Industrial Crops and Products xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Methanol-based γ-aminobutyric acid (GABA) production by genetically engineered *Bacillus methanolicus* strains

Marta Irla [a], Ingemar Nærdal [b], Trygve Brautaset [c], Volker F. Wendisch [a,*]

[a] *Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, Bielefeld, Germany*
[b] *SINTEF Materials and Chemistry, Department of Biotechnology and Nanomedicine, Trondheim, Norway*
[c] *NTNU, Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway*

ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form
14 November 2016
Accepted 27 November 2016
Available online xxx

Keywords:
Bacillus methanolicus
Thermophile
Methanol
γ-Aminobutyric acid
Fed-batch fermentation

ABSTRACT

The use of methanol as a carbon source for biotechnological processes has recently attracted great interest due to relatively low price, high abundance, high purity of methanol, and the fact that it is a non-food raw material. In this study, methanol-based production of γ-aminobutyric acid (GABA), which is a component of drugs and functional foods and is used as monomer for production of the biodegradable plastic polyamide 4, was established using recombinant *Bacillus methanolicus* strains. This was achieved by heterologous overexpression of glutamate decarboxylase genes from *Sulfobacillus thermosulfidooxidans* (*gad*St) or *Escherichia coli* (*gadB*) in methylotrophic *B. methanolicus* MGA3. Strains expressing either *gad*St or *gadB* accumulated between 0.03 and 0.4 g/L of GABA in shake flask experiments. Initially, controlled methanol fed-batch fermentations yielded low GABA concentration (0.1 g/L). However, employing a two-phase production strategy with an initial high-cell-density fermentation phase for growth and L-glutamate accumulation followed by pH reduction from 6.5 to 4.6 after 27 h for enzymatic conversion of glutamate to GABA led to 90-fold increased GABA accumulation to a final titer of 9 g/L. To the best of our knowledge, this study represents the first demonstration of methanol-based production of GABA.

1. Introduction

Gamma (γ)-aminobutyric acid (GABA) is a four-carbon non-protein amino acid, distributed in a variety of organisms, which belong to all kingdoms of life. In bacteria, decarboxylation of L-glutamate to GABA is a part of the acid stress response and possibly participates in sporulation processes (Aronson et al., 1975; Foerster and Foerster, 1973; Lin et al., 1996). In plants, GABA is involved in maintaining the C:N balance, regulation of cytosolic pH, storage of nitrogen, cell signaling and protection against various stress agents (Bouche and Fromm, 2004; Shelp et al., 1999). In mammals, GABA serves as a major inhibitory neurotransmitter and is involved in control of growth and maturation of neurons. A disrupted GABA-glutamate balance can lead to diverse disorders such as epilepsy, seizures, motoric disorders, schizophrenia, anxiety and stress (Goddard, 2016; Obata, 2013; Wong et al., 2003). Due to its tranquilizing, pain-killing and diuretic properties GABA is an interesting candidate for becoming a food additive or a drug (Bowery and Smart, 2006; Hayakawa et al., 2004). GABA is a precursor of 2-pyrrolidone, a monomer of polyamide 4 (PA4) also known as nylon 4 which is a bio-based polymer with remarkable mechanical and thermal properties resulting from its high melting point (Kawasaki et al., 2005). PA4 is degraded in soil and activated sludge by diverse microorganisms including *Pseudomonas* sp., *Fusarium solani*, *F. oxysporum*, and *Clonostachys rosea* (Hashimoto et al., 2004, 2002, 1994; Kawasaki et al., 2005; Yamano et al., 2008).

GABA is naturally produced by different lactic acid bacteria (LAB) strains such as *Lactobacillus lactis*, *L. brevis* or *L. plantarum* (Franciosi et al., 2015; Nomura et al., 1998; Tajabadi et al., 2015b). Its biosynthesis by natural isolates depends on different factors: carbon and nitrogen sources, pyridoxal phosphate (cofactor) availability, Tween-80 supplementation, initial L-glutamate concentration, temperature, pH and incubation time (Cho et al., 2007; Komatsuzaki et al., 2005; Li et al., 2010a,b; Tajabadi et al., 2015b; Villegas et al., 2016). The efficiency of natural GABA producers can be improved by overexpression of additional glutamate decarboxylase (*gad*) gene (Kook et al., 2010; Park et al., 2005, 2013; Plokhov et al., 2000; Tajabadi et al., 2015a; Le Vo et al., 2014, 2013a), supported by the overexpression of *gadC* coding for the glutamate:GABA antiporter (Le Vo et al., 2013b) and/or deletion of the GABA degradation genes (Le Vo and Kim Hong, 2012). However, neither LAB nor *Escherichia coli* naturally synthesize high concentrations of the GABA precursor L-glutamate; for this reason the fermentations carried out by these bacteria have to be sup-

