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Screening of Acetyl Donors and the Robust Enzymatic Synthesis of Acetyl-CoA by 10-Deacetylbaccatin III-10-β-*O*-acetyltransferase

Bo-Yong Zhang, Hao Wang, Ting Gong, Tian-Jiao Chen, Jing-Jing Chen, Jin-Ling Yang and Ping Zhu *

State Key Laboratory of Bioactive Substance and Function of Natural Medicines, NHC Key Laboratory of Biosynthesis of Natural Products, CAMS Key Laboratory of Enzyme and Biocatalysis of Natural Drugs, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China; zhangboyong@imm.ac.cn (B.-Y.Z.); wanghao1986117@163.com (H.W.); gongting@imm.ac.cn (T.G.); chentianjiao@imm.ac.cn (T.-J.C.); chenjingjing@imm.ac.cn (J.-J.C.); yangjl@imm.ac.cn (J.-L.Y.)
* Correspondence: zhuping@imm.ac.cn; Tel.: +86-10-6316-5197

Abstract: Acetyl-CoA is the precursor of many bio-manufacturing products and is also the hub of the cellular metabolism of energy and substances. However, acetyl-CoA is not a bulk commodity and its application is hindered due to its high cost and low yield. In this study, we screened acetyl donor candidates and utilized 10-deacetylbaccatin III-10-β-*O*-acetyltransferase (DBAT) in the synthesis of acetyl-CoA with CoASH as the acetyl acceptor. Among the tested candidates, acetylsalicylic acid methyl ester was identified to be the best acetyl donor, followed by acetyl-trans-resveratrol, acetylsalicylic acid ethyl ester, acetylsalicylsalicylic acid, and 4-acetoxyacetanilide. The enzymatic reaction conditions were optimized and the maximum yield of acetyl-CoA reached 14.82 mg/mL, which is the highest yield among all reported approaches to date. Meanwhile, 4.22 mg/mL of the by-product salicylic acid methyl ester, which is another industrial material, was produced. Additionally, a preliminary purification process for acetyl-CoA was established, in which 40 mg acetyl-CoA (HPLC purity > 98%) was acquired from the finished 20 mL reaction system (feeding 46 mg CoASH and 34 mg ASME) with a recovery rate of 86%. Our study lays the foundation for the large-scale production of acetyl-CoA by an enzymatic approach and will promote its application in different fields.

Keywords: acetyl-CoA; enzymatic production; acetyl donor; acetylsalicylic acid methyl ester; 10-deacetylbaccatin III-10-β-*O*-acetyltransferase

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1. Introduction

Acetyl-CoA is a ubiquitous substance found in organisms and participates in various metabolisms of life. It converges three nutrients (sugar, fat, and protein) into one common metabolic route—the tricarboxylic acid cycle and oxidative phosphorylation, through which these nutrients are ultimately oxidized into CO_2 and water, with the concomitant production of ATP, a high-energy phosphate compound [1]. Acetyl-CoA (also written as acetyl-S-CoA) is an acetylated form of coenzyme A (CoASH), and the acetyl group is docked on the sulfhydryl residue of CoA by a high-energy thioester bond. Currently, this product is not a bulk commodity and is costly in the market. For example, the commercial price of acetyl-CoA is over $170 per 5 mg (MilliporeSigma).

The chemical approach to acetyl-CoA production is still impractical because of the high cost and low yield at present [2–6]. In comparison, the enzymatic approach has brought great interest, with the advantages of fast reaction rates, mild reaction conditions, great specificity, and an environmentally friendly process. Several enzymatic approaches have been proposed for the preparation of acetyl-CoA. For example, acetyl-CoA could be synthesized by phosphotransacetylase from acetyl phosphate and CoASH [7]. Irving

et al. utilized carnitine acetyltransferase to prepare acetyl-CoA from acetylcarnitine and CoASH [8]. In another approach, acetyl-CoA was synthesized by acetyl-CoA synthetase from acetic acid and CoASH supplemented with ATP and Mg^{2+} as the cofactors [9]. Acetyl-CoA can also be synthesized from citric acid and CoASH, catalyzed by citric acid lyase in the presence of ATP and Mg^{2+} [10]. Additionally, using pyruvate and CoASH as the substrates, acetyl-CoA can be synthesized by the combination of pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase in the presence of NAD [11].

Although these approaches have achieved the enzymatic synthesis of acetyl-CoA, the circumstances of high cost and low efficiency have not been fundamentally changed, including the requirement of expensive cofactors and the low availability of some substrates (e.g., acetylcarnitine). Cofactors could be omitted in the reaction of acetylphosphoric acid to acetyl-CoA, but acetylphosphoric acid is unstable, making it a limiting factor for efficient production. Thus, the development of a reasonable approach to the synthesis of acetyl-CoA is of great interest.

