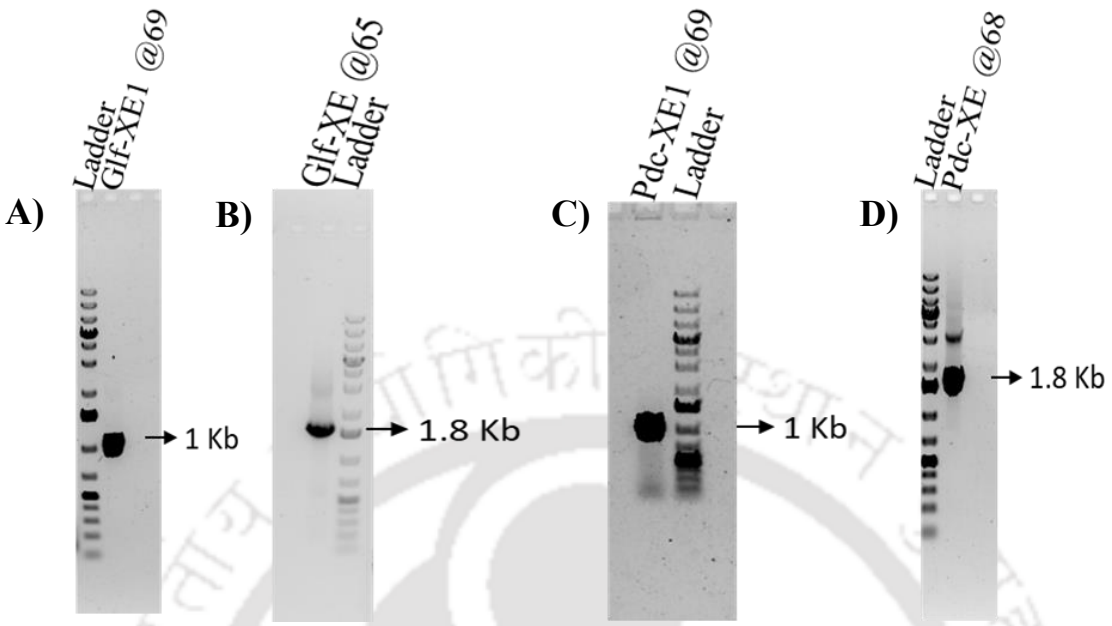


Fig. 4.3. Agarose gel electrophoresis of **A)** PCR amplified and purified partial fragment of *xylE* with primer directed mutation fused with P_{glf} at 69°C, **B)** PCR amplified and purified full length *xylE* gene using Glf-XE1 as forward primer at 65°C, **C)** PCR amplified and purified partial fragment of *xylE* with primer directed mutation fused with P_{pdc} at 69°C, and **D)** PCR amplified and purified full length *xylE* gene using Pdc-XE1 as forward primer at 68°C

Thus, six constructs were developed namely, pSRKTc-Ppdc-XE, pSRKTc-Pglf-XE, pSRKTc-Ppdc-XE*, pSRKTc-Pglf-XE*, pSRKTc-Ppdc-XFGH and pSRKTc-Pglf-XFGH (Fig. 4.4).

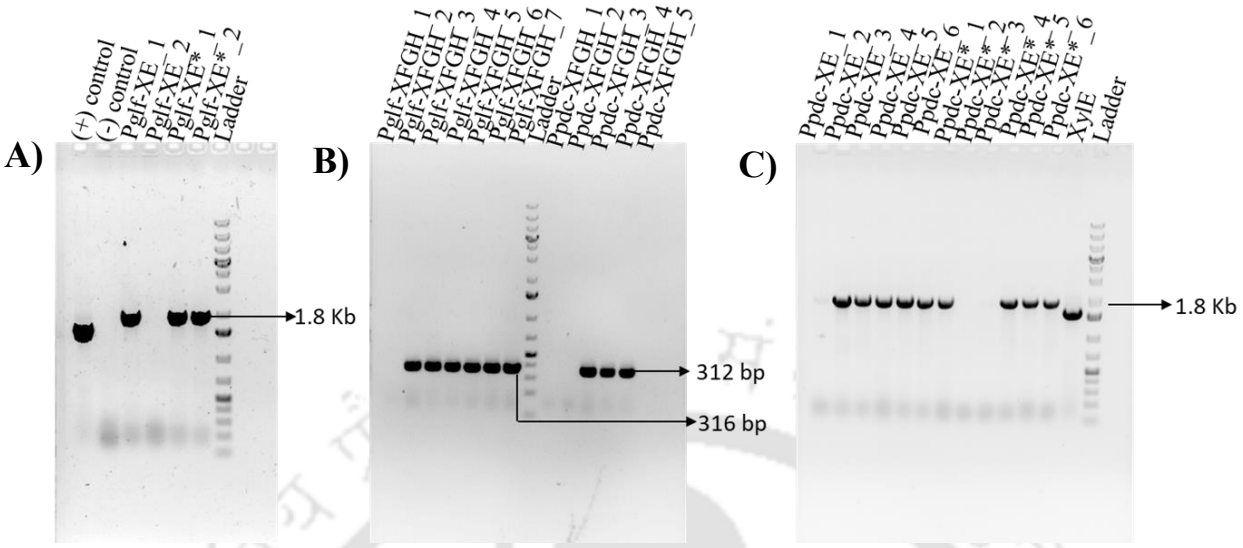


Fig. 4.4. Colony PCR and agarose gel electrophoresis analysis for clone confirmation of **A)** pSRKTc-Pglf-XE (Pglf-XE_1 and Pglf-XE_2) and pSRKTc-Pglf-XE* (Pglf-XE*_1 and Pglf-XE*_2), **B)** pSRKTc-Pglf-XFGH (Pglf-XFGH_1 to 7) and pSRKTc-Ppdc-XFGH (Ppdc-XFGH_1 to 5), and **C)** pSRKTc-Ppdc-XE (Ppdc-XE_1 to 6) and pSRKTc-Ppdc-XE* (Ppdc-XE*_1 to 6)

De-methylation (Fig 4.5) and transfection of these constructs in AD50 yielded six *Z. mobilis* mutants with xylose specific transporter were developed. The mutant strains were designated as Zm-Ppdc-XE, Zm-Pglf-XE, Zm-Ppdc-XE*, Zm-Pglf-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH (Fig 4.6A).

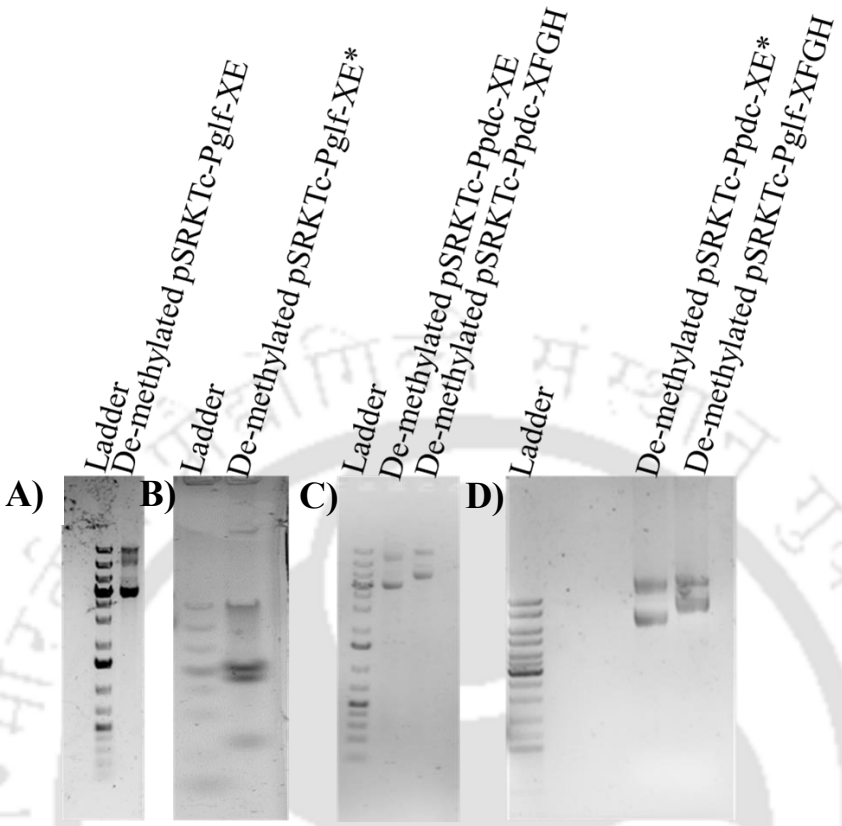


Fig. 4.5. Agarose gel electrophoresis analysis for demethylated plasmids of **A)** pSRKTc-Pglf-XE , **B)** pSRKTc-Pglf-XE*, and **C)** pSRKTc-Ppdc-XE and pSRKTc-Ppdc-XFGH, and **D)** pSRKTc-Ppdc-XE* and pSRKTc-Pglf-XFGH, extracted from *E. coli* strain JM110

The cellular localization of the transporters was analyzed using Phobius tool (Käll, Krogh, & Sonnhammer, 2007), which predicted the presence of short signal peptide in the N- terminal region of both XylE and XylFGH, responsible for membrane localization. The signal peptide sequence for XylE was identified to span over 1-23 amino acid residues. Similarly, in case of XylFGH, the signal peptide sequence was predicted to span over residues 1-15. The expression of these transporters under the influence of native promoters in AD50 was confirmed by detection of band corresponding to XylE (53 kDa), XylE*(53 kDa), and XylH (41 kDa) using ant-His antibody (Fig. 4.6B). Although, the level of expression for the transporters was not quantified, the

expression of transporter proteins under the influence of the *glf* promoter seemed to be very low, especially for construct pSRKTc-Pglf-XE and pSRKTc-Pglf-XE*, when compared to that of *pdc* promoter. A similar observation was reported regarding the functionality of native *glf* promoter, towards expressing low-level of XylE transporter in a xylose utilizing recombinant *Z. mobilis* strain (Dunn & Rao, 2014).

