Accelerated pentose utilization by *Corynebacterium glutamicum* for accelerated production of lysine, glutamate, ornithine and putrescine

Tobias M. Meiswinkel,1 Vipin Gopinath,2 Steffen N. Lindner,3 K. Madhavan Nampoothiri2** and Volker F. Wendisch1**
1Chair of Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, D-33615 Bielefeld, Germany.
2Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Trivandrum-695 019, Kerala, India.

Summary
Because of their abundance in hemicellulosic wastes arabinose and xylose are an interesting source of carbon for biotechnological production processes. Previous studies have engineered several *Corynebacterium glutamicum* strains for the utilization of arabinose and xylose, however, with inefficient xylose utilization capabilities. To improve xylose utilization, different xylose isomerase genes were tested in *C. glutamicum*. The gene originating from *Xanthomonas campestris* was shown to have the highest effect, resulting in growth rates of 0.14 h\(^{-1}\), followed by genes from *Bacillus subtilis*, *Mycobacterium smegmatis* and *Escherichia coli*. To further increase xylose utilization different xylulokinase genes were expressed combined with *X. campestris* xylose isomerase gene. All combinations further increased growth rates of the recombinant strains up to 0.20 h\(^{-1}\) and moreover increased biomass yields. The gene combination of *X. campestris* xylose isomerase and *C. glutamicum* xylulokinase was the fastest growing on xylose and compared with the previously described strain solely expressing *E. coli* xylose isomerase gene delivered a doubled growth rate. Productivity of the amino acids glutamate, lysine and ornithine, as well as the diamine putrescine was increased as well as final titres except for lysine where titres remained unchanged. Also productivity in medium containing rice straw hydrolysate as carbon source was increased.

Introduction
Lignocellulosic hydrolysates contain glucose as well as significant amounts of the pentoses xylose (5–20%) and arabinose (1–5%) (Aristidou and Penttila, 2000). Lignocellulosic hydrolysates may be obtained from agricultural wastes such as rice straw and are therefore cheap carbon sources. However, lignocellulosic hydrolysates are not fully capitalized on since several industrially relevant microorganisms are not able to utilize pentose sugars as substrates (Jeffries and Jin, 2000). Metabolic engineering of pentose utilization has been successful in some cases, e.g. of *Saccharomyces cerevisiae*, while in other cases absent or inefficient pentose utilization is still a major bottleneck to be overcome for industrial processes based on lignocellulosic biomass (Aristidou and Penttila, 2000; Becker and Boles, 2003; Karhumaa et al., 2006; Hahn-Hagerdal et al., 2007a,b).

*Corynebacterium glutamicum* as a workhorse of industrial microbiology is well known for fermentative production of amino acids and has been engineered for the production of diamines like 1,4-diaminobutane (Schneider and Wendisch, 2010) and 1,5-diaminopentane (Mimitsuka et al., 2007; Kind et al., 2010; 2011), of ketoacids such as pyruvate (Wieschalka et al., 2012) and 2-ketoisovalerate (Krause et al., 2010), diacids such as succinate (Okino et al., 2008; Litsanov et al., 2012a,b,c) and the alcohols ethanol (Inui et al., 2004) and isobutanol (Blombach et al., 2011). Traditionally, technical substrates like starch hydrolysates and molasses are used in industrial processes. The respective sugars glucose (starch hydrolysate), fructose and sucrose (molasses) are taken up and are phosphorylated by the phosphoenolpyruvate-dependent carbohydrate phosphotransferase (PTS) system or, in the case of glucose, alternatively also by *myo*-inositol permeases with subsequent phosphorylation by ATP- and/or polyphosphate-dependent glucokinas (Lindner et al., 2010; 2011). The natural substrate spectrum of *C. glutamicum* further includes sugars like ribose or maltose, alcohols like ethanol or *myo*-inositol and organic acids like acetate, citrate, lactate, propionate and pyruvate and amino acids like L-glutamate (Kramer et al., 1990; Dominguez et al., 1998; Kiefer et al., 2002; Gerstmeir et al., 2003; Eikmanns, 2005; Moon et al., 2005; Polen et al., 2005; Stansen et al., 2005; Krings et al., 2006; Frunzke et al., 2008; Kato et al., 2010; Neuner and Heinzel, 2011). Within the flexible feedstock concept, the...
substrate spectrum of C. glutamicum has been extended by metabolic engineering to allow access to starch, cellobiose, lactose, galactose and glycerol as well as succinate, fumarate and malate as carbon sources (Brabetz et al., 1991; Cadenas et al., 1992; Kotrba et al., 2003; Barrett et al., 2004; Seibold et al., 2006; Tateno et al., 2007; Rittmann et al., 2008; Youn et al., 2008; 2009).

