Appl Microbiol Biotechnol (2011) 92:985–996
DOI 10.1007/s00253-011-3478-x

Amino acid production from rice straw and wheat bran hydrolysates by recombinant pentose-utilizing *Corynebacterium glutamicum*

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Received: 9 June 2011 / Revised: 29 June 2011 / Accepted: 13 July 2011 / Published online: 28 July 2011

Abstract *Corynebacterium glutamicum* wild type lacks the ability to utilize the pentose fractions of lignocellulosic hydrolysates, but it is known that recombinants expressing the *araBAD* operon and/or the *xylA* gene from *Escherichia coli* are able to grow with the pentoses xylose and arabinose as sole carbon sources. Recombinant pentose-utilizing strains derived from *C. glutamicum* wild type or from the L-lysine-producing *C. glutamicum* strain DM1729 utilized arabinose and/or xylose when these were added as pure chemicals to glucose-based minimal medium or when they were present in acid hydrolysates of rice straw or wheat bran. The recombinants grew to higher biomass concentrations and produced more L-glutamate and L-lysine, respectively, than the empty vector control strains, which utilized the glucose fraction. Typically, arabinose and xylose were co-utilized by the recombinant strains along with glucose either when acid rice straw and wheat bran hydrolysates were used or when blends of pure arabinose, xylose, and glucose were used. With acid hydrolysates growth, amino acid production and sugar consumption were delayed and slower as compared to media with blends of pure arabinose, xylose, and glucose. The ethambutol-triggered production of up to 93±4 mM L-glutamate by the wild type-derived pentose-utilizing recombinant and the production of up to 42±2 mM L-lysine by the recombinant pentose-utilizing lysine producer on media containing acid rice straw or wheat bran hydrolysate as carbon and energy source revealed that acid hydrolysates of agricultural waste materials may provide an alternative feedstock for large-scale amino acid production.

Keywords *Corynebacterium glutamicum* · Amino acid production · Lignocellulosic hydrolysates · Pentose utilization · Renewables · Metabolic engineering

Introduction

Lignocellulosic biomass is a cheap carbon source available from agricultural wastes. Currently, it is underutilized, and its proper recycling may even be an ecological problem. Lignocellulosic biomass and agricultural wastes thus represent viable candidates as alternate carbon sources for the large-scale biotechnological production of commodity chemicals. Lignocellulose is composed mainly of cellulose (40–50%), hemicellulose (25–30%), and lignin (10–20%) (Wyman 1999). Among them, hemicellulose is a heteropolymer of various hexose (glucose, galactose, and mannose) and pentose (xylose and arabinose) sugars. Lignocellulosic hydrolysates contain not only glucose as major component, but also a significant fraction of xylose (5–20%) and arabinose (1–5%) (Aristidou and Penttila 2000). Unfortunately, relatively few native strains of industrial microorganisms can utilize pentose sugars as

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Electronic supplementary material The online version of this article (doi:10.1007/s00253-011-3478-x) contains supplementary material, which is available to authorized users.

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fermentable substrates (Jeffries and Jin 2000). The lack of pentose-utilizing industrially relevant microorganisms often is a major bottleneck to the implementation of successful industrial processes based on lignocellulosic biomass, although successful examples of metabolic engineering, e.g., of *Saccharomyces cerevisiae* for ethanol production from pentoses, have been described (Becker and Boles 2003; Aristidou and Penttila 2000; Hahn-Hagerdal et al. 2007a; Hahn-Hagerdal et al. 2007b; Karhumaa et al. 2006).

Whereas considerable efforts have been made to utilize hemicellulose for the production of organic acids like lactic acid or succinic acid and of alcohols like ethanol (Hahn-Hagerdal et al. 2007a; Kawaguchi et al. 2006), amino acid fermentation using hemicellulosic biomasses has received much less attention. *Corynebacterium glutamicum* is widely used for the fermentative production of amino acids ever since its discovery five decades ago. *C. glutamicum* has also been engineered for production of compounds derived from amino acids and their precursors such as 1, 4-diaminobutane (Schneider and Wendisch 2010) or 1, 5-diaminopentane (Mimitsuka et al. 2007), 2-ketoisovalerate (Krause et al. 2010), and isobutanol (Blombach et al. 2011). Co-utilization of carbon sources is a typical characteristic of *C. glutamicum* (Eggeling and Bott 2005; Wendisch 2006). Technical substrates such as starch hydrolysates and molasses contain glucose, fructose, and sucrose, which are imported and phosphorylated by the phosphoenolpyruvate-dependent phosphotransferase (PTS) system or via the inositol permeases (Lindner et al. 2011) with subsequent phosphorylation by ATP- and/or polyphosphate-dependent glucokinases (Lindner et al. 2010). This amino acid producing bacterium can grow aerobically on a variety of sugars (e.g., glucose, fructose, sucrose, ribose, or maltose), alcohols (myo-inositol and ethanol) or organic acids (acetate, propionate, pyruvate, L-lactate, citrate, and L-glutamate) as sole or combined carbon and energy sources (Dominguez et al. 1998; Eikmanns 2005; Gerstmeir et al. 2003; Kiefer et al. 2002; Krämer et al. 1990; Moon et al. 2005; Polen et al. 2005; Frunzke et al. 2008; Stansen et al. 2005; Kato et al. 2010; Krings et al. 2006). By metabolic engineering, the carbon substrate spectrum of *C. glutamicum* could be broadened to enable growth on starch (Cadenas et al. 1992; Seibold et al. 2006; Tateno et al. 2007), on the whey sugars lactose and galactose (Brabetz et al. 1991; Barrett et al. 2004), on glycerol (Rittmann et al. 2008), the dicarboxylates succinate, fumarate or malate (Youn et al. 2008, 2009), and on cellobiose (Kotrba et al. 2003).