In our previous work [12], we found that 10-deacetylbaccatin III-10-β-*O*-acetyltransferase (DBAT) could not only acetylate 10-deacetylbaccatin III (10-DAB) into baccatin III with acetyl-CoA as the acetyl donor, but also de-acetylate baccatin III into 10-DAB under alkaline conditions. Moreover, when CoASH was present in the reaction, the acetyl group could be transferred from baccatin III to CoASH to form acetyl-CoA [12]. Enlightened by this phenomenon, we further investigated the possibility of using DBAT to efficiently produce acetyl-CoA with a proper acetyl donor under optimized reaction conditions. In this paper, we systematically investigated the enzymatic approach to producing acetyl-CoA using DBAT as the catalyst, which included the screening of proper acetyl donors and the optimization of reaction conditions. The yield of acetyl-CoA reached a maximum of 14.82 mg/mL.

2. Results

2.1. Screening of Acetyl Donors

The chemical structures of the acetyl donor candidates are shown in Figure 1. The results of the screening are summarized in Figure 2. The highest yield of acetyl-CoA (1.12 mg/mL) was produced in the acetylsalicylic acid methyl ester (ASME, 2) group, followed by the acetyl-trans-resveratrol (ATR, 9) group under the same conditions. Other reasonable acetyl donors were acetylsalicylic acid ethyl ester (ASEE, 3), acetylsalicylsalicylic acid (ASSA, 4), and 4-acetoxyacetanilide (AOA, 10). Under the same experimental conditions, the activities of DBAT against acetylsalic acid (ASA, 1), benorilate (BL, 5), acetylsalicylic anhydride (ASAN, 6), DL-lysine acetylsalicylate (DLA, 7), and carbasalate calcium (CSC, 8) were extremely low (Figure 2). Noticeably, compared with ASME or ASEE, ASA only lacks the methyl group or ethyl group, but the yield of acetyl-CoA was significantly reduced when ASA served as the acetyl donor (Figure 2).

2.2. Optimization of Conditions for Synthesis of Acetyl-CoA with ASME as the Acetyl Donor

Firstly, we tested a series of molar concentrations of the two substrates, CoASH and ASME, on the production of acetyl-CoA. We set the concentration of CoASH at 1 mM and found that when the concentration of ASME reached 3 mM, acetyl-CoA reached its highest yield of 0.61 mg/mL (Figure 3A). Then, we set up experiments with the ratio of CoASH/ASME at 1/3. The yield of acetyl-CoA reached a maximum of 2.15 mg/mL when CoASH/ASME concentrations were set at 6 mM/18 mM (Figure 3B). Under these conditions, we tested the effects of temperature on the synthesis of acetyl-CoA and found that the maximum yield of acetyl-CoA reached 3.11 mg/mL when the temperature was set to 37 °C (Figure 3C). Next, we examined the effects of pH on the synthesis of acetyl-CoA and found that the maximum yield of acetyl-CoA reached 4.14 mg/mL when the pH was set to 7.0 (Figure 3D). Meanwhile, we found that the CoASH in the reaction solution was less than 0.60 mg/mL. The influence of potassium salts on the production of acetyl-CoA was also studied. In this experiment, CoASH/ASME concentrations were set at 7 mM/21 mM

to ensure enough supply of the substrates. The optimum concentration of $K_2HPO_4 \bullet 3H_2O$ at 16 mM (32 mM K^+) was determined to yield 5.07 mg/mL of acetyl-CoA (Figure 3E). We also studied the influence of the molar ratio of KH_2PO_4 and $K_2HPO_4 \bullet 3H_2O$ on the production of acetyl-CoA. When KH_2PO_4 and $K_2HPO_4 \bullet 3H_2O$ were set at 1.7 mM and 7.2 mM, respectively, the production of acetyl-CoA reached a maximum of 5.65 mg/mL (Figure 3F). Under these conditions, we studied the influence of the DBAT amount on the production of acetyl-CoA. We found that DBAT concentrations could be set at over 1 mg/mL, and when the DBAT concentration was 2 mg/mL, the yield of acetyl-CoA reached 5.83 mg/mL (Figure 3G). Finally, we examined the effects of reaction time and found that the production of acetyl-CoA reached its maximum of 6.12 mg/mL when the reaction time was 2 h (Figure 3H).

