Bioresource Technology 213 (2016) 239–244

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Co-expression of endoglucanase and β-glucosidase in *Corynebacterium glutamicum* DM1729 towards direct lysine fermentation from cellulose

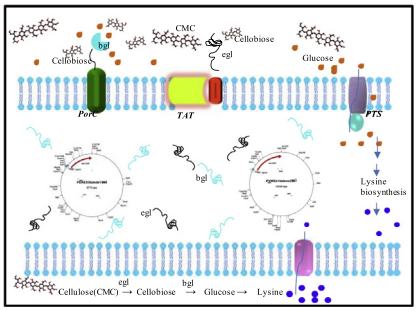
Murali Anusree [a], Volker F. Wendisch [b], K. Madhavan Nampoothiri [a,*]

[a] *Biotechnology Division, CSIR – National Institute For Interdisciplinary Science and Technology (NIIST), Trivandrum 695 019, Kerala, India*
[b] *Chair of Genetics of Prokaryotes, Bielefeld University, Universitaetsstr. 25, 33615 Bielefeld, Germany*

HIGHLIGHTS

- Heterologous expression of endoglucanase and β-glucosidase genes in *Corynebacterium glutamicum* DM1729.
- Simultaneous saccharification and fermentation of carboxymethyl cellulose and cellobiose to lysine.
- Hydrolysis of sugarcane tops and rice straw by recombinant enzymes to reducing sugars.
- Initial step toward direct conversion of lignocellulosic biomass to amino acid.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 January 2016
Received in revised form 29 February 2016
Accepted 1 March 2016
Available online 8 March 2016

Keywords:
Biorefinery
Corynebacterium glutamicum
β-Glucosidase
Endoglucanase
Lysine

ABSTRACT

The aim of the present study is the development of a consolidated bioprocess for the production of lysine with recombinant *Corynebacterium glutamicum* DM1729 strains expressing endoglucanase and β-glucosidase genes. Here, the endoglucanase genes from *Xanthomonas campestris* XCC3521 and XCC2387 and betaglucosidase gene from *Saccharophagus degradans* Sde1394 were cloned in *C. glutamicum* DM1729 and expressed either extracellularly or on cell surface. The highest β-glucosidase activity of 9 ± 0.5 U/OD_{600} of 1 and endoglucanase activity of 5.5 ± 0.8 U was obtained in *C. glutamicum* DM 1729 (pVWEx1-TAT*XCC2387*) (pEKEx3-*PorC-Sde1394*) when cellobiose (20 g/L) alone or in combination with carboxymethyl cellulose (20 g/L) was used as the carbon sources respectively. The overall efforts resulted in a lysine titre of 5.9 ± 0.5 mM. The ability of the constructs to utilize carboxymethyl cellulose and cellobiose for growth and amino acid production proves the concept of utilization of *C. glutamicum* as a biocatalyst in the lignocellulosic biorefinery.

1. Introduction

Lignocellulosic biomass is considered as the cheap, sustainable and renewable feed stock of the decade as it generally avoids competition as food and fodder (Saxena et al., 2009). Concentrated efforts are not given for lignocellulosic biomass generation as it is usually agricultural, industrial or farmyard waste or forest forage. Due to the ease of access and low cost, there was always labored efforts towards utilization of lignocellulosic biomass for production of bio based fuels and chemicals. Most of the bacterial bioproduct producers lack the inherent ability to naturally degrade the cellulose chains to assimilable sugar monomers and hence lignocellulosic biomass is underutilized in this direction. On the other hand, the feed stock has to be extensively pretreated and hydrolyzed so as to be accessible for the microbes. To fully capitalize on the available lignocellulosic feed stock for production of products of interest, the target microbes has to be engineered for

* Corresponding author. Tel.: +91 2515 366; fax: +91 2491712.
E-mail address: madhavan85@hotmail.com (K.M. Nampoothiri).

http://dx.doi.org/10.1016/j.biortech.2016.03.019
0960-8524/

heterologous expression of cellulose degrading enzymes or cellulases (Lynd et al., 2002).

Cellulose is the water insoluble plant biomaterial which is composed of repeated β-linked D-glucose units and cellulases are enzymes that degrade cellulose. Cellulases are composed of multiple enzymes acting synergistically in cellulose hydrolysis to generate monomer units. Cellulases have higher efficiencies in combined action and higher combined activity than the sum of individual activities. Endoglucanases, or Endo-1, 4-β-glucanase (EG) cleave intramolecular β-1, 4-glucosidic linkages in the amorphous regions of cellulose fiber and produces new chain ends and higher DP or lower DP cellooligosaccharides. β-Glucosidases cleave the cello oligosaccharides preferentially cellobiose and form glucose monomer units. It is the key enzyme in relieving product inhibition of endoglucanases. These enzymes form an important step for the development of a lignocellulosic biorefinery.

