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Engineered *Bacillus subtilis* for the Production of Tetramethylpyrazine,(R,R)-2,3-Butanediol and Acetoin

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Abstract: 2,3-Butanediol, acetoin and tetramethylpyrazine have a wide range of applications as important chemicals in the chemical, food and pharmaceutical fields. *Bacillus subtilis* has a very wide application potential in many industries as a food-safe grade strain and has a good performance as a potential strain for tetramethylpyrazine production. In this study, we constructed a recombinant plasmid with dual promoter to overexpress 2,3-butanediol dehydrogenase and introduced it into *Bacillus subtilis BS2*. The constructed strain (BS-ppb11) was then optimized for fermentation conditions, and the maximum concentration of 2,3-butanediol produced was 96.5 g/L, which was 36.4% higher than that of the original strain, in which the (R,R)-2,3-butanediol had a chiral purity of 94.7%. Meanwhile, BS-ppb11 produced a maximum concentration of 82.2 g/L acetoin, which was 36.7% higher than that of the original strain. Subsequently, through optimization of metabolic conditions, BS-ppb11 produced 34.8 g/L of tetramethylpyrazine in staged batch replenishment fermentation, which was 95.5% higher than the original strain and was the highest ferritin production reported to date for *Bacillus subtilis*. In addition, we introduced a photocatalytic coenzyme regeneration system in BS-ppb11 to further improve the metabolic yield of 2,3-butanediol by regulating cofactor homeostasis, which laid the foundation for the subsequent in-depth study of the related mechanism.

Keywords: tetramethylpyrazine; 2,3-butanediol; acetoin; *Bacillus subtilis*; dual promoter; photocatalytic

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1. Introduction

Nonrenewable resources, such as oil and natural gas, are becoming increasingly depleted. With rapid economic development, the demand for energy and chemicals is rapidly increasing. The production of industrial chemicals through biosynthesis has become a sustainable and green production method; this method is important for solving the energy crisis and environmental pollution, and it has received widespread attention [1,2].

Tetramethylpyrazine (TTMP), commonly known as chuanxiongqin, is an active alkaloid in the plant chuanxiong [3]. In recent decades, TTMP has been proven to have a variety of pharmacological characteristics and can be used to treat a variety of diseases, including cardiovascular, cerebrovascular and digestive system diseases; it also has significant anticancer, anti-oxidation and anti-inflammatory effects [4–7]. In addition, TTMP is a naturally occurring aroma substance found in cocoa beans, coffee, peanuts and other nutty substances, bean products and meat. With a roasted aroma, it is also an important functional substance and key characteristic aroma component in Chinese liquor, which is used to regulate the flavor and enhance the quality of liquor [8,9]. TTMP is also widely adopted by the food processing industry and is a permitted food additive [10]. At present, the production methods of TTMP mainly include plant extraction, chemical synthesis and biological fermentation [11]. Plant tissue extraction and chemical synthesis methods are impossible to achieve during large-scale production due to the complex extraction process, which can

cause environmental pollution and low product purity. The microbial fermentation method, which uses microorganisms that can obtain TTMP through mild culture conditions, is currently a hot spot of research and meets people's desire for sustainable development. The first public report of microbial synthesis of TTMP was made by foreign scientists in 1962, when Kosug [12] discovered and purified TTMP from the fermentation broth of *Bacillus subtilis*. Meng et al. overexpressed ALDS in *Bacillus licheniformis BL1* and added acetoin to the microaerobic fermentation broth to obtain a TTMP of 43.75 g/L [13]. However, these additional ingredients undoubtedly increase the cost of production. Xu et al. expressed the synthetic gene cluster of acetoin and NADH oxidase in *E. coli* and produced 16.1 g/L TTMP in optimized medium [14].

Both 2,3-butanediol (2,3-BD) and acetoin (AC) are very important biobased platform chemicals: 2,3-BD has great potential for the manufacture of printing inks, fuel additives and food additives [15] and 2,3-BD has a calorific value comparable to that of ethanol and is a potentially important fuel and fuel additive for aerospace and other related applications [16]. In addition, 2,3-BD can be used to synthesize derivatives with a wide range of applications through dehydration, dehydrogenation and esterification reactions [17]. Single-configuration (R,R)-2,3-BD not only has the basic functions of the mixed-spin 2,3-BD but also has obvious advantages in asymmetric synthesis and it is an important precursor for the synthesis of chiral reagents and chiral ligands [18–20]. (R,R)-2,3-BD is a precursor substance for pharmaceutical aspirin and advanced liquid crystal materials; it is also used as an advanced antifreeze agent due to its low freezing point property [21]. Acetoin is a pale-yellow liquid with a yogurt odor and a creamy taste. AC also has two optical isomers: (R)-AC and (S)-AC [22]. AC is usually used as a food additive to increase the aroma of food and is a natural food flavor. In the chemical synthesis industry, AC is a synthetic precursor for many chemical products [23]. AC, as a bioactive substance, can promote plant growth and has great potential for application in the field of botany and agronomy [24].

Many microorganisms can metabolize various carbon sources to produce 2,3-BD and AC, e.g., *Klebsiella* spp., *Bacillus* spp., *Serratia* spp., *E. coli*, etc. [25]. *B. subtilis* is considered a potential strain for industrial fermentation of compounds, and *B. subtilis* is endotoxin-free, with more than 95% of its naturally produced 2,3-butanediol being (R,R)-2,3-BD. Normally, in *Bacillus subtilis*, glucose is first converted to pyruvate via the glycolytic pathway (Figure 1) and then pyruvate is converted to AC via α-acetyl lactate synthase (ALS) and α-acetyl lactate decarboxylase (ALDS). A portion of AC is reduced to 2,3-BD by 2,3-butanediol dehydrogenase (BDH), a process accompanied by the consumption of NADH [26]. Another portion of AC can combine with ammonium ions to form TTMP [27]. The metabolic conditions for the synthesis of TTMP by AC and ammonium salts in *B. subtilis* are influenced by many factors, including substrate concentration, temperature and pH. It is important to explore the suitable fermentation conditions for the synthesis of TTMP in large quantities. It has been shown that BDH, also called acetoin reductase (AR), is a reversible enzyme [28]. Moreover, BDH can show different catalytic effects under different environments. Under weak acid and low oxygen environments, BDH catalyzes the conversion of AC to 2,3-BD; under weak base and high oxygen environments, BDH catalyzes the conversion of 2,3-BD to AC [29,30]. The catalytic reaction of BDH is also accompanied by the consumption of coenzymes, NADH for the reduction of AC to 2,3-BD and NAD^+ for the oxidation of 2,3-BD to AC. It has been shown that simply enhancing the key enzymes in the synthesis of the 2,3-BD and AC pathways is not very effective in increasing their yields and that the cofactors required for the enzymatic reaction process are also critical [31]. Moreover, the balance of intracellular cofactor concentrations is an important condition for maximizing enzyme catalytic efficiency [32,33].

