

# CONSTRUCTION OF A NEW SHUTTLE VECTOR PZT1 APPLICABLE TO HOSTS *ESCHERICHIA COLI* AND THE ETHANOL PRODUCING *ZYMOMONAS MOBILIS*

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**Abstract.** *Zymomonas mobilis* is a bacterium with high potential to produce ethanol via fermentation of glucose, sucrose or fructose. The need for broadening of its substrate spectrum requires the development of suitable host-vectors systems for gene cloning. Here we report the construction of a new shuttle vector, applicable to hosts *Escherichia coli* DH5 $\alpha$  and *Zymomonas mobilis* DSM424. It was based on pCR2.1-TOPO<sup>TM</sup> (*E. coli*) and contained the amplified and cloned replicon fragment (*dso ori* and *repA* gene) of 2.7 kb plasmid of *Z. mobilis*. As a reporter gene, cloned under *Plac* promoter control, was used *amyBL* encoding  $\alpha$ -amylase in *Bacillus licheniformis* strain 44MB82/G. The obtained results revealed good stability of the shuttle vector pZT1 in both hosts, as *E. coli* DH5 $\alpha$  possessed also a high amylase activity due to the extracellular expression of the reporter gene. *Z. mobilis* DSM424 was able to maintain the shuttle vector, but *amyBL* was very poorly expressed, thus indicating that *Plac* promoter is rather not suitable for gene expression in this organism.

**Key words:** Shuttle vectors, *Escherichia coli*, *Zymomonas mobilis*, Amylase

## INTRODUCTION

*Zymomonas mobilis* is a rod-shaped Gram-negative bacterium (Fig. 1), which was originally isolated from alcoholic beverages - the African palm wine, the Mexican 'pulque', and also as a contaminant of cider and beer in European countries. However, very shortly after its isolation it is considered as an alternative organism in large-scale fuel ethanol production.

Comparative laboratory and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast have indicated the suitability of *Z. mobilis* over yeasts due to the following advantages: its higher sugar uptake and ethanol yield, its lower biomass production, and its higher ethanol tolerance. In addition, *Z. mobilis* does not require controlled oxygen supply during the fermentation, and it is suitable for genetic manipulations [1]. The only limitation of *Z. mobilis* compared to the yeast is that its utilizable substrate range is restricted to glucose, fructose, and sucrose [2].

As a result, early studies on its genetic manipulation focused on extending its substrate range for ethanol production. Skotnicki et al. [3] first reported high frequency conjugal transfer of plasmids from *Escherichia coli* and *Pseudomonas aeruginosa*, and this was followed by expression of the *lacZ* gene and production of  $\beta$ -galactosidase in strains of *Z. mobilis* [4, 5]. However, the strain ZM6100 derived from this work was shown to progressively lose all plasmid markers in batch culture under non-selective conditions. Subsequently, a new strain, ZM6306, was developed in continuous culture, which showed 100% stability

for all plasmid markers when grown without selection pressure. Synthesis of  $\beta$ -galactosidase was induced in continuous culture by addition of lactose and increased ethanol production and unutilized galactose were shown [6].



Fig. 1. *Zymomonas mobilis* DSM424 cells (1000x)

Further studies to extend the substrate spectrum involved the cloning and expression of a  $\beta$ -glucosidase gene from *Xanthomonas albineans* [7] and  $\alpha$ -glucosidase gene from a *Bacillus* sp. [8], however enzyme expression levels were low. Several studies on ethanol production by wild-type strains of *Z. mobilis* on industrial starch-based raw materials have been reported. For instance, Poosaran et al. [9] evaluated a cassava-derived starch hydrolysate as a substrate.

The present study, aiming at simultaneous saccharification and fermentation of starch, describes a shuttle vector *E. coli*/*Z. mobilis* construction and cloning of  $\alpha$ -amylase gene of *B. licheniformis* under *Plac* promoter control.