A lignocellulosic biorefinery is where the lignocellulosic biomass is converted to fuels and chemicals in various steps. An efficient biocatalyst capable of multiple product production will be advantageous to the biorefinery. The supreme microbe for lignocellulosics to bioproduct conversion should have high productivities, co utilization of different sugars for efficient fermentation, broad substrate utilization range, tolerance to inhibitors generated during pretreatment, minimal byproduct formation etc. (Sukumaran et al., 2010).

Corynebacterium glutamicum is a gram positive, non spore forming rod shaped bacterium isolated from soil and used as the industrial work horse of amino acid fermentation since its discovery in the 1950 s. The microbe is also versatile in the production of multitude of products including organic acids, fuels, platform chemicals and other bioproducts. *C. glutamicm* is also engineered for substrate broadening to utilize lignocelluloses derived sugars (Gopinath et al., 2012). Under oxygen deprived conditions and high cell densities, *C. glutamicum* has also shown resistance to inhibitors for alcohol fermentation (Inui et al., 2004) which will be an added advantage in a lignocellulosic biorefinery.

Here we constructed endoglucanase and β-glucosidase expressing *C. glutamicum* for the assimilation of carboxymethyl cellulose or cellobiose for lysine fermentation. Three recombinant strains were constructed with secreted endoglucanases, and either secreted or surface displayed β-glucosidase. The rate limiting enzyme in cellulose hydrolysis is β-glucosidase which holds a very important role in cellulose hydrolysis. The substrate of β-glucosidase is the major inhibitor of endoglucanase and cellobiohydrolase at concentrations of 0.3%w/v where hydrolysis efficiencies decrease up to 50% (White and Hindle, 2000). Commercially available cellulases have low levels of β-glucosidases which necessitate the addition of high enzyme loads for increased saccharification efficiency (Sukumaran et al., 2010). Cell surface display of proteins are reported (Lee et al., 2003; Tanaka et al., 2012) to enhance their activity than in secreted form. β-glucosidases were hence displayed on the cell surface in addition to the secreted form. *porC* anchor protein was used as the anchor for cell surface display and has been successfully employed earlier in *C. glutamicum*. Porins are cell wall associated proteins in *C. glutamicum* due to the presence of mycolic acids (Bayan et al., 2003). Cell surface display of proteins were employed previously in gram positive and gram negative bacteria for complex substrate utilization (Adachi et al., 2013; Narita et al., 2006; Tateno et al., 2007a; Yao et al., 2009). Endoglucanases are expressed here only in the secreted form.

2. Methods

2.1. Bacterial strains and media

Bacterial strains and plasmids used in the study with their relevant characteristics are listed in Table 1. *E. coli* DH5α (Hanahan, 1983) was used as the host for DNA manipulation. *C. glutamicum* DM1729 (Georgi et al., 2005) and derived strains were used for recombinant protein expression and lysine fermentation.

E. coli was grown in Luria–Bertani medium at 37 °C and *C. glutamicum* was grown in LB medium at 30 °C. For recombinant selection, Luria–Bertani agar medium with either kanamycin (25–50 μg/ml) or spectinomycin (100 μg/ml) or in combination was used.

For lysine fermentation studies CGXII mineral medium (Eggeling and Bott, 2005) was used with either glucose, cellobiose, carboxymethyl cellulose as carbon sources or their blends. A loopful of cells from LB agar plates was inoculated in LB media and incubated at 30 °C and 200 rpm for 18 h. Cells were separated from this pre-culture by centrifugation and used to seed an inoculum. The inoculum after 18 h of growth was used to inoculate the CGXII production medium to an initial OD_{600} of 1. When appropriate, IPTG was added in a concentration up to 1 mM.

2.2. Plasmid Construction and general recombination methods

E. coli–C. glutamicum shuttle vectors, pEKEx3 and pVWEx1 were used as the vector backbone for plasmid construction in the study

Table 1
Bacterial strains and plasmids used in the study with their relevant characteristics.