Similarly, C. glutamicum has been engineered for growth with the pentoses arabinose and xylose and for the production of ethanol, organic acids, amino acids and diamines from arabinose and/or xylose (Kawaguchi et al., 2006; 2008; Sasaki et al., 2009, 2010; Gopinath et al., 2011; Kind and Wittmann, 2011; Schneider et al., 2011). Metabolic engineering relied on bacterial pathway genes. In Escherichia coli and other bacteria able to utilize arabinose and/or xylose, arabinose is catabolized via arabinose isomerase (encoded by araA), ribulokinase (araB) and ribulose-5-phosphate-4-epimerase (araD) while xylose catabolism requires xylose isomerase (xylA) and xylulokinase (xylB) (Lin, 1996; Hahn-Hagerdal et al., 2007a,b). Heterologous expression of araA, araB and araD from E. coli resulted in C. glutamicum recombinants able to grow with arabinose as sole source of carbon (Kawaguchi et al., 2008). When the arabinose importer gene araE from C. glutamicum ATCC31831 was expressed in addition, faster growth with arabinose entailed (Sasaki et al., 2009). In the case of xylose, heterologous expression of a single E. coli gene, xylA, was sufficient to allow growth with xylose as sole carbon source (Kawaguchi et al., 2006) since the C. glutamicum genome encodes xylulokinase (Kalinowski et al., 2003). Corynebacterium glutamicum has proven a good choice for utilizing complex mixtures of carbon sources such as hemicellulosic hydrolysates because, unlike E. coli and S. cerevisiae, C. glutamicum efficiently co-utilizes different carbon sources when present in blends (Wendisch, 2006; Arndt and Eikmanns, 2008; Blombach and Seibold, 2010; Gopinath et al., 2011). Consequently, besides proof-of-concept using pure chemicals, growth and production with hemicellulosic hydrolysates obtained, e.g. from rice straw could be achieved (Gopinath et al., 2011). In the present study, we address xylose catabolism as a possible rate-limiting step of xylose-based production by C. glutamicum.

**Results**

**Comparative analysis of recombinant C. glutamicum strains with different plasmid encoded xylose isomerases**

Corynebacterium glutamicum possesses a xylulokinase and heterologous production of E. coli xylose isomerase allowed C. glutamicum to grow with xylose, however, the observed growth rates were low (0.09 h⁻¹) as compared to growth rates, e.g. with glucose (0.32 h⁻¹), ribose (0.23 h⁻¹) or acetate (0.28 h⁻¹) (Wendisch et al., 2000; Wendisch, 2003; Netzer et al., 2004). In order to test whether xylose isomerase activity is limiting growth with xylose of C. glutamicum recombinants expressing xylA from E. coli, several recombinants were constructed expressing xylose isomerase genes from different sources. The xylose isomerase genes of well-understood model organisms, plant pathogens and strains closely to C. glutamicum related E. coli, Bacillus subtilis, Xanthomonas campestris and Mycobacterium smegmatis, were cloned into the IPTG-inducible expression vector pEKEx3 and transformed into C. glutamicum WT (Table 1C). Xylose isomerase (XI) activity measured as described in Experimental procedures was not detectable in empty vector control strains (< 0.005 U mg⁻¹) (Table 2). High and comparable XI activities were observed in crude extracts of WT(pEKEx3-xylA_Ec) (0.095 ± 0.010 U mg⁻¹) and WT(pEKEx3-xylA_CT) (0.090 ± 0.008 U mg⁻¹), while about three times less activity was found for WT(pEKEx3-xylA_Ba) (0.023 ± 0.003 U mg⁻¹) and WT(pEKEx3-xylA_Sc) (0.033 ± 0.007 U mg⁻¹). While XI activity increase due to over-expression of xylA could be detected in each case, the values are difficult to compare as a single enzyme assay was used without optimization for the enzymes of various origin.

