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Research Article

Genetically Modified *Escherichia coli* for High Yield Mixed Organic Acid Production on Lignocellulosic Biomass Derived Xylose

Poornima Rao[1], Lucy Nainan[1], Gunjan Prakash[1] and Arvind M Lali[2]*

[1]DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, A University under Section-3 of UGC Act 1956 and Maharashtra Govt.'s Elite Status and Grade `A' Centre of Excellence by MHRD, Matunga, Mumbai, India

[2]Department of Chemical Engineering, Institute of Chemical Technology, A University under Section-3 of UGC Act 1956 and Maharashtra Govt.'s Elite Status and Grade `A' Centre of Excellence by MHRD, Matunga, Mumbai, India

***Corresponding Author:** Arvind M Lali, Department of Chemical Engineering, Institute of Chemical Technology, A University under Section-3 of UGC Act 1956 and Maharashtra Govt.'s Elite Status and Grade `A' Centre of Excellence by MHRD, Matunga, Mumbai, India.

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Abstract

Lignocellulosic biomass deconstruction generates around 20 - 25 w/w% of hemicellulose derived xylose that must be exploited as a sugar platform for production of value-based chemicals. The absence of genes in the metabolic pathways for uptake and utilization of xylose in most microbes restricts its potential applicability to be used as carbon source. Rapidly evolving separation technologies dictate emerging preference for hetero-fermentation with high overall substrate based yields over lower yield homo-fermentations. *Escherichia coli* is known to metabolize both hexose and pentose sugars, and the present study describes construction and characterization of a double knock-out engineered mutant *E. coli* BW25113 *ΔadhE ΔldhA* for hetero-fermentative production of organic acids in > 90% yield on xylose majorly comprising of acetic acid. Fermentation parameters such as aeration, agitation, pH were observed to impact the xylose uptake rate, and consequently the acetate and total organic acid production rate. These investigative studies resulted in acetate yield and productivity of 0.51 ± 0.0027 g/g and 0.33 ± 0.0048 g/L.h; and a total organic acid yields and productivities of 0.92 ± 0.019 g/g and 0.61 ± 0.013 g/L.h on pure xylose. The modified strain demonstrated equivalent acetate yield of 0.51 ± 0.026 g/g and slightly reduced acetate productivity of 0.21 ± 0.011 g/L/h on lignocellulose derived xylose stream containing lignin, nitrate and furfural. Reduction in formate yield was possibly due to disproportion of formate to H_2 and CO_2 in the presence of nitrate in the lignocellulose derived xylose stream. Recovery of nitrate from the xylose fraction by employing a suitable filtration method can eliminate or reduce nitrate presence and provide higher formate yields. The study provides noteworthy insights on the practical applicability of lignocellulose derived xylose for commercial production of organic acids as important biochemical precursors.

Keywords: *Escherichia coli*; Lignocellulosic Biomass; Xylose; Organic Acids; Acetic Acid

Abbreviations

LBM: Lignocellulosic Biomass; NADH: Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H); NAD: Nicotinamide Adenine Dinucleotide; ATP: Adenosine Triphosphate; ADP: Adenosine Diphosphate; C: Carbon; LB: Luria Bertani; PCR: Polymerase Chain Reaction; vvm: Volume of Air Per Unit of Medium Per Unit of Time; rpm: Rotations Per Minute; DO: Dissolved Oxygen; PTFE: Polytetrafluoroethylene; HPLC: High Performance Liquid Chromatography; TOA: Total Organic Acids

Introduction

Organic acids are important chemical compounds having wide-ranging applications in the food, pharmaceuticals and chemical industries. Most organic acids are synthesized via the catalytic routes that largely depend on non-renewable petro-sources [1]. Low molecular weight organic acids such as formic, acetic, propionic, butyric etc. can also be produced via biological route as they are primary metabolites in microbial carbohydrate metabolism [2]. Of these, acetic acid is the largest and significant platform chemical with a global annual market of more than 15 million tons.

Currently, almost all of acetic acid is chemically synthesized via carbonylation of petro-derived methanol due to steep fall in natural gas price [3]. However, petroleum resources are not available with all countries and more importantly, there is an acute need today for developing competitive renewable routes to chemicals for climate change mitigation [4]. Lignocellulosic biomass (LBM) is abundantly available as a renewable and biodegradable, non-food substitute resource. On an average, an agricultural biomass comprises of 40 - 50% cellulose and 20 - 30% hemi-cellulose which by a combination of chemical and enzymatic actions can yield fermentable sugars majorly in the form of glucose and xylose in yields of 40 - 50g and 20g per 100g dry biomass, respectively [5]. Pre-treatment technologies can be designed for production of separate streams of the two sugars [6]. With biomass volumes being processed to the tune of several hundred tons per day at a given site, it is important that both C6 and C5 sugars are used to produce useful products. While glucose fraction is readily accepted as carbon substrate by most organisms, the pentose fraction presents a challenge. Effective utilization of the LBM derived xylose along with the glucose fraction would be an essential prerequisite to conceptualize the economics of any lignocellulose based bio-refinery process.

