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Microbial Utilization and Bioconversion of Lignocellulosic Hydrolysates

Cumulative Dissertation

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Dedicated to my family,

from ten thousand miles away

Abstract

Lignocellulosic biomass is the most abundant bio-resource on earth containing carbohydrates polymers cellulose, hemicellulose and lignin. With a number of methods including pretreatment and hydrolysis, lignocellulose-derived hydrolysates containing D-glucose, D-xylose, L-arabinose, and further sugars can be obtained. The hydrolysates consisting fermentable sugars typically display a high degree of variation depending on both the biomass source materials and process conditions, resulting in different compositions such as various concentrations of monosaccharide sugars and inhibitors.

The fermentable sugars in lignocellulosic hydrolysates have attracted many people's attention all over the world, because these fermentable sugars have a great potential to be used by microorganisms as a sole carbon source for the growth and production of bioproducts. To date, several bioproducts such as biofuels, chemicals and many high value-added products have been already investigated and produced, and the application field is still expanding. However, one of the key obstacles is that not all the microorganisms have the ability to metabolize all the different sugars in hydrolysates. Most of the known microorganisms only utilize D-glucose as the sole carbon source, which results in the energy loss of lignocellulosic biomass. Another obstacle is that the inhibitors formed during hydrolysis, limit the efficient usage of hydrolysates as a carbon source for biotechnological conversion.

To further investigate and overcome these obstacles, in this Ph.D. thesis, the following research works have been examined and summarized:

1) Investigation the sugar utilization of *Escherichia coli* strains and the production of α_{s1}-casein proteins based on lignocellulosic hydrolysates.
2) Metabolic engineering of *Pseudomonas putida* KT2440 as microbial biocatalyst for the utilization of D-xylose and L-arabinose.
3) Evaluation of different lignocellulosic hydrolysates as substrates for engineered *P. putida* KT2440 and the inhibitory effectiveness on *P. putida* KT2440.

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List of Abbreviations

α	alpha
aldA	gene encoding dehydrogenase A
araA	gene encoding L-arabinose isomerase
araB	gene encoding L-ribulokinase
araD	gene encoding L-ribulose-5-phosphate-4-epimerase
araBAD	operon including genes *xylA* and *xylB*
AFEX	Ammonia fibre/freeze explosion
ATP	Adenosine triphosphate
β	Beta
bp	Base pairs
CC	cattle casein (bovine casein)
DNA	Deoxyribonucleic
et al.	*et al. et alii* (Latin for “and others”)
eGFP	enhanced green florescent protein
E. coli	*Escherichia coli*
HC	human casein
His	Histidine

HMF	hydroxymethylfurfural
*Hind*III	a type II site-specific deoxyribonuclease restriction enzyme isolated from *Haemophilus influenzae*
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
KDa	Kilo Dalton
L	Liter
LB	Lysogeny broth
min	Minute
ml	Milliliter
mM	Millimolar
N-terminal	Amino-terminal end of a protein
*Nde*I	an endonuclease isolated from *Neisseria denitrificans*
OD	Optical density
PAGE	Polyacrylamide gel eletrophoresis
PPP	Pentose Phosphate Pathway
pH	The negative logarithm of the activity of the solvated hydronium ion
P.putida	*Pseudomonas putida*
Ptac	Tac promoter
RBS	ribosomal binding site
SDS	Sodium dodecyl sulfate
SSF	solid-state fermentation systems
SmF	submerged fermentation systems

T7	T7 RNA polymerase is and RNA polymerase from the T7 bacteriophage
talB	gene encoding transaldolase
tktA	gene encoding transketolase
*xho*I	Site-specific dexoyribonuclease restriction enzyme isolated from *Xanthomonas holciola*
*xba*I	a restriction enzyme isolated from the bacterium *Xanthomonas badril*
xylA	gene encoding xylose isomerase
xylB	gene encoding xylulokinase
xylAB	operon including genes *xylA* and *xylB*
yagF/hijhG	gene encoding D-xylonate dehydratase
yagE/hijhH	gene encoding 2-keto-3deoxy-D-xylonatedehydratase
Y_{xls}	biomass to substrate yield
μ	growth rate
μ_{max}	specific growth rate

1. Introduction

1.1. Lignocellulosic biomass

1.1.1. Lignocellulosic materials

Lignocellulosic feedstock is an abundant, renewable, sustainable and inexpensive energy source existing in the form of wastes in forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries[1,2,3,4,5]. These lignocellulosic materials (wastes) accumulate in large quantities every year, causing environmental problems[6]. In general, lignocellulosic biomass can be considered as the mass of organic material from any biological material. Therefore, there are a variety of lignocellulosic biomass resources available on our planet. These may include intact plants, plant parts (seeds, stems), plant ingredients (starch, lipids, proteins and fibre), processing by-products (distiller's grains, corn solubles), marine sources and animal by-products, municipal and industrial wastes[7,8] , as shown in Table 1.

And in all the biomass resources, straw residues (corn, wheat and rice) from agriculture residues dominate in terms of tonnage and can serve as global available feedstocks. But straw quantity varies substantially within regions, depending on climates (temperature, humidity, sunshine and availability of water)[9]. According to the data for biomass (e.g., crop production, yield, harvested area, etc.) from FAO statistics (FAOSTAT), the average values from 1994 to 2018 show that the US is the largest producer of corn. For rice, the main producers are located in Sourthern and Sourth-Eastern Asia: China, India, Indonesia, Bangladesh, and Vietnam. For wheat,

main producers are located in Southern Asia, Eastern Europe, Northern America and Eastern and Central Asia. For the European Union, wheat is by far the more dominant crop, compared to rice (http://www.fao.org/faostat/en/#data/QC/visualize). Therefore ,in Europe, wheat straw is regarded as one of the primary feedstocks for the bio-based economy given the large amount of straw produced each year[10].

Table 1 **Typical types of lignocellulosic feedstocks.** (according to Howard R. L. et.al., 2003[11])

Lignocellulosic material	Residues
Grain harvesting Wheat, rice, oats barley and corn	Straw, cobs, stalks and husks
Processed grains Corn, wheat, rice, soybean	Waste water, bran
Fruit and vegetable harvesting/processing	Seeds, peels, husks, stones, rejected whole fruit and juice
Sugar cane other sugar products	Bagasse
Oils and oilseed plants Nuts, cotton seeds, olives, soybean etc.	Shells, husks, lint, fibre, sludge, presscake, wastewater
Animal waste	Manure, other waste
Forestry-paper and pulp Harvesting of logs	Wood residuals, barks, leaves etc.
Saw-and plywood waste	Woodchips, wood shavings, saw dust
Pulp & paper mills	Fibre waste, sulphite liquor
Lignocellulose waste from communities	Old newspapers, paper, cardboard, old boards, disused furniture
Grass	Unutilised grass

The traditional uses of these lignocellulosic materials include animal feed, soil conditioner, compost, and fertiliser, some seeds for oil extraction, reuse or recycle in industries and burnt as fuel. Unfortunately, for both developing and developed countries, most of the lignocellulosic “waste” usually ends in biomass burning, which in turn causes environmental pollution problems[12]. As a result, it has attracted considerable attention as an alternative feedstock and energy resource. These raw materials can be used to create new biomaterials, which will require an intimate

understanding of the composition of the raw materials (whether it is whole plant or constituents) to help obtain the desired functional elements for bio-product production with a considerable economic potential for biotechnology industry.

1.1.2. Chemical composition of lignocellulose

Lignocellulose is the fibrous material that forms the cell walls of plants 'architecture'. Principally, cellulose forms a skeleton surrounded by hemicellulose and lignin[13] , as shown in Figure 1. It is mainly composed of three polymers: cellulose, hemicellulose and lignin[2]. Moreover, cellulose is usually the major structural polysaccharide (35-50%) of plant cell walls, followed by hemicellulose (20-35%) and lignin (10-25%)[14,15].

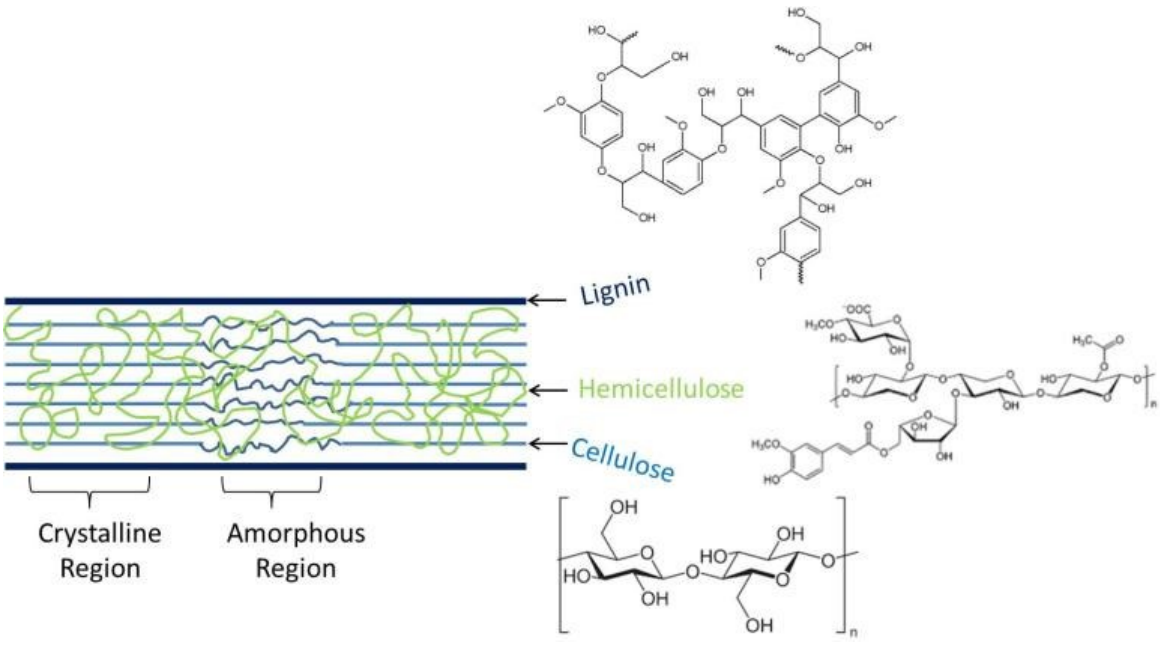

Figure 1 **Chemical composition and structure of lignocellulose**. It shows cellulose, hemicellulose and lignin fractions. (according to Bohdan Volynets et.al., 2016)

Cellulose is a high molecular weight linear homopolymer of cellobiose repeating units (two anhydrous glucose rings linked by a β-1,4 glycosidic bonds)[16], D-glucose is the only component in cellulose. Long-chain cellulose polymers are linked together by hydrogen and van der Walls bonds, which results in the cellulose being packed into microfibrils[17]. By forming these hydrogen boundaries, the chains tend to align in parallel and form crystalline structures. Therefore, cellulose microfibrils have both highly crystalline regions and less ordered amorphous regions[18,19,20,21]. More ordered or crystalline cellulose has poorer solubility and poorer degradability[22,23].

Hemicellulose is a linear and branched heterogeneous polymer usually composed of five different sugars: D-xylose, L-arabinose, D-glucose, D-galactose, D-mannose and other components such as acetic, glucuronic, and ferulic acids[24,25]. The backbone of the hemicellulose strand can be a homopolymer or a heteropolymer[26]. Depending on the major sugar residue in the backbone, the hemicelluloses have different classifications such as xylan, mannan, glucan, glucuronoxylan, arabinoxylan, glucomannan, galactomannan, galactoglucomannan, β-glucan, and xyloglucan[27]. Hemicelluloses differ from cellulose in the composition of sugar units, the presence of short chains, the branching of backbone molecules, and to be amorphous, making their structure easier to hydrolyse than cellulose[28].

Lignin is a very complex molecule, composed of units of phenylpropane, with a large three-dimensional structure connected. Three kinds of phenyl propanol exist as lignin monomers: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is tightly bound to cellulose and hemicellulose and serves to provide rigidity and cohesion to the material cell wall[29,30,31,32]. Because of its molecular configuration, lignin is extremely resistant to chemical and enzymatic degradation[33].

As a result, lignocellulose biomass contains approximately 75% of polysaccharide sugars, which makes it a potential enormous biotechnological value [34,35]. However, the content of carbohydrate polymers and lignin varies by plant species[36,37,38]. In addition, the proportions of individual plant components may also vary with age, growth stage, and other conditions. For example, hardwoods have greater amounts of cellulose, whereas wheat straw and leaves have more hemicellulose[39]. Herbaceous plants such as grasses have the lowest contents of lignin, while softwoods have the highest lignin contents. Specific to monosaccharides, the average values of the main sugar components in some lignocellulosic wastes are estimated and shown in Table 2, according to the study on bioethanol production from lignocellulosic biomass (https://www.slideserve.com/betty_james/bioethanol-production-from-lignocellulosic-biomass) and the research from Jorgensen et al.,2007[2].

Table 2 **Composition of main monosaccharides sugars in various lignocellulosic biomass**.

Raw Material	Glucose (%)	Xylose (%)	Arabinose (%)	Mannose (%)
Beet pulp	24.1	18.2	1.5	4.6
Birch	38.2	18.5	ND*	1.2
Corn stover	35.6	18.9	2.9	0.3
Cotton gin	37.1	9.4	2.3	1.1
Eucalyptus	49.5	10.7	0.3	1.3
Hybrid poplar	44.7	14.6	0.8	2.2
Pine	46.4	8.8	2.4	11.7
Rice straw	34.2	24.5	ND*	ND*
Rice hulls	38.1	14.0	2.6	3.0
Rye grass	23.9	14.7	2.8	ND
Spruce	43.4	4.9	1.1	12.0
Sugarcane bagasse	38.1	23.3	2.5	ND*
Switch grass	31.0	0.4	2.8	0.2
Wheat straw	38.2	21.2	2.5	0.3
Willow	43.0	24.9	1.2	3.2

* not determined

1.1.3. Lignocellulose hydrolysate

Lignocellulose will become an important source as fermentable substrate in the near future, because it is composed of up to 75% carbohydrates[35,40]. However, in order to use lignocellulose as a carbon source for biotechnological processes it usually has to be depolymerized since most industrially used microorganisms are not able to metabolize this compact and complex polymer. Hence, the key issue is the conversion of carbohydrates from lignocellulosic feedstocks into fermentable sugars. There are two major categories of methods employed. The fist and older method is chemical hydrolysis, which uses acids as catalysts, while the second uses enzymes called enzymatic hydrolysis[41]. The general concept of enzymatic hydrolysis involves a pretreament step that increases the digestibility of the substance, followed by enzymatic hydrolysis to release the monosaccharides[42,43], as shown in Figure 2.

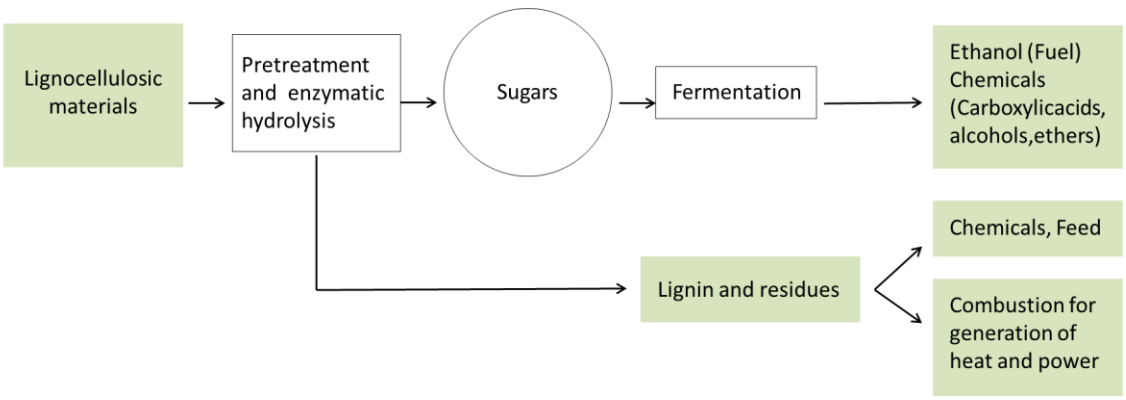

Figure 2 **Overview of the integral enzymatic hydrolysis process of lignocellulosic materials**. (according to Alessandra Verardi et.al., 2012[41])

The goal of any pretreatment technique is to break down the lignin structure, disrupt the crystalline structure of cellulose and increase the porosity of the materials,

so that the chemicals or enzymes can easily access and hydrolyse the cellulose[44]. Pretreatment must meet the following requirements: improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; avoid the degradation or loss of carbohydrates; avoid the formation of by-products' inhibitory to subsequent hydrolysis and fermentation process. Physical, physico-chemical, chemical and biological processes have been used for pretreatment of lignocellulosic materials. These methods lead to physical or chemical changes in plant biomass. Over the years a number of different technologies have been developed for pretreament of lignocellulose[44,45] , table 3 illustrates some of the most promising pretreatment categories.

Steam explosion is the most used method for pretreatment of lignocellulosic materials. In this method, the material is rapidly heated to 180-210 °C, typically 1-10 min. with high-pressure saturated steam[46,47] . And then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Due to high temperature, this process causes hemicellulose degradation and lignin transformation, which increases the potential of enzyme hydrolysis. In addition, impregnation of the material with an acid catalyst (H_2SO_4 or SO_2) can reduce time and temperature while increasing recovery, reducing inhibitors formation and improving the enzymatic hydrolysis efficiency[48,49,50]. The major advantage of steam explosion pretreatment is the low energy requirement[51]. And Steam explosion is recognized as one of the most cost-effective pretreatment processes for hardwoods and agricultural residues[52,53].

Table 3 **Pretreatment methods of lignocellulosic materials, their applications and main principles**.

Pretreatment method		Application	Main principle
Steam pretreatment and acid-catalysed pretreatment technologies	Steam (explosion pretreatment)	Straw, corn stover and hardwoods	Partial hydrolysis and solubilisation of hemicelluloses, redistribution of lignin on fibre surfaces, fractionation of fibres
	Dilute acid pretreatment		
Pretreatment under alkaline conditions	Wet oxidation	Straw, corn stover, bagasse and softwoods	Removal and partial degradation of lignin, solubilisation and oxidation of some hemicelluloses
	Ammonia fibre/freeze explosion (AFEX)	Corn stover, rice straw and switchgrass	Cleavage of lignin and partially depolymerisation of hemicelluloses and cellulose
Extractive pretreatment	Organosolv pretreatment/Alcell pulping process	Softwoods	Removal of lignin and some hemicelluloses

Dilute acid pretreatment has been successfully developed for pretreatment of lignocellulosic materials. In this way, the lignocellulosic materials are mixed with

dilute acid (H_2SO_4) and water to form a slurry, heated to the desired temperature by steam, and flashed to atmospheric pressure after a given residence time. Although dilute acid pretreatment can significantly improve the enzymatic hydrolysis, its cost is usually higher than steam explosion[54,55,56].

Lignin biodegradation could be catalysed by the peroxidase enzyme with the presence of H_2O_2. In wet oxidation, the lignocellulosic material is usually treated at 180-200 ℃ for 5-15 min with addition of an oxidative agent such as H_2O_2 or high pressure oxygen. This method has been successfully used in the pretreatment of cane bagasse and wheat straw[57,58].

The concept of AFEX is similar to steam explosion. In AFEX pretreament, lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period time, and then the pressure is swiftly reduced. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including wheat straw, wheat chaff, barely straw, corn stover, rice straw, softwoods, switchgrass and bagasse[59,60,61,62].

In Organosolv pretreatment, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahdrofurfuryl alcohol[63,64]. Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process[65]. In general, a high yield of xylose can be obtained by adding of acid. However, removal of solvents from system is necessary

because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis and fermentation.

The efficient and cost-effective hydrolysis of cellulose and hemicellulose into monosaccharides presents a challenge for their use, so attention should be focused on this step[66,67]. Among different methods of hydrolysis, enzymatic hydrolysis has become the most prominent technology for the conversion of biomass into monomer sugars for subsequent fermentation. This goal is usually achieved by using multiple enzymes in defined ratios (Table 4). Enzymes required for glucose and xylose release are considered the main enzymes, while accessory enzymes are added when those sugars are present. All the enzymes degrade the substrate in a synergistic manner, meaning that the activity of enzymes working together is higher than the addition of their individual activities. These synergistic interactions between different enzymes have been studied in order to design the optimal combination and ratio of enzymes for different lignocellulosic substrates subjected to different pretreatments.

According to the traditional enzyme classification system, the cellulolytic enzymes are dived into three classes: exo-1,4-β -D-glucanase or cellobiohydrolase (CBH) (EC 3.2.1.91) moves along the cellulose chain and cleaves cellobiose units from the ends; endo-1,4- β -D-glucanases (EG) (EC 3.2.1.4) randomly hydrolyse internal β -1,4-glucosidic bonds in the cellulose chain; 1,4- β -D-glucosidases (EC 3.2.1.21) hydrolyses cellobiose to glucose and also cleave glucose units from cellooligosaccharides. All these enzymes are cellulolytic enzymes that hydrolyse cellulose by synergistic action, eliminate obstacles and reduce product inhibition by creating new accessible sites for each other[68,69].

Table 4 **Enzymes used for hydrolysis of bonds relating to sugars of the substrate after pretreatment**.

Main enzymes	Related sugars
Exo-1,4- β-D-glucanase (EC 3.2.1.91) Cellobiohydrolase (EC. 3.2.1.176) Endo-1,4- β –D-glucanase (EC3.2.1.4) 1,4-β-glucosidase (EC 3.2.1.21)	Glucose
Endo-1,4- β –D-xylanase (EC 3.2.1.8) 1,4-β-xylosidase (EC 3.2.1.37)	Xylose
Accessory enzymes	
α-L-arabinofuranosidase (EC 3.2.1.55) Arabinase	Arabinose
Endo-1,4- β -D-mannanase (EC 3.2.1.78) 1,4- β -D-mannosidase (EC 3.2.1.25)	Mannose
Pectinase Pectatelyase	Galacturonic acid
α -D-galactosidase (EC 3.2.1.22) β-galactosidase	Galactose

The hemicellulolytic system is more complex with various side groups. The hemicellulose system includes endo-1,4-β-D-xylanase (EC 3.2.1.8) hydrolyses internal bonds in xylan chain; 1,4-β-D-xylosidases (EC 3.2.1.37) attacks xylooligosaccharides from the non-reducing end and release xylose; endo-1,4-β -D-mannanase (EC 3.2.1.78) cleaves internal bonds in mannan and 1,4-β -D-mannosidase

(EC 3.2.1.25) cleaves mannooligosaccharides to mannose. The side groups are removed by a number of enzymes : α-D-galactosidase (EC 3.2.1.22), α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl and *p*-cumaric acid esterases (EC 3.1.1.73)[27,70].

However, these methods of pretreatment and hydrolysis are nonspecific and sometimes lead to the formation of the inhibitory compounds, which can negatively impact the fermentation process[71,72]. For instance, cellulose and hemicellulose derived furan aldehydes and aliphatic as well as lignin derived phenolic compounds, as shown in Figure 3. The nature and concentration of the final inhibitory compounds vary widely with the amount of solids in the reactor, the pretreatment conditions applied and the raw materials used[73,74]. Many remediation treatments for removal of inhibitors are applied prior to fermentation, including physical (evaporation), chemical (solvent extraction, activated charcoal adsorption and use of ion exchange resins, or overlime procedures)[75]. In addition, biological detoxification has drawn the attention as an alternative to use of microorganisms and /or their enzymes to act on the specific toxic compounds in the hydrolysate and change their composition or structure to less toxic compounds. And the effectiveness of detoxification methods depends on the type of hydrolysates and also on the species of microorganism used, as each type of hydrolysate has varying degrees of toxicity, and each species of microorganism has varying degrees of tolerance[76].

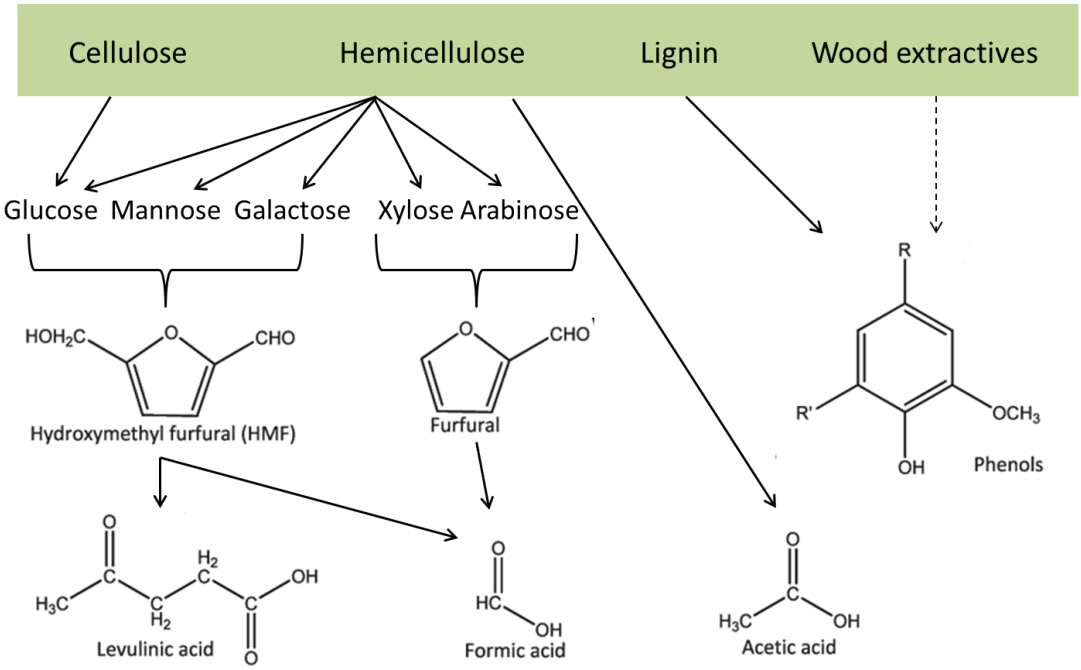

Figure 3 **Formation of inhibitors in the main routes of forming carbohydrate degradation products** (modified after Jönsson L.J. et.al., 2013[77], with permission via license CC BY 2.0, https://creativecommons.org/licenses/by/2.0).

1.2. Bioconversion of lignocellulose biomass

1.2.1. Biotechnological transformation of lignocellulosic materials

Lignocellulosic biomass wastes are particularly abundant in nature and have a potential for bioconversion into various value added biological and chemical products. There are two fermentation systems have been used to produce compounds of industrial interest from lignocellulose: solid-state fermentation systems (SSF) and submerged fermentation systems (SmF). Solid-state fermentation systems have the option to reuse lignocellulose and as advantage no need of raw material fraction before the use in the fermentation stage. But in submerged fermentation systems, the biotechnological transformation of lignocellulosic materials involves the use of biocatalysts, whole microorganisms or their enzymes or enzymes from other

organisms to synthesize or bio-transform the feedstocks into new products, followed by recovery/purification of such bio-products and any subsequent desired downstream modifications. Specifically, it requires a multi-step processes, the steps including: (i) choice of suitable biomass (collection); (ii) mechanical, chemical or biological pretreatment; (iii) hydrolysis of the polymers to produce readily metabolised molecules (usually sugars); (iv) bioavailability of these molecules for microbial growth or production (fermentation of the sugars to produce a microbial or chemical end-product); (v) downstream processing like separation/purification and modification[78], as shown in Figure 4.

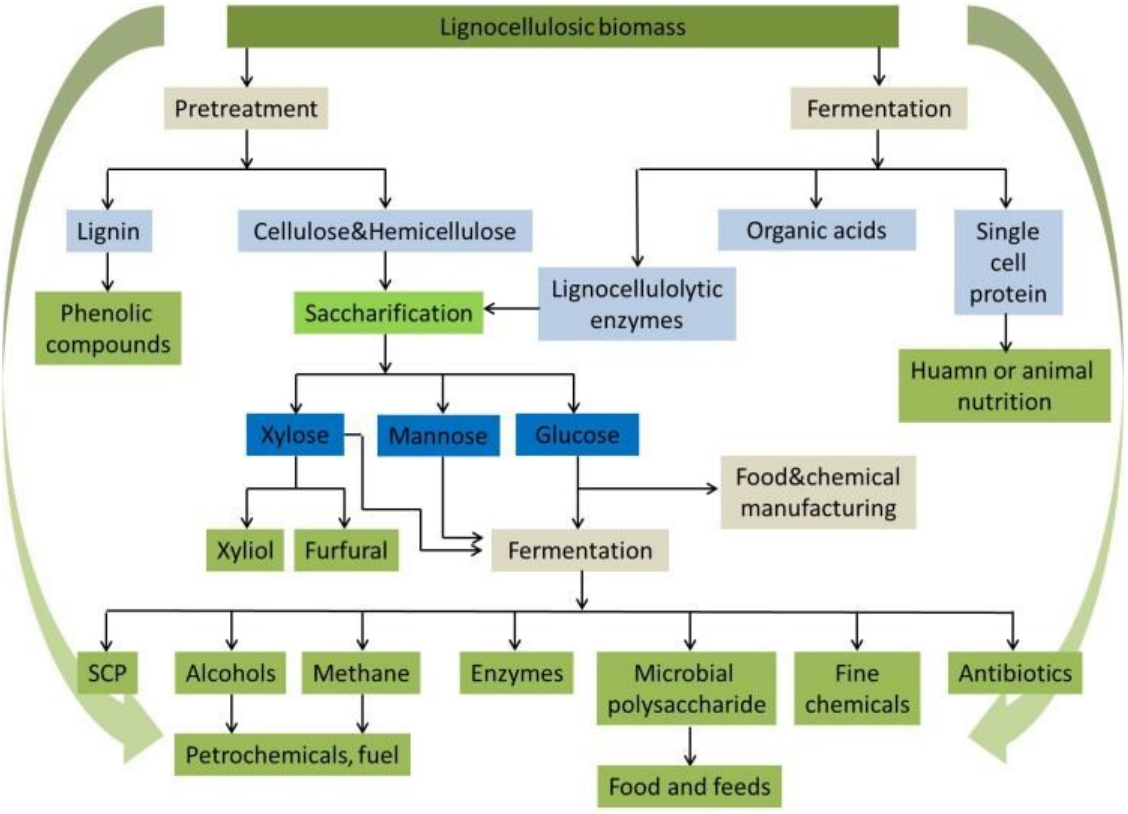

Figure 4 **The general process stages in lignocellulosic bioconversion into value-added bio-products.** (modified after Kumar A. et.al., 2016[79], with permission via license CC BY 4.0, https://creativecommons.org/licenses/by/4.0/)

The development of processes for reuse of these wastes is of great interest. Because these lignocellulosic wastes are rich in sugars and easily assimilated or absorbed by microorganisms, they are very suitable for use as raw materials for the production of fermentation relevant industrial compounds. To date, the current technology is available for all steps in the bioconversion of lignocelluloses to ethanol, other chemical products etc. However, these technologies must be improved and new technologies are developing to produce more renewable biofuel and other bio-products at prices that compete with current production costs. Besides to be used as low-cost raw materials to produce important metabolites, the reuse of lignocellulose during fermentation processes is also an environment friendly method of waste management.

1.2.2. Potential products and their applications

Bioconversion of lignocellulosic biomass (hydrolysates) into high-value products is gaining significant global attention. Market forces have demonstrated a positive effect on the natural environment and have raised the importance of renewable materials. Cellulose and hemicellulose are sugar rich fractions of interest for use in fermentation processes, because microorganisms may use the sugars for growth and production of value added compounds such as ethanol, food additives, organic acids and others[39, 27,80,81,82] , as shown in Table 5.

Biomass-derived sugars can be easily fermented to commodity chemicals and fuel ethanol by the suitable microorganisms. More than 75% of organic chemicals are produced from five major base chemicals: ethylene, propylene, benzene, toluene for the synthesis of other organic compounds[11], which in turn are used to produce a variety of chemical products including polymers and resins. The aromatic compounds

can be produced from lignin, whereas the low molecular mass aliphatic compounds can originate from the fermentation of sugars produced by cellulose and hemicellulose degradation.

Table 5 **The value-added bio-products through bioconversion of lignocellulosic biomass and their applications**.

Value-added products		Applications
Chemicals	Aromatic compounds	To produce various chemical products including polymers and resins
	Low molecular mass aliphatic compounds (e.g., ethanol, acetone, butanol, glycerol, acetic acid, citric acid, fumaric acid)	
Biofuels	Bioethanol	Petrol additive
	Biobutanol	Gasoline additive; precursor for paints, polymers, plastics
	Biomethane	production of electricity and heat
	Bio-Hygrogen	Clean and renewable energy
Other high value bioproducts	Organic acids	In chemical and pharmaceutical industries
	Amino acids	
	Vitamins	
	Bacterial and fungal polysacch arides (e.g., xanthan)	
	Vanillin	Intermediate in the chemical and pharmaceutical industries
	Xylitol	Sweetner in food
	Furfural	Use in the manufacture of furfural-phenol plastics, varnishes and pesticides

The demand for ethanol is the most significant market. Ethanol can be used as both a chemical feedstock and as an octane enhancer or petrol additive. For ethanol production, first generation is from sugars or starch, which impacts negatively on the economics of the process. Hence, the second generation has shifted towards the utilisation of residual lignocellulosic materials for lower production costs. Various lignocellulosic materials such as sugarcane bagasse, water hyacinth, rice straw, rice husk, wheat straw, corn cob and so on are tested for ethanol production[83]. For

examples, softwood is the dominant source of lignocellulose in the Northern hemisphere. In Sweden, Canada and Western USA, softwood has been the subject of interest as a raw material for fuel ethanol production[29]. Moreover, Brazil and USA produce ethanol from the fermentation of cane juice and maize, respectively. It's a very big business that 102 billion litres of fuel ethanol worldwide in 2017, of which 60 billion litres was produced in USA, 27 billion litres were produced in Brazil and 5.3 billion litres were produced in the EU (source: www.statista.com/).

Biobutanol is considered a future biofuel with the potential to replace gasoline. 1-Butanol as fuel is superior to ethanol in terms of fuel density, engine compatibility and safety[84,85]. 1-Butanol is also an important precursor for paints, polymers, and plastics[86]. A number of lignocellulosic substrates such as rice bran, corn stover, wheat bran, wheat straw, palm kernel cake, rice straw and wood chips have been used for the production of biobutanol in different studies[87,88].

Biomethane has potential to yield more energy than any other current biofuels like bioethanol and biodiesel, used for the production of electricity and heat for local needs[89,90]. Various studies showed the production of biomethane from wide range of lignocellulosic biomass such as rice straw, pulp and paper sludge, softwood spruce, birch, corn stover, wheat straw etc[91,92,93,94].

Bio-hydrogen is also considered as a viable energy option in the future. It is a clean and renewable energy carrier because its combustion produces the waters as the sole end product[95]. Different lignocellulosic biomasses including hemp, newspaper, barley straw, rice straw, corn stalk, corn cob, wheat straw, sugarcane bagasse have been utilized for the production of hydrogen[96,97,98].

High-value biological products for example organic acids, amino acids, vitamins and a number of bacterial and fungal polysaccharides, such as xanthans, are produced by fermentation using glucose as a base substrate. But theoretically these same products could be manufactured from sugars derived from lignocellulose residues. Organic acids are the natural products or at least natural intermediate of main metabolic pathways of microorganisms. They are extremely useful products because their functional groups make them suitable substrate for the synthesis of other important chemicals. Using lignocellulose as substrate, different organic acids such as citric acid, acetic acid, succinic acid, gluconic acid, etc. are produced by microbial fermentation[99,100,101,102].

Microbial polysaccharides are produced by a variety of microorganisms and due to their novel and unique physical properties; they are very useful in different industries. Microbial polysaccharides have multiple applications in different industrial fields such as food, petroleum, and pharmaceutical. Different microbial bioactive polysaccharides such as schizophyllan, pullulan, xanthan, dextran, curdlan etc. are produced by microorganisms using fermentable sugars as the substrate[103].

Vanillin and gallic acid are two of the most commonly discussed monomer potential products[104]. While vanillin is used for a variety of purposes, including intermediates used in the chemical and pharmaceutical industries for the production of herbicides, anti-foaming agents or drugs such as papaverine, levodopa and the antimicrobial trimethoprim. It is also used in household products such as air fresheners and floor waxes[104]. Xylitol and furfural are produced from hemicellulose-derived xylose[105]. Xylitol replaces sucrose in foodstuffs as a sweeteners, has odontological applications such as teeth sclerosis, remineralisation, as antimicrobials,

additionally for chewing gum and toothpaste formulations. Furfural is used to make furfural phenol plastics, varnishes and pesticides[106].

In conclusion, the bioconversion of lignocellulosic biomass is a potential sustainable approach to develop value-added bio-products, which is still expanding in different directions. And the cost effective and environmentally sustainable properties of biotechnological conversion of lignocellulosic biomass in various industrial products are very attractive and taken into a significant consideration.

1.2.3. Milk protein α_{s1}-Casein--a potential food additive

Milk is one of the first components introduced into human diet[107]. Casein is a major component of milk protein in almost all mammalian species, accounting for about 80 and 40% of the total protein in ruminant and human milk respectively[108,109].Although milk protein composition can differ from one species to another, generally the casein fraction consists of α_{s1}-, α_{s2}-, β-, and κ- casein[110].