C. glutamicum wild type (WT), which is unable to utilize the pentose sugars xylose and arabinose, has also been engineered for growth on the pentoses xylose and arabinose (Kawaguchi et al. 2006, 2008; Schneider et al. 2011). Arabinose utilization by bacteria involves arabinose isomerase (AraA), which converts arabinose to ribulose, which is phosphorylated by ribulokinase (AraB), and ribulose-5-phosphate-4-epimerase (AraD) converts ribulose-5-phosphate to xylulose-5-phosphate, an intermediate of the pentose phosphate pathway (Hahn-Hagerdal et al. 2007a,b). Unlike *Escherichia coli*, which is capable of growth on L-arabinose as the sole carbon source (Lin 1996), none of the genes of the arabinose metabolic pathway mentioned above have been identified in any of the corynebacterial genomes sequenced so far (Cerdeno-Tarraga et al. 2003; Kalinowski et al. 2003; Nishio et al. 2003; Tauch et al. 2005). A typical bacterial xylose utilization pathway includes only two steps: first, xylose is converted to xylulose by xylose isomerase (XylA), before xylulokinase (XylB) phosphorylates xylulose to xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. Most corynebacteria are unable to utilize xylose as a carbon source because no xylose isomerase encoding gene is present in any of the corynebacteria sequenced to date. The *C. glutamicum* R strain contains a functional xylulokinase (Kawaguchi et al. 2006), and heterologous expression of a xylose isomerase gene is sufficient to enable growth with xylose. *C. glutamicum* R strain has been shown to withstand pretreatment-derived inhibitors like furfural, hydroxymethyl furfural, and 4-hydroxybenzaldehyde under growth-arrested conditions (Sakai et al. 2007).

These characteristics and previous work demonstrating that genetically engineered *C. glutamicum* strains utilize arabinose and xylose when added as pure chemicals (Kawaguchi et al. 2006, 2008, 2009; Schneider et al. 2011) prompted us to analyze whether recombinant *C. glutamicum* strains may be used for amino acid production based on hemicellulosic hydrolysates. It is desirable that large-scale processes such as amino acid production make use of non-food carbon sources in order to avoid competition with the use of carbon sources such as starch and sugar in nutrition.

Materials and methods

Bacterial strains, plasmids, and culture inoculums

Bacterial strains and plasmids used in this study have been listed in Table 1. For growth experiments and L-glutamate production, *C. glutamicum* WT and derived strains were used, while *C. glutamicum* DM1729 and derived strains were used for L-lysine production (Georgi et al. 2005). A loopful of respective cells from fresh Luria–Bertani (LB) plates was inoculated into 50 ml brain heart infusion medium (Difco), and the culture was incubated at 30°C for 18 h, and the cells were removed from this preculture by centrifugation at 4°C and washed in CgXII medium (Eggeling and Reyes 2005) without carbon source, and

Table 1 Bacterial strains and plasmids used in this study

Strain/ Plasmid	Relevant characteristics	References
E. coli		
DH5α	F*thi*-1 *endA*1 *hsdr*17(r-, m-) *supE*44 _*lacU*169 (ϕ80*lacZ*_M15) *recA*1 *gyrA*96 *relA*1	Hanahan (1983)
MG1655	K-12, wild type, CGSC6300	CGSC
JW3537-1	*xylA* deletion mutant, KEIO collection	CGSC
C. glutamicum		
ATCC13032	Wild type (WT)	Kinoshita et al. (1957)
DM1729	*lysC* ^{P458S}, *hom*^{V59A}, *pyc* ^{T311I}	Georgi et al. (2005)
Plasmids		
pVWEx1	Kan^{R}, P_{tac}, *lacI*^{q}	Peters-Wendisch et al. (2001)
pEKEx3	Spec^{R}, P_{tac}, *lacI*^{q}	Stansen et al. (2005)
pVWEx1-*araBAD*	Kan^{R}, pVWEx1 carrying *araBAD* from *E. coli* MG1655	Schneider et al. (2011)
pEKEx3-*xylA*	Spec^{R}, pEKEx3 carrying *xylA* from *E. coli* MG1655	This study

was used to inoculate the CgXII production medium to an optical density at 600 nm (OD_{600}) of 1, with either (blends of) glucose, arabinose, or xylose, or acid rice straw or wheat bran hydrolysates as carbon source(s). *E. coli* MG1655 and DH5α were cultivated in LB medium or on LB agar plates at 37°C. *E. coli* strain DH5α was used as host for cloning. When appropriate, kanamycin was used at a concentration of 25–50 μg/ml, spectinomycin at a concentration of 100 μg/ml, and isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations up to 1 mM.

Chromosomal DNA from *E. coli* MG1655 was isolated as described (Eikmanns et al. 1994). Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Transformation of *E. coli* was performed using the rubidium chloride method (Hanahan 1983), while *C. glutamicum* was transformed by electroporation as described (Eggeling and Reyes 2005). The *xylA* gene used in this study was amplified from genomic DNA from *E. coli* K12 MG1655 using xylA_fw_SmaI_RBS_E.coli 5′-ACGC**CCCGGG***AAAGAGATATAGAT*ATGCAAGCCTATTTTGAC-3′ (the *Sma*I restriction site is given in bold, the region containing the ribosomal binding site in italics) and xylA_rev_SmaI_E.coli 5′-CTGTTCGACAAATAA**CCCGGG**ACG-3′ (the *Sma*I restriction site is given in bold) primers. The overexpression vector pEKEx3-*xylA* was obtained by cloning of the resulting PCR product into the vector pEKEx3 (Stansen et al. 2005) using the *Sma*I restriction site within the multiple cloning site of the vector. Pentose-utilizing *C. glutamicum* strains were constructed by transforming *C. glutamicum* WT and the L-lysine producing strain DM1729 with plasmid pVWEx1-*araBAD* (Kawaguchi et al. 2006, 2008, 2009; Schneider et al. 2011) for heterologous expression of *araBAD* from *E. coli* and/or with plasmid pEKEx3-*xylA*. Plasmid pVWEx1-*araBAD* has previously been shown to be functional (Kawaguchi et al. 2006, 2008, 2009; Schneider et al. 2011) and complementation of *E. coli* JW3537-1, the *xylA* deletion mutant in the KEIO collection, revealed that plasmid pEKEx3-*xylA* is functional (data not shown). The respective empty vector control strains were constructed for comparison.