Conventional bio-production of acetic acid is a two-step process involving glucose fermentation using *Saccharomyces cerevisiae* for ethanol production as first step followed by oxidation of ethanol either catalytically or by *Acetobacter sp*. in the second step. The aforementioned traditional acetic acid fermentation restricts to glucose as the only carbon source for acetate production with glucose largely coming from corn or sugar industry. However, with expected emergence of biomass derived sugars in coming decades, use of C5 sugars for chemical production will gain importance. Thus, while C6 sugars may be used for a large variety of chemicals, it may become important to use concomitantly produced xylose for possible high volume chemicals even though limited by the inability of most microorganisms to use C5 sugar substrates. For example, with a significant global focus on production of second generation ethanol from LBM, the inability of *S. cerevisiae* to utilize pentose sugars led many a workers to construction of genetically modified xylose utilizing *S. cerevisiae* strains. However, while many constructs have been seen to result in low ethanol yields on xylose with xylitol obtained as the primary product, some have been more successful albeit these giving relatively low volumetric productivities [7-10]. On the other hand, non-*Saccharomyces* yeasts like *Scheffersomyces stipites*, *Candida shehate*, *Kluyveromyces marxiamus* etc. are also known to utilize pentose sugars for ethanol production but provide yields and productivities that are not enough for large scale ethanol production [11-13]. In such scenarios and from a commercial stand-point, it becomes imperative that xylose be used to produce chemicals other than ethanol. Organic acids, primarily C2 to C4 acids, may find better utilization of xylose sugars from biomass if produced in high yields. These facts and limitations of native yeast system with C5 sugars, have driven this work for engineering micro-organisms that can utilize pentose sugars and are able to produce organic acids.

Two step bio-production of acetic acid result in yields of around 50% w/w on sugars using the best *Acetobacter* strains reported with a large proportion of sugars being 'wasted' in metabolism [3,14,15]. *Moorella thermoacetica*, a homo-fermentative strain and has been reported to provide > 90% yields of acetic acid on sugars. However, being an extremely sensitive obligate anaerobe, its industrial scale up is extremely challenging. *Escherichia coli* is on the other hand, a robust gram negative facultative anaerobe known to readily utilize multitude of carbon sources including C5 sugars. On account of being hetero-fermentative *E. coli* employs mixed acid fermentation pathway producing acetate, lactate, ethanol, formate and succinate as end products. For *E. coli* to be used as a predominantly acetic acid producer at commercial scale, it is necessary to genetically modify the strain to channel bulk of carbon flux towards acetic acid.

In conjunction with metabolic engineering, fermentation process parameters need careful optimization to maximize the growth, organic acid profile, and production rate during scale-up. It is important to implement a rational fermentative approach to a metabolically engineered strain to improve the performance of the modified organism. Organic acid fermentations are governed by pH

control since pH influences multiple factors like NADH/NAD ratio as well as ATP levels and its rate of formation [16]. In addition, oxygen is also an important nutrient that influences micro-organisms growth, maintenance, and metabolite production [17-19].

This report is on the study that was focused on designing and characterizing the efficiency of a genetically engineered *E. coli* strain for mixed acids production on xylose. A parallel successful project on novel reactive separation of individual organic acids from fermentation broths indicated that production of mixed acids shall become lucrative on overall return on sugar basis [20]. The work was also aimed at developing scalable upstream fermentation strategies for the metabolically engineered *E. coli* strain using LBM derived xylose for enhanced production of mixed organic acids.

Materials and Methods

Bacterial strain, inoculum development and media components

E. coli BW25113 was used as the host strain. *E. coli* BW25113 ΔadhE (Single knock out) and *E. coli* BW25113 *ΔadhE ΔldhA* (double knock out) strains were engineered strains developed at the molecular biology laboratory of the DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai [21]. Cultures were grown in sterile Luria Bertani (LB) broth (Hi-Media Laboratories, Mumbai) at 37ºC and 200 rpm. The host and transformants *E. coli* were preserved as glycerol stock (50% v/v) at -80ºC.

