Biocatalysis and Agricultural Biotechnology 13 (2018) 213–218

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Galacto-oligosaccharide hydrolysis by genetically-engineered alpha-galactosidase-producing *Pseudomonas chlororaphis* strains☆

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ARTICLE INFO

Keywords:
Biodegradable polymer
Biosurfactant
Raffinose
Stachyose

ABSTRACT

Various *Pseudomonas chlororaphis* strains have been shown to produce rhamnolipid, poly(hydroxyalkanoate), and antifungal compounds for plants. Ability to metabolize galacto-oligosaccharides would allow *P. chlororaphis* to use soy molasses as a low-cost fermentation feedstock. In this study, genetically engineered *P. chlororaphis* strains expressing a *Streptomyces coelicolor* α-galactosidase (*α-gal*) were constructed. In recombinant *P. chlororaphis* [chr::AG], the *α-gal* was integrated into the chromosome. *P. chlororaphis* [pBS-dAG], however, contains a truncated *α-gal* (coding for the N-terminal catalytic domain of the enzyme) on an expression vector. Real-time RT-qPCR showed 1,438-fold higher *α-gal* gene expression in [pBS-dAG] than [chr::AG]. In agreement with qPCR study, the results of an enzyme assay using *p*-nitrophenyl-α-galactopyranoside (p-NP-α-Gal) as a chromogenic substrate also showed that the cell extracts of [pBS-dAG] contained ca. 8-times higher p-NP-α-Gal-hydrolyzing activity than that of [chr::AG]. The cell extracts of [pBS-dAG] were also demonstrated to hydrolyze raffinose (32.7 ± 4.1% of the initial amount remained in the reaction mixture) > melibiose (65.4 ± 7.9%) > stachyose (72.8 ± 11.9%). The incubation of an EDTA-permeabilized (1.5 μM, 28 °C, 200 rpm shaking, 20 min) *P. chlororaphis* [pBS-dAG] whole-cell preparation with 0.5% (w/v) raffinose in a Medium E* for 7 days resulted in the reduction of the carbon source to 0.14% (w/v), or 28% relative to the initially added amount, and the biomass reached a value of 0.46 g CDW (cell dry weight)/l. In contrast, EDTA-permeabilized wild-type *P. chlororaphis* did not hydrolyze the 0.5% (w/v) raffinose in the medium, and the final biomass yield was 0.26 g CDW/l.

1. Introduction

The genus *Pseudomonas* includes countless species and strains of bacteria that are important to medical field and industrial sector. Among these species, *P. chlororaphis* is an interesting and valuable organism because of its metabolic capabilities to produce highly valued microbial products such as the antimicrobial biosurfactant rhamnolipids (Gunther et al., 2005; Solaiman et al., 2015), ecologically friendly bioplastic poly(hydroxyalkanoates) (Chung and Rhee, 2012; Solaiman et al., 2014a), and agriculturally important antifungal compounds (Thomashow et al., 1990; Dowling and O'Gara, 1994; Calderón et al., 2015). Genetic engineering could provide a straightforward and convenient approach to improve or add metabolic capabilities to *P. chlororaphis*. To this end, we have constructed and demonstrated the use of a gene-expression vector to successfully deliver heterologous genes by electroporation method into *P. chlororaphis* (Solaiman et al., 2015, 2016).

In this paper, we describe a study to construct genetically engineered *P. chlororaphis* strains to enable the organism to utilize galacto-oligosaccharides (GOs) as carbon source for cell growth and/or as substrates for the synthesis of its microbial products. Commonly found GOs in agricultural commodities or byproducts are raffinose, stachyose, and melibiose (Kuo et al., 1988). Of particular interest to us are the soy molasses byproduct stream generated from the processing of soybeans – the foremost vegetable oilseed crop produced by the United States (USDA-ERS, 2017a), and the beet sugar molasses byproduct – a high volume agricultural commodity in the United States with an estimated annual production volume of 4.6 Million Metric Tons in 2016/17 (USDA-ERS, 2017b). We had previously described our studies on the use of soy molasses as an economically attractive fermentative feedstock for the biosynthesis of sophorolipid biosurfactant by *Candida bombicola* (Solaiman et al., 2007) and of poly(hydroxyalkanoate) biopolymer by *P. corrugata* (Solaiman et al., 2006). Other researchers had also studied the use of soy molasses and other related soy-processing