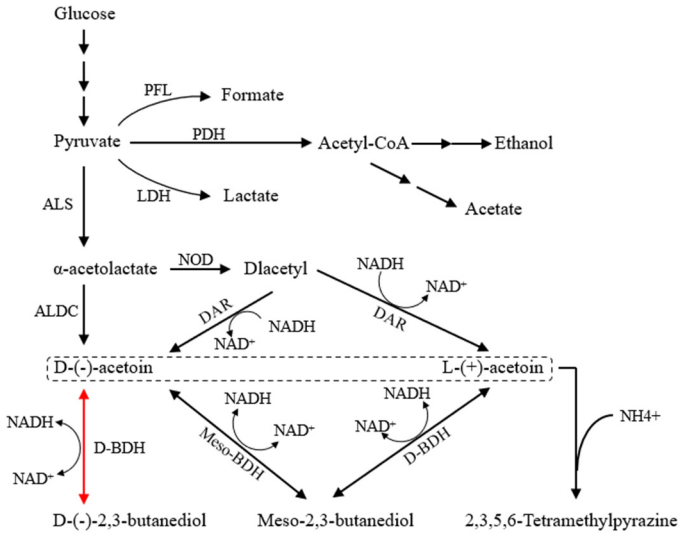

Figure 1. Synthetic pathways of 2,3-BD, acetoin and TTMP in *Bacillus subtilis*.

In recent years, photoactive nanoparticles, such as cadmium sulfide, gold nanoclusters and indium phosphide nanoparticles, have been used to construct functional bio-inorganic hybrid systems for the synthesis of simple metabolites in bacteria or yeast. In biohybrid systems, light-absorbing materials typically convert light into light energy and generate electrons that can directly regulate specific biological pathways in organisms, such as CO_2 fixation and NADH production [34–36] (Figure 2). Some studies have shown that AuNPs have good biocompatibility, high extinction coefficient and catalytic activity, indicating their potential for the construction of bio-inorganic hybrid systems [37]. Zhang's group introduced the photocatalyst AuNCs in *M. thermoacetica*; after four days of system operation, the biocompatible AuNCs increased the cumulative yield of acetic acid by 14% [34]. Li combined *C. zofingiensis* with efficient light-trapping AuNPs to construct a microbial hybrid system in which AuNPs could enter the interior of *C. zofingiensis*, prompting a carotenoid yield of 10.7 ± 1.2 mg/L, 42.7% higher than that of natural microalgae [38].

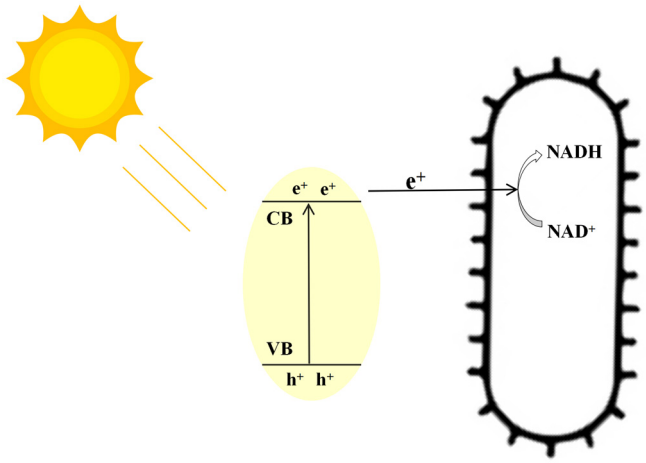

Figure 2. Photocatalytic materials for the synthesis of NADH.

In this study, we constructed a recombinant strain overexpressing BDH using a dual promoter expression system with *B. subtilis BS2* as the starting strain, and measured and optimized the ability of the recombinant strain to produce 2,3-BD and acetoin by fermentation. Based on this, we conducted single-factor tests on the conditions (ammonium addition time, ammonium concentration, temperature and pH) for the production of TTMP by the recombinant strain in order to explore the optimal production conditions, and finally

increased the concentration of TTMP. In addition, we introduced a NADH regeneration system with gold nanoclusters as the material in the recombinant strain and increased the concentration of 2,3-BD in the light fermentation mode, demonstrating the availability of photocatalytic materials in *B. subtilis*.

2. Materials and Methods

2.1. Strains, Plasmids, and Primers

Bacillus subtilis BS2, the strain used in this study, was maintained by the State Key Laboratory of Microbiology, School of Biological Engineering, Qilu University of Technology. The plasmids and primers used in this study are described in Table 1. The enzyme preparation used in this study was purchased from Nanjing Vazyme Biotechnology Co.

Table 1. Plasmids and Primers.

Plasmids and Primers	Description	Source
pBE980a	Kmr; P43 promoter; expressing vector	Laboratory stock
pBE980a-Plaps	pBE980a carries Plaps gene	This study
pBE980a-Plaps-bdhA	pBE980a carries Plaps, bdhA gene	This study
pBE980a(P43)-Plaps-bdhA	pBE980a carries Plaps, bdhA gene, deletes P43 gene	This study
pUC-sp	Kmr; carries Plaps gene	This study
bdhA-F	ggtacccggggatcctctagaATGAAGGCAGCAAGATGGCA	This study
bdhA-R	tggaattgtgctgaagctagcTTAGTTAGGTCTAACAAGGATTTTGACTT	This study
PBE980a-P-F	tcaagcttttgcctcgagctcGAGCTCTCAGGAGCATTTAACCTAA	This study
PBE980a-P-R	tgccttcattctagaggatccGGATCCCGTTCATGTCTCCTT	This study
PBE980a-bdhA-F	ggtacccggggatcctctagaATGAAGGCAGCAAGATGGCA	This study
PBE98a-bdhA-R	tggaattgtgctgaagctagcTTAGTTAGGTCTAACAAGGATTTTGACTT	This study
PUC-Plaps-F	caggtcgactctagaggatccCGTTCATGTCTCCTTTTTTATGTACTG	This study
PUC-Plaps-R	tcaagcttttgcctcgagctcTCAGGAGCATTTAACCTAAAAAAGC	This study

The gold nanoclusters were provided by the Department of Bioengineering, Qilu University of Technology.

