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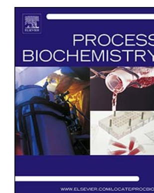

Rational design and medium optimization for shikimate production in recombinant *Bacillus licheniformis* strains

Siqi Zuo[a], Jun Xiao[a], Yuan Zhang[b], Xiaodong Zhang[a], Christopher T. Nomura[c], Shouwen Chen[a,*], Qin Wang[a,*]

[a] Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, Environmental Microbial Technology Center of Hubei Province, Hubei Key Laboratory of Industrial Biotechnology, College of Life Sciences, Hubei University, Wuhan 430062, PR China
[b] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, PR China
[c] Department of Chemistry, The State University of New York College of Environmental Science and Forestry (SUNY-ESF), Syracuse, NY 13210, USA

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ABSTRACT

Shikimate is a key intermediate for the synthesis of an anti-influenza drug sold under the trade name Tamiflu®. The metabolic pathway of shikimate has been extensively explored in *Escherichia coli*. This paper presents a rational strategy via cumulative disruption and overexpression of rate-limiting genes in *Bacillus licheniformis*, which is a GRAS (Generally Recognized as Safe) strain with high potential for the production of shikimate. The enhanced production of shikimate was achieved by the deletion of shikimate kinase (AroK) and pyruvate kinase (Pyk) as well as overexpression of transketolase (Tkt), 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase (AroA), and shikimate dehydrogenase (AroD) respectively and the amount of byproducts was reduced significantly. Furthermore, the medium was optimized by an orthogonal test, and the titer of shikimate (21.8 g/L) produced by the strain WX-02Δ*aroK*Δ*pyk*/pHY300-*aroD* increased by 56.8% compared to the original medium. This work revealed a new GRAS platform for the production of shikimate. These findings also suggest that *B. licheniformis* has great potential for the production of aromatic compounds of the extended shikimate pathway.

1. Introduction

Shikimate is a key chiral precursor for the synthesis of oseltamivir-phosphate, an anti-influenza drug sold under the trade name Tamiflu® [1]. Tamiflu® is used to treat common seasonal influenza A and B virus infections and for the treatment of both the avian virus type H5N1 and A/H1N1 influenza infections [2]. In 2009, it was reported that Tamiflu® sales was 3.5 billion dollars, with a production capacity of up to 33 million treatments per month and 400 million packages per year [3]. Although, current commercial production is mainly achieved via extraction from the *Illicium* plant, the isolation process is cumbersome and costly [4]. As an alternative process, microbial fermentation from inexpensive carbon source like glucose [5] or glycerol [6] for shikimate production has drawn more and more attention.

The shikimate pathway is the common route leading to the biosynthesis of aromatic amino acids and other aromatic compounds, which exists in bacteria, several eukaryotic organisms, and plants [7]. The synthesis of shikimate begins with the basic metabolites phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) which are converted by four enzymes in the shikimate biosynthesis pathway. As an intermediate, the concentration of shikimate in microorganisms is usually very low. Several microorganisms, especially *Escherichia coli*, have been modified by genetic manipulation and metabolic engineering approaches to produce shikimate [3,8,9].

Although *E. coli* has proven to be a model organism for these modifications, *Bacillus licheniformis,* a GRAS (Generally Recognized as Safe) strain, is an organism that has many benefits as a potential production strain for shikimate. *B. licheniformis* has been used as an industrial producer of bacitracin [10] and poly-γ-glutamate [11,12] and has recently been shown to produce high levels of other valuable chemical feedstocks such as acetoin [13] and 2, 3-butanediol [14,15]. In this study, the metabolic flow of *B. licheniformis* WX-02 (CCTCC M208065) [16] was modified to produce high levels of shikimate. This was accomplished by deleting genes (*pyk* and *aroK*) and overexpressing *aroD*, *aroA* and *tkt* respectively (Fig. 1). In addition to the aforementioned genetic modifications, the growth medium was also optimized for shikimate production. This study established an efficient system for the production of high shikimate titers with relatively lower amounts of

* Corresponding authors.
E-mail addresses: chenshouwen@hubu.edu.cn (S. Chen), qin.wang@hubu.edu.cn (Q. Wang).

