Ethanol Production in Actinomycetes after Expression of Synthetic *adh*B and *pdc*

Uwe Hardter^, Marta Luzhetska^, Sandra Ebeling and Andreas Bechthold*

Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften, Pharmazeutische Biologie und Biotechnologie, Stefan-Meier-Straße 19, 79104 Freiburg, Germany

Abstract: Streptomyces strains are producing many important secondary metabolites, in many cases used as pharmaceutical drugs. Though the usage of these bacteria for the production of fuel has never been described. We could show, that the expression of two artificial ethanologenic genes of *Zymomonas mobilis* in different actinomycetes strains resulted in the production of ethanol. The synthetic genes *adh*B and *pdc* encoding an alcohol dehydrogenase and a pyruvate decarboxylase, respectively, were expressed in eight different *Streptomyces* strains. Best production was obtained using *Streptomyces coelicolor* A3(2) harboring both genes. By variation of the cultivation conditions, the amount of ethanol produced by the strain could be increased up to 2.6 g/l.

Keywords: Ethanol production, *Streptomyces*, synthetic biology, actinomycetes, alcohol dehydrogenase, pyruvate decarboxylase.

INTRODUCTION

The introduction of genes into an organism, allowing the development of novel biological functions, is defined as synthetic biology [1]. Important areas for synthetic biology approaches are biomedicine, the synthesis of biopharmaceuticals, the production of chemicals and biomaterials, the environment and energy. Energy sources, which derive in some way from biomass, are gaining great popularity and scientific importance [2]. For example, the world ethanol production increased around threefold in the last ten years from about 30,000 $x10^6$ liter in the year 2000 up to more than 80,000 x 10^6 liter in 2010 [3]. Ethanol is an important biofuel, which has considerable advantages in terms of sustainability, lower greenhouse gas emissions, and cost reduction opposite to fossil fuels. Bioethanol is mostly produced from corn-starch, especially in the U.S.. Main producers are *Zymomonas mobilis*, *Saccharomyces cerevisiae* and engineered *Escherichia coli* strains [2, 4], producing maximum ethanol amounts between 40 – 90 g/l. However, these microorganisms industrially used for large scale production of biofuels, currently are not able to utilize non-edible lignocellulosic feedstock but depend on the fermentable monosaccharide glucose. Thereby, the current biofuel production proves only marginally profitable and is furthermore disputed in ethical regard. Thus, intensive research effort has been employed to realize the use of lignocellulosic feedstock in biofuel production [5]. One approach to reach this goal is to introduce cellulolytic ability into common ethanol producing organisms as described by Linger *et al*. [7] who successfully expressed cellulolytic enzymes from *Acidothermus cellulolyticus* in *Z. mobilis* [6, 7]. Another strategy would be to express ethanol generating enzymes in bacteria which are known as cellulose utilizing organisms.

Streptomyces is a genus of the Actinobacteria with more than 500 species. Most of these species are producers of various secondary metabolites which have been developed as pharmaceutical drugs in many cases. In our opinion these bacteria are very valuable organisms for the production of biofuels. They have the capacity to withstand relatively low pH, high temperature, high sugar, salt and ethanol concentrations, as well as various other harsh conditions, which could be used to develop an advanced biocatalyst and improve the commercial competitiveness of fuel ethanol production. In addition, actinomycetes produce a broad range of interesting enzymes which allow the degradation of complex glycans, such as cellulose and hemicellulose. This might help to overcome the current problem of shortage of feedstock supply when starch or sugar is used.

As *Streptomyces* strains are not known to produce ethanol by nature, introduction of genes allowing the strains to perform an ethanologenic pathway is essential. Application of synthetic biology approaches in actinomycetes is still under development [8]. In this study we describe the application of synthetic biology to *Streptomyces*. Two synthetic genes were introduced into different *Streptomyces* strains enabling the strains to produce ethanol.

MATERIALS AND METHODOLOGY

Strains, Plasmids and Culture Conditions

Escherichia coli DH5α and *E. coli* XL1 blue (Life Technologies, Carlsbad) were used for propagating plasmid clones. Plasmids were passed through *E. coli* ET12567 (dam-, dcm-, hsds-, Cm+) to generate demethylated DNA

*Address correspondence to this author at the Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften, Pharmazeutische Biologie und Biotechnologie, Stefan-Meier-Straße 19, 79104 Freiburg, Germany;
Tel: (+49) 761-203-8371; Fax: (+49) 761-203-8383;
E-mail: andreas.bechthold@pharmazie.uni-freiburg.de
^Contributed equally

[9]. *E. coli* ET 12567 carrying the RP4 derivative pUZ8002 was used for intergeneric conjugations from *E. coli* to *Streptomyces*. *E. coli* strains were grown under standard conditions [10] on Luria-Bertani (LB) medium containing hygromycin (50 µg/ml), apramycin (50 µg/ml), carbenicillin (50 µg/ml) or kanamycin (30 µg/ml) for antibiotic selection as required. Exconjugands were grown on MS agar containing adequate antibiotics for selection.