Strain	Description	Reference
E. coli DH5α	*Fthi-1 endA1 hsdr17* (*r-*, *m-*) *supE44 _lacU169* (*ϕ80lacZ_M15*) *recA1 gyrA96 relA1*	Hanahan (1983)
C. glutamicum DM1729	*lysC P458S*, *homV59A*, *pyc T311I*	Georgi et al. (2005)
Corynebacterium variabile		DSM
Xanthomonas campestris		ATCC
Saccharophagus degradans		ATCC
DM1729 (pVWEx1-*TAT-XCC2387*)	DM 1729 harbouring (pVWEx1-*TAT-XCC2387*)	This study
DM1729 (pVWEx1-*TAT-XCC3521*)	DM 1729 harbouring (pVWEx1-*TAT XCC3521*)	This study
DM1729 (pEKEx3-*TAT-Sde1394*)	DM 1729 harbouring (pEKEx3-*TAT-Sde1394*)	This study
DM1729 (pEKEx3-*porC-Sde1394*)	DM 1729 harbouring (pEKEx3-*PorC-Sde1394*)	This study
Plasmids		
pVWEx1	*KanR*, *Ptac*, *lacIq*	Peters-Wendisch et al. (1998)
pEKEx3	*SpecR*, *Ptac*, *lacIq*	Stansen et al. (2005)
pVWEx1-*TAT-XCC2387*	Vector for endoglucancase expression (*XCC2387* from *Xanthomonas campestris*)	This study
pVWEx1-*TAT-XCC3521*	Vector for endoglucancase expression (*XCC3521* from *Xanthomonas campestris*)	This study
pEKEx3-*TAT-Sde1394*	Vector for β-glucosidase expression (*Sde 1394* from *Saccharophagus degradans*)	This study
pEKEx3-*porC-Sde1394*	Vector for β-glucosidase expression (*Sde 1394 f*rom *Saccharophagus degradans*) using *porC* anchor protein	This study

(Peters-Wendisch et al., 1998; Stansen et al., 2005). The betaglucosidase and endoglucanase genes were amplified from the respective genomic DNA sequences by KOD PCR (KOD, Novagen, Darmstadt, Germany). For the present study, six betaglucosidase genes were amplified i.e. *Corynebacterium variabile* DSM 44702 – *Cvar 2943* (bgl B), *Xanthomonas campestris pv. campestris* str. ATCC 33913-*XCC1090* (bglX) with the secretory signal, *XCC1090* (bglX), *XCC1250* (bglX), *XCC1404* (bglS), *XCC3814* (bgl X) and *Saccharophagus degradans str.* ATCC 43961–*Sde1394* (bglB). Similarly, five endoglucanase genes were identified from *X. campestris* pv. *campestris* str. ATCC 33913 i.e. *XCC0026*, *XCC0027*, *XCC0028*, *XCC2387* and *XCC3521*. The amplified fragments along with RBS and TAT secretion signal was cloned in the vector using Gibson assembly for the expression of enzymes in the secreted form.

Of the endoglucanase and β-glucosidase genes described above, the plasmids constructed with *Sde 1394*, *XCC2387 & XCC3521* showed quantifiable enzyme activities and were selected for further studies. Hence the plasmids for endoglucanase expression were named pVWEx1-*TAT-XCC2387* and pVWEx1-*TAT-XCC3521*. The plasmid for bgl expression on the cell surface with the *por C* anchor was also constructed similarly, and the resultant plasmid was named pEKEx3-*porC-Sde1394* and the secreted form was pEKEx3-*TAT-Sde1394*.

Standard molecular biology reactions including PCR, restriction digestion and ligation, were executed as described previously (Sambrook and Russell David, 1989). Gibson assembly in the isothermal reaction buffer has been applied for the construction of plasmids, in addition to the ligation reaction (Gibson et al., 2009). PCR products were purified using PCR purification kit or Min Elute PCR purification kit (QIAGEN, Hilden, Germany). *E. coli* transformation was performed using RbCl method (Hanahan, 1983) and *C. glutamicum* was transformed via electroporation (Van der Rest et al., 1999) at 2.5 kV, 200 Ω, and 25 μF. All cloned DNA fragments were confirmed by sequencing for integrity.

2.3. Enzyme expression

The cloned enzymes were under the control of IPTG inducible promoter. The cells were cultured in the CGXII minimal medium with cellobiose or carboxymethyl cellulose as carbon sources for 96 h and sampled at 24 h intervals. Induction was done at the time of inoculation, with IPTG up to 1 mM concentration. Either the cell free supernatant or cell pellet was used for further analysis of enzyme expression.

