

## Article

# Efficient Production of 3'-Sialyllactose by Single Whole-Cell in One-Pot Biosynthesis

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**Abstract:** Sialyllactose (SL) is one of the most important acidic oligosaccharides in human milk, which plays an important role in the health of infants. In this work, an efficient multi-enzyme cascade was developed in a single whole cell to produce 3'-SL. We constructed two compatible plasmids with double cloning sites to co-express four genes. Different combinations were assessed to verify the optimal catalytic ability. Then, the conversion temperature, pH, and stability under the optimal temperature and pH were investigated. Moreover, the optimal conversion conditions and surfactant concentration were determined. By using the optimal conditions (35 °C, pH 7.0, 20 mM polyphosphate, 10 mM cytidine monophosphate (CMP), 20 mM MgCl<sub>2</sub>), 25 mL and 4 L conversion systems were carried out to produce 3'-SL. Similar results were obtained between different volume conversion reactions, which led the maximum production of 3'-SL to reach 53 mM from 54.2 mM of sialic acid (SA) in the 25 mL system and 52.8 mM of 3'-SL from 53.8 mM of SA in the 4 L system. These encouraging results demonstrate that the developed single whole-cell multi-enzyme system exhibits great potential and economic competitiveness for the manufacture of 3'-SL.

**Keywords:** multi-enzyme; single whole cell; human milk oligosaccharides; 3'-sialyllactose; CTP regeneration



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## 1. Introduction

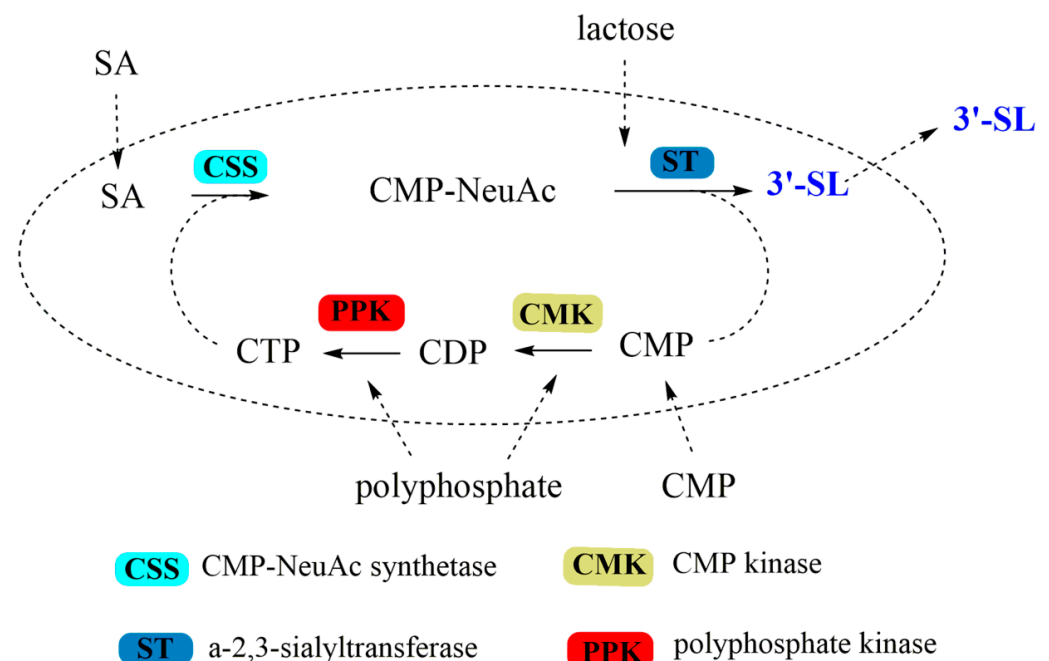
Breastfeeding is the gold standard of infant nutrition, and human milk oligosaccharides (HMOs) are unique and important bioactive ingredients in human milk [1,2]. SL is one of the most abundant and representative acidic oligosaccharides, which accounts for about 10–30% of total HMOs [3]. SL has important physiological functions in human health, such as gut maturation, resistance to gut pathogens, and prebiotic effects [4,5]. According to the position of the glycoside bond between SA and lactose, SL is divided into 3'-SL and 6'-SL [6]. At present, other rich HMOs have been used in infant formula [7], but SL has not been widely used due to technical limitations. Therefore, research on the efficient preparation of SL has important application significance.

Enzyme-catalyzed synthesis is one of the methods for preparing 3'-SL and has a wide range of application prospects [8]. Two kinds of enzymes are involved in the catalytic reaction, trans-sialidase [9,10] and sialyltransferase [11,12]. The former transfers the sialic acid of the donor to the lactose to form 3'-SL. This catalytic reaction does not require an additional energy donor, but trans-sialidase can only recognize  $\alpha$ -2, 3-bound sialic acid of the donor [13], resulting in low substrate utilization and low yield. In contrast, sialyltransferase can also transfer the sialic acid monomer to lactose, but sialic acid must be in active form,

e.g., cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-sialic acid) [14]. Therefore, using SA as a substrate requires CMP-sialic acid synthetase for preparation of activated intermediates. Several research works have tried to use sialyltransferase to produce 3'-SL. Endo [15] used five types of permeabilized cells to prepare 3'-SL. Although 52 mM of 3'-SL was obtained after 11 h of reaction and the actual conversion rate was rather high, a large number of cell additions and cumbersome operations limited further industrial applications of 3'-SL. Moreover, the fusion expression of CMP-sialic acid synthetase and sialyltransferase was used to prepare 3'-SL [16]. After 7 days of reaction in the 2.2 L system, 48.6 mM of 3'-SL was obtained from 165 mM of SA. It can be clearly seen that the reaction time was long and the conversion rate was low. Additionally, the more expensive substrate phosphoenolpyruvate (PEP) was used to regenerate cytidine triphosphate (CTP) from CMP. Therefore, a fast, efficient, and low-cost method for preparing 3'-SL is an important prerequisite for industrialization.