The fermentation medium used for the studies was a modified M9 minimal medium suited for the growth and product formation using *E. coli* BW25113. Modified minimal medium included Na_2HPO_4 6 g/L; KH_2PO_4 3 g/L; NaCl 0.5 g/L; NH_4Cl 1 g/L; $MgSO_4.7H_2O$ 0.24 g/L; $CaCl_2.2H_2O$ 0.014 g/L; xylose between 10 - 20 g/L; and yeast extract 2 g/L. All chemicals were obtained from Hi-Media Laboratories, Mumbai, India. For inoculum development, revived culture in LB broth was transferred to the modified M9 minimal medium followed by incubation at 37ºC and 200 rpm. For bioreactor cultivation, 10% of the culture in modified M9 minimal medium was taken as inoculum.

Lignocellulosic biomass hydrolysate preparation

Lignocellulosic biomass derived C5 sugar hydrolysate samples were obtained using the in-house developed platform technology [22]. In this process, the lignocellulosic biomass is fractionated to components by a two-step acid-alkali hydrolysis. The xylose stream used in the study was obtained by treating comminuted rice straw residue (< 500 µm) at 5% biomass loading with 0.5% HNO_3. The reaction was carried out in a high pressure autoclave (Parr Instruments). Nitrogen was introduced into the reactor set up until a pressure of 10 bar was attained to avoid evaporation of nitric acid at high temperatures. Reactor was heated to around 130ºC and maintained at the set temperature for 30 minutes. The reaction mixture was then cooled and filtered through a nylon blotting cloth (BSS 400, 38 µm). The filtrate was thereafter neutralized with calcium carbonate and the supernatant was analyzed for carbohydrates using HPLC and was used as such for fermentations.

Construction of knockout mutants

E. coli BW25113 strain was used to generate deletion mutants. All bacterial strains and plasmids used to construct deletion mutants are listed in table 1. The λ-Red and flippase recombinase (FLP) mediated site-specific recombination method was used to create knockout strains [23]. Primers used for gene deletion and confirmation are listed in table 2. A template DNA was prepared by amplifying the kanamycin region from pKD13 using polymerase chain reaction (PCR). This amplified product containing the flanking regions of the gene to be knocked was purified and its concentration was estimated on a nanodrop spectrophotometer (Genova Nano, Jenway, UK). The strain containing the helper plasmid pKD46 was electroporated with the amplified PCR product. To confirm the gene deletion, colony PCR was performed using confirmation primers (locus specific primers and primers from within the kanamycin gene). The flippase recognition target (FRT) flanked antibiotic resistant gene was eliminated by transforming with pCP20 (which is temperature sensitive plasmid and has flippase recombinase). Colonies were grown at 43°C, purified and then screened for loss of all antibiotic resistances.

Strains/ Plasmids	Relevant characteristics	Reference
BW25113	$lacI^q$ rrn-B_{T14} $\Delta lacZ_{WJ16}$ hsdR514 $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	Lab stock
BW25113/*Δadh*	BW25113/ Δ*adhE*	This study
BW25113/*Δldh*	BW25113/ Δ*ldhA*	This study
BW25113/*Δldh Δadh*	BW25113/*ΔldhA ΔadhE*	This study
pKD46	*bla_exo* low-copy vector containing red recombinase and temperature-conditional pSC101 replicon	[23]
pKD13	Kan^R template plasmid	[23]
pCP20	Amp^R, Cm^R, FLP recombinance	[23]

Table 1: Strains and plasmids used to generate deletion mutants.

Sr. No.	Primers	Sequence (5'-3')
1	adhE del N	CAGATGATTTACTAAAAAAGTTTAA-CATTATCAGGAGAGCATTATG ATTC-CGGGGATCCGTCGACC
2	adhE del C	TTTATGTTGCCAGACAGCGCTACT-GATTAAGCGGATTTTTTCGCTTTTTT TGTAGGCTGGAGCTGCTTCG
3	ldhA del N	TTAGTAGCTTAAATGTGATTCAACAT-CACTGGAGAAAGTCTT ATG ATTCCGGGGATCCGTCGACC
4	ldhA del C	CCTGGGTTGCAGGGGAGCGGCAAGAT-TAAACCAGTTCGTTCGGGCA TGTAGGCTGGAGCTGCTTCG
5	Conf adhE N	AGGTCTGAATCACGGTTAGC
6	Conf adhE C	GCAGAAAGCGTCAGGCAGTGT
7	Conf ldhA N	GTTCTACCATGCCGACGTTCA
8	Conf ldhA C	TGCTGTAGCTGTTCTGGCGTAA
9	K1	CAGTCATAGCCGAATAGCCT
10	K2	CGGTGCCCTGAATGAACTGC

Table 2: Primer sequences used for gene deletion and confirmation.