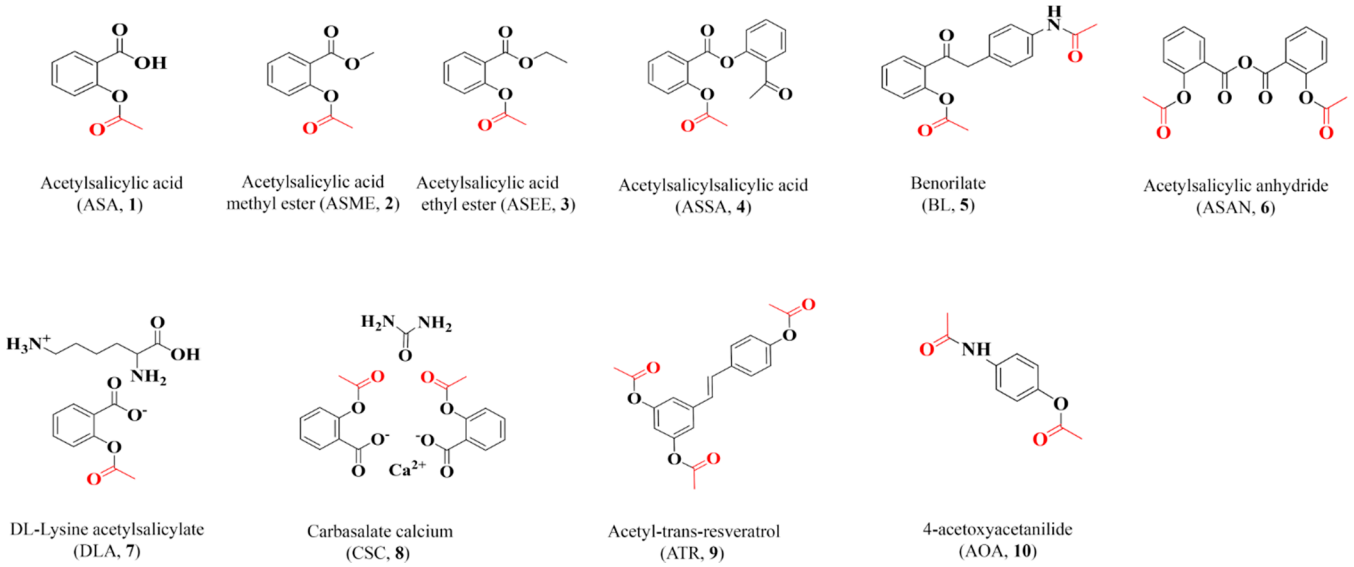

Figure 1. Chemical structures of the acetyl donor candidates.

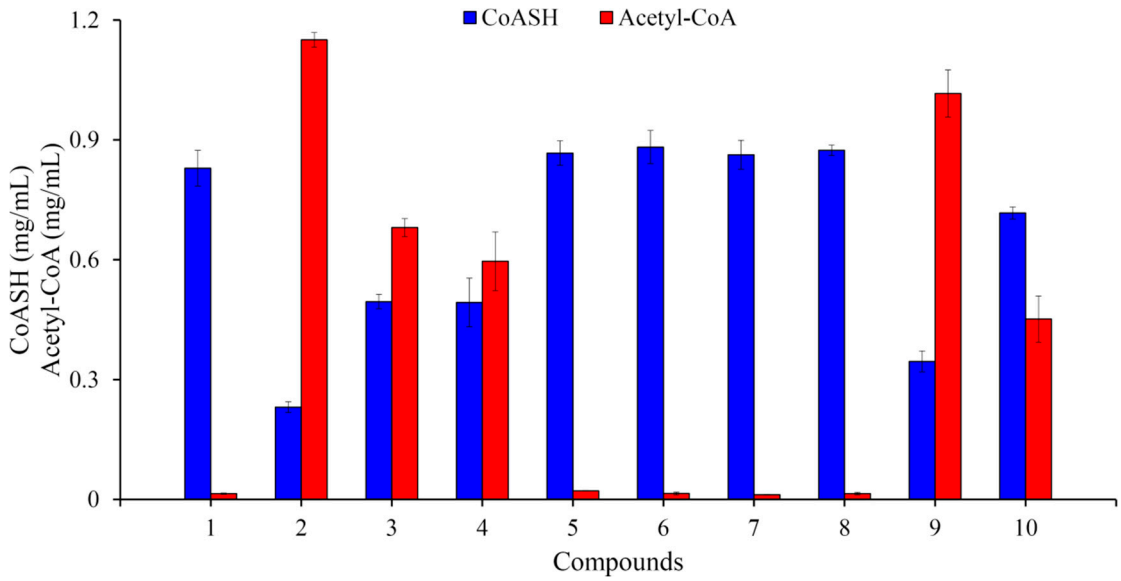

Figure 2. Screening of acetyl donors. 1, Acetylsalicylic acid (ASA); 2, acetylsalicylic acid methyl ester (ASME); 3, acetylsalicylic acid ethyl ester (ASEE); 4, acetylsalicylsalicylic acid(ASSA); 5, benorilate(BL); 6, acetylsalicylic anhydride (ASAN); 7, DL-lysine acetylsalicylate (DLA); 8, carbasalate calcium (CSC); 9, acetyl-trans-resveratrol (ATR); 10, 4-acetoxyacetanilide(AOA). The data represent the means ± SD, n = 3.