CTP is the important substrate for preparing CMP-sialic acid, but its high price makes it unrealistic to directly use as the substrate for the production of 3'-SL. Several CTP regeneration pathways have been proposed. Sun-Gu Lee [17] used CMP kinase and acetate kinase as the catalyst to produce CTP from CMP and acetyl phosphate. Wang [18] utilized CMP kinase, polyphosphate kinase, and nucleoside-diphosphate kinase to synthesize CTP from CMP, adenosine triphosphate (ATP), and polyphosphate. However, these CTP regeneration systems require either more expensive substrates or more types of enzymes. In contrast, due to the fact that the substrate used was cheap and easily available, the combination of CMP kinase and polyphosphate was considered to be the most promising method [19], which was adopted in this study.

In this study, the previously constructed multi-cell enzyme system [20] was reconstructed into a single recombinant strain. Two compatible plasmids were used to complete the generation of 3'-SL and regeneration of CTP (Figure 1). More importantly, transformation conditions were optimized, and the optimal conversion system was conducted in 25 mL and 4 L systems to investigate the production of 3'-SL.



**Figure 1.** Diagram of single whole-cell multi-enzyme catalytic pathway.

## 2. Materials and Methods

### 2.1. Materials

3'-SL was purchased from Carbosynth (Carbosynth, China), SA was obtained from CASOV (Wuhan, China), and CMP was purchased from Huaren (Wuhu, China). All other chemicals used in the study were commercially available and were of analytical grade. Molecular biology reagents used in this study are listed in Table S1.

### 2.2. Plasmids and Strains

The pET-22b (+) plasmid harboring the gene encoding CMP-sialic acid synthetase,  $\alpha$ -2, 3-sialyltransferase, CMP kinase, and polyphosphate kinase was previously constructed [20]. The gene of CMP-sialic acid synthetase was amplified with *NcoI* and *NotI* restriction sites by using plasmid pET-CSS as template. Then, *NcoI* and *NotI* were used to digest amplified fragments and pCOLADuet-1. Double enzyme fragments were recovered from agarose gel and ligated by DNA ligase. The ligation products were transformed into competent cells and spread on the plate containing  $Km^R$  for screening. The positive transformants were confirmed by PCR verification and sequencing. After the verification, the plasmid pCOLADuet-CSS was obtained. The gene of  $\alpha$ -2, 3-sialyltransferase was also cloned and inserted into the second multiple cloning sites of pCOLADuet-CSS between the restriction sites of *NdeI* and *XhoI* to achieve recombinant expression plasmid pCOLADuet-CSS-ST. The same method was used to obtain recombinant plasmids pCOLADuet-ST-CSS, pETDuet-PPK-CMK, and pETDuet-CMK-PPK. The flow chart of the different plasmid constructions is shown in Figures S1–S4.

All constructed plasmids were transformed into *E. coli* BL21 Star (DE3)  $\Delta lacZ \Delta nanETKA$ , constructing the following strains: *E. coli*/pCSPC (pCOLADuet-CSS-ST/pETDuet-PPK-CMK), *E. coli*/pCSCP (pCOLADuet-CSS-ST/pETDuet-CMK-PPK), *E. coli*/pSCPC (pCOLADuet-ST-CSS/pETDuet-PPK-CMK), and *E. coli*/pSCCP (pCOLADuet-ST-CSS/pETDuet-CMK-PPK). The positive single clone in the double-resistant ( $Km^R$  and  $Amp^R$ ) plate was picked, cultured, and stored for use.

Chromosomal gene disruption of the host strain was carried out with the  $\lambda$ red homology recombination method [21], with a slight modification. Transformants carrying pKD46 were grown in 5 mL LB cultures with ampicillin and L-arabinose at 30 °C, and then electrocompetent cells were produced by centrifuging and washing with ice-cold 10% glycerol. PCR products with the FRT-flanked resistance gene were gel purified and suspended in ddH<sub>2</sub>O. Electroporation was conducted by using an electroporator (MicroPulser, Bio-Rad). Shocked cells were added to 1 mL LB culture, incubated at 37 °C for 2 h, and then spread onto agar to select  $Km^R$  transformants. After PCR verification, positive mutants were transformed with pCP20 and selected at 30 °C. The colony was cultured at 42 °C and tested for the loss of  $Km^R$ . Strain *E. coli* BL21 Star (DE3)  $\Delta lacZ$  was previously constructed [20]. Gene cluster *nanETKA* in the *E. coli* BL21 Star (DE3)  $\Delta lacZ$  genome was disrupted by homology recombination. *E. coli* BL21 Star (DE3)  $\Delta lacZ nanETKA$  was used as the expression strain for protein expression. Plasmids and strains are shown in Table 1.