2.4. Enzyme assay

2.4.1. Qualitative assay

For qualitative determination of endoglucanase and β-glucosidase activities, either CMC or cellobiose agar plates were streaked with the transformants and incubated at 30 °C for 48 h. Qualitative determination of endoglucanase expression was done on CMC agar plates showing clearance zone of hydrolyzed polysaccharide when flooded with 0.1% congo red followed by 1 M NaCl (Teather and Wood, 1982); or trypan blue (Tsuchidate et al., 2011). β-Glucosidase activity was determined by formation of zones with iodine reagent on cellobiose agar plates. Endoglucanase activity was also determined by CMC agar plates flooded with iodine (Kasana et al., 2008).

2.4.2. Quantitative assay

For β-glucosidase activity measurement, the *C. glutamicum* DM1729 cells were separated by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellet was washed with 1X PBS twice and resuspended in 50 mM citrate buffer. The cell pellet fraction and the cell free supernatant were used to determine β-glucosidase activity. Endoglucanase activity was determined by measuring the glucose yield from 2% (w/v) carboxymethylcellulose (CMC) using a modified protocol (Xiao et al., 2005). The assay was performed at 50 °C for 30 min. β-Glucosidase activity was determined by measuring the concentration of p-nitrophenol (pNP) liberated from p-nitrophenyl β-d-glucopyranoside (pNPG) in 10 min at 50 °C. Colorimetric detection was performed with a Shimadzu UV160 A spectrophotometer at 540 nm for endoglucanase and 400 nm for β-glucosidase, respectively. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or p-nitrophenol per minute.

2.5. Pretreatment of biomass and enzymatic hydrolysis

Rice straw and sugarcane tops, obtained locally were used for pretreatment. Rice straw is the aerial vegetative part of the rice plant. After harvesting, it is either burned or left in the field or used as livestock feed. Sugarcane tops are the leaves of sugarcane plant and are usually burned after harvesting sugarcane. The milled and processed biomass was subjected to dilute acid pretreatment with 4%w/w sulfuric acid at 121 °C for 60 min. The biomass so obtained was neutralized after cooling with 10 N sodium hydroxide and air dried for hydrolysis. The recombinant strains were incubated in CGXII medium at 30 °C for 48 h and the supernatant was obtained by centrifugation at 6000 rpm for 10 min at 4 °C. The supernatant thus obtained was used as the enzyme mixture for biomass hydrolysis. The reaction mixture consisted of 2%w/v substrate in the enzyme solution and hydrolysis continued for 72 h at 30 °C. The reducing sugars were analysed by DNS assay at 540 nm after obtaining the supernatant by centrifuging at 6000 rpm for 10 min.

2.6. Simultaneous saccharification and fermentation

Pure polysaccharides like carboxymethyl cellulose and cellobiose individually or in combination at a concentration of 2%w/v were used as carbon source for fermentation in CGXII medium. Fermentation was carried out in CGXII medium at 30 °C for 72 h in both cases.

3. Results and discussion

3.1. Recombinant C. glutamicum DM1729 expressing endoglucanase and β-glucosidase genes

Engineering of microbes for direct substrate utilization by heterologous expression of enzymes so as to broaden the substrate spectrum is crucial in the development of a microbial biorefinery. In this direction, several enzymes has been successfully expressed in *C. glutamicum* (Adachi et al., 2013; Seibold et al., 2006; Tateno et al., 2007b). *C. glutamicum* is an excellent host for heterologous protein expression considering the limited extracellular protease activity and efficient secretion mechanisms for proteins (Geisseler and Horwath, 2008).

Of the six betaglucosidases *Sde1394* gave heterologous expression in *C. glutamicum* DM 1729 and among the endoglucanases *XCC2387* & *XCC3521* showed heterologous enzyme expression. Thus in this study, the recombinant strains obtained were, (1) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) (2) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*) and (3) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-TAT-*Sde1394*). All the three recombinant *C. glutamicum DM1729* strains were evaluated for its endoglucanase and β-glucosidase expression and lysine fermentation. Two of the recombinant strains namely *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*) and *C. glutamicum*

DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*) had both the enzymes in secreted form and *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) had endoglucanase in secreted form and betaglucosidase in cell surface expressed form using the *porC* anchor. Porin for cell surface expression of heterologous proteins has been successfully demonstrated earlier (Tateno et al., 2009). *C. glutamicum* has TAT and Sec secretion systems. TAT secretion system used in the study for the expression of proteins in the secreted form, transports fully folded proteins in the cytoplasm (Dilks et al., 2003) and is reported to be industrially efficient. Moreover the feasibility of choosing the TAT system is corroborated by the earlier reports stating that *Corynebacteria* transports more proteins through the TAT system than other related genera like *Mycobacterium* and *Streptomyces* (Schaerlaekens et al., 2004).

