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## Temperature Screening Of Trisaccharide (GlcNAc?1, 3Gal?1, 4Glc) Synthesis In Recombinant Escherichia Coli By Fermentation

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TEMPERATURE SCREENING OF TRISACCHARIDE (GlcNAc $\beta$ 1, 3Gal $\beta$ 1, 4Glc)  
SYNTHESIS IN RECOMBINANT *ESCHERICHIA COLI* BY FERMENTATION

by

CHI ZHANG

Under the Direction of Peng George Wang, PhD

ABSTRACT

This study reported an improved method of trisaccharide (GlcNAc $\beta$ 1, 3Gal $\beta$ 1, 4Glc) synthesis by fermentation of recombinant *Escherichia Coli*. The first part was designed for an induction temperature study. We want to compare the level of enzyme activity at different temperature and select the best condition to repeat the induction temperature in a fermenter to improve the production yield. First, the trisaccharide was synthesized at different induction temperature by direct fermentation (4L flask) of *E. coli* JM109 (DE3) harboring the LgtA-pET22b plasmid. The bacterial growth and trisaccharide production were compared. Second, the best condition for bacterial growth and induction of recombinant enzyme expression was repeated in a fermenter to improve its production yield. After simple two-step purification, final products were obtained with purity above 90% and all products were characterized by MALDI-TOF and HPLC.

INDEX WORDS: Fermentation, Chemoenzymatic, *Escherichia Coli*, Human Milk  
Oligosaccharide, Induction Temperature

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

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May 2018

## **DEDICATION**

Firstly, I would like to thank myself and family, especially to my mother and father for their love, encouragement, and more importantly, the financial support from them. My entire family gave me enough support to let me live outside of my country comfortably and peacefully.

To all my friends and teachers, thank you all for having me here, and it was a grateful two years. I experienced it and more importantly, I survived and made it.

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Eventually, I would like to thank every person I met in my life so far, no matter good or bad, I have always learned something from each of you. Thank you all.

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## 1 INTRODUCTION

Recent studies have shown that human milk oligosaccharides (HMOs) are a unique and irreplaceable component found in human breast milk. HMOs are a mixture of indigestible oligosaccharides composed of glucose, galactose, fucose, sialic acid and N-acetyl-glucosamine<sup>[1, 2]</sup>. It's essential to understand HMOs because it's natural exist in the human body and more importantly, HMOs have several positive factors for the development of infants. For example, the human milk has more beneficial oligosaccharides than other milk sources, which supports infant's immune system, and encourages the growth of beneficial bacteria in infant's digestive system<sup>[3, 4]</sup>. Furthermore, some manufactured fucosylated HMOs are already available on the market, such as, 2-fucosyllactose (2-FL, Fuc $\alpha$ 1-2Gal $\beta$ 1-4Glc) and are added as supplement in infant formula such as *Similac*. The other fucosylated HMOs showed potential value in pharmaceuticals. Recent publications reported that lacto-N-fucopentaose III (LNFP III, Gal $\beta$ 1-4(Fuc $\alpha$ 1-3) GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) could reduce central nervous system inflammation and experimental allergic encephalomyelitis severity<sup>[5, 6]</sup>. Therefore, based on above potentials and benefits of HMOs, the approach for large-scale synthesis of HMO is highly demanded<sup>[7, 8]</sup>.

From previous reports and publications, there are three strategies for oligosaccharide synthesis in large-scale production. (1) Enzymatic synthesis, (2) chemical synthesis, and (3) whole-cell synthesis or living cell factory<sup>[9, 10]</sup>. All three strategies have their advantages, however, for synthesis of more complex oligosaccharides, chemical synthesis and enzymatic synthesis are still facing some weaknesses<sup>[11, 12]</sup>.

Chemical synthesis of simple carbohydrates, such as 2-fucosyllactose (2-FL, Fuc $\alpha$ 1-2Gal $\beta$ 1-4Glc) have been reported and achieved in large scale. However, for more complex structures, multistep of protection, deprotection processes, and relatively low production yield could affect the variable cost and efficiency of chemical synthesis<sup>[13, 14]</sup>. On the other hand, enzymatic

synthesis was used as a suitable option because of its high stereoselectivity, high efficiency and under mild conditions, but the complex structure of oligosaccharide will be limited by enzyme sources, and fussy enzyme purification processes<sup>[15, 16]</sup>.