In all the types of casein proteins, two α- and one β-type casein have the properties of phosphoproteins, which present as stable calcium phosphate protein complexes termed micelles. And a major role for the non-phosphorylated κ- casein is to stabilize those micelles. Therefore, caseins have the functions not only to serve as a major source of amino acids for the suckling infant, but also to transport phosphate and calcium in sufficient amounts to support growth of bones. Moreover, it has been proposed that the binding of calcium phosphate to the caseins could protect the mammary gland from pathological calcification[111]. In addition, it was discovered that the casein proteins are also rich in lysine. Lysine is an essential amino acid in humans

and one in which many plant sources are lacking, so casein extracts can be an effective nutritional supplement for cereals[112].

Alpha-casein is a major component (~50%) of bovine casein. Among them, α_{s1}-casein is the most abundant protein in cow's milk, accounting for 34% of the total milk proteins. Bovine α_{s1}-casein is a 199 amino acid residue single stranded linear phosphoprotein with only minor amount of secondary structure and lacquer disulphide bonds, which leads to a reduction in tertiary interactions[113,114]. However, human casein was previously thought to lack the α-casein subunit. Then it was found by Cavaletto *et al* in 1990 that human casein also contains very small amounts of α-casein (α_{s1}-casein), accounting for only 0.06% of the total protein content in human milk[115]. This pinpoints the difficulty of obtaining large amount of native human α_{s1}-casein.

Casein has attracted considerable interests for a long time. Particularly those of human and bovine origin have potential uses in pharmaceutical preparations, because casein-derived peptides have been found to exhibit different biological activities such as opiate, antithrombotic, antihypertensive and immunomodulatory activity[116,117]. And on the other hand, since recently 'synthetic green food' or food for vegetarian people has been attractive for people's attention, casein proteins have a promising potential usage as foodstuff or food additives in food industries due to their biological functions.

α_{s1}-casein like protein was used to be separated and purified from bovine and human milk[118]. Subsequently, in the 1990s it has been reported that the production of α_{s1}-casein using recombinant DNA technology by isolating and cloning cDNA coding for α and β caseins[108,109,119].

1.3. Carbohydrate metabolism in bacteria

1.3.1. Screening of bacterial organisms for sugar metabolism

All bacteria must utilize the energy sources in their environment to produce ATP. ATP is required for all of the biosynthetic processes that bacteria use for their maintenance and reproduction[120,121]. Bacteria produce enzymes that oxidize environmental energy, but the energy used by different bacteria depends on the particular enzymes that each bacterium produces[122]. Considering a certain extracellular environment, bacteria only need to be amplified by part of the enzymes encoded by the genome, so it can be differentially to regulate gene expression. For example, in the absence of a specific substrate, the genes encoding the enzymes required for subsequent uptake and metabolism are often repressed[123,124].

Heterotrophic bacteria often use carbohydrates as energy source. Many bacteria use glucose, a monosaccharide or simple sugar, because many bacteria have the enzymes needed to degrade and oxidize this sugar. Fewer bacteria can naturally use other monosaccharides (xylose, arabinose and mannose), complex carbohydrates such as disaccharides (lactose or sucrose) or polysaccharides (starch). For example, *Escherichia coli* is capable of metabolizing a wide range of substrates, including hexoses and pentoses[125]. *Z. mobilis* can ferment glucose, fructose and sucrose, but not pentose sugars. *Clostridia* are able to ferment a variety of polysaccharides, oligosaccharides and monomeric sugars like hexoses (glucose, fructose, galactose, and mannose) and pentoses (arabinose and xylose)[126]. *Streptomyces padanus* MITKK-103 is an actinomycete closely related to *Rhodococcus*, can also efficiently utilize xylose as a sole carbon source[127].

As most common laboratory strains, *E. coli* are well investigated. The ability to utilize hexoses as well as pentose makes this microorganism an interesting host for biofuel production from lignocellulosic biomass, such as ethanol[128], isopropanol[129], n-butanol[130], n-propanol[131], isobutanol, 2-*methyl*-1-butanol and 3-*methyl*-1-butanol[132] etc. are reported. Furthermore, the production of fatty-acid-derived fuels and isoprenoid based fuels have been also studied[133]. As stated in the previous chapter, many chemical compounds and high-value bioproducts (organic acids, amino acids, vitamins, bacterial polysaccharides etc.) are also produced based on lignocellulosic biomass by *E. coli* strains.

Moreover, *E. coli* strains are also commonly used in other industrial processes such as recombinant protein production. Because they can quickly and easily grow on inexpensive substrates and can be simply modified by a variety of molecular tools, they have been well-established as a cell factory and become the most popular expression platform. The ability to express and purify the desired recombinant proteins in a large quantity allows them to be used in the development of industrial enzymes and biopharmaceuticals. To date, more than 150 recombinant proteins to be used as pharmaceuticals have been licensed by FDA (Food and Drug Administration) [134], such as insulin[135,136], interferons (IFN-α, -β and -γ), growth hormones and antibodies. And the industrial enzyme (proteases, amylases, lipases, cellulases and pectinases) market is also expanding [137,138].

1.3.2. Metabolic pathways of D-xylose

To achieve an economically viable manufacturing process from lignocellulosic biomass, the knowledge of pentose utilization is required. Therefore, it is important to identify the metabolic pathways of xylose and arabinose.

For prokaryotes, there are three different pathways investigated for the catabolism of D-xylose: An isomerase pathway (PPP pathway) is typically used[139], and there are also two oxidative pathways known as Weimberg and Dahms pathways, respectively[140,141,142],.

In the isomerase pathway, the enzyme xylose isomerase converts D-xylose directly into D-xylulose. D-xylulose is then phosphorylated to D-xylulose-5-phosphate as in the oxido-reductase pathway[143], as shown in Figure 5. Therefore, the genes *xylA* indicating enzyme Xylose isomerase and *xylB* indicating enzyme Xylulokinase are required in these steps to continue further in the Pentose Phosphate Pathway.

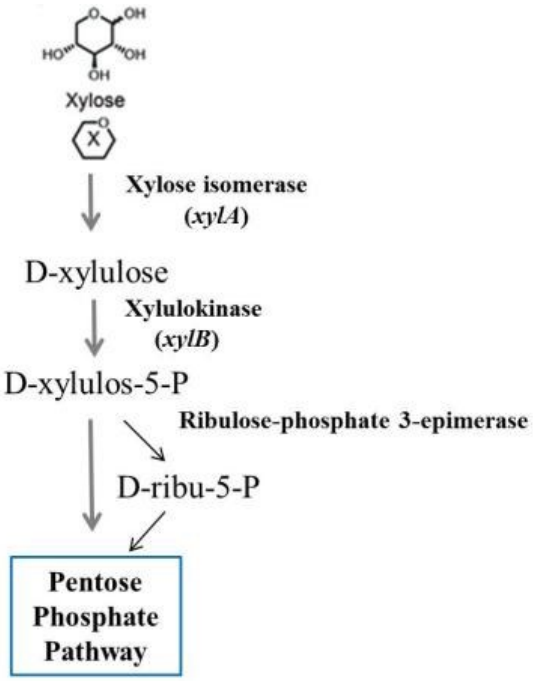

Figure 5 **Isomerase pathway (Pentose Phosphate Pathway) for the metabolism of D-xylose**. (according to Jared W. Wenger et.al., 2010, available via license CC BY 4.0, https://creativecommons.org/licenses/by/4.0/)

As shown in figure 6, the Weimberg pathway is an oxidative pathway in which D-xylose is oxidized to D-xylono-lactone by a D-xylose dehydrogenase and then

lactone is hydrolysed to D-xylonic acid by a lactonase. The xylonate dehydratase splits off from the water molecule and produces 2-keo-3-deoxy-xylonate. The second dehydratase forms 2-keto glutarate semialdehyde, which is subsequently oxidized to 2-ketoglutarate[144].

The Dahms pathway begins with the Weimberg pathway, but the 2-keto-3-deoxy-xylonate is split by an aldolase to pyruvate and glycoladehyde[145].

Consequently, xylose is taken up directly for catabolism via the isomerase and oxo-reductive pathway, both of which provide fuel for pentose phosphate pathway, the Weimberg and Dahms pathways require an initial conversion of xylose to xylonate, which is then taken up by the cells.

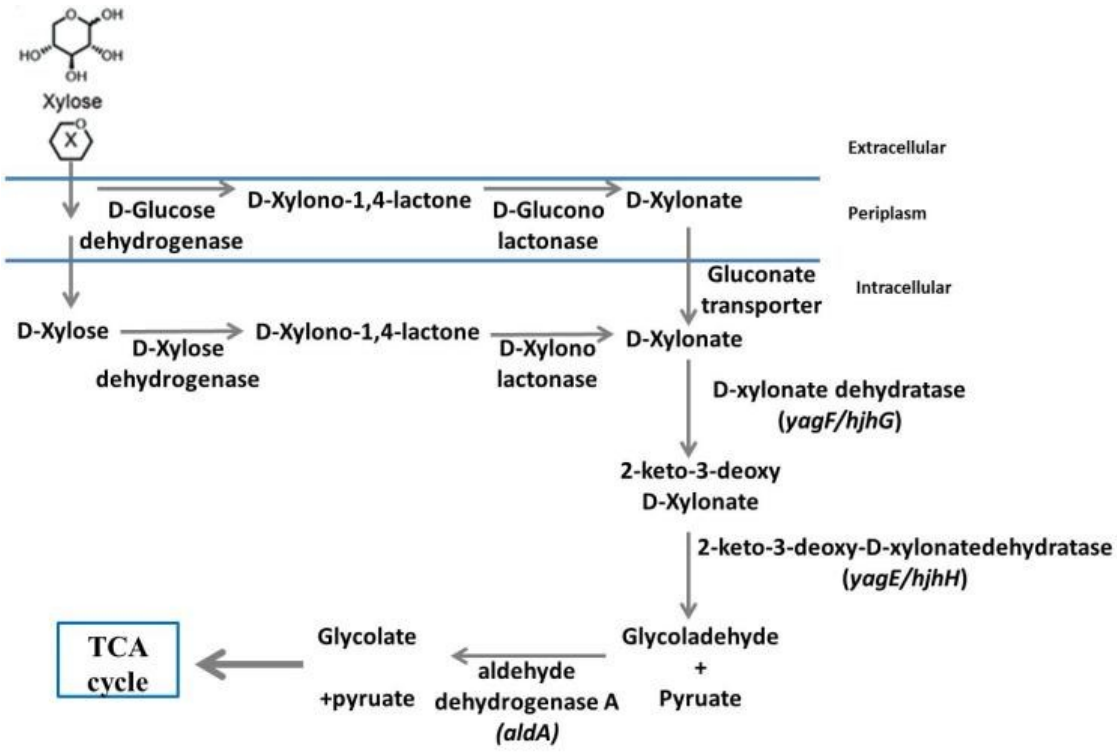

Figure 6 **The Weimberg/Dahms pathways for utilization of D-xylose**. (according to A. Stephen Dahms, 1974[145])

1.3.3. Metabolic pathways of L-arabinose

Arabinose is an aldopentose (a monosaccharide) containing five carbon atoms, and including an aldehyde (CHC) functional groups. Due to biosynthetic reasons, most saccharides are almost always more abundant in nature as the 'D'-form, or structurally analogous to D-glyceraldehyde. However, L-arabinose is actually more common in nature than D-arabinose and it has been found in nature that they are components of biopolymers such as hemicellulose and pectin.

The L-arabinose operon, known as the *araBAD* operon, has been the subject of many biomolecular studies. The operon directs the catabolism of arabinose in *E. coli*, and it is dynamically activated in the presence of arabinose and in the absence of glucose[146].

The structural genes encoding arabinose catabolic enzymes are *araB*, *araA* and *araD* (collectively referred as *araBAD*). The *araB* gene encodes an L-arabinose isomerase, which catalyses the isomerization between L-arabinose and L-ribulose. The *araA* gene encodes ribulokinase that catalyses the phosphorylation of (L/D)-ribulose 5-phosphate. The *araD* gene encodes an L-ribulose-5-phosphate 4-epimerase, which catalyses the epimerization between L-ribulose 5-phosphate and D-xylulose 5-phosphate. D-xylulose 5-phosphate and D-ribulose-5-phosphate are metabolites in the pentose phosphate pathway (PPP), as shown in Figure 7.

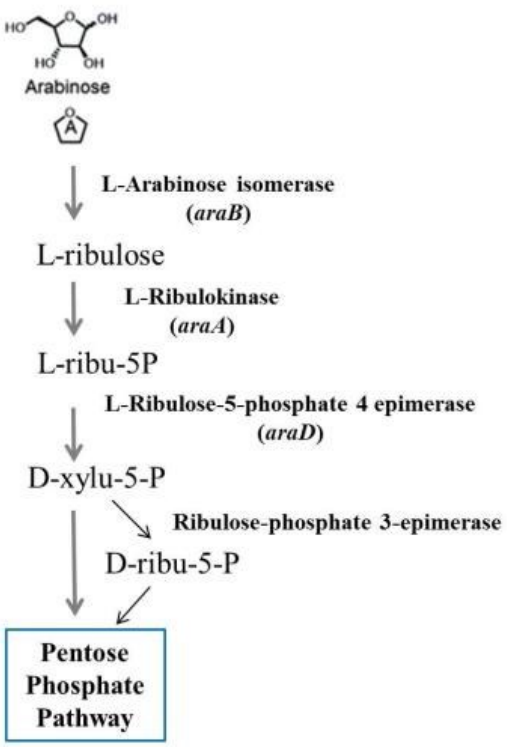

Figure 7 **Metabolism route of L-arabinose in Pentose Phosphate Pathway**. (according to Petschacher B. et. al., 2008, available via license CC BY 2.0, https://creativecommons.org/licenses/by/2.0/)

The *araBAD* operon has both positive and negative regulation and is allosterically activated. The regulator gene is *araC*, which is located upstream of the L-arabinose operon and encodes the arabinose-responsive regulatory protein AraC. The genes *araC* and *araBAD* are transcribed in opposite directions from the *araBAD* promoter (P_{BAD}) and *araC* promoter (*Pc*) respectively. The operators are *araO* and *aral*, they are located between the *araC* and *araBAD*. The *araO* and *aral* are DNA-binding sites that induce expression when occupied by AraC, as shown in Figure 8.

This means that the *ara* operon is regulated by AraC protein. If arabinose is absent, the dimeric AraC protein represses the structural gene by binding to *araO* and *aral* and the DNA forms a loop. The loop prevents RNA polymerase from binding to the promotor of *ara* operon, thereby blocking transcription. When arabinose is present, arabinose binds AraC and prevents AraC from interaction. This breaks the DNA loop.

The two AraC-arabinose complexes bind to *araO* and *aral* sites which promotes transcription. So AraC acts as an activator when arabinose is present.

Figure 8 **Structural elements of araBAD operon** (according to https://en.wikipedia.org/wiki/L-arabinose_operon).

1.3.4. Applications of genetic engineering bacteria strains for pentose utilization

As an alternative raw material in industrial technology, lignocellulosic substrates have received more and more attention, with the production of chemicals and biofuels as a front runner[147,148]. After glucose, L-arabinose and D-xylose are the next most abundant sugars found in plant biomass. However, not many suitable microbial organisms exist in nature, which are able to ferment other monosaccharides rather than glucose. Therefore, a key step will be the engineering of strains capable of efficiently fermenting these three most abundant lignocellulosic sugars.

Various approaches have been applied to introduce or enhance xylose metabolism in bacterium, as shown in Table 6. *Pseudomonas* strains were engineered for xylose metabolism. Meijnen and colleagues integrated the orthologous xylose degradation pathway into *Pseudomonas putida* S12 by overexpressing the original xylose operon of *xylXABCD* (*Caulobacter crescentus*) encoding for the Weimberg pathway[149]. The introduction of the *xylXABCD* operon enabled growth of *P. putida* S12 on xylose at a growth rate of 0.21 h^{-1}. Thereafter, an operon of *xylAB* (*Escherichia coli*) encoding for the isomerase pathway was also introduced into *P. putida* S12[150,151]. Integration of

xylAB operon from *E. coli* resulted only in the slow growth of *P. putida* S12 on xylose (0.01 h^{-1}). Even by the introduction of an additional xylose transporter, the growth of the engineered *P. putida* S12 could not be improved.

In addition, the *xylAB* operon from *E. coli* was also introduced into *P. putida* KT2440 by LeMeur and colleagues[152]. The strain was able to grow on xylose with a growth rate of 0.24 h^{-1}. It has been also shown that there was no need to integrate an additional xylose uptake system into *P. putida* KT2440, and it was hypothesized that xylose is either taken up by the glucose uptake system or by a phosphenolpyruvate-dependent phosphotransferase system. Recently, Pavel Dvořák et al. also engineered *Pseudomonas putida* EM42, a robust strain derived from *P. putida* KT2440, to metabolize cellobiose and xylose to co-utilize these two carbohydrates. *P. putida* EM 42 expressing the intracellular β -glucosidase BglC is able to utilize 5 g L^{-1} cellobiose as a sole carbon source. It was demonstrated that *P. putida* uses its native transport routes for cellobiose uptake. For xylose metabolism, operon *xylAB* was isolated from *E. coli* BL21(DE3), and a pentose transporter XylE was selected and cloned downstream of the *xylAB* fragment to form the synthetic *xylABE* operon[153].

Corynebaterium glutamicum is a gram-positive bacterium, which was also engineered for xylose assimilation. *Escherichia coli* gene *xylA* coding xylose isomerase, either alone or in combination with *E. coli* gene *xylB* were integrated into *Corynebaterium glutamicum.* Both recombinant strains were able of growth on xylose as a sole carbon source. But the one having both *xylA* and *xylB* genes grew faster than the strain only having *xylA* gene. Moreover, lactic acid and succinic acid were produced in both glucose and xylose containing medium. Although the production

rate of lactic acid from xylose was lower than that from glucose, the production rate of succinic acid were comparable in both cases[154].

R. opaus PD630 is an oleaginous bacterium that accumulates large amounts of triacylglycerols (TAGs), which can be processed into advanced liquid fuels. To develop economically feasible process for fuel production from lignocellulosic biomass, heterologous genes *xylA* and *xylB* from *Streptomyces lividans* TK23 were introduced into *R. opaus* PD630. And the engineered strain is capable of high-cell-density cultivation at high xylose concentrations, and substantiated its suitability for TAG production[155].

Bacillus subtilis is a gram-positive bacterium found in soil and the gastrointestinal tract of ruminants and humans. It was engineered to metabolize glucose and xylose as substrates for acetoin production[156]. The genes *xylA* and *xylB* from *Escherichia coli*, encoding xylose isomerase and xylulokinase were integrated into *B. subtilis*.

Moreover, the introduction of arabinose metabolism strains has been also investigated. For instance, the ethanologenic bacterium *Zymomonas mobilis* was expanded to utilize L-arabinose as a carbon source. The genes *araA* (encoding L-arabinose isomerase), *araB* (L-ribulokinase), *araD* (L-ribulose-5-phosphate-4-epimerase), *talB* (transaldolase) and *tktA* (transketolase) were isolated from *Escherichia coli* and introduced into *Z. mobilis*. The engineered strain achieved a high ethanol yield in the cultivation with L-arabinose[157].

Corynebaterium glutamicum was also metabolically engineered by introducing *araBAD* operon from *Escherichia coli* to broaden its substrate utilization. The

engineered strain was able to grow on L-arabinose and produce succinic, lactic and acetic acids[158].

Table 6 **Genetic engineering bacteria strains for pentose utilization**.

Engineering strain	pathways	Target genes	Source strains	Metabolism sugars	Production	References
Pseudomonas putida S12	Isomerase pathway	*xylAB*	*Escherichia coli*	Glucose D-xylose L-arabinose	Theoretical chemicals	Jean-Paul Meijnen et al., 2008
Pseudomonas putida S12	Oxidative D-xylose catabolic pathway	*xylXABCD* operon	*Caulobacter crescentus*	D-xylose	Theoretical chemicals	Jean-Paul Meijnen et al., 2009
Pseudomonas putida KT2440	Isomerase pathway	*xylAB*	*Escherichia coli* W3110	Glucose D-xylose	mcl-PHAs	Sylvaine Le Meur et al., 2012
Pseudomonas putida KT2440	Isomerase pathway	*xylABE*	*Escherichia coli* BL21(DE3)	D-xylose Glucose/ Cellobiose	Theoretical chemicals	Pavel Dvořák et al., 2018
Corynebacterium glutamicum	Isomerase pathway	*xylA xylAB*	*Escherichia coli*	Glucose D-xylose	Organic acids	Hideo Kawagucji et al., 2006
R. opaus PD630	Isomerase pathway	*xylAB*	*Streptomyces lividans* TK23	Glucose D-xylose	TAGs	Kazuhiko Kurosawa et al., 2013
Bacillus subtilis	Isomerase pathway	*xylAB*	*Escherichia coli*	Glucose D-xylose	acetoin	Tao Chen et al., 2013
Zymomonas mobilis	araBAD operon	*araBAD talB tktA*	*Escherichia coli*	Glucose L-arabinose	ethanol	Kristine Deanda et al., 1996
Corynebacterium glutamicum	araBAD operon	*araBAD*	*Escherichia coli*	Glucose L-arabinose	Organic acids	Hideo Kawagucji et al., 2008

1.3.5. *Pseudomonas putida* KT2440

Pseudomonad is a genus of ubiquitous bacteria belonging to the gamma subclass of the proteobacteria. These bacteria are involved in important metabolic activities in the environment, including the recycling of element and the degradation of biogenic and xenobiotic pollutants[159].

Pseudomonas putida KT2440 strain is the most characteristic strain of Pseudomonas saprophyte, maintaining its ability to survive and function in the environment[160,161]. This bacterium is the plasmid-free derivate of a toluene-degrading bacterium. It originally designated *Psedomonas arvilla* strain mt-2 and subsequently reclassified as *P. putida* mt-2[161],[162]. *Pseudomonas putida* is a robust microorganism with a comparatively low sensitivity towards inhibitors[163,164,165,166], which will be beneficial to the growth and production of bioproducts based on lignocellulosic hydrolysates.

Strain KT2440 has high metabolic potential utilization, because it is the first Gram-negative soil bacterium to be certified as a safety strain by the Recombinant DNA Advisory Committee and is the preferred host for cloning and gene expression for Gram-negative soil bacteria[167]. And strain KT2440 is classified as biosafety level I according to the American Type Culture Collection. Furthermore, the complete and annotated genome sequence of *P. putida* KT2440 is available[168]. Therefore, it has become a laboratory workhorse worldwide as well as a valued cell factory for biotechnological processes[169,170,171]. In addition, its high resistance to endogenous and exogenous stresses makes it tolerant to industrially relevant chemicals (ethanol, p-coumaric acid, toluene) and to biomass hydrolysis by-products (furfural, benzoate,

acetic acid) at concentrations that will be inhibitory to other microbial platforms[172,173,174,175]. The properties of strain *P. putida* increase its value for the biotechnological recycling of lignocellulosic feedstocks. Therefore, using synthetic and biological approaches, carbon from lignocellulose hydrolysates can be streamlined towards valuable chemicals has been reported in *P. putida*, such as mcl-PHA[176], terpenoids[176], coronatines[177], rhamnolipids and others[169,164]. In particular, the production of the biosurfactant rhamnolipid has been successfully performed[178,179,180,181,182,183,184].

2. Motivation

This study is designed from the perspective of scientists working in the field of lignocellulose bioconversion. And the purpose of this study is to remind ourselves and other scientists working in related areas of lignocellulose research about the enormous economic potential of the bioprocessing of lignocellulosic raw materials generally regarded as “waste”, and secondly to highlight some of the modern approaches which potentially could be used to tackle the major impediments in using hydrolysates derived fermentable sugars by microorganisms.

This focus of this study is to highlight important aspects of lignocellulose biotechnology with emphasis on demonstrating the potential value from an application rather than basic research perspective. In this dissertation, the following tasks will be fulfilled:

1) The possibility of utilizing fermentable sugars from lignocellulosic hydrolysates by microorganisms.
2) The production of valuable products based on bioconversion of lignocellulosic hydrolysates.
3) Development of engineering microorganism strains as microbial catalyst for the utilization of lignocellulosic hydrolysates.
4) Evaluation of the microbial catalyst for the potential and feasibility in conversion of lignocellulosic hydrolysates.

3. Summary of publications

3.1. Investigation of sugar utilization in *Escherichia coli* strains and the production of α_{s1}-casein protein based on lignocellulosic hydrolysates

3.1.1. Summary I

Lignocellulosic biomass accumulates in a large amount annually. Lignocellulosic biomass can be broadly divided into primary biomass, waste biomass and energy crops. Primary biomass includes all naturally occurring land plants such as trees, bushes and grass. Waste biomass is a as low-value by-product of various industrial sectors such as agriculture (corn stover, bagasse, straw etc.) and forestry (waste from sawmills and paper mills). Energy crops (switch grass, elephant grass etc.) are high yield lignocellulosic biomass and can be used as raw materials for the production of second generation biofuels[185]. In this study, we focus on the waste biomass, because the traditional treatment of the waste biomass is usually burning, which results in a big global environment problem. Another outstanding point is that lignocellulose mainly consists of carbohydrates polymers including cellulose, hemicellulose and lignin. Due to the composition of lignocellulosic biomass, the usage of lignocellulosic biomass as alternative feedstocks (carbon sources) for the industrial biotechnology attracts people's attention and has been developed in the past years.

D-glucose is the only component in cellulose while the composition of hemicellulose varies among different bioresources[186,31,23]. Pentose (such as D-xylose and L-arabinose) is the

predominant sugar in hemicellulose, accounting for 25% of the total amount, but hemicellulose can also contain hexoses like D-glucose, D-mannose, and D-galactose[187,43,188]. While cellulose is mainly used for other industrial applications, 60 billion tons of hemicellulose is almost completely unused every year. However, both cellulose and hemicellulose can be hydrolysed into hydrolysates containing sugars by pretreatment and hydrolysis. Therefore, to avoid energy loss and take good advantage of lignocellulosic biomass is an important and outstanding project.

There is an obstacle to use them as substrates for bioprocess, since not all the microorganisms are naturally able to use pentose sugars as well as hexose sugars. It is known that *Escherichia coli* strains are capable to metabolize both pentose and hexose sugars. There are several approaches have been used to copy the metabolic flexibility of *Escherichia coli* strains into different bacteria by genetic manipulation and metabolic engineering methods[189,190]. However, there is nearly no study on the different sugars' utilization by *Escherichia coli* strains. Therefore, in this work, we have firstly investigated the sugars metabolism of four different common laboratory *Escherichia coli* strains and a probiotic *E. coli* strain: *E. coli* DH5α, *E. coli* K12-MG1655, *E. coli* K12-W3110, *E. coli* BL21(DE)3 and *E. coli* Nissle 1917. Hence, the successful utilization of three main sugars D-glucose, D-xylose and L-arabinose by all the five strains is confirmed.

These sugars provided by lignocellulosic hydrolysates can potentially be utilized for the growth of *Escherichia coli* strains, and the further aim is the bioconversion of lignocellulosic hydrolysates into valuable products. Various value-added products including biochemical compounds, fine chemicals, food additives, and enzymes have been reported in the bioprocesses based on lignocellulosic biomass[191,192]. Taking into consideration that *E. coli* strains are one of the best bacterium used for recombinant protein production. They have

been well-established as a cell factory and become the most popular expression platform, because it can be simply modified by a variety of molecular tools and have the ability to express and purify the desired recombinant proteins in a large quantity. Consequently, in this study we combined the two dominant features in *Escherichia coli* strains: the DNA recombinant technology and utilization of lignocellulosic sugars. First of all, we chose a florescent protein eGFP as a model protein, examined the protein production under both lac and T7 promoters on different monosaccharide sugars in different *Escherichia coli* strains, plasmid map is shown in Figure 9, picture was created with software SnapGene version 5.3.2, GSL Biotech LLC.

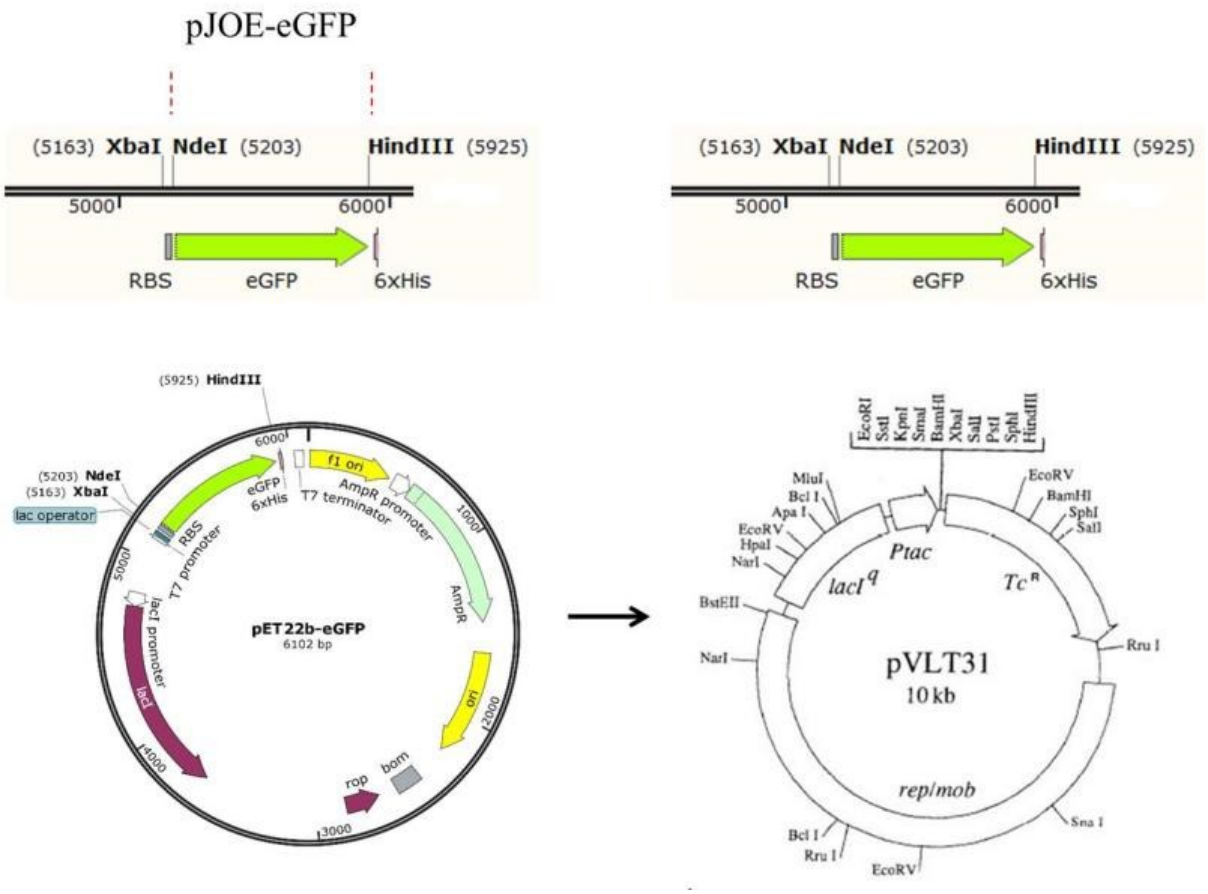

Figure 9 **Plasmid map of the constructed eGFP production vectors pET22b-*egfp* and pVLT31-*egfp***.

With the successful production of eGFP protein, furthermore, synthesised genes encoding bovine α_{s1}-casein and human α_{s1}-casein were cloned into vector pET22b(+) for expression in *E. coli* BL21(DE)3, as shown in Figure 10 (picture was created with software SnapGene version 5.3.2, GSL Biotech LLC). Except single sugars, an artificial wheat straw hydrolysate as well as a real hydrolysate of wheat straw lignocellulose were also used to investigate the growth of recombinant *E. coli* BL21(DE)3 strains and the production of α_{s1}-caseins. The purpose of choosing α_{s1}-casein proteins is that caseins are the major component of milk protein, and milk is one of the first food components into diet. So caseins are definitely the best nutritional option for new-born infants. Moreover, α_{s1}-caseins have the characteristics of phosphoproteins, so they have the potential to serve as a main source of amino acids and transport sufficient amount of calcium and phosphate. As a result, casein proteins have a great potential to be used as food additive in biotechnological food industry. In our study, wheat straw hydrolysate was selected as a model for inspection, because wheat straw is the most advantageous lignocellulosic raw material in Europe in terms of the quantity of the biomass available every year.

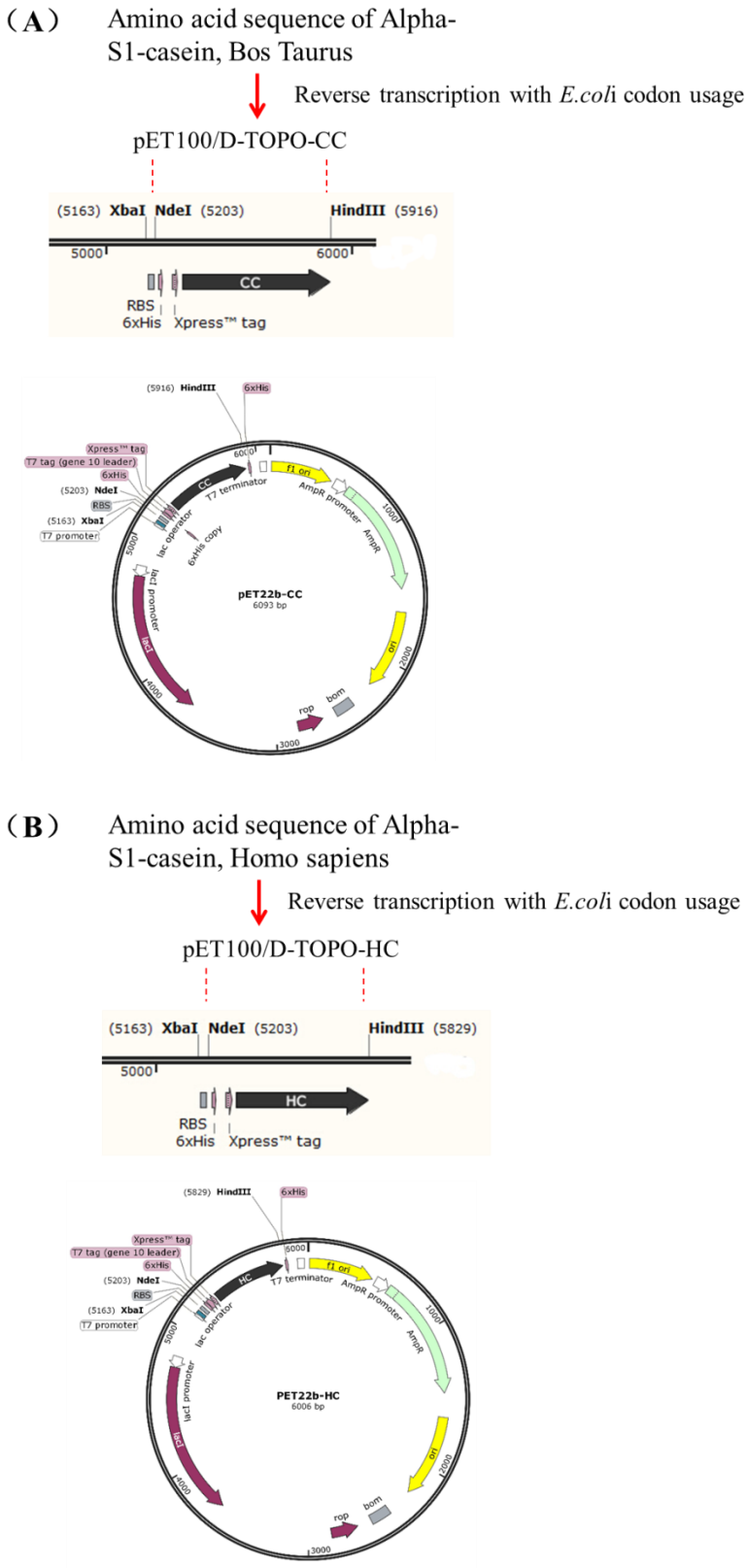

Figure 10 **Plasmid map of the constructed αs1-casein production vectors pET22b-*CC* and pET22b-*HC***.

In conclusion, this study successfully displayed the efficient utilization of the carbon sources derived from lignocellulosic biomass by *Escherichia coli* strains and showed the feasibility of bioconversion of lignocellulosic 'waste' to a potential food additive protein α_{s1}-casein.