Bioreactor cultivation

Batch fermentation runs were carried out in a 5L water cooled jacketed glass bioreactor (Biostat® B plus, Sartorius Stedim) equipped with turbidity (Fundalux II, Sartorius Stedim), temperature and pH probe (Easyferm Plus K8 200, Hamilton) for continuous monitoring of optical density, temperature and pH, respectively. The working volume for the fermentation runs was maintained at 2L at all times. The bioreactors were sterilized by autoclaving at 121ºC for 20 minutes. Reactor pH was controlled at 6.8 by automated addition of alkali (2N NaOH or aqueous ammonia). Fermentations were carried out at controlled temperature of 37ºC, with 0.325 vvm aeration and agitation of 200 rpm with standard Rushton turbine. Aeration was maintained by sparging sterilized compressed air using a polytetrafluoroethylene (PTFE) filters (Midistart 2000, Sartorius Stedim).

Process parameter optimization

Different aeration and agitation rate combinations of 0vvm/200rpm, 0.325vvm/250rpm, 0.625vvm/250rpm and 0.82vvm/300rpm were studied. The pH and temperature were maintained at 6.8 and 37ºC, respectively. For studying effect of pH, fermentation was carried out at uncontrolled pH, and also at different near neutral pH values of 6.2, 6.8 and 7.5 during which aeration and agitation maintained at 0.625vvm and 25rpm, respectively at a temperature of 37ºC. The optimized fermentation parameters study was conducted at a pH of 6.8, aeration of 0.625vvm, agitation of 250 rpm at 37ºC temperature.

Analytical methods

Sugars and organic acids in samples were estimated on High Performance Liquid Chromatography (HPLC) (Agilent 1100 series) with an Aminex 87H column (Bio-Rad Laboratories) at 65˚C and equipped with an RI detector (G1362A) at 35˚C with 5mM H_2SO_4 as mobile phase at the flow rate of 0.6 mL/min; with ChemStation/EZchrome software used for data processing. Concentrations were estimated using HPLC grade standards procured from Sigma-Aldrich. For sample preparation, samples were filtered through 0.2 µm pore sized nylon filters (Axiva Sichem Biotech, Delhi) to obtain a cell free filtrate.

Results and Discussion

Development of a double knock out *E. coli* BW25113 strain

E. coli BW25113, a K-12 strain, was chosen as host primarily for enhancing the yield of acetic acid on xylose. The K12 strain has an active acetate pathway and hence was preferred over the B type strain [24]. *E. coli* also has a native xylose assimilation pathway whereby D-xylose is converted into xylulose-5 phosphate, and further to glyceraldehyde-3 phosphate which enters the glycolysis pathway and is subsequently converted to acetyl-CoA (Figure 1). The theoretical yield of acetate on xylose is 0.67 g/g.

Figure 1: *E. coli* central metabolic pathway with *ldh* A and *adhE* knockouts, having acetate as the principal product from xylose metabolism (Orange indicates the pathways name. Bold red cross symbol indicates that the pathways have been blocked. Gene names as shown in italics- *ldhA*, lactate dehydrogenase; *pflA*, pyruvate formate lyase; *adhE*, alcohol dehydrogenase; *ptA*, phosphotransacetylase; *ackA*, acetate kinase).

E. coli BW25113 converts sugars to biomass and organic acids majorly in the form of acetate and formate, while ethanol yields are comparable to that of acetate [25]. The Acetyl-CoA node is an important high energy intermediate pool in the central metabolic pathway that controls the ratio of flow of flux towards different metabolites. To channelize the flow towards acetate production from Acetyl CoA (AcCoA node), the competing pathways need to be down-regulated or deleted. The alcohol dehydrogenase gene deletion mutant (*adhE* gene knock out) was thus constructed using the lambda red recombination strategy [23]. It has been previously reported that in BW25113 Δ*adhE* mutant the acetate yield was seen to be more than 200 times that of alcohol indicating that partition of flux at AcCoA node is indeed channeled towards acetate production [26].

Depleted oxygen conditions favor the production of lactic acid at the pyruvate node. Therefore, in order to prevent lactate accumulation, genes leading to formation of lactate were also deleted. Thus, the double deletion mutant *E. coli* BW25113 *ΔadhE ΔldhA,* with alcohol and lactate dehydrogenase deletion (Figure 1) was created with simultaneous antibiotic marker removal making it a marker free strain [21].