According to the third strategy, using whole-cell synthesis or living cell factory, a Japanese company *Kyowa Hakko Kogyo Co* has achieved a 100 gram-scale oligosaccharide production by using cheap starting materials and engineered bacteria for the production of oligosaccharides<sup>[17, 18]</sup>. Similarly, our group achieved trisaccharide(GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) synthesis by using “superbug technology”, which contained recombinant enzyme expression in *Escherichia Coli*. Even so, both *Kyowa Hakko Kogyo*’s method and superbug technology were still limited on complex oligosaccharide production and could not synthesize functional HMOs in only one batch. Based on current challenges we noticed that changing enzymatic reaction conditions and take advantages of a fermenter could improve the production of the HMOs starting structure, trisaccharide(GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc)<sup>[19, 20]</sup>. As presented in Figure 1, the trisaccharide synthesis start with lactose, and an engineered *E. coli* expressing a  $\beta$ 1–3-N-acetylglucosaminyltransferase from *Neisseria meningitidis* (NmLgtA or LgtA) for the enzymatic reaction of trisaccharide(GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) synthesis. In this study, HMOs starting structure were successfully synthesized in engineered *E. coli* at the studied induction temperature, and the best condition was repeated in a fermenter to achieve an improved result. Lastly, the product was purified by two simple step purifications and characterized by MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry) and HPLC-ELSD (High Performance Liquid Chromatography-Evaporative Light Scattering Detector)<sup>[21, 22]</sup>.

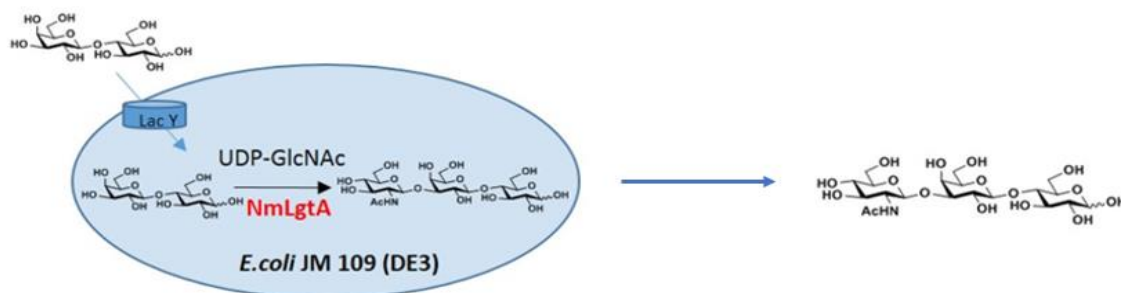


Figure 1. A trisaccharide synthesis process by “super bug” technology.

## 1.1 Purpose of the Study

Overall, the purpose of this study is to synthesize a large amount of trisaccharide using the best condition and easiest method. First, the best induction temperature was selected for 4L flask reaction. Second, repeat the selected induction temperature in a fermenter to prove the utility of this method, and use minimal medium to increase production yield and efficiency. Third, use two-step purification to purify the product and ended with purity above 90%.

## Materials and Methods

### 1.2 Microorganism and materials

Engineered *E. coli* strain JM109 (DE3) and LgtA-pET22b plasmid were prepared and stored at Dr. Peng Wang's lab. Bio-Gel P2 Gel (fine) was from Bio-Rad Laboratories. Activated charcoal and other reagents were from Sigma.

### 1.3 Media and solutions

LB (Luria-Bertani) medium and minimal medium were used in this experiment. LB medium and minimal medium were used for preparation of the seed culture of *E. coli* strain JM109 (DE3). LB medium was used as seed medium and flask medium, and minimal medium was used as fermenter medium and fed medium which contained (KH<sub>2</sub>PO<sub>4</sub> 7.8 g/L, Citric acid 1 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.33 g/L, Trace metal solution 1 ml/L, Glycerol 22 g/L, MgSO<sub>4</sub> 1 g/L, CaCl<sub>2</sub> 40 mg/L, Ampicillin 100 mg/L)<sup>[23, 24]</sup>.