2.2. Construction of Recombinant Strains and Validation of Enzyme Activity

The plasmid pBE980a-Plaps with dual promoter (Plaps, P43) was constructed by cloning the promoter Plaps gene fragment from pUC-sp by PCR and ligating it to the plasmid pBE980a. The gene encoding 2,3-butanediol dehydrogenase, *bdhA*, was cloned from *Bacillus subtilis BS2* by PCR and ligated to the recombinant plasmid pBE980a-Plaps to complete the construction of the recombinant plasmid pBE980a-Plaps-bdhA, and the plasmids expressing *bdhA* with the single promoter Plaps or P43 were used as controls. The recombinant plasmids were transformed into *Bacillus subtilis BS2* by the Spizizen method, and the transformants were cultured to extract the plasmids for verification of the target genes by PCR.

The original strain and the recombinant strain were incubated in 200 mL LB medium containing 50 μg/mL kanamycin at 37 °C for 12 h. Then, the supernatant was removed by centrifugation and the bacterial cells were washed three times with 0.1 mol/L PBS buffer and finally resuspended in PBS buffer. The cell resuspension was crushed with an ultrasonic cell crusher, the cell debris was removed by centrifugation and the upper layer of enzyme solution was retained.

Briefly, for the oxidation reaction, a mixture containing 100 mM substrate 2,3-BD, 4 mM NAD^+, 50 mM potassium phosphate buffer (pH 7.0) and 10 μL enzyme solution in a final volume of 1 mL was reacted at 37 °C for 1 min, and the difference in OD_{340} from the beginning to the end of the reaction was recorded. The reduction reaction was carried out in 1 mL of a mixture containing 100 mM substrate AC, 0.2 mM NADH, 50 mM potassium phosphate buffer (pH 7.0) and 10 μL enzyme solution at 37 °C for 1 min, and the difference in OD_{340} from the beginning to the end of the reaction was recorded.

2.3. Shake Flask Fermentation and Bioreactor Fermentation Methods

A single colony of the recombinant strain was added to 200 mL of LB medium containing 50 μg/mL kanamycin and incubated at 37 °C for 8 h as a seed solution. The seed solution was added to the fermentation medium at 10% inoculum. The medium formulation was as follows: 5 g/L yeast extract, 20 g/L corn pulp dry powder, 70 g/L glucose, 14 g/L K_2HPO_4, 6 g/L KH_2PO_4, 5 g/L urea and 8 g/L trisodium citrate. Shake flask incubation was carried out in a constant temperature incubator at 37 °C with a speed of 200 r/min. The pH value was controlled by adding HCl/NaOH solution. The 5 L bioreactor cultivates bacteria with a seed inoculum of 10% and a loading volume of 2 L. The pH is controlled by HCl/NaOH solution, and the aeration volume is controlled by a compressor. The fermentation process is supplemented with substrate.

2.4. Construction and Fermentation of Engineered Photocatalytic System Bacteria

The gold salt compound was first reduced to Au^0 using a reducing agent, and small-sized gold nanoclusters were synthesized with sulfhydryl groups as ligands. Then, an amino acid short peptide was wrapped on the surface of the gold nanoclusters to make a photocatalytic material. BS-ppb11 was cultured to the logarithmic phase by adding gold nanoclusters, which were absorbed by BS-ppb11 through cytocytosis, thus constructing a photocatalytic complex system of engineered BS-ppb12 bacteria. The prepared bacteria were observed using scanning transmission electron microscopy (HAADF-STEM, FEI SuperX Quad) at 80 kV and scanned with an energy dispersive spectrometer (EDS) operating system with Bruker Esprit software. EDS is able to excite characteristic X-rays of different elements and to discriminate them in different colors.

Because gold nanoclusters themselves can have some inhibitory effect on bacterial growth, we first measured the growth of BS-ppb11 in five media containing different concentrations of gold nanoclusters. Gold nanocluster reagents with concentrations of 0%, 12%, 20%, 28%, 36% and 44% were added to the basic medium, BS-ppb11 was added to the medium at 2% inoculum, and the optimum gold nanocluster concentration was selected by incubating the bacteria at 37 °C and 200 r/min for 30 h and measuring their density every 5 h.

After selecting the appropriate concentration of gold nanoclusters, the fermentation of the engineered bacterium BS-ppb12 in the photocatalytic system was carried out using glucose as the carbon source. BS-ppb12 was inoculated into 30 mL of fermentation medium containing 70 g/L glucose at 10% inoculum, the incubation temperature was 37 °C, the magnetic stirring speed was 200 r/min, and the pH was 7. A xenon lamp was used to continuously irradiate the shake flask for 10 h per day, and the distance between the light source and the shake flask was kept constant at 10 cm.

2.5. Analysis Method

Glucose was detected by HPLC. The fermentation broth was centrifuged at 12,000 rpm for 10 min, and the supernatant was taken and diluted appropriately for immediate liquid chromatographic analysis. The instrument model was Shimadzu LC-20A, the detector model was RID-20A, and the column model was Shimadzu Inertsil NH2 (4.6 mm × 250 mm × 5 μm). The detection conditions were as follows: column temperature was 26 °C, mobile phase was 75% acetonitrile solution, injection volume was 10 μL, isocratic elution and analysis time was 10 min.

GC was used for the simultaneous detection of (R,R)-2,3-butanediol, acetoin and tetramethylpyrazine with a Shimadzu GC-2030. The fermentation broth was centrifuged at 12,000 rpm for 10 min, and the supernatant was extracted and treated with ethyl acetate at an extraction volume ratio of 1:1. The extract obtained was subjected to GC analysis. The column was Shimadzu Stabilwax (30 m × 0.32 mm × 0.5 μm). The detection conditions were as follows: initial column temperature was 80 °C with a retention time of 1 min followed by incremental 10 °C temperature increases to 180 °C; injector and detector temperature was 250 °C; injection volume was 1 μL, split injection and split ratio was 50:1

and the FID detector was used. (R,R)-2,3-BD detection was performed on a Shimadzu HP-chiral-20b column with the same detection conditions as above.