To check the performance of the C. glutamicum strains harbouring the different XI genes growth experiments in CgXII minimal medium with 100 mM xylose as sole carbon source were performed (Fig. 1A). All recombinant strains expressing xylose isomerase genes were able to grow with xylose as sole carbon source (Fig. 1A). Corynebacterium glutamicum WT(pEKEx3-xylA_Ec) showed the fastest growth (0.144 ± 0.001 h⁻¹) and reached the highest biomass concentration (3.37 ± 0.12 gCDW l⁻¹), followed by WT(pEKEx3-xylA_Ba) (0.118 ± 0.007 h⁻¹; 1.29 ± 0.15 gCDW l⁻¹), WT(pEKEx3-xylA_CT) (0.093 ± 0.003 h⁻¹; 1.29 ± 0.05 gCDW l⁻¹) and WT(pEKEx3-xylA_SC) (0.090 ± 0.005 h⁻¹; 2.79 ± 0.05 gCDW l⁻¹). Thus, heterologous expression of the xylose isomerase gene from X. campestris improved xylose-utilization by recombinant C. glutamicum significantly reducing generation times from 7.7 h to 4.8 h.

**Comparative analysis of recombinant C. glutamicum strains overexpressing endogenous or heterologous xylulokinase genes**

Corynebacterium glutamicum WT contains xylulokinase, however, xylulokinase activities determined as described in Experimental procedures were low in crude extracts of C. glutamicum WT, the empty vector control strain and of all recombinants expressing only a heterologous xylose
isomerase gene (between 0.013 and 0.024 U mg⁻¹) (Table 2). Ectopic expression xylA from X. campestris was combined either with overexpression of endogenous xylB or with overexpression of xyulokinase genes from E. coli or B. subtilis (Table 1C). Xyulokinase (XK) activity was not increased significantly in strain WT(pPEKEx3-xylAEC; xylBBS10) (Table 2). In contrast, ectopic expression of xylB from E. coli and overexpression of endogenous xylB increased XK activity in crude extracts about 25-fold. To test the effect of xylB overexpression in addition to xylA overexpression, growth of C. glutamicum strains overproducing the different XK’s along with XI from X. campestris in CgXII minimal medium with 100 mM xylose as sole carbon source was compared (Fig. 1B). The control strain WT(pPEKEx3-xylAEC) reached a lower biomass concentration (3.37 ± 0.12 gCDW l⁻¹) and grew with a slower growth rate (0.144 ± 0.001 h⁻¹) than the strains overproducing XK in addition. Corynebacterium glutamicum WT(pPEKEx3-xylAEC-xylBBS10) grew fastest (0.199 ± 0.009 h⁻¹) and reached the highest biomass concentration (4.87 ± 0.53 gCDW l⁻¹) followed by WT(pPEKEx3-xylAEC-xylBBS10) (0.189 ± 0.001 h⁻¹; 4.82 ± 0.33 gCDW l⁻¹) and WT(pPEKEx3-xylAEC-xylBBS10) (0.162 ± 0.001 h⁻¹; 5.30 ± 0.22 gCDW l⁻¹). Thus, heterologous expression of the endogenous xyulokinase gene from C. glutamicum in addition to the xylose isomerase gene from X. campestris further improved xylose utilization significantly reducing generation times from 4.8 h to 3.5 h.
Amino acid and diamine production from xylose by the improved strain