### 1.4 Experimental method

#### 1.4.1 Enzyme expression of LgtA

For growth of *E. coli* harboring LgtA-pET22b, use one 125 ml flask, and two 4-L flasks, wash them very carefully. Weight 1.25g LB medium in the 125-ml flask, fill up with 50 ml water. Weight 50g LB medium in the 4-L flask, and fill up with 2L water. Carefully seal the flasks with aluminum wraps and tape sterilization indicator on each flask; send them to high-pressure steam sterilization pot and make sure the indicator turned black after sterilization.

After sterilization, cool down to room temperature and add 5ul of the seed *E. coli* and 50 ul (100 mg/ml) antibiotic to 125 ml flask under bacteria-free environment. Transfer the 125ml

flask to 37°C shaker and incubate overnight. After 24h transfer all the culture from 125 ml flask and evenly distribute to two 4-L flasks, add 2ml antibiotic under bacteria-free environment. Shake well and place the 4-L flasks in 37°C shaker and incubate for 3h. After 3h, the liquid turned from clear to cloudy, place both flask at room temperature and let it cool down to room temperature. At this stage, the growth of E. coli in 4-L flasks reaches the OD600nm range of 0.6-0.8. Table 1 and Figure 2 summarized the growth rate of E. coli in LB medium.

Table 1 Enzyme growing table

Time	Flask 1	Flask 2
1h	0.063	0.070
3h	0.281	0.322
4h	0.624	0.671

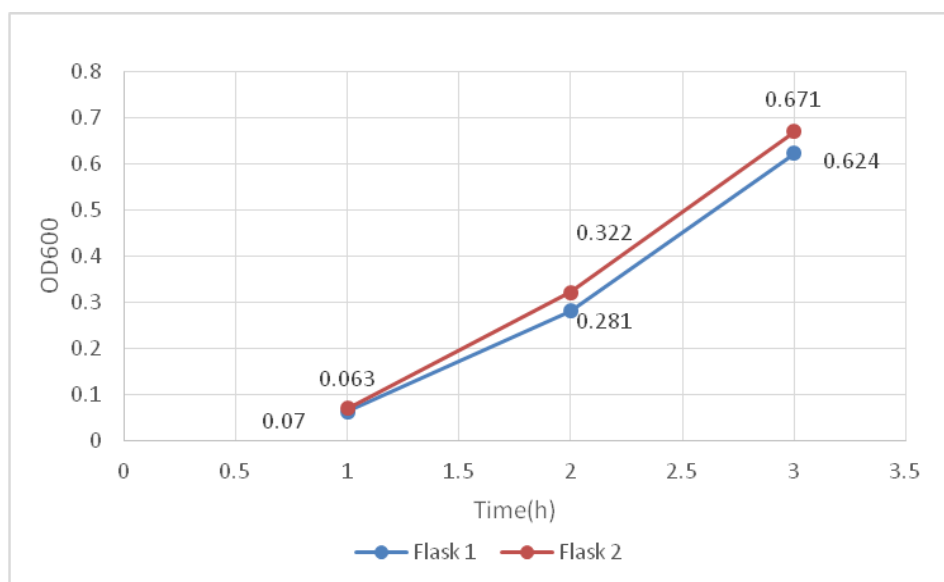


Figure 1. E. coli growth chart

#### 1.4.2 Enzymatic synthesis of trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc)

At this step, enzymatic synthesis of trisaccharide is ready to proceed, more importantly, besides using the enzymatic reaction at 16 °C (the condition used from previous studies) we also want to do a temperature comparison study at three different temperatures 16°C, 20 °C and 24 °C to explore at what temperature the growth of bacteria is highest, but without losing enzyme activity after expression is induced. Prepare three sets of 4-L flasks (2 flasks per set) that contained the bacteria culture (OD600nm 0.6-0.8). Add 30g prepared lactose solution and isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) into each flask under bacteria free environment. Transfer all three sets flasks to three shakers with temperature at 16°C, 20 °C and 24 °C, respectively. Shake overnight and collect flasks. Table 2 and Figure 2 summarized OD600 values of E. coli at different induction temperatures.

