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A combinational optimization method for efficient synthesis of tetramethylpyrazine by the recombinant *Escherichia coli*

Youqiang Xu[a,b,1], Chunyan Xu[a,b,1], Xiuting Li[a,b,*], Baoguo Sun[a,c], Ahmed Alaa Eldin[d], Yingmin Jia[d]

[a] Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology & Business University (BTBU), Beijing 100048, China
[b] Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology & Business University (BTBU), Beijing 100048, China
[c] Beijing Laboratory for Food Quality and Safety, Beijing Technology & Business University (BTBU), Beijing 100048, China
[d] School of Food and Chemical Engineering, Beijing Technology & Business University (BTBU), Beijing 100048, China

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ABSTRACT

2,3,5,6-Tetramethylpyrazine (TMP) is a functional chemical applied for curing cardiovascular and cerebrovascular diseases, and also a famous compound for adding food flavours. In this study, a combinational optimization method was used for realizing the efficient production of TMP. First, a combined engineering way was used to generate a recombinant strain designated *E. coli* BL15, which contained the selection of acetoin biosynthetic gene cluster and fine tuning of NADH oxidase expression. Second, medium formulation and aeration rate were optimized, and *E. coli* BL15 could efficiently convert the renewable substrate glucose to acetoin, one precursor of TMP, with 68.4 g/L by fed-batch fermentation, the record of acetoin production using *E. coli*. Thereafter, the preferred ammonium salt (the other precursor) and pH values of the initial solution were investigated for the production of TMP, respectively. Finally, 16.1 g/L TMP was achieved using diammonium hydrogen phosphate within 40 min under an initial pH value of 7.5.

1. Introduction

2,3,5,6-Tetramethylpyrazine (TMP, also named ligustrazine,) is a biologically active chemical originally isolated from the rhizome of a Chinese herbal medicine named *Chuanxiong* (*Ligusticum wallichii*) [1]. TMP has been identified to have pharmacological effects, which is recognized as an effective drug for cardiovascular and cerebrovascular diseases [2]. Besides, TMP is one kind of the main pyrazines detected in many fermented foods and drinks, such as Whiskey, Chinese *Baijius* (Chinese liquor), vinegar and soybean-based fermented foods [3,4]. TMP is generally considered as a safe substance and widely used in the food industry as a flavour ingredient for nutty, roasted and toasted tonalities [5].

Technologies for pyrazine production have been developed *via* Maillard reaction, which was a non-enzymatic chemical reaction between amino acids and reducing sugars [6]. However, such synthetic methods often lead to a high price of TMP because of the costs of the substrates, and also other drawbacks such as lots of byproducts [1]. In addition, consumers prefer natural products, especially when the chemical is applied to medicines or foods with concerns about the health. Therefore, attentions have been paid for the development of biotechnological processes for TMP synthesis [2,7]. Several microorganisms were shown to have the metabolic potential to biosynthesize TMP, including *Bacillus subtilis*, *B. licheniformins*, *Corynebacterium glutamicum*, and *Lactococcus lactis* [2,7–9]. These species could metabolically synthesize 3-hydroxy-2-butanone (also named acetoin, AC), which could be condensed with ammonia to produce TMP [10] (Fig. 1), an alternative way for the chemical reaction with moderate reaction conditions and less byproducts. However, attempts to use these microorganisms are us0ually hampered on commercial scale by the low concentration of TMP in the fermentation broth, resulting in a high cost of downstream processing [1]. The limiting step in microbial TMP production is the low concentration of AC, and attentions have focused on breeding of strains, optimization of medium formulation and culture conditions to enhance AC production [11].

AC can be metabolically synthesized using pyruvate as the direct substrate which originated from glycolysis (Fig. 1). First, Pyruvate is catalyzed to α-acetolactate by α-acetolactate synthase (AlsS), and followed by α-acetolactate decarboxylase (AlsD) to produce AC [12]. Two moles of pyruvate could be produced from one mole

* Corresponding author at: School of Food and Chemical Engineering, Beijing Technology and Business University. No. 11, Fucheng Road, Haidian District, Beijing 100048, China.
E-mail address: lixt@btbu.edu.cn (X. Li).
[1] These authors contribute equally to this work.