Previously, we showed production of amino acids like L-glutamate and L-lysine as well as the diamine putrescine from xylose minimal medium by strains harbouring the basic xylose utilization plasmid pEKEx3-xylAEc (Gopinath et al., 2011). The improved plasmid pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G} was transformed into the model lysine producer DM1729, the model ornithine producer ORN1 and the model 1,4-diaminobutane producer PUT21. L-glutamate production in CgXII minimal medium with 100 mM xylose as sole carbon source by \textit{C. glutamicum} WT(pEKEx3-xylAEc) and by WT(pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G}) was triggered by ethambutol addition and the improved strain reached higher titres (14.5 \pm 0.1 mM as compared with 0.8 \pm 0.1 mM) and exhibited an increased productivity (29.7 \pm 0.2 as compared with 1.6 \pm 0.3 mg l\(^{-1}\) h\(^{-1}\), Fig. 2). Lysine production by DM1729(pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G}) was characterized by a volumetric productivity improved from 25.5 \pm 0.8 to 35.4 \pm 1.4 mg l\(^{-1}\) h\(^{-1}\). The volumetric ornithine productivity by ORN1(pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G}) was higher than that of the control (43.2 \pm 4.3 as compared with 14.8 \pm 2.2 mg l\(^{-1}\) h\(^{-1}\)) and higher ornithine concentrations were achieved (19.6 \pm 1.9 mM as compared with 9.4 \pm 1.4 mM). Also putrescine production was faster (27.8 \pm 2.0 as compared with 15.7 \pm 1.2 mg l\(^{-1}\) h\(^{-1}\)) and titres rose from 12.9 \pm 1.0 mM to 15.1 \pm 1.1 mM. Taken together, all recombinants carrying the improved plasmid pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G} showed significantly increased volumetric productivities in medium with pure xylose.

Amino acid production on rice straw hydrolysate

To characterize L-glutamate and L-lysine production from hemicellulosic hydrolysates in particular rice straw hydrolysate (52 mM glucose, 203 mM xylose, 55 mM arabinose) derivatives of \textit{C. glutamicum} WT or L-lysine model producer DM1729 harbouring either empty vectors, pVWE\textsubscript{X1}-araBAD and pEKEx3-xylAEc or pVWE\textsubscript{X1}-araBAD and pEKEx3-xylA\textsubscript{Xc}-xylBC\textsubscript{G} were used. The empty vector control utilized glucose for biomass formation and amino acid production, while the pentose-utilizing recombinants grew to higher biomass concentrations and produced more L-glutamate and L-lysine as they utilized arabinose and xylose in addition to glucose (Fig. 3). In case of L-glutamate the empty vector control reached 16 \pm 5.4 mM and a volumetric productivity of 98.1 \pm 33.1 mg l\(^{-1}\) h\(^{-1}\) in contrast to the pentose-utilizing strain WT(pVWE\textsubscript{X1}-araBAD)(pEKEx3-xylAEc) with 39 \pm 1.9 mM L-glutamate and a productivity of 79.7 \pm 3.9 mg l\(^{-1}\) h\(^{-1}\). The strain improved for xylose utilization reached a comparable level of L-glutamate at 37 \pm 5 mM and the highest productivity at 113.4 \pm 15.3 mg l\(^{-1}\) h\(^{-1}\). As expected for growth-coupled L-glutamate production the specific
productivities were similar [around 2.6, 3.0 and 3.1 mg gCDW h\(^{-1}\) for the empty vector control, WT(pVWEx1-araBAD)(pEKEx3-xylAEc) and WT(pVWEx1-araBAD)(pEKEx3-xylAXc-xylBCg) respectively].

As observed in the L-glutamate production experiment, product formation and productivity strongly depends on the ability to utilize the pentose fraction of the rice straw hydrolysate due to the expression of araBAD and xylA and/or xylA along with xylB. Therefore empty vector control reached 8.3 ± 1.5 mM L-lysine and a volumetric productivity of 50.6 ± 1.5 mg l\(^{-1}\) h\(^{-1}\) in contrast to the pentose-utilizing strain DM1729(pVWEx1-araBAD) (pEKEx3- xylAx-xylBcg) with clearly increased 47 ± 5 mM L-lysine and a productivity of 95.4 ± 10.2 mg l\(^{-1}\) h\(^{-1}\). The strain improved for xylose utilization reached a similar level of L-lysine at 47.5 ± 2 mM together with the highest productivity at 144.7 ± 6.1 mg l\(^{-1}\) h\(^{-1}\). As L-lysine production was growth-coupled the specific productivities were similar [around 2.6, 3.0 and 3.1 mg gCDW h\(^{-1}\) for the empty vector control, DM1729 (pVWEx1-araBAD) (pEKEx3-xylAEc) and DM1729 (pVWEx1-araBAD) (pEKEx3-xylAx-xylBcg) respectively].