### 2.3. Biocatalyst Preparation

The recombinant *E. coli* BL21 Star (DE3)  $\Delta lacZ \Delta nanETKA$  was cultured in a 5 L fermenter. The cells were grown at 37 °C in 3 L fermentation medium (peptone 12 g/L, yeast extract 8 g/L, K<sub>2</sub>HPO<sub>4</sub> 4 g/L, NaCl 3 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 2.5 g/L, glycerin 10 g/L, citric acid 2.1 g/L; feeding substrates: glycerol 400 g/L, peptone 30 g/L, yeast extract 50 g/L). The cell was induced with 0.2 mM IPTG at 20 °C for 18 h after starting the feeding. The recombinant wet cells were harvested by using a laboratory centrifuge at 4 °C (8000 rpm, 10 min).

**Table 1.** Strains, genes, and plasmids used in the study.

	Description	Reference or Source
Strains		
<i>E. coli</i> BL21 Star (DE3) $\Delta lacZ\Delta nanETKA$	F- ompT hsdSB (rB-, mB-) gal dcm rne131 (DE3) $\Delta lacZ\Delta nanETKA$	This study
Genes		
<i>css</i>	CMP-sialic acid synthetase from <i>Neisseria meningitidis</i> (U60146.1)	[16]
<i>st</i>	$\alpha$ -2, 3-sialyltransferase from <i>Neisseria gonorrhoeae</i> (U60664.1)	[11]
<i>cmk</i>	CMP kinase from <i>Escherichia coli</i> (X00785.1)	[22]
<i>ppk</i>	Polyphosphate kinase from <i>Escherichia coli</i> (CP043942.1)	[23]
Plasmids		
pCOLADuet-ST-CSS	pCOLADuet-1 containing <i>st</i> and <i>css</i>	This study
pCOLADuet-CSS-ST	pCOLADuet-1 containing <i>css</i> and <i>st</i>	This study
pETDuet-CMK-PPK	pETDuet-1 containing <i>cmk</i> and <i>ppk</i>	This study
pETDuet-PPK-CMK	pETDuet-1 containing <i>ppk</i> and <i>cmk</i>	This study

#### 2.4. Optimization of Reaction Conditions

The optimization experiments were carried out in a 100 mL flash shaker with 25 mL mixtures. The effect of temperatures on the 3'-SL content in the range of 25–45 °C was compared. The bioconversion at different pH ranging from 5.0 to 10.0 was conducted, including 50 mM of sodium acetate buffer (pH 5.0–6.0), 50 mM of Tris-HCl (pH 7.0–8.0), and 50 mM of glycine-NaOH (pH 9.0–10.0). The catalytic stability of the bioconversion system at optimum temperature and pH was also evaluated.

The optimal addition of the cell extracts was investigated by varying the wet weight cells from 10 to 50 g/L. In addition, the effect of different concentrations of CMP, MgCl<sub>2</sub>, and polyphosphate on the content of 3'-SL was assessed. The substrate concentrations were set at 0 mM, 5 mM, 10 mM, 20 mM, 40 mM, and 60 mM.

Furthermore, to simplify the catalytic process, the effect of different surfactants of Triton X-100, Tween-20, sodium dodecyl sulfate (SDS), and cetyl trimethyl ammonium bromide (CTAB) on 3'-SL biosynthesis was investigated. Finally, the effect of the Triton X-100 concentration from 0.2 to 1.6% on 3'-SL content was studied. All the other components were fixed at 50 mM SA, 60 mM lactose, 20 mM polyphosphate, 10 mM CMP, and 20 mM MgCl<sub>2</sub>. The samples were taken after 2 h, and 3'-SL content was detected by HPLC.

#### 2.5. Enzyme Activity Assays

The activity of CSS was measured with 0.2 M Tris-HCl (pH 8.5), 20 mM MgCl<sub>2</sub>, 5 mM SA, 5 mM CTP, and an enzyme sample. The reaction was performed at 37 °C for 10 min.

The activity of ST was measured with 0.2 M Tris-HCl (pH 8.5), 20 mM MgCl<sub>2</sub>, 5 mM SA, 5 mM CTP, and 10 mM lactose. Both CSS and ST were used to start the reaction, and the reaction was performed at 37 °C for 30 min.

The CMK activity reaction mixture included 50 mM Tris-HCl (pH 8.0), 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 10 mM ATP, and 5 mM CMP. The PPK activity reaction mixture included 50 mM Tris-HCl (pH 8.0), 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 5 mM ADP, and 10 mM polyphosphate. The reactions were performed at 30 °C for 30 min.

One unit of enzyme activity was defined as the amount that catalyzes the formation of 1  $\mu$ mol target product per min.

## 2.6. Production of 3'-SL

The reaction was performed with 25 mL and 4 L of mixtures in a 100 mL flash shaker and 5 L fermenter, respectively. The reaction mixture contained 50 mM SA, 60 mM lactose, 20 mM MgCl<sub>2</sub>, 20 mM polyphosphate, 20 mM CMP, 40 g/L recombinant cells, and 0.8% (*v/v*) Triton X-100. The reaction in the 25 mL system was incubated at 35 °C in a water bath with a magnetic stirrer, and in the 4 L system, it was automatically controlled by the temperature at 35 °C. The reaction system was maintained at pH 7.0 using 4 N NaOH. A reaction sample was taken every 2 h, and the concentrations of SA and 3'-SL were analyzed by HPLC. All the experiments above were performed in triplicates.