* Corresponding author at: Universitätsstr. 25, 33615 Bielefeld, Germany.
E-mail address: volker.wendisch@uni-bielefeld.de (V.F. Wendisch).

http://dx.doi.org/10.1016/j.indcrop.2016.11.050
0926-6690/

plemented with this compound. To tackle this issue, *E. coli* was engineered for overproduction of L-glutamate directly from glucose (Pham et al., 2016, 2015). To our knowledge, the highest GABA titer achieved so far by engineered *E. coli* strain is 1.3 g/L from 10 g/L glucose (Pham et al., 2015). *Corynebacterium glutamicum* is an industrial workhorse for the production of amino acids, in particular of L-glutamate (Kimura, 2003) and has been engineered for glucose-based production of GABA (Heider and Wendisch, 2015). Heterologous expression of the glutamate decarboxylase gene *gadB2*, the GABA:glutamate antiporter gene *gadC,* and the regulator gene *gadR* from *L. brevis* in *C. glutamicum* led to accumulation of about 2 g/L of GABA within 72 h (Shi and Li, 2011). The titer was increased to 7 g/L (after 84 h) when the activity range of glutamate decarboxylase was broadened towards a near-neutral pH by mutagenesis (Shi et al., 2014) or to 19 g/L (after 84 h cultivation) by co-expression of two glutamate decarboxylase genes *gadB1* and *gadB2* from *L. brevis* (Shi et al., 2013). Overexpression of *E. coli*-derived glutamate decarboxylase gene *gadB* in *C. glutamicum* yielded 8 g/L of GABA after 96 h (Takahashi et al., 2012). Changing the genetic background of *C. glutamicum* by deleting *pknG* coding protein kinase G increased GABA production to 30 g/L during 120 h fermentation (Okai et al., 2014). As consequence of engineering of glutamate decarboxylase from *E. coli* towards activity at neutral pH the GABA titers of 39 g/L were achieved in this host (Choi et al., 2015). Finally, a *C. glutamicum* strain was constructed that produced 71 g/L of GABA during 70 h of fermentation under a two-stage pH control strategy. The latter strain lacked genes *argB*, *proB* and *dapA* encoding enzymes involved in the formation of the by-products L-arginine, L-proline and L-lysine, and it had two copies of the *gad* gene from *L. plantarum* inserted into its genome (Zhang et al., 2014). Besides glutamate decarboxylase-based production of GABA, a new metabolic route to GABA via putrescine has been described in *C. glutamicum* and the highest volumetric productivity reported so far for fermentative production of GABA from glucose in shake flasks was achieved (Jorge et al., 2016a,b).

Methanol has recently attracted interest as a potential feedstock for biotechnological processes due to its numerous advantages over conventional carbon sources such as availability, chemical purity and lack of competition with food industry (Linton and Niekus, 1987; Müller et al., 2015; Ochsner et al., 2015; Schrader et al., 2009). To date, methanol is mostly produced from syngas; however, a lot of progress has been made in last years in development of alternative, renewable routes for methanol synthesis for example from crude glycerol which is a by-product in production of biodiesel from plant-derived triglycerides (Haider et al., 2015). For this reason methanol is expected to emerge as a base of methanol bio-economy in the near future (Olah, 2013).

In the present study, we have established methanol-based production of GABA by recombinant *Bacillus methanolicus*. A wild-type strain of this Gram-positive, endospore-forming, thermophilic and methylotrophic bacterium is known to secrete up to 60 g/L of the GABA precursor L-glutamate during high-cell-density fed-batch methanol fermentation (Heggeset et al., 2012; Schendel et al., 2000) and 0.8 g/L of L-glutamate in shake flaks cultivations (Krog et al., 2013). The overexpression of heterologous glutamate decarboxylase genes led to conversion of L-glutamate to GABA in the two phase production process.