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https://doi.org/10.1016/j.bcab.2017.12.008
Received 25 September 2017; Received in revised form 5 December 2017; Accepted 20 December 2017
Available online 21 December 2017
1878-8181/ Published by Elsevier Ltd.

byproducts as fermentation feedstocks for the production of microbial products (Hou et al., 2015; Loman et al., 2016; Karp et al., 2016; Cheng et al., 2017). The survey of 20 soybean genotypes showed that the three major sugars of this oilseed are sucrose (4.62 ± 0.17 mg/g soybean), raffinose (0.91 ± 0.04 mg/g), and stachyose (3.43 ± 0.02 mg/g) (Hou et al., 2009). The relative abundance of these sugars in the beans was fully reflected in the soy molasses (Solaiman et al., 2006). Metabolic capability to utilize GOs is therefore imperative in order to perform fermentation using soy molasses. α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) (α-Gal) is the key enzyme to breakdown the GOs by cleaving off the galactose unit at the reducing end of the oligosaccharides. Organisms belonging to the genus *Pseudomonas*, including *P. chlororaphis*, are seldom found to possess α-Gal activity (Katrolia et al., 2014). In a previous study, we had used a *Streptomyces coelicolor* α-galactosidase (*α-gal*) gene (Kondoh et al., 2005) as a heterologous marker to successfully demonstrate the function of a newly constructed expression vector in *P. chlororaphis* (Solaiman and Swingle, 2010). It is generally considered, however, that the integration of a heterologous gene into the chromosome of the host organisms would confer segregational stability in comparison to episomally replicating plasmid-borne recombinant. A chromosomal-integrant recombinant could also be stably maintained without the need to apply selection pressure such as the addition of antibiotics. In the present study, we therefore adapted a genetic transformation system to insert the *S. coelicolor α-gal* gene into the chromosomal DNA of *P. chlororaphis*. We further conducted a systematic characterization of the two recombinant *P. chlororaphis* strains containing, respectively, a plasmid-borne and a chromosomally integrated *α-gal* gene, leading to the confirmation of the ability of the *P. chlororaphis* [pBS-dAG] transformant to degrade galacto-oligosaccharides. The study laid down the foundation for future development of *P. chlororaphis* strains capable of metabolizing soybean GOs and the associated sugars such as sucrose and fructose to support production of bioproducts.

2. Material and methods

2.1. Bacterial strains and growth conditions

P. chlororaphis NRRL B-30761 (Gunther et al., 2007) was a patent strain deposited in the ARS Culture Collection (Peoria, IL). Commercially available competent *E. coli* strains such as but not limited to DH5α (Invitrogen, Carlsbad, CA) and STELLAR (Clonetech Labs, Mountain View, CA) were purchased and used in routine plasmid propagation and maintenance. Plasmids pET28-*α-gal* and pET28-Δ*α-gal* containing a *Streptomyces coelicolor α*-galactosidase (*α-gal*) and its N-terminal catalytic domain (Δ*α-gal*), respectively, were supplied by Prof. H. Kobayashi (Kondoh et al., 2005). The construction of the expression vector pBS29-P2-Δ*α-gal* was described in Solaiman and Swingle (2010). The suicide delivery vector pUC18-mini-Tn7T-LAC and a helper plasmid pTNS-2 used for inserting a copy of the heterologous *α-gal* gene into the chromosomal DNA of *P. chlororaphis* were a kind gift of Prof. H.P. Schweizer (Choi et al., 2005). Bacteria were cultured in LB medium (tryptone, 1% w/v; yeast extract, 0.5% w/v; NaCl, 0.5%) at 30 °C (*P. chlororaphis*) or 37 °C (*E. coli*) with orbital shaking at 200–250 rpm. LB-Agar medium was prepared in LB by adding 1.0–1.2% Bacto™ agar to the liquid medium. Recombinant bacteria were appropriately selected, cultured, or maintained in culture media containing tetracycline (Tc; 12 μg ml^{-1}), carbenicillin (Cb; 50 μg ml^{-1}), kanamycin (Km; 35 μg ml^{-1}), or gentamycin (Gm; 35 μg ml^{-1}).