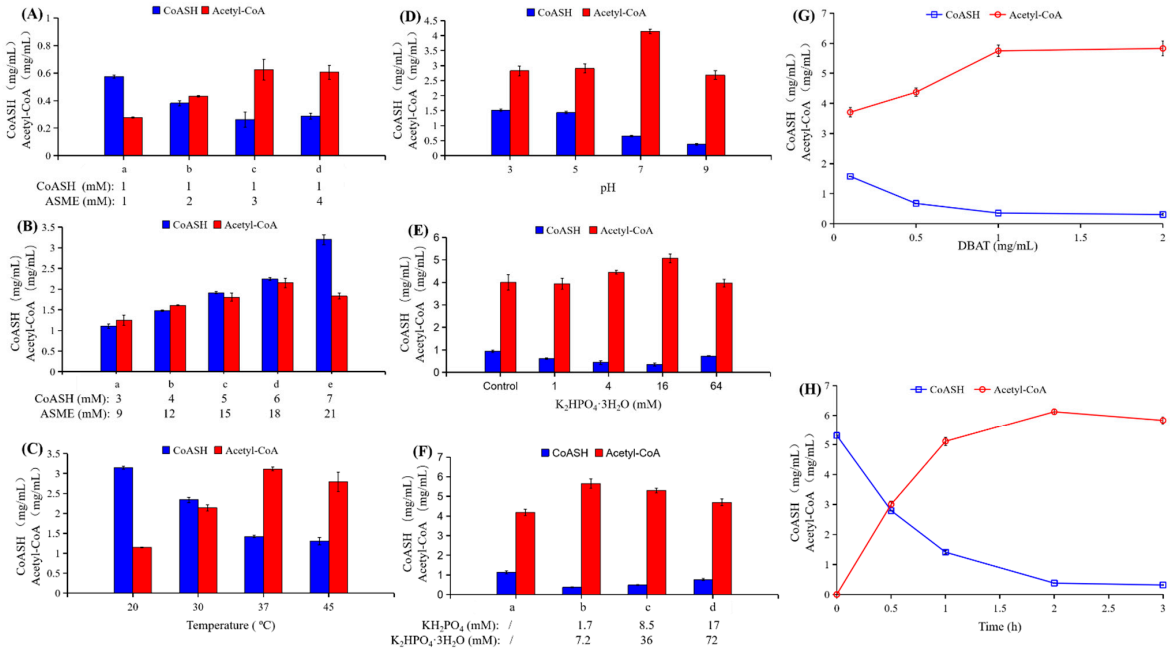

Figure 3. Optimization of DBAT-mediated acetyl-CoA in vitro biosynthesis with ASME as the acetyl donor. (**A**) The influence of different molar concentrations of CoASH and ASME on the production of acetyl-CoA (DBAT = 1 mg/mL). (**B**) The influence of different molar concentration combinations of CoASH/ASME on the production of acetyl-CoA. The molar ratio of CoASH/ASME was defined as 1/3 (DBAT = 1 mg/mL). (**C**) The influence of different temperatures on the production of acetyl-CoA (CoASH/ASME = 6 mM/18 mM, DBAT = 1 mg/mL). (**D**) The influence of different pH levels on the production of acetyl-CoA (CoASH/ASME = 6 mM/18 mM, DBAT = 1 mg/mL, at 37 °C). (**E**) The influence of different $K_2HPO_4 \bullet 3H_2O$ concentrations on the production of acetyl-CoA, (CoASH/ASME = 7 mM/21 mM, DBAT = 1 mg/mL, at 37 °C, pH 7.0). (**F**) The influence of different concentration combinations of $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ on the production of acetyl-CoA, (CoASH/ASME = 7 mM/21 mM, DBAT = 1 mg/mL, at 37 °C, pH 7.0). (**G**) The influence of different DBAT concentrations on the production of acetyl-CoA, (CoASH/ASME = 7 mM/21 mM, $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, at 37 °C, pH 7.0). (**H**) The influence of different reaction times on the production of acetyl-CoA, (CoASH/ASME = 7 mM/21 mM, $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, DBAT = 2 mg/mL, at 37 °C, pH 7.0). The data represent the means ± SD, n = 3.

2.3. Re-Optimization by Increasing the Concentrations of CoASH/ASME and DBAT

As shown in Figure 3H, the substrate of CoASH was almost exhausted after 2 h. Thus, we further increased the supply of CoASH/ASME to evaluate the catalytic capacity of DBAT on the synthesis of acetyl-CoA. We kept the ratio of CoASH/ASME at 1/3, and the yields of acetyl-CoA were positively correlated with the concentrations of CoASH/ASME. When concentrations of CoASH/ASME were at 25 mM/75 mM the yield of acetyl-CoA was 12.69 mg/mL (Figure 4A). Then, when we increased the DBAT concentration to 3 mg/mL, the yield of acetyl-CoA reached 13.59 mg/mL (Figure 4B). The optimal reaction time was further determined by setting the time to 0.5 h, 1.0 h, 1.5 h, and 2 h, respectively, and the yield of acetyl-CoA reached its maximum of 14.82 mg/mL at 1.5 h (Figure 4C). Additionally, the concentration of the by-product salicylic acid methyl ester (SME) reached 4.22 mg/mL after 1.5 h (Figure 4D). This by-product can be used for other purposes, such as the preparation of extraction solvent perfumes, cosmetics, food preservatives, and drugs [13–17]. The HPLC-MS profiles of CoASH/acetyl-CoA and ASME/SME after 1.5 h are shown in Figure 4E,F, respectively.

