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DESIGNING A NEW RECOMBINANT STRAIN WITH ADDITIONAL COPY NUMBER OF DSZ CLUSTER TO ENHANCE BIODESULFURIZATION ACTIVITY IN *PSEUDOMONAS* AERUGINOSA ATCC 9027*

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Abstract – The combustion of sulfur-rich fossil fuels leads to the release of acid rain-causing sulfur dioxide into the environment. The aim of this study was to enhance the efficiency of biodesulfurization using *Pseudomonas aeruginosa* through the duplication of the *dsz* cluster in this organism. One copy of the dsz cluster was inserted by a tri-parental mating method into the chromosome of *Pseudomonas aeruginosa* ATCC 9027 strain, and another copy in the form of a plasmid was cloned under control of a *tac* promoter. The recombinant *Pseudomonas* sp. was able to desulfurize dibenzothiophene more efficiently than the strains which contain only one copy of *dsz* cluster. Efficiency of dibenzothiophene (DBT) desulfurisation was measured through the release of 2-hidroxybiphenyle (2-HPB).This is the first time that an increase in desulfurisation activity through gene duplication has been shown.

Keywords – Biodesulfurization, *Pseudomonas aeruginosa*, dibenzothiophene

1. INTRODUCTION

The combustion of sulfur-compounds in petroleum leads to the release of sulfur dioxide, which is a principal cause of acid rain. In industrial applications, the specific cleavage of the carbon-sulfur bonds is very important as the calorific value is retained [1]. Removal of inorganic sulfur from fossil fuels is possible by physical, chemical or biological means. Physical and chemical methods are expensive [2], meanwhile biodesulfurization (BDS) is a process which is cheap and economic for the desulfurization of fossil fuel [1-3]. Till now, several *Rhodococcus* and *Corynebacterium* strains have been reported to remove sulfur from DBT without ring destruction [4].

Rhodococcus erythropolis IGTS8 was the first strain in which biodesulfurization was characterized at the molecular level [1].

In order to enhance biodesulfurization, recombinant *Pseudomonas* strains have been designed with modifications made to the desulfurization operon because they have a high tolerance to solvents [1]. Part of this tolerance is due to the production of a rhamnolipid biosurfactant [1, 5]. This industrial trait also increased the DBT desulfurization rate when the *dsz* cluster was engineered into a rhamnolipid-producing *Pseudomonas aeruginosa* PG201 [1].

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In this study, the *dsz* cluster under control of a *tac* promoter was cloned into the rhamnolipid-producing strain *Pseudomonas aeruginosa* ATCC9027 at a high copy number in order to produce strains capable of increased biodesulfurization.

2. MATERIALS AND METHODS

a) Plasmids and bacterial strains

The bacterial strains used are *Escherichia coli* HB101 [6], *Pseudomonas aeruginosa* EGSOX, *P. putida* KT2442 (pESOX3), *Pseudomonas aeruginosa* ATCC 9027, and the plasmid used are pRK600, pESOX3, pESOX4, pVLT31, pBSL118 [1].

b) Media and growth conditions

The sulfur-free medium used in this study was a modification of the standard basal salt medium (BSM): (g/*l*) $KH_2PO_4.6H_2O$ (2.44), Na_2HPO_4 (5.57), NH_4Cl (2.0), $MgCl_2.2H_2O$ (0.2), $CaCl_2.2H_2O$ (0.001), $FeCl_3.6H_2O$ (0.001), $MnCl_2.4H_2O$ (0.004). Glycerol (6.4 ml/*l*), or occasionally citrate (2 g/*l*), was added after sterilisation of the medium [7].

Pseudomonas and *Escherichia coli* strains were grown in Luria-Bertani (LB) (Difco) at 30ºC and 37ºC, respectively. Transformants were selected on LB agar supplemented with 1.5% (w/v) agar containing ampicillin (50 µg/ml), or tetracycline (10 µg/ml). Transconjugants were incubated at 30ºC.

c) Gibb's assay

This assay was accomplished according to the method of Oldfield *et al*. [8]. Briefly, bacteria were cultured in LB medium. The bacteria were harvested, the pellet washed twice in 50 mM HEPPS, pH 8.0, and resuspended in it to obtain an OD_{600} of 1.0. Substrate (25 µM final concentration of DBT, from a 40 mM stock solution in acetone) was added to 40 ml fresh cell suspension. A 1.0 ml of the sample culture supernatant was collected on 0,12,24,36,48,60,82 hour time intervals and stored at 4ºC. Gibbs reagent (10 µl of a 10 mM stock solution in acetone) was then added to each cuvette. A blank solution (50 mM HEPPS, pH 8.0) plus 10 µl Gibbs reagent) was also prepared. All samples were incubated overnight at 30ºC for full colour development and the A_{610} was measured.