## MATERIALS AND METHODS

### Media and cultivation conditions

*E. coli* DH5 $\alpha$  strain was cultured at 37°C in Luria-Bertani (LB) medium (Scharlau Chemie S.A., Barcelona, Spain). For LB plates 1.5% agar (Oxoid, Thermo Fisher Scientific, Hampshire, UK) was used. The antibiotics ampicillin and kanamycin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added.

*Z. mobilis* DSM424 was cultured at 30°C, in medium, containing (g/l): yeast extract – 10, bacto peptone – 10, glucose – 20.

The strains were cultivated on a rotary shaker. They were maintained at 4°C, or frozen at -80°C with 20% (w/w) glycerol.

### Isolation of genomic and plasmid DNA and PCR

Total genomic DNA was isolated from 24 h-old cells of *Z. mobilis* DSM424 with GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, USA), following manufacturer's recommendations.

Plasmid purifications were either by the maxi-preparative alkaline procedure (Sambrook and Russel 2001) or by GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific Inc.).

For PCR amplification of *ori/rep* region of pZM2 of *Z. mobilis* was used primer pair F 5'GTTCA

ATCTAGACCTGCCTTATCTCTTTCCCCAG3'  
and

R'GTTCAATCTAGACAAACCTAAAGATTGCA TAGTTCGGC3' with introduced *Xba*I site (underlined). For PCR amplification, PCR mixture of GeneAmp® High Fidelity PCR System (Applied Biosystems, CA, USA) in total volume 50  $\mu$ l, at final concentrations of primers 0.5 pmol/ $\mu$ l and template genomic DNA of *B. licheniformis* 44MB82/G - 2 ng/ $\mu$ l.

PCR was performed in QB-96 Satellite Gradient thermal cycler (LKB Vertriebs GmbH, Vienna, Austria) under the following temperature profile: 94 °C for 3.5 min, 35 cycles consisting of 94 °C for 1 min, 55 °C for 45 sec, 72 °C for 2 min, followed by final elongation at 72 °C for 5 min. The corresponding PCR products were visualized in 1% agarose gel.

Plasmid construct pCRamyBL, containing amylase encoding gene of *Bacillus licheniformis* was obtained previously [10].

### Bacterial transformation and amylase secretion assay

For *E. coli* DH5 $\alpha$  transformation was used TransformAid™ bacterial transformation Kit (Fermentas, Thermo Fisher Scientific Inc).

The transformation of *Z. mobilis* was by electroporation in buffer, containing 0.3 M sucrose and 0.2 mm cuvettes, using BioRad Micropulser. The successful pulse parameters were 2.9 mV and 5.4 msec.

Qualitative analysis of the ability of transformants to secrete amylase was performed at LB-plates, containing 0.5% soluble starch. After 24 h of cultivation at 37°C, the colonies were exposed to iodine vapours. Clear yellow color zones surrounded the positive clones.

## RESULTS AND DISCUSSION

*Zymomonas mobilis* is an ethanol-producing bacterium currently considered a major candidate organism for bioethanol production. The main problem of the species from biotechnological point of view lies in its inability to convert complex carbohydrate polymers like starch to ethanol. To solve this problem, genetic manipulation of *Z. mobilis* DSM424 for broadening the utilizable range of substrates has been attempted.

To be stably maintained, every shuttle vector must contain *ori* (double stranded origin) and *rep*-genes, typical for the different hosts – *E. coli* and *Z. mobilis* and insuring the plasmid replication.

Successful bifunctional construct was obtained by insertion of 1016 bp fragment carrying *dso* and *rep*-gene of the plasmid pZM2 (2749 bp) of *Z. mobilis* [11] into *Xba*I site of pCRamyBL vector (Fig. 2). The obtained shuttle construct was named pZT1 (Fig. 3).

The recombinant clones were selected in *E. coli* host by their resistance to ampicillin (50  $\mu$ g/ml). After isolation of plasmid DNA and restriction analysis, several *E. coli* DH5 $\alpha$  clones were selected for further analysis – *Xba*I treatment (Fig. 4A) and sequencing. They were used for transformation of *Z. mobilis* DSM424.