Screening of agricultural residues

In Kerala, India, locally available agricultural residual substrates, such as rice straw, rice husk, wheat bran, sugarcane bagasse, wood powder (*Atrocarpus myrestus*), reed, bamboo, and cotton stalk, were screened to find out the pentose sugar percentage in each of them and to use the best hemicellulolytic substrate for the hydrolysis to generate maximum pentose sugars and to use it for amino acid fermentation. Laboratory Analytical Protocol by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2008) was used for the composition analysis of the above samples. In brief, 0.3 g of biomass was mixed with 72% H_2SO_4 and kept in shaking water bath at 30°C for 3 h. After incubation in the water bath, the samples were diluted to 4% H_2SO_4 with distilled water and autoclaved at 121°C for 1 h. The acid soluble portion was assayed for carbohydrate composition (glucose, xylose, and arabinose) by high-performance liquid chromatography (HPLC) (see below).

Acid hydrolysis of agricultural residues

Hydrolysis has been carried out with different pretreatment agents such as H_2SO_4, HCl, and NaOH. Out of this, H_2SO_4 was the most efficient pretreatment agent at 121°C. Other pretreatment parameters, like substrate loading percentage, temperature, retention time, and reagent concentration, were optimized with H_2SO_4 (data not shown). Optimized pretreatment conditions for rice straw and wheat bran hydrolysis were found to be with 4% (*w*/*v*) H_2SO_4 at 134°C for 35 min with 37.5% substrate loading. Under these conditions, 1.9 g solids remained when 7.5 g of wheat bran were hydrolysed and 4.7 g solids remained from 7.5 g rice straw. Under the chosen conditions, hemicellulose was readily hydrolysed, while sulfonation of cellulose precluded its efficient hydrolysis.

Fermentation

C. glutamicum cultivations for amino acid production were carried out in 500-ml Erlenmeyer flasks with 50 ml medium

at 30°C and 120 rpm. Selective antibiotics were added to the production medium in case of plasmid harboring cultures. To trigger L-glutamate production in all strains, ethambutol (500 μg/ml) was added to the production medium (Radmacher et al. 2005). The genetically defined L-lysine-producing strain DM1729, which differs from *C. glutamicum* WT by point mutations in the aspartokinase gene *lysC*, the homoserine dehydrogenase gene *hom*, and the pyruvate carboxylase gene *pyc* (Georgi et al. 2005), was transformed with the following plasmid combinations pVWEx1 and pEKEx3, pVWEx1-*araBAD* and pEKEx3, pVWEx1 and pEKEx3-*xylA*, as well as pVWEx1-*araBAD* and pEKEx3-*xylA*. The resulting strains were cultured in CgXII minimal medium supplemented with the hydrolysates as carbon source for L-lysine production. Plasmids were induced with 1 mM IPTG, and the samples were removed at different intervals for the determination of biomass, amino acid production, and sugar utilization.

Analytical methods

Growth was followed by measuring the OD_{600} with UV 160A spectrophotometer (Shimadzu). The biomass concentration was calculated from OD_{600} values using an experimentally determined correlation factor of 0.25 g cell dry weight (DW) $l^{-1}$for OD_{600}=1 (Wendisch et al. 2000). The culture samples were centrifuged (10,000×*g*, 4°C; 10 min), and the resulting clear supernatant was used for various analysis. Quantitative determination of L-lysine and L-glutamate in the supernatants was carried out by reversed phase HPLC as described (Georgi et al. 2005). Various sugar concentrations were determined by high-performance liquid chromatography using Schimadzu HPLC system equipped with a refractive index detector and an HPX-87P column (Bio-Rad, USA) operating at 85°C with water as mobile phase at a flow rate of 0.6 ml/min.

Results

Screening and hydrolysis of agricultural residues

The composition analysis of several agricultural residues showed the highest xylose concentration in rice straw (37%) and the highest arabinose concentration in wheat bran (21%) (Table 2). Hemicellulosic hydrolysates were prepared by acid treatment of various agricultural residues (see "Materials and methods"). CgXII minimal medium with acid rice straw hydrolysate was prepared to contain 42 g/l of carbohydrate (40 mM glucose, 166 mM xylose, and 66 mM arabinose) as carbon source. Similarly, CgXII minimal medium with acid wheat bran hydrolysate was prepared to contain 41 g/l of carbohydrate (125 mM glucose, 62 mM xylose, and 64 mM arabinose) as carbon source. For comparison, a xylose/arabinose/glucose minimal medium was prepared by adding glucose, arabinose, and xylose as pure chemicals to CgXII medium (60 mM glucose, 200 mM xylose, and 120 mM arabinose). The xylose/arabinose/glucose minimal medium contained a higher total carbohydrate concentration (60 g/l) to compensate for osmotic effects due to the non-carbohydrate fractions of the acid rice straw and wheat bran hydrolysates. These media were used for growth and amino acid production experiments.