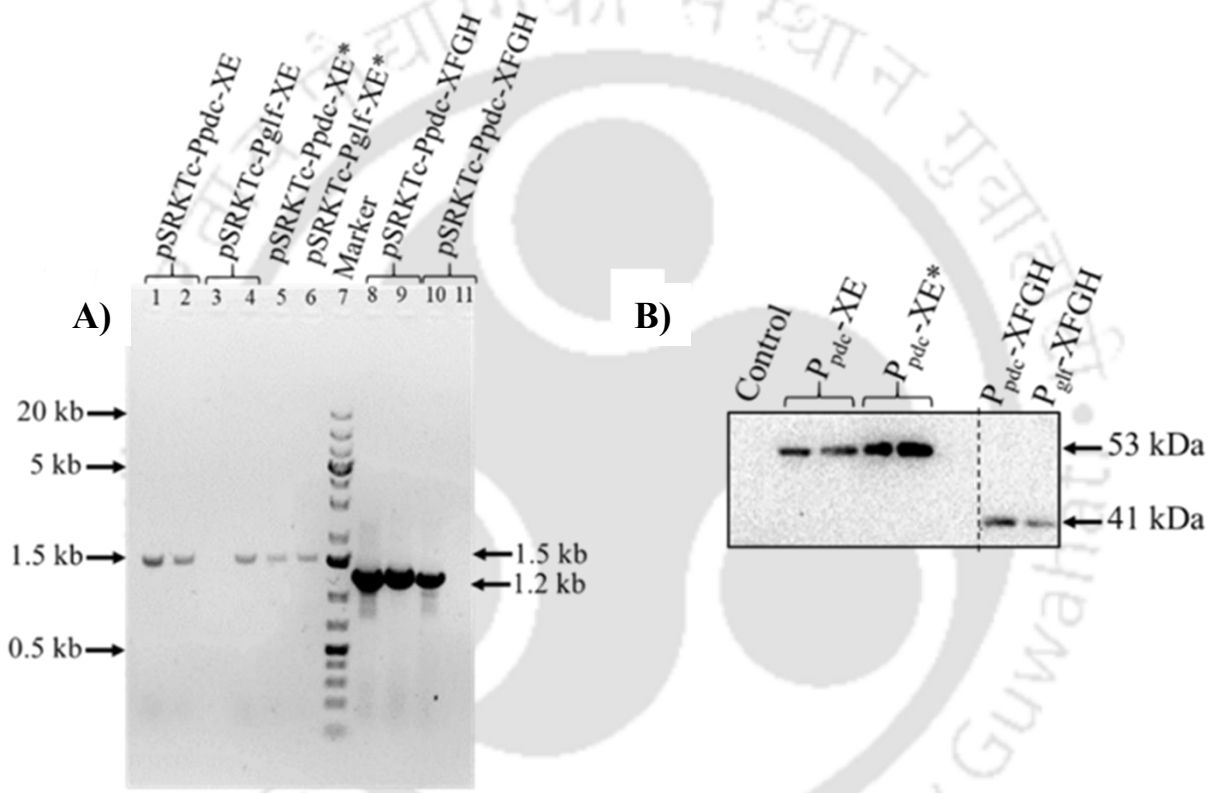


Fig. 4.6 Agarose gel electrophoresis analysis for **A)** molecular confirmation of cloned xylose specific transporter genes in pSRKTc vector from AD50 cells, where lane 1 & 2 represent cloned *Ppdc-xylE*, lane 3 & 4 represent cloned *Pglf-xylE*, lane 5 represents cloned *Ppdc-xylE**, lane 6 represents cloned *Pglf-xylE**, lane 7 represents the DNA marker, lane 8 & 9 represent cloned *Ppdc-xylFGH* and lane 10 & 11 represent cloned *Pglf-xylFGH*, and **B)** shows heterologous expression of the transporter proteins, XylE, XylE* and XylFGH under the influence of *Ppdc* and *Pglf*

promoters, detected using western blotting technique, wherein, control represents the cell lysate from untransformed AD50 cells.

4.3.2 Recombinant *Z. mobilis* strains with heterologous xylose specific transporters exhibit improved xylose utilization in presence of glucose

AD50, developed through ALE of ZW658 under high xylose concentration, exhibited highly efficient xylose fermentation, when cultivated in medium containing only xylose (Sarkar et al., 2020). AD50 also demonstrated efficient utilization of xylose in presence of glucose, albeit, with moderate delay in xylose utilization rate as compared to that of glucose. Since, the exquisite aim of this study is to develop an efficient xylose and glucose co-utilizing strain, with comparable effectiveness, which might facilitate the implementation of the strain in lignocellulosic ethanol production, we specifically, evaluated the phenotypic response of the six engineered *Z. mobilis* strains i.e. Zm-Ppdc-XE, Zm-Pglf-XE, Zm-Ppdc-XE*, Zm-Pglf-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH in media supplemented with dual sugar mixture comprising of 5% (w/v) glucose and 5% (w/v) xylose.. Various phenotypic responses such as growth, extent and rate of sugar utilization, ethanol synthesis and xylitol production by these recombinant strains were compared with that of AD50 and ZW658, considered as the controls. It is essential to note that the strains Zm-Pglf-XE and Zm-Pglf-XE* did not show any visible improvement in none of the aforementioned phenotypic attributes, when compared to AD50, which could probably be due to insufficient expression of the heterologous xylose-transporter under the influence of the *glf* promoter (Fig. 4.6B). Therefore, further discussion focusses only on characterization and comparison of rest of the four recombinant strains, AD50 and ZW658 (Fig. 4.7). Heterologous expression of the xylose specific transporters did not result in significant variation in growth of the strains as compared to AD50. The maximum DCW obtained for all the engineered *Z. mobilis*

strains was recorded to be within narrow range of 3.57 to 3.75 g L^{-1}, similar to that of AD50 (Fig. 2A). However, in line with AD50, all recombinant strains exhibited significant growth improvement with respect to ZW658 (Fig. 4.7A).

With regard to utilization of sugars, all four recombinant strains were observed to exhibit interesting phenotypic response. Similar to AD50, all these recombinant strains could completely utilize glucose within 12 h of cultivation (Fig. 4.7B), albeit with different specific uptake rates. Intriguingly, the transporter genes expressed in these engineered strains were specific to xylose (Dunn & Rao, 2014; Farwick et al., 2014; Hasona, Kim, Healy, Ingram, & Shanmugam, 2004; Zhao et al., 2020), however, the specific glucose uptake rates were found to be elevated by approximately two fold in all the recombinant strains as compared to that of AD50 (Table 4.3). The enhanced glucose uptake rate could be rationalized by the availability of glf transporter, which is a native promiscuous glucose transporter in *Z. mobilis* with the ability to transport xylose with lesser efficiency, towards glucose. The expression of xylose specific transporters in the evolved *Z. mobilis* strain AD50, might have nullified the competitive inhibition, on glf transporter protein, which glucose had encountered in presence of xylose in the medium. The specific glucose uptake rate was found to be highest at 2.7 g g^{-1} h^{-1} in case of Zm-Ppdc-XE, followed by Zm-Pglf-XFGH (2.5 g g^{-1} h^{-1}), Zm-Ppdc-XFGH (2.49 g g^{-1} h^{-1}) and Zm-Ppdc-XE* (2.38 g g^{-1} h^{-1}) (Fig 4.8A). Unlike glucose, overall xylose uptake rates for four recombinant strains and AD50 remained unaltered at an average value of 0.546 g g^{-1} h^{-1} (Table 4.3). However, most desirable phenotypic trait of improved xylose consumption in presence of glucose, both, in terms of rate and extent of xylose utilization was achieved via heterologous expression of xylose specific transporters in AD50. For instance, highest upregulation in specific xylose uptake rate in presence of glucose was recorded for Zm-Ppdc-XFGH (2.04 g g^{-1} h^{-1}) (Fig 4.8B) when equated to AD50 (Table 4.3).

Similar upliftment in specific xylose uptake rate in presence glucose was observed for other recombinant strains Zm-Ppdc-XE* (1.97 g g^{-1} h^{-1}), Zm-Ppdc-XE (1.9 g g^{-1} h^{-1}) and Zm-Pglf-XFGH (1.7 g g^{-1} h^{-1}), as well (Table 4.3). Further, phenomenon of near co-utilization of glucose and xylose in Zm-Ppdc-XFGH was evident from comparable specific xylose and glucose uptake rate of 2.04 g g^{-1} h^{-1} and 2.49 g g^{-1} h^{-1}, respectively, during dual sugar phase of the fermentation (until glucose was present in the fermentation medium). Besides, improvement in rate of utilization of both the sugars, further increase in extent of xylose utilization was observed for all four recombinant strains as compared to AD50. While AD50 utilized 90.3% of the total xylose, present in the fermentation medium within 24 h, Zm-Ppdc-XE, Zm-Ppdc-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH showed greater extent of xylose utilization during the same period of time corresponding to 96.4%, 96.3%, 96.1% and 95.5%, respectively (Fig. 4.7C). Interestingly, Zm-Zm-Ppdc-XE, Zm-Ppdc-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH demonstrated a shorter fermentation time and within the first 18 h consumed 81.8%, 86%, 86% and 77.5 %, respectively, of the total xylose as compared to only 71.4% by AD50 (Fig. 4.7C). Therefore, out of four recombinant strains, Zm-Ppdc-XFGH emerged to be the best in terms extent of sugar utilization and attribute of co-utilization of both the sugars.