3.1.2. Graphical Abstract Summary I

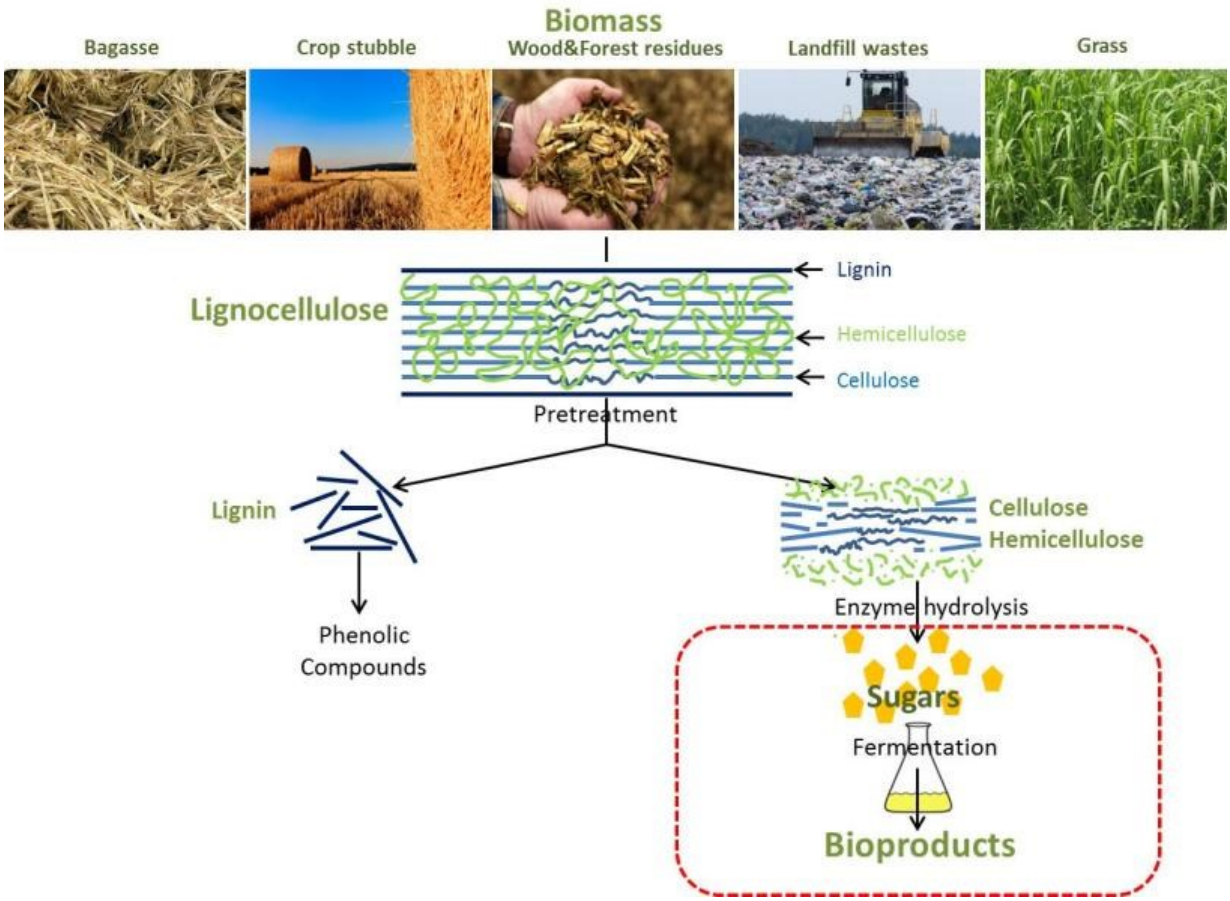

Figure 11 **Scheme of publication I**. Simplified overview of bioconversion of lignocellulosic biomass. The main composition of lignocellulose is cellulose and hemicellulose, which can be further hydrolysed into fermentable sugars in the form of hydrolysates. Thus, microorganisms can use these fermentable sugars as carbon source to produce high-value bioproducts.

3.1.3. Publication I: Bioconversion of lignocellulosic 'waste' to high-value food proteins: Recombinant production of bovine and human α_{S1}-casein based on wheat straw lignocellulose

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Y. Wang: constructed the recombinant strains, conducted the Erlenmeyer flasks cultivations and the protein analysis experiments, wrote and co-revised the manuscript

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ORIGINAL RESEARCH

Bioconversion of lignocellulosic 'waste' to high-value food proteins: Recombinant production of bovine and human α_{S1}-casein based on wheat straw lignocellulose

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Abstract

Lignocellulosic biomass is the most abundant bio-resource on earth, mainly composed of D-glucose, D-xylose and L-arabinose. It is widely considered to be a promising alternative feedstock for biotechnological processes. Here we evaluated its potential to be the carbon source for growth of broadly distributed and well-established *Escherichia coli* laboratory and protein expression strains as well as a classic probiotic *E. coli* strain. *E. coli* DH5α, *E. coli* K12-MG1655, *E. coli* K12-W3110, *E. coli* BL21(DE3) and *E. coli* Nissle 1917 were cultivated in mineral media containing single lignocellulosic sugar components. Sugar consumption in these cultures and growth parameters of the different strains were characterized. enhanced green fluorescent protein (eGFP) was chosen as a first easy to measure and prominent model recombinant target protein to demonstrate lignocellulose-dependent recombinant protein production in *E. coli*. To open new production routes for high value food proteins based on lignocellulose, structural genes encoding bovine α_{S1}-casein and human α_{S1}-casein were synthesized, cloned and then expressed in an *E. coli* T7 expression system in different media based on single sugars and a synthetic wheat straw mixture. Successful recombinant production of both bovine and human α_{S1}-caseins in *E. coli* under these experimental conditions was demonstrated and quantified by densitometric analysis after protein separation in polyacrylamide gels. Finally, efficient casein production in *E. coli* based on a real hydrolysate obtained by steam explosion of wheat straw lignocellulose in a bioreactor-based batch production process was successfully demonstrated. We believe that this proof-of-concept presented here is a promising starting point to open new routes for the production of food or feed proteins with high nutritional and economic value. As such, a valorization of bulk residual biomass like lignocellulose is envisioned as a key support of a growing and truly sustainable bioeconomy.

KEYWORDS

bioeconomy, D-xylose, *Escherichia coli*, hydrolysate, L-arabinose, Lignocellulose, wood sugar, α_{S1}-casein

1 | INTRODUCTION

Promising economic and ecologic options arising from the use of renewable energies and resources have meanwhile raised attention all over the world, due to the mostly undoubted rising global challenges as posed to mankind by the climate change, decline of environmental integrity and diversity, energy prices, energy long-run supply problems and impacts of these challenges also on human health (Hansen et al., 2006; McMichael et al., 2006; Schröter et al., 2005). A renewable source of considerable importance is lignocellulosic 'waste', which accumulates in large quantities every year in the form of residues from agriculture, forestry, energy crops, as well as residues from paper-pulp industries, timber industries and many agro-industries (Saini et al., 2015). Among these basic resources, straw residues (from corn, wheat and rice) dominate in terms of tonnage and can be expected to serve as global available feedstocks, but available quantities of straw substantially vary within different regions of the world. According to statistical data provided by the Food and Agriculture Organization of the United Nations (FAO) concerning the production of biomasses (e.g. crop production, yields, harvest areas, etc.) it is shown that in average from 1994 to 2018 the USA is the largest producer of corn, accounting for 52% of the currently 1.05 billion tons globally produced and the People's Republic of China with 20% of global production being on the second place (FAOSTAT, www.fao.org/faostat). With respect to annual global production of rice of about 750 million tons, Asia is the primary production region with over 90% of the global production coming from the world largest harvest areas. For the global production of wheat (about 750 million tons) Asia (43%) and Europe (32%) are the primary production regions. Statistical data for the annually available quantity of biomass suggest that wheat straw is potentially the most favourable lignocellulosic resource in Europe. Lignocellulosic feedstocks are mainly composed of cellulose, hemicellulose and lignin (McKendry, 2002). Cellulose is an unbranched homo-polysaccharide consisting of D-glucopyranosyl units. In contrast, hemicelluloses are branched hetero-polysaccharides consisting of both hexose (D-glucose, D-mannose and D-galactose) and predominantly pentose sugars (D-xylose and L-arabinose). As a result, lignocellulose biomass contains approximately 75% of polysaccharide sugars (Bayer et al., 2007). Different technical processes such as water steam explosion or organosolv focus on conversion and release of these lignocellulosic carbohydrates via pretreatment and subsequent enzymatic hydrolysis to gain the so-called lignocellulose hydrolysates (Alvira et al., 2010; Domínguez de María et al., 2015; Mosier et al., 2005; Taherzadeh & Karimi, 2008). During these processes aimed to obtain fermentable sugars, several potentially inhibitory molecules for microbial growth are formed as by-products. These include cellulose or hemicellulose-derived furan aldehydes and aliphatic acids as well as lignin-derived phenolic compounds. The complex profile of different products of pretreatment processes along with potentially inhibitory substances has been reviewed on several occasions (Chandel et al., 2011; Horlamus, Wang, et al., 2019; Sun, & Cheng, 2002). Therefore, lignocellulose biomass has been suggested to be the most suitable feedstock to provide monosaccharides, which then can serve as carbon sources for biotechnological processes based on (optimized) microbial biocatalysts in novel fermentative routes (Horlamus, Wang, et al., 2019; Mussatto & Teixeira, 2010; Peters, 2006; Wang et al., 2019).

Most bacterial organisms can only utilize glucose due to the lack of enzymes needed for degradation and oxidation of different sugars. Fewer bacteria can naturally utilize more "exotic" other monosaccharides like xylose, arabinose and mannose, complex carbohydrates such as disaccharides (lactose, cellobiose and xylobiose) or even polysaccharides (starch, xylan and cellulose). As most common laboratory strains, *Escherichia coli* strains have the ability to utilize hexoses as well as pentoses, which makes this microorganism an interesting host not only for traditional applications, but also in the context of novel metabolic pathways and the mentioned non-traditional carbon sources (Calero & Nikel, 2019; Idalia & Bernardo, 2017; Singh & Mishra, 1995). More importantly, *E. coli* has been the workhorse in microbiology for decades including an exceptional role as a cell factory and it has become the most popular expression platform, because it can quickly and easily grow on inexpensive substrates and can simply be modified by a variety of molecular tools (Rosano & Ceccarelli, 2014; Sharma & Chaudhuri, 2017). The ability to express desired recombinant proteins in large quantities allows it to be used in the development of industrial enzymes and biopharmaceuticals with to date more than 150 recombinant pharmaceutical proteins that have been licensed by Food and Drug Administration (Ferrer-Miralles et al., 2009). Insulin is probably the most prominent example (Leader et al., 2008; Rosenfeld, 2002), but interferons (IFN-α, -β and -γ), growth hormones and antibodies can also be considered as breakthrough products coming from *E. coli*-based processes. Another field in *E. coli* biotechnology

is the production of industrial enzymes like proteases, amylases, lipases, cellulases and pectinases which are of high relevance in special but expanding market (Sanchez & Demain, 2011; Sarmiento et al., 2015).

Milk is the first and basic food for mammals including humans and still it can be regarded as the best nutritional option for new-born infants. Within the milk proteins as important constituents casein is the major component in almost all mammalian species, accounting for up to 80% of the total protein in bovine milk (Kim et al., 1997). In general, the milk casein fraction consists of α_{s1}-, α_{s2}-, β-, and κ-casein (Wal, 1998). The two α-type and the single β-type caseins are characterized by the formation of aggregates in the form of micelles in solution and their ability to sequester up to 5% of their dry weight as Ca^{2+} (Koczan et al., 1991). One of the biological functions of the casein for the young is to serve as a main and extremely well-balanced source of essential amino acids with a biological value (BV) higher than chicken egg and soya (Chanat et al., 1999; Hoffmann, & Falvo, 2004). The BV is a measure of how efficiently an absorbed food protein can be used for protein biosynthesis by the organism compared to protein from chicken egg as the reference value. In bovine milk α_{S1}-casein is the most abundant protein, accounting for 34% of the total milk proteins. Initially discussed as being absent in humans it was reported later that human casein also contains α-casein (α_{S1}-casein), although it is present only in very small amounts accounting for only 0.06% of the total protein content in human milk (Cavaletto et al., 1994). With an increasing population preferring a vegetarian nutritional life style in the western world, casein proteins are not only considered as ideal nutrients from milk, but also have a promising potential use as healthy food additives in food industries due to its biological functions and especially its particular amino acid composition. In previous studies, α_{S1}-casein-like milk protein was used to be isolated and purified from bovine and human milk (Rasmussen et al., 1995). However, casein isolated from milk is not acceptable for those who prefer vegan nutrition. Thus, subsequently, in the 1990s the successful production of casein proteins using recombinant DNA technology by isolating and cloning genes encoding bovine/human α and β caseins from cDNA library was reported (Kim et al., 1997).

In this study, four prominent *E. coli* laboratory strains and a probiotic strain were chosen as examples to examine their capability to grow on single sugars representing the main components of lignocellulosic biomasses. *E. coli* DH5α is one of the most commonly used strains for cloning experiments and plasmid maintenance due to its high transformation efficiency and *recA* mutation to avoid heterologous recombination (Chan et al., 2013). The *E. coli* K12 strains MG1655 and W3110 are among the oldest *E. coli* laboratory strains and were cured from the F plasmid and phage lambda (Bachmann, 1972; Jensen, 1993). MG1655 was also chosen for the first published genome sequence of *E. coli* K12 (Blattner et al., 1997). For highly efficient protein production using the T7 expression system, the strain BL21(DE3) was generated especially by knock-out of two key proteases and is the most used protein overexpression strain in laboratories world-wide (Studier & Moffatt, 1986). In contrast, the probiotic strain *E. coli* Nissle 1917 is a well-established medical product available as Mutaflor® (Ardeypharm). Moreover, with the fluorescent protein eGFP a first model for the ability of each strain to produce proteins based on these sugars was tested. As the main objective of this study we have also demonstrated the recombinant production of bovine and human α_{S1}-casein proteins on sugar mixtures emulating lignocellulosic hydrolysates from wheat straw residues as sole carbon sources. Finally, efficient casein production in *E. coli* based on a real hydrolysate of wheat straw lignocellulose in a bioreactor-based batch production process was successfully demonstrated. The animal-free decoupling of food production from traditional agriculture and the introduction of biotechnological food proteins represents a novel food technology that will contribute to global health, food security and sustainability. Especially zoonoses such as the current Coronavirus disease 2019 but also salmonellosis and others stress the need for animal-free alternatives of high-value protein supply in combination with climate-neutral production. We believe that this proof-of-concept presented here may be a promising starting point to open general new routes for the production of food or feed proteins with high nutritional and economic value based on bulk residual biomass like lignocellulose for the support of a growing and truly sustainable bioeconomy.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was also used for cloning procedures and plasmid maintenance.

2.2 | DNA manipulations

DNA manipulations were carried out using established methods as described in Sambrook and Russell (2001). Restriction enzymes and T4 DNA ligase were obtained from Thermo Fisher Scientific and used as recommended. Plasmid DNA was isolated with QIAprep spin miniprep kit (Qiagen). DNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA fragments were recovered from agarose gels by using a QIAEXII gel extraction kit (Qiagen). *E. coli* cells were transformed with

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source/Reference
Strains		
Escherichia coli DH5α	F^- λ^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*(r_K^-, m_K^+)	DSM-6897/Grant et al. (1990)
E. coli K12-MG1655	F^- λ^- *rph-1*	DSM-18039/Guyer et al. (1981)
E. coli K12-W3110	F^- λ^- IN(*rrnD-rrnE*)1 *rph-1*	DSM-5911/Kohara et al. (1987)
E. coli BL21(DE3)	F^- *dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(DE3)	NEB/Studier and Moffatt (1986)
E. coli Nissle 1917	Wild type	Mutaflor (Ardeypharm)
E. coli DH5α+pVLT31-*egfp*	*E. coli* DH5α containing pVLT31-*egfp*	This study
E. coli K12-MG1655+pVLT31-*egfp*	*E. coli* K12-MG1655 containing pVLT31-*egfp*	This study
E. coli K12-W3110+pVLT31-*egfp*	*E. coli* K12-W3110 containing pVLT31-*egfp*	This study
E. coli BL21(DE3)+pET22b-*egfp*	*E. coli* BL21(DE3) containing pET22b-*egfp*	This study
E. coli Nissle 1917+pVLT31-*egfp*	*E. coli* Nissle 1917 containing pVLT31-*egfp*	This study
E. coli BL21(DE3)+pET22b-*bc*	*E. coli* BL21(DE3) containing pET22b-*bc*	This study
E. coli BL21(DE3)+pET22b-*hc*	*E. coli* BL21(DE3) containing pET22b-*hc*	This study
Plasmids		
pET22b(+)	*bla lacI* P_{T7}	Novagen/Studier et al. (1990)
pVLT31	*rep mob lacI*^{q} P_{tac} Tc^R	de Lorenzo et al. (1993)
pJOE4056.2	*bla*, *cer*, *rop*, *rhaP*$_{BAD}$, *egfp*	Wegerer et al. (2008)
pET100/D-TOPO-*bc*	*bla lacI* P_{T7} containing *bovine casein*	Gene Art (Regensburg, Germany)
pET100/D-TOPO-*hc*	*bla lacI* P_{T7} containing *human casein*	Gene Art
pET22b-*egfp*	pET22b with *NdeI*/*Hin*dIII fragment containing *egfp*	This study
pVLT31-*egfp*	pVLT31 with *XbaI*/*Hin*dIII fragment containing *egfp*	This study
pET22b-*bc*	pET22b with *NdeI*/*Hin*dIII fragment containing *bovine casein*	This study
pET22b-*hc*	pET22b with *NdeI*/*Hin*dIII fragment containing *human casein*	This study

the resulting recombinant plasmids (Table 1) using a standard protocol (Hanahan, 1983).

2.3 | Media and growth conditions

Escherichia coli strains were grown at 37°C in either lysogenic broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; pH 7.0), Wilms-KPi medium or wheat straw hydrolysate medium. The adapted Wilms-KPi medium (Wilms et al., 2001) was composed of a phosphate buffer system (6.58 g/L K_2HPO_4, 1.64 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4CL, 2 g/L Na_2SO_4, 25 g/L $MgSO_4{\cdot}7H_2O$; pH 7.4) supplemented with 3 ml/L of a trace element solution (0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $MnSO_4{\cdot}H_2O$, 13.92 g/L $FeCL_3{\cdot}6H_2O$, 10.05 g/L ethylenediaminetetraacetic acid Titrieplex III, 0.18 g/L $CoCL_2{\cdot}6H_2O$, 0.662 g/L $CaCL_2{\cdot}2H_2O$) and 0.01 g/L thiamine HCl. All components were sterilized using a 0.2 µm membrane filter, except for the phosphate buffer, which was sterilized by autoclaving. A quantity of 10 g/L D-glucose, D-xylose or L-arabinose was used as sole carbon source. In artificial wheat straw hydrolysate medium, a total sugar amount of 10 g/L with the same composition as in typical wheat straw hydrolysates was added: 6.29 g/L D-glucose, 3.30 g/L D-xylose and 0.41 g/L L-arabinose (Schläfle et al., 2017). In bioreactor cultivations a dried and milled wheat straw was used as substrate. Based on steam explosion process, hemicellulose and lignocellulose components were disrupted and further degraded into monosaccharides by an enzymatic hydrolysis process over 5 days (Schläfle et al., 2017). Particles were removed by centrifugation and the supernatant was sterilized through filtration (Nalgene Rapid Flow 0.2 µm, Thermo Fisher Scientific). After quantification of the carbohydrate monomers the hydrolysates were used as carbon source for the bioreactor cultivations. For the selection of recombinant strains, 100 µg/ml ampicillin or 10 µg/ml tetracycline was added to the media. For induction of gene expression, 0.5 mM of isopropyl β-D-thiogalactoside

(IPTG) was supplemented when the cultures were grown to a density of approximately OD_{600} of 0.5–0.8.

For shaking flasks experiments precultures of 15 ml LB medium in 100-ml Erlenmeyer flasks were inoculated with a single colony from an LB agar plate and incubated overnight in a incubator shaker (New Brunswick Scientific) at 37°C and 150 rpm. Main cultures with 15 ml of defined medium in 100-ml Erlenmeyer flasks were inoculated to an OD_{600} of 0.1 from the precultures. For bioreactor cultivations first precultures were cultivated for 8 h in 10 ml LB medium (100-ml flasks) before a volume of 100 µl cell suspension was used for the inoculation of the seed culture with 25 ml Wilms-KPi medium (250-ml flasks) containing 10 g/L glucose for further 8 h of cultivation. The bioreactor cultivation was carried out as duplicate in a 2 L bioreactor (Labfors 4; Infors AG) using 600 ml Wilms-KPi medium including 380 ml of pretreated lignocellulose hydrolysate, resulting in concentration of 7.5 g/L glucose and 5.5 g/L xylose. Aeration rate was set to 0.2 vvm with pO_2 set at 20% regulated by stirring speed. The temperature was set to 37°C and pH was maintained at 7.4 using 1 M H_2SO_4 and 1 M NaOH. After inoculation with seed culture medium to an OD_{600} of 0.1, cultivation was conducted for 18 h. Heterologous gene expression was induced at OD 0.6 using a final concentration of 0.5 mM ITPG.

2.4 | Analytical methods

Cell growth was determined densitometrically by measuring the optical density at 600 nm. The culture samples were centrifuged at 4°C and 15,000 *g* for 5 min. Cell pellets and supernatant were stored at −20°C for later analyses.

For the shaking flasks experiments, the culture supernatants were analysed for residual sugars by using the D-Glucose assay kit, D-Xylose assay kit and L-Arabinose/D-Galactose assay kit (Megazyme). Carbohydrate analysis of the samples from the bioreactor was conducted by separation with a HPTLC system (CAMAG), followed by staining with diphenylamin-aniline (DPA) reagent, heating for 20 min at 120°C and detection at 620 nm. DPA reagent was prepared by 2.4 g diphenylamine and 2.4 g aniline in a mixture of 200 ml methanol and acidification with 20 ml phosphoric acid (85%). The eluent used for chromatography was a mixture of 85:15 (v/v) acetonitrile/water on silica gel glass plates (Silica Gel 60; Merck).

For the verification of protein production, 200 µl of Bugbuster Mastermix (Merck) was added to the pellets and incubated for 15 min on a shaking platform for cell disruption. Samples were used as whole cell extracts or cell debris were separated from the supernatant by centrifugation at 21,000 *g*. For quantification of the α_{S1}-casein, Laemli buffer (Bio-Rad) was added to the samples in proportion to the OD measured during cultivation and heated at 95°C for 15 min. For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), samples were diluted in 1:20 ratio and applied on conventional 12% polyacrylamide gels or on Mini Protean TGX Gels (Bio-Rad). Gels were stained with coomassie blue staining solution (Thermo Fisher Scientific) and photographed using a gel documentation system (Bio-Rad).

For the measurement of fluorescence, three aliquots of 100 µl of each suspension were added to 96 well flat-bottom polystyrene microplates (Sarstedt), and the fluorescence was measured with a Tecan infinite f200 (Tecan), at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The background fluorescence was determined by using each corresponding *E. coli* strain culture carrying the empty vector. The relative fluorescence (%) was calculated according to the highest measurement.

Quantification of protein expression was performed by densitometry scanning. The concentrations of the α_{S1}-caseins were determined by comparing the densities of the bands to the standard casein (α_{S1}-casein from bovine milk, C6780; Merck) using the ImageJ software (www.imagej.net/ImageJ1; Schneider et al., 2012). The background from the empty vector was subtracted in the calculation. Error bars for quantitative data for casein during bioreactor cultivation were obtained from the largest observed relative measurement error from all independent biological experiments and transferred to all individual measurements as relative error bars.

Graphical and regression analysis was performed using scientific graphing and data analysis software (SigmaPlot 13.0; Systat Software Inc.).

3 | RESULTS

3.1 | Growth of *E. coli* strains on glucose, xylose and arabinose

Traditionally *E. coli* is cultivated on D-glucose in laboratories world-wide. However, most if not all typical *E. coli* strains are known to be capable to grow also on other sugars like D-xylose and L-arabinose as sole carbon sources, which are the predominant sugars of lignocellulose especially in grasses and thus do not represent a direct competitor to food production. With the aim to verify this as a base for potential applications using lignocellulosic biomass as sustainable and cost-efficient carbon source, the growth behaviour of various plasmidless and recombinant *E. coli* strains on different sugars was examined. Prominent *E. coli* laboratory strains, namely DH5α as a standard laboratory strain for cloning experiments, K12-MG1655 and K12-W3110 as two of the oldest laboratory strains and BL21(DE3) as the typical overexpression strain, as well as the probiotic strain *E. coli* Nissle 1917 were cultivated in minimal medium containing 10 g/L glucose, xylose or arabinose as sole carbon source. Samples for measurement of growth performance and sugar

consumptions were taken every 3 h (Figure 1). Additionally, the values of the final optical densities after 37 h of cultivation and the timepoints, when each sugar was completely consumed, are listed in Table 2. As expected, the commonly used laboratory strains *E. coli* DH5α and BL21(DE3) reached the highest cell density growing on glucose, which was about 19% respectively 12% higher in comparison to their growth on xylose and arabinose. Surprisingly, the growth of the *E. coli* strains MG1655 and W3110 as two more 'wild typical' laboratory strains with less genomic modifications and the probiotic *E. coli* Nissle 1917 wild type strain was 18%, 14% and 19%, respectively, higher on xylose in comparison to the averaged growth on glucose and arabinose. In all cases, fastest consumption was observed for glucose followed by xylose and arabinose, but there were also differences in the duration a specific *E. coli* strain needed to completely deplete the different sugars. Once more, the laboratory strains DH5α and *E. coli* BL21(DE3) showed a conspicuous different behaviour compared to the other three strains and needed a longer time of about 20–25 h to consume all the sugars. In comparison, the other *E. coli* strains depleted all the sugars 5.5 h faster on average.

3.2 | Heterologous protein production on glucose, xylose and arabinose using enhanced green fluorescent protein as an example

For demonstrating the ability to produce recombinant proteins based on the different lignocellulosic sugars, the enhanced green fluorescent protein (eGFP) was chosen as a model protein. This well-established reporter protein originally isolated from the jellyfish *Aequorea victoria* (Shimomura et al., 1962) is commonly used to provide reliable information about the effectiveness of gene expression in various host organism. Therefore, the plasmid pJOE4056 was hydrolysed using *Nde*I

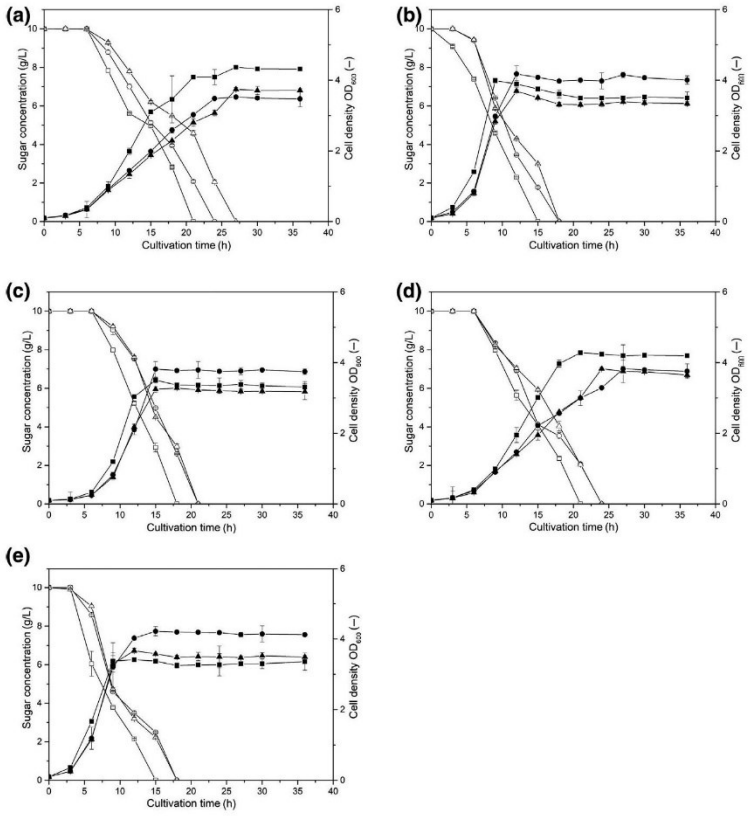

FIGURE 1 Growth performance of different *Escherichia coli* strains on glucose, xylose and arabinose. The strains *E. coli* DH5α (a), *E. coli* K12-MG1655 (b), *E. coli* K12-W3110 (c), *E. coli* BL21(DE3) (d) and *E. coli* Nissle 1917 (e) were cultivated in Wilms-KPi medium containing 10 g/L of different monosaccharides. The bacterial growth (filled symbols) and the sugar consumption (empty symbols) are presented for the cultivation on glucose (squares), xylose (circles) and arabinose (triangles) as sole carbon source. The symbols indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations

TABLE 2 Conclusion of final optical densities of plasmidless and recombinant *Escherichia coli* strains on different carbon sources after 37 h of cultivation and the timepoints when each sugar was completely consumed

	Plasmidless strain			Empty vector			*egfp* expression		
***E. coli* strain**	**Glucose**	**Xylose**	**Arabinose**	**Glucose**	**Xylose**	**Arabinose**	**Glucose**	**Xylose**	**Arabinose**
DH5α									
Optical density after 37 h	4.3 ± 0.02	3.5 ± 0.01	3.7 ± 0.05	5.7 ± 0.04	4.5 ± 0.16	4.7 ± 0.03	6.0 ± 0.16	5.0 ± 0.16	5.1 ± 0.06
Sugar consumed after	21 h	24 h	27 h	21 h	24 h	24 h	24 h	27 h	27 h
MG1655									
Optical density after 37 h	3.5 ± 0.17	4.0 ± 0.12	3.3 ± 0.06	4.1 ± 0.11	4.3 ± 0.02	4.1 ± 0.15	4.7 ± 0.08	4.7 ± 0.21	4.4 ± 0.23
Sugar consumed after	15 h	18 h	18 h	15 h	18 h	18 h	15 h	18 h	18 h
W3110									
Optical density after 37 h	3.3 ± 0.16	3.7 ± 0.08	3.2 ± 0.03	4.5 ± 0.1	4.9 ± 0.05	4.3 ± 0.25	5.7 ± 0.13	5.1 ± 0.3	5.1 ± 0.28
Sugar consumed after	18 h	21 h	21 h	18 h	24 h	21 h	18 h	21 h	21 h
BL21(DE3)									
Optical density after 37 h	4.2 ± 0.02	3.8 ± 0.01	3.7 ± 0.07	5.6 ± 0.04	6.8 ± 0.07	4.9 ± 0.04	7.1 ± 0.11	8.0 ± 0.01	6.7 ± 0.21
Sugar consumed after	21 h	24 h	24 h	21 h	24 h	24 h	21 h	24 h	24 h
Nissle 1917									
Optical density after 37 h	3.4 ± 0.14	4.1 ± 0.04	3.5 ± 0.04	5.3 ± 0.15	7.1 ± 0.16	6.4 ± 0.18	6.3 ± 0.16	7.4 ± 0.06	7.4 ± 0.14
Sugar consumed after	15 h	18 h	18 h	15 h	18 h	18 h	15 h	18 h	18 h

and *Hind*III to receive the *egfp* gene for subcloning into the pET22b vector, which was equally digested. Subsequently, the resulting pET22b-*egfp* containing *egfp* under the control of a T7 promoter was hydrolysed by *Xba*I and *Hind*III for subcloning of *egfp* including the ribosomal binding site (RBS) of pET22b into the pVLT31 shuttle vector. In the resulting plasmid pVLT31-*egfp* the transcriptional regulation of *egfp* is controlled by an IPTG inducible P_{tac} for expression in *E. coli* strains, which do not harbour a T7 RNA polymerase in their genome. The resulting recombinant strains *E. coli* DH5α+pVLT31-*egfp*, *E. coli* K12-MG1655+pVLT31-*egfp*, *E. coli* K12-W3110+pVLT31-*egfp*, *E. coli* BL21(DE3)+pET22b-*egfp* and *E. coli* Nissle 1917+pVLT31-*egfp* and respective strains containing the corresponding empty vectors were cultivated in minimal medium containing 10 g/L glucose, xylose or arabinose as sole carbon source. Samples were also taken every 3 h for measurement of cell densities and sugar consumption. Additionally, samples were taken before induction as well as 12 and 24 h after induction to verify the successful expression of *egfp* by SDS-PAGE and fluorescence measurements (Figure 2).

In general, the curve progression of recombinant *E. coli* strains was quite similar related to growth performances and sugar consumption in comparison to the plasmidless strains in the earlier experiment, wherefore we decided to provide the figure in the Supporting Information section (Figure S1). However, the finally OD_{600} values attained as well as the timepoints when each sugar was completely consumed are listed in comparison to the other cultivation in Table 2. Surprisingly, all recombinant strains reached significantly higher optical densities, while the timepoints, when all sugars were completely consumed, were almost identical to the experiments with the plasmidless strains. Moreover, the growth of strains harbouring the empty vectors was on average increased by 40%, but strains expressing the *egfp* gene even reached a 61% increased final optical density. The greatest increase was recorded for the growth of *E. coli* Nissle 1917 expressing *egfp*, which could even more than double its growth cultivated on arabinose in comparison to the wild type strain. Astonishingly, in this experiment the strain *E. coli* BL21 reached slightly higher optical densities on xylose than on glucose, but

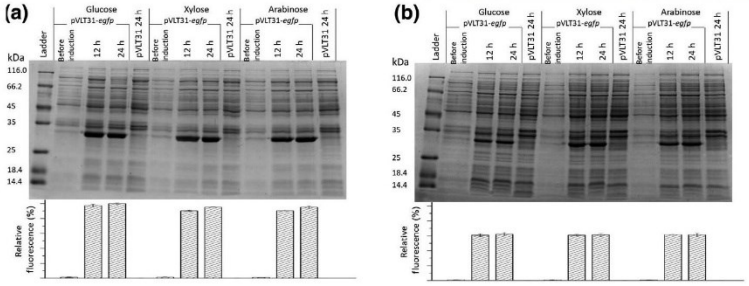

FIGURE 2 Determination of eGFP production in different recombinant *Escherichia coli* strains by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence measurement. The strains were cultivated in Wilms-KPi medium containing 10 g/L of different monosaccharides. The results for *E. coli* DH5α+pVLT31-*egfp* (a), and *E. coli* Nissle 1917+pVLT31-*egfp* (b) are shown to be exemplary, other data are deposited as Supporting Information. Samples of eGFP protein from different time points are shown: before induction, 12 and 24 h after induction. Samples of empty vector taken 24 h after induction serve as control. The relative fluorescence of the eGFP protein is shown corresponding to the bands on SDS-PAGE. The values indicate the averages of the results of triplicate measurements. Error bars represent the SDs

in general all strains showed very effective growth on all three sugars.

The successful expression of *egfp* under both promoters (P_{T7} in pET22b and P_{tac} in pVLT31) in all the *E. coli* strains on each single sugar was verified using SDS-PAGE. As exemplarily shown in Figure 2, in the samples taken 12 h after induction from every single strain independent of the used sugar distinct eGFP bands are already visible, indicating a sufficient amount of the protein production. Moreover, there were nearly no differences in the intensity of the bands for each single strain cultivated on the different sugars. However, with the corresponding fluorescence measurements, the differences between each strain were determined (Figure S2). In particular, the strain *E. coli* DH5α showed the highest relative fluorescence, which was set to 100%, followed by the other *E. coli* strains with 70%–90%. Only *E. coli* Nissle 1917 showed the lowest relative fluorescence of about 60%, but a successful expression of *egfp* based on typical sugars from lignocellulose was given in all cases.

3.3 | Production of bovine and human α_{S1}-casein on single sugars and artificial wheat straw hydrolysate

For the realization of a potential casein biosynthesis based on lignocellulosic hydrolysates, relevant expression vectors harbouring respective genes were constructed. For the heterologous production of α_{S1}-casein variants, the efficient T7 expression system was chosen, with the pET22b expression vector and *E. coli* BL21(DE3) as host organism, which also showed highly effective growth on all single sugars in the earlier experiments. The genes for α_{S1}-casein from bovine and human resources were reverse translated from their amino acid sequences (http://www.uniprot.org/uniprot/P02662 and http://www.uniprot.org/uniprot/P47710) and optimized for codon usage of *E. coli*. The gene sequence for bovine α_{S1}-casein and the human α_{S1}-casein has sizes of 645 and 558 bp respectively. As additional elements a His-affinity tag (six consecutive histidine residues) and an enterokinase recognition site were added to be present at the N-terminus of the final protein. Both synthetic genes, designated as *bc* for bovine and *hc* for human α_{S1}-caseins, respectively, were individually synthesized and provided in the pET100/D-TOPO vectors by Gene Art (Regensburg, Germany). Their sequences are given in the Supporting Information section (Figure S3). The received plasmids were hydrolysed with *Nde*I and *Hin*dIII to obtain fragments including the encoding region and additional functional sites and subsequently ligated into the pET22b expression vector, which was similarly digested. The resulting recombinant expression plasmids were designated as pET22b-*bc* and pET22b-*hc*, with the α_{S1}-casein genes under the control of a T7 promotor.

The cultivation of the recombinant strains *E. coli* BL21(DE3)+pET22b, *E. coli* BL21(DE3)+pET22b-*bc* and *E. coli* BL21(DE3)+pET22b-*hc* on 10 g/L glucose, xylose and arabinose as sole carbon sources was carried out as described before. All the recombinant strains also showed successful growth on the three single sugars and curve progressions are comparable with the earlier experiments with the BL21(DE3) strain (Table 3), thus corresponding graphs are provided in the Supporting Information section (Figure S4).

Specifically, the *E. coli* BL21(DE3) strain harbouring the empty vector reached nearly identical optical densities as before. Remarkably, also in this experiment the recombinant strain expressing the target protein, in this case the two α_{S1}-casein variants, showed better growth on all three sugars and reached on average 19% higher optical density as the strain containing the empty vector.