**Discussion**

The newly engineered strain WT(pEKEx3-xylAx-xylBcg) was shown to grow significantly faster (0.199 ± 0.009 h\(^{-1}\)) on minimal medium containing xylose as sole carbon source compared with the previously described strain WT(pEKEx3-xylAx) (0.090 ± 0.005 h\(^{-1}\)) (Gopinath et al., 2011) expressing xylA from E. coli only. A first improvement was already achieved by expressing different xylA
genes, where \( \text{xybA} \) from \( X. \text{campestris} \) performed best (0.144 ± 0.001 h\(^{-1}\)). By additional production of xylulokinase from different organisms further growth acceleration was observed with the fastest growing strain mentioned above. These findings let to the construction of production strains for lysine, glutamate, ornithine and putrescine for optimized utilization of xylose. The newly engineered xylose utilization strains showed a significantly higher volumetric productivity (L-glutamate: 29.7 ± 0.2 mg l\(^{-1}\) h\(^{-1}\); L-lysine: 35.4 ± 1.4 mg l\(^{-1}\) h\(^{-1}\); L-ornithine: 43.2 ± 4.3 mg l\(^{-1}\) h\(^{-1}\); putrescine: 27.8 ± 2.0 mg l\(^{-1}\) h\(^{-1}\)) compared with production strains using the previously reported (Gopinath et al., 2011) xylose utilization plasmid (L-glutamate: 1.6 ± 0.3 mg l\(^{-1}\) h\(^{-1}\); L-lysine: 25.5 ± 0.8 mg l\(^{-1}\) h\(^{-1}\); L-ornithine: 14.8 ± 2.2 mg l\(^{-1}\) h\(^{-1}\); putrescine: 15.7 ± 1.2 mg l\(^{-1}\) h\(^{-1}\)) compared with production strains using the previously reported (Gopinath et al., 2011) xylose utilization plasmid (L-glutamate: 1.6 ± 0.3 mg l\(^{-1}\) h\(^{-1}\); L-lysine: 25.5 ± 0.8 mg l\(^{-1}\) h\(^{-1}\); L-ornithine: 14.8 ± 2.2 mg l\(^{-1}\) h\(^{-1}\); putrescine: 15.7 ± 1.2 mg l\(^{-1}\) h\(^{-1}\)). Also during growth and production on rice straw hydrolysate a clear increase in volumetric productivity was observed for strains carrying pEKEx3-xylA\(_{xc}\)-xylB\(_{sc}\) (L-glutamate: 113.4 ± 15.3 mg l\(^{-1}\) h\(^{-1}\); L-lysine: 144.7 ± 6.1 mg l\(^{-1}\) h\(^{-1}\)) compared with strains with pEKEx3-xylA\(_{sc}\) (L-glutamate: 79.7 ± 3.9 mg l\(^{-1}\) h\(^{-1}\); L-lysine: 95.4 ± 10.2 mg l\(^{-1}\) h\(^{-1}\)) and in case of lysine production as well compared with the empty vector control strain, which is only capable of utilizing the glucose part of rice straw hydrolysate. As expected for growth-coupled amino acid production the specific productivities normalized to the biomass concentrations were similar.

Engineering for a better use of second-generation feedstock like rice straw hydrolysate, one possible bottleneck regarding catabolism of those carbon sources was successfully dealt with in this work. Further possible bottlenecks are transport of the carbon sources into the cell and the process of breaking the poly- and oligomeric sugars into their monomeric compounds to make them accessible for the producing microorganisms. Concerning the later point in this study a mild sulfuric acid treatment was used to hydrolysate the rice straw (Gopinath et al., 2011). A potential formation of typical fermentation inhibitors, e.g. 5-HMF or weak acids, may result in slower growth and lower production (Palmqvist et al., 1999; Zaldivar and Ingram, 1999; Zaldivar et al., 1999; 2000; Klinke et al., 2004; Heer and Sauer, 2008; Gopinath et al., 2011) and could be an aim to analyse in more detail in future studies for further optimization. However it was already described that in case of ethanol production by growth-arrested cells, typical inhibitors like organic

![Fig. 3. Product concentrations (A, C) and volumetric productivities (B, D) for L-glutamate (A, B) and L-lysine (C, D) production in CgXII medium containing rice straw hydrolysate. Corynebacterium glutamicum strains with empty vectors, pVWE1-araBAD and pEKE3-xylA\(_{sc}\)-xylB\(_{sc}\) or pVWE1-araBAD and pVWE3-xylA\(_{xc}\)-xylB\(_{sc}\) were analysed. L-glutamate was produced with WT (hatched bars) and L-lysine with DM1729 (open bars). Data represent mean values and experimental imprecision of two independent cultivations.](image-url)
acids, phenolic inhibitors or furans did not substantially disturb *C. glutamicum* (Sakai *et al.*, 2007). In principal overcoming inhibition can be achieved by different ways, e.g. by simple resistance to the inhibitory substances due to efflux pump or prevention of uptake, by degradation of the relevant substances (Koopman *et al.*, 2010) or by simply not creating inhibitors during processing of the substrates.