Table 2 Sugar composition of different agricultural residues

Agricultural residues	Xylose (%)[a]	Arabinose (%)[a]	Glucose (%)[a]
Rice straw[b]	37±0.8	11±1	16±1.3
Cotton	36±1.5	12±0.9	10±1.5
Rice husk	31±2	ND	26±0.6
Reed	30±2	9±1	9±0.7
Wheat bran[b]	30±1.9	21±2	24±0.5
Sugarcane bagasse	28±2.4	7±0.3	10±2
Bamboo	23±0.8	8±1	6±0.2
Saw dust	15±2	5±2.1	3±1.3

[a] The monomeric sugar content is given as weight percent of the acid-soluble fraction of the indicated agricultural residues according to the LAP002 protocol (Sluiter et al. 2008)

[b] Rice straw containing the highest xylose fraction among the tested agricultural wastes is highlighted as well as wheat bran containing the highest arabinose fraction

L-Glutamate production using acid rice straw and wheat bran hydrolysates

The empty vector control strain *C. glutamicum* WT (pEKEx3)(pVWEx1) and the pentose-utilizing recombinants WT(pEKEx3)(pVWEx1-*araBAD*), WT(pEKEx3-*xylA*)(pVWEx1), and WT(pEKEx3-*xylA*)(pVWEx1-*araBAD*) were grown in CgXII medium with either acid hydrolysates of rice straw or wheat bran or with the xylose/arabinose/glucose minimal medium (Table 3), and L-glutamate production was triggered by ethambutol addition as described previously (Radmacher et al. 2005; Stansen et al. 2005). All strains could grow with acid rice straw and wheat bran hydrolysates (Table 3). In all media tested, the empty vector control utilized glucose for biomass formation and L-glutamate production, while the pentose-utilizing recombinants grew to higher biomass concentrations and produced more L-glutamate as they utilized xylose and/or arabinose in addition to glucose (Table 3). In medium with acid rice straw hydrolysate, for example, the empty vector control strain grew to an OD of 12±2 and produced 16±3 mM L-glutamate, while the

Table 3 Fermentation data of cultivations on the different media

Strain	Biomass formation (OD_{600})	Glucose consumption (mM)	Xylose consumption (mM)	Arabinose consumption (mM)	Lysine production (mM)	Glutamate production (mM)
Rice straw hydrolysate (40 mM glucose, 166 mM xylose, 66 mM arabinose)						
WT (pEKEx3)(pVWEx1)	12±2	40	0	0	0	16±3
WT (pEKEx3)(pVWEx1-*araBAD*)	20±3	40	0	61	0	55±4
WT (pEKEx3-*xylA*)(pVWEx1)	33±2	40	162	0	0	77±5
WT (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	42±2	40	162	62	0	93±4
DM1729 (pEKEx3)(pVWEx1)	15±2	40	0	0	11±2	0
DM1729 (pEKEx3)(pVWEx1-*araBAD*)	23±2	40	0	61	23±2	0
DM1729 (pEKEx3-*xylA*)(pVWEx1)	33±2	40	162	0	32±2	0
DM1729 (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	42±2	40	162	62	42±2	0
Wheat bran hydrolysate (125 mM glucose, 62 mM xylose, 64 mM arabinose)						
WT (pEKEx3)(pVWEx1)	20±3	125	0	0	0	48±5
WT (pEKEx3)(pVWEx1-*araBAD*)	36±2	125	0	58	0	78±4
WT (pEKEx3-*xylA*)(pVWEx1)	36±2	125	58	0	0	80±4
WT (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	46±3	125	58	59	0	96±4
DM1729 (pEKEx3)(pVWEx1)	20±2	125	0	0	22±2	0
DM1729 (pEKEx3)(pVWEx1-*araBAD*)	40±2	125	0	58	29±2	0
DM1729 (pEKEx3-*xylA*)(pVWEx1)	39±2	125	58	0	28±2	0
DM1729 (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	48±2	125	58	59	42±3	0
Xylose/arabinose/glucose minimal medium (60 mM glucose, 200 mM xylose, 120 mM arabinose)						
WT (pEKEx3)(pVWEx1)	10±1	53	20	0	0	3±1
WT (pEKEx3)(pVWEx1-*araBAD*)	17±1	56	12	116	0	10±1
WT (pEKEx3-*xylA*)(pVWEx1)	27±1	55	189	11	0	6±1
WT (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	20±1	54	97	83	0	17±1
DM1729 (pEKEx3)(pVWEx1)	12±1	50	18	0	12±1	0
DM1729 (pEKEx3)(pVWEx1-*araBAD*)	30±1	42	57	113	22±1	0
DM1729 (pEKEx3-*xylA*)(pVWEx1)	37±1	50	182	11	52±2	0
DM1729 (pEKEx3-*xylA*)(pVWEx1-*araBAD*)	76±3	50	194	113	61±1	0

arabinose-utilizing strain grew to an OD of 20±3 and produced 55±4 mM L-glutamate, the xylose-utilizing strain grew to an OD of 33±3 and produced 77±5 mM L-glutamate, and the strain utilizing both pentoses grew to an OD of 42±2 and produced 93±4 mM L-glutamate (Table 3 and Fig. 1). In the experiments with acid rice straw hydrolysate, WT(pVWEx1-*araBAD*)(pEKEx3-*xylA*) showed a carbon-normalized product yield of 0.34 mol carbon in L-glutamate per mole of carbon in the sugars and a specific growth rate of 0.10 h^{-1} (compare to Fig. 1). A similar pattern was observed with respect to yields and growth rates using acid wheat bran hydrolysate (compare to Fig. S1).