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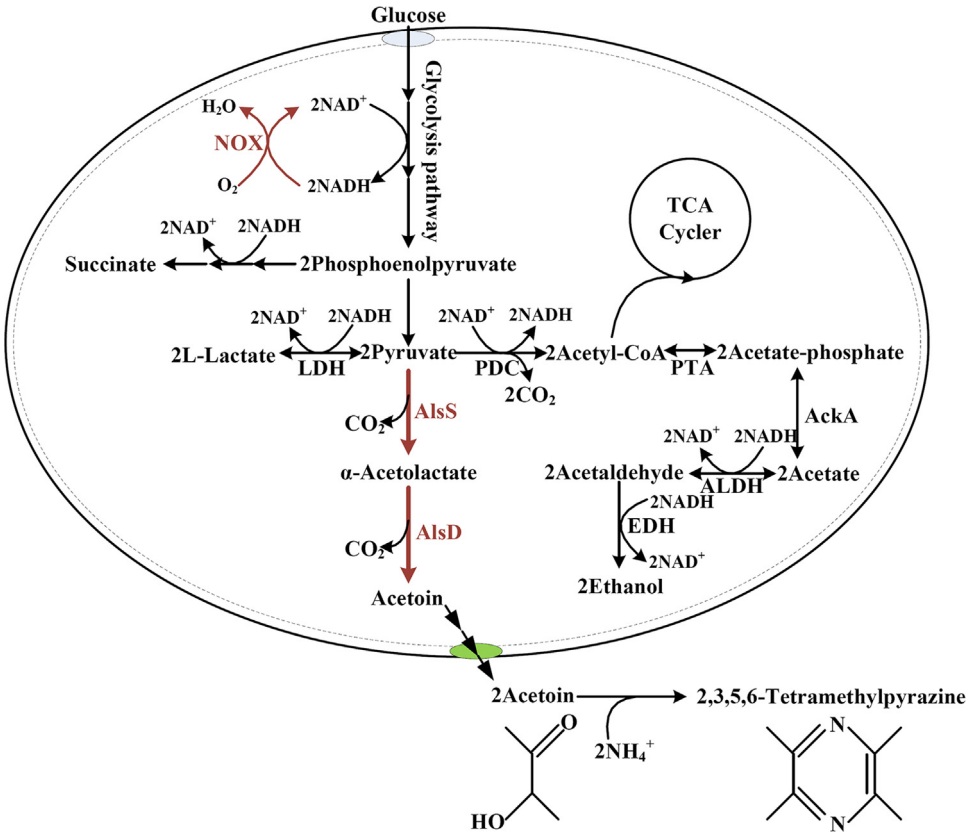

Fig. 1. Metabolic engineering of *E. coli* for 2,3,5,6-tetramethylpyrazine production. AlsS, α-acetolactate synthase; AlsD, α-acetolactate decarboxylase; NOX, NADH oxidase; LDH, lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; PTA, phospho-transacetylase; AckA, acetate kinase; EDH, ethanol dehydrogenase; ALDH, aldehyde dehydrogenase.

of glucose, and two moles of NADH are generated. Through the formation of one mole of AC from two moles of pyruvate, no NADH is consumed, which leads to the cofactor imbalance. Although the formation of byproducts such as succinate, lactate, acetate and ethanol could consume NADH, but it affects the production yield and also increases the difficulty of product purification. Several studies have been focused on solving the problem, such as the usage of electron acceptors or enzymes for rebalancing NAD^+/NADH [13–15]. Among the above methods, a water forming NADH oxidase is chosen to rebalance of *in vivo* cofactors NAD^+ and NADH in this work, which is identified from *Lactobacillus brevis*, and could be used in the AC synthetic process for improving the efficiency of AC production [15].

A suitable host strain is required for the efficient AC production. More recently, most of the studies have used genetically engineered wild type strains, which have limitations due to the lack of mature genetic engineering tools. *Escherichia coli* is a good alternative, since many genetic engineering tools have been developed [16]. The heterologous expression of genes will bring metabolic burden to the host cells, for example, the unsuitable expression of NOX will lead to the abnormal of NAD^+/NADH ratio *in vivo*, which greatly influence cell growth and metabolism [17]. Besides, the codon preference is a bottleneck for gene expression in *E. coli* [18]. Therefore, the selection of a compatible AC synthetic pathway gene cluster is crucial as well as the fine tuning of NOX expression for the construction of recombinant strains.

In this study, we carried out a combinational optimization method for TMP production. First, the AC synthetic pathway gene clusters from different bacteria were cloned and the recombinant strains were compared for their AC productions in order to select a preferred one. Second, the expression of gene *nox* was regulated by using promoters P_{T7} and P_{tac} and two different ribosome binding sites, respectively, and the translational efficiency was verified by altering the distance between the ribosome binding site and the initiation codon. Third, the fermentation medium formulation was optimized using the orthogonal test. Thereafter, the AC production was carried out using fed-batch fermentation with different aeration rates. Finally, different kinds of ammonium salts and the pH values of initial solutions were investigated to establish the efficient biological TMP synthetic method.

2. Materials and methods

2.1. Strains and media

Strains used in this study were listed in Table 1. *E. coli* DH5α was used for vector propagation. *E. coli* BL21(DE3) was used as the host strain for AC production. *Bacillus amyloliquefaciens* F3302, *B. licheniformis* F0022, *B. subtilis* F3303, *Enterobacter cloacae* F1001 and *Klebsiella pneumoniae* F3472 were all isolated from the *Daqus* used for brewing of Chinese liquor (Unpublished work). The genomes of the strains were extracted and used as the templates for the amplification of AC biosynthetic gene operons through polymerase chain reaction (PCR). pET-28a(+) was used as the vector for gene expression. LB broth was used as the culture medium during the construction of the recombinant strains, which contained 5.0 g/L yeast extract, 10.0 g/L tryptone and 10.0 g/L NaCl. The fermentation medium was used for AC production which contained 10.0 g/L yeast extract, 3.0 g/L $CH_3COONa{\cdot}3H_2O$, 0.60 g/L KH_2PO_4, 0.40 g/L $MgSO_4$, 0.10 g/L $MnSO_4$, 0.060 g/L $FeSO_4$. The pH was adjusted to 7.0 before sterilization. Thereafter, sterilized glucose solution (800 g/L) was added to a final concentration of 80 g/L glucose before inoculation.