Production cultures of *Streptomyces* strains: *S. cyanogenus* S136 [11], *S. sp*. Tü 6071[12], *S. fradiae* Tü 2717 [13], *S. cinnamonensis* [14] *S. collinus* [15], *S. coelicolor* CH999 [16], *S. lividans* 1326 [17], *S. coelicolor* M1154 [18] and *S. coelicolor* A3(2) [17] were grown at 28°C in 100 ml of liquid medium. HA medium (yeast extract, 4 g/l; malt extract, 10g/l; D-glucose, 4 g/l; $CaCl_2$,1 M), TSB medium (tryptic soy broth, 30 g/l), NL111 medium (Lab lemco meat extract, 20 g/l; malt extract, 100g/l; $CaCO_3$ 10g/l), NL 19 medium (D-mannitol, 20 g/l; soy flour,20g/l) and E1 medium (starch 20 g/l; D-glucose, 20 g/l; yeast extract, 2.5 g/l; pharmamedia cottonseed flour, 3 g/l; $CaCO_3$, 1g/l; NaCl, 2 g/l; $KHPO_4$, 1 g/l; $MgSO_4$, 1 g/l) containing appropriate antibiotics for selection had been tested for best production levels, which could be obtained using NL 111 medium.

For cultivation under "semi-anaerobic" conditions flasks were closed using parafilm.

GENETIC METHODS

Isolation of plasmid and chromosomal DNA, conjugation and other gene cloning techniques for actinomycetes were performed using protocols that have been described by Kieser *et al*. [14].

RNA was isolated using the SV Total RNA Isolation System from Promega GmbH, Mannheim, Germany. For RT PCR the QuantiTect Rev. Transcription Kit from Qiagen, Hilden, Germany was used.

The manipulation of *E. coli* DNA and other standard molecular biology procedures were performed as described previously [10]. Restriction enzymes were purchased from Promega GmbH, Mannheim, Germany.

All products were used according to manufacturers` instructions.

PLASMID CONSTRUCTION

Assembling of the nucleotide sequence of *adh*B and *pdc* according to the actinomycetes codon usage was performed by GenScript Corporation, Piscataway, NJ, USA. *Fdh*, as well adapted to the actinomycetes codon usage, was purchased from Mr. Gene GmbH, Regensburg, Germany. All three genes were ligated into pUC57, resulting in pUC57-*adh*B, pUC57-*pdc*, and pUC57-*fdh*, respectively. As expression vector, pUWL-H-tnp [19] was used. Gene *adh*B was cloned into the *Hind*III and *Xba*I sites of pUWL-H-tnp, resulting in pUWL-H-*adh* (tnp gene was removed by this cloning step). For construction of pUWL-H-*adh-pdc,* pUC-57-*pdc* was restricted by *Spe*I. The fragment containing *pdc* was ligated into the *Xba*I site of pUWL-*H-adh*. For coexpression of *adh*B, *pdc*, and *fdh,* pUWL-H-*adh-pdc-fdh* was used. The plasmid was generated by ligation of *fdh* into pUWL-H-*adh-pdc* using *Hind*III and *Cla*I restriction sites.

ETHANOL EXTRACTION AND ANALYSIS

After 5 days of incubation, cultures were centrifuged. Mycelium was separated from supernatant and dried for 48 hours at 60 °C. Dry weight was measured subsequently. Average mass of mycelium in cultures of *S. coelicolor* A3(2) x *adh-pdc* (*S. coelicolor* A3(2) containing pUWL-H-*adh-pdc*) was 15,4 g/100ml under aerobic conditions and 15,0 g/100ml under "semi-anaerobic" conditions. In case of *S. coelicolor* x pUWL-H, we measured 16,8 g/100ml under aerobic conditions and 15,0 g/100 ml under "semi-anaerobic" conditions, respectively.

