Journal of Biotechnology 163 (2013) 217–224

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

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

Production of L-lysine on different silage juices using genetically engineered *Corynebacterium glutamicum*

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ARTICLE INFO

Article history:
Received 26 March 2012
Received in revised form 27 July 2012
Accepted 31 July 2012
Available online 9 August 2012

Keywords:
Grass silage
Corn silage
Amino acid production
Overexpression
Fermentation
Oxygen limitation

ABSTRACT

Corynebacterium glutamicum, the best established industrial producer organism for lysine was genetically modified to allow the production of lysine on grass and corn silages. The resulting strain *C. glutamicum* $lysC^{fbr}$ dld_{Psod} pyc_{Psod} $malE_{Psod}$ fbp_{Psod} $gapX_{Psod}$ was based on earlier work (Neuner and Heinzle, 2011). That mutant carries a point mutation in the aspartokinase (*lysC*) regulatory subunit gene as well as overexpression of D-lactate dehydrogenase (*dld*), pyruvate carboxylase (*pyc*) and malic enzyme (*malE*) using the strong Psod promoter. Here, we additionally overexpressed fructose 1,6-bisphosphatase (*fbp*) and glyceraldehyde 3-phosphate dehydrogenase (*gapX*) using the same promoter. The resulting strain grew readily on grass and corn silages with a specific growth rate of 0.35 h^{-1} and lysine carbon yields of approximately 90 C-mmol (C-mol)$^{-1}$. Lysine yields were hardly affected by oxygen limitation whereas linear growth was observed under oxygen limiting conditions. Overall, this strain seems very robust with respect to the composition of silage utilizing all quantified low molecular weight substrates, e.g. lactate, glucose, fructose, maltose, quinate, fumarate, glutamate, leucine, isoleucine and alanine.

1. Introduction

European green biorefinery concepts are usually based on silage guaranteeing a constant supply over the year (Hanegraaf et al., 1998; McDonald et al., 1991). Ensiling is the process of preserving the wet plant material applying anaerobic conditions, either in a storage silo or wrapped in plastic. After harvest, the green plant material is chopped and left to wither up to a dry mass content of approximately 30% followed by the removal of oxygen by compression assuring anaerobic conditions. Lactic acid bacteria convert a large fraction of water soluble carbohydrates into lactic acid. The low pH (3–4) and the anaerobic conditions prevent coliform bacteria and clostridia from spoiling the crop (McDonald et al., 1991). On a large scale, silage is either used for the production of biogas or as animal feed (Kromus et al., 2004). In the case of biogas production it seems interesting to convert part of the easily accessible compounds into more valuable products, e.g. amino acids, and use the remainder for biogas production. We aimed at the production of lysine using *Corynebacterium glutamicum* (Eggeling and Bott, 2005). Since *C. glutamicum* is a GRAS organism, fermentation broth containing lysine and *C. glutamicum* could be used as feed supplement for monogastric animals in close-by chicken and pig farming (Leuchtenberger et al., 2005). A second alternative might be a direct combination of lysine fermentation of silage to enrich silage with lysine. Only small increases in lysine content improves the biological value measured by the protein efficiency ratio significantly (Belitz et al., 2001). This makes lysine the most used amino acid in animal feed supplementation (Belitz et al., 2001) improving the nitrogen uptake, enhancing growth (Leclercq, 1998) and decreasing the release of nitrogen into the environment.

Dominating compounds in sugar beet and corn silages are sucrose and starch, respectively, whereas grass silage does not have a major component. Lactic acid itself is a valuable intermediate for the manufacture of biodegradable polymers (Södergard and Stolt, 2002) but recovery costs from silage are very high (Datta et al., 1995). Therefore, we try to use it as an carbon source for lysine production (Neuner and Heinzle, 2011).

C. glutamicum, a gram positive, non-pathogenic soil bacterium grows aerobically on various carbohydrates and organic acids as carbon sources (Liebl et al., 1991) but only poorly on racemic lactate (Neuner and Heinzle, 2011). Applying metabolic network analysis using elementary modes (Kjeldsen and Nielsen, 2009; Kromer et al., 2006; Melzer et al., 2009; Neuner and Heinzle, 2011; Schuster et al., 1999) to guide genetic engineering, *C. glutamicum* was engineered to grow on racemic lactate and on carbohydrates and mixtures thereof and at the same time producing lysine (Neuner and Heinzle, 2011).

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http://dx.doi.org/10.1016/j.jbiotec.2012.07.190

Table 1
Molecular and biological tools and strains constructed starting from *C. glutamicum* ATCC 13032.

Strain	Modifications	Reference
C. glutamicum $LysC^{fbr}$	Exchange T311I in the *lysC* gene	BASF
C. glutamicum $LysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld, pyc* and *malE* genes by the promoter of the *sod* gene	Neuner and Heinzle (2011)
C. glutamicum $LysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld, pyc, malE, fbp* genes by the promoter of the *sod* gene	This work
C. glutamicum $LysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}gapX_{Psod}$ Referred to as *C. glutamicum* strain SL	lysC T311I + Exchange of the natural promoter of the *dld, pyc, malE, fbp, gapX* genes by the promoter of the *sod* gene	This work
E. coli DH5α	F^- *endA1, hsdR17 (vk⁻ mk⁺) supE44, thi-I* λ^- *recAl gyrA96 rel A1,* Δ *(lac ZYA-argF)U169* F*80d lacZ* ΔM15	Hanahan (1983)
E. coli NM522	*supE thi-1 Δ(lac-proAB)Δ(mcrB-hsdSM) 5(rK⁻ mK⁺) [F' proAB lacl*q *ZΔM15]*	Stratagene
Plasmid		
pClik int *sacB*	Vector for integrative, allelic replacement by homologuous recombination, non-replicative in *C. glutamicum*, Kan^R, *sacB*	BASF