## 2.7. Analytical Method

The concentrations of SA and 3'-SL were analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with a UV detector and the detection wavelength was 210 nm. A TSK-Gel Amide-80 column was used, and 10 mM ammonium formate (pH 4.0) and acetonitrile at a ratio of 30:70 were used as a mobile phase. The flow rate was set at 1.0 mL/min.

Quantitative analysis of CDP and ATP was performed using HPLC (LC-16, Shimadzu, Kyoto, Japan), which was equipped with a UV detector at 271 nm and a Zorbax C18 column. The mobile phase was 0.6% phosphate-triethylamine (pH 6.6), and the methanol ratio was 89:11. Quantitative analysis of CMP-NeuAc was detected at 210 nm, and the mobile phase was 20 mM pH 8.0 phosphate buffer. The samples were detected at 30 °C at a flow rate of 0.6 mL/min. All tested samples were boiled for 2 min and centrifuged at 12,000 rpm for 5 min. The supernatant was filtered with a 0.22-micron filter membrane and diluted to a certain concentration before testing. The relative content of 3'-SL was calculated as follows:

$$\text{Relative content of 3'-SL} = \frac{\text{3'-SL content of each condition in the experimental group}}{\text{The highest content of 3'-SL in the experimental group}} \times 100\%$$

## 2.8. Statistical Analysis

The relative content of 3'-SL, the concentration of 3'-SL, and relative activity were evaluated statistically. Evaluation of statistical significance ( $p < 0.05$ ) was calculated according to the one-sample *t*-test.