2. Materials and methods

2.1. Strains, plasmids, and primers

Bacterial strains and plasmids used in this study are listed in the Supplementary Table S1. The *E. coli* strain DH5α was used as general cloning host and MG1655 was source for cloning of the *gadB* gene. All primers (Metabion or BASF SE) used in this research are listed in the Supplementary Table S1.

2.2. Molecular cloning

The *E. coli* competent cells were prepared according to the calcium chloride protocol as described in Chan et al. (2013). All standard molecular cloning procedures were carried out as described in Sambrook and Russell (2001) or according to manuals provided by producers. Chromosomal DNA of *E. coli* and *Corynebacterium terpenotabidum* was isolated as described in Eikmanns et al. (1994). The genomic DNA (gDNA) of *S. thermosulfidooxidans*, *Bacillus megaterium*, and *L. brevis* was obtained from German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen; DSMZ). PCR product purification and gel extraction were performed with the NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel). GeneJET Plasmid Miniprep Kit from (Thermo Fisher Scientific) was used for plasmids isolation. Restriction enzymes were produced by Thermo Fisher Scientific. The DNA amplification was carried out with ALLin™ HiFi DNA Polymerase (highQu). Antarctic Phosphatase from New England Biolabs was used for the dephosphorylation of plasmid DNA. The DNA fragments were joined either with Rapid DNA Ligation Kit (Roche) or by the means the isothermal DNA assembly (Gibson et al., 2009). The plasmids pTH1mp-*gad*^{Bm} and pTH1mp-*gad*^{Ct} were constructed by amplifying the respective *gad* gene from gDNA with primers as described in Table S1. The PCR product and pTH1mp plasmid were digested with PciI and KpnI, and both fragments were ligated. The plasmids pTH1mp-*gadB,* pTH1mp-*gadB*^{TM}, pTH1mp-*gad*St, pTH1mp-*gadB1*^{Lb}, pTH1mp-*gadB2*^{Lb}, pTH1mp-*gadB3*^{Lb} were constructed by amplifying respective *gad* gene from gDNA with primers as described in Table S1, and joining the resulting PCR product with PciI and BamHI digested pTH1mp by means of the isothermal DNA assembly method. For the plasmids pTH1mp-*gadB1*^{Ao}, pTH1mp-*gadB3*^{Ao} the synthetic genes were codon optimized for *B. methanolicus* by GeneArt (Thermo Fisher Scientific). The sequences of the codon optimized genes are available in the Supplementary material. The colony PCR was done with Taq polymerase (New England Biolabs). All cloned DNA fragments were confirmed by sequencing. The competent cells of *B. methanolicus* were prepared and the electroporation was performed as described before (Jakobsen et al., 2006).

2.3. Media and conditions for shake flask cultivations

E. coli strains were cultivated at 37 °C in Lysogeny Broth (LB) or on LB agar plates supplemented with antibiotics when necessary. For transformations *B. methanolicus* strains were cultured at 50 °C in SOBsuc (SOB medium supplemented with 0.25 M sucrose). For GABA production screening experiments, recombinant *B. methanolicus* strains were grown in MVcM minimal medium with 200 mM methanol supplemented with 5 μg/ml chloramphenicol and 20 μM pyridoxal 5′-phosphate (PLP) and no yeast extract (Jakobsen et al., 2009). The medium optimized for L-glutamate and GABA production was MVcM medium in which the final magnesium concentration was reduced from 1 mM to 0.04 mM, 200 μM PLP and no yeast extract were added.

2.4. Determination of amino acid concentration

For the analysis of amino acids concentrations, 1 ml of the culture sample was taken from the bacterial cultures and centrifuged for 10 min at 13,000*g*. Extracellular amino acids were quantified by means of high-pressure liquid chromatography (1200 series; Agilent Technologies Deutschland GmbH). The samples underwent pre-column derivatization with *ortho*-phthaldialdehyde (OPA),

Please cite this article in press as: Irla, M., et al., Methanol-based γ-aminobutyric acid (GABA) production by genetically engineered *Bacillus methanolicus* strains. Ind. Crops Prod. (2016), http://dx.doi.org/10.1016/j.indcrop.2016.11.050