In addition, samples for analysing the effective production of α_{S1}-caseins in the recombinant strains on the three

TABLE 3 Conclusion of final optical densities of recombinant *Escherichia coli* BL21(DE3) strains on different carbon sources after 37 h of cultivation and the timepoints when each sugar was completely consumed

	Empty vector				Bovine casein expression				Human casein expression			
	Glucose	Xylose	Arabinose	Hydrolysate	Glucose	Xylose	Arabinose	Hydrolysate	Glucose	Xylose	Arabinose	Hydrolysate
E. coli BL21(DE3)												
Optical density after 37 h	5.5 ± 0.07	6.8 ± 0.27	4.6 ± 0.09	6.8 ± 0.15	6.5 ± 0.08	7.6 ± 0.07	5.3 ± 0.1	7.7 ± 0.06	6.4 ± 0.12	7.5 ± 0.08	6.4 ± 0.08	7.6 ± 0.03
Sugar consumed after	15 h	24 h	24 h	Glu: 12 h Xyl: 18 h Ara: 9 h	15 h	24 h	24 h	Glu: 12 h Xyl: 18 h Ara: 9 h	15 h	24 h	24 h	Glu: 12 h Xyl: 18 h Ara: 9 h

different sugars by SDS-PAGE were taken prior to the induction of gene expression and 6, 12 and 24 h after induction. As shown in Figure 3, the bovine and human α_{S1}-caseins were both successfully produced on glucose, xylose and arabinose and the bands from the samples on various sugars at specific time points showed comparable intensities. Both proteins are already visible as dominant bands in SDS-PAGE at 6 h after the induction indicating their efficient biosynthesis. Samples taken before induction from all the strains and 24 h after induction of the empty vector served as control.

To investigate the possible utilization of lignocellulosic hydrolysates by the recombinant *E. coli* BL21(DE3) strains, we first applied an artificial hydrolysate with a typical sugar composition as in wheat straw hydrolysate (6.29 g/L glucose, 3.30 g/L and 0.41 g/L arabinose). Due to its high annual tonnage, especially in Europe, wheat straw could become an excellent feedstock provided through hydrolysis for biotechnological applications.

All recombinant *E. coli* strains were able to grow on these artificial straw hydrolysates with high cell densities (Figure 4; Figure S5; Table 3). The low amounts of arabinose were always consumed first and, surprisingly, already parallel to the utilization of glucose as the presumed preferred carbon source. However, the utilization of xylose started not until glucose was completely consumed. Again, the *E. coli* strains expressing the α_{S1}-casein variants showed better growth performance and reached about 13% higher optical density than the strain carrying the empty vector.

The successful production of bovine α_{S1}-casein and human α_{S1}-casein proteins was again verified using SDS-PAGE (Figure 5). Samples were taken prior to the induction of gene expression and 6, 12 and 24 h after induction. The dominant bands of both casein variants are visible and had higher intensities as corresponding bands in the empty vector indicating the efficient production of casein based on the artificial hydrolysates.

Figures 3 and 5 display the successful production of bovine α_{S1}-casein and human α_{S1}-casein proteins on each single sugar and artificial wheat straw hydrolysate. For determining the casein concentrations for both variants at each timepoint additional SDS-PAGE was carried out containing casein standard samples in known concentrations (Table 4). In this way, amounts of the expressed proteins under different cultivation conditions were densitometrically calculated using the ImageJ software (Schneider et al., 2012). The densities of the bands from the empty vector were subtracted in the calculations as background.

For the production of both α_{S1}-casein variants, there are some characteristics in common. Almost all cultures achieved the highest protein concentration already after 12 h of induction of gene expression or with only marginal further increases within the next 12 h. The achieved amounts of bovine casein were nearly identical using the different single sugars as carbon source, but increased by about 58% when this strain was cultivated on artificial wheat straw hydrolysates. In contrast,

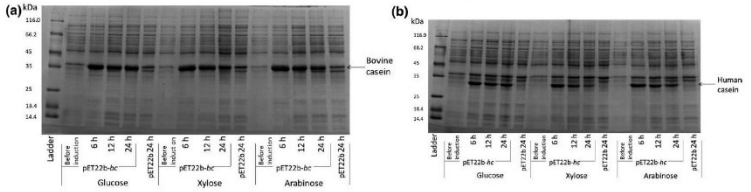

FIGURE 3 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of bovine and human α_{S1}-casein production in different recombinant *Escherichia coli* strains. The production of recombinant bovine α_{S1}-casein (a) and human α_{S1}-casein (b) expressed in *E. coli* BL21(DE3) from the cultivation in Wilms-KPi medium containing 10 g/L of different monosaccharides. Samples of bovine α_{S1}-casein and human α_{S1}-casein are shown from different time points: before induction, 6, 12 and 24 h after induction. Samples of empty vector 24 h after induction are shown as a control

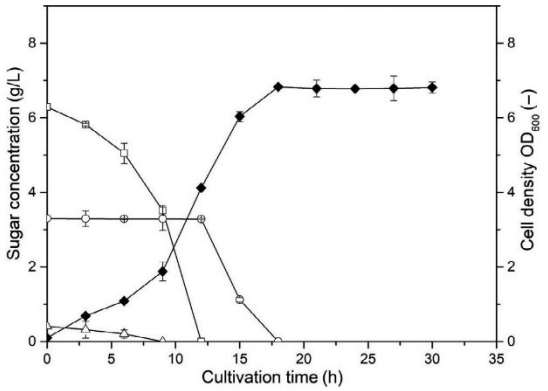

FIGURE 4 Growth performance of α_{S1}-casein expressing recombinant *Escherichia coli* BL21(DE3) strains on artificial wheat straw hydrolysate. The strains were cultivated in Wilms-KPi medium containing artificial wheat straw hydrolysate with 10 g/L as total amount of sugars (6.29 g/L glucose, 3.30 g/L and 0.41 g/L arabinose). The results for *E. coli* BL21(DE3)+pET22b are shown to be exemplary, other data are deposited as Supporting Information. Growth curves are shown as filled diamonds. The consumption of glucose (empty squares), xylose (empty circles) and arabinose (empty triangles) is presented. The symbols indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations

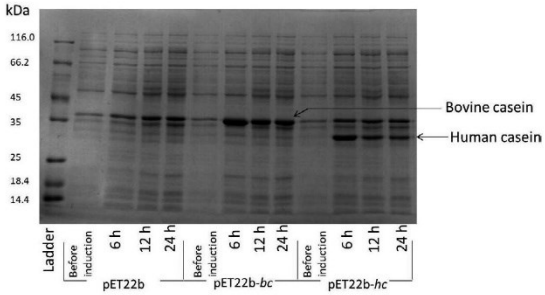

FIGURE 5 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant bovine α_{S1}-casein and human α_{S1}-casein expressed in recombinant *Escherichia coli* BL21(DE3) strains. The strains were cultivated in Wilms-KPi medium containing artificial wheat straw hydrolysate with a total sugar amount of 10 g/L and a composition of 6.29 g/L glucose, 3.30 g/L xylose and 0.41 g/L arabinose. Samples of empty vector, bovine α_{S1}-casein and human α_{S1}-casein are shown from different time points: before induction and 6, 12 and 24 h after induction

the biosynthesis of human casein is quite similar on glucose as single sugar and the sugar mixture, but the concentrations were about 44% higher than on xylose and arabinose. In general, these data indicate that mixtures of sugars as typical for lignocellulosic hydrolysates are preferable carbon sources for the successful biosynthesis of target proteins like in this case α_{S1}-caseins for recombinant *E. coli* strains.

3.4 | Application of real wheat straw hydrolysates for the production of human α_{S1}-casein

The final proof, that lignocellulosic hydrolysates serve as efficient and sustainable carbon source for the production of α_{S1}-casein, a bioreactor cultivation using *E. coli* BL21(DE3) expressing the gene for human α_{S1}-casein was applied using real wheat straw hydrolysates obtained from steam explosion followed by enzymatic hydrolysis process (Figure 6). The recombinant strain exhibited exponential growth following a short adaptation time to the lignocellulose hydrolysate-based medium resulting in maximum specific growth rates of up to 0.87 1/h after 6 h. The IPTG-based induction of heterologous gene expression was performed after 5 h of cultivation. As a consequence, a drop in growth rate was observable after 7 h. Casein was detected in the insoluble protein aggregate fraction after cell disruption, rising to a maximum of 1.45 g/L after 9 h. About 2 h post induction, a sharp decrease in specific growth rate was observed, which correlates with the onset of protein formation. With the depletion of glucose after approximately 10 h, xylose metabolization started. Cellular dry weight increased to a maximum of 4.8 g/L while casein concentration decreased to approximately 0.7 g/L after complete consumption of all metabolizable sugars in the medium.

TABLE 4 Concentration of bovine and human α_{S1}-casein proteins in the medium at different time points during cultivation on glucose, xylose and arabinose as single sugars as well as artificial wheat straw hydrolysate

	Glucose			Xylose			Arabinose			Artificial wheat straw hydrolysate		
Time (h)	6	12	24	6	12	24	6	12	24	6	12	24
Bovine casein (g/L)	0.51	0.72	0.67	0.21	0.29	0.74	0.33	0.68	0.69	0.61	1.03	1.13
Human casein (g/L)	0.65	0.95	0.89	0.27	0.50	0.57	0.38	0.58	0.76	0.72	0.97	0.96

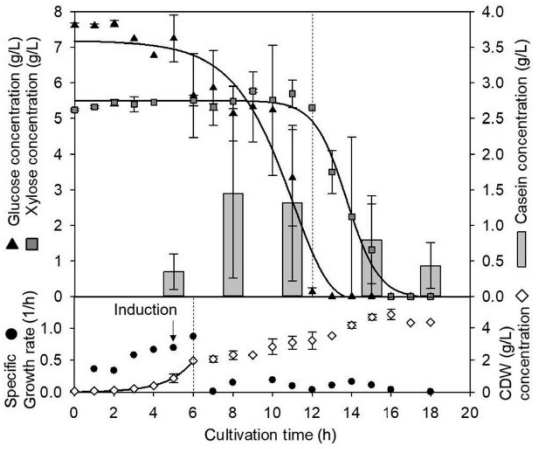

FIGURE 6 Time-course of biomass concentration (empty diamonds), glucose and xylose concentrations (filled triangles and squares) during cultivation of *Escherichia coli* BL21 Gold (DE3) pET22b during bioreactor cultivation on wheat straw hydrolysates. Casein formation is indicated by grey bars and specific growth rates (filled circles) along with vertical lines indicate changes in growth behaviour and change in sugar consumption respectively

4 | DISCUSSION

Its broad metabolic versatility enables *E. coli* to grow especially on multiple different sugars including various hexoses and pentoses. These sugars constitute the main components in lignocellulosic biomass, a sustainable and world-wide available carbon source as wood, food and agricultural residues (Anwar et al., 2014; Jørgensen et al., 2007; Lange, 2007; Naik et al., 2010; Van Dyk et al., 2013). Other bacterial organisms typically used in biotechnological applications are often incapable for utilizing pentoses and require previous genomic modifications to grow on lignocellulosic sugars (Chen et al., 2013; Kawaguchi et al., 2008; Le Meur et al., 2012; Wang et al., 2019), especially if the target product is difficult to produce in *E. coli*, e.g. biosurfactants (Bator et al., 2020; Cabrera-Valladares et al., 2006; Wittgens & Rosenau, 2018). On the other hand some more 'exotic' organisms, e.g. *Cellvibrio japonicus* are even able to metabolize complex lignocellulosic polymers, but they are not sufficiently developed and thus represent less convenient hosts for biotechnological applications (Gardner, 2016; Horlamus, Wittgens, et al., 2019).

In this study, different *E. coli* strains exhibited effective growth on single glucose, xylose and arabinose sugars as the main components of lignocellulosic hydrolysates from grasses with only minor differences. Surprisingly, the traditional *E. coli* strains K12-MG1655, W3110 and the probiotic Nissle 1917 grew better on xylose than on glucose and arabinose and consumed all the sugars faster in comparison to the present commonly used laboratory strains DH5α and BL21(DE3). Presumably, these modern strains underwent some undocumented modifications probably as side effects of a sort of evolutionary process during generations of laboratory treatments, which led to the different growth behaviour. Adaptive laboratory evolution is an efficient tool to force organisms and strains to improve particular desired properties like pH or solvent tolerance, acceptance of exceptional carbon sources and altered growth rates (Du et al., 2020; LaCroix et al., 2015; Meijnen et al., 2008; Mohamed et al., 2019). Our observations for the laboratory strains could be explained by these processes, since glucose is the most common carbon source used in biotechnology (Wendisch et al., 2016) and slower bacterial growth can be beneficial for the effective biosynthesis of recombinant proteins (Papaneophytou & Kontopidis, 2014; Yee & Blanch, 1992).

The utilization of different sugars is hierarchically organized in *E. coli*, which is visible during cultivation on sugar mixtures and real lignocellulosic hydrolysates. Based on the native regulatory mechanisms, glucose represents the preferred carbon source, while the utilization of xylose did not begin until glucose was completely consumed. The reason for this is the transcriptional regulation of the *xylAB* and *araBAD* operons, which are responsible for the metabolization of xylose and arabinose, respectively, by transcription factors of the AraC/XylS family and the cAMP-CRP interaction (Gallegos et al., 1997). Surprisingly, the small amounts of arabinose decreased already parallel to the utilization of glucose. Perhaps, arabinose is not already metabolized simultaneous with glucose, but already taken up by the fast increasing numbers of cells in this growth phase. *E. coli* possesses specific transporters for the uptake of xylose and arabinose encoded by *xylE* and *araE* (xylose/arabinose:H^+ symporters) as well as *xylFGH* and *araFGH* (xylose/arabinose ABC

transporters), whose expressions are not repressed by AraC/XylS in contrast to the operons for metabolizing these sugars (Gallegos et al., 1997).

The high effective production of recombinant proteins based on lignocellulosic sugars was initially demonstrated using the fluorescence reporter eGFP as a prominent model. Astonishingly, the recombinant *E. coli* strains, those expressing eGFP rather than those harbouring the corresponding empty vector, reached significantly higher cell densities during the cultivations. Usually, the selection pressure by the added antibiotics and the constitutive expression of antibiotic resistance genes in response as well as the general plasmid maintenance induce a stress response, especially when an additional target protein is highly expressed (Hoffmann & Rinas, 2004; Hoffmann et al., 2002). Such improved growth performances of recombinant strains were frequently described, but the reasons are very speculative (Yee & Blanch, 1992). However, the response to the high expression of recombinant protein includes an extensive reprogramming of gene expression patterns and down regulation of several housekeeping genes (Hoffmann et al., 2002), which probably provides more energy resources for other cellular functions.

This improved growth behaviour was further observed during the heterologous expression of bovine and human α_{S1}-casein in *E. coli* BL21(DE3). In addition to its highlyefficient protein biosynthesis depending on the T7 expression system, this host was chosen because it lacks the Lon and the OmpT proteases (Studier & Moffatt, 1986). Furthermore, the successful production of other recombinant milk proteins was also reported for this strain (García-Montoya et al., 2013; Goda et al., 2000; Hansson et al., 1993; Ponniah et al., 2010; Simons et al., 1993; Wang et al., 1989). However, this strain still possesses further proteases like the ATP-dependent Clp proteases, which are known to degrade a variety of proteins through multiple site cleavage (Thompson et al., 1994). The α_{S1}-caseins are known to be inherently sensitive to proteases in general, since they have only less secondary structures and lack disulphide bonds (Kumosinski et al., 1991), which explains the reduction in casein concentration after the initial spike during the fermentation process. With a pK_a of 4.6, α_{S1}-casein is highly soluble in alkaline or strongly acidic environments, but less soluble under the applied cultivation conditions (Post et al., 2012) and a high content of proline is increasing the hydrophobicity of casein (Gordon et al., 1950). Therefore, the synthesized casein was mainly involved in the formation of inclusion bodies, which have been reported for other mammalian recombinant proteins such as insulin and interleukin in *E. coli*, too (Chrunyk et al., 1993; Williams et al., 1982). Furthermore, in its natural environment, α_{S1}-casein folding is typically aided by other structural milk proteins, inorganic ions and hydrophobic substances, which results in a micellar structure (Dalgleish, 1998). Therefore, a deviation from its natural folding pattern in a high-efficiency heterologous expression system was expected. Regardless of its reported masses of 24.53 kDa for bovine and 21.67 kDa for bovine casein, corresponding bands were detected at approximately 35 kDa on the SDS gel. This effect is caused by the additional N-terminal elements, which increase the masses by 3.90 kDa, and local high negative charges, leading to an expended structure in the presence of SDS (Creamer & Richardson, 1984).

The factors affecting the capacity and cost associated with the production of a recombinant protein using *E. coli* was investigated in recent studies (Cardoso et al., 2020; Ferreira et al., 2018). The authors report prices between 35$ and 350$ per kg of protein, with the nitrogen source being the dominant part of total cost breakdown. Sugar production costs for hydrolysates gained via steam exploded straw can be estimated with 0.43$ per kg sugar (Baral, & Shah, 2017). It should be considered however, that these studies assume optimized steps of the process chains, especially downstream processing, which has a major influence of the final product price on its own. Furthermore, it should be noted, that for human casein or other difficult to obtain products, competitive pricing of heterologous production is less in the focus as biotechnological production is the only reasonable method of obtaining.

In conclusion, this approach successfully demonstrated how valuable (food) proteins can be synthesized based on bulk residual biomass in a cost-effective and economically efficient bioprocesses to achieve a truly sustainable bioeconomy in the future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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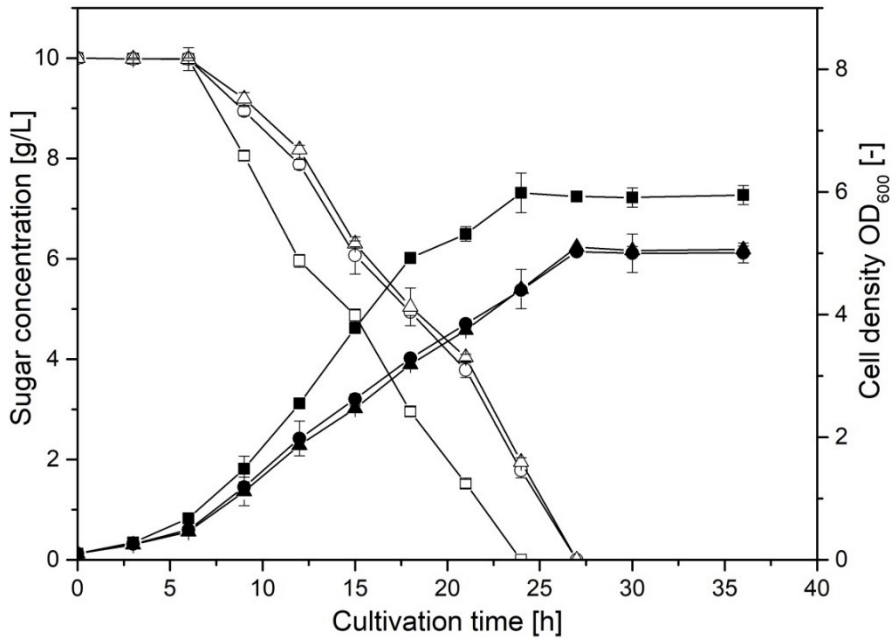
Supplementary information

Fig. S1A



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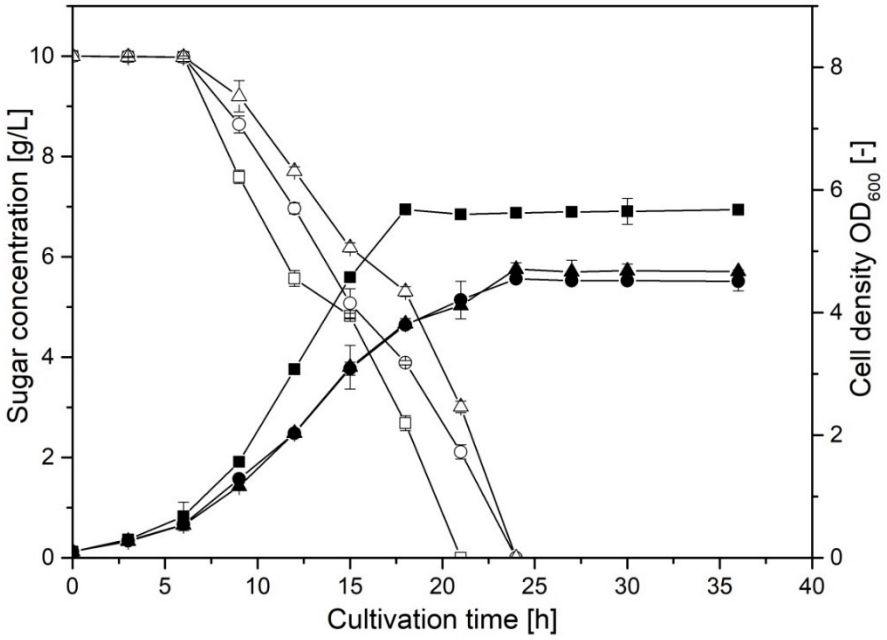
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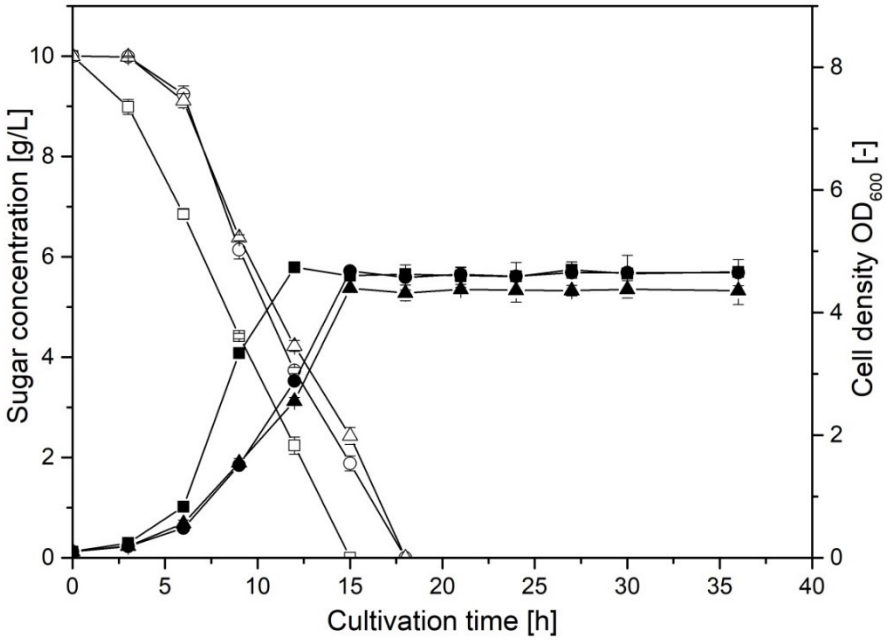
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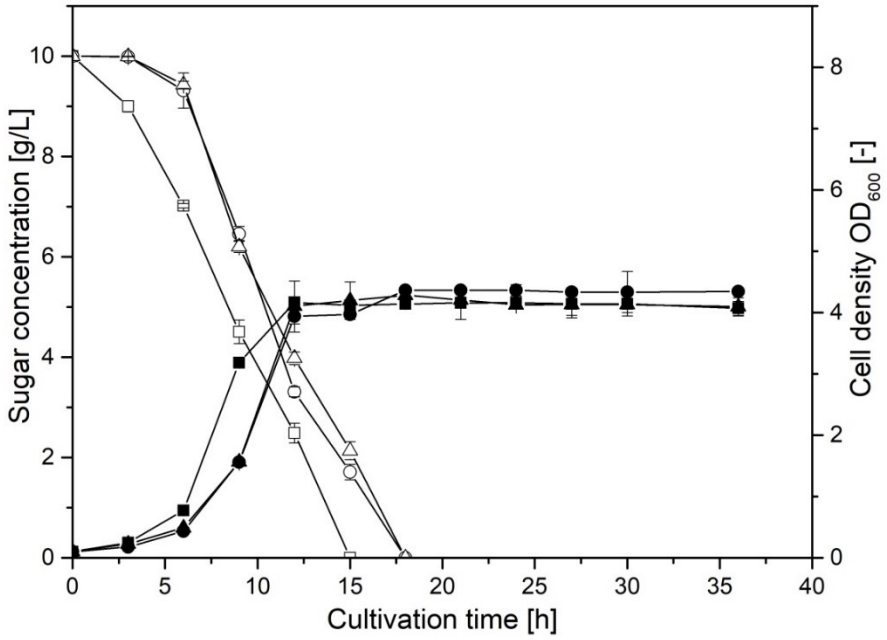
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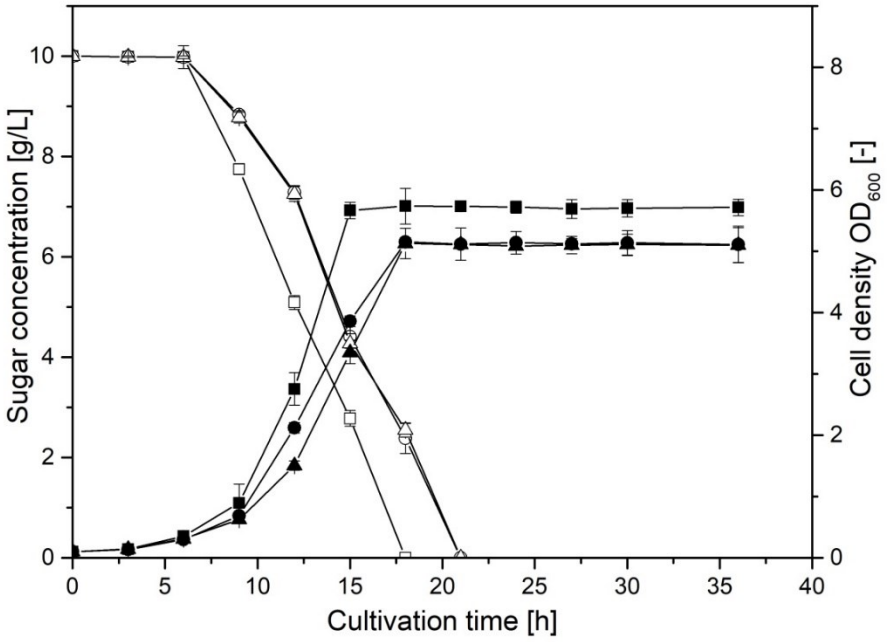
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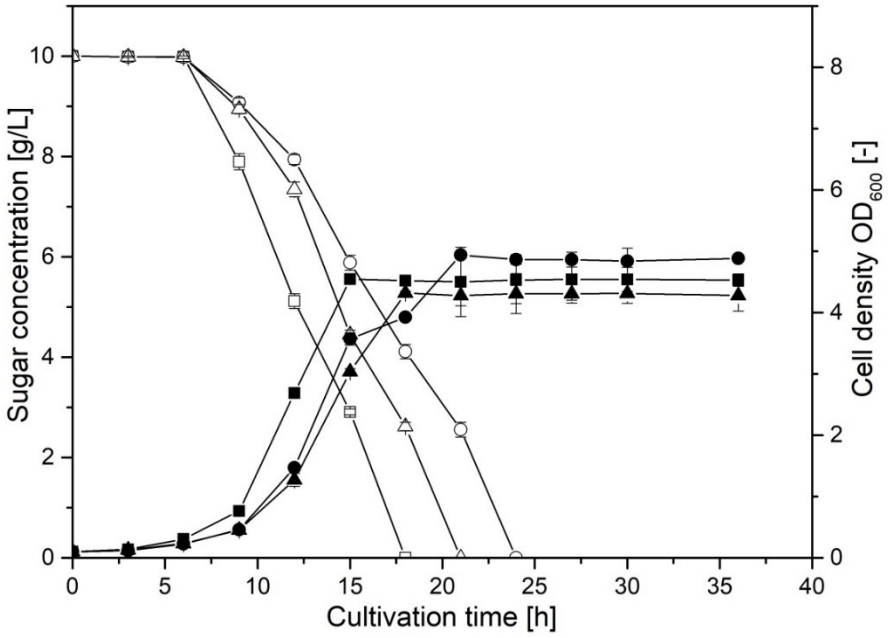
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Fig. S1G

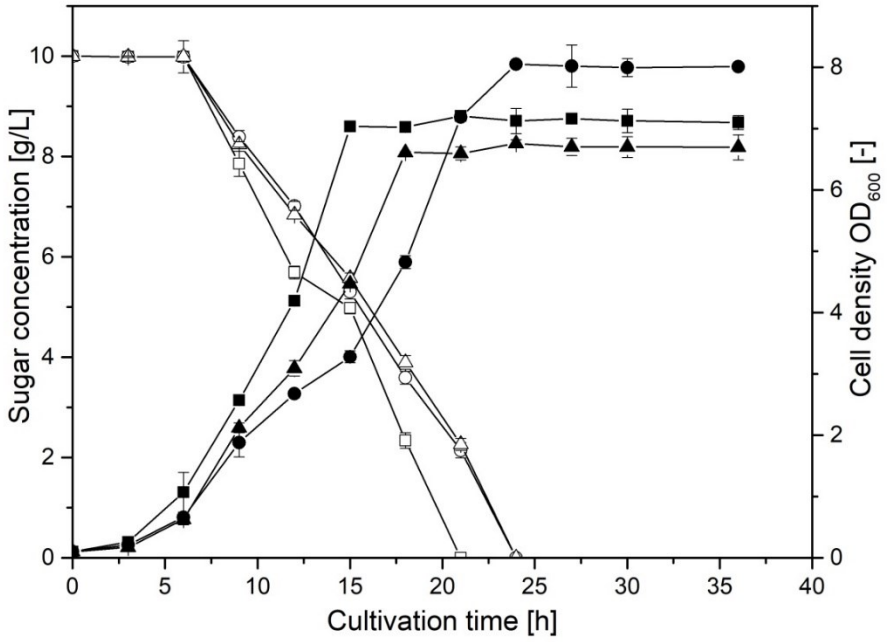
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Fig. S1H

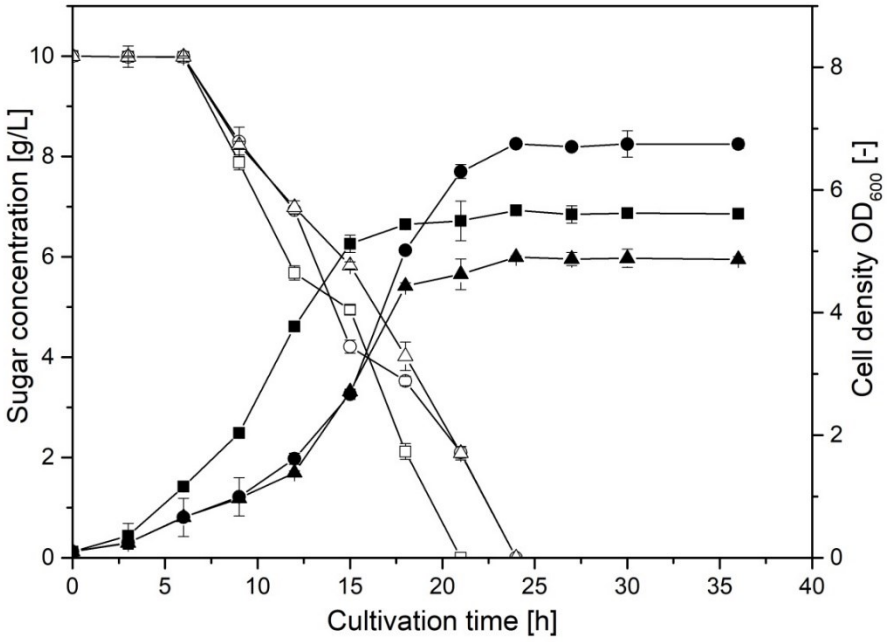
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Fig. S1I

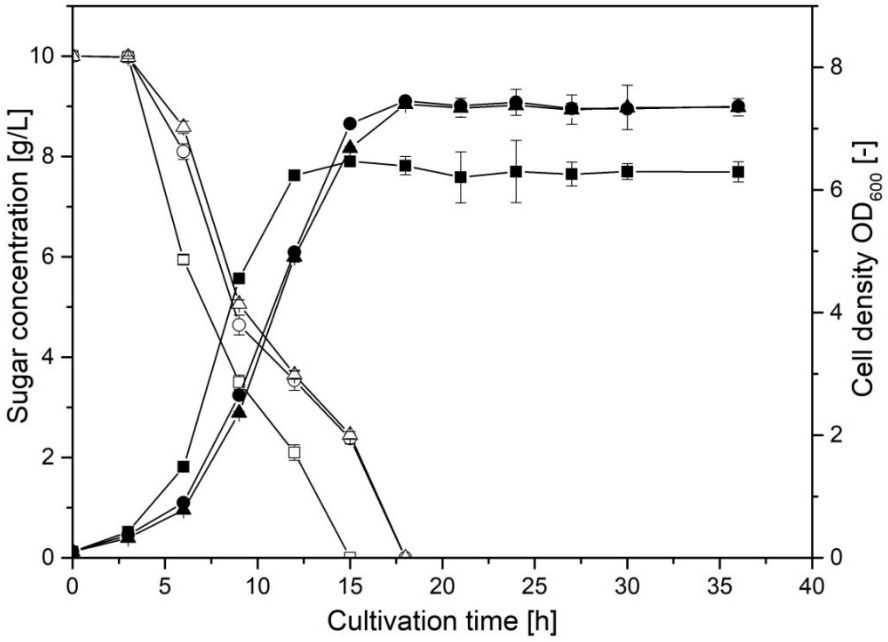
Sugar concentration [g/L]
Cell density OD600 [-]
Cultivation time [h]
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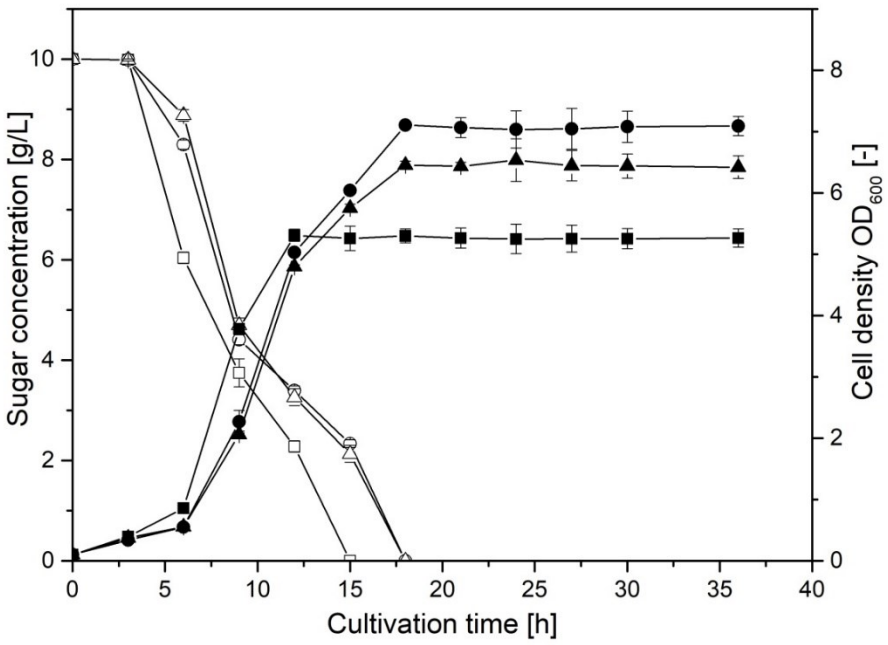
Fig. S1J



Supplementary figure 1 Growth performance of different recombinant *egfp* expressing *E. coli* strains. The strains *E. coli* DH5α+ pVLT31-*egfp* (A), *E. coli* K12-MG1655+pVLT31-*egfp* (C), *E. coli* K12-W3110+pVLT31-*egfp* (E), *E. coli* BL21(DE3)+pET22b-*egfp* (G) and *E. coli* Nissle 1917+pVLT31-*egfp* (I) were cultivated in Wilms-KPi medium containing 10 g/L of different monosaccharides. The same strains containing the corresponding empty vectors pVLT31 or pET22b are shown as controls (B, D, F, H, J). The bacterial growth (filled symbols) and the sugar consumptions (empty symbols) are presented for the cultivations on glucose (squares), xylose (circles) and arabinose (triangles) as sole carbon source. The symbols indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations.