For selection of positive *Z. mobilis* clones was used their resistance to kanamycin (200  $\mu$ g/ml). By the reason that the strain contained several native plasmids, *Xba*I digest would be non-informative. Therefore, the presence of pZT1 in this host was proved by PCR amplification of *amyBL* gene with a template total plasmid content of the respective clone (Fig. 4, B). The obtained results revealed the presence and stability of the shuttle vector pZT1 in both hosts.

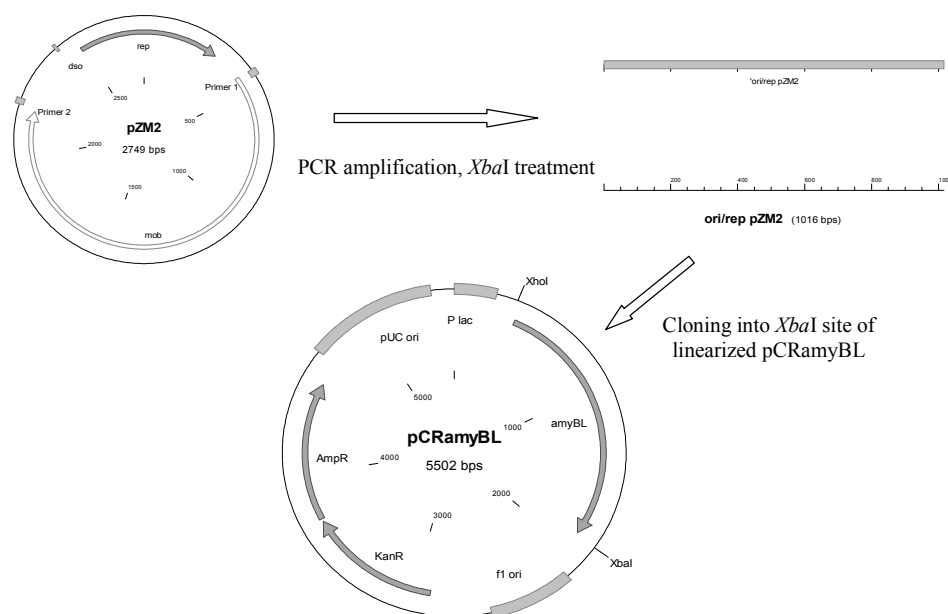


Fig. 2. Cloning scheme. The region, containing *dso* and *rep*-gene of the plasmid pZM2 (2749 bp) of *Z. mobilis* was inserted into *Xba*I site of pCRamyBL vector.

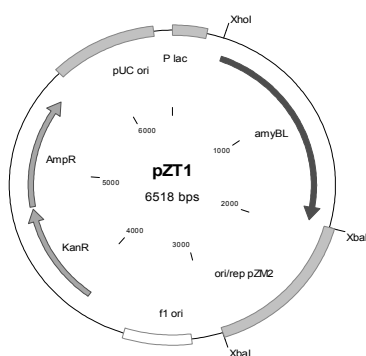


Fig. 3. Structural map of the shuttle vector pZT1.

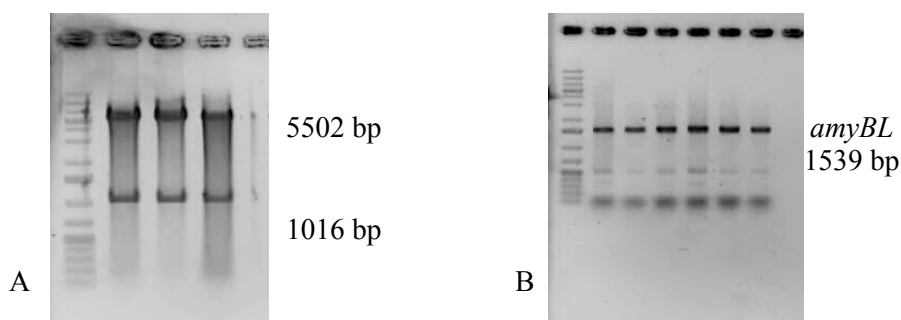


Fig. 4. Analysis of the recombinant clones in *E. coli* and *Z. mobilis*.