HPLC was used to detect lactic acid, acetic acid and formic acid. The fermentation broth was centrifuged at 12,000 rpm for 10 min, and the supernatant was taken and diluted appropriately for immediate liquid chromatographic analysis. The instrument model was Shimadzu LC-20A, the detector model was CBM-20A, the detection wavelength was 210 nm, and the column model was shim-pack GIST C18-AQ (4.6 mm × 150 mm × 5 μm). The detection conditions were as follows: the column temperature was 35 °C, the mobile phase was 0.5 mmol/L sulfuric acid solution, flow rate was 0.5 mL/min, isocratic elution, and the analysis time was 20 min.

Biomass concentration was determined by measuring the optical density (OD) at 600 nm using an ultraviolet spectrophotometer (UV-2350, Unic).

Statistical analysis was performed using Origin 2021 software, and results were expressed as the average value with its standard deviation.

3. Results and Discussion

3.1. Determination of Enzyme Activity and Optimization of Culture Conditions of Recombinant Strains

After completing the construction and validation of the recombinant strains, the strain carrying the dual promoter was named BS-ppb11, the strain with single promoter P43 was named BS-43A and the strain with single promoter Plaps was named BS-LA26. To determine the activity of BDH in the recombinant strains, BS2, BS-ppb11, BS-43A and BS-LA26 were incubated in LB medium containing 10% glucose at 37 °C for 24 h. The activity of the enzyme solution was measured after the cells were broken and collected. When AC was used as a substrate, AC underwent a reduction reaction and reacted with NADH to produce 2,3-BD. The results showed (Figure 3) that BS-ppb11 had the highest enzyme activity among the four bacterial strains, and its enzyme activity was increased by 44.2% when compared to the original strain BS2. When 2,3-BD was used as a substrate, 2,3-BD underwent oxidation and reacted with NAD^+ to produce AC. The results showed that the enzyme activity of BS-ppb11 was increased by 25.6% when compared to that of the original strain and was also significantly higher than that of the other two strains with a single promoter. All the above results indicate that strains overexpressing BDH with dual promoters can obtain higher enzymatic activity, which lays the foundation for subsequent fermentation production.

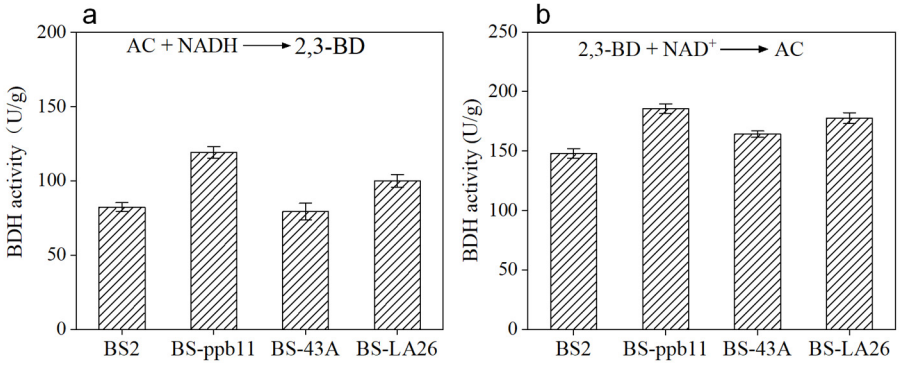

Figure 3. Activity of BDH when catalyzing 2,3-BD and acetoin. (**a**) BDH catalyzes acetoin to produce 2,3-BD and consumes NADH; (**b**) BDH catalyzes 2,3-BD to produce acetoin and consumes NAD^+.

To establish suitable conditions for the fermentation of BS-ppb11 for the production of 2,3-BD and AC, a series of pH and aeration experiments were conducted. Fermentation was carried out at 37 °C with a speed of 200 rpm and a substrate of 140 g/L glucose. To determine the effect of pH on BS-ppb11, fermentation was performed at pH 6, 6.5, 7, 7.5 and 8. For AC (Figure 4), the concentration increased with increasing pH; the highest

concentration was found at pH 7.5, and its concentration decreased with the continual increase in pH. For 2,3-BD, the concentration gradually increased as the pH decreased, but the concentration did not increase when the pH decreased to 6. We conclude that the engineered bacterium BS-ppb11 is more biased to produce AC under weakly acidic conditions and more biased to produce 2,3-BD under weakly alkaline conditions; this is consistent with previous studies. To determine the effect of aeration on BS-ppb11, fermentation was conducted at aerations of 0.5, 1, 1.5, 2 and 2.5 L/min. The fermentation results showed that as the aeration increased, the oxygen concentration in the fermentation system increased correspondingly, leading to a gradual increase in the concentration of AC. However, when fermentation was performed using lower aerations, the engineered bacterium BS-ppb11 was more inclined to produce 2,3-BD. As such, we conclude that in environments with higher levels of oxygen, the fermentation system is more prone to oxidation reactions, corresponding to the production of AC. In a lower oxygen environment, reduction reactions are more likely to occur, corresponding to the production of 2,3-BD.

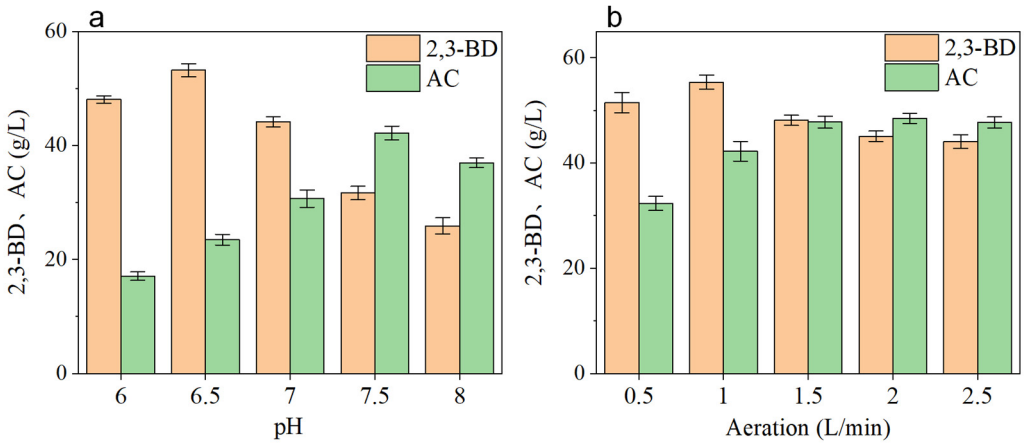

Figure 4. Effect of pH and aeration on the production of 2,3-BD and AC by BS-ppb11, (**a**) Effect of different pH on the production of 2,3-BD and AC by BS-ppb11; (**b**) Effect of different aeration on the production of 2,3-BD and AC by BS-ppb11.