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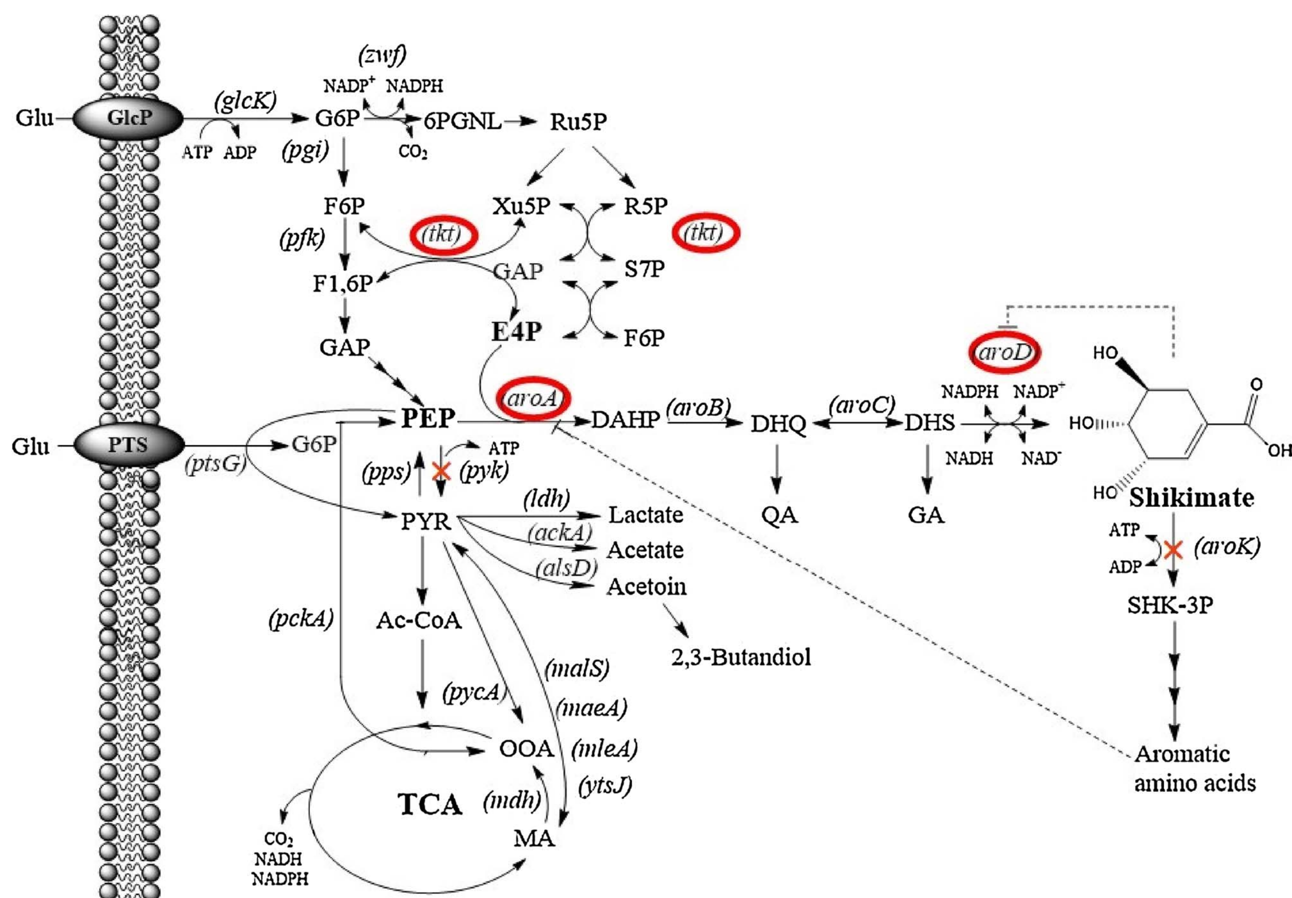