L-Lysine production using acid rice straw and wheat bran hydrolysates

To characterize L-lysine production from hemicellulosic hydrolysates by derivatives of the L-lysine model producer strain DM1729, the empty vector control strain *C. glutamicum* DM1729(pEKEx3)(pVWEx1) and the pentose-utilizing recombinant strains DM1729(pEKEx3)(pVWEx1-*araBAD*), DM1729(pEKEx3-*xylA*)(pVWEx1), and DM1729(pEKEx3-*xylA*)(pVWEx1-*araBAD*) were grown in CgXII medium with either acid hydrolysates of rice straw or wheat bran or with the xylose/arabinose/glucose minimal medium (Table 3). As observed in the L-

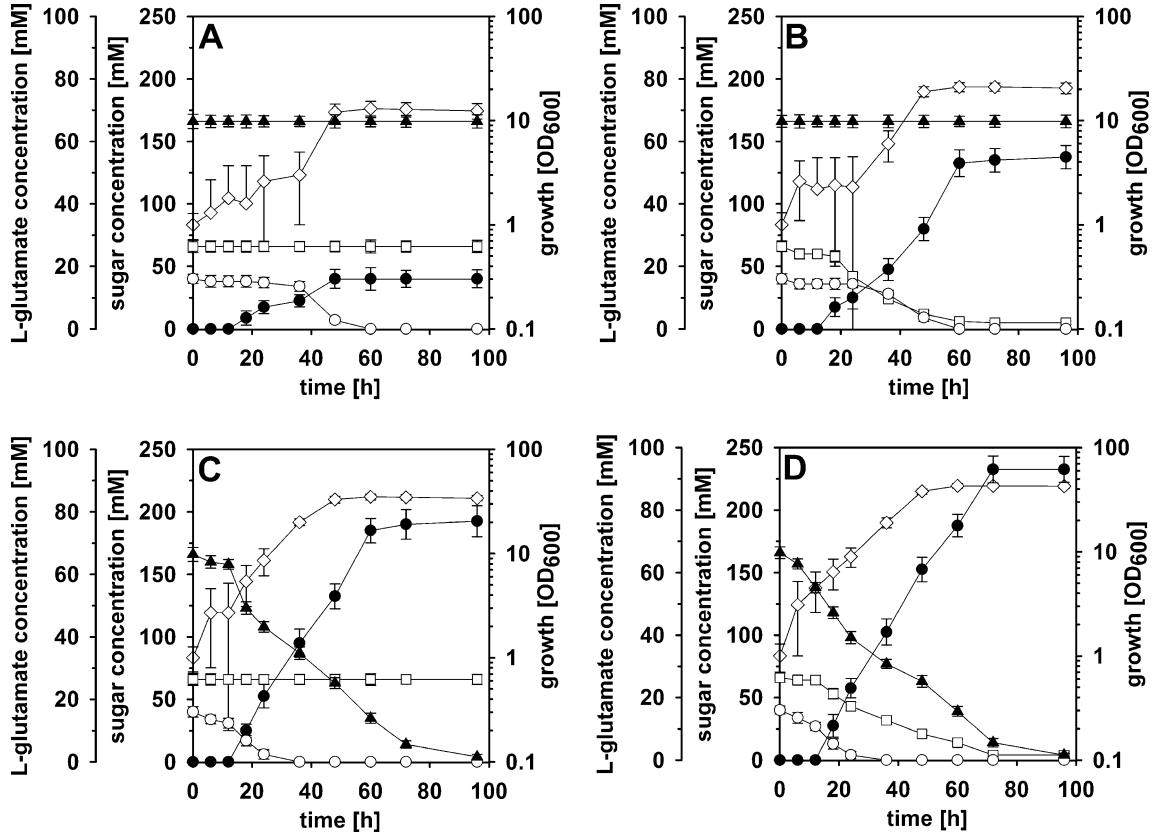

Fig. 1 L-Glutamate production by different *C. glutamicum* strains with rice straw hydrolysate. **a** WT(pEKEx3)(pVWEx1); **b** WT (pEKEx3)(pVWEx1-*araBAD*); **c** WT(pEKEx3-*xylA*)(pVWEx1); **d** WT(pEKEx3-*xylA*)(pVWEx1-*araBAD*). Xylose concentrations in the culture supernatant are represented by *filled triangles*, arabinose concentrations by *open squares*, glucose concentrations by *open circles*, L-glutamate concentrations by *filled circles*, and OD_{600} by *open diamonds*. Ethambutol was added at the beginning of the experiment. Averages and standard deviations for three or more replicates are reported

glutamate production experiments, growth and product formation from the pentose fractions of the hydrolysates depended on the presence of heterologously expressed *araBAD* and/or *xylA* whereby all pentose-utilizing strains reached higher final biomass and L-lysine concentrations than the empty vector controls strains (Table 3). For example, in medium with acid rice straw hydrolysate, the arabinose-utilizing strain grew to a higher OD (23±2) than the empty vector control strain (OD of 15±2) and produced more L-lysine (23±2 mM as compared to 11±2). As the xylose concentration in acid rice straw hydrolysate exceeded the arabinose concentration, the xylose-utilizing strain formed more biomass (OD of 33±2) and produced more L-lysine (32±2 mM), while the highest biomass formation (OD of 42±2) and L-lysine production (42± 2 mM) was observed for the strain utilizing both xylose and arabinose (Table 3 and Fig. 2). The carbon-normalized product yields of the control and the arabinose-utilizing strains (0.27±0.15 and 0.25±0.06 mol-c L-lysine per mol-c monosaccharide) were higher than those of the strains utilizing xylose or both pentoses (0.18±0.03 and 0.19± 0.02 mol-c L-lysine per mol-c monosaccharide, Fig. 3). However, the total amount of substrate consumed differed strikingly (7.2 g l^{-1} for the control and 41 g l^{-1} for the strain utilizing both pentoses), which may affect product yields.

The maximal specific growth rates in acid rice straw hydrolysate determined in the experiments depicted in Fig. 2 were 0.11 h^{-1} for the empty vector control strain, 0.10 h^{-1} for DM1729(pVWEx1-*araBAD*), 0.09 h^{-1} for DM1729 (pEKEx3-*xylA*), and 0.07 h^{-1} for DM1729(pVWEx1-*araBAD*)(pEKEx3-*xylA*). The volumetric productivities observed with rice straw hydrolysate were about twofold higher for the strain utilizing both pentoses (0.085±0.004 g h^{-1} l^{-1}) than that of the control strain (0.043±0.004 g h^{-1} l^{-1}), while the volumetric productivities of the strains utilizing only a single pentose were intermediate (Fig. 3). Taken together, the L-lysine production experiments (Figs. 2 and S2) revealed that the recombinant pentose-utilizing strains are superior to the parent strain with respect to production of L-lysine from acid rice straw and wheat bran hydrolysates.