3.2. Production of 2,3-BD and AC by Batch Replenishment Fermentation with the BS-ppb11

To verify the ability of BS-ppb11 to produce 2,3-BD and AC, *BS2*, BS-ppb11, BS-43A and BS-LA26 were cultured in shake flasks. The fermentation broth was supplemented with glucose solution to maintain the substrate concentration; the fermentation cycle was 10 days. The results showed (Table 2) that the rate of glucose consumption by the engineered strain BS-ppb11 with dual promoter expressing BDH was slightly elevated when compared to that of the other three strains and showed better results for both products, 2,3-BD and AC. For fermentation by BS-ppb11, the maximum concentration of AC was 53.3 g/L, which was a 36.7% increase when compared to that produced by the original strain *BS2*; the maximum concentration of 2,3-BD was 58.8 g/L, which was a 36.4% increase when compared to that produced by *BS2*. BS-ppb11 also performed better than the other two single promoter strains expressing BDH. For formic acid, acetic acid and lactic acid, BS-ppb11 produced several by-product concentrations that decreased to varying degrees when compared to *BS2*, with the more pronounced being formic acid and lactic acid. The above results indicate that metabolic flow can be enhanced by upregulating the expression genes of key enzymes of the 2,3-BD and AC synthesis pathways, which can reduce the intensity of the branched pathway, and that gene expression at different intensities also affects the rate of product synthesis. We found that thanks to the high activity of BDH, BS-ppb11 was able to produce high concentrations of both 2,3-BD and AC in culture, which also provides a basis for controlling the metabolic direction through the control of enzymatic reactions and subsequently.

Table 2. BS-ppb11 shake flask fermentation characterization.

Fermentation Characterization	Strains			
	BS2	**BS-ppb11**	**BS-43A**	**BS-LA26**
Glucose (g/L)	190 ± 1.5	191 ± 1.8	190 ± 2.0	187 ± 1.4
Glucose consumption rate	0.99 ± 0.01	1.14 ± 0.02	1.05 ± 0.02	1.06 ± 0.01
2,3-BD	43.1 ± 0.9	58.8 ± 1.0	47.4 ± 1.1	33.0 ± 1.1
2,3-BD yield	0.23 ± 0.02	0.35 ± 0.02	0.29 ± 0.01	0.19 ± 0.02
Acetoin	39.0 ± 1.1	53.3 ± 1.3	43.9 ± 1.2	52.8 ± 1.1
Acetoin yield	0.21 ± 0.02	0.32 ± 0.01	0.26 ± 0.03	0.31 ± 0.03
Formic acid	4.4 ± 0.12	2.9 ± 0.15	4.2 ± 0.2	4.5 ± 0.11
Acetic acid	7.7 ± 0.21	4.5 ± 0.16	5.5 ± 0.14	6.2 ± 0.16
Lactic acid	12.3 ± 0.23	6.3 ± 0.23	8.2 ± 0.25	8.8 ± 0.21

To further increase the product concentration, BS-ppb11 was incubated in a batch replenishment fermentation mode in a 5 L benchtop fermenter with a loading volume of 2 L. The initial glucose concentration was 70 g/L, and the glucose solution was replenished every 12 h to maintain the concentration of residual sugars, for a total of six replenishments. The results showed (Figure 5) that when BS-ppb11 was cultured to produce AC, the concentration of AC reached 82.2 g/L with a yield of 0.76 g/L-h, and the concentration of 2,3-BD as a byproduct was 15.6 g/L at 108 h.

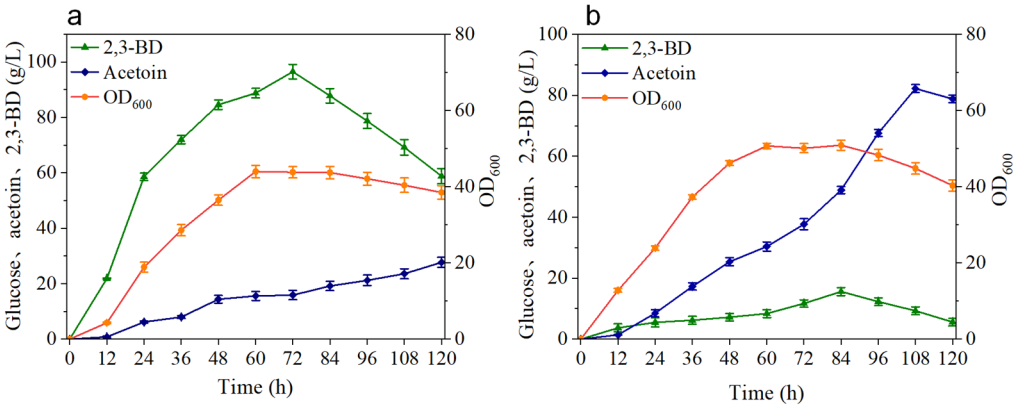

Figure 5. 5 L bioreactor culture BS-ppb11 to produce 2,3-BD or AC, (**a**) BS-ppb11 fermentation production 2,3-BD; (**b**) BS-ppb11 fermentation production AC.

When 2,3-BD was the main product, the highest concentration of 2,3-BD reached 96.5 g/L with a yield of 1.34 g/L-h at 72 h, in which the chiral purity of (2R,3R)-2,3-BD was 94.7%. The concentration of 2,3-BD then started to decrease, probably because of the gradual conversion of 2,3-BD to AC; the concentration of AC slowly increased in the late stage of 2,3-BD production. When the substrate glucose was depleted, the bacteria actively consumed 2,3-BD and AC to maintain growth, so both 2,3-BD and AC showed different rates of decrease, which proved that 2,3-BD and AC could also be used as carbon sources to provide essential nutrients for cell growth.