The expressed cellulases were able to degrade the polysaccharides and produce the reducing sugars for efficient utilization by the recombinant *C. glutamicum* DM1729. This shows that the enzymes were transported out of the cells efficiently. The vector map for plasmid encoding representative endoglucanase (a) and betaglucosidase (b) is depicted in Fig. 1a and b respectively.

3.2. Enzyme activities of endoglucanases and β-glucosidases

The enzyme activities were qualitatively determined using chromogenic reaction to iodine, trypan blue and congo red. All the recombinant strains were able to grow on the respective substrates viz carboxymethyl cellulose and cellobiose. The positive chromogenic reaction was shown by clear zone of polysaccharide hydrolysis around the colonies whereas the parent strain was unable to produce clearance zones. Endoglucanase activity was shown by clearance zones with congo red, trypan blue and iodine whereas positive β-glucosidase was shown by reaction with iodine.

The endoglucanase and β-glucosidase activities of the recombinant *C. glutamicum* strains were quantitatively evaluated using standard assay protocols. The endoglucanase and β-glucosidase activities of all the recombinant strains and the parent strain are depicted in Fig. 2. Generally, *C. glutamicum* 13032 and derived strains lack the ability to assimilate cello oligosaccharides like cellobiose. Unlike the wild type strain, *C. glutamicum* R is reported to utilize salicilin, arbutin and methyl β-glucoside by direct uptake through the PEP-dependant phosphotransferase system (PTS_{bglF1} & PTS_{bglF2}) but was unable to utilize cellobiose. Point mutations in the *bglF1* gene in the PTS extended the substrate utilization spectrum of *C. glutamicum* R to cellobiose (Kotrba et al., 2003) and simultaneous utilization of cellobiose, glucose and xylose (Sasaki et al., 2008). We have followed heterologous expression of β-glucosidase genes for cellobiose utilization. The highest β-glucosidase activity of 9 ± 0.5 U/OD_{600} of 1 was observed in *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) in medium containing cellobiose (20 g/L) as the carbon source and in the medium with both carboxymethyl cellulose and cellobiose in combination, the β-glucosidase activity dropped to 0.5 ± 0.2 U/OD_{600} of 1. Considering the other two strains with secreted β-glucosidases, the activities are higher in the cell surface expressed form. *C. glutamicum* has been engineered for endoglucanase expression and glucan hydrolysis using *Clostridium thermocellum* endoglucanase (Tsuchidate et al., 2011). In the present study, endoglucanase activities were highest in DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) having 5.5 ± 0.8 U and for the other two strains highest activities were obtained in carboxymethyl cellulose based medium. In all the cases, the parental strain did not show endoglucanase or β-glucosidase activities. The enzyme activities in media where either cellobiose or carboxymethyl cellulose alone was used as carbon source are described in Table 2.

3.3. Biomass hydrolysis

The hydrolysis of pretreated biomass with the secreted enzymes of *C. glutamicum* recombinants yielded reducing sugars. Sugarcane tops was more assessable to the recombinant enzymes than rice straw which is evident from the reducing sugars produced. The recombinant strain I [DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*)] and strain III [DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)] showed similar reducing sugar concentrations (5.2 ± 0.26 mg/g substrate and 5.4 ± 0.27 mg/g substrate) with sugarcane tops as the substrate and with rice straw the highest reducing sugar concentration obtained was 4.7 ± 0.23 mg/g substrate. The results are described in Fig. 3.

3.4. Lysine fermentation by endoglucanase and β-glucosidase expressing C. glutamicum

Lysine fermentation was carried out in CGXII medium with cellobiose and carboxymethyl cellulose either in combination or alone. The lysine titres and growth of the recombinants were depicted in Fig. 4. There was a general decline in the lysine titre after 48 h, except in *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*), where the maximum production was in 96 h of fermentation reaching 5.9 ± 0.5 mM. The highest lysine titres for the other two strains were 2.8 ± 0.5 mM under the same fermentation condi-