In this work, the lysine producing strain *C. glutamicum* $lysC^{fbr}$ dld_{Psod} $pyc_{Psod}malE_{Psod}$ (Neuner and Heinzle, 2011) was further genetically modified and tested for efficient lysine production on different silage juices. We focused on the three main carbon sources, glucose, fructose and lactate. However, other sugars and organic acids existing in silage juices (Wiseman and Irvin, 1957) were also analyzed. Besides earlier identified and tested modifications (Neuner and Heinzle, 2011) we studied two additional targets for overexpression that were predicted by the same model and method but using lactate, glucose and fructose as substrates. The gluconeogenic enzyme fructose 1,6-bisphosphatase (*fbp*) was already earlier found being beneficial for lysine production on carbohydrates (Becker et al., 2005, 2011; Rittmann et al., 2003) and therefore was constitutively overexpressed. Furthermore, glyceraldehyde 3-phosphate dehydrogenase that was also predicted as beneficial for lysine production was overexpressed. We studied growth and lysine production of the resulting strain on different silage juices and under different cultivation conditions and found substantial lysine production even under oxygen limitation as was already observed previously (Ensari and Lim, 2003).

2. Materials and methods

2.1. Pre-treatment of silage juice

The silages we used were provided by the Lehr- und Versuchsanstalt für Viehhaltung, Hofgut Neumühle (Münchweiler an der Alsenz, Germany). The silage juices were obtained by using a HP2H tincture press (Fischer Maschinenfabrik GmbH, Neuss, Germany). We applied filtration with stericups (Merck Millipore, Darmstadt, Germany) and pasteurization, using different temperatures for different periods of time. When using filtration with 0.22 μm pore size, the silage juices were centrifuged previously at maximum speed (16,000 × *g*) for 10 min to remove the majority of the particles from the suspension. The pH was adjusted to a value of 7 using 30% ammonia solution or 2 N NaOH. Heat pre-treatment tests were only made on grass silage. After the heat pre-treatment, silage juice was plated out on LB and CM agar plates and incubated at 30 °C and 37 °C for one week. Colony formation was observed. Subsequently, the treated juice was used for growth experiments with *C. glutamicum* mutants.

2.2. Strains, plasmids and recombinant DNA methods

All mutants were designed on basis of the lysine producing strain *C. glutamicum* ATCC 13032 $lysC^{fbr}$ (BASF AG, Ludwigshafen, Germany) with deregulated lysine biosynthesis (allelic replacement of the *lysC* gene with a *lysC*T311I gene) (Kim et al., 2006). Overexpression of the genes *fbp* and *gapX* in *C. glutamicum* was achieved by cloning the open reading frame (ORF) of the mentioned genes under the control of the strong constitutive promoter of the *sod* gene, encoding superoxide dismutase (NCgl2826).

Escherichia coli DH5α was used for the amplification of the genetic constructs. Application of the methylation pattern of *C. glutamicum* to the genetic constructs was performed using *E. coli* NM522, containing the pTc plasmid as an expression vector for the DNA – methyltransferase of *C. glutamicum*. The integrative plasmid pClik int *sacB*, carrying a kanamycine resistance and the *sacB* gene as selective markers was used for introducing the genetic modifications. Transformation of the organism with the plasmid and selection for kanamycine resistance yielded transformants with genome integrated plasmid DNA. Integration of the plasmid DNA occurred via a single crossover homologous recombination. The second recombination was detected and selected via the *sacB* positive selection system (Jäger et al., 1992). Sucrose resistant, kanamycine sensitive clones were tested for the presence of the mutation by PCR. In addition, sequencing of the resulting PCR product was performed (GATC, Konstanz, Germany). Detailed information about all bacterial strains and plasmids used in this study, their relevant characteristics and their sources are listed in Table 1. Used primers are listed in Table 2.

For the construction and purification of plasmid DNA standard protocols were applied. Chromosomal DNA from *C. glutamicum* was obtained using the Instant Bacteria DNA Kit (Analytic Jena, Jena, Germany). Plasmids from *C. glutamicum* were isolated using the HiSpeed® Midi Kit (Quiagen, Hilden, Germany). Plasmids from *E. coli* were recovered using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Munich, Germany). Oligonucleotide synthesis was carried out by Sigma (Munich, Germany). All PCR

Table 2
Primer sequences used for verification of the mutant strains and the PCR fragment size of the wild type and mutant alleles.

Genetic modification	Primer sequence	PCR fragment size	PCR fragment size (bp)
$P_{sod}gapX$	Fw: 5′-ATCGACGCGTTCGCAGCCGGCGGCCTTTCAACCTCCG-3′	WT	1218
	Rw: 5′-CGATCTCGAGCGCCAGCGGCCGGTGTTGTCTACCACGACG-3′	$P_{sod}dld$	1410
$P_{sod}fbp$	Fw: 5′-AGTTGCATGATCAGTCATTGCGCGCGCTTCC-3′	WT	1377
	Rw: 5′-AGTCTGTCCACCAGCTGTCCAAGCTGCAGGAATAC-3′	$P_{sod}pyc$	1569