3.3. Staged Batch Replenishment Fermentation of BS-ppb11 for TTMP Production

TTMP is produced by the combination of its precursor substance AC with ammonium ions. In previous studies, the most suitable ammonium salt for TTMP synthesis was diammonium hydrogen phosphate. Therefore, the concentration and addition time of diammonium hydrogen phosphate have important effects on the synthesis of TTMP. Because of the special physical properties of TTMP, the pH and temperature of the culture environment also have an effect on the presence of TTMP. To evaluate the ability of BS-ppb11 to produce TTMP, we incubated it in a 5 L bioreactor. Different concentrations

of diammonium hydrogen phosphate were added in the middle of fermentation, and the pH and temperature were adjusted. The results showed (Figure 6b) that the concentration of AC gradually increased from the early stage of fermentation and could maintain a stable upward trend; when the fermentation reached 120 h, the yield of B-even in-laid reached the highest concentration of 75.4 g/L. From this point its concentration showed a decreasing trend, because the fermentation entered the late stage, the bacterial metabolic activity decreased, the substrate utilization ability decreased and the product synthesis ability decreased. Therefore, we selected 120 h as the time point for the addition of diammonium hydrogen phosphate. As shown in Figure 6a, the maximum TTMP produced by BS-ppb11 without the addition of ammonium salt was only 2.7 g/L. As the concentration of ammonium salt increased, the concentration of the product in the fermentation system also gradually increased. This indicates that the fermentation-generated ethyl, even in-lactate, combined with ammonium to produce TTMP, and that when the concentration of ammonium salt was 30 g/L, the highest concentration of TTMP produced by BS-ppb11 was 16.6 g/L. However, continuing to increase the concentration of $(NH_4)_2HPO_4$ did not result in a corresponding increase in product yield; instead, there was a decreasing trend. This trend could be attributed to the increase in osmotic pressure of the fermentation system due to the high concentration of NH_4^+; the excessive high osmotic pressure would directly affect the growth activity of bacteria and the secretion of products, ultimately leading to a decrease in TTMP concentration. The results showed (Figure 6c) that the concentration of TTMP produced by BS-ppb11 increased gradually with the increase of pH, proving that the pH was favorable for microorganisms to synthesize TTMP using ammonium and ethyl coupling in the fermentation system. When the pH increased to 7.8, BS-ppb11 was able to produce 18.3 g/L of TTMP; the continued increase of pH did not increase the product yield accordingly, so it was concluded that the optimum conversion pH of BS-ppb11 for TTMP in the middle of fermentation was 7.8. At the initial fermentation temperature of 37 °C, the concentration of tetramethylpyrazine synthesized by BS-ppb11 was 16.3 g/L (Figure 6d). With the increase of temperature, the concentration of TTMP tended to increase slowly; when the temperature increased to 40 °C, the concentration of TTMP in the fermentation system reached the maximum value of 19.1 g/L. The continued increase in temperature did not continue to raise the concentration of TTMP, but showed a decreasing trend. The results showed that after adding ammonium salt in the middle of fermentation, slightly increasing the fermentation temperature could promote the efficiency of converting BS-ppb11 into TTMP and help to increase the concentration of TTMP in the fermentation broth; however, too high a temperature would affect the synthesis reaction of TTMP, probably because the higher temperature was no longer suitable for the growth of bacteria, leading to the reduction of their vital activities, intracellular enzyme activity and metabolic capacity.

To further enhance TTMP production by BS-ppb11, we used a staged batch replenishment fermentation culture system. Fermentation was performed in a 5 L benchtop fermenter with a 2 L loading volume. The initial glucose concentration was 70 g/L, and the glucose solution was replenished every 12 h to maintain the concentration of residual sugars, for a total of six replenishments. The focus of the first stage of fermentation was to generate sufficient AC, so fermentation was conducted at 37 °C and pH 7.5. The results showed (Figure 7) that the AC concentration increased rapidly in the first stage of fermentation, reaching a maximum of 71.4 g/L AC at 120 h. The TTMP concentration was only 3.6 g/L at this time due to the lack of ammonium ions necessary for synthesis. In the second stage of fermentation, the temperature was raised to 40 °C, the pH was adjusted to 7.8 and the fermentation broth was supplemented with diammonium hydrogen phosphate to a concentration of 30 g/L. After that, the concentration of AC started to decrease and the concentration of TTMP increased significantly, proving that AC was converted into TTMP in large quantities. At the 216th hour, the combined concentration of TTMP reached 34.8 g/L. The TTMP yield in the second stage of fermentation was 0.26 g/L-h; the conversion rate of AC was 67.4%.

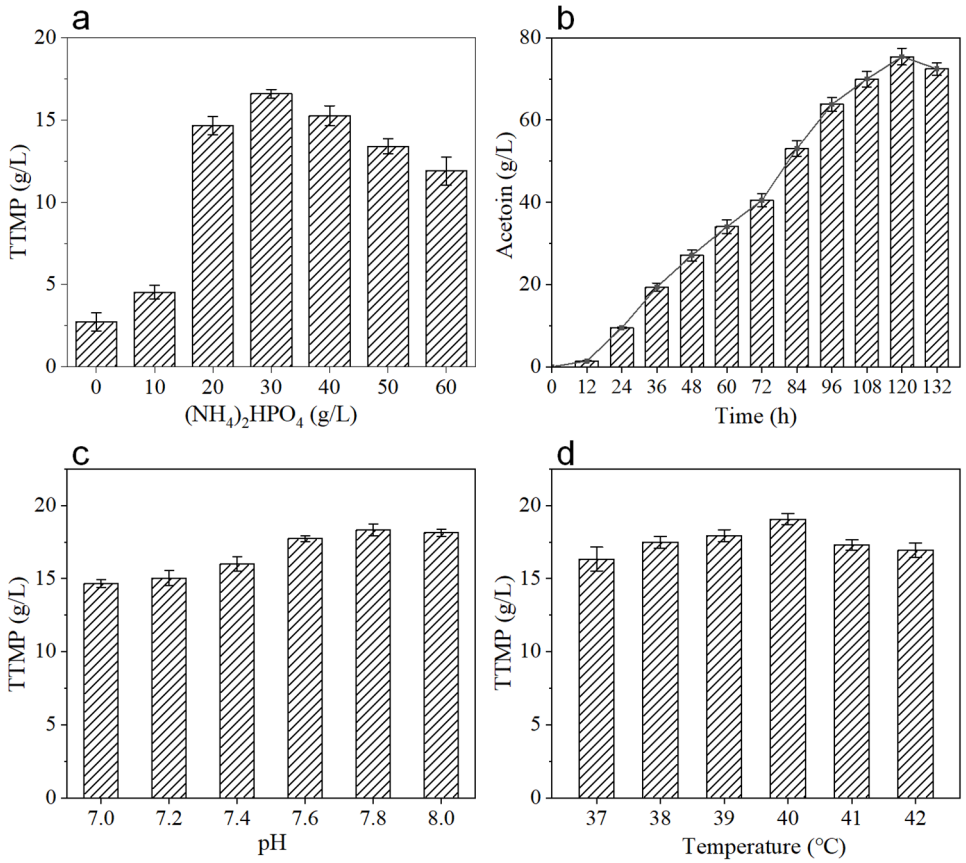

Figure 6. Effect of different factors on the production of TTMP by BS-ppb11 in shake flask fermentation, (**a**) Effect of the concentration of $(NH_4)_2HPO_4$ on the production of TTMP from BS-ppb11; (**b**) Concentration trend of AC produced by BS-ppb11; (**c**) Effect of pH on the production of TTMP from BS-ppb11; (**d**) Effect of temperature on the production of TTMP from BS-ppb11.