Dealing with the potential bottleneck of transport, the xylose and/or arabinose transporting system in the used *C. glutamicum* wild-type strain ATCC13032 is still unknown and therefore the heterologous expression of xylose and/or arabinose transport systems, e.g. *araE* (Sasaki *et al.*, 2009), might result in faster substrate uptake and also higher productivity. *AraE* from *C. glutamicum* ATCC31831 might be a promising target for transport optimization because this uptake system accepts both arabinose and xylose and allows growth on even very low arabinose or xylose concentrations (Sasaki *et al.*, 2009) and the donor strain is closely related to the used *C. glutamicum* ATCC13032.

With the potential for further improvements this study has clearly shown the ability of the already industrially intensively used *C. glutamicum* (Eggeling and Bott, 2005; Wendisch, 2006) to play a key role in utilization of second-generation feedstocks with respect to a wide product spectra reaching from products like amino acids to products like fine chemicals as diamines, e.g. putrescine.

**Experimental procedures**

**Microorganisms and cultivation conditions**

*E. coli* strain DH5α (Hanahan, 1985) was used for cloning and was cultivated in lysogeny broth medium (LB) (Sambrook *et al.*, 1989). *Corynebacterium glutamicum* strains used in this work are wild-type strain ATCC13032 (WT) (Abe *et al.*, 1989), L-lysine producing model strain DM1729 (Georgi *et al.*, 2010) and was cultivated in lysogeny broth medium (LB) (Sambrook *et al.*, 1989). Pre-cultures of *C. glutamicum* putrescine producing strain PUT21 (Schneider *et al.*, 2012) was harvested by centrifugation (10 min; 3220 g) and stored at 4°C until use.

**Enzyme activity measurements**

Enzyme activity measurements were analysed in crude extracts of *C. glutamicum* (Guyer *et al.*, 1981). Cells were inoculated from LB overnight cultures to an OD₆₀₀ of 0.5 in 50 ml of LB medium containing 1 mM IPTG. Cells were harvested by centrifugation at a final OD₆₀₀ of 4 and stored at −20°C until use.

Xylose isomerase and xylulokinase activity was measured by the determination of NADH using sorbitol dehydrogenase in the case of xylose isomerase and pyruvate kinase as well as lactate dehydrogenase in case of xylulokinase. Xylose isomerase assays were carried out at 30°C in a total volume of 1 ml containing 100 mM TRIS/HCl, pH 7.5, 10 mM MgCl₂, 0.23 mM NADH and sorbitol dehydrogenase (1 U) (Brat *et al.*, 2009), in the case of xylulokinase the assay contained pyruvate kinase (6.8 U), lactate dehydrogenase (9.9 U), 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1 mM ATP, 2 mM MgCl₂ and 50 mM TRIS/HCl, pH 7.5 (éliasson *et al.*, 2000). Tests were started by addition of ω-xylose (2 M) or ω-xululose (167 mM) respectively. Enzymatic activities are displayed in μmol min⁻¹ mg⁻¹, defined as one unit (U). Continuous measurements were carried out using a Shimadzu UV-1650 PC photometer (Shimadzu, Duisburg, Germany).
Protein concentrations in crude extracts were determined using Bradford reagents (Sigma, Taufkirchen, Germany) and concentrations were calculated against bovine serum albumin standards.

**Acid hydrolysis of agricultural residues**

Hydrolysis of rice straw has been carried out as described before (Gopinath et al., 2011).

**Determination of amino acid and diamine concentrations**

Amino acids L-lysine, L-glutamate and L-ornithine were quantified via HPLC as described previously (Georgi et al., 2005). Putrescine was quantified via HPLC as described before (Schneider et al., 2012).

**Conflict of interest**

None declared.

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