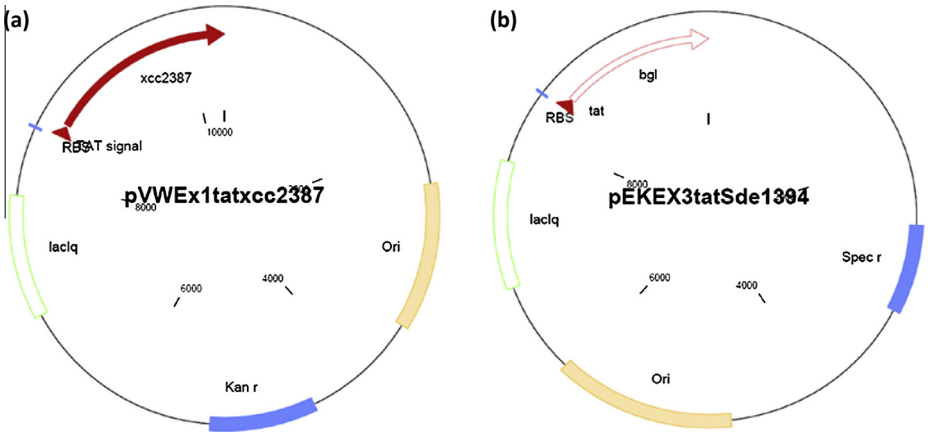


Fig. 1. (a) The vector pVWEx1 harbouring the endoglucanase gene *XCC2387* (b) The vector pEKEx3 harbouring β-glucosidase gene *Sde1394*.

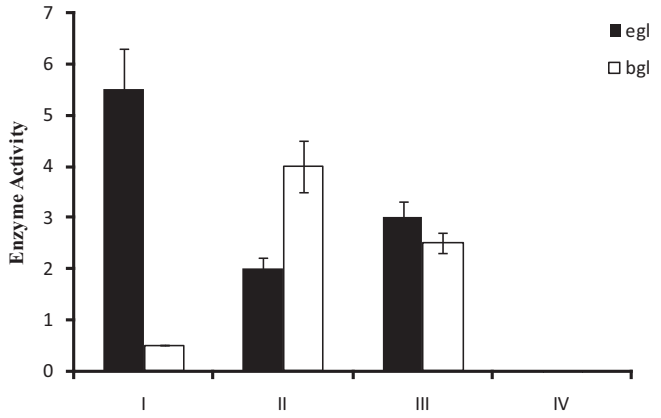


Fig. 2. Ezyme activities of the *C. glutamicum* recombinants in CGXII medium with 20 g/L CMC and 20 g/L cellobiose. The solid column represents the endoglucanase activity and open column represent the β-glucosidase activity. The strains I-IV represents *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*) and empty vector control. The enzymes activities are represented in U except for strain I, where the β-glucosidase activity is represented as U/OD_{600} of 1.

Table 2
Enzyme activities and lysine titre in the media supplemented with either 20 g/L CMC or 20 g/L cellobiose.

Bacterial strains	Strain I	Strain II	Strain III
Fermentation in CGXII supplemented with 20 g/L CMC			
β-glucosidase activity (U or U/OD_{600} of 1)	8 ± 0.4	0 ± 0.5	5 ± 0.3
Endoglucanase activity (U)	2.5 ± 0.5	4.5 ± 0.2	3.2 ± 0.8
Lysine titre (mM)	2.1 ± 0.12	1.7 ± 0.09	2.1 ± 0.12
Fermentation in CGXII supplemented with 20 g/L cellobiose			
β-glucosidase activity (U or U/OD_{600} of 1)	9 ± 0.5	0 ± 0.5	3 ± 0.5
Endoglucanase activity (U)	1.5 ± 0.4	1.5 ± 0.1	2 ± 0.13
Lysine titre (mM)	2.8 ± 0.12	1.4 ± 0.1	4.8 ± 0.2

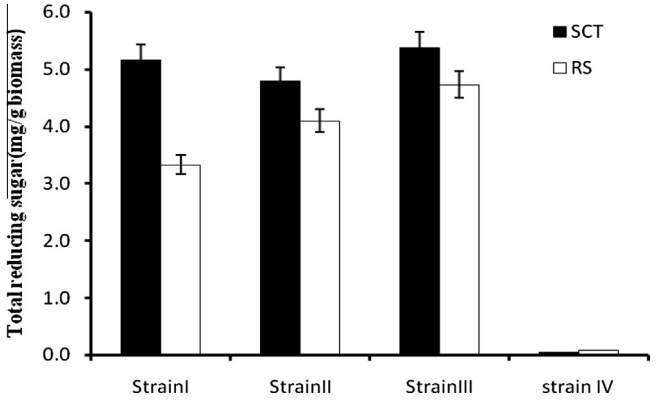


Fig. 3. Reducing sugars produced from the conversion of pretreated sugarcane tops and rice straw. The closed column represent the reducing sugars produced per gram of acid pretreated sugarcane tops and open column represent reducing sugars produced per gram of acid pretreated rice straw after hydrolysis at 30 °C for 72 h. The strain I, II, III & IV are *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*) and empty vector control.

tions. Lysine production by the other strains in the media supplemented with either carboxymethyl cellulose or cellobiose alone is shown in Table 2.