Fig. 1. The constructed metabolic pathway for enhanced shikimate titer in recombinant *B. licheniformis.* Inactivated genes are indicated with a cross and plasmid-expressed genes are circled (details in Materials and methods). Dotted lines show feedback inhibition for related enzymes. Abbreviations: Glu, glucose; GlcP, glucose/mannose: H+ symporter; G6P, glucose-6-P; F6P, fructose 6-phosphate; F1,6P, fructose 1,6-two phosphate; GAP, glyceraldehyde 3-phosphate; 6PGNL, 6-phosphogluconolactone; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; Xu5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; Ac-CoA, acetyl-coenzyme A; MA, malic acid; OAA, oxaloacetate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; QA, quinic acid; GA, gallic acid; SHK-3P, shikimate 3-phosphate; Genes and coded enzymes: *zwf*, glucose-6-phosphate 1-dehydrogenase; *pgi*, phosphoglucose isomerase; *pfk*, 6-phosphofructokinase I; *tkt*, transketolase I; *ptsG*, phosphotransferase system (PTS) glucose-specific enzyme IICBA component; *pyk*, pyruvate kinase; *pps*, phosphoenolpyruvate synthase; *ldh*, L-lactate dehydrogenase; *ackA,* acetate kinase; *alsD,* alpha-acetolactate decarboxylase; *pycA,* pyruvate carboxylase; *pckA,* phosphoenolpyruvate carboxykinase; *mdh,* malate dehydrogenase; *malS,* malate dehydrogenase (decarboxylating); *maeA,* malate dehyrogenase isozyme; *mleA,* malate dehydrogenase; *ytsJ,* malate dehydrogenase isozyme YtsJ; *aroA*, DAHP synthase isoenzymes; *aroB*, DHQ synthase; *aroC*, DHQ dehydratase; *aroD*, shikimate dehydrogenase; *aroK*, shikimate kinase.

byproducts and also provided potential for production of valuable aromatic compounds in *B. licheniformis.*

2. Materials and methods

2.1. Bacterial strains, media, and cultivation conditions

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. licheniformis* seed cultures were maintained in 250 mL flasks containing 50 mL LB medium at 37 °C, 180 rpm on a rotary shaker. The following antibiotics were added as appropriate to growth media: 25 μg/mL kanamycin, 50 μg/mL ampicillin and 20 μg/mL tetracycline. For shikimate production, cells were grown at 37 °C, 230 rpm with the fermentation medium consisted of (per liter) glucose 50 g, sodium citrate 10 g, yeast powder (ANGEL YEAST CO.,LTD) 2 g, K_2HPO_4 5.32 g, KH_2PO_4 6.40 g, $MgSO_4$ 0.60 g, $MnCl_2$ 0.005 g, $CaCl_2$ 0.003 g, $FeSO_4$ 0.003 g, $(NH_4)_2SO_4$ 4 g, pH 7.2.

2.2. Determination of cell growth and glucose consumption

In order to measure cell growth, 2 mL aliquots of fermentation broth were periodically collected from cell cultures and centrifuged at 12,000*g* for 5 min. The supernatants were removed and cell pellets were resuspended in the same volume of water for determination of cell density at 600 nm (OD_{600}) using a spectrophotometer. The concentrations of residual glucose in the spent media were measured using a SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P.R. China).

2.3. Construction of the gene deletion mutants of B. licheniformis

The vectors for two gene (*aroK, pyk*) deletion mutants were constructed based on the previous method [17]. Briefly, based on the nucleotide sequences surrounding the *aroK* gene of *B. licheniformis* WX-02, the primers (*aroK*-A-F/R, *aroK*-B-F/R) (Table 2) were designed to amplify the upstream (A) and downstream (B) homologous arms of *aroK* gene. The two fragments were ligated by spliced overlap extension PCR (SOE-PCR) with the primers *aroK*-A-F and *aroK*-B-R. The fused fragment was digested with *Spe* I and *Sac* I, and inserted into T2(2)-ori vector [14] to generate the plasmid T2Δ*aroK*. Since *pyk* gene is a key metabolic gene and is difficult to knock out, the spectinomycin resistance gene (*spc*^{r}) flanked by the upstream and downstream homologous arms of *pyk* gene was inserted into T2(2)-ori to generate the plasmid T2Δ*pyk:spc*.

Transformation of *B. licheniformis* WX-02 with the knock-out plasmid T2Δ*aroK* was performed via electroporation based on

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