A) pZT1, isolated from *E. coli* DH5 $\alpha$  and digested with *Xba*I.

B) PCR amplification of pZT1-containing plasmid DNA isolations from *Z. mobilis* clones.

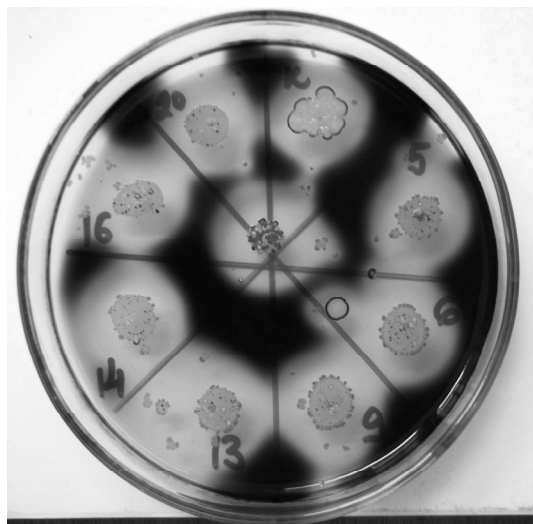


Fig. 5. *E. coli* DH5α recombinant colonies, cultured on starch-agar plates. Clear haloes surround the positive clones.

The subsequent analysis of the expression of amylase-encoding reporter gene showed that *E. coli* DH5α was able to produce great amount of extracellular amylase, evident by yellow haloes around colonies (Fig. 5).

*Z. mobilis* recombinants grew well on starch-containing media, but showed clearing only behind the colonies. The gene *amyBL* was very poorly expressed, thus indicating that *Plac* promoter is rather not suitable for gene expression in this organism. However, the stable maintenance of pZT1 in *Z. mobilis* made it good shuttle vector platform for cloning of powerful promoters, deriving from this host.

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## КОНСТРУИРАНЕ НА НОВ СОВАЛКОВ ВЕКТОР PZT1 С ГОСТОПРИЕМНИЦИ *ESCHERICHIA COLI* И ЕТАНОЛ-ОБРАЗУВАЩИЯ *ZYMONOMAS MOBILIS*

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**Резюме.** *Zymomonas mobilis* е бактерия с огромен потенциал като продуцент на етанол. Субстратният му спектър, обаче, е твърде ограничен – ферментира само глюкоза, захароза и фруктоза. Нуждата от разширяването му изисква създаването на подходящи системи за генно клониране. В настоящата работа е конструиран нов совалков вектор с гостоприемници *Escherichia coli* DH5α и *Zymomonas mobilis* DSM 424.

Векторът е произведен на pCR2.1-ТОРО™ (*E. coli*) и съдържа амплифициран и клониран (*dso ori* и *repA* gene) на 2.7 kb плазмид на *Z. mobilis*. Като репортерен ген в совалката под контрола на промотора *Plac* е клониран генът *amyI*, кодиращ  $\alpha$ -амилаза в *Bacillus licheniformis* щам 44MB82/G. Получените резултати показват добра стабилност на совалковият вектор pZT1 и в гостоприемника, като при *E. coli* DH5 $\alpha$  се наблюдава и силна амилазна активност поради успешната извънклетъчна експресия на репортерния ген. *Zymomonas mobilis* DSM 424 поддържа pZT1 в клетката, но не се наблюдава амилазна активност, което показва, че промоторът *Plac* не е подходящ за генна експресия в този микроорганизъм.

**Ключови думи:** совалкови вектори, *Escherichia coli*, *Zymomonas mobilis*, амилаза

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