Fig. S2A

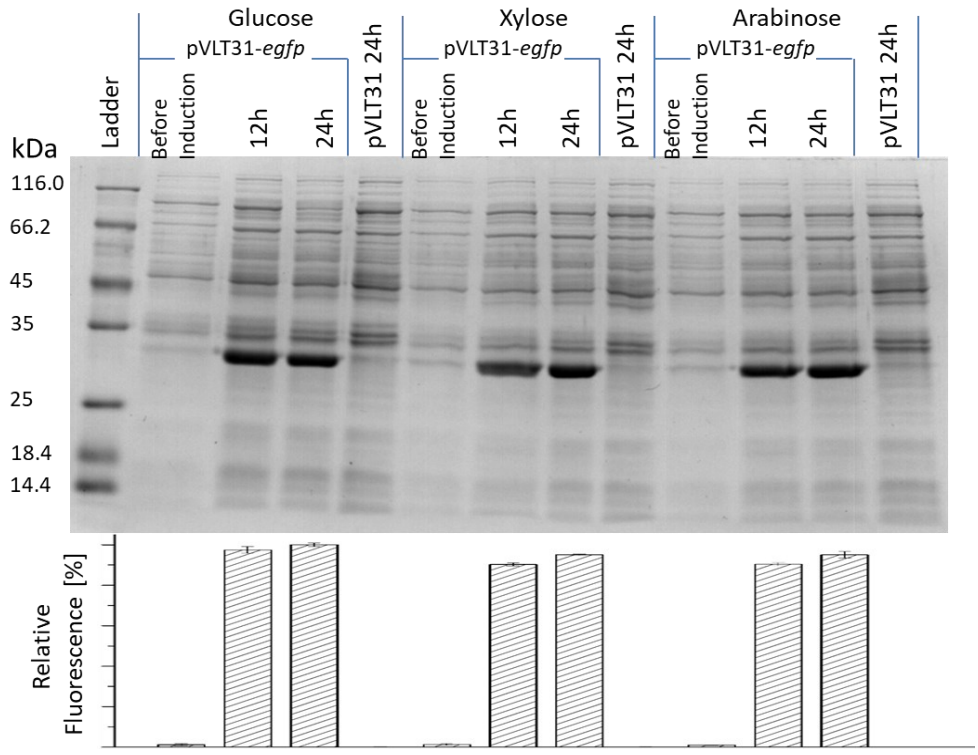


Fig. S2B

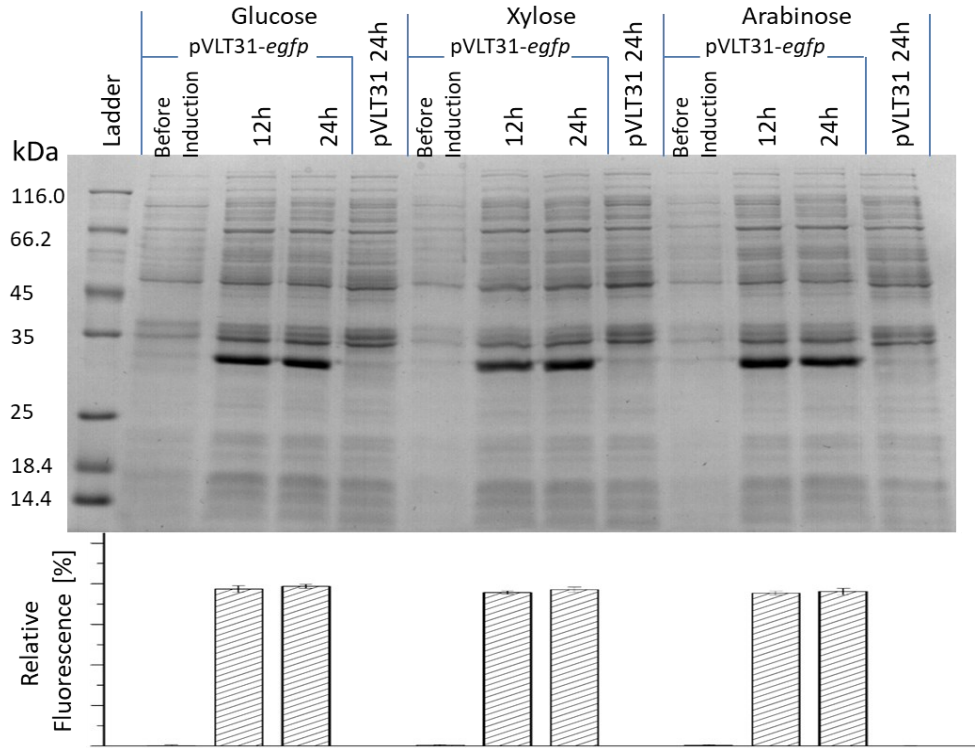


Fig. S2C

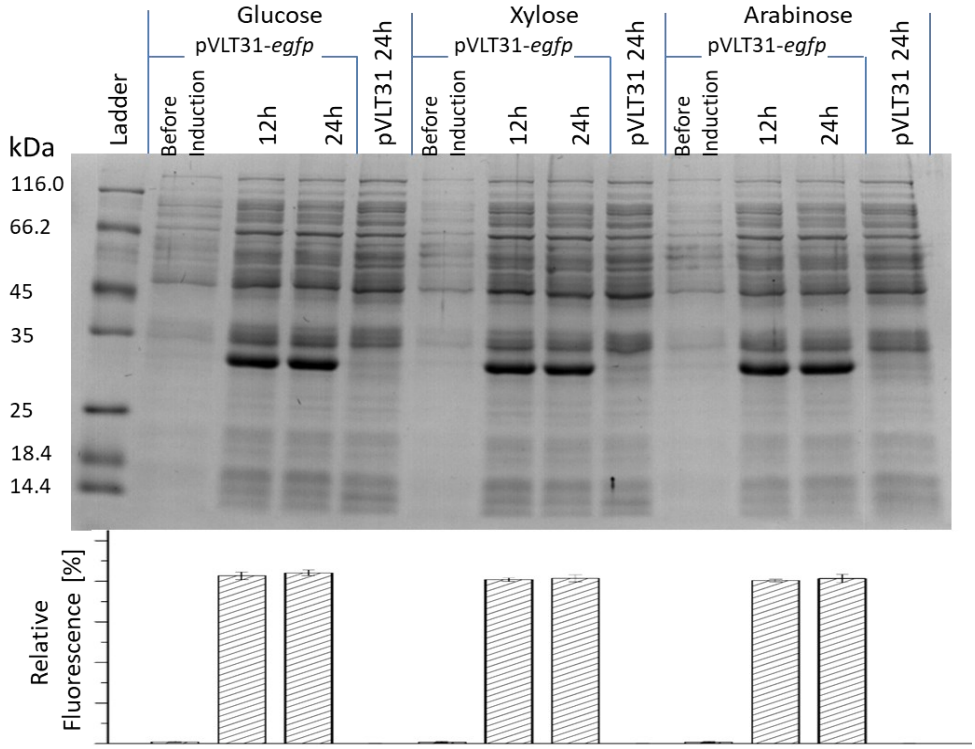


Fig. S2D

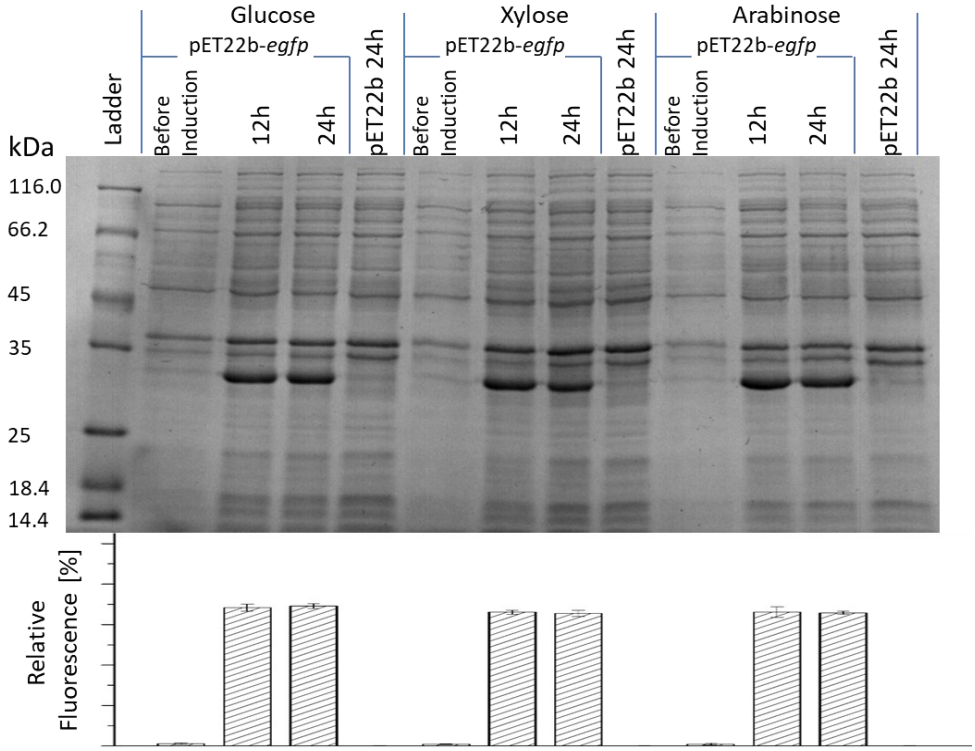
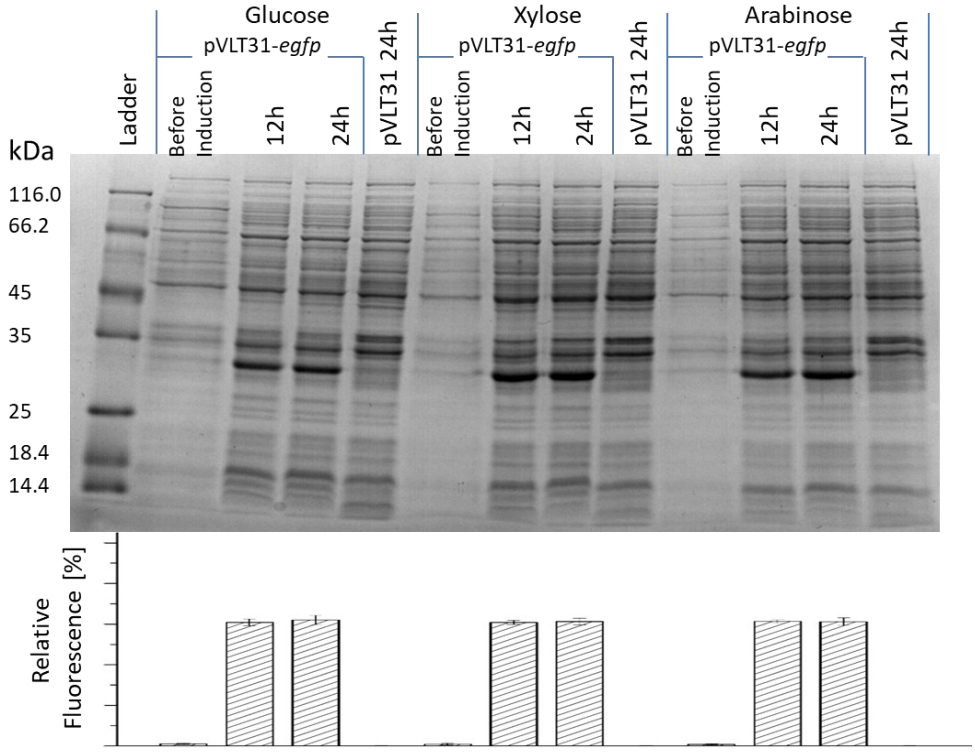


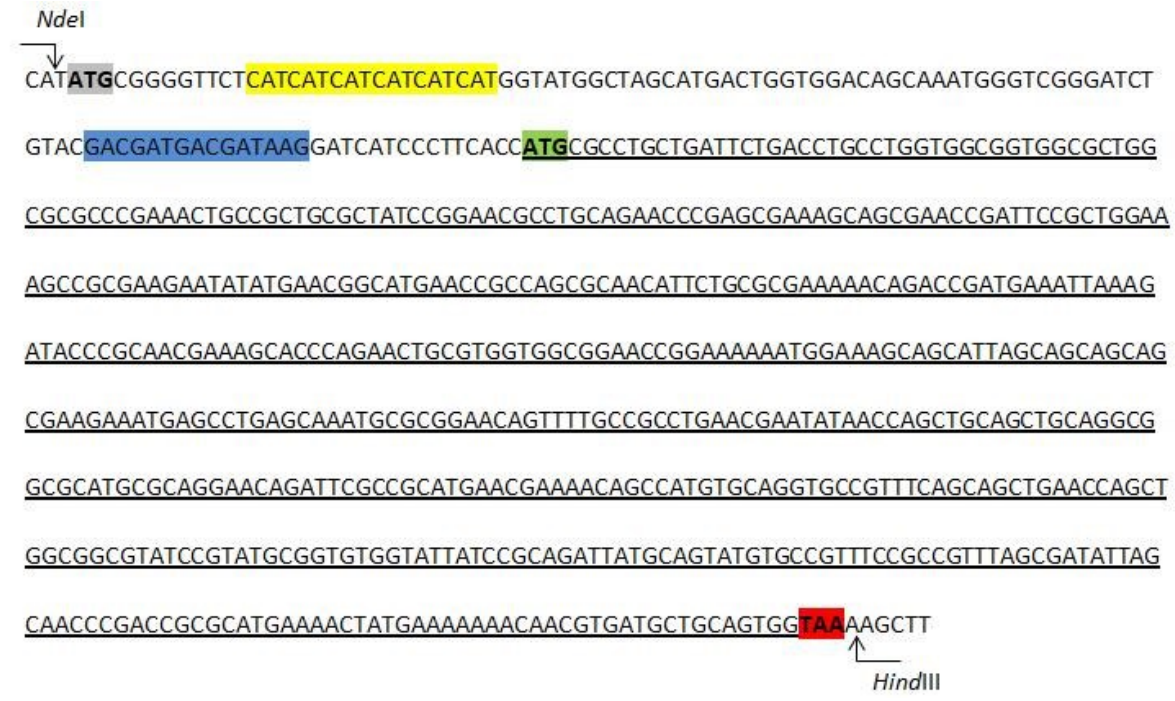
Fig. S2E



Supplementary figure 2 Determination of eGFP production in different recombinant *E. coli* strains by SDS-PAGE and fluorescence measurement. The strains *E. coli* DH5α+pVLT31-*egfp* (A), *E. coli* K12-MG1655+pVLT31-*egfp* (B), *E. coli* K12-W3110+pVLT31-*egfp* (C), *E. coli* BL21(DE3)+pET22b-*egfp* (D) and *E. coli* Nissle 1917+pVLT31-*egfp* (E) were cultivated in Wilms-KPi medium containing 10 g/L of different monosaccharides. Samples of eGFP protein from different time points are shown: before induction, 12 and 24 hours after induction. Samples of empty vector taken 24 hours after induction serves as a control. The relative fluorescence of the eGFP protein is shown corresponding to the bands on SDS-PAGE. The values indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations.

Fig. S3A

Fig. S3B



Supplementary figure 3 DNA sequences of the synthetic fragments of α_{S1}-casein from *Bos taurus (bovine)* (A) and *Homo sapiens (human)* (B). The encoding sequences of α_{S1}-casein genes are underlined, including the start codon (bold and green highlighted) and the stop codon (bold and red highlighted). The sequences of other N-terminal elements are marked as followed: an initial ATG is marked in grey colour and bold, His_6- tag is marked in yellow and an enterokinase recognition site is marked in blue. Recognition sites for the endonucleases *Nde*I and *Hind* III and their restriction patterns are marked with arrows.

Fig. S4A

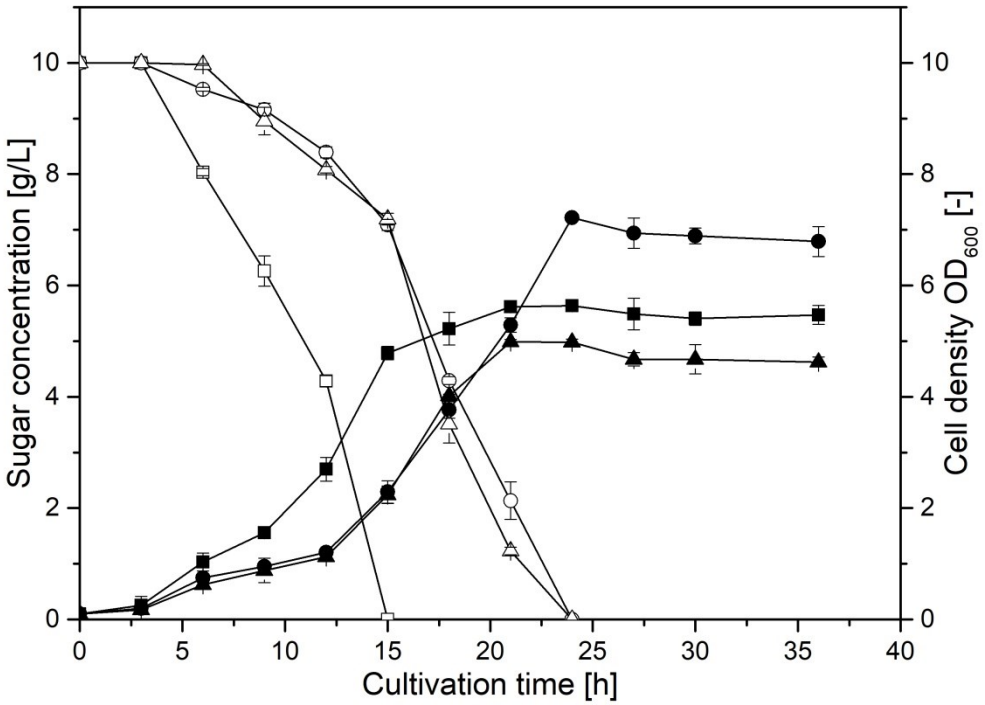

Sugar concentration [g/L]
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Fig. S4B

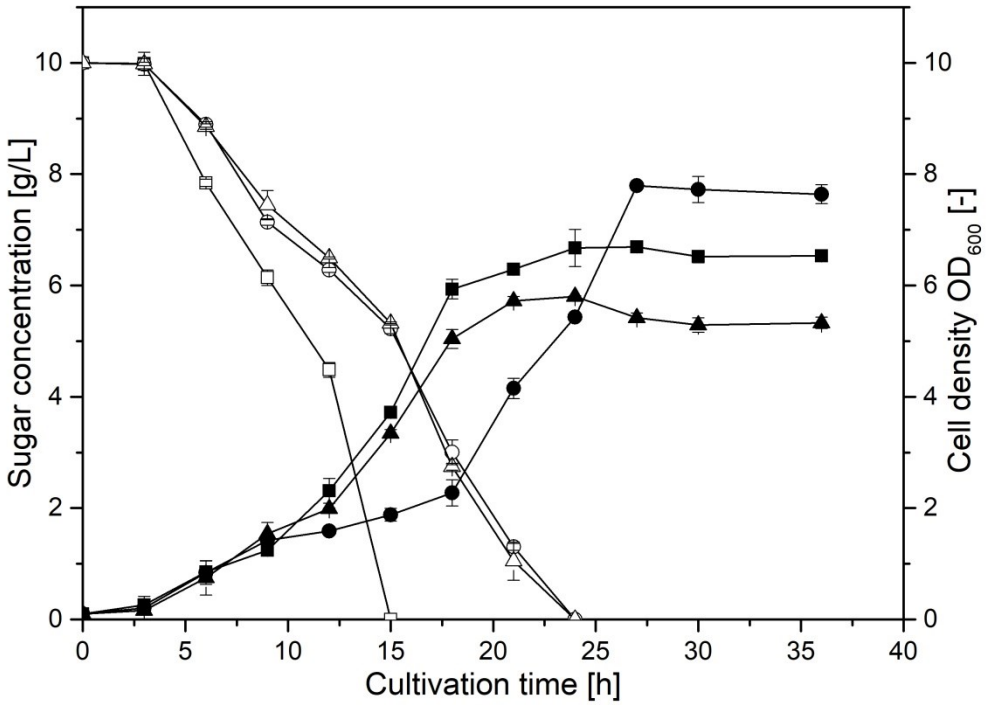
Sugar concentration [g/L]
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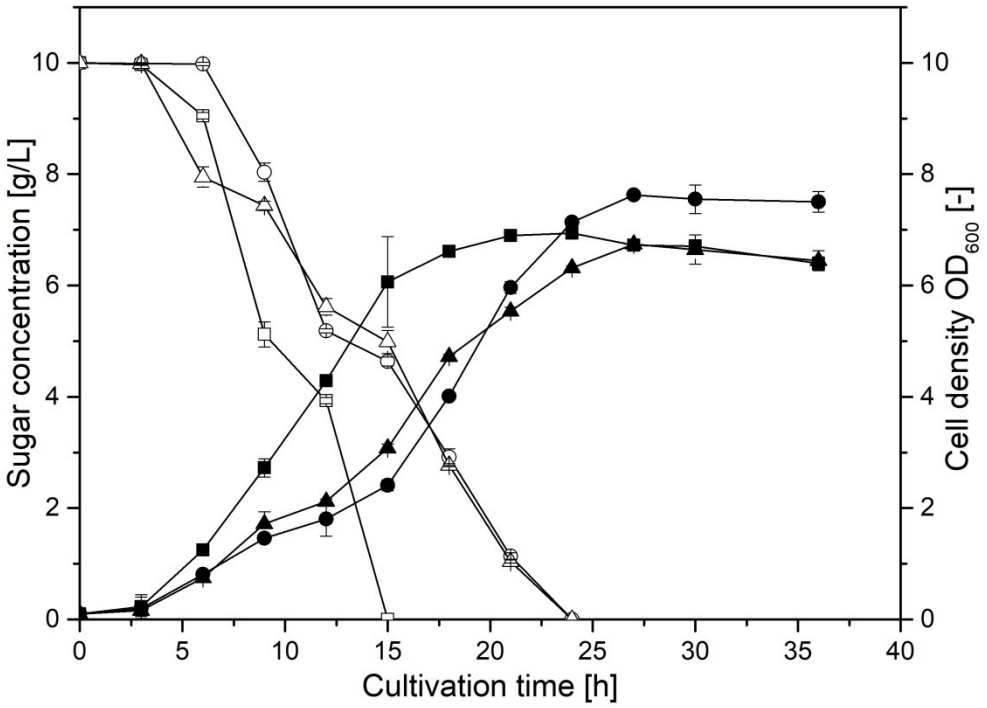
Fig. S4C



Supplementary figure 4 Growth performances of different recombinant α_{S1}-casein expressing *E. coli* strains. The strains *E. coli* BL21(DE3)+pET22b (A), *E. coli* BL21(DE3)+pET22b-*bc* (B) and *E. coli* BL21(DE3)+pET22b-*hc* (C) were cultivated in Wilms-KPi medium containing 10 g/L of different monosaccharides. The bacterial growth (filled symbols) and the sugar consumptions (empty symbols) are presented for the cultivations on glucose (squares), xylose (circles) and arabinose (triangles) as sole carbon source. The symbols indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations.

Fig. S5A

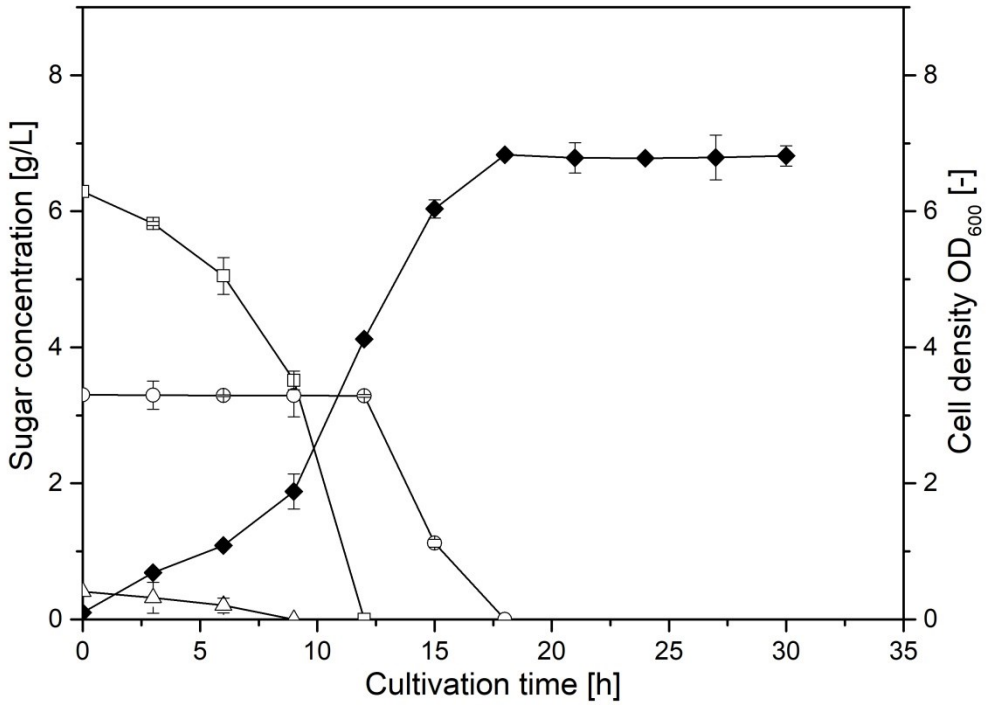

Sugar concentration [g/L]
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Fig. S5B

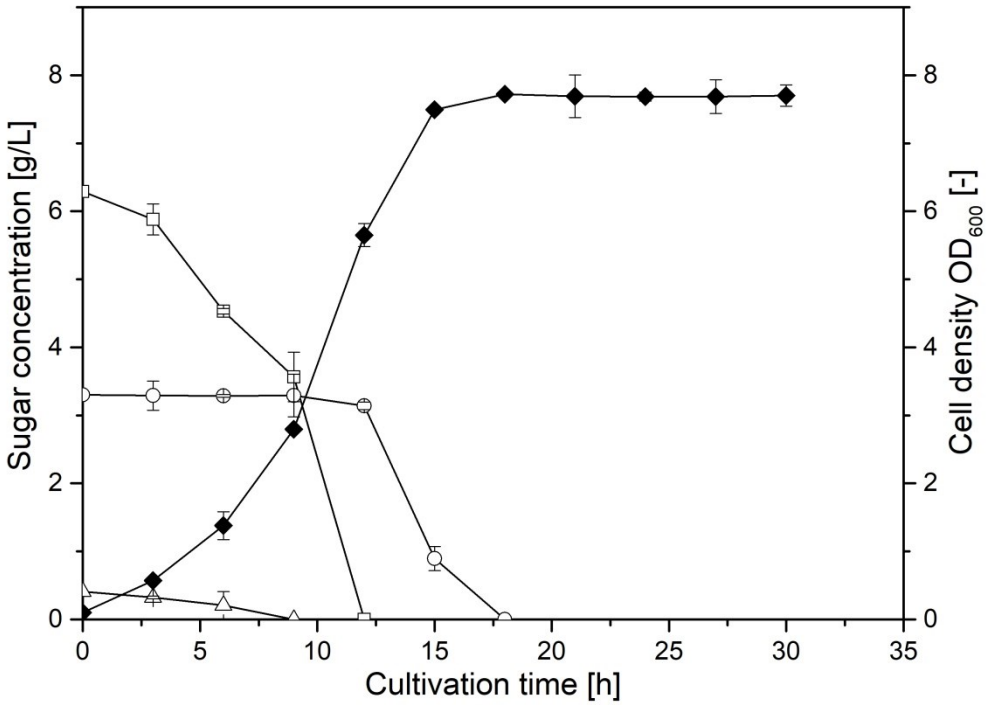
Sugar concentration [g/L]
Cell density OD600 [-]
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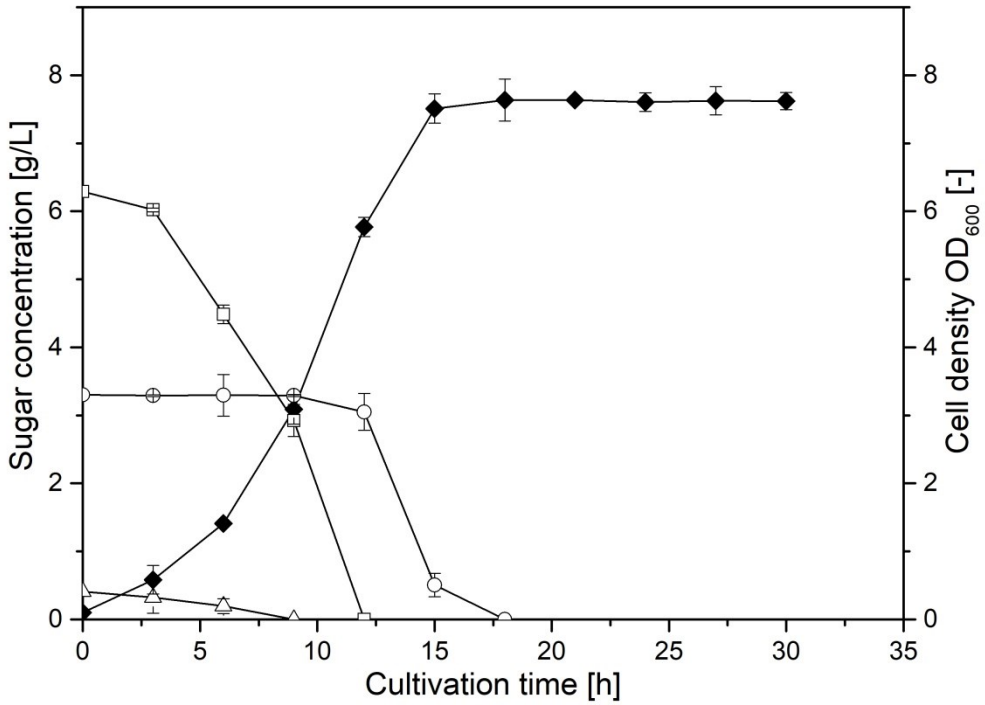
Fig. S5C



Supplementary figure 5 Growth performance of α_{S1}-casein expressing recombinant *E. coli* BL21(DE3) strains on artificial wheat straw hydrolyzate. The strains *E. coli* BL21(DE3)+pET22b (A), *E. coli* BL21(DE3)+pET22b-*bc* (B) and *E. coli* BL21(DE3)+pET22b-*hc* (C) were cultivated in Wilms-KPi medium containing artificial wheat straw hydrolyzate with10 g/L as total amount of sugars (6.29 g/L glucose, 3.30 g/L and 0.41 g/L arabinose). Growth curves are shown as filled diamonds. And the consumption of glucose (empty squares), xylose (empty circles) and arabinose (empty triangles) are presented. The symbols indicate the averages of the results of triplicate measurements. Error bars represent the standard deviations.

3.2. Metabolic engineering of *Pseudomonas putida* KT2440 as microbial biocatalyst for the utilization of D-xylose and L-arabinose

3.2.1. Summary II

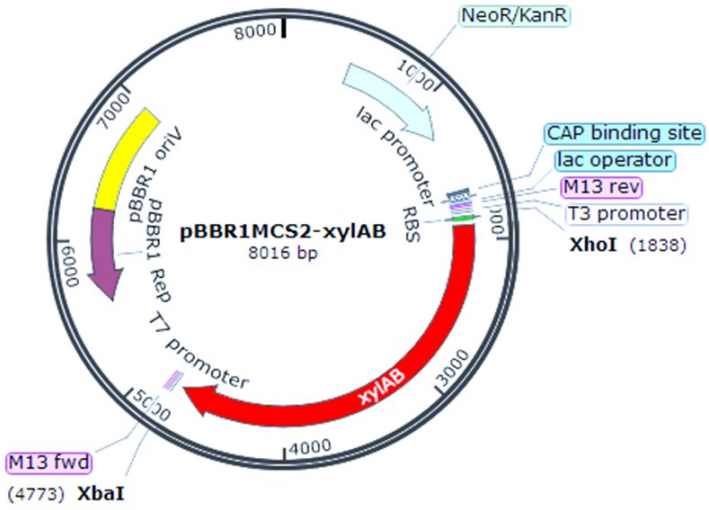
Lignocellulose has to be depolymerized into fermentable sugars in order to be used as a carbon source for biotechnological processes. And an advanced method is pretreatment and enzymatic hydrolysis. During these processes, inhibitors are normally formed. For example, furan aldehydes and aliphatic acids can be formed during the hydrolysis of cellulose and hemicellulose. Phenolic compounds can be formed during the hydrolysis of lignin. There are many methods such as physical, chemical and biological treatment to remove the inhibitors. But the effectiveness of detoxification varies from the original lignocellulosic materials[193]. Then it becomes an obstacle for some species of the microorganisms to utilize hydrolysates as carbon source, because different microorganisms have various degrees of tolerance.

Therefore, in this study we selected *Pseudomonas putida* KT2440 for further investigation, which is a robust microorganism. It displays a comparatively low sensitivity toward inhibitors[194,164,165,166]. However, *P. putida* KT2440 is not capable of utilizing pentose sugars like D-xylose and L-arabinose, so it cannot get fully use of the fermentable sugars in hydrolysates. While the complete and annotated genome sequence for *P. putida* KT2440 is available[168]. Consequently, to apply genetic manipulation and metabolic engineering methods to introduce D-xylose and L-arabinose metabolism pathway from *Escherichia coli* strains into *P. putida* KT2440 is a good idea and performed in this work.

The pentose phosphate pathway (PPP) is the preferred biochemical pathway for the metabolism of D-xylose and L-arabinose found in many bacteria[195,196]. Both D-xylose and L-

arabinose enter the PPP pathway through D-xylulose 5 phosphate[197]. According to the previous studies, heterologous expression of *xylA* indicating xylose isomerase and *xylB* indicating xylulokinase is a suitable strategy to enable the growth on D-xylose as sole carbon source in foreign species. For the utilization of L-arabinose, a group of three genes, *araB* (ribulokinase), *araA* (L-arabinose isomerase), and *araD* (L-ribulose phosphate 4-epimerase) is required in foreign species. Hence, we successfully designed two recombinant plasmids containing operons *xylAB* and *araBAD* from *E. coli* respectively, which are named pBBR1MCS2-*xylAB* and pBBR1MCS2-*araBAD*, as shown in Figure 12 (picture was created with software SnapGene version 5.3.2, GSL Biotech LLC).

（A）



（B）

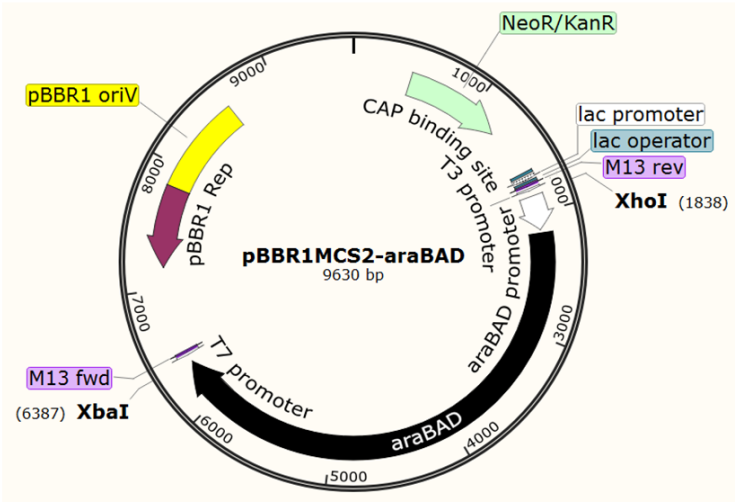


Figure 12 **Plasmid map of constructed vectors pBBR1MCS2-*xylAB* and pBBR1MCS2-*araBAD***.

Although introduction of D-xylose metabolism pathway by expressing *xylA* and *xylB* have been already performed in different bacteria strains, such as *Zymomonas mobilis*[198], *Corynebacterium glutamicum*[154], *Bacillus subtilis*[156], and even *Pseudomonas putida*[152,150,153]. The growth behaviour of our recombinant strain *P. putida* pBBR1MCS2_*xylAB* exhibited a significant high efficiency of D-xylose utilization without inserting external genes for xylose uptake, with much higher cell density, maximal specific growth rate and biomass yield compared to other engineered strains. In addition, the new expression strain *P. putida* pBBR1MCS2_*araBAD* presented also high efficiency of L-arabinose utilization. And to our knowledge, this is a first study describing the implementation of a dedicated arabinose metabolism pathway in *Pseudomonas putida*. Moreover, after transformation of operon *xylAB* or *araBAD* into *P. putida*, the recombinant strains can grow immediately on either xylose or arabinose, not like in the previous report that an adaption process over 36 generations was necessary for engineered *P. putida* strain S12 to improve the growth rate.

It has been reported that a recombinant *P. putida* S12 strain engineered for xylose metabolism also showed non-specific activity of *xylA* and *xylB* on L-arabinose[150]. Interestingly, we also observed the same phenomena in our study by cultivation *P. putida* pBBR1MCS2_*xylAB* on arabinose and *P. putida* pBBR1MCS2_*araBAD* also on xylose. The results demonstrated that not only *xylAB* can mediate growth on arabinose but also operon *araBAD* can allow *P. putida* to grow on D-xylose. It elucidates that there is a "cross-reaction" between D-xylose and L-arabinose metabolism.

Moreover, the successful growths and efficiency sugar utilization of two recombinant strains on different mixes of sugars were also inspected. Finally in this study, wheat straw hydrolysate was further tested as a model to demonstrate the potential usage of lignocellulosic hydrolysate. As a result, this study created recombinant *Pseudomonas putida* strains, which not only achieved the xylose and arabinose metabolism ability from *E. coli*, but also benefit from its own features like multiple metabolisms and robustness against numerous organic compounds. All these advantages make it become a great candidate as a potential microbial catalyst based on lignocellulosic hydrolysates bioprocess in the biotechnological industry.

3.2.2. Graphical Abstract Summary II

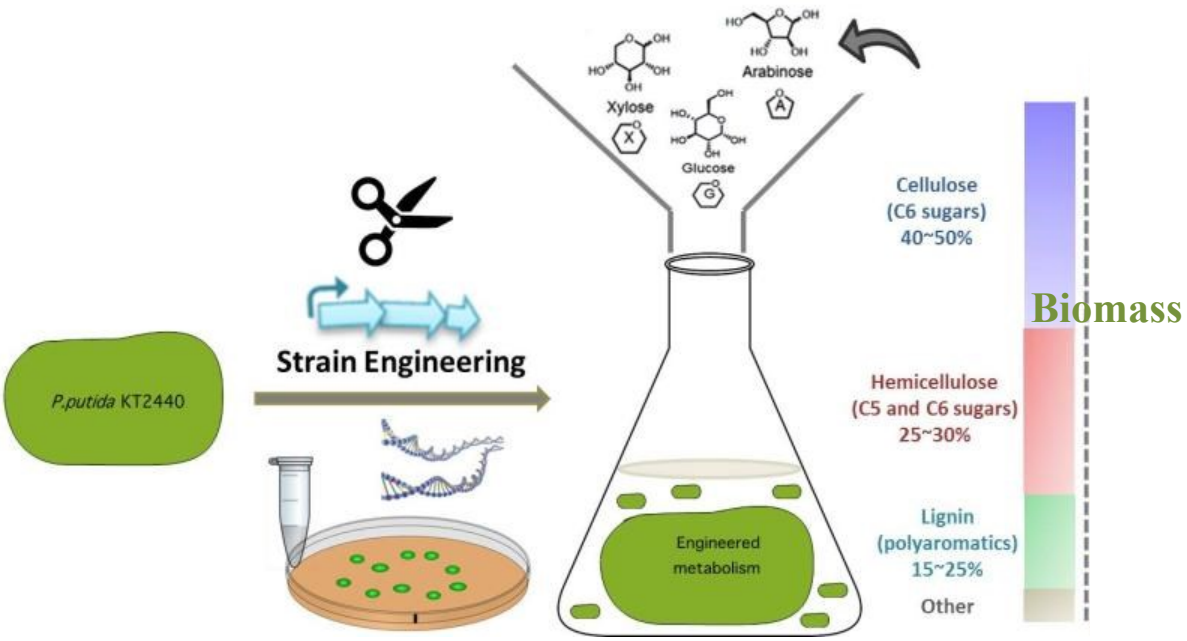


Figure 13 **Scheme of publication II**. *Pseudomonas putida* KT2440 was metabolically engineered for utilization of pentose sugars (D-xylose and L-arabinose) from lignocellulosic hydrolysates.

