ABSTRACT

Malic acid has a wide variety of applications in different industries and its demand has been increasing over the years. At present, malic acid is mainly produced by chemical methods which lead to various environmental sustainability concerns. Since it is naturally synthesized in the cell via TCA pathway, microorganisms offer an eco-friendly and cost-effective alternative for malic acid production. Additional advantages of microbial production include synthesis of pure L-form of malic acid. Several studies have been conducted earlier in different organisms. This study was an attempt to produce malic acid by genome engineering in *Zymomonas mobilis*.

The pyruvate decarboxylase enzyme, which constitutes significantly high amount of total protein in *Z. mobilis* and thus responsible for high production of ethanol, was selected for deletion to divert the flux towards malic acid. The replication and stability of pBBR1 and RK2 replicon was established in *Z. mobilis*. Recombineering was used and the *pdc* gene was replaced in the genome by a kan^R cassette by homologous recombination in the presence of a pSIM plasmid containing pBBR1 replicon and lambda *red* genes. The deletion of *pdc* gene was confirmed by PCR using region specific and gene specific primers and Southern blotting and hybridization. As a result of *pdc* gene deletion, malic acid production increased compared to that in wild type strain and more than 50% of theoretical yield was obtained. But the deletion resulted into disturbed redox balance due to which growth was hampered. The Δ*pdc* mutant cells were also found to be shrunken.

As an alternative strategy, gene encoding malic enzyme from *Escherichia coli* (*Ecmae*) was expressed in *Z. mobilis* under different promoters. To select the appropriate promoters, genome-wide analysis of promoters was conducted and -10 and -35 box consensus sequences of *Z. mobilis* promoters were predicted. The P*chap*, P*pap* and P*pdc* promoters from *Z. mobilis* were selected. The strength of these promoters were determined and compared with P*tac*

promoter in *E. coli* by cloning gfp_{uv} gene downstream to them. The expression of gfp_{uv} was studied with respect to growth at different pH and temperatures. Based on the results, P*chap* and P*pdc* promoters from *Z. mobilis*, and P*tac* promoter from *E. coli* were used to express *Ecmae* gene in *Z. mobilis* to increase malic acid production. The mae^{+} recombinants were obtained by recombineering-based genomic integration of P*chap-mae*, P*tac-mae* and P*pdc-mae* sequences. Maximum malic acid yield was obtained in P*pdc-mae* recombinant, followed by P*tac-mae* and P*chap-mae* recombinants. In P*pdc-mae* recombinant, the yield of malic acid obtained in shake flask was ~31% of theoretical, while ~37% of theoretical yield was obtained in a batch fermenter, which were much higher than that in the wild type strain. This is the first report demonstrating the use of lambda *red* genes based recombineering for deletion as well as integration of genes. The methodology developed and the mutants of *Z. mobilis* constructed in the present study can be used for several other metabolic engineering applications.