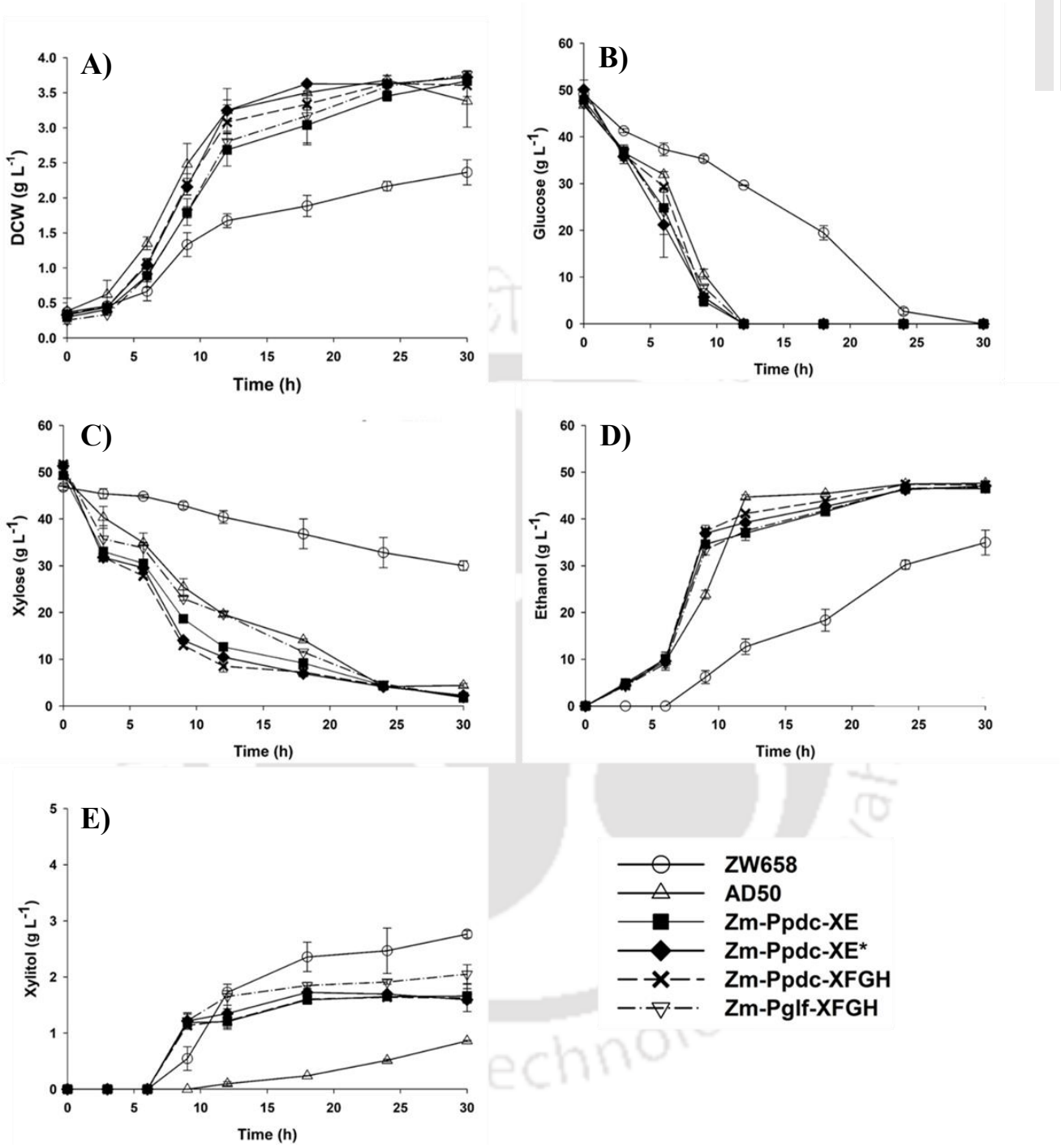


Fig. 4.7 Dynamic profiles of **A)** growth, **B)** glucose utilization, **C)** xylose utilization, **D)** ethanol production, and **E)** xylitol production by ZW658, AD50, Zm-Ppdc-XE, Zm-Ppdc-XE*, Zm-Ppdc-

XFGH and Zm-Pglf-XFGH, when cultivated on mixed carbon source of 5% (w/v) glucose and 5% (w/v) xylose. The data are presented as mean ± standard error

Table 4.3 Kinetic parameters for utilization of sugars and ethanol production for engineered *Z. mobilis* strains compared to ZW658 and AD50

		Specific sugar uptake rate (g g^{-1} h^{-1})		
Strain	**Initial sugar concentration (%, w/v)**	**Glucose**	**Xylose**	
			Overall	**In presence of glucose€**
ZW658	5% Glucose + 5% Xylose	0.57± 0.01	0.17± 0.04	0.13± 0.02
AD50	5% Glucose + 5% Xylose	1.24± 0.03	0.55± 0.03	1.37± 0.04
Zm-Ppdc-XE	5% Glucose + 5% Xylose	2.7± 0.04	0.53± 0.02	1.9± 0.05
Zm-Ppdc-XE*	5% Glucose + 5% Xylose	2.38± 0.02	0.56± 0.03	1.97± 0.06
Zm-Ppdc-XFGH	5% Glucose + 5% Xylose	2.49± 0.05	0.54± 0.02	2.04± 0.04
Zm-Pglf-XFGH	5% Glucose + 5% Xylose	2.5± 0.02	0.55± 0.04	1.7± 0.03
		Ethanol		
		Yield (g g^{-1})	**Titer (g L^{-1})**	**Overall Productivity (g L^{-1} h^{-1})**
ZW658	5% Glucose + 5% Xylose	0.42± 0.002	29.7± 0.09	0.62± 0.002
AD50	5% Glucose + 5% Xylose	0.49± 0.002	47.7± 0.12	1.98± 0.005
Zm-Ppdc-XE	5% Glucose + 5% Xylose	0.48± 0.003	46.5± 0.19	1.92± 0.003
Zm-Ppdc-XE*	5% Glucose + 5% Xylose	0.47± 0.001	47.1± 0.15	1.93± 0.005
Zm-Ppdc-XFGH	5% Glucose + 5% Xylose	0.472± 0.003	47.4± 0.6	1.97± 0.004
Zm-Pglf-XFGH	5% Glucose + 5% Xylose	0.473± 0.002	46.7± 0.19	1.94± 0.002

€ until glucose was present in the fermentation media

Implementation of directed metabolic engineering strategy, to heterologously express xylose-metabolizing genes in *Z. mobilis*, led to the construction of various engineered strains, with enhanced xylose utilization capabilities (Dunn & Rao, 2014; Gao, Zhang, Mcmillan, & Kompala, 2002; Mohagheghi et al., 2002; M. Zhang et al., 1995). However, advantage of improved xylose utilization by these strains was realized only when xylose was present as sole carbon source in the culture media. Often, dynamic profile of xylose utilization of the engineered strains, in binary sugar mixture, revealed that the xylose consumption initiated only after complete or after consumption of atleast 50% of glucose in the fermentation medium, portraying a sequential utilization of the sugars (Agrawal et al., 2011; Dunn & Rao, 2014; Gao et al., 2002; Mohagheghi et al., 2002; M. Zhang et al., 1995). Therefore, those strains failed to efficiently co-utilize xylose, when glucose was also present in dual sugar supplemented medium. Subsequently, this characteristic exhibited by the genetically modified *Z. mobilis* strains, led to protracted fermentation time and incomplete xylose utilization. It is noteworthy to mention that the coherent application of targeted metabolic engineering along with ALE has also transpired into the development of efficient *Z. mobilis* strains, which could metabolize xylose faster as compared to the mutant *Z. mobilis* strains, modified using directed metabolic engineering strategies only (Agrawal et al., 2011; Dunn & Rao, 2015; Mohagheghi et al., 2015; Sarkar et al., 2020; Viitanen et al., 2008). A3, an adapted *Z. mobilis* strain, developed by Agrawal et al. (Agrawal et al., 2011) has been contemplated as one of the encouraging *Z. mobilis* strain for dual sugar fermentation, yet it could not efficiently ferment xylose, in presence of glucose. Inferior xylose uptake or transport has also been recognized as one of the key bottlenecks in xylose utilization in *Z. mobilis* (Dunn & Rao, 2014). Two potential approaches to address this limitation have been: (i) heterologous expression of *E. coli* xylose transporter, XylE, and (ii) over expression of native promiscuous