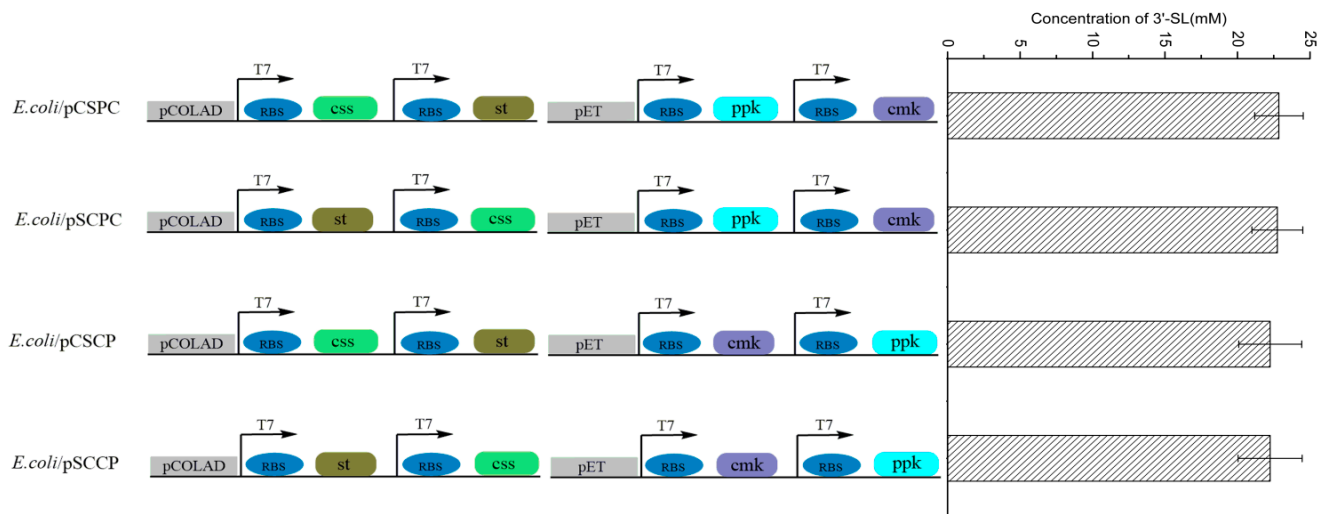
## 3. Results

### 3.1. Single-Cell Construction

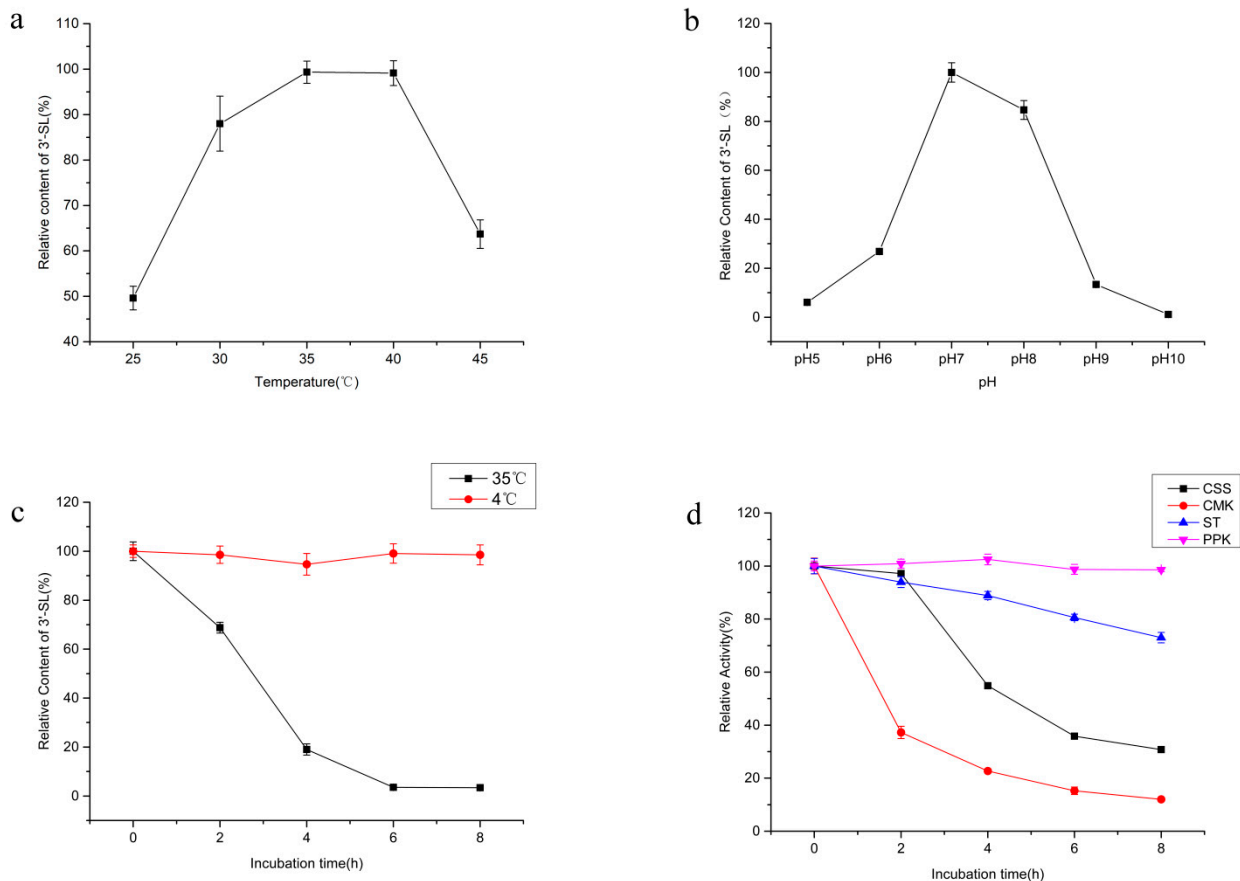
To investigate the overexpression of *css*, *st*, *cmk*, and *ppk* affecting the 3'-SL synthesis, two compatible plasmids, pETDuet-1 and pCOLADuet-1, were used to express four genes in a recombinant *E. coli* strain by using the promoter from the bacteriophage T7. As shown in Figure 2, based on the different positions of the gene in the double cloning site, four recombinant strains were constructed. However, the best combination was *E. coli*/pCSPC, and the catalytic content of 3'-SL was  $22.8 \pm 1.7$  mM. In contrast, the worst combination was *E. coli*/pSCCP, which resulted in  $22.2 \pm 2.2$  mM of 3'-SL. No obvious difference in 3'-SL content was observed between different expression systems ( $p > 0.05$ ). Due to strain *E. coli*/pCSPC having the highest average value, the strain *E. coli*/pCSPC was selected for the subsequent study. This shows that the adjustment of the genes in the plasmid containing the double cloning site did not significantly affect the protein expression.

### 3.2. Optimization of Biotransformation Temperature and pH

Optimization of biotransformation was conducted to increase 3'-SL production. As seen in Figure 3a, when the temperature was lower than 35 °C, with the increase in reaction temperature, 3'-SL showed a gradually increasing trend. 3'-SL decreased with the temperature over 40 °C, and there was no significant difference in 3'-SL content between 35 and 40 °C ( $p > 0.05$ ). Considering energy consumption, 35 °C was chosen as the optimal temperature.



**Figure 2.** Optimization of 3'-SL production by different combinations of plasmids.



**Figure 3.** Effect of temperature and pH on the conversion. (a) Effect of temperature on the conversion; (b) effect of pH on the conversion; (c) catalytic stability under different temperatures; (d) stability of enzyme activity under optimal temperature.

The biotransformation showed the maximum production of 3'-SL at pH 7.0 (Figure 3b). When the pH of the biotransformation system was adjusted to 5.0, the relative content of 3'-SL dropped sharply. When the pH reached 10.0, the relative concentration of 3'-SL was only 2% of the highest concentration ( $p < 0.01$ ). Therefore, an appropriate pH is essential to 3'-SL formation, and a higher or lower pH could seriously affect the content of the product.