Table 2 OD600 values of E. coli culture at different induction temperatures

Induction Temperature °C	Culture 3h at 37°C	Induction 22h
16	0.864	1.791
20	0.791	1.915
24	0.687	1.985

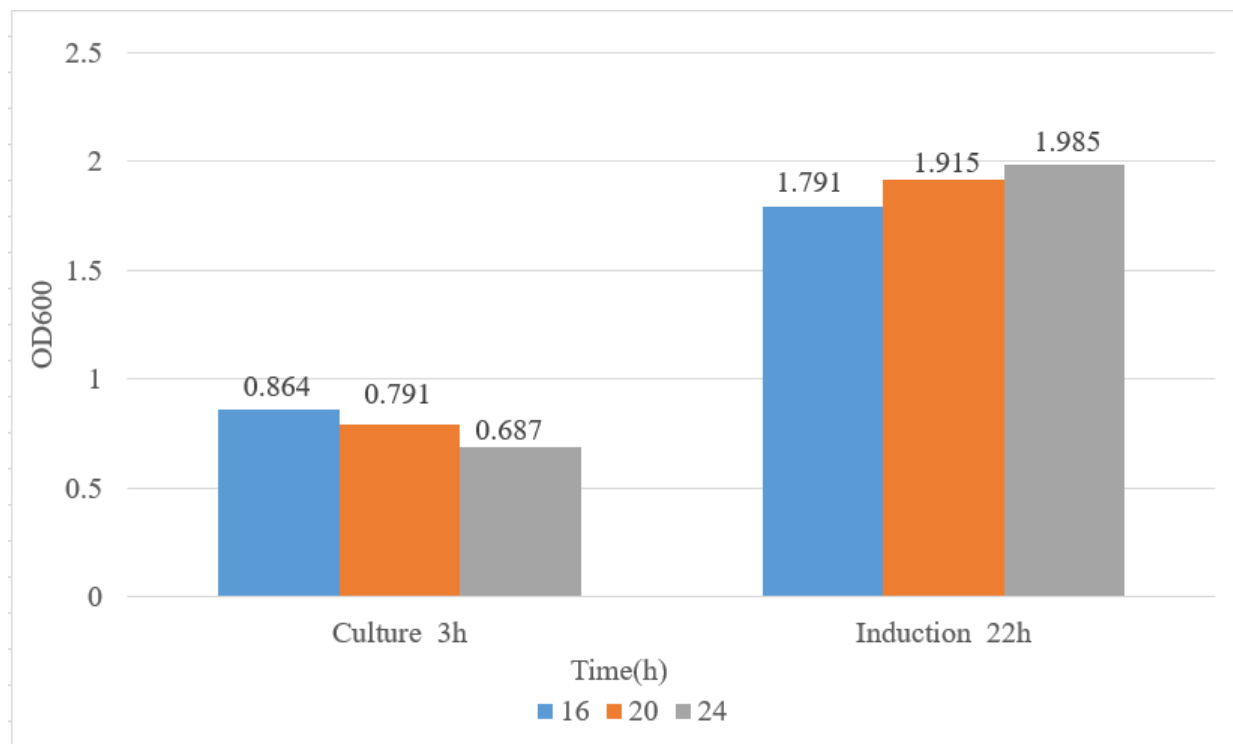


Figure 2. OD600 values of E. coli culture at different induction temperatures

#### 1.4.3 Repeated Enzymatic Synthesis of trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) in a Fermentation

First, 1.25 g minimal medium was weighted and transferred to a 125-ml flask, fill the flask with 50 ml water. Carefully seal the flask with aluminum wraps and sterilize using a high-pressure steam sterilization pot. After sterilization, cool the flask to room temperature. When it reached room temperature, add 5ul of the seed E. coli and 50 ul of antibiotic to the 125-ml flask under bacteria-free environment. Transfer the 125ml flask to 37°C 180 rpm shaker and shake overnight<sup>[25, 26]</sup>.

Carefully disconnect tubes and capes from the fermentation cylinder, wash the cylinder and other necessary flasks with ddH<sub>2</sub>O. Dry the cylinder and pour prepared minimal medium into the cylinder and flasks. Lastly, adjust pH of the buffer solution and close all lids and wrap all the connections with aluminum and send to an autoclave. After sterilization, start the system and stirring the medium until pH level and temperature stabilized (usually take one day). Next

transfer 50ml E. coli culture into the cylinder and keep stirring at 37°C overnight, after stirring overnight, add prepared lactose solution and isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) into the cylinder and change the temperature control to 24 °C. Results are shown in Table 3 and Figure 4.