glucose transporter, Glf in *Z. mobilis* (Dunn & Rao, 2014). Heterologous expression of low-affinity xylose specific transporter (XylE) and over expression of Glf, in *Z. mobilis*, led to the development of strain 31821 (pKLD4) and 31821 (pKLD5). Improvement in extent and rate of xylose utilization, resulting from these two approaches, were realized only at high concentrations of xylose (Dunn & Rao, 2014). Besides, extent and rate of glucose utilization by these *Z. mobilis* recombinant strains were greater compared to that of xylose, depicting preferential utilization of glucose over xylose and in turn, sequential utilization of sugars. Therefore, successful development of *Z. mobilis* strain competent to co-utilize glucose and xylose remains a major challenge in the field. To that end, we recently reported an efficient xylose fermenting *Z. mobilis* strain, AD50, developed through a systematic ALE approach, exhibiting higher specific xylose uptake rate in presence of glucose as compared to other *Z. mobilis* strains engineered previously (Sarkar et al., 2020). The ALE strategy evidenced to be emphatically beneficial to conquer the previously reported bottlenecks such as strictly sequential sugar utilization, incomplete xylose utilization, and extended fermentation time. Although, AD50 proved to be an efficient glucose and xylose utilizing strain, however, the fermentation time of xylose still lagged behind that of glucose. With the aim of addressing the gap in the current state of the art, different xylose specific transporters were introduced and screened in AD50, as a suitable platform. AD50 endowed with xylose specific transporters has been substantiated to be potential biocatalyst, demonstrating improved xylose consumption in presence of glucose. The significantly improved xylose utilization by the engineered *Z. mobilis* strains, even in presence of glucose leading to considerable reduction in fermentation time, certainly highlights the efficacy of the selected xylose transporters, towards xylose uptake by the recombinant strains. In accordance with the objective of recombineering AD50 towards simultaneous utilization of glucose and xylose, Zm-Ppdc-XFGH evinced to be a

novel strain, exhibiting the best phenotypic response in that regard. Comparative literature analysis indicated that the maximum specific xylose uptake rate in presence of glucose of Zm-Ppdc-XFGH were comparable or better than the other ethanol producing strains, including both genetically modified *Z. mobilis* and state-of-the-art *S. cerevisiae* strains, reported in the literatures (Table 4.4). However, efficient co-fermentation of xylose in presence of glucose could not be achieved for the previously reported strains. Hence, this study manifested into the development of an enhanced biocatalyst with the potential to effectively co-utilize xylose and glucose. The superior phenotypic response exhibited by engineering XylFGH could be attributed to its high affinity towards xylose (Zhao et al., 2020) as compared to that of XylE. Structurally, XylF, which is the periplasmic D-xylose binding receptor of XylFGH transporter system, has two aromatic amino acid residues Trp 16 and Trp 183 forms a stacking interaction with xylose, whereas two planar polar residues Asp 89 and Asp 236 binds xylose by creating hydrogen bonds. An important aspect of XylF is its xylose inducible transcription, which is unaffected by the presence of glucose (Erbeznik, Strobel, Dawson, & Jones, 1998). Moreover, *Ppdc* has been reported to be one of the strongest native promoters in *Z. mobilis* (Y. Yang et al., 2019). Thus, cumulative effect of expression of an efficient transporter under the control of a strong promoter led to the construction of an enhanced xylose-glucose co-utilizing strain Zm-Ppdc-XFGH.

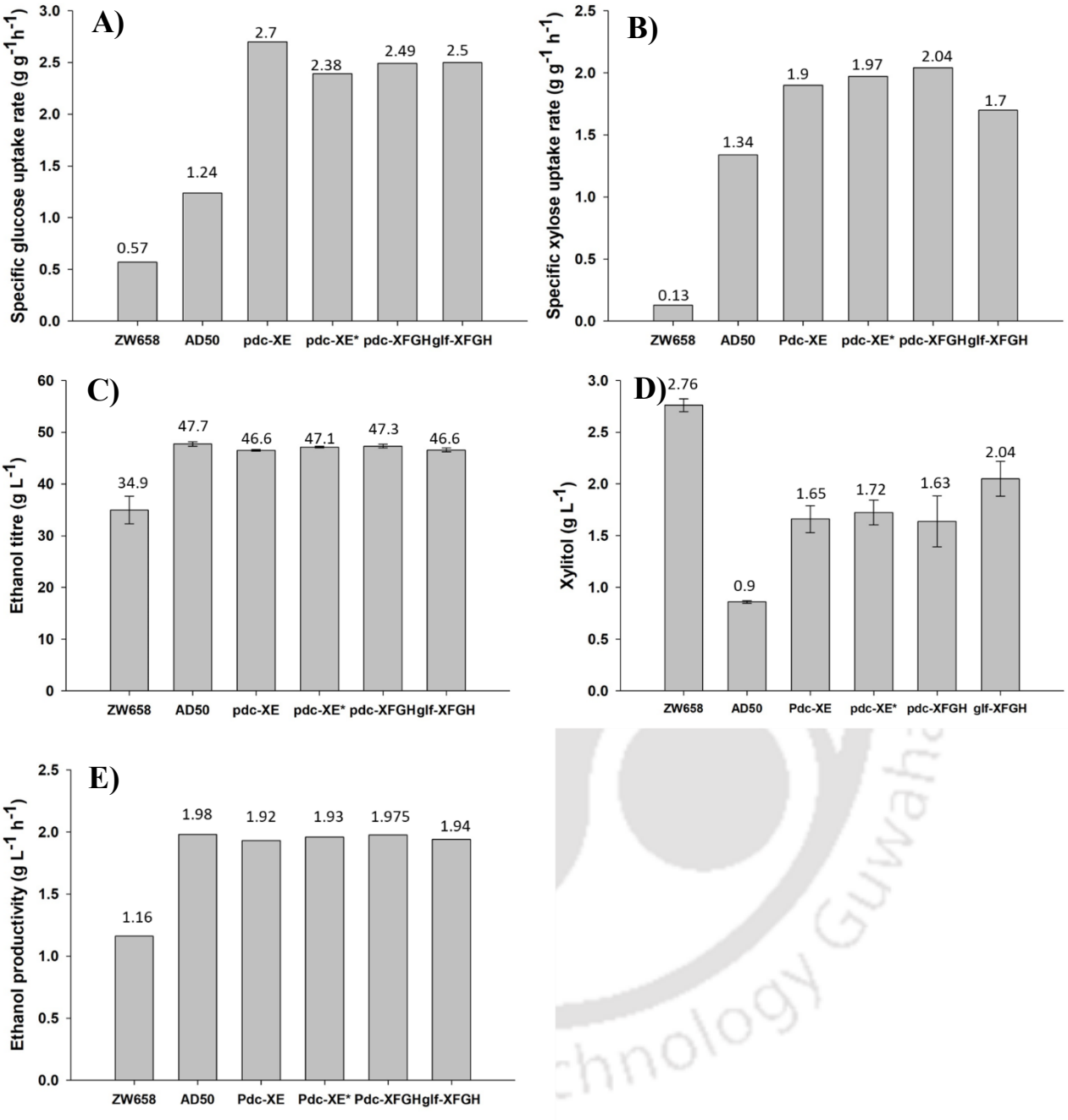


Fig. 4.8 Comparative analysis of **A)** specific glucose uptake rate, **B)** specific xylose uptake rate (until glucose is present), **C)** ethanol titer, **D)** xylitol titer, and **E)** ethanol productivity by ZW658, AD50, Zm-Ppdc-XE, Zm-Ppdc-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH, when cultivated on mixed carbon source of 5% (w/v) glucose and 5% (w/v) xylose

4.3.3 Ethanol and xylitol biosynthesis profile in the recombinant strains

Heterologous expression of xylose specific transporters in AD50 did not alter ethanol titer produced by the engineered strains (Fig. 4.7D). The ethanol titer achieved from all the four recombinant strains was found to be in the range of 46.6-47.3 g L^{-1}, comparable to that of AD50 (Table 4.3). The overall ethanol productivity for all the four mutants (1.92-1.97 g L^{-1} h^{-1}**)** were also found to be similar to AD50 (1.98 g L^{-1} h^{-1}) (Table 4.3). Out of four mutants, Zm-Ppdc-XFGH exhibited the highest ethanol productivity of 1.97 g L^{-1} h^{-1} (Fig. 4.8E). Comparative literature analysis suggested that the ethanol titer and ethanol productivity of Zm-Ppdc-XFGH were comparable or better than the other strains reported in the literatures, except ZW658 and *S. cerevisiae* STXQ, owing to higher ethanol titer obtained through fermentation of very high initial sugar concentrations (Table 4.4). A higher ethanol titer of 66 g L^{-1} was obtained when ZW658 was grown in a sugar mixture of 10% glucose and 8% xylose (Viitanen et al., 2008). The strain ZW658 was not able to utilize xylose efficiently and hence, the higher ethanol titer was a result of utilization of primarily glucose supplemented in the media at higher concentration. Similarly, *S. cerevisiae* STXQ exhibited an ethanol titer of 120.6 g L^{-1} when grown in a sugar mixture of 16% glucose and 9.5% xylose (Liu, Huang, & Geng, 2018). However, the ethanol yield was observed to be comparable for the transporter mutants to that of *S. cerevisiae* STXQ (0.47 g g^{-1}) (Liu et al., 2018). Although, in terms of ethanol productivity, *S. cerevisiae* STXQ was better, however, the binary sugar co-utilization proved to be superior in case of the *Z. mobilis* transporter mutants. Interestingly, in engineered *Z. mobilis* it has been observed that under lower sugar concentrations the kinetic attributes such as sugar uptake rates and product formation efficiency decreases (Dunn & Rao, 2014; Mussatto & Roberto, 2003). Similarly, for *S. cerevisiae* STXQ, when tested under low glucose and xylose concentrations, the sugar uptake rates as well as the ethanol productivity

also observed to be reduced significantly (Liu et al., 2018). The ethanol yield was observed to be slightly lower in case of the transporter mutants as compared to that of AD50, which can be attributed to increased xylitol titer showcased by the developed strains (Fig. 4.7E).