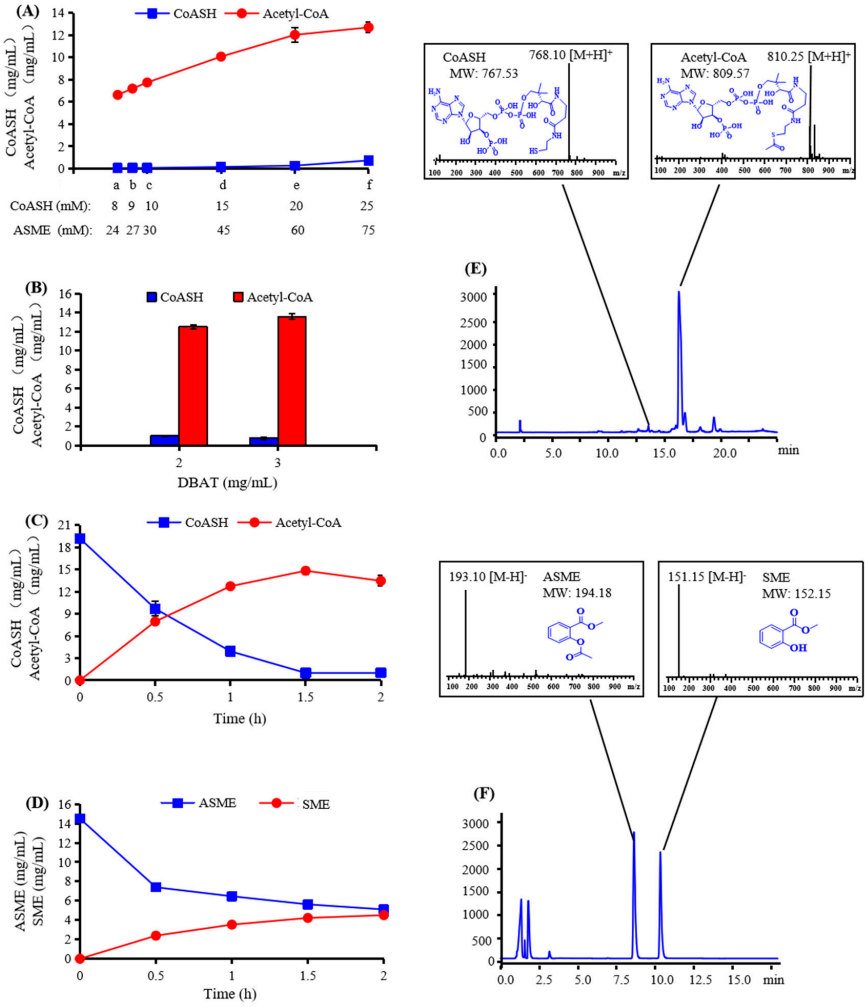

Figure 4. Re-optimization by increasing the concentrations of CoASH/ASME and DBAT. (**A**) Increasing the concentrations of CoASH/ASME for the production of acetyl-CoA. The molar ratio of CoASH/ASME was defined as 1/3 ($KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, DBAT = 2 mg/mL, at 37 °C, pH 7.0). (**B**) The influence of different DBAT concentrations on the production of acetyl-CoA, (CoASH/ASME = 25 mM/75 mM, $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, at 37 °C, pH 7.0). (**C**) The time-production curve of acetyl-CoA based on the HPLC analysis, (CoASH/ASME = 25 mM/75 mM, $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, DBAT = 3 mg/mL, at 37 °C, pH 7.0). (**D**) The time-production curve of SME based on the HPLC analysis (CoASH/ASME = 25 mM/75 mM, $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ = 1.7 mM/7.2 mM, DBAT = 3 mg/mL, at 37 °C, pH 7.0). (**E**) The HPLC profile of CoASH and acetyl-CoA at 1.5 h. (**F**) The HPLC profile of ASME and SME at 1.5 h. The data represent the means ± SD, n = 3.

2.4. Kinetic Study of DBAT

The kinetic parameters of DBAT against ASME, ATR, ASEE, ASSA, and AOA were determined and the results are listed in Table 1. The Km values of DBAT against ATR (4.80 μM), ASEE (4.07 μM), ASA (3.79 μM), and AOA (4.35 μM) were at least 2.5-fold higher than that of ASME (1.59 μM), while the turnover number (kcat) of DBAT against ASME (13.08 min^{-1}) was apparently higher than those of ATR (11.05 min^{-1}), ASEE (8.55 min^{-1}), ASA (6.29 min^{-1}), and AOA (5.23 min^{-1}). Consequently, DBAT exhibited the highest catalytic efficiency (kcat/Km) against ASME (8.32 $min^{-1}\mu M^{-1}$), followed by ATR (2.38 $min^{-1}\mu M^{-1}$), ASEE (2.11 $min^{-1}\mu M^{-1}$), ASA (1.68 $min^{-1}\mu M^{-1}$), and AOA (1.24 $min^{-1}\mu M^{-1}$).