2.2. Molecular biological procedures

All molecular biological procedures were performed in principles as described (Ausubel et al., 1987). Enzymes used in these procedures were purchased from commercial sources. Plasmids and genomic DNA samples were isolated using commercial kits such as GenElute Plasmid Prep Kit and GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO). Oligonucleotides used as primers for PCR amplification and nucleotide-sequence determination were custom-ordered (Sigma-Aldrich). Transformation of chemically competent *E. coli* and of *P. chlororaphis* was carried out according to supplier's instructions and by electroporation (Solaiman and Swingle, 2010), respectively. Nucleotide sequence determination was performed using an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp, Carlsbad, CA).

2.3. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays

The expression of cloned genes in *P. chlororaphis* transformants was studied using a real-time RT-qPCR procedure. Briefly, 1–2 bacterial colonies on an agar plate were used as inoculum to prepare an overnight-culture (5-ml LB medium + antibiotic as appropriate). The next day, a fresh culture was prepared in same medium at a 1/20th-dilution using the overnight-culture as inoculum. At ca. 3 h of incubation when cells were at a log-phase growth, 1 ml of the culture was transferred to a fresh tube and centrifuged (13,400 g, 2 min, RT) to collect the cells. Total RNA was isolated from the cell pellet using a RiboPure-Bacteria (Ambion, ThermoFisher Scientific, Philadelphia, PA) rapid RNA isolation kit according to manufacturer's protocol. After a DNase I treatment, the cDNA was then synthesized from the total RNA using a SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen, ThermoFisher Scientific) according to procedures supplied by the manufacturer. The oligonucleotide primers used in the qPCR reactions to amplify genes of interest were designed using NCBI/Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed on 26-October-2016) (Supplement Table S1). RT-qPCR of the cDNA was performed on a 96-well microplate using the QuantiNova SYBR Green PCR Kit (QIAGEN, Valencia, CA). A Realplex^2 Mastercycler epgradient S instrument and accompanied built-in software (Eppendorf, Hauppauge, NY) were used to monitor the reactions and to analyze the data. Reactions without reverse transcriptase were used as negative controls. The ΔCt, ΔΔCt, and 2^{-fx} values were calculated and used to evaluate relative levels of gene expression (Pfaffl, 2001).

2.4. In vitro α-galactosidase activity assays

α-Galactosidase enzyme (α-Gal) activity of wild-type and recombinant *P. chlororaphis* was determined using a chromogenic substrate p-nitrophenyl α-*D*-galactopyranoside (p-NP-α-gal; Sigma-Aldrich). A colony of bacterial cells from an agar plate was used to inoculate 2 ml of LB media. The culture was incubated at 30 °C and 200 rpm for an overnight period (~16 h). The entire culture was added as an inoculum into 50 ml of an appropriate culture medium and antibiotic in a 125-ml Erlenmeyer flask. Cells were grown at 30 °C and 150–200 rpm for 3–4 days. Cell culture density was measured by absorbance at 600 nm in a 10-mm path-length cuvette. Culture was centrifuged (5000 g, 15 min, 4 °C) to separate the spent medium (i.e., supernatant) and the cells (i.e., pellet). The supernatant was concentrated using a centrifugal filtration device (Amicon Ultra 0.5 Centrifugal 3 K; Sigma-Aldrich), and the retentate fraction was used to determine the extracellular α-Gal enzyme activity present in the culture broth. The cell pellet was lysed using the CelLytic B Cell Lysis Reagent (Sigma-Aldrich), and the lysate was used to determine the intracellular α-Gal enzyme activity of the cells. α-Galactosidase enzyme activity was assayed on a 96-well microplate using p-NP-α-gal as a substrate based on a previously described method (Kondoh et al., 2005). Briefly, 80 μl of a 12.5 mM p-NP-α-gal solution (in 0.1 M HEPES pH 7) was mixed with 20 μl of the test sample in the well of the microplate. The microplate was incubated for 15–30 min at 60 °C and 300 rpm-shaking in a ThermoMixer C (Eppendorf). The reaction was terminated by adding 100 μl of freshly prepared 0.2 M Na_2CO_3 solution. The reaction product (i.e., p-NP) was determined by absorbance reading at wavelength of 400 nm