The xylitol titer for Zm-Ppdc-XFGH was quantified to be 1.63 g L^{-1} as opposed to 0.9 g L^{-1} of final xylitol concentration shown by AD50 (Fig. 4.7E). Similarly, higher xylitol titer was recorded for Zm-Ppdc-XE, Zm-Ppdc-XE*, and Zm-Pglf-XFGH, which corresponded to 1.66 g L^{-1}, 1.7 g L^{-1} and 2.04 g L^{-1}, respectively as compared to that of AD50 (Fig. 4.8D). This metabolic behavior of the recombinant strains confirmed a transferal in the xylose flux towards xylitol, which could be due to the significant augmentation in the degree and rate of xylose utilization. Engineering efficient xylose transporter in evolved *Z. mobilis* strain, under a strong promoter, led to ameliorated kinetic attributes in terms of co-utilization of xylose and glucose and subsequent ethanol synthesis by the engineered *Z. mobilis* strain Zm-Ppdc-XFGH, makes it a potential biocatalyst to be considered for commercial applications.

Table 4.4 Comparison of maximum specific xylose uptake rate, ethanol titer, ethanol yield and maximum ethanol productivity of potential ethanol producing strains when grown on dual substrate mixture of glucose and xylose

Ethanol producing strains	Initial sugar concentration (%, w/v)	Maximum specific xylose uptake rate (g g^{-1} h^{-1})	Ethanol titer (g L^{-1})	Ethanol yield (%)	Overall ethanol productivity (g L^{-1} h^{-1})	References
Zm-Ppdc-XFGH	**5 % Glucose - 5% Xylose**	**5.66**	**47.4**	**92.5**	**1.97**	**This study**
Zm-Ppdc-XE*	**5 % Glucose - 5% Xylose**	**5.6**	**47.1**	**92.3**	**1.93**	**This study**
Zm-Ppdc-XE	**5 % Glucose - 5% Xylose**	**3.6**	**46.5**	**94.1**	**1.92**	**This study**
Zm-Pglf-XFGH	**5 % Glucose - 5% Xylose**	**3.5**	**46.7**	**92.7**	**1.94**	**This study**
AD50	5 % Glucose - 5% Xylose	3.94	47.7	96	1.98	(Sarkar et al., 2020)
CP4 (pZB5)¥	2.5 % Glucose- 2.5 % Xylose	-	24.22	95	0.8	(M. Zhang et al., 1995)
AX101¥	4 % Glucose - 4 % Xylose - 2 % Arabinose	-	42.8	84	0.6	(Mohagheghi et al., 2002)
39676/pZB4L¥	4% Glucose – 4% Xylose	2.9	37.09	85	1.22	(Gao et al., 2002)
C25¥	4% Glucose – 4% Xylose	1.6	38.9	96.5	1.29	(Gao et al., 2002)
ZW658Ψ	10% Glucose – 8% Xylose	-	66	71.9	-	(Viitanen et al., 2008)
A3¥	5 % Glucose - 5% Xylose	1.8	49.9	96.6	1.53	(Agrawal et al., 2011)

31821 (pKLD4)¥	5 % Glucose - 5% Xylose	0.82	39.6	90.1	0.43	(Dunn & Rao, 2014)
S. cerevisiae XUSEA¥	4 % Glucose – 2 % Xylose	-	29.1	92.1	0.8	(Hoang Nguyen Tran, Ko, Gong, Um, & Lee, 2020)
S. cerevisiae SR8N¥	7 % Glucose – 4 % Xylose	-	47	84.3	1.6	(G. C. Zhang, Turner, & Jin, 2017)
S. cerevisiae STXQ	16 % Glucose – 9.5 % Xylose	-	120.6	94	5.26	(Liu et al., 2018)

‖ *Ethanol titer and yield was calculated based on the graphs shown in (Viitanen et al., 2008)*

¥ *Overall ethanol productivity was calculated based on the graphs shown in (Agrawal et al., 2011; Dunn & Rao, 2014; Gao et al., 2002; Hoang Nguyen Tran et al., 2020; Mohagheghi et al., 2002; G. C. Zhang et al., 2017; M. Zhang et al., 1995)*

4.3.4 Screening of divalent metal ions (Cu^{2+} and Zn^{2+}) based on its effect on growth, glucose-xylose utilization, ethanol titer and xylitol titer of Zm-Ppdc-XFGH

The engineered *Z. mobilis* strain Zm-Ppdc-XFGH manifested into an excellent glucose-xylose co-utilizing strain, exhibiting highest specific xylose uptake rate in presence of glucose, as compared to other contemporary glucose-xylose and ethanol producing strains developed till date. However, even though the rate and percentage of xylose utilization was observed to have increased for Zm-Ppdc-XFGH, the ethanol titer, yield and productivity was found to be similar as compared to AD50. The xylitol titer is significantly higher in case of Zm-Ppdc-XFGH (1.63 g L^{-1}) as compared to that of AD50 (0.9 g L^{-1}). Increase in xylitol production could be rationalized by the transference of the xylose flux towards xylitol as opposed to ethanol in Zm-Ppdc-XFGH. Thus, in order to reduce the formation of xylitol, which might be anticipated with the subsequent enhancement in ethanol titer, yield, and productivity, Zm-Ppdc-XFGH was subjected to two different strategies of media engineering. Two divalent metal ions Cu^{2+} and Zn^{2+}, which have been reported to inhibit xylose reductase (enzyme converting xylose to xylitol), were screened as media supplements, with an aim to decrease xylitol production by the mutant *Z. mobilis* strain Zm-Ppdc-XFGH. Based on earlier studies undertaken, which divulges the effect of these metal ions on xylose reductase, considering both *in vitro* and *in vivo* conditions, a range of concentrations of these metals were screened. Eventually, the dynamic profiles of Zm-Ppdc-XFGH was obtained in terms of growth, substrate utilization and product formation with three and five different concentrations of zinc and copper respectively, supplemented in the fermentation medium. Out of the three concentrations screened, supplementation of 0.5 mM Zn^{2+} in the cultivation media of Zm-Ppdc-XFGH, led to the decrease in xylitol titer from 1.62 g L^{-1}, in case of control, to 0.45 g L^{-1} in presence of zinc, however, the rate and extent of xylose utilization as well as the growth rate was found to be highly compromised. The specific xylose uptake rate of Zm-Ppdc-XFGH

in presence of 0.5 mM Zn^{2+} was evaluated to be 1.21 g g^{-1} h^{-1}, which was significantly low as compared to that of the control batch (2 g g^{-1} h^{-1}). Similarly, the extent of xylose utilization decreased to 89.7% from that of 96.1% in case of control batch, with subsequent decrease in ethanol titer (44.6 g L^{-1}) in presence of zinc (Fig. 4.9). Further, at concentrations higher than or equal to 1 mM, Zn^{2+} completely inhibited the growth of the mutant *Z. mobilis* strain. Such phenotypic response could be ascribed to the typical phenomenon of zinc toxicity on *Z. mobilis* strain at higher concentration (McDevitt et al., 2011).

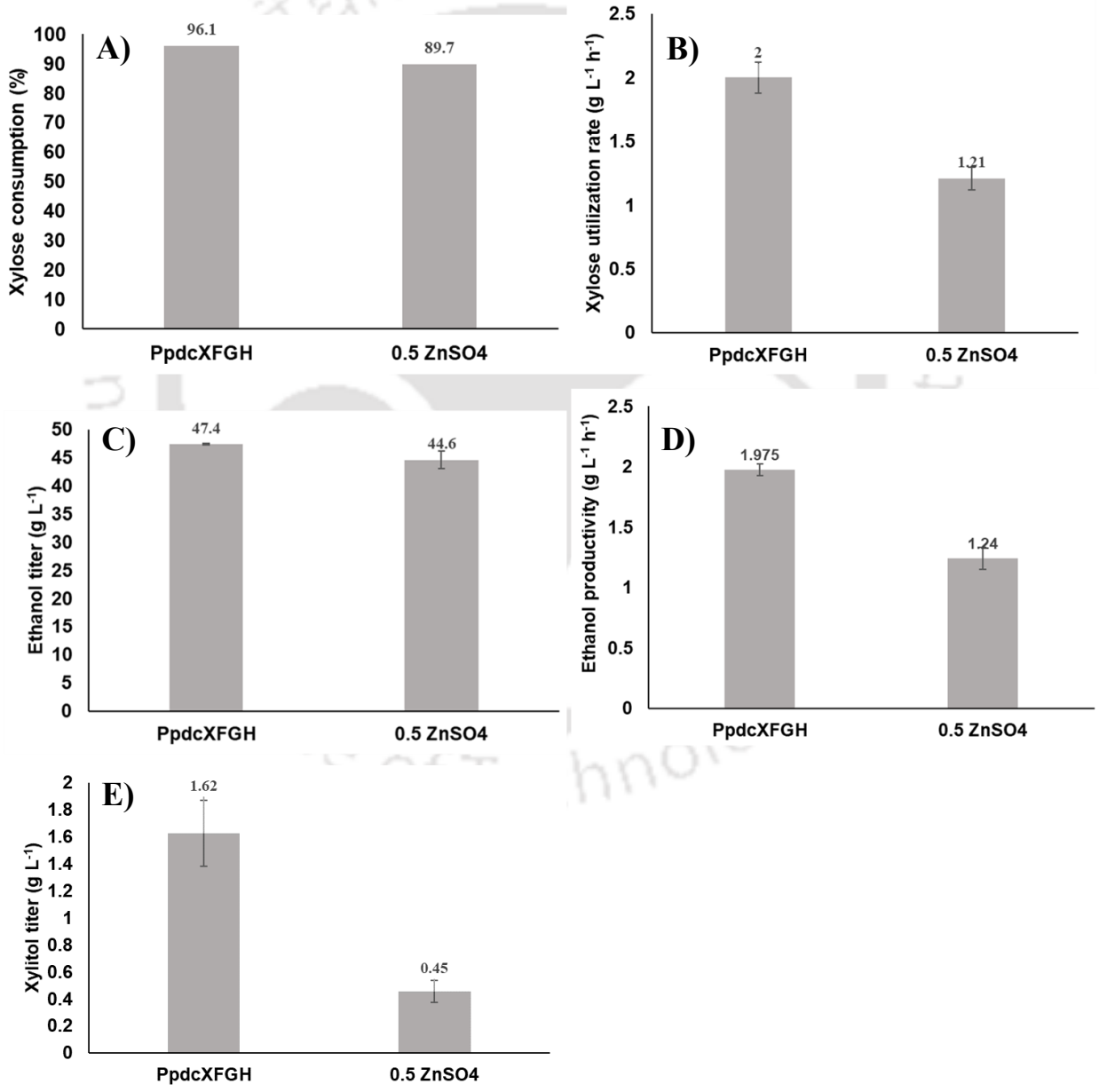


Fig. 4.9 Comparative analysis of **A)** extent of xylose consumption, **B)** specific xylose uptake rate (until glucose is present), **C)** ethanol titer, **D)** ethanol productivity, and **E)** xylitol titer by Zm-Ppdc-XFGH, when cultivated on mixed carbon source of 5% (w/v) glucose and 5% (w/v) xylose, in presence or absence of Zn^{2+}