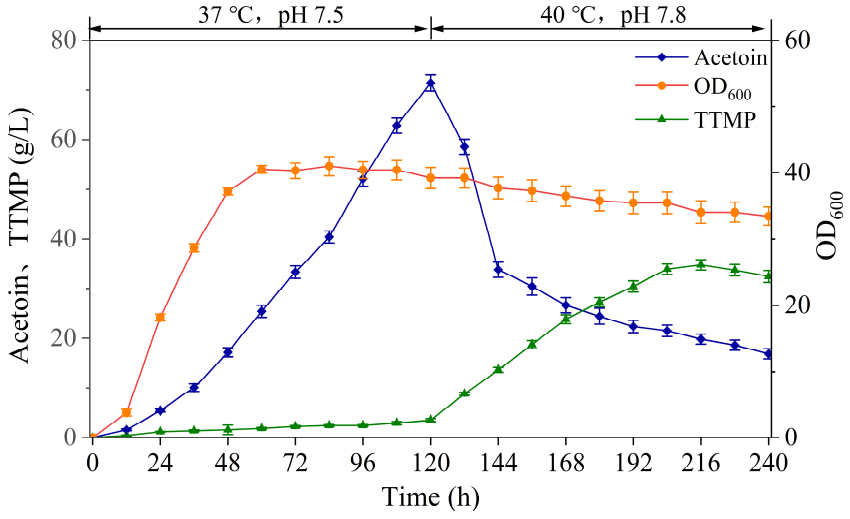

Figure 7. BS-ppb11 for TTMP production in a 5 L bioreactor.

3.4. Light Fermentation of Engineered Photocatalytic System Bacteria BS-ppb12

For the fermentation experiments of the photocatalytic composite system engineered bacterium BS-ppb12, we first tested the effect of five concentrations of gold nanoclusters on bacterial growth. The results showed (Figure 8) that bacterial growth was inhibited to different degrees as the concentration of gold nanoclusters was increased; with concentrations

of 12% and 20%, near normal growth of bacteria was observed, but the inhibition effect of the gold nanoclusters gradually increased as the concentration continued to increase. Observation of the bacteria by HAADF-STEM and EDS showed (Figure 9) that the bacterial cells contained a large number of gold nanoclusters; it also demonstrated that the gold nanoclusters were effectively absorbed. To ensure the normal growth of the engineered bacteria and the effectiveness of the photocatalytic material, a concentration of 20% gold nanoclusters was selected for subsequent experiments.

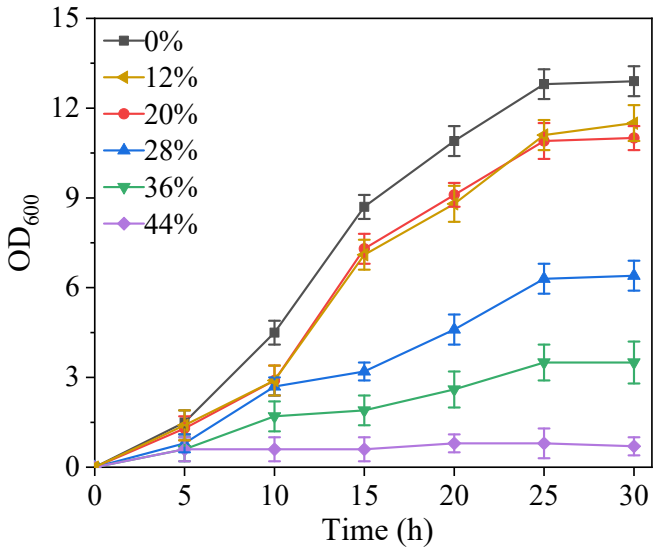

Figure 8. Effect of gold nanoclusters on bacterial growth.

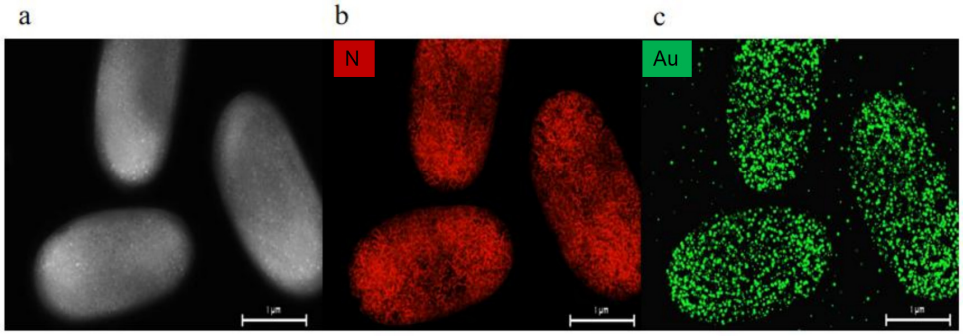

Figure 9. Observation of the photocatalytic composite system engineered bacterium BS-ppb12 by HAADF-STEM and EDS. (**a**) HAADF-STEM image of BS-ppb12. EDS mapping of the region in (**b**,**c**), showing the elements Au (**c**) and N (**b**) across the entire cel. Scale bars for (**a**–**c**), 1 um.

In light fermentation (Figure 10), the rate of glucose consumption by the photocatalytic strain BS-ppb12 was slightly increased when compared to that of the control strain BS-ppb11, which does not perform fermentation in light. The highest 2,3-BD produced by 100 h of fermentation was 33.5 g/L, which was a 26.4% increase in 2,3-BD production when compared to that of BS-ppb11. Apparently, the time point of AC production by photocatalytic bacteria was significantly delayed, and the concentration decreased by 60.2%, proving that, under irradiation with a xenon light source, the gold nanoclusters inside BS-ppb12 cells could absorb light energy into electrons, which promoted the conversion of NAD^+ to NADH and helped to convert a portion of AC into 2,3-BD.