Batch fermentation profile was checked to ascertain the effect of double knockout mutant *E. coli* BW25113 *ΔadhE ΔldhA*, for growth and organic acids production. Fermentation was carried out on glucose and xylose independently.

Comparison of organic acid yields and productivities on glucose and xylose

E. coli BW25113 *ΔadhE ΔldhA* was evaluated for its efficiency to grow and produce organic acids on glucose and xylose to compare the performance on both the substrates and determine the suitability of xylose as an alternative to glucose. Figure 2 depicts the organic acid yields attained on glucose and xylose as substrates. Figure 3 represents the organic acids productivities and substrate uptake rates on glucose and xylose. With glucose, the acetate, formate and succinate yields were 0.53 g/g, 0.28 g/g and 0.03 g/g respectively, while the acetate and total organic acid productivities attained were 0.22 g/L/h and 0.35 g/L/h, respectively. Xylose gave the acetate, formate and succinate yields of 0.50 g/g, 0.35 g/g and 0.058 g/g, respectively, while the acetate and total organic acid productivities achieved were 0.19 g/L/h and 0.35 g/L/h. Yields and productivities of acetate and formate on xylose were comparable to respective yields and productivities on glucose. The substrate uptake rate of 0.382 g/L/h on xylose was also comparable to that of glucose at 0.408 g/L/h. Fermentation process parameters were thereafter optimized for organic acids production potential of the double knock out mutant on xylose.

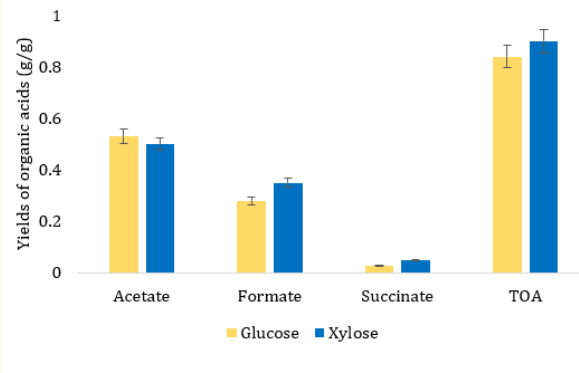

Figure 2: Yields of acetate, formate, succinate and total organic acids (TOA) on glucose and xylose individually as carbon source using *ldhA* and *adhA* knockout *E. coli*; (Yellow bar, yields on glucose; Blue bar, yields on xylose).

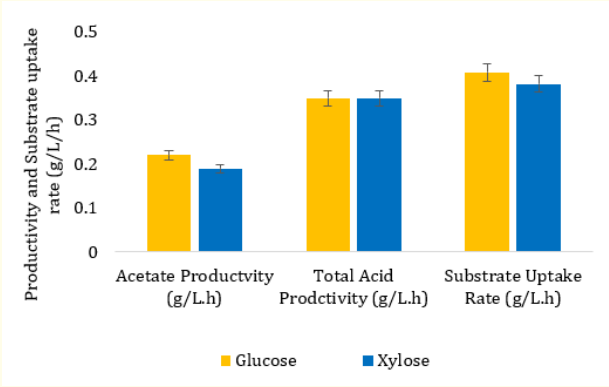

Figure 3: Acetate and total acid productivities and substrate uptake rates of *ldhA* and *adhE* knockout *E. coli* on glucose and xylose (Yellow bar, productivities on glucose; Blue bar, productivities on xylose).

Effect of aeration/agitation on organic acid formation

E. coli BW25113 *ΔadhE ΔldhA* is a double knock out mutant in which both the knocked-out genes code for dehydrogenases (*ldhA*

and a*dhE*) that play a vital role in re-oxidising the reducing equivalents generated during glycolysis. On account of deletion of these genes, provision of external electron acceptors becomes essential for maintaining the redox balance at the pyruvate node. Oxygen is one of the primary terminal electron acceptors in aerobic fermentation, and which can be provided by aerating the fermentation medium. Aeration and agitation thus become critical for this double knock out strain.