Apart from zinc, effect of copper supplementation in production media of Zm-Ppdc-XFGH was also assessed. Five different concentrations were evaluated keeping 0.5 mM, as the highest concentration, supplemented in the medium to avoid metal toxicity on the engineered *Z. mobilis* strain. While zinc at concentration of 0.5 mM effectively regulated the xylitol production bringing its titer down to 0.45 g L^{-1}, however, copper, even at lowest screened concentration 0.05 mM, negatively affected the growth and substrate utilization by the engineered *Z. mobilis* strain. The highest rate of specific xylose uptake observed was 1.17 g g^{-1} h^{-1}, which was significantly low as compared to the control batch (1.99 g g^{-1} h^{-1}) (Fig. 4.10B). Apart from rate, extent of xylose utilization was also reduced to 86.7% in case of lowest supplemented concentration of copper (Fig. 4.10A). Although, the xylitol production was moderately modulated at 0.1 mM $CuSO_4$ leading to lower xylitol titer (1.09 g L^{-1}) as compared to the control condition, however, the ethanol titer (46.6 g L^{-1}), productivity (1.29 g L^{-1} h^{-1}) and yield was also compromised in presence of copper (Fig. 4.10).

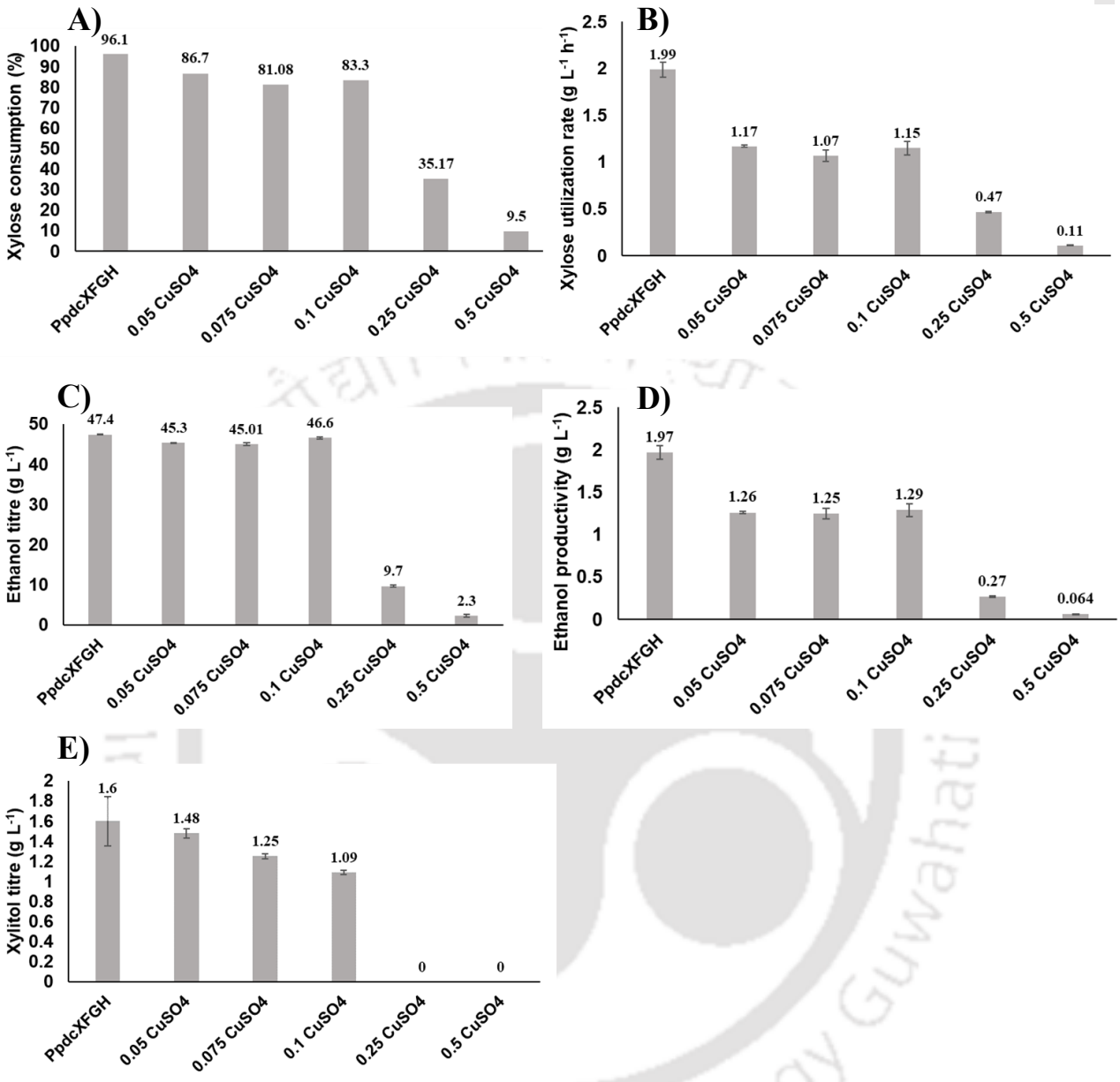


Fig. 4.10 Comparative analysis of **A)** extent of xylose consumption, **B)** specific xylose uptake rate (until glucose is present), **C)** ethanol titer, **D)** ethanol productivity, and **E)** xylitol titer by Zm-Ppdc-XFGH, when cultivated on mixed carbon source of 5% (w/v) glucose and 5% (w/v) xylose, in presence or absence of Cu^{2+}

No observable growth, substrate utilization or product formation was observed at a concentration of 0.5 mM CuSO4. Hence, supplementation of copper or zinc in the culture

medium of Zm-Ppdc-XFGH, to curtail xylitol production, did not transpire into a successful strategy.

4.3.5 Performance evaluation of Zm-Ppdc-XFGH under scale-up condition in a bioreactor

The performance of the selected transporter mutant strain, Zm-Ppdc-XFGH, was assessed, in terms of growth, substrate utilization and product formation, under scale-up condition. Zm-Ppdc-XFGH was cultivated in an automated 7.5 L bioreactor (Brunswick, Eppendorf, Germany) with a working volume of 3 L RM medium supplemented with 5% (w/v) of glucose and 5% (w/v) of xylose (Fig. 5.1). The maximum optical density (OD_{600}) of biomass, obtained for the selected strain was 8.1 corresponding to 3.8 g L^{-1} DCW (Fig. 5.1A), a marginal increase from air-tight bottle. Interestingly, the pH profile of the fermentation medium was observed to vary within the range of 5.8 to 4.2, which emphasized on the viability of Zm-Ppdc-XFGH in a range of different pH (Fig. 5.1A). Zm-Ppdc-XFGH completely consumed glucose within first 12 h of the cultivation period at a specific uptake rate of 2.6 g g^{-1} h^{-1} (Fig. 5.1B). 92% of the xylose, present in the fermentation medium was consumed with an overall specific uptake rate of 0.53 g g^{-1} h^{-1} (Fig. 5.1B). Similar to the response observed in air-tight bottle, 85.7% of the total xylose present in the medium was utilized within 18 h of the fermentation period, thus indicating a consequential decrease in the xylose fermentation time. The extent of co-utilization by Zm-Ppdc-XFGH was assessed by quantifying the specific xylose uptake rate during the dual sugar phase of fermentation, and it was recorded to be 2.1 g g^{-1} h^{-1}, comparable to that of glucose. Further, an increase in ethanol titer from 47.4 g L^{-1} obtained in airtight bottle to 48.5 g L^{-1} (Fig. 5.1C) was achieved with an overall ethanol productivity of 1.98 g L^{-1} h^{-1}. The maximum ethanol productivity was determined to be 4.01 g L^{-1} h^{-1} with a yield of 97% of the maximum theoretical yield. The xylitol titer for Zm-Ppdc-XFGH was quantified to be 1.44

g L^{-1} as opposed to 1.63 g L^{-1} of final xylitol concentration obtained when it was cultivated in 100 mL of medium in an airtight bottle (Fig. 5.1D).