Table 3 OD600 values of E. coli culture in the fermenter

E. coli growth time (h)	OD600
0	0.063
2	0.100
3.5	0.243
20	5.00
22	16.82 (add IPTG to start induction)
23.5	23.29
43.5	30.64
46	30.95

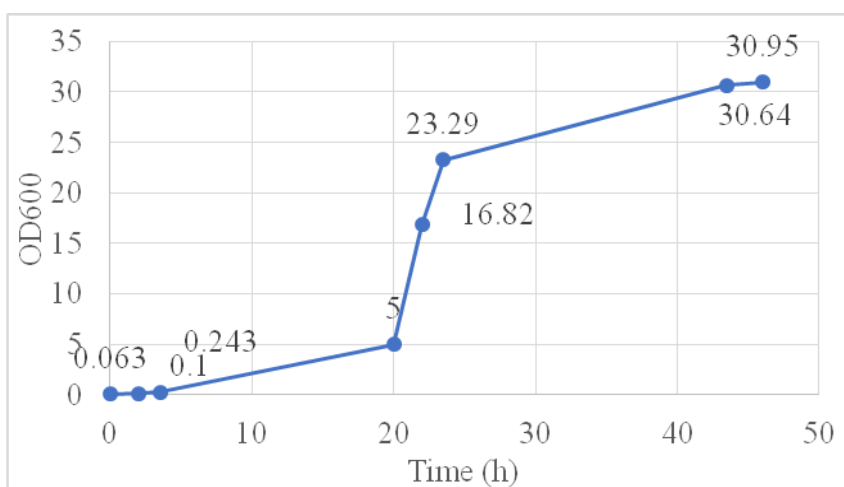




Figure 3 OD600 values of E. coli culture in the fermenter

#### 1.4.4 Trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) purification and analysis

Our trisaccharide purification process has two steps. The first step is purification using activated charcoal (Figure 4). First, collect the cells from 2-L medium and transfer all the liquids to 2-L centrifuge bottles, centrifuge at 4,000 rpm for 30min. After centrifugation, collect supernatant and save the sample for analysis. Collect the pellet and dilute with 25 ml ddH<sub>2</sub>O into 50ml centrifuge tube and boil the tube in boiling water for 20min, then centrifuge again at 7,000 rpm for 30 mins and collect supernatant in 50 ml tube.

Next weigh 40g charcoal and 20g silica gel, mix them well and pack into a column (Biotage SNAP Cartridge KP-Sil 100g). Activate the charcoal column with 800ml methanol and wash with 600ml ddH<sub>2</sub>O, then load samples on the surface of charcoal and wait until the sample absorbed into charcoals. Wash with 600ml ddH<sub>2</sub>O again and 600ml 5% ethanol. Lastly, elute with 800ml 50% ethanol and collect the eluted sample<sup>[27, 28]</sup>.

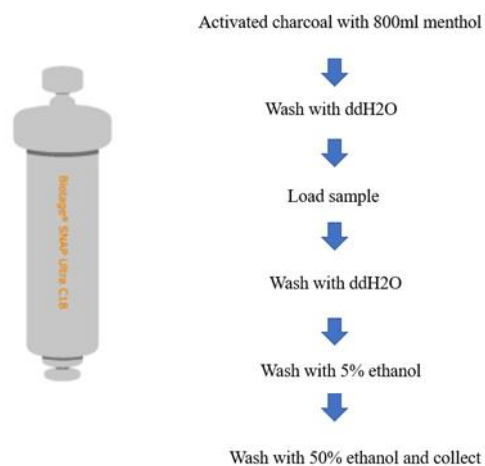


Figure 4 trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) purification step 1

Collect the 800ml compound and concentrate to about 3ml, then rinse with 2ml water and make sure that the total volume is less than 5ml. The next step is P2 column purification (P2 is a size exclusion purification method). Before loading samples, make sure the P2 column is washed and rinsed overnight through water. When loading, carefully load sample on the surface of the P2 gel and wait until the liquid fully loaded into the gel, and fill up with water and let samples run through the entire column and collect the fractions. Figure 5 illustrates the principle of P2 column in the purification process<sup>[29]</sup>.