3.2.3. Publication II: Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources

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*corresponding author

[†] contributed equally

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Author contributions:

Y. Wang: constructed the recombinant strains, conducted the cultivation experiments, wrote and co-revised the manuscript

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DOI: 10.1111/gcbb.12590

ORIGINAL RESEARCH

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose, and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources

Yan Wang[1*] | **Felix Horlamus**[2*] | **Marius Henkel**[2] | **Filip Kovacic**[3] | **Sandra Schläfle**[4] | **Rudolf Hausmann**[2] | **Andreas Wittgens**[1,5,6] | **Frank Rosenau**[1,5,6]

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Abstract

Lignocellulosic biomass is the most abundant bioresource on earth containing polymers mainly consisting of D-glucose, D-xylose, L-arabinose, and further sugars. In order to establish this alternative feedstock apart from applications in food, we engineered *Pseudomonas putida* KT2440 as microbial biocatalyst for the utilization of xylose and arabinose in addition to glucose as sole carbon sources. The D-xylose-metabolizing strain *P. putida* KT2440_*xylAB* and L-arabinose-metabolizing strain *P. putida* KT2440_*araBAD* were constructed by introducing respective operons from *Escherichia coli*. Surprisingly, we found out that both recombinant strains were able to grow on xylose as well as arabinose with high cell densities and growth rates comparable to glucose. In addition, the growth characteristics on various mixtures of glucose, xylose, and arabinose were investigated, which demonstrated the efficient co-utilization of hexose and pentose sugars. Finally, the possibility of using lignocellulose hydrolysate as substrate for the two recombinant strains was verified. The recombinant *P. putida* KT2440 strains presented here as flexible microbial biocatalysts to convert lignocellulosic sugars will undoubtedly contribute to the economic feasibility of the production of valuable compounds derived from renewable feedstock.

KEYWORDS

biocatalyst, D-xylose, hemicellulose hydrolysate, L-arabinose, metabolic engineering, *Pseudomonas putida* KT2440

*Contributed equally.

1 | INTRODUCTION

The development of alternative feedstocks as carbon sources for the industrial biotechnology is one of the major goals to achieve cost-effective and economically efficient bioprocesses, since the price for raw materials especially those of the carbon sources represents a significant proportion of total production costs. Due to insufficient global food supply, the use of feedstocks, which can primarily be used also for food production, is at least ethically questionable and not a preferable basis for the establishment of a truly sustainable bioeconomy. Nevertheless, numerous current biotechnological production processes mostly depend on glucose as carbon source (Wendisch et al., 2016). The conflict between food and biotechnology and the resulting demand to create ethically less problematic processes, which also offer a promising potential for increasing positive socio-economical perception and acceptance by customers of biotechnological products, alternative carbon sources like lignocellulosic biomass, have moved into the focus of attention as renewable and thus sustainable raw materials with a considerable economic potential for industrial biotechnology. An obvious advantage is the fact that they can be recovered from forestry and agro-industrial waste or agricultural residuals (Anwar, Gulfraz, & Irshad, 2014; Mussatto & Teixeira, 2010). Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin containing different polymers. D-Glucose is the only component in cellulose while the composition of hemicelluloses highly varies among different bioresources (Himmel et al., 2010; Shahzadi et al., 2014; Taherzadeh & Karimi, 2008). Pentoses like D-xylose and L-arabinose are the predominant sugars in hemicelluloses and make up to 25% of the total sugar amount in lignocelluloses especially in hardwoods and grasses like wheat, corn, and rice, thereby representing a worldwide available bioresource, but hemicellulose can also contain hexoses like D-glucose, D-mannose, and D-galactose (Brodeur et al., 2011; Kumar, Barrett, Delwiche, & Stroeve, 2009; Lee, 1997). While cellulose is primarily used for other industrial applications, 60 billion tons of hemicelluloses remain almost completely unused every year, which can be hydrolyzed into sugar containing hydrolysates by chemical or enzymatic hydrolysis. This is a prerequisite to use them as substrates for bioprocesses, since typically used microorganisms in industrial biotechnology are naturally unable to use polymers directly (Sun & Cheng, 2002; Xu, Sun, Liu, & Sun, 2006). However, these sugars provided in lignocellulosic hydrolysates can potentially be utilized for the growth of microorganisms and can be converted into different valuable products including biochemical compounds, fine chemicals, food additives, and enzymes (Asgher, Ahmad, & Iqbal, 2013; Iqbal & Asgher, 2013). However, the natural limited metabolic flexibility of many industrial-relevant microorganisms for the use of uncommon carbon sources impedes the efficient utilization of pentose sugars (Kim & Gadd, 2009).

Therefore, several approaches have been used to address this challenge by genetic manipulation and metabolic engineering in different bacteria (Aristidou & Penttilä, 2000; Nieves, Panyon, & Wang, 2015). The pentose phosphate pathway (PPP) is the preferred biochemical route for metabolizing xylose and arabinose present in numerous bacteria. Both xylose and arabinose enter the PPP through D-xylulose 5-phosphate as an intermediate (Stincone et al., 2015). For establishing a xylose degrading pathway in foreign species, heterologous expression of *xylA* (xylose isomerase) and *xylB* (xylulokinase) is a suitable strategy to enable growth on xylose as sole carbon source, which has been successfully performed in various bacteria like *Zymomonas mobilis* (Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995), *Corynebacterium glutamicum* (Kawaguchi, Verte, Okino, Inui, & Yukawa, 2006), *Bacillus subtilis* (Chen, Liu, Fu, Zhang, & Tang, 2013), and *Pseudomonas putida* (Le Meur, Zinn, Egli, Thöny-Meyer, & Ren, 2012; Meijnen, Winde, & Ruijssenaars, 2008). Therefore, D-xylose is converted to D-xylulose 5-phosphate through D-xylulose (Gu et al., 2010; Kawaguchi et al., 2006). For the utilization of L-arabinose, a group of three genes, *araB* (ribulokinase), *araA* (L-arabinose isomerase), and *araD* (L-ribulose phosphate 4-epimerase), is necessary, which mediates the conversion of L-arabinose though L-ribulose and L-ribulose 5-phosphate to D-xylulose 5-phosphate (Deanda, Zhang, Eddy, & Picataggio, 1996; Xiong, Wang, & Chen, 2016). This *araBAD* operon has been successfully integrated and heterologously expressed in *C. glutamicum* (Kawaguchi, Sasaki, Vertès, Inui, & Yukawa, 2008) to enable its growth on L-arabinose.

In this present study, we chose *P. putida* KT2440 as a host for generating optimized expression strains by heterologous expression of the *xylAB* and *araBAD* operons to enlarge the available substrate spectrum for this remarkable platform organism. *P. putida* KT2440 has developed into an excellent and robust workhorse for the expression of heterologous genes (Loeschcke & Thies, 2015; Martins Dos Santos, Heim, Moore, Strätz, & Timmis, 2004), possesses an outstanding tolerance toward numerous organic compounds and has been extensively studied for the biosynthesis of biotechnological relevant products, for example, rhamnolipids (Cha, Lee, Kim, Kim, & Lee, 2008; Tiso et al., 2016, 2018; Wittgens et al., 2017, 2018, 2011). Its genome has been completely sequenced, which provides complete insights into its metabolic potential (Nelson et al., 2002; Poblete-Castro, Becker, Dohnt, Santos, & Wittmann, 2012), and especially in Germany, the strain KT2440 is of great importance, since it is the only *P. putida*, which remained in the biosafety level 1 (S1) being a key prerequisite for its use in many industrial applications (BVL, 2012). According to a previous study, *P. putida* KT2440 lacks part of the PPP and is unable for utilizing xylose and arabinose,

but carries the *oprB* gene encoding the outer membrane protein D1, which is responsible for the uptake of xylose and arabinose (Henkel et al., 2012). The growth behaviors of engineered *P. putida* KT2440 strains were investigated in detail during cultivation experiments on glucose, xylose, or arabinose as sole carbon sources as well as on mixtures of these sugars and finally real hemicellulose hydrolysates, to investigate the potential of efficiently utilizing of this cost-effective and renewable feedstock.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

Pseudomonas putida KT2440 (Nelson et al., 2002), *Escherichia coli* DH5α (Grant, Jessee, Bloom, & Hanahan, 1990), and *E. coli* K-12 strain MG1655 (Blattner et al., 1997) were routinely cultivated in lysogenic broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 120 rpm orbital shaking and 30°C for *P. putida* and 37°C for *E. coli*, respectively. Growth experiments using wild-type and engineered *P. putida* strains were carried out in 250-ml baffled Erlenmeyer flasks filled with 25 ml of adapted Wilms-KPi medium (Wilms et al., 2001) containing 13.15 g/L K_2HPO_4, 3.28 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$, 1 g/L NH_4Cl, 4 g/L Na_2SO_4, 50 g/L $MgSO_4{\cdot}7H_2O$ supplemented with 3 ml/L of a trace element solution consisting of 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $MnSO_4{\cdot}H_2O$, 13.92 g/L $FeCl_3{\cdot}6H_2O$, 10.05 g/L EDTA, 0.18 g/L $CoCl_2{\cdot}6H_2O$, 0.662 g/L $CaCl_2{\cdot}2H_2O$, and 10 g/L thiamin HCl. A total amount of 10 g/L D-glucose, D-xylose, L-arabinose, or equal mixtures of these sugars were added to the medium as carbon source.

Hydrolysates were obtained from dried and milled wheat straw, which was first treated in a steam explosion process followed by an enzymatic hydrolysis process carried out for 5 days without using any additives (Schläfle, Tervahartiala, Senna, & Kölling-Paternoga, 2017). These wheat straw hydrolysates containing almost exclusively monomers of D-glucose, D-xylose, and L-arabinose were added to the adapted Wilms-KPi medium complying with a total sugar concentration of 10 g/L, and artificial straw hydrolysates were prepared from single sugars imitating this composition.

Pre-cultures were prepared from glycerol stocks using a total volume of 50 µl stock solution in 25 ml LB medium. Main cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.1 using cells harvested by centrifugation for 10 min at 5,000 *g*.

2.2 | Construction of recombinant plasmids

Genomic DNA of *E. coli* strains DH5α and K-12 MG1655 were isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The amplification of the 2.8-kb *xylAB* operon from *E. coli* DH5α and of the 4.3-kb *araBAD* operon from *E. coli* K-12 strain MG1655 was performed by standard PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt a. M., Germany) according to the manufacturer's instructions. The DNA sequences of the primers, obtained from Eurofins Genomics (Ebersberg, Germany), were GTGAAATAACATACTCGAGCAACTGAAAGG and CCCACCCGGTCTAGAAGGGGATAA for *xylAB* and CTTTTCTCGAGCCCACCATTC and GGTTTCTCTAGATTGGCTGTGG for *araBAD*, respectively. The two resulting PCR products were hydrolyzed using restriction enzymes *Xho*I and *Xba*I and subsequently ligated using T4 DNA ligase with the pBBR1MCS-2 expression vector (Kovach et al., 1995) hydrolyzed with the same enzymes. All enzymes were used as recommended by the supplier (Thermo Fisher Scientific, St. Leon-Rot, Germany). *E. coli* DH5α cells were transformed with the resulting recombinant plasmids pBBR1MCS-2_*xylAB* and pBBR1MCS-2_*araBAD* using a standard protocol (Hanahan, 1983). Transformation of *P. putida* KT2440 was performed by electroporation after Choi, Kumar, and Schweizer (2006). Agar plates and liquid media were supplemented with 50 µg/ml kanamycin for selection of positive cells. Recombinant *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* strains were additionally screened using solid Wilms-KPi medium plates containing and 10 g/L xylose or arabinose after electroporation.

2.3 | Analytical methods

Cell growth was determined densitometrically by measuring the OD_{600} using a spectral photometer. Culture supernatants were analyzed for sugar concentrations after removing the cells by centrifugation for 5 min at 15,000 *g* and 4°C using the D-Glucose Assay Kit, D-Xylose Assay Kit, and L-Arabinose/D-Galactose Assay Kit (Megazyme, Wicklow, Ireland). The formation of xylonate and arabinoate was determined according to Hofmann et al. (2018).

For the analysis of growth, graphs were created with SIGMAPLOT 13.0 (Systat, San Jose, CA, USA), and a logistic equation with four parameters was used to fit the data. Specific growth rate (μ), maximal specific growth rate (μ_{max}), and biomass to substrate yield (Y_{xls}) were calculated according to the derivation of the polynomial fitting. A maximal standard deviation was applied for all the measurements.

3 | RESULTS

3.1 | D-Xylose and L-arabinose as carbon sources for *P. putida* KT2440

The wild-type strain *P. putida* KT2440 is not able to utilize D-xylose and L-arabinose as sole carbon sources according

to its genetic background (Henkel et al., 2012; Nelson et al., 2002). This was confirmed here by the cultivation of *P. putida* KT2440 in minimal medium containing glucose in comparison with growth experiments using xylose or arabinose as sole carbon sources (Table 1). With glucose, *P. putida* KT2440 reached a significant high cell density ($OD_{600} = 12.1$) with a maximal specific growth rate of 0.61 hr^{-1} and a biomass to substrate yield (Y_{xls}) of 0.37 g/g. In contrast, no growth could be detected after cultivation in either xylose or arabinose containing media after 34 hr. However, in this time the xylose concentration decreased by about 33%, indicating a considerable consumption of xylose. In the same time, an increasing amount of xylonate could be detected, which corresponds to the consumed xylose amount (data not shown). In contrast, a similar depletion of arabinose did not occur during the cultivation.

With the intention to provide *P. putida* KT2440 with efficient pathways for the utilization of xylose and arabinose—which, in addition to glucose, represent the most abundant carbohydrates in lignocelluloses—the dedicated operons *xylAB* and *araBAD* of *E. coli*, respectively, were amplified from *E. coli* chromosomal DNA and subsequently cloned into the pBBR1MCS-2 shuttle vector under transcriptional regulation of the plasmid-encoded *lac*-promoter (P^{lac}). Due to the lack of a functional *lac*-operon and especially the absence of the *lac*-inhibitor (LacI) in *P. putida* KT2440, the expression of the operons controlled by P^{lac} occurs constitutively omitting the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The resulting recombinant plasmids were finally transferred into *P. putida* yielding the two expression strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD*, respectively. A *P. putida* KT2440 strain harboring the pBBR1MCS-2 empty vector served as a control and showed a growth performance similar to the *P. putida* wild type on glucose with an $OD_{600} = 12.6$, a maximal specific growth rate of 0.58 hr^{-1}, and a biomass yield of 0.34 g/g (Table 1). As expected, this strain did not show any detectable growth after cultivation on xylose or arabinose, but the xylose concentration decreased by 21% while the xylonate concentration increased as observed for the wild type.

Next, the recombinant strain *P. putida* KT2440_*xylAB* was cultivated using one of the three sugars each as the sole carbon source (Table 1; Supporting Information Figure S1a). In contrast to the wild-type and the *P. putida* strain containing the empty vector, this strain was able to grow on xylose and reached an OD_{600} of 9.8, what is similar to its growth on glucose ($OD_{600} = 9.4$). The calculated maximal specific growth rate of 0.39 hr^{-1} on xylose was half as much than on glucose (0.98 hr^{-1}), while the biomass yield was in comparable ranges (xylose: 0.30 g/g, glucose: 0.29 g/g).

It has been reported that a recombinant *P. putida* Sl2 strain engineered for xylose metabolism showed also unspecific activity of XylA and XylB toward L-arabinose (Meijnen et al., 2008). In our experiments, we could confirm this finding for *P. putida* KT2440_*xylAB* as well, since this strain was also able to grow with arabinose as single carbon source (Table 1, Supporting Information Figure S1a) and reached an OD_{600} of 9.0 with $\mu_{max} = 0.65$ hr^{-1} and $Y_{xls} = 0.27$ g/g comparable to glucose and xylose.

The decreasing sugar concentrations during the cultivation revealed that xylose was consumed from 6 to 22 hr similar to its glucose counterpart proving the presence of a functional and efficient xylose utilization pathway in the recombinant *P. putida* KT2440_*xylAB* strain. In contrast, significant depletion of arabinose was observed in later stages starting from 16 hr and rapidly decreasing until 22 hr.

With the aim to evaluate and potentially improve this basic arabinose utilization and to get deeper understanding in the mechanism behind this "cross-reaction" of the hypothetic unspecific XylAB activities, which we suppose to be responsible for this, we constructed and characterized a

TABLE 1 Growth parameters of different *Pseudomonas putida* strains using various carbon sources

	***P. putida* WT**			***P. putida*_pBBR1MCS-2**			***P. putida_xylAB***			***P. putida_araBAD***		
	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}	OD_{600}	μ_{max}	Y_{xls}
Glucose	12.1	0.61	0.37	12.6	0.58	0.34	9.4	0.98	0.29	11.5	0.54	0.35
Xylose	0	0	0	0	0	0	9.8	0.39	0.30	6.9	0.40	0.20
Arabinose	0	0	0	0	0	0	9.0	0.65	0.27	8.4	0.66	0.26
Glucose/xylose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.4	0.54	0.26	6.8	0.49	0.21
Glucose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.0	0.59	0.24	8.4	0.62	0.26
Xylose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.5	0.54	0.26	7.3	0.66	0.25
Glucose/xylose/arabinose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.4	0.72	0.26	5.9	0.45	0.18
Real hydrolysates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.3	0.68	0.34	7.4	0.55	0.22
Artificial hydrolysates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.5	0.58	0.35	8.6	0.58	0.26

Note. μ_{max}: maximal specific growth rate (hr^{-1}); n.d.: not determined; OD_{600}: optical density at 600 nm; Y_{xls}: biomass to substrate yield (g/g).

P. putida KT2440 strain carrying a heterologous *araBAD* operon from *E. coli* yielding the new expression strain *P. putida* KT2440_*araBAD*. To our knowledge, this is the first study describing the implementation of a dedicated arabinose metabolism pathway. The new strain was cultivated under the same conditions in the presence of glucose, xylose, or arabinose as its counterpart carrying the *xylAB* operon in the experiments described earlier in this chapter (Table 1, Supporting Information Figure S1b). Unexpectedly, this strain was not only able to grow on glucose and arabinose, but also on xylose. This demonstrates that not only XylAB can mediate growth on arabinose but also that a similar cross- or side reaction exists vice versa allowing *P. putida* to grow on D-xylose enabled by the arabinose-dedicated operon. The growth performance of *P. putida* KT2440_*araBAD* on glucose was similar to both strains described earlier with an $OD_{600} = 11.5$, a maximal specific growth rate of 0.54 hr^{-1} and a biomass yield of 0.35 g/g. However, the lag phase was significantly prolonged, especially in the experiment using xylose, but, astonishingly, also with arabinose resulting in a later start of the exponentially growth phase for both strains between 16 and 20 hr after inoculation. Nevertheless, the *P. putida* KT2440_*araBAD* strain reached a higher cell density of 8.4 with $\mu_{max} = 0.66\ hr^{-1}$ and $Y_{xls} = 0.26$ g/g on L-arabinose compared to the cultivation on xylose ($OD_{600} = 6.9$, $\mu_{max} = 0.40\ hr^{-1}$, $Y_{xls} = 0.20$ g/g) demonstrating a better performance of *P. putida* KT2440_*araBAD* on its expected "preferred" substrate L-arabinose compared to D-xylose.

In a further experiment, the strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* were cultivated without selective pressure toward plasmid maintenance and thereby cured from their plasmids pBBR1MCS-2_*xylAB* and pBBR1MCS-2_*araBAD*, respectively. This procedure resulted in a complete loss of their ability to utilize xylose and arabinose (data not shown).

In conclusion and without any further attempts toward strain optimization, these results already suggest *P. putida* KT2440_*xylAB* to be a suitable expression strain of choice for the efficient utilization of glucose, xylose, and arabinose.

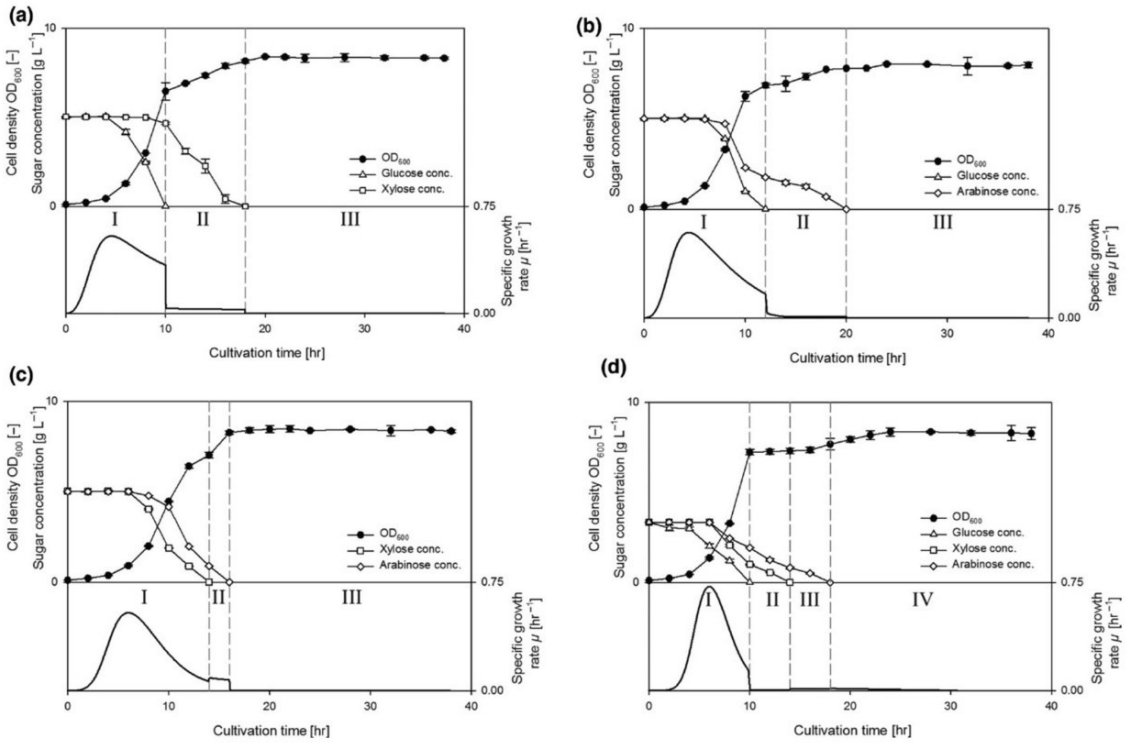

FIGURE 1 Growth performance of *Pseudomonas putida_xylAB* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*xylAB* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (b), xylose/arabinose (c), and glucose/xylose/arabinose (d). The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

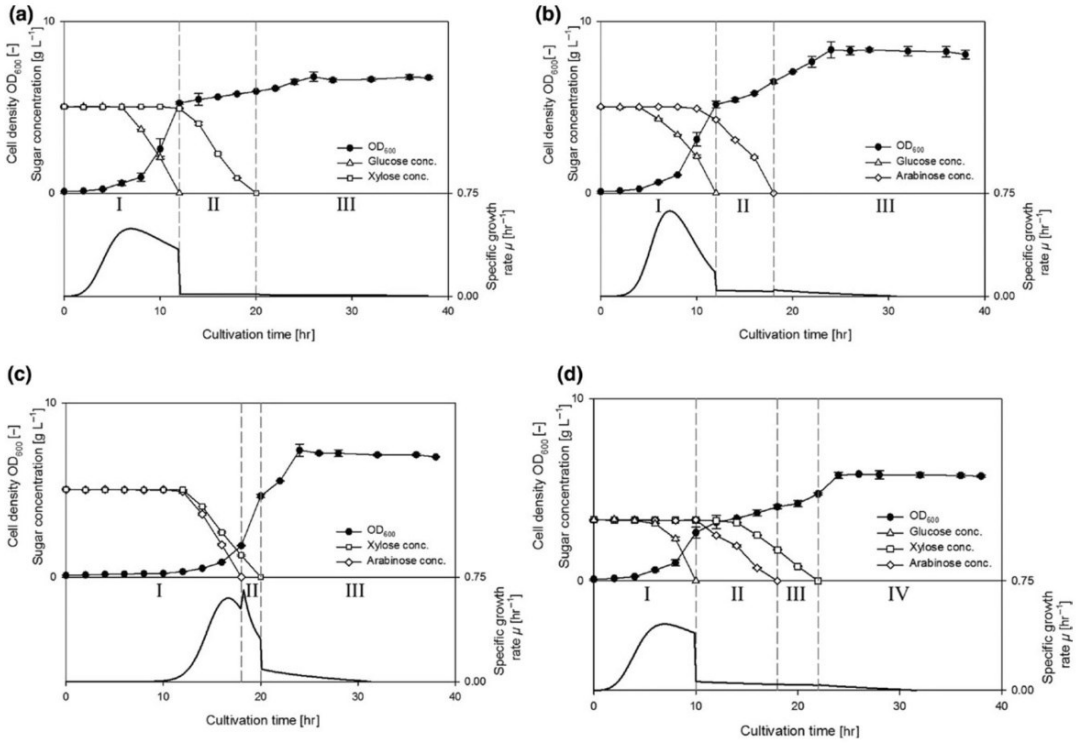

FIGURE 2 Growth performance of *Pseudomonas putida_araBAD* using mixtures of sugars as carbon source. The strain *P. putida* KT2440_*araBAD* (filled circles) was cultivated in Wilms-KPi medium containing mixtures of sugars with a total amount of 10 g/L equally distributed on glucose/xylose (a), glucose/arabinose (b), xylose/arabinose (c), and glucose/xylose/arabinose (d). The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

3.2 | Growth characteristics of recombinant *P. putida* strains on mixtures of sugars

After the verification and characterization of an effective growth of recombinant *P. putida* strains expressing either *xylAB* or *araBAD* with the single sugars glucose, xylose, or arabinose as sole carbon sources, the growth performance on mixtures of two or three of these sugars was investigated.

Both strains showed efficient growth on all sugar mixtures with cell densities and maximal specific growth rates comparable to those on the single sugars. The strain *P. putida* KT2440_*xylAB* reached the following similar values on all different sugar compositions: glucose/xylose: $OD_{600} = 8.4$, $\mu_{max} = 0.54\ hr^{-1}$, $Y_{xls} = 0.26$ g/g; glucose/arabinose $OD_{600} = 8.0$, $\mu_{max} = 0.59\ hr^{-1}$, $Y_{xls} = 0.24$ g/g; xylose/arabinose $OD_{600} = 8.5$, $\mu_{max} = 0.54\ hr^{-1}$, $Y_{xls} = 0.26$ g/g; and glucose/xylose/arabinose $OD_{600} = 8.4$, $\mu_{max} = 0.72\ hr^{-1}$, $Y_{xls} = 0.26$ g/g (Figure 1, Table 1). This performance again revealed the efficient growth of *P. putida* KT2440_*xylAB* on all these three sugars. In contrast, *P. putida* KT2440_*araBAD* exhibited different growth performance on the various sugar combinations (Figure 2, Table 1). Its growth on glucose/xylose ($OD_{600} = 6.8$, $\mu_{max} = 0.49\ hr^{-1}$, $Y_{xls} = 0.21$ g/g) was significantly lower than on glucose/arabinose ($OD_{600} = 8.4$, $\mu_{max} = 0.62\ hr^{-1}$, $Y_{xls} = 0.26$ g/g) confirming a better growth on the supposed specific sugar (L-arabinose). However, the growth on xylose/arabinose ($OD_{600} = 7.3$, $\mu_{max} = 0.66\ hr^{-1}$, $Y_{xls} = 0.25$ g/g) was slightly higher than on glucose/xylose indicating a more efficient utilization when only pentoses were present in the culture media instead of a mixture of hexose and pentose. This hypothesis is supported by the relatively low growth on a mixture of glucose, xylose, and arabinose, where *P. putida* KT2440_*araBAD* reached an OD_{600} of 5.9 and a maximal specific growth rate of $0.45\ hr^{-1}$ and a biomass to substrate yield of 0.18 g/g.

The growth curves for both strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* on all

sugar mixtures show multiple growth phases (indicated with Roman numbers) along with significant high maximal specific growth rates at the beginning followed by very low ones (Figures 1 and 2, Table 1) based on the switch in metabolic response toward the different carbon sources. Using mixtures of two sugars, the first growth phases abruptly end after 10 hr, when the first sugar is (almost) completely consumed and the growth ceases completely after 20 hr, when also the second sugar was consumed. Exceptions are only the cultivations on xylose/arabinose mixtures where the postponed stops of the first growth phases occurred between 14 and 18 hr followed by reduced second phases. Notably, using glucose/xylose mixtures the utilization of xylose only started after the total consumption of glucose (Figures 1a and 2a, Table 1), while utilization of arabinose already started when the concentration of glucose was decreased, but did not yet reach zero (Figures 1b and 2b, Table 1). When *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD* were cultivated on the xylose/arabinose mixture, the consumption of both sugars started almost simultaneously, whereby each strain metabolized its specific sugar a bit faster than the second one (Figures 1c and 2c, Table 1). When both strains were cultivated on a mixture composed of all three sugars, first glucose was utilized and completely consumed after 10 hr, followed by xylose and arabinose after 18–22 hr (Figures 1d and 2d, Table 1). Surprisingly, *P. putida* KT2440_*xylAB* started catabolism of xylose and arabinose shortly after metabolizing of glucose began, while in the case of *P. putida* KT2440_*araBAD* the glucose was totally consumed before utilization of both other sugars started. Here again, apart from the consumption of glucose, which is obviously the preferred carbon source, both *P. putida* strains favor its specific pentose sugar and consumed it faster than the remaining one.

In conclusion, both recombinant *P. putida* strains totally consumed all provided sugars, whereby each stain preferred either xylose or arabinose next to glucose as its more specific sugar. However, growth profiles suggest that *P. putida* KT2440_*xylAB* is rather qualified for more effective utilization of these three sugars.

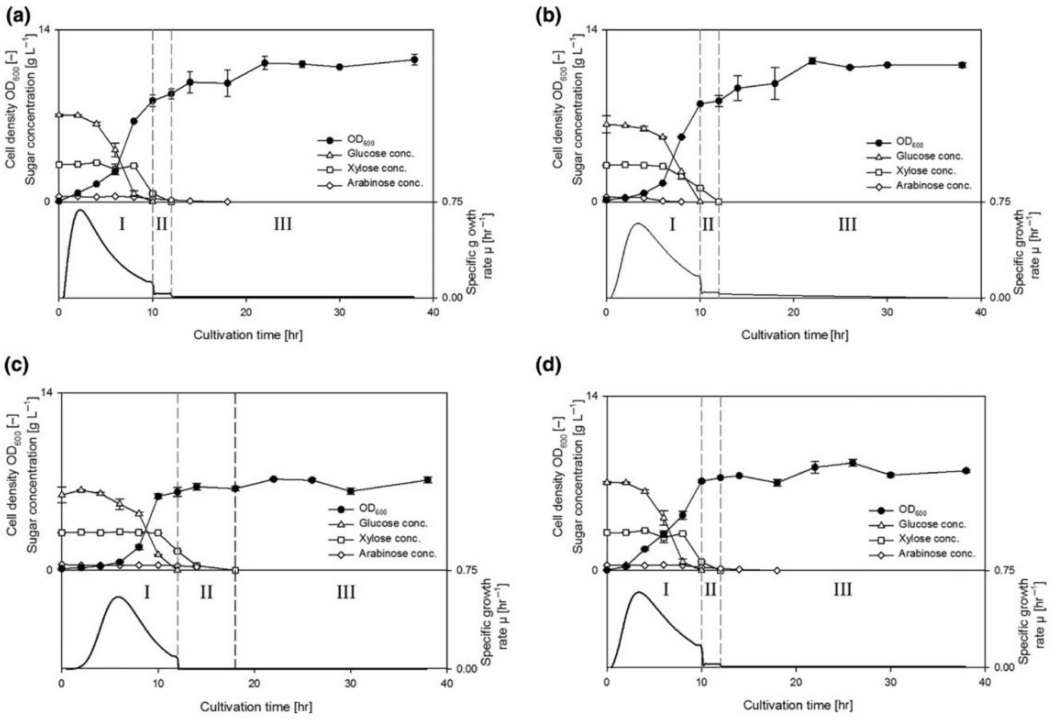

FIGURE 3 Growth performance of recombinant *Pseudomonas putida* using wheat straw hydrolysate as carbon source. The strains *P. putida* KT2440_*xylAB* (a, b) and *P. putida* KT2440_*araBAD* (c, d) (filled circles) were cultivated in Wilms-KPi medium containing real wheat straw hydrolysate (a, c) or artificial hydrolysate with the identical sugar composition (b, d). The growth curves are shown as a smoothed red line. The consumptions of glucose (empty triangles), xylose (empty squares), and arabinose (empty diamonds) are presented. The specific growth rates of each experiment are separately shown in the lower diagrams, and single growth phases are indicated with Roman numbers. The data points are averages of the results of duplicate measurements. The error bars represent the standard deviations

3.3 | Lignocellulosic hydrolysates as substrate for recombinant *P. putida*

Finally, the ability to utilize real lignocellulosic hydrolysates made from wheat straw was tested using the recombinant strains *P. putida* KT2440_*xylAB* and *P. putida* KT2440_*araBAD*. Artificial hydrolysates containing only glucose, xylose, and arabinose were used in comparison with identical concentrations as the real model mixture (Figure 3, Table 1). Both strains were capable to completely utilize the provided glucose, xylose, and arabinose sugars. The real lignocellulosic hydrolysates also contain minimal amounts of additional sugars like mannose or galactose, which are negligible for this present approach. *P. putida* KT2440_*xylAB* reached nearly identical maximal optical densities of 11.3 and 11.5 (Figure 3a,b, Table 1) on the two substrates in comparison with an OD_{600} of 7.4 and 8.6 of *P. putida* KT2440_*araBAD* (Figure 3c,d, Table 1). However, both strains reached comparable maximal specific growth rates when cultivated with real hydrolysates instead of pure sugar mixtures. Surprisingly, the shift to exponential growth of *P. putida* KT2440_*araBAD* on the real hydrolysate takes longer than in all other cases once more underlining *P. putida* KT2440_*xylAB* as a highly suitable strain for efficient utilization of lignocellulosic hydrolysates and its containing sugars.

4 | DISCUSSION

The *P. putida* KT2440 wild type and the strain harboring the empty vector were unable to grow with D-xylose or L-arabinose as sole carbon source. However, a decreasing concentration of xylose could be observed during the cultivation, which is most likely caused by the activity of glucose dehydrogenase (Gcd). Gcd oxidizes xylose to xylonate, which is a dead-end product in the metabolism of *P. putida* and cannot be utilized further (Hardy, Teixeira De Mattos, & Neijssel, 1993; Meijnen et al., 2008). In contrast, no depletion of arabinose was determined indicating the absence of a respective specific enzyme activity and/or a profound incompatibility of arabinose with the specificity of Gcd.

After transformation of *P. putida* KT2440 with *xylAB* or *araBAD* harboring plasmids, the recombinant strains were immediately able to grow on xylose and arabinose without implementation of an adaptation process. In *E. coli,* specific transporters are responsible for the uptake of xylose and arabinose encoded by *xylE* and *araE* (xylose/arabinose:H^+ symporters) or *xylFGH* and *araFGH* (xylose/arabinose ABC transporters). *P. putida* do not encode homologous genes, but possess at least the outer membrane protein D1 (*oprB*) for the transport through the other membrane. According to Meijnen et al. (2008), the sugar transport does not influence its effective utilization indicating an existing unspecific transport mechanism for these sugars into the cytoplasm. However, the growth curves for these experiments show extended lag phases in comparison with the cultivation with glucose indicating the need for a comprehensive metabolic switch to utilize these unusual sugars, possibly depending on regulatory mechanism or slower metabolic fluxes especially in the pentose phosphate pathway. It was earlier reported that successful utilization of xylose after expression of *xylAB* in *P. putida* strain S12 needed a considerably laborious so-called "laboratory evolution" over 36 generations to improve the growth rate from 0.01 to 0.35 hr^{-1} (Meijnen et al., 2008), while this procedure is obviously not required for *P. putida* KT2440 confirming the work by Le Meur et al. (2012), where such an unadapted strain reached a maximal specific growth rate of 0.24 hr^{-1}. The higher growth rate in this present experiment ($\mu_{max} = 0.39\ hr^{-1}$) is probably based on different plasmid characteristics of pBBR1MCS in comparison with the pVLT used in earlier experiments, which shows a much lower copy number and contains an IPTG-dependent *tac*-promoter due to a plasmid-encoded LacI instead of the constitutive P^{lac} in this experiment.