Increasing aeration and agitation speed reduced fermentation run time indicating a strong correlation between these process parameters and substrate uptake rate. Figure 4 and 5 illustrate the effect of aeration/agitation on organic acid yields and productivities, respectively. Highest overall organic acids yield (0.91 g/g) and productivity (0.66 g/L/h) were attained with the xylose uptake rate of 0.654 g/L/h at 0.625vvm aeration and agitation speed at 250 rpm. The over-all fermentation run time was thus reduced significantly under these conditions. A further increase in the aeration and agitation speed led to increase in substrate uptake rate (0.715 g/L/h), but negatively affected the acetate and formate yields (Figure 4). Acetate and formate are major products of incomplete oxidation of sugars in presence of limited oxygen. The probable reason for reduced acetate and formate yields at higher oxygen levels is due to oxic conditions in the fermentation broth which leads to conversion of pyruvate to acetyl- CoA and CO_2 in the presence of pyruvate dehydrogenase which is active only under oxic conditions. These results are in unison with the earlier reports that describe oxygen as one of the important nutrients in fermentation [17-19]. In view of the acids yields and productivities, 0.625 vvm aeration and 250 rpm agitation was considered optimum for batch studies with the engineered *E. coli* for further process augmentation.

Figure 4: Effect of aeration/agitation on acetate, formate and succinate yields in *ldhA* and *adhE* knockout *E. coli* (Red bar, Acetate yield; Green bar, Formate yield; Light blue bar, Succinate Yield).

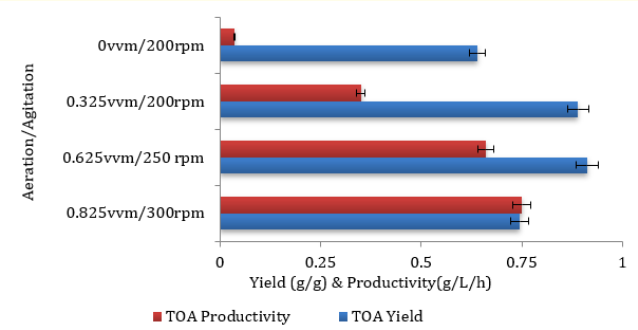

Figure 5: Effect of aeration/agitation on total organic acid yields and productivities of *ldhA* and *adhE* knockout *E. coli* (Red bar, Total organic acid productivity; Blue bar, Total organic acid yield).

pH studies

The pH value of the fermentation broth is known to play a critical role in dictating the organic acid profile and governs cell growth rate and substrate uptake take. Organic acid fermentation leads to drop in the pH that is offset by the addition of appropriate base to run the fermentation at near neutral pH. Neutral pH however leads to the formation of acid salts which results in added downstream steps for recovery of the organic acids. Acids if produced at low pH in un-dissociated form bring about ease in downstream processing which can reduce the over-all process cost [26]. Thus, fermentation was attempted at uncontrolled pH and the metabolic profile was studied under uncontrolled pH conditions. Drop in pH below 5.1 negatively affected the xylose uptake and led to growth cessation. The primary reason for this is diffusion of organic acids in their un-dissociated form into *E. coli* cellular membranes which leads to drop in the internal cytoplasmic pH [27-29]. This resulted in incomplete utilization of xylose and lowered the overall organic acid yield to 0.596 g/g and productivity to 0.13 g/L/h, which was 52% and 400% lower than at fermentation run with pH controlled at 6.8.

The fermentation runs were then carried at different controlled pH conditions by addition of alkali to maintain the required pH. From Figure 6 it can be noted that highest individual acid yields of on xylose was attained at pH 6.8 and 7.5 with no residual xylose. It can also be noted that the acetate yield at uncontrolled pH was marginally higher when compared to conditions at which pH was maintained. But this difference in acetate yield can be considered irrelevant in the light of very low substrate consumption under uncontrolled pH conditions. Formation of other acids was

also negatively affected at this uncontrolled pH condition. Figure 7 represents the total organic acid yields and productivities attained at different pH conditions. In view of complete xylose consumption, (Figure 6) pH 6.8 was considered as optimum for highest total organic acid yield and productivity obtained (Figure 7). However, it was noted that fermentation carried out at pH values lower than 6.8 resulted in reduced formate yields, and consequently the total organic acid yields and productivities were also reduced. This is possibly due to the induction of formate hydrogen lyase (FHL) which in turn is responsible for the disproportionation of formate to CO_2 and H_2. Transcription of FHL complex has been reported to be dependent on acidic pH in the growth medium and on the presence of formate and is not active at pH above 6.8 [29].

Figure 6: Organic acid production and xylose consumption at different pH in ldhA and adhE knockout *E. coli* (Red bar, Acetate yield; Green bar, Formate yield; Light blue bar, Succinate yield; Violet closed triangle, residual xylose concentration).

Figure 7: Total organic acid yields and productivities attained on fermentation at different pH conditions using *ldhA* and *adhE* knockout *E. coli* (Blue bar, Total organic acid yield; Red bar, Total organic acid productivity).