d) Southern blot transfer and hybridization

DNA hybridization was performed according to the method described by Sambrook *et al*. [6]. Genomic DNA from a recombinant strain was digested and separated on a 0.7% agarose gel, then incubated in 0.25 M HCl at 20ºC for 10 min, in the denaturation solution(1.5M NaCl, 0.5M NaOH) for 45 min and in the neutralization solution(1M Tris PH:7.5, 1.5M NaCl) for 30 min. The hybridization between the Dig-labeled probe and immobilized DNA on the membrane was carried out using the procedure as described by Sambrook *et al*. [6].

3. RESULTS

The tri-parental filter mating method was done using *E. coli* CC118λpir (pESOX4) as the donor, *E. coli* HB101 (pRK600) as the helper, and *P. aeruginosa* ATCC9027 as the recipient. *P. aeruginosa* ATCC9027: mini Tn*5*Km (*ptac*: *dsz*) transconjugants that isolated and confirmed by an assay for sulfur bioavailibility and Gibb's assay, designated as *P. aeruginosa* PTSOX. The result of Southern blot of chromosomal DNA from this construct is shown in Fig. 1. The results here confirmed that the

dsz cluster was functional and stably inserted in a single copy into the chromosome of *P. aeruginosa* ATCC9027.

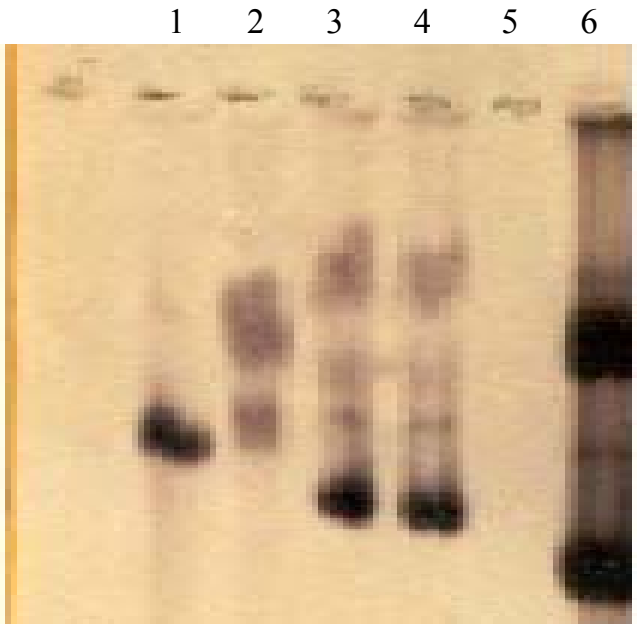

Fig. 1. Southern hybridization experiment, Blots of chromosomal DNA from *Pseudomonas aeruginosa* EGSOX (Lane 1and 2) and pESOX4 plasmid (lane 6) are as positive controls, blots of chromosomal DNA from *Pseudomonas aeruginosa* ATCC 9027 (PTSOX) (Lane 3 and 4), blot of chromosomal DNA from *Pseudomonas aeruginosa* ATCC9027 (Lane 5) as a negative control, *Rhodococcus erythropolis* IGTS8 *dsz* operon was used as probe

For an additional copy number, the pESOX3 plasmid was selected to clone into *P. aeruginosa* ATCC9027 (PTSOX). To this end, *E. coli* DH5α (pESOX3) was used as the donor, *E. coli* HB101 (pRK600) was used as the helper and the *P. aeruginosa* ATCC9027 (PTSOX) was used as recipient in a tri-parental mating.

P. aeruginosa ATCC9027 (PTSOX) cells harboring plasmid pESOX3, containing a copy of the *dsz* cluster in the chromosome and another copy carried on a plasmid, were chosen and named *P. aeruginosa* ATCC9027 (PTSOX34).

The time course for DBT desulfurization, for all the recombinant strains, was carried out by measuring the release of 2-HBP from DBT using Gibb's assay method. DBT desulfurization by the recombinant strains *P. aeruginosa* ATCC9027 (PTSOX) and *P. aeruginosa* ATCC9027 (PTSOX34), in comparison to that of the wild-type strain *Rhodococcus* sp. strain FMF, as a positive control and *P. aeruginosa* ATCC9027 as a negative control are shown in Fig. 2. After 12 hours of incubation, the wild-type strain of *Rhodococcus* demonstrated less degradation of DBT compared to the recombinant strains. After 84 hours there was little overall difference in the amount of 2-HBP produced by the reombinant *P. aeruginosa* strains, although with two copy numbers it was consistently more productive. The parental strain of *P. Aeruginosa* ATCC 9027 showed no production of 2-HBP at all. Sulfur bioavailability assays [8], indicated that DBT was being used as a sulfur source and not as the sole carbon source by this organism.