The maximum growth rate for *P. putida_xylAB* (Table 1, Supporting Information Figure S1a) was calculated at the time of very low optical densities (around 0.1). Therefore, it should be noted that measurement errors in this region contribute disproportionally to errors in the specific growth rate, which results in an unusually high growth rate of 0.98 hr^{-1}.

In some experiments with mixtures of sugars especially if arabinose was present, the growth curves further increased although the primary carbon source was already consumed (Figures 2 and 3). The probable reason for this additional growth is the formation of by-products like intracellular deposited polyhydroxyalkanoates, whose production is well described and which serves as carbon and energy store (Poirier, Nawrath, & Somerville, 1995), as well as arabinoate, which is produced in significant amounts in cultures grown under similar experimental conditions (data not shown).

Unexpectedly, the strain *P. putida_araBAD* was not only able to grow on glucose and its supposedly preferred sugar L-arabinose, but showed also unspecific activity toward D-xylose. Nevertheless, *P. putida_araBAD* showed a better growth behavior on L-arabinose compared to D-xylose, which reasonably can be considered to depend on the higher specificity of the Ara-enzymes toward the sugars they are expected to be specific for. A similar cross- or side reaction was already reported for XylA and XylB toward L-arabinose (Meijnen et al., 2008), which was confirmed by our findings. Possibly, in *P. putida* KT2440 the flow of L-arabinose into the pentose phosphate pathway follows an alternative route involving one or more putative enzymes for the conversion of L-ribulose into D-xylulose, which will subsequently be phosphorylated by XylB. However, the exact metabolic route for utilization of L-arabinose by *P. putida* KT2440_*xylAB* needs to be elucidated.

The fact that plasmid curing restored the wild-type phenotype proved the dependency of the results on the presence of the *xylAB* and *araBAD* operons and that these were essential for both, the growth on and the consumption of xylose and arabinose and thereby excluded the possibility that the observed effects were based on mutations in the genome rather than on the plasmid-encoded metabolic enzymes.

The specificity of heterologous proteins certainly explains that the *xylAB*-expressing strain prefers xylose instead of arabinose and the other way around for *P. putida* KT2440_*araBAD*, but this is likely not the reason for different growth profiles of these strains. In *E. coli,* the expression of the *xylAB* and *araBAD* is transcriptionally regulated by transcription factors of the AraC/XylS family, which are widely distributed in gammaproteobacteria (Gallegos, Schleif, Bairoch, Hofmann, & Ramos, 1997). In this study, both operons are controlled by a *lac*-promoter generally uncoupled from native regulatory mechanisms, but conceivably a homologous transcription factor in *P. putida* is able to bind and regulate the heterologous operons. Furthermore, the catabolism of the various sugars used in this study occurs via different metabolic pathways, whose interactions could be responsible for differences in the growth behaviors. The hexose glucose is metabolized through the Entner–Doudoroff pathway, and pentoses like xylose and arabinose are metabolized via the pentose phosphate pathway. The switch between these two metabolic pathways depending on the currently available carbon source could be one reason for the growth curves shapes. In addition, Meijnen, Winde, and Ruijssenaars (2012) report about extensive changes in expression levels in genes involved in both pathways, which are possibly responsible for differences in the utilization of xylose and arabinose.

In this present study, we used wheat straw hydrolysate as a model to demonstrate the potential usage of different lignocellulosic hydrolysates for the growth of recombinant *P. putida* KT2440. Moreover, a wide variety of raw materials including other grain straws, grasses, sugarcane bagasse, miscanthus as well as hard and soft woods are potential bioresources, which can be used as carbon sources after pretreatment and hydrolysis (Hahn-Hägerdal, Galbe, Gorwa-Grauslund, Lidén, & Zacchi, 2006; Lavarack, Griffin, & Rodman, 2002; Mussatto & Teixeira, 2010; Saha, 2003). In future, large-scale processes will be developed for the production of valuable products based on lignocellulosic biomass, to move on from research level to commercial applications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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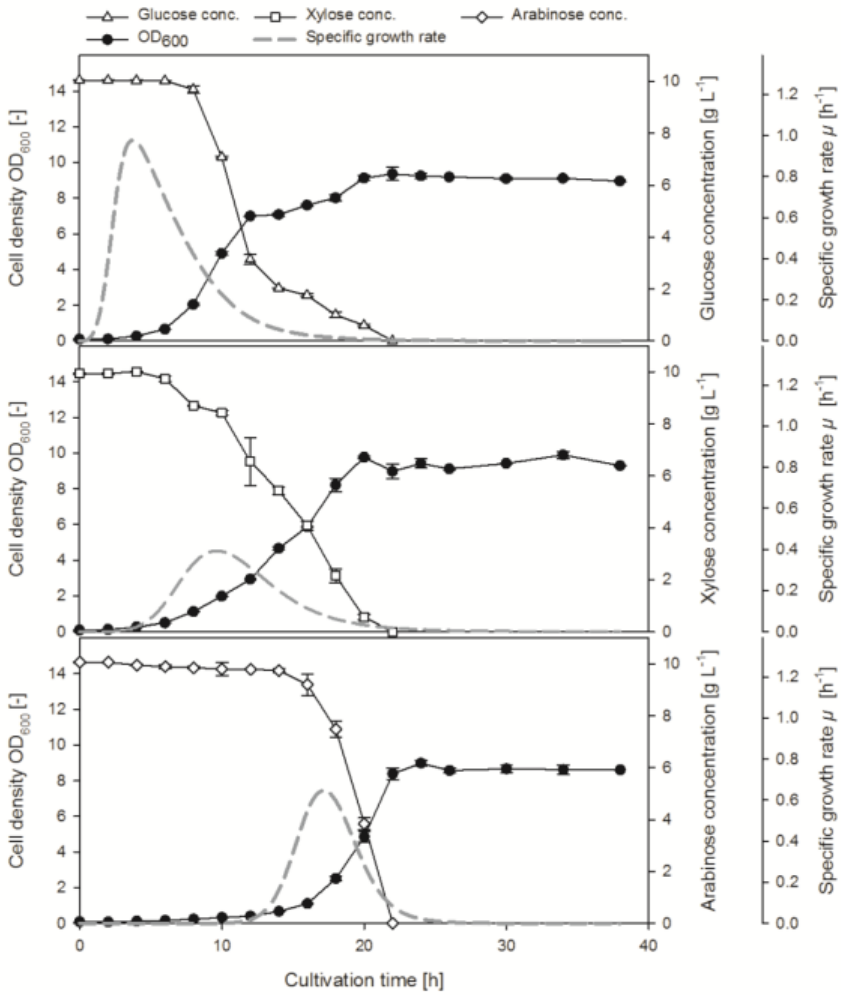
Supporting information

Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose and arabinose: hemicellulose hydrolysates and their major sugars as sustainable carbon sources

Yan Wang[+], Felix Horlamus[+], Marius Henkel, Filip Kovacic, Sandra Schläfle, Rudolf Hausmann, Andreas Wittgens, Frank Rosenau

[+]contributed equally

Fig. S1A


Glucose conc.
Xylose conc.
Arabinose conc.
OD600
Specific growth rate
Cell density OD600 [-]
Glucose concentration [g L-1]
Xylose concentration [g L-1]
Arabinose concentration [g L-1]
Specific growth rate μ [h-1]
Cultivation time [h]

2

Fig. S1B

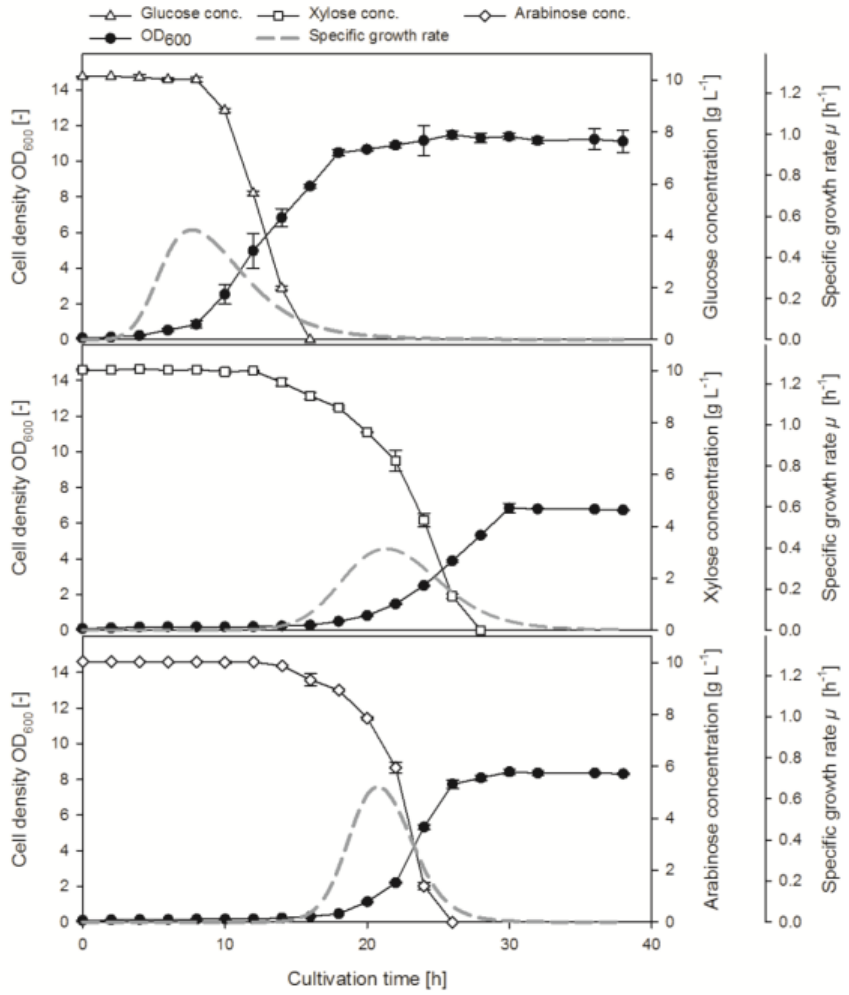


Fig. S1 Growth performance of recombinant *P. putida* using different sugars as carbon source. The strains *P. putida* KT2440_*xylAB* (A) and *P. putida* KT2440_*araBAD* (B) (filled circles) were cultivated in Wilms-KPi-Medium with 10 g L^{-1} glucose (upper diagrams), xylose (middle diagrams) or arabinose (lower diagrams) as the sole carbon source. The consumptions of glucose (empty triangles), xylose (empty squares) and arabinose (empty diamonds) are presented. The specific growth rates are shown as dashed curves. The symbols indicate the

averages of the results of duplicate measurements. The error bars represent the standard deviations.

3.3. Evaluation of different lignocellulosic hydrolysates as substrates for engineered *P. putida* KT2440 and the inhibitory effectiveness on *P. putida* KT2440

3.3.1. Summary III

In our previous study, upon introduction of the *xylAB* and *araBAD* operon, the resulting strains *P. putida* pBBR1MCS2_*xylAB* and *P. putida* pBBR1MCS2_*araBAD* were able to metabolize both xylose and arabinose[199]. Among them, recombinant strain *P. putida* pBBR1MCS2_*xylAB* displayed better growth behaviour, resulting in quite similar growth rates compared to the growth on D-glucose. Therefore, the growth profiles indicate that *P. putida* pBBR1MCS2_*xylAB* is rather qualified to utilize the three sugars more effectively. Moreover, the cultivation of two engineering strains on wheat straw hydrolysate also underlined *P. putida* pBBR1MCS2_*xylAB* is a highly suitable strain for efficient utilization of lignocellulosic hydrolysates.

Continuously in this work, we firstly investigated the growth performance of *P. putida* pBBR1MCS2_*xylAB* on different resources of hydrolysates, including: a). cellulose and hemicellulose from beech; b). cellulose and hemicellulose from spruce; c). hemicellulose from rice hulls; d). cellulose and hemicellulose from miscanthus; e). cellulose and hemicellulose from wheat straw; f). cellulose from beech; g) hemicellulose from beech. Different resources were hydrolysed using various methods, resulting diverse concentrations of monosaccharide sugars and inhibitors. Thus, the different growth performances of *P. putida* pBBR1MCS2_*xylAB* revealed that the preferred hydrolysates for *Pseudomonas putida*

strain, meaning the further information about the tolerance or resistance of *Pseudomonas putida* strain are primarily obtained.

Furthermore, the representatives of inhibitors existing in most of the hydrolysates were selected to examine deeply the inhibitory effects toward *P. putida* pBBR1MCS2_*xylAB*, including: a). formic acid; b). acetic acid; c). furfural; d). hydroxymethylfurfural (HMF)[200,77,33]. The results showed that *P. putida* KT2440 is relative tolerant to formic acid and acetic acid, but is very sensitive to HMF and furfural content. These consequences highly demonstrated the key criterion for applying lignocellulosic hydrolysates as a carbon source for *P. putida* KT2440, meaning it is more clear in selecting lignocellulosic hydrolysates or in choosing the hydrolysis methods in order to apply *P. putida* KT2440 as a host.

During the cultivation with hydrolysates, a diauxic-like grown pattern was observed with a non-simultaneous consumption of different sugars. As a potential strategy to overcome this issue, a fed-batch cultivation was implemented, resulting both glucose and xylose were consumed as carbon source by *P. putida* KT2440. As a result, a solution as fed-batch process has been confirmed for simultaneous consumption of all sugars, which makes the application of *P. putida* KT2440 in a high-efficiency bioprocess within the frame of bio-based economy.

In conclusion, this study evaluated and demonstrated the possibility of applying *P. putida* KT2440 as a platform for utilization of lignocellulose hydrolysates, which moved further from a research level to biotechnological industry.

3.3.2. Graphical Abstract Summary III

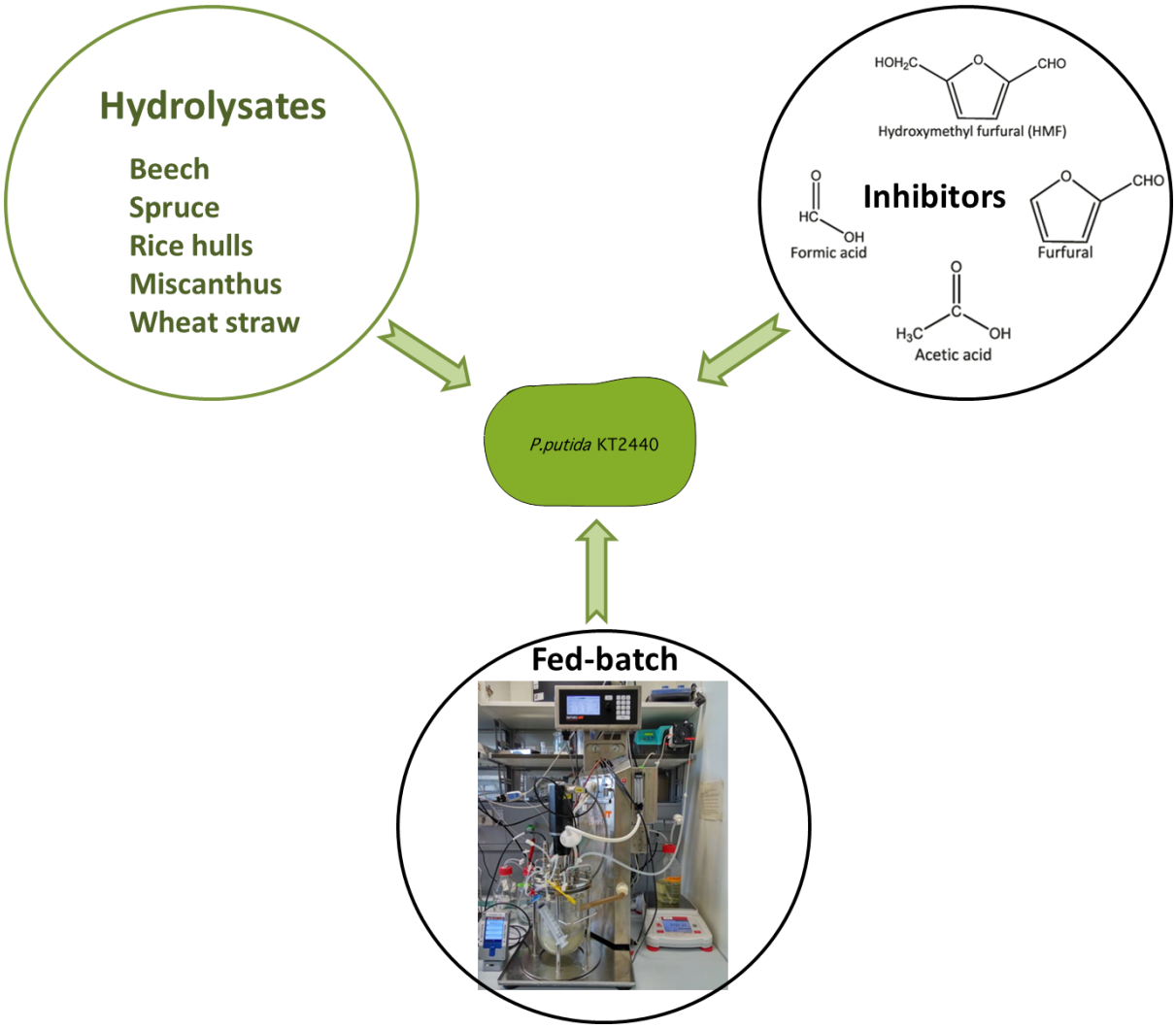


Figure 14 **Scheme of publication III.** Different resources of lignocellulosic hydrolysates, four main inhibitors and a fed-batch process were tested on the engineered strain *P. putida* KT2440. To reveal its tolerance and improve further the growth profile, which gives a deep evaluation of applying *P. putida* KT2440 in the bio-based economy.

3.3.3. Publication III: Potential of biotechnological conversion of lignocellulose hydrolysates by *Pseudomonas putida* KT2440 as a model organism for a bio-based economy

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Author contributions:

Y. Wang: provided the engineered strain, co-conducted the fed-batch bioreactor cultivations, discussed and co-revised the manuscript

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ORIGINAL RESEARCH

GLOBAL CHANGE BIOLOGY BIOENERGY WILEY

Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as a model organism for a bio-based economy

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Abstract

Lignocellulose-derived hydrolyzates typically display a high degree of variation depending on applied biomass source material as well as process conditions. Consequently, this typically results in variable composition such as different sugar concentrations as well as degree and the presence of inhibitors formed during hydrolysis. These key obstacles commonly limit its efficient use as a carbon source for biotechnological conversion. The gram-negative soil bacterium *Pseudomonas putida* KT2440 is a promising candidate for a future lignocellulose-based biotechnology process due to its robustness and versatile metabolism. Recently, *P. putida* KT2440_*xylAB* which was able to metabolize the hemicellulose (HC) sugars, xylose and arabinose, was developed and characterized. Building on this, the intent of the study was to evaluate different lignocellulose hydrolyzates as platform substrates for *P. putida* KT2440 as a model organism for a bio-based economy. Firstly, hydrolyzates of different origins were evaluated as potential carbon sources by cultivation experiments and determination of cell growth and sugar consumption. Secondly, the content of major toxic substances in cellulose and HC hydrolyzates was determined and their inhibitory effect on bacterial growth was characterized. Thirdly, fed-batch bioreactor cultivations with hydrolyzate as the carbon source were characterized and a diauxic-like growth behavior with regard to different sugars was revealed. In this context, a feeding strategy to overcome the diauxic-like growth behavior preventing accumulation of sugars is proposed and presented. Results obtained in this study represent a first step and proof-of-concept toward establishing lignocellulose hydrolyzates as platform substrates for a bio-based economy.

KEYWORDS

bioconversion, bioeconomy, biomass, biorefinery, hemicellulose, hydrolysis, lignocellulose, *Pseudomonas putida* KT2440

Felix Horlamus and Yan Wang contributed equally to this work.

1 | INTRODUCTION

Lignocellulose is a potential key carbon resource for a future bio-based economy as it is the most abundant renewable raw material on earth. Furthermore, it is not a direct competitor to food production as it accumulates in large quantities as waste in the wood, food, and agricultural industry (Anwar, Gulfraz, & Irshad, 2014; Jørgensen, Kristensen, & Felby, 2007; Lange, 2007; Naik, Goud, Rout, & Dalai, 2010; van Dyk, Gama, Morrison, Swart, & Pletschke, 2013). Lignocellulose is the structural framework of woody plant cell walls and consists mainly of lignin, cellulose (CE), and hemicellulose (HC). The amorphous heteropolymer lignin, consisting of phenylpropane units, is mainly interesting for material sciences and as a source for aromatic polymers (Upton & Kasko, 2016). CE consists of glucose linked by β-1,4 glucosidic bonds and most microorganisms are able to metabolize its depolymerization product glucose. On the other hand, the HC fraction remains mostly unused although approximately 60 billion tons of HCs are produced annually (Gatenholm & Tenkanen, 2003; Shahzadi et al., 2014; Wyman, 1994; Xu, Sun, Liu, & Sun, 2006). HCs are a group of heterogeneous polysaccharides consisting of different monomers such as D-xylose, D-mannose, D-arabinose, D-glucose, and sugar acids. In contrast to CE, the structure of HCs differs from plant to plant (Gatenholm & Tenkanen, 2003; Hendriks & Zeeman, 2009; Timell, 1967). In hardwoods like beech (Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003; Lu, Yamauchi, Phaiboonsilpa, & Saka, 2009; Teleman, Tenkanen, Jacobs, & Dahlman, 2002) and grasses (*Poaceae*), such as miscanthus (Schläfle, Tervahartiala, Senn, & Kölling-Paternoga, 2017), corn (Jørgensen et al., 2007), or wheat (Jørgensen et al., 2007; Schläfle et al., 2017), xylose is the dominant monosaccharide in HC. In softwood like fir and spruce (Hoyer, Galbe, & Zacchi, 2009; Tengborg et al., 1998), mannose is the predominant monosaccharide component. In order to use lignocellulose as a carbon source for biotechnological processes it usually has to be depolymerized since most industrially used microorganisms are not able to metabolize this compact and complex polymer. A common method is chemically or enzymatically catalyzed hydrolysis (Sun & Cheng, 2002; Taherzadeh & Karimi, 2007a, 2007b; van Dyk & Pletschke, 2012). Hydrolysis is usually preceded by pretreatment such as water steam explosion or organosolv treatment (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010; Domínguez de María, Grande, & Leitner, 2015; Mosier et al., 2005; Taherzadeh & Karimi, 2008). During these processes, inhibitors are formed, for instance, CE- and HC-derived furan aldehydes and aliphatic acids as well as lignin-derived phenolic compounds. Usually less toxic substances are formed in catalytic processes like hydrolysis than in thermochemical depolymerization processes such as pyrolysis, but nevertheless this may lead to issues when applied in biological systems (Arnold, Moss, Henkel, & Hausmann, 2017; Jönsson, Alriksson, & Nilvebrant, 2013; Palmqvist & Hahn-Hägerdal, 2000). The inhibitor concentrations showed great fluctuation between different hydrolyzates, as summarized by (Chandel, da Silva, & Singh, 2011). Acetic acid concentration varies from 0.4 g/L (Alriksson, Cavka, & Jönsson, 2011) to 5.45 g/L (Chandel & Singh, 2011), furfural concentration from 0.15 g/L (Nigam, 2001) to 2.2 g/L (Qian et al., 2006), and hydroxymethylfurfural (HMF) concentration from 0.07 g/L (Villarreal, Prata, Felipe, & Almeida E Silva, 2006) to 3.3 g/L (Alriksson et al., 2011). This, however, can be countered with an adjusted process technology, for example, milder hydrolysis methods to decrease inhibitor formation or separation techniques to remove inhibitors. However, this either leads to lower yields of fermentable sugar or requires additional process steps. For this reason, a promising solution could be the utilization of robust microorganisms like *Pseudomonas putida* which display a comparatively low sensitivity toward inhibitors (Martins Dos Santos, Heim, Moore, Strätz, & Timmis, 2004; Poblete-Castro, Becker, Dohnt, Dos Santos, & Wittmann, 2012; Roma-Rodrigues, Santos, Benndorf, Rapp, & Sá-Correia, 2010; Santos, Benndorf, & Sá-Correia, 2004; Segura et al., 2005). *P. putida* KT2440 is a plasmid-free derivative of the strain *P. putida* mt-2 isolated in Japan (reviewed by Nakazawa, 2003). This gram-negative, ubiquitous, saprophytic soil bacterium has become a remarkable workhorse for biotechnical processes (Loeschcke & Thies, 2015; Martins Dos Santos et al., 2004). As an example, it is a suitable host for the production of the biosurfactant rhamnolipid (Arnold, Henkel, et al., 2019; Beuker, Barth, et al., 2016, Beuker, Steier, et al., 2016; Cha, Lee, Kim, Kim, & Lee, 2008; Tiso et al., 2017; Wittgens et al., 2011, 2017, 2018). Unlike many other *Pseudomonads*, *P. putida KT2440* is classified as biosafety level 1 according to American Type Culture Collection. Furthermore, the complete and annotated genome sequence for *P. putida KT2440* is available (Nelson et al., 2002). Its versatile metabolism and robustness against numerous organic compounds (Martins Dos Santos et al., 2004; Nelson et al., 2002; Poblete-Castro et al., 2012) makes it a candidate for a next-generation lignocellulose biorefinery strain. However, *P. putida* KT2440 wild type is not able to metabolize the main HC sugars, xylose and arabinose, as it lacks the enzymes xylose isomerase (*xylA*), xylulose kinase (*xylB*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate 4-epimerase (Henkel et al., 2012). In the recent past, *P. putida* KT2440 has been engineered toward metabolization of HC sugars (Dvořák & de Lorenzo, 2018; Meijnen, Winde, & Ruijssenaars, 2008; Wang et al., 2019). Similarly, in this study, strain *P. putida* KT244_*xylAB* carrying the plasmid pBBR1MCS-2 with the *xylAB* operon from *Escherichia coli* DH5α was used. Upon introduction of the *xylAB* operon, the resulting strain was able to metabolize xylose and arabinose

resulting in similar growth rates compared to glucose (Wang et al., 2019).#AuthorQueryReply##AuthorQueryReply## AuthorQueryReply#

Building on this, the intent of the current study was to evaluate *P. putida* KT2440 as a platform model organism for bioconversion of different lignocellulose hydrolyzates. Firstly, hydrolyzates of different origins were evaluated as potential carbon sources for the developed HC sugar metabolizing *P. putida* by cultivation experiments. Secondly, the content of major toxic substances in CE- and HC hydrolyzates was determined and their inhibitory effect on bacterial growth was characterized. Thirdly, a fed-batch cultivation strategy in a bioreactor with hydrolyzate as the carbon source was proposed. Results obtained in this study represent a first step and proof-of-concept toward establishing lignocellulose hydrolyzates as platform substrates for a bio-based economy.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

If not stated otherwise, all chemicals were purchased from Carl Roth GmbH. HMF was obtained from AVA Biochem BSL AG.

2.2 | Hydrolyzates

As potential carbon sources, hydrolyzates from different sources were evaluated. The main differences between each hydrolyzate are related to the manufacturing process, the applied raw material as well as the utilized lignocellulose fractions:

- For hydrolysis with diluted sulfuric acid, CE and HC were hydrolyzed simultaneously with beech (hydrolyzate a) and spruce (hydrolyzate b). Hydrolysis was performed in a fixed bed reactor loaded with biomass in chip size. The reactor was heated up to the reaction temperature of 180°C with a constant water flow. When the reaction temperature was reached, 0.05 mol/L sulfuric acid was introduced to the reactor and the hydrolyzate was constantly removed.
- The two-step acid hydrolysis included a high concentration of hydrochloric acid (32% and 28%), HC fraction, and rice hulls (hydrolyzate c; Green Sugar AG) as described previously (Green Sugar AG, 2018).
- Steam explosion followed by enzymatic hydrolysis included CE/HC, miscanthus (hydrolyzate d), and wheat straw (hydrolyzate e) as described previously (Schläfle et al., 2017).
- For the organosolv process followed by enzymatic hydrolysis, CE fraction (hydrolyzate f), HC fraction (hydrolyzate g), and beech were used as described in Dörsam et al. (2017).

Initially, the pH of the hydrolyzates was measured (SevenCompact, Mettler-Toledo GmbH) and adjusted to 7.0 using a 10 M sodium hydroxide solution. Later, samples were centrifuged (12,000 × g, 20°C, 10 min) with Heraeus Multifuge X3 (Thermo Fisher Scientific GmbH) and subsequently the supernatant was sterile filtered (Rotilabo®-syringe filters, 0.22 µm pore size; Carl Roth GmbH). Finally, the hydrolyzates were concentrated or diluted to a concentration of 100 or 150 g/L.

2.3 | Strains and plasmids

Pseudomonas putida KT2440 wild type (Nelson et al., 2002) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures listed under strain number 6125. *P. putida* KT2440 pBBR2MCS-2_*xylAB* contains the *xylAB* operon from *E. coli* DH5α (Grant, Jessee, Bloom, & Hanahan, 1990). The operon encodes for the genes *xylA* (xylose isomerase) and *xylB* (xylulose kinase), which are required for metabolizing the HC monosaccharides. The construction of the plasmid is described in detail by Wang et al. (2019).

2.4 | Media

Preculture was performed with lysogenic broth (LB) medium: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl; pH 7.0. All other cultivations were carried out with adapted Wilms medium (Wilms et al., 2001) together with an adapted phosphate buffer system (Beuker, Steier, et al., 2016): 500 g/L 100 mM KP_i buffer (13.15 g/L K_2HPO_4, 3.28 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$, 1 g/L NH_4Cl, 4 g/L Na_2SO_4, 50 g/L $MgSO_4 \cdot 7H_2O$), 3 ml/L trace element solution (0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$, 0.1 g/L $MnSO_4 \cdot H_2O$, 13.92 g/L $FeCl_3 \cdot 6H_2O$, 10.05 g/L EDTA Titriplex III, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 0.662 g/L $CaCl_2 \cdot 2H_2O$, pH 2), and 0.01 g/L thiamin HCl. A total amount of 10 g/L D-(+)-glucose, D-(+)-xylose, L-(+)-arabinose, equal mixtures of these sugars, or lignocellulose hydrolyzates were added to the medium as carbon source. For strains with pBBR2MCS-2 plasmid, kanamycin with a concentration of 50 µg/ml was added to the medium as a selection marker.

The medium was modified for bioreactor cultivations. The batch medium contained a lower sugar concentration of 5 g/L. As a carbon source, wheat straw hydrolyzates were used. The feed medium had a higher sugar concentration of 150 g/L and a higher nitrogen concentration (NH_4Cl: 2.51 g/L, $(NH_4)_2SO_4$: 25.1 g/L).

2.5 | Cultivation (Erlenmeyer flasks)

For the preculture, 25 ml LB medium was inoculated with 100 µl glycerol stock solution. After overnight cultivation, 25 ml

Wilms–KP_i medium were inoculated with an initial optical density of 0.1 at 600 nm (OD_{600}). The cultivations took place in 250-ml Erlenmeyer baffled flasks at 30°C and 120 rpm in an incubation shaker (Innova 44R, Eppendorf AG). For storage, cultures were mixed with glycerol (25% v/v) and frozen at −80°C.

2.6 | Cultivation (bioreactor)

2.6.1 | Equipment

The experiments were performed in a 2 L bench-top bioreactor (Labfors 4; Infors AG). The process control and the recording of the results were carried out with a bioreactor control software IRIS (Infors AG). Temperature was kept constant at 30°C. The pH value was determined with a pH sensor (EasyFerm Bio K8224; Hamilton Company) and was adjusted to 7.0 with 4 M phosphoric acid and 4 M sodium hydroxide solution. Oxygen partial pressure (pO_2) of the medium was measured with an optical probe (VisiFerm DO 225; Hamilton Company) and controlled to 30% by adjusting stirring rate (300–1,250 rpm) and aeration with compressed air (0.1–0.5 vvm). In addition, carbon dioxide and oxygen content of exhaust gas was measured with a gas analyzer (INFORS HT; Infors AG). Feed medium was added with the laboratory peristaltic tube pump (model 323Du/D; Watson-Marlow Fluid Technology Group) and controlled via MATLAB (The MathWorks, Inc.).

2.6.2 | Experimental setup (fed-batch)

Five hundred milliliters of Wilms–KP_i medium was inoculated with a starting OD_{600} of 0.1 and the feed was started when all sugars were consumed as indicated by a rise in pO_2. A specific growth rate μ of 0.44/hr was applied for calculation of the feed rate to allow for exponential growth (Beuker, Barth, et al., 2016). Antifoam agent (Contraspum A4050; Zschimmer & Schwarz GmbH & Co. KG Chemical Factories) was added to the medium in case of excessive foaming as required.

2.7 | Analytical methods

2.7.1 | Cell density

Cell density was determined immediately after sampling. The optical density at 600 nm (OD_{600}) was measured via a cell density meter (CO8000; Biochrome) and samples were diluted as required with saline solution (0.9%).

2.7.2 | Monosaccharides

Samples were centrifuged (5 min, 4°C, 4,700 rpm) and the supernatant was used to measure D-glucose, D-xylose, L-arabinose, and D-mannose content. This was carried out with the following enzyme assays: D-glucose (R-Biopharm AG), D-Xylose Assay Kit, L-Arabinose/D-Galactose Assay Kit, and D-Mannose/L-Fructose/D-Glucose Assay (Megazyme u.c., Co.).

2.7.3 | Formic acid, acetic acid, furfural, HMF

The quantitative analysis of formic acid, acetic acid, furfural, and HMF was performed via high-performance liquid chromatography.

The furan compounds HMF and furfural were separated at 20°C in a Lichrospher 100 RP-18 column (Merck). As the mobile phase, a water–acetonitrile eluent (9:1 v/v) at a flow rate of 1.4 ml/min was used, and a UV detector was operated at 290 nm. Formic acid and acetic acid were analyzed with an Aminex HPX-87H column (Bio-Rad) at a temperature of 25°C. The eluent was 0.004 mol/L sulfuric acid at a flow rate of 0.65 ml/min and detection was performed by refractive index detector and diode array detector.

2.8 | Inhibitory effect of formic acid, acetic acid, furfural, and HMF on the growth of *P. putida* KT2440

Pseudomonas putida KT2440 wild type and *P. putida* KT2440_*xylAB* were cultivated in Wilms–KP_i medium as described above. In addition, formic acid, acetic acid, furfural, and HMF were added in different concentrations to the medium, respectively. The pH value of the medium was adjusted to 7.0 with 10 M sodium hydroxide after addition of the inhibitors.

2.9 | Software for graphical analysis, biological replicates, and measurement error

Creation of graphs and graphical analysis of measured data were performed using scientific graphing and data analysis software (SigmaPlot; Systat Software Inc.). All data, if not stated otherwise, were obtained as duplicates from at least two independent biological experiments, and measurement results are presented as mean ± *SD*.

3 | RESULTS

3.1 | Growth performance of *P. putida* KT2440_*xylAB* with lignocellulose monosaccharides

Pseudomonas putida KT2440_*xylAB* was able to metabolize all main lignocellulose monosaccharides (Wang et al., 2019). Based on these results, an evaluation of different

TABLE 1 Monosaccharide concentration and inhibitory concentration in lignocellulose hydrolyzates of different origin

Hydrolyzate	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Mannose (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)
Sulfuric acid, CE/HC, beech (a)	7.5	2.3	0.2	ND	1.8	1.9	2.0	4.8
Sulfuric acid, CE/HC, spruce (b)	7.8	0.4	0.1	1.7	0.1	0.1	0.6	2.4
Hydrochloric acid, HC, rice hulls (c)	2.5	5.9	1.6	ND	ND	ND	0.1	ND
Steam explosion and enzymes, CE/HC, miscanthus (d)	7.5	2.3	0.2	ND	ND	0.5	0.2	ND
Steam explosion and enzymes, CE/HC, wheat straw (e)	6.8	3.0	0.2	ND	ND	0.2	0.2	ND
Organosolv process and enzymes, CE, beech (f)	7.9	2.1	ND	ND	ND	0.3	ND	ND
Organosolv process and enzymes, HC, beech (g)	1.2	8.0	0.8	ND	1.3	2.3	0.1	ND

Note: All hydrolyzates were adjusted to a total monosaccharide concentration of 10 g/L.
Abbreviations: CE, cellulose; HC, hemicellulose; ND, not detectable.

lignocellulose hydrolyzates (Table 1) for use as carbon sources in biotechnological processes with *P. putida* KT2440_*xylAB* was performed (Figure 1; Table 2).