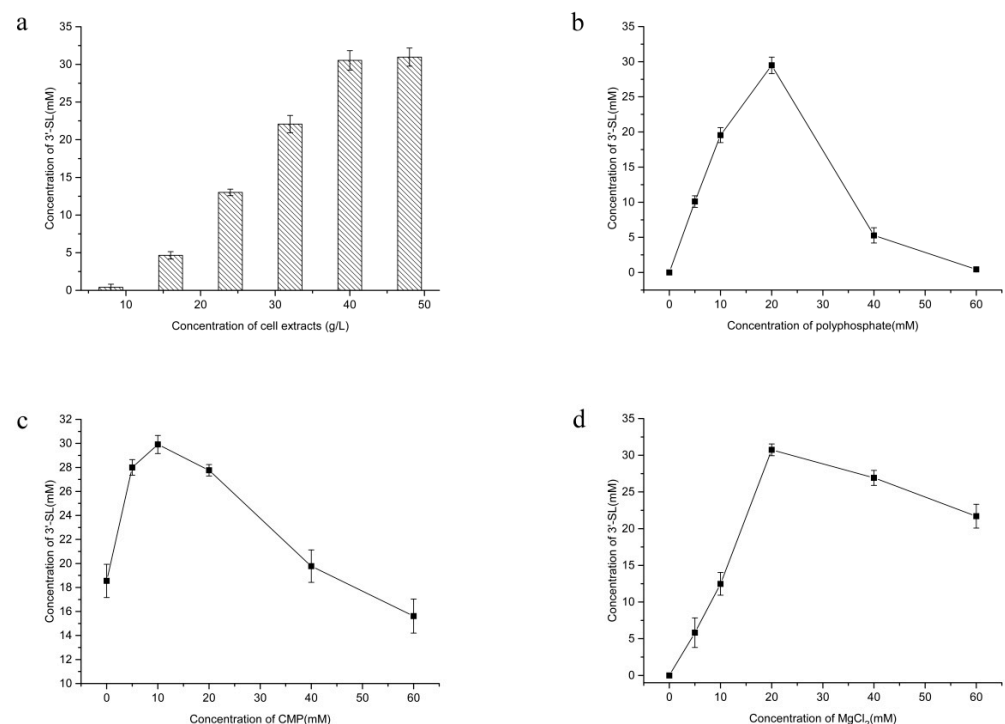
In addition, the catalytic stability at different temperatures was also investigated. As shown in Figure 3c, 4 °C and 35 °C represent the cell extracts that were placed at pH 7.0 at

4 °C and 35 °C for 8 h, respectively. After the cell extracts were placed at pH 7.0 and 4 °C for 8 h, no significant loss of catalytic activity was observed compared with 0 h ( $p > 0.05$ ). However, under the condition of pH 7.0 and 35 °C, the catalytic activity of the cell extracts was gradually lost with the time increase. At 6 h, the relative catalytic activity was only 3% of the initial catalytic activity ( $p < 0.01$ ). This shows that the multi-enzyme catalytic system has a poor thermal stability.

Next, we investigated which enzyme or enzymes caused poor thermal stability. To study the thermal stability of a single enzyme, the expression host containing a single enzyme was used. pET-CSS, pET-ST, pET-CMK, and pET-PPK recombinant plasmids were constructed in the previous study [20] and transformed into four single strains, which formed four single-gene expression strains. As depicted in Figure 3d, CSS, ST, CMK, and PPK represent the relative activities of CMP-sialic acid synthetase, sialyltransferase, CMP kinase, and polyphosphate kinase, respectively. Using the single-gene expression strains to investigate the thermal stability of each enzyme, it was found that the thermal stability of different enzymes changed at the optimal catalytic temperature. Among them, PPK has the best thermal stability, and there was no obvious loss of enzyme activity after 8 h of incubation compared with 0 h ( $p > 0.05$ ). CMK has the worst thermal stability. The catalytic activity was only 11% of the initial catalytic activity after 8 h of incubation ( $p < 0.01$ ), while the enzyme activity of CSS was only 30% of the initial activity ( $p < 0.01$ ). Therefore, the poor stability of more than one enzyme leads to the poor stability of the entire catalytic system.

### 3.3. Optimization of Cell Extracts, Polyphosphate, CMP, and $MgCl_2$

Different concentrations of cell extracts were assessed to determine the optimal cell extract addition (Figure 4a). With the number of cell extracts increased, the 3'-SL content increased accordingly, but there was no significant difference in 3'-SL content at 40 g/L and 48 g/L cell extract additions ( $p > 0.05$ ); therefore, it was considered that the optimal amount of cell extracts was 40 g/L.



**Figure 4.** Effect of concentration of cell extracts (a), polyphosphate (b), CMP (c), and  $MgCl_2$  (d) on 3'-SL production.

Polyphosphate provides phosphate for CTP regeneration, meaning an appropriate concentration of polyphosphate helps to promote the accumulation of 3'-SL. When the

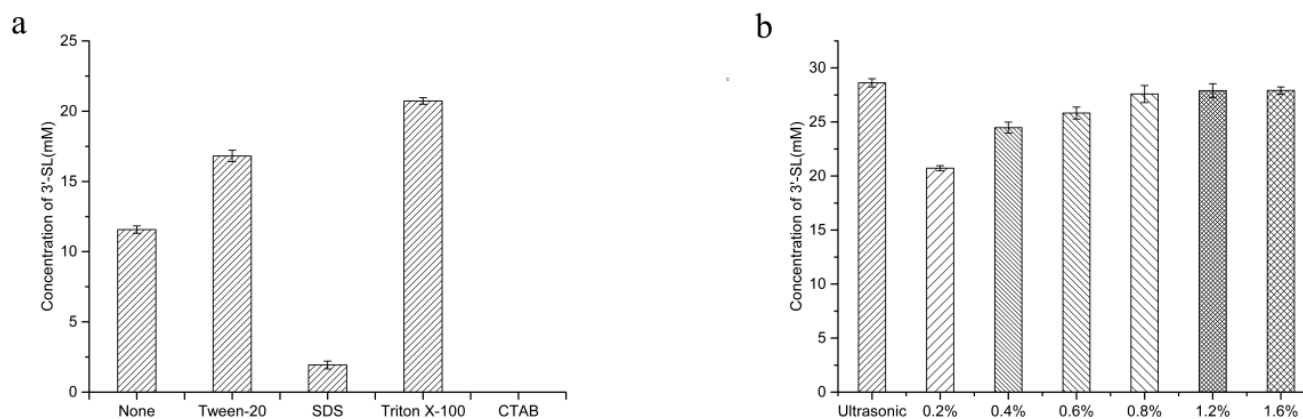
amount of polyphosphate added exceeded 20 mM, the 3'-SL content decreased significantly compared with the 20 mM addition ( $p < 0.01$ ). This may be due to excessive polyphosphate forming a chelate with magnesium ions, thereby affecting the formation of 3'-SL. The amount of 20 mM of polyphosphate was determined as the optimal addition according to the different additions of polyphosphate (Figure 4b).