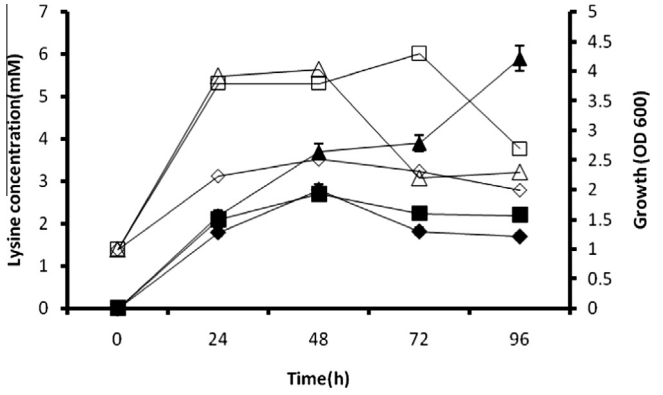


Fig. 4. Lysine fermentation and growth of *C. glutamicum* mutants in20 g/L cellobiose and 20 g/L carboxymethyl cellulose. Closed diamond represent lysine produced and open diamond represent growth at OD_{600} of strain I [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*)]; closed square represent lysine produced and open square represent growth at OD_{600} of strain II [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*)]; closed triangle represent lysine produced and open triangle represent growth at OD_{600} of strain III [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)].

4. Conclusion

C. glutamicum strains co-expressing cellulases were constructed which demonstrated lysine fermentation. The strains also efficiently saccharified pretreated biomass like sugarcane tops and rice straw to reducing sugars depicting that the heterologous cellulases functioned in synergy. For better utilization of cellulose for high yields of bio products, cellulases like cellobiohydrolases need to be co expressed. Since the hemicelluloses fractions in the lignocellulosic biomass produces pentose sugars as the monomeric units, introduction of the cellulases into pentose utilizing constructs that we already developed will lead to the complete utilization of lignocellulosic biomass leading towards *C. glutamicum* based biorefinery for lysine production.

Conflict of interest

Authors declare that there is no conflict of interest.

Acknowledgements

Author MA acknowledge the research grant from DST. KMN and VFW thank DST-DAAD project based personnel exchange programme. The author MA also thanks Tobias Meiswinkel, Christian Matano and Sophie Loismann for their contributions and the molecular biology expertise and research facilities provided at the Wendisch lab, University of Bielefeld, Germany.

References

Adachi, N., Takahashi, C., Ono-Murota, N., Yamaguchi, R., Tanaka, T., Kondo, A., 2013. Direct L-lysine production from cellobiose by *Corynebacterium glutamicum* displaying beta-glucosidase on its cell surface. Appl. Microbiol. Biotechnol. 97 (16), 7165–7172.

Bayan, N., Houssin, C., Chami, M., Leblon, G., 2003. Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. J. Biotechnol. 104 (1), 55–67.

Dilks, K., Rose, R.W., Hartmann, E., Pohlschröder, M., 2003. Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. J. Bacteriol. 185 (4), 1478–1483.

Eggeling, L., Bott, M., 2005. Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, Florida.

Geisseler, D., Horwath, W.R., 2008. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. Soil Biol. Biochem. 40 (12), 3040–3048.

Georgi, T., Rittmann, D., Wendisch, V.F., 2005. Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: roles of malic enzyme and fructose-1, 6-bisphosphatase. Metab. Eng. 7 (4), 291–301.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6 (5), 343–345.

Gopinath, V., Murali, A., Dhar, K.S., Nampoothiri, K.M., 2012. *Corynebacterium glutamicum* as a potent biocatalyst for the bioconversion of pentose sugars to value-added products. Appl. Microbiol. Biotechnol. 93 (1), 95–106.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166 (4), 557–580.

Inui, M., Kawaguchi, H., Murakami, S., Vertès, A.A., Yukawa, H., 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. 8 (4), 243–254.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr. Microbiol. 57 (5), 503–507.

Kotrba, P., Inui, M., Yukawa, H., 2003. A single V317A or V317M substitution in Enzyme II of a newly identified β-glucoside phosphotransferase and utilization system of *Corynebacterium glutamicum* R extends its specificity towards cellobiose. Microbiology 149 (6), 1569–1580.