Table 1. Kinetic parameters of DBAT against several acetyl donors for the synthesis of acetyl-CoA.

Substrates	V_{max} (μM/min)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}μM^{-1}$)
ASME	26.56 (±0.75)	1.59 (±0.15)	13.08 (±0.0.37)	8.33 (±1.84)
ATR	22.43 (±2.07)	4.80 (±1.08)	11.05 (±1.02)	2.38 (±0.45
ASEE	17.36 (±1.55)	4.07 (±0.93)	8.55 (±0.77)	2.11 (±0.37)
ASA	12.76 (±0.64)	3.79 (±0.50)	6.29 (±0.32)	1.68 (±0.32)
AOA	10.61 (±1.09)	4.35 (±1.12)	5.23 (±0.54)	1.24 (±0.36)

2.5. Preparation of Acetyl-CoA

To minimize the consumption of the substrate CoASH, the optimized reaction conditions obtained in Figure 3 were used for the production of acetyl-CoA. The 20 mL reaction system contained 1.7 mg/mL ASME (9 mM), 2.3 mg/mL CoASH (3 mM), 1.7 mM KH_2PO_4, 7.2 mM $K_2HPO_4•3H_2O$, and 2 mg/mL DBAT in 100 mM Tris-HCl buffer with a pH of 7.0. The reaction was kept at 37 °C for 2 h. The yield of acetyl-CoA was 2.3 mg/mL (CoASH conversion rate of 94.78%) with 0.12 mg/mL of residual CoASH. A total of 3 g of extract was obtained by lyophilization. From this extract, 40 mg of purified acetyl-CoA (HPLC purity > 98%) was acquired by gel column chromatography on Sephadex LH-20, with a recovery rate of 86% (the theoretical amount of acetyl-CoA was 46 mg, detected by HPLC). The flow chart of acetyl-CoA purification is shown in Figure S1.

2.6. The ^1H NMR Data of Acetyl-CoA

^1H NMR (D_2O, 500 MHz), $[M+H]^+$ 810.25, δ: 0.83 (s, 3H), 0.96 (s, 3H), 2.36 (s, 3H), 2.45 (t, *J* = 6.5 Hz, 2H), 3.00 (t, *J* = 6.5 Hz, 2H), 3.34 (t, *J* = 6.5 Hz, 2H), 3.47 (t, *J* = 6.5 Hz, 2H), 3.63 (m, 1H), 3.88 (m, 1H), 4.05 (s, 1H), 4.27 (m, 2H), 4.61 (brs, 1H), 4.82–4.89 (m, 2H), 6.22 (d, *J* = 6.0 Hz, 1H), 8.44 (s, 1H), 8.69 (s, 1H) [18].

3. Discussion

Acetyl-CoA is the precursor of many bio-manufacturing products. It is also the hub of the cellular metabolism of energy and substances, thus it exerts a pivotal role in living organisms. However, its application is hindered due to its high cost and low yield. To enzymatically synthesize acetyl-CoA by DBAT with CoASH as the acetyl acceptor, we screened a total of ten potential acetyl donors, all of which are active phenolic esters, making the acetyl group easier to dissociate from the acetyl donor and to form the thioester with CoASH. We found that the best candidate was ASME, followed by ATR, ASEE, ASSA, and AOA (Figure 2). Specifically, although the backbone structure of ASA is the same as that of ASME, and both of them are featured with electron-withdrawing groups at the ortho-position (Figure 1), the catalytic efficiency of DBAT against ASA was much lower than that of ASME (Figure 2, Table 1). The possible reason for this is that the formation of a hydrogen bond between the hydrogen of the carbolic group and the phenolic oxygen in ASA decreases the reactivity. The mechanism behind this phenomenon needs to be further investigated.

Using ASME as the acetyl donor, the yield of acetyl-CoA reached a maximum of 14.82 mg/mL, while the yield of SME reached 4.22 mg/mL (Figure 4).

In addition to the availability of cheaper acetyl donors, another advantage of this approach is that some precious cofactors, such as ATP and NAD, can be omitted in this enzymatic reaction, which greatly reduces the cost and lays a solid foundation for the large-scale production of acetyl-CoA. While we were preparing this manuscript for submission, one group reported a yield of 3.89 mg/mL using O-acetyltransferase to synthesize acetyl-

CoA in vitro with diacerein as the acetyl donor [19], which is still far lower than the aforementioned yield. To the best of our knowledge, 14.82 mg/mL is the highest yield of acetyl-CoA among all reported approaches to date [2–11,18,19].