Additionally, CMP is an important substrate for CTP regeneration, and the concentration of CMP has an important impact on 3'-SL yield and production cost. As shown in Figure 4c, when the concentrations of CMP were lower than 10 mM, the content of 3'-SL increased with the increase in CMP concentration. With the concentration of CMP increased, the yield of 3'-SL gradually decreased when the concentration of CMP was higher than 10 mM. Additionally, the content of 3'-SL in the 10 mM CMP addition was significantly higher than that at 5 mM and 20 mM ( $p < 0.05$ ). Therefore, the optimal CMP concentration was 10 mM. It can be seen that about 18 mM of 3'-SL was produced in the conversion system without adding CMP. This may be due to the use of RNA degradation products in cell extracts for CTP synthesis, which is consistent with the result of the multi-bacterial catalytic system [20]. Due to the reduction in the number of cell extracts, a lower CMP addition was used, and a higher 3'-SL catalytic yield was obtained, which was of great significance to reduce the cost of catalysis.

Different concentrations of  $MgCl_2$  were set to verify the effect on the 3'-SL content (Figure 4d). The 3'-SL content first increased and then decreased with the increase in the  $MgCl_2$  concentration. When the  $MgCl_2$  concentration was 20 mM, the 3'-SL content reached the maximum value, which was obviously higher than that of 10 mM and 40 mM ( $p < 0.05$ ). The results indicate that the optimal  $MgCl_2$  concentration was 20 mM.

### 3.4. Optimization of Cell Permeability

Surfactants can change cell permeability and increase the transfer rate of intracellular and extracellular substances [24]. Triton X-100 had the highest 3'-SL production, which was 44% higher than the control group ( $p < 0.01$ ) and 19% higher than Tween-20 ( $p < 0.01$ ) (Figure 5a). However, the 3'-SL content of the CTAB and SDS groups was lower than that of the control group, which may be due to the protein denaturation caused by these two surfactants. Moreover, the production of 3'-SL treated by Triton X-100 was still lower than that in the ultrasonic group. This may be due to the low amount of surfactant added, which led to cell permeability not being completely released. Therefore, Triton X-100 was selected as the optimal surfactant for further investigation.



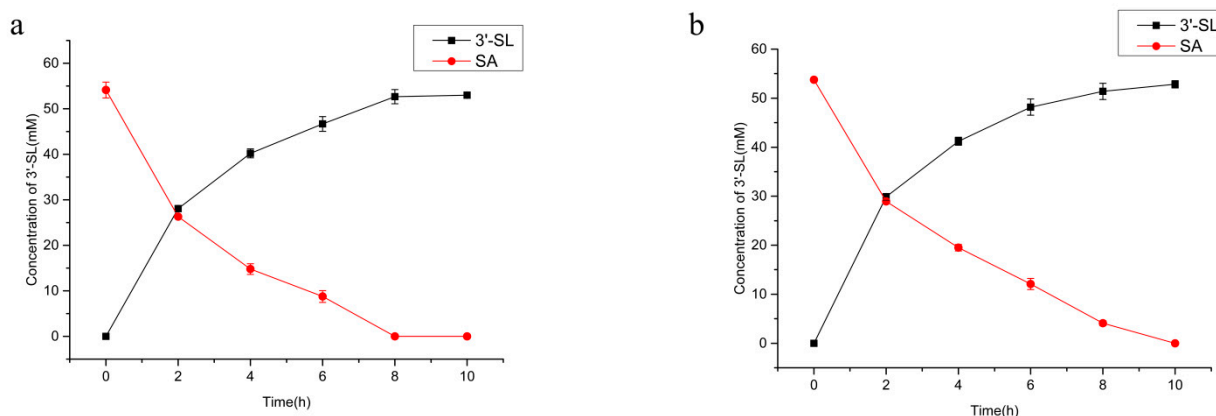
**Figure 5.** Effect of surfactants on 3'-SL concentration. (a) Effect of different surfactants on 3'-SL concentration; (b) effect of different contents of Triton X-100 on 3'-SL concentration.

The 3'-SL content gradually increased as the content of Triton X-100 increased; however, the 3'-SL content hardly increased when the amount of Triton X-100 was higher than 0.8%. The concentration of 3'-SL in the 1.2% or the 1.6% Triton X-100 addition was not

significantly higher than that in the 0.8% addition ( $p > 0.05$ ) (Figure 5b). Therefore, the optimal concentration of Triton was determined to be 0.8%. Comparing with the ultrasonic group, the 3'-SL content of the experimental group with Triton X-100 was 97% of its 3'-SL content, indicating that the addition of Triton X-100 did not obtain fully permeable whole cells. Overall, the addition of Triton X-100 was able to obtain a higher content of 3'-SL than the control group, which could be used to improve cell permeability and produce 3'-SL.