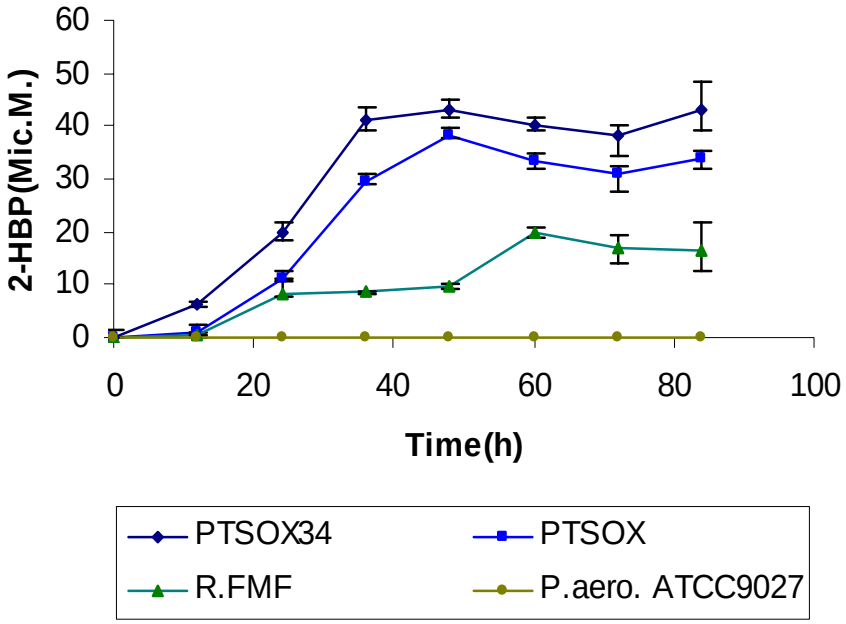

Fig 2. Standard Gibbs assay. DBT utilization by the parental strain *Pseudomonas aeruginosa* ATCC9027 (●) as a negative control, *Pseudomonas aeruginosa* ATCC9027 (PTSOX34) (◆), *Pseudomonas aeruginosa* ATCC9027 (PTSOX) (■), the wild type strain *Rhodococcus* sp. strain FMF (▲)

4. DISCUSSION

One of the major problems with using bacteria for biodesulfurization is that oil is toxic to the bacterial species used. Bacteria capable of desulfurization have to be able to function in a highly hydrophobic environment and have to be able to withstand the solvents used in the separation process. The *P. aeruginosa* strain used in this study has several advantages for future industrial applications, such as a higher level of solvent tolerance than similar microorganisms [1, 5]. In addition, *P. aeruginosa* ATCC9027 is known to produce a rhamnolipid biosurfactant, which accelerates the two-phase separation step in the biodesulfurization process through increased emulsification, wetting, and phase separation, and a reduction in viscosity [1]. The most studied bacterium capable of desulfurization is *Rhodococcus erythropolis*, which is hydrophobic in nature and not resistant to solvents [3]. This organism has been well characterised at the molecular level and the genes involved in desulfurization, the dsz cluster have been described [7].

In this paper, we report for the first time that increasing the copy number of the increase in the *dsz* gene cluster increases the desulfurization activity of organisms with potential use as biocatalyts. Although the location of genes (chromosomal or plasmid) make no significant difference in DBT metabolism, increasing the copy number of the genes involved leads to a small but reproducibe increase in desulfurization. DBT metabolism as measured by the production of 2-HBP in the recombinant strain *P. aeruginosa* ATCC 9027 (PTSOX34), which has *dsz* genes in its chromosome and on a plasmid, has the highest level of desulfurization, whilst that containing chromosomal gene *P. aeruginosa* ATCC 9027 (PTSOX) showed a reproducibly lower activity. The strain with no *dsz* genes was unable to grow in Gibbs assay conditions.

While it has been seen that increasing the copy number of the dsz cluster in *P. aeruginosa* would increase the desulfurization rate, this is not the case seen for strains that contained either a plasmid-located copy of dsz cluster or a chromosomally-located copy of the cluster.

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