The metabolic profile of genetically engineered *E. coli* BW25113 *ΔadhE ΔldhA* (double knock out mutant) attained at optimized conditions of pH 6.8, 0.625vvm aeration and 250rpm agitation is represented in figure 8. The yields and productivities of 0.51 ± 0.0027 g/g and 0.33 ± 0.0048 g/L.h for acetate and 0.92 ± 0.019 g/g and 0.61 ± 0.013 g/L.h for total organic acids were achieved at these optimized conditions. The acetate yield attained was 76% that of the theoretical yield (0.67 g/g). The specific growth rate (μ) of the engineered strain was 0.603 h^{-1} with a generation of time of 1.10h. It was witnessed that the organism had the ability of re-utilizing the organic acids on complete consumption of xylose to maintain the cell growth and viability. The assimilation of acetate and formate after xylose consumption is due to the reversibility of pyruvate formate lyase (PFL) reaction which is also responsible for acetate and formate formation under anaerobic conditions [30]. Two strategies were therefore of interest: (a) Stopping fermentation just as xylose gets to complete depletion, or (b) operate the system in a continuous flow mode. A continuous fermentation system supplemented with a microfiltration membrane module for cell recycle and continuous removal of products is considered as a viable way to control and maintain the system under optimal operating conditions preventing the aforesaid problem of product re-utilization [31].

Figure 8: Growth and metabolic profile of genetically modified *E. coli* at optimized process conditions using *ldhA* and *adhE* knockout *E. coli.* (Blue closed diamond, xylose concentration; Red closed square, acetate concentration; Green closed triangle, formate concentration; Light blue closed circle, succinate concentration; orange closed diamond, Turbidity).

The carbon distribution at optimized cultivation conditions was evaluated based on yields of individual acids obtained on xylose as substrate and is represented in figure 9. It was calculated that

of the total of 0.369 moles of carbon contributed by xylose, 0.188, 0.09 and 0.018 moles of carbon were distributed between acetate, formate and succinate production, respectively. Around 0.026 moles of carbon were lost in the form of CO_2, while un-accounted carbon moles (0.047) can be attributed to biomass production.

Figure 9: Carbon balance based on the yields of acids obtained at batch fermentation mode (Red, Acetate; Green, Formate; Light blue, succinate; Violet, CO_2; Yellow, biomass).

To the best of our knowledge, this is the first study of a genetically engineered *E. coli* strain and its fermentation optimization on xylose for organic acids, majorly acetate production. Causey., *et al*. in 2003 reported an engineered homo-acetate strain *E. coli* TC36 constructed by multiple gene deletions in a sequential format that deactivated oxidative phosphorylation (Δ*atpFH*), disordered tricarboxylic acid pathway (Δ*sucA*) and removed the native fermentation pathways (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ *adhE*). The strain produced acetate at 68% of theoretical yield (0.67g of acetate/g) on glucose in batch mode. With modification in the nutrient feeding approach the yield was improved to around 86% of theoretical yield 0.57g of acetate/g of glucose [32]. The remainder carbon was diverted towards growth or lost in the form of CO_2. In comparison, the marker free strain constructed in this study had much limited gene deletions, was adapted and characterized for acetate production on xylose with a yield of 0.51 g/g of xylose. Bulk of the balance carbon was diverted towards formate production instead of CO_2 liberation and gave a total acid yield of 0.92 g/g. Thus, in terms of sugar value and carbon economy, the engineered strain reported in the present study performs better on a C5 sugar. And although a mixed acid stream is obtained, individual acids can be recovered using the indigenously developed patented separation technology from this laboratory [20]. This integrated technology results in the formation of mixed acid esters directly from the fermentation broth. These esters can be easily separated to high purities by fractional distillation before being hydrolyzed to respective acids in high yields and purities.

Lignocellulosic biomass (LBM) derived xylose hydrolysate study

Lignocellulosic biomass sugars majorly comprise of hexose sugars in the form of glucose, and pentose sugars in the form of xylose. Using another indigenously developed technology, rice straw can be fractionated in two step acid (0.5% nitric acid) and alkali (12.5% ammonia) treatment, followed by enzymatic hydrolysis, results in generation of a glucose stream, and a xylose rich stream that also contains ~ 0.45% lignin. With glucose being a universal substrate, the focus of this study was on effective utilization of the xylose rich stream. The 0.5% HNO_3 treated LBM derived xylose rich hydrolysate fraction was obtained from comprised of 0.7 - 0.8% xylose, 0.4 - 0.45% lignin, 0.25% nitrate and less than 0.001% furfural. In order to assess the suitability of the hydrolysate for acetate production, the developed strain was adapted on this lignin and nitrate containing xylose stream.