DBAT was originally found in *Taxus* species and has been characterized as a key enzyme to specifically convert 10-deacetylbaccatine III (10-DAB) into baccatin III by acetylating the C10, one of the crucial steps in the biosynthesis of Taxol [20–23]. Currently, the chemical semi-synthesis of Taxol is initiated by 10-DAB, thus DBAT has great potential in the acetylation step of 10-DAB. In our previous study, we constructed the acetyl-CoA and DBAT hybrid metabolic pathway in *E. coli* for the acetylation of 10-deacetylbaccatin III to baccatin III [12]. Meanwhile, we found that DBAT could also catalyze the deacetylation of baccatin III under alkaline conditions, in which the acetyl group could be transferred to CoASH [12]. In addition, we demonstrated that a K^+ ion could improve the deacetylation of baccatin III and increase the production of 10-DAB and acetyl-CoA [12]. Therefore, in the present work, we further investigated the influence of potassium salts on acetyl-CoA production. We found that 1.7 mM KH_2PO_4 and 7.2 mM $K_2HPO_4 \bullet 3H_2O$ could maximally increase the production of acetyl-CoA (Figure 3F).

In brief, our present study demonstrates that DBAT is a robust enzyme that can be used in the large-scale production of acetyl-CoA by using ASME as the acetyl donor and CoASH as the acetyl acceptor. Our method is to hopefully turn acetyl-CoA into a bulk commodity. Additionally, the by-product salicylic acid methyl ester (SME), which is extensively used in the synthesis of extraction solvent [13], perfumes [14], cosmetics [15], food preservatives [16], and drugs [17], can be simultaneously produced using our method. Thus, our method will further increase the revenue of production.

4. Materials and Methods

4.1. Strains and Plasmids

The bacterial host BL21(DE3) (TransGen Biotech Beijing, Beijing, China) and the recombinant plasmid pCWori-*dbat* (preserved in our laboratory) were used for preparing the DBAT.

4.2. Chemicals and Media

Salicylic acid methyl ester (SME) and all acetyl donor candidates, including acetylsalicylic acid (ASA, 1), acetylsalicylic acid methyl ester (ASME, 2), acetylsalicylic acid ethyl ester (ASEE, 3), acetylsalicylsalicylic acid (ASSA, 4), benorilate (BL, 5), acetylsalicylic anhydride (ASAN, 6), DL-Lysine acetylsalicylate (DLA, 7), carbasalate calcium (CSC, 8), acetyl-trans-resveratrol (ATR, 9), and 4-acetoxyacetanilide (AOA, 10) were purchased from Shanghai Bepharm Science & Technology Co., Ltd. Acetyl-CoA and CoASH standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl β-D-thiogalactopyranoside (IPTG) and ampicillin sodium salt were purchased from Inalco Spa, Milano, Italy (Inalco, USA). All other chemicals were of analytical grade unless otherwise indicated.

4.3. Screening of Acetyl Donors and Optimization of Reaction Conditions

The preparation of the recombinant DBAT and the methods of analyzing acetyl-CoA were described by Wang, et al [12,24].

CoASH, Acetyl-CoA, ASME, and SME were analyzed by HPLC on a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with LC-20AT pumps, SPD-M20A detector.

CoASH and acetyl-CoA were analyzed by an OSAKA SODA CAPCELL PAK C18 AQ column (5 μm, 4.6 mm × 150 mm) monitoring at 254 nm and 30 °C. Mobile phases used were solvent A (Na_3PO_4 buffer solution, pH 5.5) and solvent B (Na_3PO_4 buffer solution (pH 5.5):acetonitrile = 4:1, *v*/*v*) with a flow rate of 1 mL/min. The following gradient was used: 0–5 min, 3–18% B; 5–7.5 min, 18–28% B; 7.5–12.5 min, 28–40% B; 12.5–18 min, 40–42% B; 18–19 min, 42–97% B.

ASME and SME were analyzed by an OSAKA SODA CAPCELL PAK ADME HR column (5 μm, 4.6 mm × 150 mm) monitoring at 230 nm and 30 °C. Mobile phases used were solvent A (water) and solvent B (acetonitrile) with a flow rate of 1 mL/min. The following gradient was used: 0–10 min, 25–62% B; 10–11 min, 62–90% B; 11–12 min, 90–25% B; 12–18 min, 25–25% B.

Screening for the optimal acetyl donors was carried in a reaction volume of 200 μL containing 1 mg/mL DBAT, 1.7 mM (1.3 mg/mL) CoASH, and 5.1 mM acetyl donor in 100 mM Tris-HCl buffer, pH 6.0. One of the following compounds—ASA, ASME, ASEE, ASSA, BL, ASAN, DLA, CSC, ATR, or AOA (Figure 4)—was added as an acetyl donor. The reaction was performed at 30 °C for 3 h.