Compared to L-lysine production from a blend of glucose, arabinose, and xylose as pure chemicals (xylose/arabinose/glucose minimal medium; Fig. S2), hemicellulo-

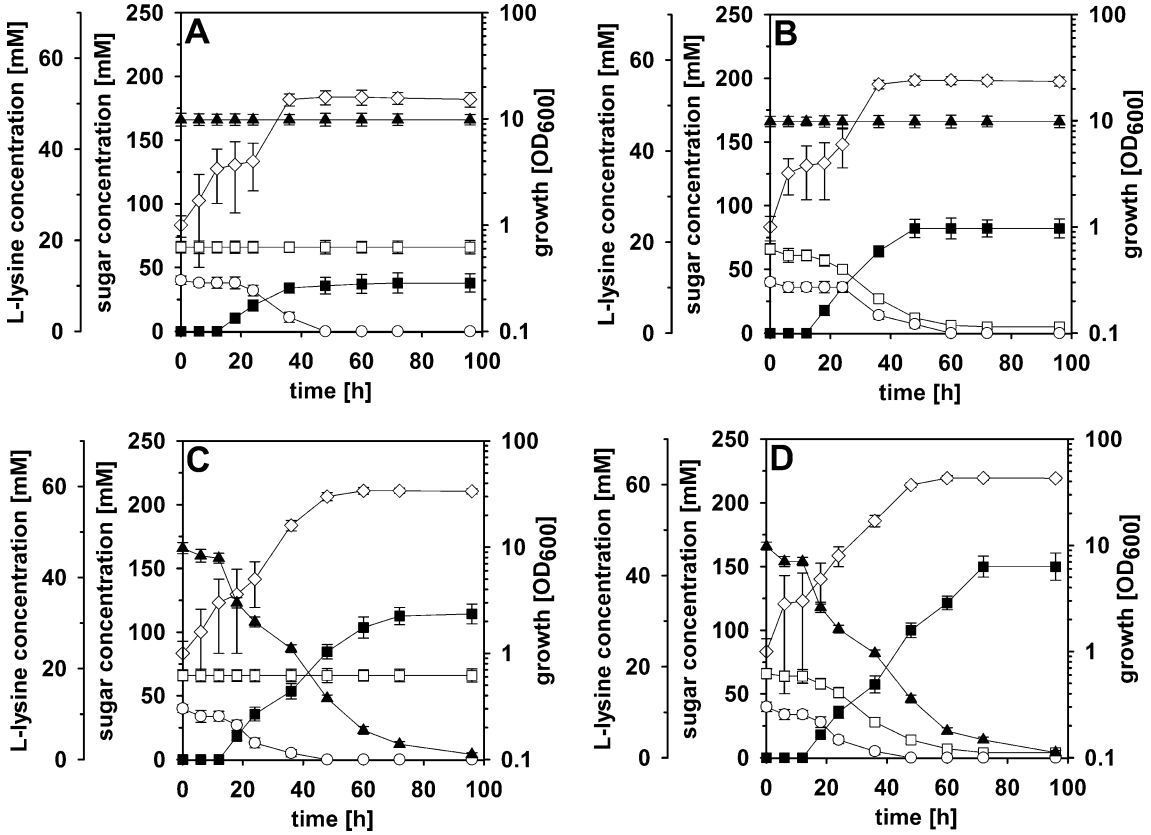

Fig. 2 L-Lysine production by different *C. glutamicum* strains with rice straw hydrolysate. **a** DM1729(pEKEx3)(pVWEx1); **b** DM1729 (pEKEx3)(pVWEx1-*araBAD*); **c** DM1729(pEKEx3-*xylA*)(pVWEx1); **d** DM1729(pEKEx3-*xylA*)(pVWEx1-*araBAD*). Xylose concentrations in the culture supernatant are represented by *filled triangles*, arabinose concentrations by *open squares*, glucose concentrations by *open circles*, L-lysine concentrations by *filled squares*, and OD_{600} by *open diamonds*. Averages and standard deviations for three or more replicates are reported

sic hydrolysate-based L-lysine production was slower. This may be exemplified by growth and L-lysine production of strain DM1729(pVWEx1-*araBAD*)(pEKEx3-*xylA*) on the synthetic medium with the blend of glucose, arabinose, and