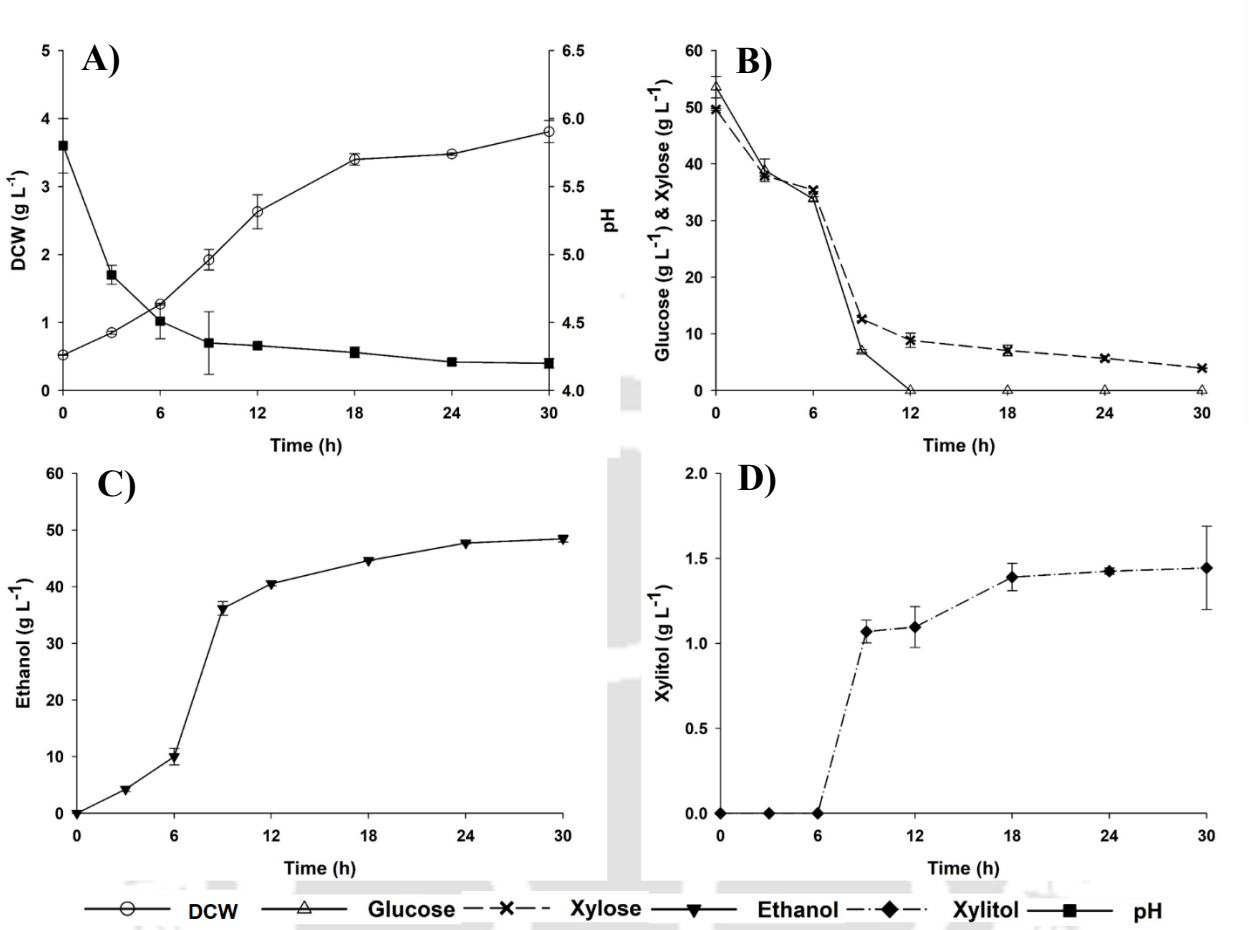


Fig. 4.11 Dynamic profiles of **A)** growth and pH, **B)** glucose utilization and xylose utilization, **C)** ethanol production, and **D)** xylitol production by Zm-Ppdc-XFGH when cultivated on mixed carbon source of 5% (w/v) glucose and 5% (w/v) xylose under scale up conditions

Comprehensive performance analysis of an industrial strain under scale-up condition is imperative before recognizing its potential at a commercial scale. Primarily, assessment of substrate utilization and product formation profiles of previously reported engineered xylose-fermenting *Z. mobilis* strains, depicted a sequential utilization of sugar with considerable by-product formation at bioreactor scale (Agrawal, Mao, & Chen, 2011; Gao, Zhang, Mcmillan, & Kompala, 2002; Mohagheghi, Evans, Chou, & Zhang, 2002; Viitanen et al., 2008). Although, complete consumption of glucose was prevalent for all these engineered xylose-

fermenting strains, incomplete xylose utilization was marked in certain instances (Mohagheghi et al., 2002; Viitanen et al., 2008). The extent of xylose utilization by these engineered strains was observed to be within a range of 60% to 96% of the total xylose concentration, supplemented in the medium. However, their respective specific xylose uptake rates were measured to be considerably lower compared to that of glucose, under scale-up conditions (Agrawal et al., 2011; Gao et al., 2002; Mohagheghi et al., 2002; Viitanen et al., 2008). This might be rationalized as a negative impact of absence of efficient xylose specific transporters in *Z. mobilis*. In addition, the ethanol yield was observed to vary significantly from 72% to 97% of the maximum theoretical yield in these strains, mainly due to significant by product formation. An evolved *Z. mobilis* strain, A3 exhibited satisfactory performance in terms of ethanol formation with an yield of 96.6% of the maximum theoretical yield, in a bioreactor set up; however, it was notably cultivated in lower working volume of cultivation medium (500 mL) (Agrawal et al., 2011). Detailed evaluation of the results in the ensued study, point towards a pragmatic outcome of a directed metabolic engineering strategy, that kindles into a prompt and augmented xylose flux towards enhanced ethanol biosynthesis mitigating the inhibition posed in presence glucose on xylose uptake, by the recombinant strains especially Zm-Ppdc-XFGH, which could be extrapolated to scale-up conditions.

4.4 Conclusion

Directed metabolic engineering was exploited for heterologous expression of xylose specific transporter genes namely *xylE*, *xylFGH* and mutated *xylE* (*xylE**) from *E. coli*, under the influence of two different promoters (*Ppdc* and *Pglf*), into evolved AD50 to improve its xylose uptake rate, in presence of glucose, and subsequent enhancement in ethanol production.

- ✓ The four engineered strains namely Zm-Ppdc-XE, Zm-Ppdc-XE*, Zm-Ppdc-XFGH and Zm-Pglf-XFGH showed enhanced xylose uptake rate as compared to AD50.

- ✓ The xylose uptake rate increased from 1.34 g g^{-1} h^{-1} to maximum of 2.04 g g^{-1} h^{-1} by Zm-Ppdc-XFGH.
- ✓ Increased xylose uptake rate was coupled with higher xylitol titer of 1.6 g L^{-1} for Zm-Ppdc-XFGH.
- ✓ Divalent metal ions (Zn^{2+} and Cu^{2+}) supplementation was used as a strategy to reduce xylitol production.
- ✓ Zn^{2+} supplementation could reduce xylitol titer from 1.6 g L^{-1} to 0.45 g L^{-1} but the rate and extent of xylose utilization was compromised.
- ✓ A 3 L culture of Zm-Ppdc-XFGH was cultivated in the fermenter.
- ✓ 92% of the total xylose and 100% of the total glucose, present in the medium, was utilized.
- ✓ The maximum ethanol productivity was 4.01 g L^{-1} h^{-1} with a titer of 48.5 g L^{-1}.
- ✓ The xylitol titer was observed to be 1.4 g L^{-1}.
- ✓ The pH of the media varied within a range of 5.8 to 4.2.

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CHAPTER 5
CONCLUSIONS

"It's more fun to arrive a conclusion than to justify it."

Malcolm S. Forbes, *American entrepreneur*

CHAPTER 5

Conclusions

Bioethanol has garnered considerable scientific attention as a prospective alternative to fossil resources, which are still exploited as primary energy sources. Lignocellulosic biomass based ethanol production, as an alternative to the conventional food crop based bioethanol production, indisputably stimulates sustainability. However, competitive alternative route of biomass based bioethanol synthesis from microorganisms had several impediments in terms of utilization of all biomass-derived sugars, before realizing this process at commercial scale. With the overall objective of developing a suitable and potential platform for efficacious utilization of two of the primary biomass derived sugars (glucose and xylose), the present study was executed with *Zymomonas mobilis* as the biological platform. Initially, two strains of *Z. mobilis*, namely *Z. mobilis* ATCC 31821 (ZM4) and *Z. mobilis* ATCC ZW658 (ZW658), were characterized in terms of their growth, substrate utilization and ethanol production on two types of carbon sources i.e. glucose and/or xylose. The aim of the strain characterization pertained to the aim of selecting a suitable platform for genetic manipulation directed towards simultaneous utilization of glucose and xylose. Although, ZM4 outperformed ZW658 in terms of rate & extent of glucose utilization and ethanol yield, titer, productivity, however, ZM4 being a wild type strain could not metabolize xylose. Directed metabolic engineering of crucial xylose assimilating genes *xylA* (codon optimized from *Lactobacillus brevis*) & *xylB* (from *E. coli*) and xylose metabolizing genes namely *talB* & *tktA* (both from *E. coli*) in ZM4 did not proved to prolific in terms of its xylose utilization ability. This could be reasoned as the unstable nature of the host plasmid used for the genetic manipulation and subsequent lack of stable expression