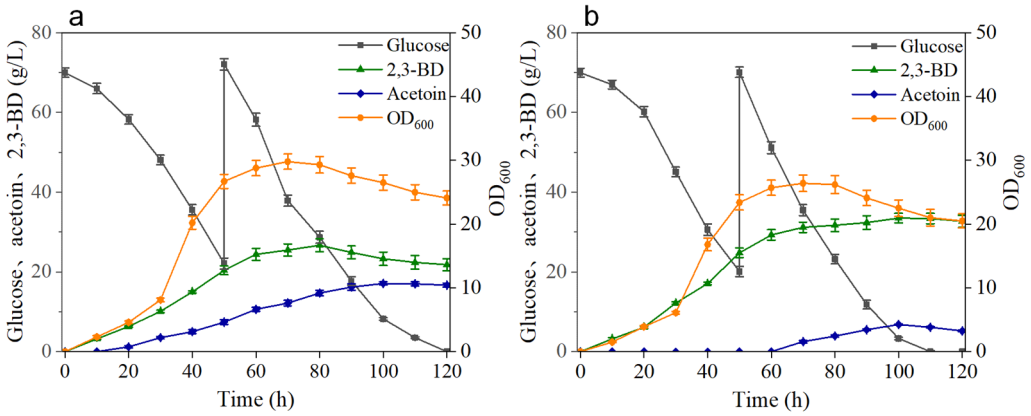

Figure 10. BS-ppb11 and BS-ppb12 fermentation for the production of 2,3-BD. (**a**) BS-ppb11 fermentation in no-light mode to produce 2,3-BD; (**b**) BS-ppb12 fermentation in light mode to produce 2,3-BD.

Figure 11 shows the accumulation of BS-ppb11 by-products. Lactic acid also requires NADH in the synthesis pathway of *Bacillus*, so the concentration of lactic acid increases to a certain extent during light fermentation, while there is no significant change in the pathway that does not require the participation of coenzymes such as formic acid. The experimental results above demonstrate that microbial cells can be used as "catalysts" and gold nanoclusters as light absorbers to generate photogenerated electrons to regulate intracellular redox homeostasis, thus affecting the catalytic activity and efficiency of key enzymes and achieving effective regulation of metabolic pathways. It should be noted that the effectiveness of gold nanoclusters requires suitable concentrations, so a rational approach to the use of gold nanoclusters should be based on a better biocompatibility and achieved by exploring suitable binding modes and binding concentrations.

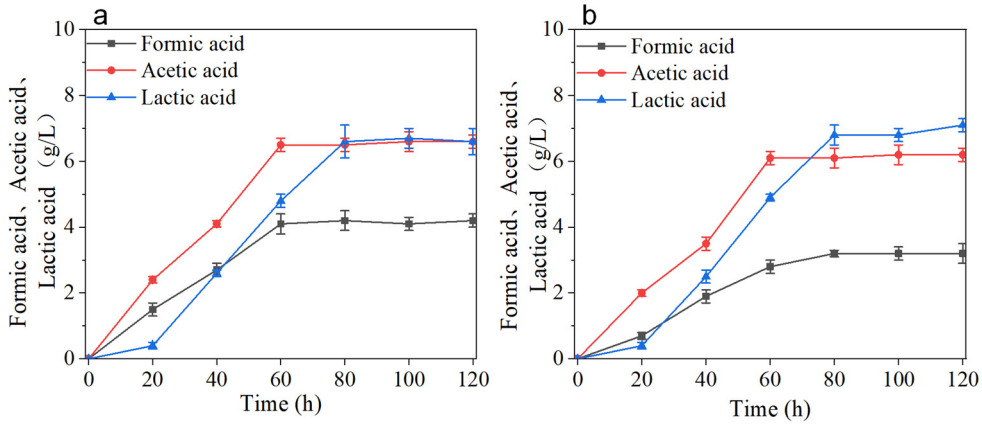

Figure 11. Accumulation of by-products during fermentation production of BS-ppb11 and BS-ppb12. (**a**) Accumulation of formic acid, acetic acid and lactic acid during fermentation production of BS-ppb11; (**b**) Accumulation of formic acid, acetic acid and lactic acid during fermentation production of BS-ppb12.

4. Conclusions

We explored the fermentation conditions of BS-ppb11 and came up with culture conditions suitable for 2,3-BD and AC, respectively. We also obtained higher concentrations of the corresponding products under different conditions.

In this study, *Bacillus subtilis* was used as the target to explore culture conditions suitable for 2,3-BD and AC and to obtain higher concentrations of the corresponding products under different conditions after overexpression of 2,3-butanediol dehydrogenase by dual promoter.

The maximum content of 2,3-BD obtained with BS-ppb11 was 96.5 g/L at a pH of 6.5 and an aeration of 1 L/min; the chiral purity of (R,R)-2,3-BD was 94.7%. The maximum content of AC was 82.2 g/L at a pH of 7.5 and a aeration of 2 L/min. Based on the high concentration of AC, we optimized the process of TTMP production from BS-ppb11; the results showed that the fermentation of BS-ppb11 for 120 h, supplemented with 30 g/L of diammonium hydrogen phosphate, raising the pH to 7.8 and the temperature to 40 °C, resulted in a final product of 34.8 g/L, which is the highest record of TTMP obtained by biological methods so far. By constructing a photocatalytic coenzyme regeneration system, the yield of 2,3-BD produced by BS-ppb12 was increased by 26.4%, demonstrating the feasibility of photocatalytic materials combined with microorganisms for the synthesis of organic compounds. The engineered strain BS-ppb11 can carry out selective production of tetramethylpyrazine, (R,R)-2,3-butanediol and acetoin in higher yields under the synergistic regulation of different processes; this can save energy and reduce emissions to achieve large-scale green high-value synergistic production. All results indicate that *Bacillus subtilis* is a promising strain for increased production of TTMP, acetoin and 2,3-BD, and that the use of BDH as a key hub for the coproduction of the three products is desirable but is based on precise metabolic regulation.

Author Contributions: W.M. and W.Q. designed and supervised the research work and guided the experiments. X.H. and H.L. conceived and designed the study. L.S., Y.L. (Yuan Lin) and J.S. carried out the experiment and analyzed the data. Y.G. and Y.L. (Yonghong Lin) wrote the manuscript and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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