To analyze the influence of different molar concentrations of CoASH and ASME on acetyl-CoA production, the CoASH concentration was set at 1 mM and ASME concentrations were tested at 1 mM, 2 mM, 3 mM, and 4 mM. The molar ratio of CoASH/ASME was defined as 1/3, and the molar concentration combinations of CoASH/ASME were tested at 3 mM/9 mM (2.3 mg/mL vs. 1.7 mg/mL), 4 mM/12 mM (3.1 mg/mL vs. 2.3 mg/mL), 5 mM/15 mM (3.8 mg/mL vs. 2.9 mg/mL), 6 mM/18 mM (4.6 mg/mL vs. 3.5 mg/mL), and 7 mM/21 mM (5.4 mg/mL vs. 4.1 mg/mL), respectively. To optimize the temperature, the reactions were tested at 25 °C, 30 °C, 37 °C, and 45 °C. The 200 μL reaction contained 6 mM CoASH and 18 mM ASME.

To optimize the reaction pH, the pH values were tested at 3.0, 5.0, 7.0, and 9.0. The 200 μL reaction contained 6 mM CoASH and 18 mM ASME and the temperature was set at 37 °C.

The influence of potassium salts on enzyme efficiency was analyzed. The 200 μL reaction contained 7 mM CoASH and 21 mM ASME, the temperature and pH were set at 37 °C and pH 7.0, respectively. Firstly, we set the $K_2HPO_4 \bullet 3H_2O$ concentrations at 1 mM, 4 mM, 16 mM, and 64 mM. Then, we analyzed the influence of the $KH_2PO_4/K_2HPO_4 \bullet 3H_2O$ pair on enzyme efficiency. The concentration combinations were set at 1.7 mM/7.2 mM, 8.5 mM/36 mM, 17 mM/72 mM, and 34 mM/144 mM.

The concentrations of DBAT were tested at 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL, respectively. The 200 μL reaction contained 7 mM CoASH, 21 mM ASME, 1.7 mM KH_2PO_4, and 7.2 mM $K_2HPO_4 \bullet 3H_2O$ in 100 mM Tris-HCl buffer, pH 7.0.

Under the optimized conditions, the optimal reaction time was further determined. DBAT was set at 2 mg/mL and the reaction was tested at 0.5 h, 1 h, 2 h, and 3 h, respectively.

In order to increase the productivity of acetyl-CoA, the molar concentration combinations of CoASH/ASME were further increased to 8 mM/24 mM (6.1 mg/mL vs. 4.7 mg/mL), 9 mM/27 mM (6.9 mg/mL vs. 5.2 mg/mL), 10 mM/30 mM (7.7 mg/mL vs. 5.8 mg/mL), and 15 mM/45 mM (11.5 mg/mL vs. 8.7 mg/mL), 20 mM/60 mM (15.3 mg/mL vs. 11.7 mg/mL), and 25 mM/75 mM (19.2 mg/mL vs. 14.6 mg/mL), respectively. The DBAT concentrations were set at 2 mg/mL and 3 mg/mL, respectively. Again, the reaction time was further determined by testing at 0.5 h, 1 h, 1.5 h, and 2 h, respectively, with 3 mL/mL of DBAT. Finally, the 200 μL reaction contained 25 mM CoASH, 75 mM ASME, 1.7 mM KH_2PO_4, 7.2 mM $K_2HPO_4 \bullet 3H_2O$ in 100 mM Tris-HCl buffer, pH 7.0.

4.4. Enzyme Kinetic Analysis

The kinetic parameters against ATR, ASME, ASSA, ASEE, and AOA were determined. The reaction mixtures containing 100 mM Tris-HCl buffer (pH 7.0), an acetyl donor (0.00125–16 mM), 0.1 mg/mL DBAT, 1 mM CoASH, 1.7 mM KH_2PO_4, and 7.2 mM $K_2HPO_4 \bullet 3H_2O$ in a final volume of 200 μL were incubated at 37 °C for 0.5 h. The reactions were terminated by adding 300 μL methanol. The kinetic data were processed via a proportional weighted fit using a nonlinear regression analysis program based on Michaelis–Menten enzyme kinetics [25].

The experiments were performed at least 3 times.

4.5. Purification of Acetyl-CoA

The acetyl-CoA reaction solution was freeze-dried, then purified by gel column chromatography (Sephadex LH-20, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) eluted with water to achieve acetyl-CoA.

5. Conclusions

In conclusion, using 75 mM ASME as the acetyl donor and 25 mM CoASH as the acetyl acceptor, DBAT can efficiently catalyze the formation of acetyl-CoA with a yield of 14.82 mg/mL, accompanying 4.22 mg/mL of the by-product salicylic acid methyl ester. Additionally, a preliminary purification process for acetyl-CoA was established with a recovery rate of 86% (HPLC purity > 98%). Our results lay the foundation for the large-scale production of acetyl-CoA by an enzymatic approach and will promote its application in different fields.

Supplementary Materials: Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal11101240/s1, Figure S1: Flow chart of acetyl-CoA purification.

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