As a reference, cultivation with glucose as a sole carbon source was performed (Figure 1h; Table 2). The cells reached a maximum OD_{600} of 12.7, with a maximal specific growth rate μ_{max} of 0.7/hr and biomass to substrate ratio Y_{XIS} of 0.41 g/g. Glucose was completely consumed. With beech and spruce hydrolyzates treated with sulfuric acid (hydrolyzates a + b), no cell growth and no decrease in the sugar concentration could be measured over the entire cultivation period of 48 hr (Figure 1a,b; Table 2). *P. putida* KT2440_*xylAB* had a similar growth performance with rice hull samples hydrolyzed with hydrochloric acid (hydrolyzate c) as with glucose with a maximum OD_{600} = 11.5, μ_{max} = 0.4/hr and Y_{XIS} = 0.41 (Figure 1c; Table 2). The bacteria showed a diauxic-like growth pattern. First, *P. putida* KT2440_*xylAB* metabolized mainly glucose (phase I) and when glucose was almost consumed the strain metabolized xylose and arabinose (phase II) and at the end only small amounts of arabinose remain in the medium (phase III). Next, hydrolyzates depolymerized via steam explosion and enzymes were applied (Figure 1d,e; Table 2). With miscanthus (hydrolyzate d), maximum OD_{600} = 11.3, μ_{max} = 0.4/hr, and Y_{XIS} = 0.38 was detected. Using wheat straw (hydrolyzate e), slightly higher growth rates were measured (OD_{600} = 11.7, μ_{max} = 0.5/hr and Y_{XIS} = 0.38). In both cases, first glucose (phase I) and then xylose and arabinose were consumed (phase II). The highest values with OD_{600} = 12.6, μ_{max} = 0.7/hr, and Y_{XIS} = 0.42 were obtained with the CE fraction of the organosolv process (figure f, Table 2). The strain metabolized glucose first and then xylose. No growth could be verified applying the HC fraction of the organosolv process (figure g, Table 2).

The growth parameters of all cultivation experiments are summarized in Table 2. As a comparison, cultivations of the wild type and cultivations with glucose as carbon source are presented. As expected, the maximum OD_{600} of *P. putida* KT2440 wild type (12.7) and *P. putida* KT2440_*xylAB* (12.4) were very similar using glucose as carbon source. With the CE fraction of the organosolv process (hydrolyzate f), *P. putida* KT2440_*xylAB* reached a slightly higher maximum OD_{600} of 12.6 as the wild type strain with a maximum OD_{600} of 11.5. Overall, the recombinant strain displayed higher growth rates than the wild-type strain with hydrolyzates containing HC monosaccharides. This was particularly evident with the pure HC samples (hydrolyzate c) as the wild type strain reached a maximum OD_{600} of 4.1 and the *xylAB* strain reached a maximum OD_{600} of 11.5. For better comparability, the relative carbon hydrolyzate conversion index (RCHC) was defined. It is defined as the ratio of biomass of respective cultivation to the cultivation with *P. putida KT2440* wild type and glucose as carbon source. Furthermore, this index was calculated to obtain system-independent data, which facilitates the comparability of other work on carbon sources/hydrolyzates and gene constructs. With *P. putida* KT2440_*xylAB* the highest RCHC was calculated with hydrolyzate f (100%) followed by hydrolyzate e (93%), hydrolyzate c (90%), and hydrolyzate d (90%; Table 2).

3.2 | Inhibitors

The inhibitory effect of major toxic substances in lignocellulose hydrolyzates on the growth of *P. putida* KT2440 was investigated.

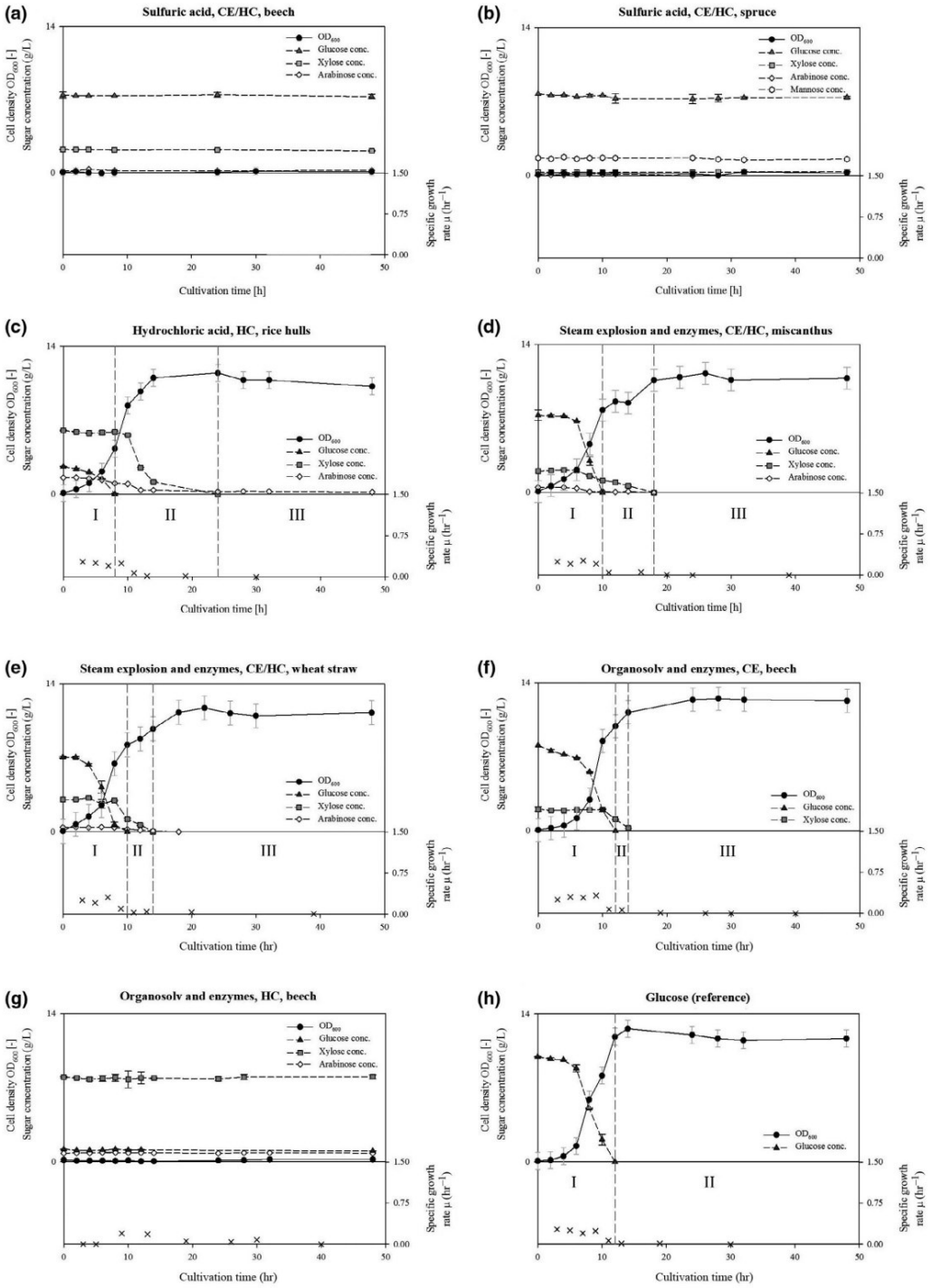

FIGURE 1 Time course of cultivation of *Pseudomonas putida* KT2440_*xylAB* with different lignocellulose hydrolyzates as carbon source. The specific growth rates are separately shown in the lower diagrams and single growth phases are indicated with roman numbers: phase I, mainly consumption of the first sugar (glucose); phase II, mainly consumption of the second (xylose) and third sugar (arabinose); phase III, consumption of the third sugar (arabinose). Subfigures indicated by lowercase letters correspond to the applied hydrolyzate (Table 2). CE, cellulose; HC, hemicellulose

TABLE 2 Growth parameter of cultivations of *Pseudomonas putida* KT2440 with different hydrolyzates as carbon source

Substrate	OD_{max}	Bacterial biomass (g/L)	Max. spec. growth rate μ_{max} (1/hr)	$Y_{X|S}$ (g/g)[a]	RCHC (%)[b]
P. putida KT2440 (wild type)					
Glucose (reference)	12.7 ± 0.4	4.2	0.6	0.42	100
Sulfuric acid; CE/HC; beech (a)	0.1 ± 0.1	—	—	—	—
Sulfuric acid; CE/HC; spruce (b)	0.1 ± 0.0	—	—	—	—
Hydrochloric acid; HC; rice hulls (c)	4.1 ± 0.1	1.4	0.4	0.14	33
Steam explosion and enzymes; CE/HC, miscanthus (d)	9.5 ± 0.6	3.2	0.5	0.32	76
Steam explosion and enzymes; CE/HC; wheat straw (e)	8.4 ± 0.6	2.8	0.5	0.28	67
Organosolv process and enzymes, CE, beech (f)	11.5 ± 0.4	4.2		0.42	100
Organosolv process and enzymes, HC, beech (g)	0.1 ± 0.0	—	—	—	—
P. putida KT2440_*xylAB*					
Glucose (reference)	12.4 ± 0.3	4.1	0.7	0.41	98
Sulfuric acid; CE/HC; beech (a)	0.2 ± 0.1	—	—	—	—
Sulfuric acid; CE/HC; spruce (b)	0.2 ± 0.0	—	—	—	—
Hydrochloric acid; HC; rice hulls (c)	11.5 ± 0.7	3.8	0.4	0.41	90
Steam explosion and enzymes; CE/HC, miscanthus (d)	11.3 ± 0.5	3.8	0.4	0.38	90
Steam explosion and enzymes; CE/HC; wheat straw (e)	11.7 ± 0.8	3.9	0.5	0.39	93
Organosolv process and enzymes, CE, beech (f)	12.6 ± 0.3	4.2		0.42	100
Organosolv process and enzymes, HC, beech (g)	0.1 ± 0.0	—	—	—	—

Abbreviation: RCHC, relative carbon hydrolyzate conversion.

[a]Bacterial biomass (X) to substrate (S) ratio, substrate: 10 g/L carbohydrate.

[b]Ratio of biomass of respective cultivation to the cultivation with *P. putida* KT2440 (wild type) and glucose as carbon source.

Maximum achieved biomass of *P. putida* KT2440 wild type decreased slightly already upon addition of formic acid starting at a concentration of 1 g/L from a maximum OD_{600} 12.3 to 11.5 (Figure 2a). At the highest applied formic acid concentration of 10 g/L the cells reached a maximum OD_{600} of 8.4. The recombinant strain was more sensitive to formic acid than the wild type. Cell growth decreased upon addition of formic acid starting at a concentration of 1 g/L (maximum OD_{600} = 10.5). At 5 g/L formic acid the maximum OD_{600} was 4.7 and at 10 g/L the maximum OD_{600} was 2.9. Acetic acid had no negative influence on the growth of the two *P. putida* KT2440 strains (Figure 2b) until the highest applied acid concentration of 10 g/L. Furthermore, the strains metabolized acetic acid and partially used it for growth (Arnold, Tews, Tews, Kiefer, Henkel, & Hausmann, 2019). In detail, compared to cultivation without acetic acid (maximum OD_{600} = 12.4), the wild type reached a maximum OD_{600} of 14.6 and 15.1 at acetic acid concentrations of 2.5 and 10 g/L, respectively. *P. putida* KT2440_*xylAB* showed similar growth characteristics as the wild type strain and reached a maximum OD_{600} of 12.3, 15.2, and 15.9 at acetic acid concentrations of 0, 2.5, and 10 g/L, respectively. The addition of acetic acid had no effect on the length of the lag phase.

Furfural causes an extension of the lag phase in low concentrations and a reduction in cell growth in high concentrations (Figure 2c). There was hardly any difference between wild type and recombinant strain. The lag phase was extended starting from a threshold of 0.4 g/L furfural concentration (lag phase = 6 hr) and at a concentration of 4 g/L, cell growth started with a delay of 24 hr after inoculation. No growth was detectable over a period of 5 days at a concentration of 5 g/L furfural (data not shown). Similar to furfural, the addition of HMF resulted in an extension of the lag phase and, at a higher concentration, in a decrease in cell growth (Figure 2d). In detail, the lag phase of *P. putida* KT2440 wild type was prolonged starting from a threshold value of 1.2 g/L HMF concentration (lag phase = 4 hr) and the lag phase lasted for 8 hr at an HMF concentration of 2.4 g/L and approx. 52 hr at an HMF concentration of 4.8 g/L. A decrease in the maximum OD_{600} could be observed only at higher HMF concentrations starting at 2.8 g/L with a maximum OD_{600} of 7.7. No growth could be detected at the highest applied HMF concentration of 5.2 g/L. *P. putida* KT2440_*xylAB* proved to be slightly more resistant to HMF. When HMF was added, the lag phases were also extended, but to a lesser extent than for the wild type. In addition, the maximum OD_{600} almost did not decrease up to HMF concentrations of 4.8 g/L and remained constant at approx. 12.0. As with the wild type,

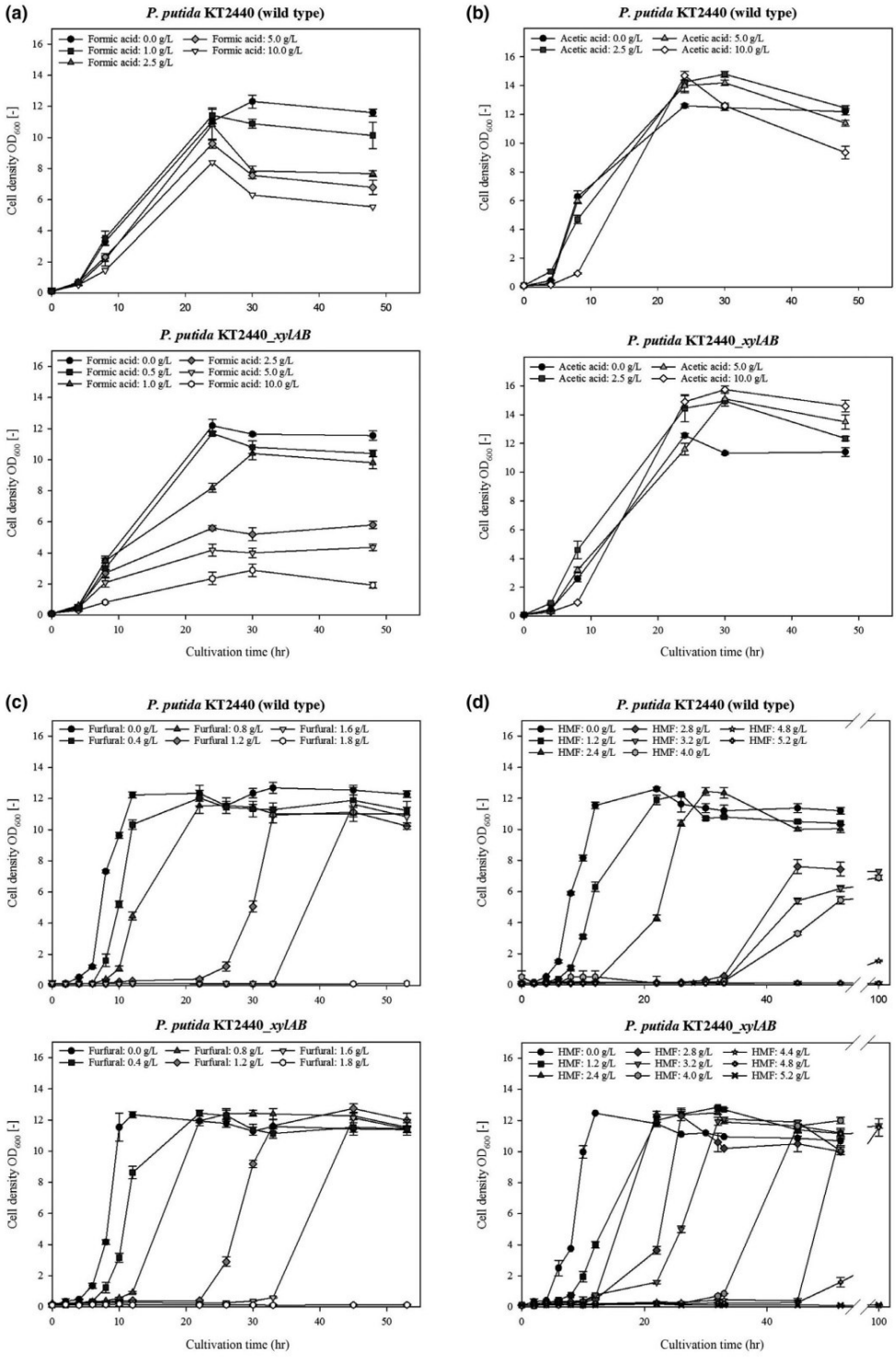

FIGURE 2 Time courses of OD_{600} during cultivation with *Pseudomonas putida* KT2440_*xylAB* and wild type by using glucose as a primary carbon source and adding formic acid (a), acetic acid (b), furfural (c), and hydroxymethylfurfural (HMF; d) to the medium

no growth was detected at HMF concentration of 5.2 g/L over a period of 5 days.

3.3 | Fed-batch bioreactor cultivations with lignocellulose hydrolyzates as carbon source

Pseudomonas putida KT2440_*xylAB* was able to grow with several hydrolyzates and reached comparable growth rates as with glucose. Building on this, a fed-batch process was performed in a 2 L bioreactor with wheat straw hydrolyzates as carbon sources. Furthermore, the results of the shaking flask cultivations led to the hypothesis that the recombinant strain only metabolizes xylose once glucose has been consumed. To confirm this hypothesis, fed-batch experiments were performed.

Observed growth shows a preferential metabolization of glucose, followed by xylose and lastly arabinose (Figure

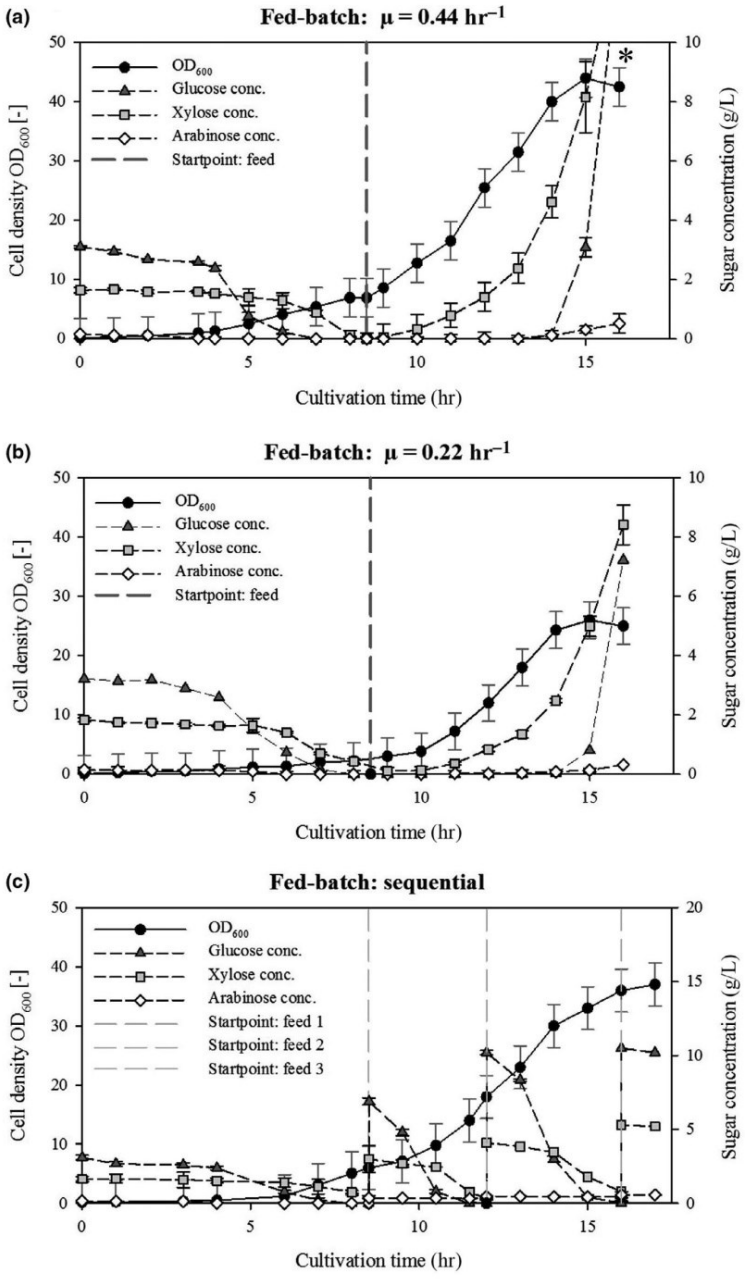

FIGURE 3 Time courses of OD_{600} and sugar concentration during fed-batch bioreactor cultivations with *Pseudomonas putida* KT2440_*xylAB*. (a) Calculated growth rate of $\mu = 0.44$/hr, (b) $\mu = 0.22$/hr, (c) sequential feeding. *Concentration after 16 hr: glucose 23.9 g/L, xylose 13.2 g/L

3a), in accordance with previous observations (Figure 1). The feed was started when glucose and xylose were almost consumed. As expected, the strains almost did not metabolize xylose and arabinose in the presence of glucose. Xylose was accumulated in the medium and after 15 hr a concentration of 8.2 g/L could be detected.

To solve the issue of sugar accumulation, a lower growth rate of $\mu = 0.22$/hr was applied for the next experiment (Figure 3b) to potentially enable uptake of xylose and arabinose. However, similar results regarding sugar accumulation were obtained. As a consequence, feed medium was sequentially added in the next fed-batch experiment. This feed medium was only added when xylose was almost depleted (Figure 3c). The applied feed profile did indeed lead to complete metabolization of relevant sugars xylose and arabinose. Furthermore, during metabolization of xylose, growth could be detected and the strain grew without any relevant lag phase. A maximum OD_{600} of 35 was achieved after 15 hr.

4 | DISCUSSION

4.1 | Lignocellulose hydrolyzates as carbon source for *P. putida* KT240_*xylAB*

Pseudomonas putida KT2440_*xylAB* reached similar growth rates with miscanthus and wheat straw hydrolyzates treated with steam explosion and enzymes (hydrolyzate d + e) as compared with glucose. However, it should be noted that for the combination of steam explosion and enzymatic hydrolysis, depolymerization of CE and HC to sugars is incomplete and may be optimized in the future. For the applied wheat straw, conversion of CE (38%) and HC (41%) to the respective monosaccharides is reported (Schläfle et al., 2017). However, if more severe process conditions for steam explosion are applied to obtain a better enzymatically convertible substrate, this may result in increased levels of HMF and furfural, which may have a negative effect on the applicability of the hydrolyzate. In this case, a view into polymer-degrading microorganisms could be worthwhile, including suitable yeasts, fungi, or specialized bacteria such as *Cellvibrio japonicus* (Gardner, 2016; Gardner & Keating, 2010; Horlamus et al., 2019). Furthermore, a genome-edited derivative of *P. putida* KT2440 was recently constructed, which was able to use cellobiose as the carbon source (Dvořák & de Lorenzo, 2018).

The results with hydrochloric acid-depolymerized samples (hydrolyzate c) are especially interesting, since only HC-derived sugars were used. As such, conversion of contained sugars to biomass is significantly lower for the wild type, as no conversion of xylose and arabinose is observed. In a future study, performance of these hydrolyzates could also be investigated for bioreactor cultivations.

When applying the hydrolyzates obtained after digestion with sulfuric acid (hydrolyzate a + b) as the carbon source, no growth of *P. putida* KT2440_*xylAB* and wild type was detected. This hydrolysis process was initially designed for a nonbiochemical value chain. The goal was to obtain high sugar concentrations in the hydrolyzate after short reaction times and to convert the sugars in further reaction steps to furfural and HMF. Consequently, the chosen hydrolysis reaction conditions were chosen were more severe compared to hydrolysis processes designed for fermentable sugar production. Therefore, more fermentation inhibitors are present in the hydrolyzate of this sulfuric acid hydrolysis process. High concentrations of furfural and HMF were identified as main critical points for *P. putida* KT2440. Besides individual inhibitory effects, the combined action of different inhibitors is still unknown for *P. putida* KT2440 and could be a further target of research. This is apparent for hydrolyzate b, where the HMF and furfural are each not present at critically inhibiting concentrations, yet, no growth could be observed.

For detoxification of hydrolyzates, many different physical, chemical, and biochemical processes have been developed and summarized (Chandel et al., 2011; Jönsson et al., 2013). The high concentrations of HMF and furfural do not have to be a disadvantage, as both are important basic building block chemicals (Steinbach, Kruse, & Sauer, 2017). For this reason, a worthwhile process strategy could be to combine chemical and biotechnological methods. Furfural and HMF should be separated from hydrolyzates firstly to obtain them as marketable products and secondly to get detoxified hydrolyzates which can be applied as the carbon source for microorganisms.

The CE fraction of the organosolv process (hydrolyzate f) was a highly suitable carbon source and similar growth rates as with glucose were achieved. No growth could be measured with the HC fraction (hydrolyzate g), although the measured organic acid or furfural aldehyde concentrations were not critically high. A potential reason for that could be lignin-derived phenolic compounds, which are not determined in this study but should be a topic for future research, because phenols are also well-known fermentation inhibitors (Palmqvist & Hahn-Hägerdal, 2000).

In a previous study, beech hydrolyzates from the same organosolv process as applied in this study were used as carbon source for organic acid production with filamentous fungi (Dörsam et al., 2017). In contrast to *P. putida* KT2440, *Aspergillus oryzae* was able to grow with HC hydrolyzates as the carbon source. A final malic acid concentration of 5.8 g/L and an overall production rate of 0.03 g/(L*hr) in a bioreactor cultivation with 99.5 g/L HC sugars was reported. *Rhizopus delemar* was more sensitive to inhibitors than *A. oryzae* and a production of organic acids with the HC fraction as the carbon source was not possible.

4.2 | Inhibitors

Pseudomonas putida KT2440_*xylAB* and wild type proved to tolerate acetic acid up to the highest measured concentration

of 10 g/L after neutralization of the medium. This is a major advantage for applying this strain as host for lignocellulose biorefinery since most hydrolyzates contain acetic acid below a concentration of 10 g/L originating from HC (Chandel et al., 2011; Palmqvist & Hahn-Hägerdal, 2000). Both strains were however less tolerant to formic acid than to acetic acid and growth was weakened starting from a concentration of 1.0 g/L.

As most hydrolyzates contain only a small amount of formic acid clearly below 1.0 g/L (Chandel et al., 2011; Palmqvist & Hahn-Hägerdal, 2000), this is an issue only in high-efficiency fed-batch bioprocesses, where an accumulation of formic acid after prolonged feeding would occur. Furthermore, the recombinant strain was more sensitive to formic acid than the wild type. It should be noted that the additional plasmid and the expression of additional genes represents a metabolic burden for the bacteria. This may not have a serious effect under moderate cultivation conditions but may lead to reduced growth in the presence of another stress factor. Furfural and HMF are identified as critical components, since a negative influence on growth could be identified starting from a furfural concentration of approx. 0.4 g/L and an HMF concentration of 1.2 g/L. This was not reflected in a deterioration in growth rates, but in an extension of the lag phase. This is consistent with results obtained in a study in which a lag phase of 24 hr was observed during the cultivation of *P. putida* KT2440 with lignocellulose hydrolyzates supplemented with 2 g/L HMF and 1 g/L furfural. An explanation for this is the metabolization of furfural aldehydes to less toxic dead-end alcohol counterparts (furfuryl alcohol and HMF furfuryl alcohol) practiced by many microorganisms, which has been reported in the past (Guarnieri, Ann Franden, Johnson, & Beckham, 2017). Furthermore, in theory, part of the highly reactive furfural and HMF could have formed less toxic macromolecules over time of the cultivation. However, under the applied experimental conditions, it was verified that this was not the case (data not shown).

In case of microbial biorefinery, organic acids and furan aldehydes are mainly discussed as inhibitors, although they consist of carbon to a considerable extent. As a consequence, a future lignocellulose strain should not only have a high resistance to these compounds but also be able to utilize them as carbon source. *P. putida* KT2440_*xylAB* and wild type were able to metabolize acetic acid. With an amount of 2.5 g/L, the maximum OD_{600} increased from 12.4 to 14.6. Since *P. putida* KT2440 wild type is not able to use furfural and HMF as substrates for growth, Guarnieri et al. (2017) engineered a strain via genomic integration of the *hmf* gene cluster. Consequently, the strain metabolized HMF and furfural via the intermediate 2-furoic acid. This constructed strain grew on HMF and furfural up to a concentration of 1 g/L. In summary, the results show that the HMF and furfural content in hydrolyzates is a key criterion for applying lignocellulosic hydrolyzates as a carbon source for *P. putida* KT2440.

4.3 | Fed-batch cultivation in a bioreactor

During cultivation with hydrolyzate, a diauxic-like grown pattern with a nonsimultaneous consumption of different sugars was observed. With sufficient carbon supply, the recombinant strain metabolized glucose but almost no decrease in xylose and arabinose could be detected. Considering an envisioned high-efficiency bioprocess, this is an issue that needs to be addressed in terms of complete consumption of sugars and carbon efficiency as well as potentially inhibitory effects of accumulation sugars.

Reduction of added sugars by a decrease in applied growth rate of exponential feeding did not lead to a significant consumption of xylose and arabinose confirming the presence of a diauxic-like growth behavior. As a potential strategy to overcome this issue, a stepwise fed-batch process was investigated. It was shown that not only glucose but also xylose as a carbon source was consumed by the microorganisms. Another possibility to overcome this issue could be to employ a bacterial consortium with glucose-negative strains for metabolizing HC sugars or engineered strains for simultaneous consumption of sugars (Dvořák & de Lorenzo, 2018). Le Meur, Zinn, Egli, Thöny-Meyer, & Ren (2012) transformed *P. putida* KT2440 with *xylAB* genes and then carried out bioreactor cultivation with xylose and octanoic acid. However, only limited biomass concentrations of 2.7 g/L were reported while the production of medium-chain length polyhydroxyalkonoates was the focus of this study.

For application of *P. putida* within the frame of a biobased economy, a fed-batch process for simultaneous consumption of all contained sugars is envisioned. In the future, along with engineered processes or strains, a model could be developed to evaluate the process economically and ecologically. Altogether, this study represents a first step and proof-of-concept toward establishing *P. putida* KT2440 as a platform for bioconversion of lignocellulose hydrolyzates.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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4. Conclusion and Outlook

In this study, to achieve the microbial conversion of lignocellulosic materials to bioproducts, we firstly constructed engineered *E.coli* strain producing potential food additive protein α_{s1}-caseins. Due to the successful casein production based on wheat straw hydrolysate, a first step and proof-of-concept toward DNA recombinant biotechnology as a tool for bioconversion from lignocellulosic "waste" to high-value bioproducts are established. In the future research, more biological or pharmaceutical valuable proteins could be designed and produced through this way.

Moreover, taking into consideration of the inhibitors exiting in lignocellulosic hydrolysates, more tolerant strain *Pseudomonas putida* was selected for the utilization of hydrolysates. Thus, two recombinant strains *P. putida* pBBR1MCS2_*xylAB* and *P. putida* pBBR1MCS2_*araBAD* were successfully engineered. Both strains are capable of metabolising its own aiming pentose sugar D-xylose or L-arabinose with a high efficiency. More interestingly, a "cross-reaction" between xylose and arabinose pathways is hypothesised, which means the two engineered strains can utilize both xylose and arabinose as carbon source. In the future study, the exact metabolic route for utilization of D-xylose and L-arabinose by *P. putida* KT2440 needs to be elucidated to explain this "cross-reaction"

It should be noted that the additional plasmid and the expression of additional genes represent a metabolic burden for the bacteria. This may not have a serious effect under moderate cultivations but may lead to reduced growth in the presence of another stress factor. As a result, to integrate the operons *xylAB* and *araBAD* into the genome of *P. putida* KT2440

instead of using heterogonous plasmids might be a better solution, which is taken into consideration for next step in our research. In addition, except pentose phosphate pathway (PPP) there are other metabolism pathways for D-xylose, which are called Weimberg and Dahms pathway. It is worth to introduce this alternative xylose metabolism pathway also into *P. putida* KT2440, it might result in a more efficient xylose utilization strain.

Furthermore, several hydrolysates from different resources and hydrolysed by various methods have been applied to our engineered strain *P. putida* KT2440 pBBR1MCS2_*xylAB*, which showed clearly the preferred hydrolysates of *P. putida* KT2440. This result gives us a clear concept in applying our recombinant *P. putida* KT2440 as a microbial catalyst for lignocellulose metabolism. To further investigate the tolerance or resistance of this strain, the most common exiting inhibitors formic acid, acetic acid, furfural, and hydroxymethylfurfural (HMF) were chosen to test on *P. putida* KT2440. Results showed furfural and HMF content in hydrolysates is a key criterion for applying lignocellulosic hydrolysates as a carbon source for *P. putida* KT2440. There are several possibilities to solve this problem. Firstly, a process strategy could be considered to separate furfural and HMF from hydrolysates to obtain them as chemical products, and at the same time hydrolysates are detoxified to be used as carbon source for microorganisms. Secondly, furfural and HMF consist also of carbon to a considerable content, which can be used as carbon source. So engineering method such as genomic integration of the *hmf* gene cluster into *P. putida* KT2440 can be studied in the future.

The growth profiles of *P. putida* KT2440 showed a diauxic like growth behaviour with a non-simultaneous consumption of different sugars. We have already applied fed-batch process to overcome this problem. Another possibility to overcome this issue in the future

study could be creating glucose-negative *P. putida* KT2440 strain. This strain will have the ability to utilise hemicellulose hydrolysates more efficiently.

To accomplish the aim of bioconversion, a final product should be designed and produced suitable for *P. putida* KT2440. For example, the genes of caseins could be introduced into other *P. putida* suitable vectors. It has been reported that valuable chemicals such as mcl-PHA, terpenoids, coronatines and specially rhamnolipids have been successfully produced in *P. putida* strains, so in the future the energy source for *P. putida* to produce these chemicals can be based on lignocellulosic hydrolysates. Moreover, once the advanced microbial catalyst for value-added products is established, more resources of lignocellulosic materials could be applied according to the availability of the raw materials, such as grain straws, grasses, sugarcane bagasse, miscanthus and so on.

In the future, along with metabolism engineering biotechnology and genetic manipulation technology, a model could be developed to evaluate the process economically and ecologically. In addition, large-scale processes will be developed to produce valuable products based on lignocellulosic biomass, thereby moving from research level to commercial applications. Eventually, the bio-based economy will be accomplished.

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List of Publications

1. **Yan Wang, Dennis Kubiczek, Felix Horlamus, Heinz Fabian Raber, Till Hennecke, Daniel Einfalt, Marius Henkel, Rudolf Hausmann, Andreas Wittgens, Frank Rosenau**. Bioconversion of lignocellulosic 'waste' to high value food proteins: recombinant production of bovine and human α_{S1}-casein based on wheat straw lignocellulose. *GCB Bioenergy.*2021; 13:640-655. doi: 10.1111/gcbb.12791

2. **Felix Hoalamus, Yan Wang, David Steinbach, Maliheh Vahidinasab, Andreas Wittgens, Frank Rosenau, Marius Henkel, Rudolf Hausmann**. Potential of biotechnological conversion of lignocellulose hydrolyzates by *Pseudomonas putida* KT2440 as a model organism for a bio-based economy. *GCB Bioenergy.*2019; 00:1-14. doi: 10.1111/gcbb.12647

3. **Yan Wang, Felix Horlamus, Marius Henkel, Filip Kovacic, Sandra Schläfle, Rudolf Hausmann, Andreas Wittgens, Frank Rosenau**. Growth of engineered *Pseudomonas putida* KT2440 on glucose, xylose and arabinose: Hemicellulose hydrolysates and their major sugars as sustainable carbon sources. *GCB Bioenergy*.2019; 11: 249-259. doi: 10.1111/gcbb.12590

4. **LIU Rui, WANG Yan, MA Yudan, WU Yi, GUO Yi, XU Li**. Effects of the Molecular Weight of PLGA on Degradation and Drug Release In vitro from an mPEG-PLGA Nanocarrier. *Chem. Res. Chin. Univ*., 2016, 32(5), 848-853. doi: 10.1007/s40242-016-6155-x.

5. **Guo-Bin Ding, Yan Wang, Yi Guo, Li Xu**. Integrin αvβ3-Targeted Magnetic Nanohybrids with Enhanced Antitumor Efficacy, Cell Cycle Arrest Ability, and Encouraging Anti-Cell-Migration Activity. *ASC Appl. Mater. Interfaces* 2014, 6, 16643-16652. dx. doi. org/10.1021/am503359.

6. **WANG Yan, WANG Xiao-Ying, LÜ Yan-Yun, XU Wen-Bin, GUO Yi, XU Li** Preparation of Magnetic Drug Carriers with Evodiamine and Fe_3O_4 Nanoparticles Co-encapsulated. *Chemical Journal of Chinese Universities*. 2013, 34(12), 2866-2870. doi: 10.7503/cjcu20130807.

7. **DING Guo-bin, LIU Hui-ying, WANG Yan, LÜ Yan-yun, WU Yi, GUO Yi, XU Li**. Fabrication of a Magnetite Nanoparticle-loaded Polymeric Nanoplatform for Magnetically Guided Drug Delivery. *Chem. Res. Chin. Univ*. 2013, 29(1), 103-109. doi: 10.1007/s40242-013-2134-7.

Scientific Conference Participation

1. Participated in the 2nd International Bioeconomy Congress | Stuttgart-Hohenheim (Germany) September 2017

2. Participated in the 5th Joint Conference of the DGHM & VAAM (VAAM Annual Meeting 2017/ 69th Annual Meeting of the DGHM) | Würzburg (Germany) March 2017

3. Participated in the 3nd Symposium on Innovative Polymers for Controlled Delivery (SIPCD) | Suzhou (China) September 2014

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Statement

I declare that the present thesis is the result of my own work and that all sources of information and support used for its achievement have been mentioned in the text and in the references. All citations are explicitly identified, all figures contain only the original data and no figure was edited in a way that substantially modified its content. All exiting copies of the present thesis are identical regarding their form and contents.

Ulm, Dec 2020

Yan Wang