### 3.5. Production of 3'-SL

Based on the optimized reaction conditions, a different conversion system was conducted to verify the production of 3'-SL. In the 25 mL conversion system, the 3'-SL content increased with time, while the substrate content gradually decreased (Figure 6a). After an 8-h reaction, the substrate was completely consumed, and the 3'-SL content reached the maximum. An amount of 53.0 mM of 3'-SL was achieved from 54.2 mM of SA, which resulted in a high substrate conversion rate of 97.9%. According to the reaction time and product yield, the productivity rate was 6.63 mM/h. Since no obvious substrate loss was found, the conversion rate was the actual conversion rate calculated from the substrate content.



**Figure 6.** Time course of 3'-SL production under optimal conditions. (a) Time course of 3'-SL production in 25 mL transformation system; (b) time course of 3'-SL production in 4 L transformation system.

According to the result of the 25 mL conversion system, the 4 L conversion system in the 5 L fermenter was also investigated. The result was similar to the 25 mL conversion system, in which the reaction could completely convert the substrate into 3'-SL. An amount of 52.8 mM of 3'-SL was achieved from 53.8 mM of SA, and the substrate conversion rate was 98.1%. The chromatogram of the HPLC of the 4 L conversion system is demonstrated in Figure S5. It is obvious from the chromatogram that with the increase in the catalytic time, the peak of SA gradually decreased to disappear, while the peak of SL gradually increased. Although the expansion of the reaction volume has no obvious effect on the 3'-SL yield and substrate conversion rate, it can be seen that the time for the complete conversion of the substrate in the 4 L conversion system was 2 h longer than the 25 mL conversion volume, which resulted in the productivity rate being 5.28 mM/h. This may be due to the stirring speed having a certain influence on the catalytic efficiency in a larger conversion system. In conclusion, although the 25 mL and 4 L conversion systems have similar catalytic yields and substrate conversions, the 25 mL catalytic system has obvious advantages in terms of the productivity rate.

At present, it has been reported in the literature that the highest yield of 3'-SL achieved is 52 mM [15]. Although the catalytic production did not increase significantly, 98.1% of the actual conversion of sialic acid was obtained in a 4 L conversion system, which was the largest conversion volume and substrate conversion. The research results have important guiding significance for the scale-up of 3'-SL.

#### 4. Discussion

Enzyme-catalyzed synthesis systems are increasingly being used to produce fine chemicals or pharmaceutical products [25–27]. Several routes have been developed for biosynthesizing 3'-SL, and sialyltransferase can be used to produce relatively high-content products, which was considered to be one of the potential catalysts for industrialization. Considering the specificity of substrate utilization and the economics of product preparation, the utilization of CMP-sialic acid synthetase and construction of a cofactor regeneration of an expensive substrate were considered to be reasonable solutions. A similar CTP regeneration system was also used in the enzymatic synthesis of 3'-SL, which also contained CMP kinase and polyphosphate kinase [28]. The experiment used multi-cell and multi-enzyme coupling catalysis; however, the yield of 3'-SL was low and an expensive cofactor still needed to be added. In contrast, the multi-enzyme system in a single whole cell simplified the manufacturing process and exhibited the potential for industrial manufacture.

In our study, efficient multi-enzyme whole-cell catalysis for the production of 3'-SL in engineered *E. coli* was developed. Compatible plasmids were used to co-express key enzymes to increase the production of target products, which are widely used in multi-enzyme expression [29,30]. Based on the optimized conversion conditions, different volume transformation experiments were performed, and a relatively high yield and high conversion rate were obtained. However, the thermal stability of CMP kinase and CMP-sialic acid synthetase was poor, which suggests that improving the thermal stability of the enzyme may increase the catalytic efficiency and reduce the reaction time. Currently, the thermal stability modification experiment of the enzyme is in progress, and the catalytic verification experiment will be carried out.

#### 5. Conclusions

In summary, a multi-enzyme single whole-cell route for the production of 3'-SL was developed in this study. The catalytic process can be divided into two parts: product generation and cofactor regeneration. The product generation includes CMP-sialic acid synthetase and sialyltransferase, which was constructed to generate 3'-SL from SA. The cofactor regeneration was designed for the CTP formation from CMP, which includes CMP kinase and polyphosphate kinase. To obtain the optimal catalytic effect, conversion conditions were optimized. In the 25 mL and 4 L conversion systems, 53.0 mM and 52.8 mM of 3'-SL were obtained, which lead to conversion rates of 97.9% and 98.1%, respectively. This multi-enzyme system in the single whole-cell process provides the foundation for industrial-scale production of 3'-SL.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/pr9060932/s1>, Figure S1: Flow chart of recombinant plasmid pCOLADuet-CSS-ST construction, Figure S2: Flow chart of recombinant plasmid pCOLADuet-ST-CSS construction, Figure S3: Flow chart of recombinant plasmid pETDuet-PPK-CMK construction, Figure S4: Flow chart of recombinant plasmid pETDuet-CMK-PPK construction, Figure S5: Chromatogram of HPLC of 4 L conversion system at different time, Table S1: Molecular biology reagents used in this study.

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