Lee, S.Y., Choi, J.H., Xu, Z., 2003. Microbial cell-surface display. Trends Biotechnol. 21 (1), 45–52.

Lynd, L.R., Weimer, P.J., Van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66 (3), 506–577.

Narita, J., Okano, K., Kitao, T., Ishida, S., Sewaki, T., Sung, M.-H., Fukuda, H., Kondo, A., 2006. Display of α-amylase on the surface of Lactobacillus casei cells by use of the PgsA anchor protein, and production of lactic acid from starch. Appl. Environ. Microbiol. 72 (1), 269–275.

Peters-Wendisch, P.G., Kreutzer, C., Kalinowski, J., Pátek, M., Sahm, H., Eikmanns, B. J., 1998. Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the pyc gene. Microbiology 144 (4), 915–927.

Sambrook, J., Russell David, W., 1989. Molecular Cloning: A Laboratory Manual. Cold spring harbor laboratory press, Vol. 3.

Sasaki, M., Jojima, T., Inui, M., Yukawa, H., 2008. Simultaneous utilization of D-cellobiose, D-glucose, and D-xylose by recombinant *Corynebacterium glutamicum* under oxygen-deprived conditions. Appl. Microbiol. Biotechnol. 81 (4), 691–699.

Saxena, R., Adhikari, D., Goyal, H., 2009. Biomass-based energy fuel through biochemical routes: a review. Renew. Sustain. Energy Rev. 13 (1), 167–178.

Schaerlaekens, K., Van Mellaert, L., Lammertyn, E., Geukens, N., Anné, J., 2004. The importance of the Tat-dependent protein secretion pathway in Streptomyces as revealed by phenotypic changes in tat deletion mutants and genome analysis. Microbiology 150 (1), 21–31.

Seibold, G., Auchter, M., Berens, S., Kalinowski, J., Eikmanns, B.J., 2006. Utilization of soluble starch by a recombinant *Corynebacterium glutamicum* strain: growth and lysine production. J. Biotechnol. 124 (2), 381–391.

Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J.-L., Wendisch, V.F., 2005. Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. Appl. Environ. Microbiol. 71 (10), 5920–5928.

Sukumaran, R.K., Surender, V.J., Sindhu, R., Binod, P., Janu, K.U., Sajna, K.V., Rajasree, K.P., Pandey, A., 2010. Lignocellulosic ethanol in India: prospects, challenges and feedstock availability. Bioresour. Technol. 101 (13), 4826–4833.

Tanaka, T., Yamada, R., Ogino, C., Kondo, A., 2012. Recent developments in yeast cell surface display toward extended applications in biotechnology. Appl. Microbiol. Biotechnol. 95 (3), 577–591.

Tateno, T., Fukuda, H., Kondo, A., 2007a. Direct production of L-lysine from raw corn starch by *Corynebacterium glutamicum* secreting Streptococcus bovis α-amylase using cspB promoter and signal sequence. Appl. Microbiol. Biotechnol. 77 (3), 533–541.

Tateno, T., Fukuda, H., Kondo, A., 2007b. Production of L-Lysine from starch by *Corynebacterium glutamicum* displaying α-amylase on its cell surface. Appl. Microbiol. Biotechnol. 74 (6), 1213–1220.

Tateno, T., Hatada, K., Tanaka, T., Fukuda, H., Kondo, A., 2009. Development of novel cell surface display in *Corynebacterium glutamicum* using porin. Appl. Microbiol. Biotechnol. 84 (4), 733–739.

Teather, R.M., Wood, P.J., 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43 (4), 777–780.

Tsuchidate, T., Tateno, T., Okai, N., Tanaka, T., Ogino, C., Kondo, A., 2011. Glutamate production from β-glucan using endoglucanase-secreting *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 90 (3), 895–901.

Van der Rest, M., Lange, C., Molenaar, D., 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. 52 (4), 541–545.

White, T.C., Hindle, C.D., 2000. Genetic constructs and genetically modified microbes for enhanced production of beta-glucosidase. Google Patents.

Xiao, Z., Storms, R., Tsang, A., 2005. Microplate-based carboxymethylcellulose assay for endoglucanase activity. Anal. Biochem. 342 (1), 176–178.

Yao, W., Chu, C., Deng, X., Zhang, Y., Liu, M., Zheng, P., Sun, Z., 2009. Display of α-amylase on the surface of *Corynebacterium glutamicum* cells by using NCgl1221 as the anchoring protein, and production of glutamate from starch. Arch. Microbiol. 191 (10), 751–759.