of the xylose metabolizing proteins. Hence, the selected strain i.e. ZW658, which is a recombinant xylose fermenting strain was considered for further modification towards efficient xylose utilization. ZW658 was subjected to extended Adaptive Laboratory Evolution (ALE) involving 50 transfers, carried out over a period of 200 days. The strain was grown under strict selection pressure of increasing xylose concentration starting from 30 g L^{-1} to 100 g L^{-1}. The evolved strain (designated as AD50) showed utilization of 42.73% of the supplemented xylose that corresponded to 42.73 g L^{-1} of xylose as compared to that of 14.66% i.e. 14.66 g L^{-1} of xylose in case of ZW658. AD50 could produce a maximum of 11.7 g L^{-1} of ethanol utilizing 42.73 g L^{-1} of xylose in media containing 100 g L^{-1} of xylose, as compared to 4.49 g L^{-1} in case of ZW658 in presence of 80 g L^{-1} of xylose. However, there was incomplete xylose utilization in all the concentrations tested. Thus, optimization of process parameters and growth conditions of AD50 led to further improvisation of the strain's performance. Addition of pure N_2 gas along with optimum nutrient mixing, showed drastic increase in overall rate of xylose utilization (0.53 g g^{-1} h^{-1}) by the strain and increased production of ethanol 49 g L^{-1} as compared to 12.65 g L^{-1} in case of ZW658, cultivated under similar cultivation conditions (10% (w/v) of xylose). As per the primary objective of this study, the performance of AD50 was evaluated in a dual substrate mixture of glucose and xylose, both supplemented in media at concentration of 5% (w/v), to analyse its efficiency towards co-fermentation of xylose in presence of glucose. AD50 exhibited 1.65 times increase in the overall specific xylose utilization rate when compared with the parent strain. It also exhibited one of the highest maximum specific xylose uptake rate of 6.9 g g^{-1} h^{-1}, when compared to other reported strains till date. Further, the strain displayed exceptional performance in terms of co-fermentation of xylose in presence of glucose with specific xylose and glucose utilization rate of 1.34 g g^{-1} h^{-1} and 1.24 g g^{-1} h^{-1}, respectively. Ethanol titer, productivity and yield was quantified to be 47 g L^{-1}, 1.96 g L^{-1} h^{-1}, and 0.49 g g^{-1}, respectively, which was remarkably better than that of ZW658. High throughput (Next-gen)

sequencing revealed novel mutations in xylose assimilating, metabolizing, and crucial regulatory pathway genes, which substantiate the exceptional phenotypic response of AD50 in terms of co-utilization of glucose and xylose. Enzyme activity assays were carried out to validate the performance of the strain with high confidence.

In our second objective, an array of xylose specific transporter containing plasmids were constructed using AD50 as host organism. XylE, the low-affinity xylose transporter from *Escherichia coli*, XylE* (a mutant variant of XylE), XylFGH, the ABC type transporter system from *E. coli*, was expressed in an evolved xylose-utilizing strain of *Z. mobilis* (AD50). Out of the six potential constructs developed, Zm-Ppdc-XFGH was considered as the best glucose xylose co-utilizing strain with highest specific xylose uptake rate of 2.04 g g^{-1} h^{-1} (in presence of glucose), which is 52.2% higher as compared to the parent strain AD50. Although, the rate and percentage of xylose utilization was observed to have increased for Zm-Ppdc-XFGH, the ethanol titer, yield and productivity was found to be similar as compared to AD50. This could be explained by the evidently increased xylitol titer in case of Zm-Ppdc-XFGH (1.63 g L^{-1}) as compared to AD50 (0.9 g L^{-1}). This could be the result of a shift in the xylose flux towards xylitol as opposed to ethanol. Cu^{2+} and Zn^{2+} which are known to inhibit xylose reductase (converts xylose to xylitol), were screened as a media supplement, to decrease xylitol production by the mutant *Z. mobilis* strain Zm-Ppdc-XFGH. 0.5 mM Zn^{2+} supplementation in the cultivation media of Zm-Ppdc-XFGH, reduced the formation of by-product i.e. xylitol (0.45 g L^{-1}) as compared to Zm-Ppdc-XFGH without zinc supplementation (1.62 g L^{-1}), however, the rate and extent of xylose utilization as well as the growth rate was found to be compromised. Similarly, growth, substrate utilization, the ethanol titer, productivity and yield was also compromised in presence of copper. Finally, the performance of the evolved and genetically manipulated strain, Zm-Ppdc-XFGH, was demonstrated in a bioreactor and evaluated in terms of commercial application and sustainability.

Engineering significance

Ethanol biosynthesis, though realized as a competent alternative to transport fuel in the 20th century, however, it has not met with significant industrial success all over the world and is hindered by major practically relevant bottlenecks. The primary obstacle being the inefficient utilization of pentose sugars (xylose & arabinose) from cheap alternative substrate to food crops i.e., lignocellulosic hydrolysate, especially in presence of hexose sugar (glucose). The ensued study ignites an anticipation towards a plausible step to overcome the obstruction, rendering the overall strategy industrially lucrative.

The major technological advances as proposed and demonstrated are:

- Development of an organically evolved ethanol producing *Z. mobilis* strain, with significant improvement in extent of xylose utilization and ethanol titer, yield, productivity through adaptive laboratory evolution based genetic engineering strategy.
- Elucidating the potential candidate genes involved in maneuvering the xylose metabolizing capability of the evolved strain. Differential activities of the key metabolic enzymes aided in decoding the major regulators responsible for transpired xylose metabolism and ethanol production, in lieu of addressing the void in unknown metabolic regulations.
- Annotating the effect of various xylose transporters under two different strong promoters, in the evolved *Z. mobilis* strain, towards co-utilization of xylose in presence of glucose. It helped to shed light on the best performing strain's potential of growth, substrate utilization and solvent production on dual carbon source, highlighting the significance and relevance of the approach undertaken towards future applicability of the strain for ethanol production via mitigating the expensive and conventionally used substrates for ethanol fermentation.
- The assessment of strain's performance in terms of glucose & xylose co-fermentation and subsequent production of ethanol in large scale set up, demonstrated in a bioreactor. The

steady and reproducible kinetic parameters marked an intriguing nucleation towards the industrial employment of the developed strain.

Future Prospects

Future scope of the work

The future prospects of this study include:

- ✓ Evaluating the performance of the engineered strain Zm-Ppdc-XFGH in lignocellulosic hydrolysate based media, obtained by hydrolyzing different types of low cost lignocellulosic biomass.
- ✓ Process engineering strategies can be explored to improve the substrate utilization rate coupled with enhance ethanol productivity.
- ✓ Techno-economic feasibility of the ethanol biosynthesis process.
- ✓ To unveil the mechanism behind the efficient dual substrate utilization by Zm-Ppdc-XFGH, transcriptomic and proteomic studies can be undertaken.

The present study is significant and can be extrapolated to industrial application for the production of ethanol, by utilizing lignocellulosic biomass as a sustainable low cost substrate.

List of Publications

Publications from thesis work:

1. **Sarkar, P**., Mukherjee, M., Goswami, G., & Das, D. (2019). Adaptive Laboratory Evolution induced novel mutations in *Zymomonas mobilis* ATCC ZW658: A potential platform for co-utilization of glucose and xylose. *Journal of industrial microbiology & biotechnology*
2. **Sarkar, P**., Goswami, G., Mukherjee, M & Das, D. (2021). Heterologous expression of xylose specific transporter improves xylose utilization by recombinant *Zymomonas mobilis* strain in presence of glucose. *Process Biochemistry*

Publications from collaborative work:

1. Mukherjee, M., **Sarkar, P**., Goswami, G., & Das, D. (2019). Regulation of butanol biosynthesis in *Clostridium acetobutylicum* ATCC 824 under the influence of zinc supplementation and magnesium starvation. Enzyme and Microbial Technology. (Equal author contribution)

List of Conferences / Workshop

List of conferences

1. **Payel Sarkar**, Mayurketan Mukherjee, Gargi Goswami and Debasish Das. "Adaptive evolution of *Z. mobilis* ATCC ZW658 directed towards efficient xylose utilization". DBT National Workshop on Bioenergy-2018, 6-7th July 2018, held at Indian Institute of Technology, Roorkee, India

2. **Payel Sarkar**, Gargi Goswami and Debasish Das. "Development of a metabolically engineered *Zymomonas mobilis* for efficient utilization of pentose sugar". Bioprocessing INDIA 2017, 9-11th December, held at Indian Institute of Technology, Guwahati, India. (Secured Best Poster)

3. **Payel Sarkar** and Debasish Das. "Metabolic Engineering of *Zymomonas mobilis* for efficient substrate utilization and enhanced ethanol production: A Review". Research Conclave 2017, held Indian Institute of Technology, Guwahati.

4. Anwesha Purkayastha Mayurketan Mukherjee, **Payel Sarkar**, Saumya Ahlawat, Mehak Kaushal and Debasish Das. "Unravelling the structure of Spo0A towards understanding its role in butanol biosynthesis from *Clostridium acetobutylicum*". 57th International Annual Conference of The Association of Microbiologists of India, held at Guwahati University, Guwahati, India.

5. **Payel Sarkar**, Anwesha Purkayastha and Debasish Das. "Different aspects of metabolic engineering of *Zymomonas mobilis* for substrate utilization". Reflux 2017, held at Indian Institute of Technology, Guwahati.

List of workshop

1. Received Certificate of appreciation for organizing a workshop on Polymerase Chain Reaction (PCR). Research Conclave (2016). Indian Institute of Technology Guwahati.

Vitae

The author was born in a Bengali household on 8th September, 1987 in Kolkata, West Bengal, India. She successfully completed her school education in the year 2006, appearing the higher secondary examination from B. D Memorial Institute School under Central Board of Secondary Education (CBSE). She completed her B. Tech and M. Tech from West Bengal University of Technology in the year 2010 and 2012, respectively.

Payel Sarkar joined the Ph.D. program under the supervision of Dr. B Anand in July, 2012 and later continued under the supervision of Prof. Debasish Das at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati in July, 2016. She successfully completed all the academic courses, examinations and presented her Synopsis seminar on 26th February, 2020 before the Doctoral Committee.