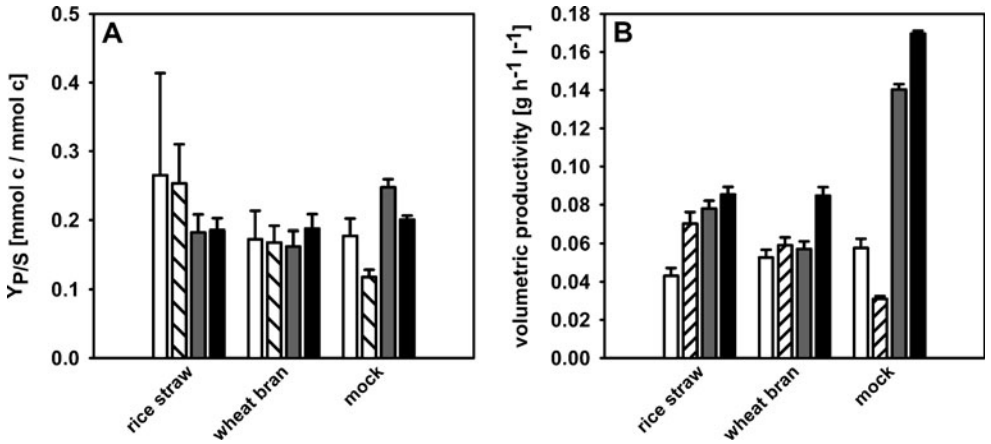

Fig. 3 Yields and volumetric productivities of L-lysine production experiments in different media and with different *C. glutamicum* strains. Minimal medium with hydrolysates of rice straw or wheat bran as well as xylose/arabinose/glucose minimal medium (mock) has been used. **a** Product yields expressed in mmol carbon (c) in L-lysine per mmol-c substrate. **b** Volumetric productivities expressed in $g\ h^{-1}\ l^{-1}$. Averages and standard deviations from triplicate experiments are given in *white columns* for DM1729(pEKEx3)(pVWEx1); in *hatched columns* for DM1729(pEKEx3)(pVWEx1-*araBAD*); in *gray columns* for DM1729(pEKEx3-*xylA*)(pVWEx1); and in *black columns* for DM1729(pEKEx3-*xylA*)(pVWEx1-*araBAD*)

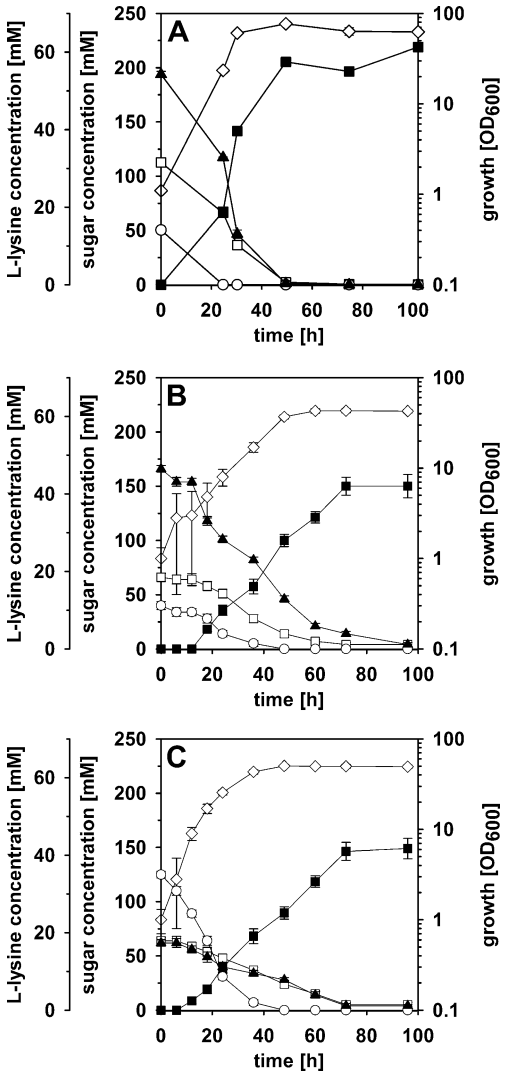

Fig. 4 L-lysine production with xylose/arabinose/glucose minimal medium (**a**), rice straw hydrolysate (**b**; data taken from Fig. 2d) and wheat bran hydrolysate (**c**) with *C. glutamicum* DM1729(pEKEx3-*xylA*)(pVWEx1-*araBAD*). Xylose concentrations in the culture supernatant are represented by *filled triangles*, arabinose concentrations by *open squares*, glucose concentrations by *open circles*, L-lysine concentrations by *filled squares*, and OD_{600} by *open diamonds*. Averages and standard deviations for three or more replicates are reported

xylose (Fig. 4a), with acid rice straw hydrolysate (Fig. 4b) and with acid wheat bran hydrolysate (Fig. 4c). The stationary growth phase was reached earlier (30 h) in the xylose/arabinose/glucose minimal medium than with the acid rice straw and wheat bran hydrolysates (48 h, Fig. 4). Similarly, the final L-lysine concentrations were reached earlier in the xylose/arabinose/glucose minimal medium than with the acid rice straw and wheat bran hydrolysates (50 h as compared to 72 h, Fig. 4). In addition, the substrates were exhausted earlier in the xylose/arabinose/glucose minimal medium than with the acid rice straw and wheat bran hydrolysates (Fig. 4). As a consequence, the volumetric L-lysine productivity for DM1729(pEKEx3-*xylA*)(pVWEx1-*araBAD*) (Fig. 3b) was higher in the xylose/arabinose/glucose minimal medium (0.170±0.001 g h^{-1} l^{-1}) than in media with acid hydrolysates of rice straw (0.085±0.004 g h^{-1} l^{-1}) and of wheat bran (0.085±0.004 g h^{-1} l^{-1}).

Discussion

Minimal media with acid hydrolysates of, e.g., rice straw or wheat bran support growth of *C. glutamicum* WT with the glucose fraction of the hydrolysates being utilized completely for biomass formation. However, the maximal specific growth rate of 0.38 h^{-1} for *C. glutamicum* WT in glucose minimal medium (Wendisch et al. 2000) is clearly higher than that of the empty vector control strains used here in hydrolysate-based media (Figs. 1, 2, S1 and S2), which were about 0.10–0.20 h^{-1}. This might be due in part to the higher osmolality of the hydrolysate-based media as compared to glucose minimal medium because the growth rate of the control strain in the xylose/arabinose/glucose minimal medium (i.e., when arabinose and xylose were present besides glucose, but could not be utilized) was also lower than in the glucose minimal medium. In addition, the presence of inhibitors in hemicellulosic hydrolysates may as well play a role leading to reduced growth and production rates in hydrolysate-based media (see below).