Expression of introduced synthetic genes *adh*B and *pdc* was proved by RT PCR following RNA isolation

For ethanol analysis an internal standard (0.03 ml 1-propanol) was added to 30 ml of the supernatant. Extraction was performed with 10 ml ethyl acetate. After addition of sodium sulphate, samples were analyzed by GC/MS using a HP6890 series GC-system coupled to an Agilent 5973 Network Mass Detector. As column we used a capillary tube (Optima 1), 30 mx 25 mm, coated with 25µm dimethylsiloxan. As carrier gas, helium was used and the flow was 1ml/min. The temperature program started at 60°C and was held for 2 min, followed by a ramp of 10°C/min to 220°C, which was held for 10 min. The final temperature of 270°C was reached by a second ramp of 55°C/min and was held for 5 min. The injector and detector temperatures were set to 280°C, the injection volume was 1.0µl and the split was 10:1. Mass spectra were recorded. The acquisition mode was a MID (Multiple Ion Detector) monitoring ions *m/z* 31 and 45 for ethanol and 1-propanol (internal standard). Quantification was performed measuring the SIM area of the respective *m/z* 31 peak. Calibration of the system was performed using ethanol in a concentration of 0,1‰ to 5‰ (V/V) in ethyl acetate.

RESULTS AND DISCUSSION

Pyruvate is a common intermediate of the primary metabolism of many organisms. Two steps, catalyzed by a pyruvate decarboxylase and an alcohol dehydrogenase, are required to convert pyruvate into ethanol [20]. *Streptomyces* strains are not known to produce ethanol and the reason for this might be the absence of functional pyruvate decarboxylase and alcohol dehydrogenase genes [21, 22]. In contrast to many other bacteria the DNA of actinomycetes is very GC rich, with a GC content of 70-80% [17]. Very often AT rich genes cannot be expressed in actinomycetes efficiently, as the supply of tRNAs complementary to rare codons like UUA, UUU or AUA is not guaranteed, resulting in small amounts of protein and/or misincorporated amino acids [23, 24]. In order to coexpress a pyruvate decarboxylase gene (*pdc*) together with a NADH dependent alcohol dehydrogenase gene (*adh*B), two artificial GC-rich genes were generated. *Pdc* and *adh*B, both from *Z. mobilis, w*ere used as templates. 204 of 386 codons in *adh*B and 269 of 569 codons in *pdc* are considered rare codons as they are found with a frequency of less than 1% in the *S. coelicolor* A3(2) genome [24]. We adapted these genes to the *S. coelicolor* codon

usage, assuring a higher translational efficiency in Streptomycetes as these bacteria prefer G and C nucleotides in the wobble position [17]. By substituting 255 nucleotides within 1152 of the whole gene, a synthetic alcohol dehydrogenase gene (*adh*B) was designed *in silico* and synthesized by Gene Script Corporation, Piscataway, NJ, USA. For the synthetic pyruvate decarboxylase gene, 385 of 1707 bp were changed. Artificial genes were surrounded by suitable restriction enzyme sites, allowing the construction of pUWL-H-*adh-pdc*. In this plasmid, expression of both genes is controlled by the strong ermE promoter. pUWL-H-*adh-pdc* was introduced into *S. cyanogenus* S136, *S. sp.* Tü 6071, *S. fradiae* Tü 2717, *S. cinnamonensis, S. collinus, S. coelicolor* CH999, *S. lividans* 1326 and *S. coelicolor* A3(2) by intergeneric conjugation with *E. coli* harboring this plasmid.

Exconjugands of these strains were cultivated in NL19-, NL111-, E1- und HA medium. After 5 days ethanol production was not detectable in extracts of strains cultivated in NL19- and E1-medium. However, it was detectable in extracts of *S. coelicolor* A3(2) x *adh-pdc* (20mg/l) cultivated in HA medium and in extracts of *S. coelicolor* A3(2) x *adh-pdc* (510 mg/l), *S. fradiae* Tü 2717 x *adh-pdc* (158 mg/l), *S. cyanogenus S136* x *adh-pdc* (64 mg/l), *S. coelicolor* CH999 x *adh-pdc* (10 mg/l), and *S. lividans* 1326 x *adh-pdc* (2 mg/l) cultivated in NL111 medium. As the most promising results were obtained with S. *coelicolor* A3(2) x *adh-pdc* it was subsequently used for further experiments.

We observed that ethanol production could be increased to 1.5 g/l when D-glucose in a concentration of 4g/l was added to the medium and when the strain was cultivated for six days. Even more, when the strain was cultivated in a flask which was closed by parafilm to minimize ethanol evaporation and to achieve lower oxygen concentration in cultures ("semi-anaerobic" conditions) ethanol production was at 2.6 g/l (Fig. **1**). Pyruvate in a concentration of 1 mol/l did not influence ethanol formation (data not shown).

The enzyme Fdh from *Candida boindinii*, a formate dehydrogenase, is influencing the intracellular concentrations of both NADH and NAD^+ [25]. Expression of *fdh*, which was adapted to the *S. coelicolor* codon usage as described for the other two genes, led to a significantly higher ethanol production in *E. coli* [25, 26]. In our studies coexpression of *adh*B, *pdc* and *fdh* was performed in *S. coelicolor* A3(2) and ethanol production after addition of formate in different concentrations was investigated. We could not obtain higher ethanol amounts in any experiment (data not shown).