The modified *E. coli* strain could grow under previously optimized conditions for pure xylose, and also produce acetate on the LBM derived xylose hydrolysate in the presence of compounds like lignin and nitrate. Figure 10 depicts the metabolic profile obtained using LBM derived hydrolysate as sole carbon source. Figure 11 illustrates the comparative yields and productivities of acetate on commercial and LBM derived xylose. Acetate yields using the LBM xylose hydrolysate (0.51 g/g) was equivalent to that of acetate yield obtained using commercial xylose (0.51 g/g). However, presence of lignin and nitrate did affect the xylose uptake rate (0.406 g/L/h, compared to 0.654 g/L/h control) which reflected in reduced acetate productivities (Figure 11). The lower acetate productivity in LBM hydrolysate (0.21 g/L/h as compared to pure xylose control, 0.33 g/L/h) can be attributed to presence of potent inhibitors like phenolic compounds, lignin monomers, furfural, aldehydes and nitrate that can affect the cell growth and sugar uptake [33,34]. Growth inhibition of *E. coli* due to presence of lignin derived aromatic like vanillic acid, catechol and vanillin has been recently reported [35]. The extent of inhibition by aromatics depends on the concentration of the lignin monomers. However, the exact mechanism of inhibition is not known.

Figure 10: Metabolite profile of double knockout mutant of *E. coli* using LBM xylose hydrolysate in batch mode (Blue closed diamond, xylose concentration; Red closed square, acetate concentration; Light blue closed circle, succinate concentration).

Figure 11: Comparative analysis of yields and productivities of acetate on LBM derived and commercial xylose using a double knockout mutant of *E. coli* (Blue bar, acetate yields; Red bar, acetate productivities).

Formate production was found to be completely inhibited in *E. coli* grown in LBM derived xylose hydrolysate. The possible explanation for absence of formate production could be the presence of nitrate in the xylose fraction. It has been reported that in presence of nitrate, *E. coli* opts for nitrate respiration [28]. The FDH-N enzyme is abundant when the cells are grown anaerobically in the presence of nitrate and the enzyme is consequently responsible for formate cleavage to CO_2 as highlighted in the figure 12. This was further confirmed by growing *E. coli* in presence of commercial xylose supplemented with 40 mM ammonium nitrate (equivalent to nitrate in xylose hydrolysate). Metabolite profiling with commercial xylose in presence of nitrate also showed the absence of formate formation (Figure 13), providing an experimental confirmation of nitrate inhibition. The nitrate in the LBM xylose hydrolysate originates from using nitric acid treatment for pretreating the biomass. Removal of nitrate from the hydrolysate by some means can help formate formation and restore total organic acids yields.

Figure 12: Fate of formate in presence of nitrate and under acidic conditions. PFL: Pyruvate formate lyase; FHL: Formate hydrogen lyase; FDH-N: Formate dehydrogenase-N.

Figure 13: Metabolite profile of *ldhA* and *adhA* knockout *E.coli* using commercial xylose with 40 mM nitrate (Blue closed diamond, xylose concentration; Red closed square, acetate concentration; Green closed triangle, formate concentration; Light blue closed circle, succinate concentration).

Conclusion

This study is a first of its kind wherein, a genetically modified *E.coli* strain has been grown on the LBM derived xylose fraction for acid production, paving path towards potential realization of the concept of LBM based biorefinery capable of manufacturing multiple products from separated sugar and lignin streams from any given biomass. Hetero-fermentative production of mixed organic acids majorly comprising of acetate was established using a genetically engineered *E. coli* strain BW25113 *ΔadhE ΔldhA* on xylose. Fermentation process parameters affecting the organic acids yields and productivity were optimized for this strain. A total mixed acid yield in excess of 0.90 g/g xylose at productivities that hold promise for further enhancement through advanced fermentation strategies was attained using pure xylose as a substrate. LBM derived xylose as substrate resulted in comparable acetate yields, however, presence of nitrate in the fraction, affected the formate yields. Removal of nitrate from the LBM xylose fraction can help restore the total organic yields comparable to that on pure xylose. Novel separation technologies that are evolving for cost effective and scalable separation and recovery of individual organic acids from mixed acids broth will ensure the process to be commercially lucrative.

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Author's Contribution

Experiments were planned by PR, LN, GP and AML. Experiments were performed by PR and LN. Data was analysed by PR, LN, GP and AML. Manuscript is written by PR, GP, LN and AML. Funding for the work was facilitated by AML.

Conflict of Interest

None.

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