Glucose is co-utilized with arabinose and xylose by the recombinant *C. glutamicum* strains not only when the substrates were present as pure chemicals but also when present in acid hydrolysates from agricultural residues (Figs. 1, 2, S1 and S2). Co-utilization of pentoses and hexoses by *C. glutamicum* has been observed in this and previous studies (Kawaguchi et al. 2006, 2008; Sasaki et al. 2008; Schneider et al. 2011) for several but not all (Buschke et al. 2011) strains tested. Efforts towards engineering co-utilization of glucose, arabinose, and xylose by *S. cerevisiae* were made for a long time and have led to recent successes (Karhumaa et al. 2006; van Maris et al. 2006), but until today, alternative solutions are followed as well, e.g., sequential fermentation of rice straw hydrolysate first by *S. cerevisiae*, followed by heat inactivation, and finally by fermentation of the xylose fraction by *Pichia stipitis* (Li et al. 2011). Simultaneous utilization of various carbon sources by *C. glutamicum* (Dominguez et al. 1997; Eggeling and Bott 2005; Engels et al. 2008; Lee et al. 1998; Wendisch 2006; Wendisch et al. 2000) is a hallmark of this bacterium setting it apart from yeasts, *E. coli* and *B. subtilis*, which typically show sequential utilization of substrates present in blends and this growth pattern is often accompanied by a diauxic growth lag. Very few exceptions to substrate co-utilization have been described for *C.*

glutamicum (e.g., glucose being utilized prior to ethanol (Arndt et al. 2008; Arndt and Eikmanns 2008) or prior to glutamate (Kronemeyer et al. 1995)). Thus, *C. glutamicum* lends itself as ideal candidate for processes based on lignocellulosic or hemicellulosic hydrolysates and other second-generation substrate blends.

While co-utilization of pentoses and glucose was observed using acid hydrolysates as well as a blend of the pure chemicals, glucose, arabinose, and xylose growth and substrate utilization were slower in media based on the acid hydrolysates, which might contain growth inhibitors. It is known for *E. coli* and yeasts that compounds present in lignocellulosic hydrolysates such as acetic acid, hydroxymethylfurfural, or furfural inhibit growth (Klinke et al. 2004; Palmqvist et al. 1999; Zaldivar and Ingram 1999; Zaldivar et al. 1999, 2000; Heer and Sauer 2008). It has been described for *C. glutamicum* that a number of organic acids, furan, and phenolic inhibitors did not affect ethanol production by growth-arrested *C. glutamicum* notably, which was contributed primarily to the growth-arrested conditions (Sakai et al. 2007). While it is known that acetic acid reduces the growth rate of *C. glutamicum* to some extent when added to glucose minimal medium, *C. glutamicum* can utilize acetic acid efficiently as sole or combined source of carbon and energy (Gerstmeir et al. 2003; Wendisch et al. 2000). By contrast, the growth sensitivities of *C. glutamicum* to furfural and hydroxymethylfurfural were found to be similar to those of yeasts (e.g., *S. cerevisiae* CBS 1200, *Candida shehatae* ATCC 22984 or *P. stipitis* NRRL Y 7124) (Sakai et al. 2007). Thus, the slower growth and amino acid production observed using media based on hemicellulosic hydrolysates may at least in part be due to the presence of furfural and/or hydroxymethylfurfural. Recently, a (hydroxymethyl)furfural degrading bacterium was isolated, and the genes for enzymes of the involved pathway, which had been proposed previously based on biochemical evidence (Koenig and Andreesen 1990), have been identified (Koopman et al. 2010). The latter study opened the way to in situ detoxification of lignocellulosic hydrolysates either using sequential or co-fermentation of *Cupriavidus basilensis* with the biotechnologically relevant microorganisms (yeast, *E. coli*, and *C. glutamicum*) or by metabolic engineering of the biotechnologically relevant microorganisms using the genes for 5-(hydroxymethyl)furfural degradation from *C. basiliensis* (Koopman et al. 2010).

To increase growth and amino acid production based on hemicellulosic hydrolysates by recombinant *C. glutamicum*, the capacities to import arabinose and/or xylose and to catabolize them to intermediates of the central metabolism may be engineered. Faster pentose catabolic flux may be obtained by increasing and/or balancing heterologous gene expression levels, by making use of genes taken from pentose-utilizing bacteria other than *E. coli* or by implementing the fungal pentose utilization pathways as, e.g., present in *P. stipitis*. Increased pentose uptake rates may be achieved by transport engineering, e.g., by employing the L-arabinose transporter AraE from *C. glutamicum* strain ATCC31831 (Sasaki et al. 2009). This uptake system apparently accepts both L-arabinose and xylose as substrates (Sasaki et al. 2009). However, as AraE is not encoded in the genome of *C. glutamicum* WT, other hitherto-unknown uptake systems ensure at least a basal pentose transport capacity. Gene regulatory engineering is also conceivable to improve pentose utilization, as it was observed in this and previous studies (Kawaguchi et al. 2006, 2008; Schneider et al. 2011) that glucose slowed pentose utilization, indicating some sort of glucose repression. Presumably, this type of glucose repression affects expression of the endogenous hitherto-unknown pentose transporter gene(s) rather than plasmid-borne expression of the heterologous genes for arabinose and xylose catabolism.

Notwithstanding the potential to further improve recombinant pentose-utilizing *C. glutamicum* strains, this study has shown that *C. glutamicum*, a work-horse of biotechnology (Eggeling and Bott 2005; Wendisch et al. 2006), may be used for biotechnological processes based on agricultural residues by capitalizing on its property to simultaneously utilize several carbon substrates such as the pentose and hexose fractions of hemicellulosic hydrolysates.

Acknowledgements Work in the laboratories of the corresponding authors was supported in part by grants from the Department of Biotechnology (DBT), New Delhi, India, and from the International Bureau (IB) of the Federal Ministry of Education and Research (BMBF), Germany (IND 07/030) under the Indo-German bilateral program.

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