Also when *adh*B and *pdc* were expressed in *S. coelicolor* M1154, which has been described as suitable producing host for secondary metabolites [18], ethanol production was comparable to the production in *S. coelicolor* A3(2) x *adh-pdc* (data not shown).

Metabolic engineering, as well as synthetic biology have been aiming at creating industrial methods for the production of compounds and usually exploit rational model-supported, computed approaches for the design of experiments. This approach has resulted in some very successful industrial projects with some model bacterial systems, in particular *E. coli* and *Bacillus subtilis*, where normal cellular metabolism may be altered to produce nonnatural metabolites or to shift the balance of synthesis toward a key metabolite of interest leading to novel production systems for vitamins and fine chemicals [1]. While metabolic engineering is widely used for the generation of novel natural products like antibiotics and anticancer drugs, the application of synthetic biology to actinomycetes has not been described very often. In our study we were able to introduce two artificial genes into *S. coelicolor* A 3(2), allowing the strain to produce ethanol up

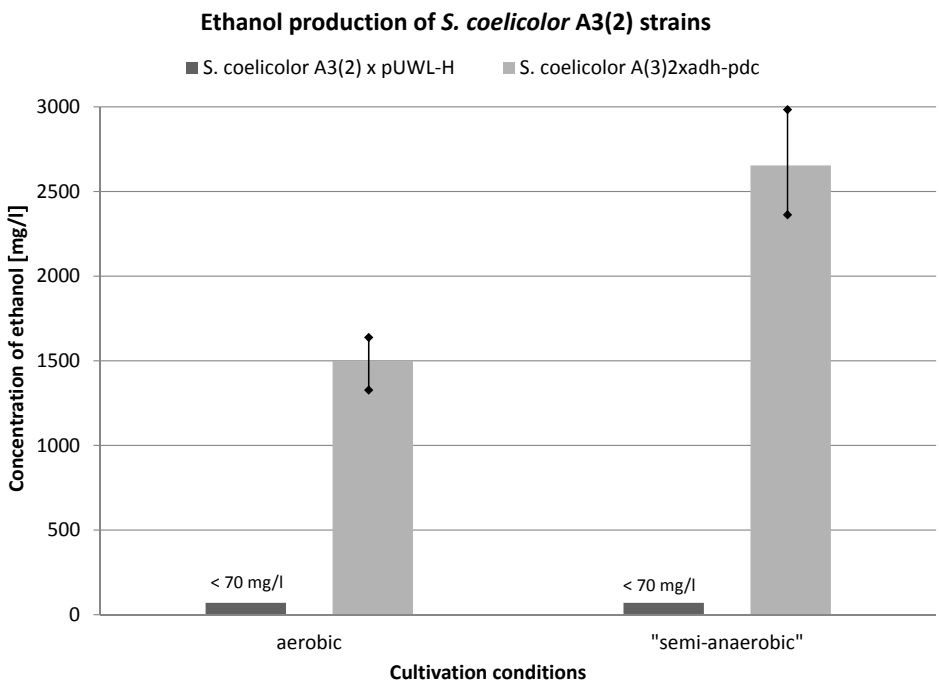

Fig. (1). Ethanol production of *S. coelicolor* A3(2) strains. Average amounts of ethanol produced in 10 individual experiments with *S. coelicolor* A3(2) x *adh-pdc* and *S. coelicolor* A3(2) x pUWL-H (2) under aerobic conditions and under "semi-anaerobic" conditions, respectively. The minimal ethanol concentration detectable by our methods was 70 mg/l.

to 2.6 g/l. Results of this study show that *Streptomyces* strains are promising candidates for synthetic biology approaches. It is probable, that Streptomyces strains should be able to produce much more ethanol. We think that one very promising strategy is to knock out genes encoding for enzymes of competing pathways of pyruvate as well as acetyl-CoA metabolism in order to provide more of these important intermediates for the synthesis of ethanol. The genus of *Streptomyces* contains a huge variety of species providing great opportunities to find better fitted strains for the production of ethanol than *S. coelicolor*.

We could show that *S. coelicolor* tolerates ethanol concentrations in the medium up to 3% [V/V], thus productions of 30 ml/l or 24 g/l, respectively, should be possible. Even if higher production levels could be achieved, ethanol could be removed from the media like frequently performed in current proceedings. Future work will indicate whether this proposal can be confirmed and these bacteria can be enhanced to feasible hosts for the production of biofuels.

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CONFLICT OF INTEREST

None declared.

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