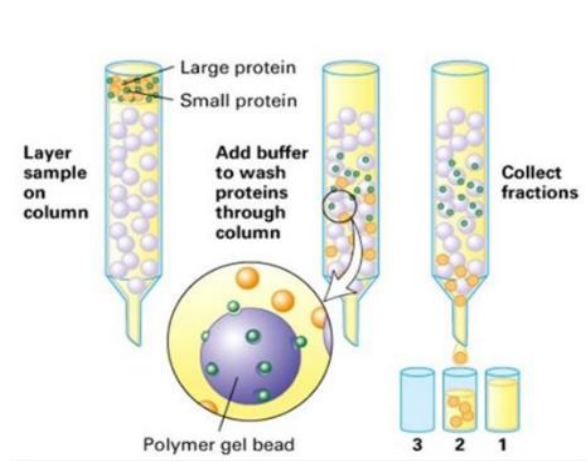


Figure 5 Final purification step

## 2 RESULTS AND DISCUSSION

Without purification, when running through the HPLC the peak for trisaccharide produced from the cytosol of *E. coli* grown at all three induction temperatures was found at 20min. By comparing the peak area, the induction temperature at 24°C gave the highest trisaccharide concentration (Figure 6).

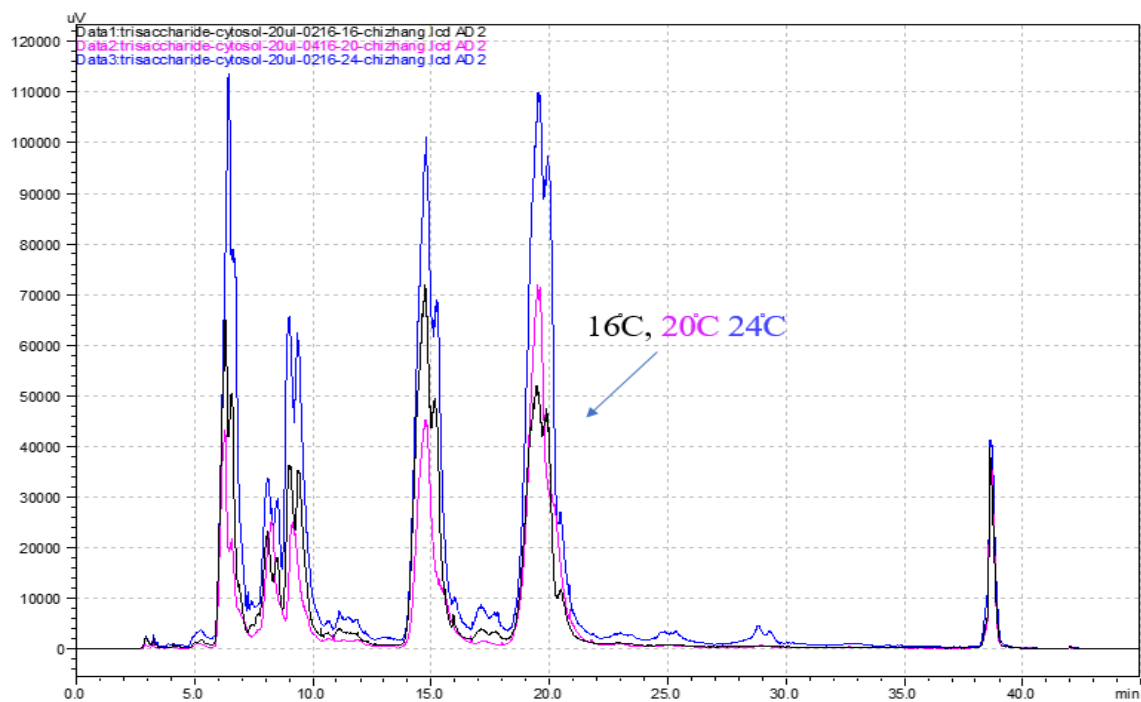


Figure 6 HPLC result of trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) (cytosol)

However, the HPLC result from medium alone showed a very low concentration of trisaccharide (Figure 7). Therefore, the most enzymatic reaction of trisaccharide synthesis was happening in the cytosol and the trisaccharide remained in the cytosol.

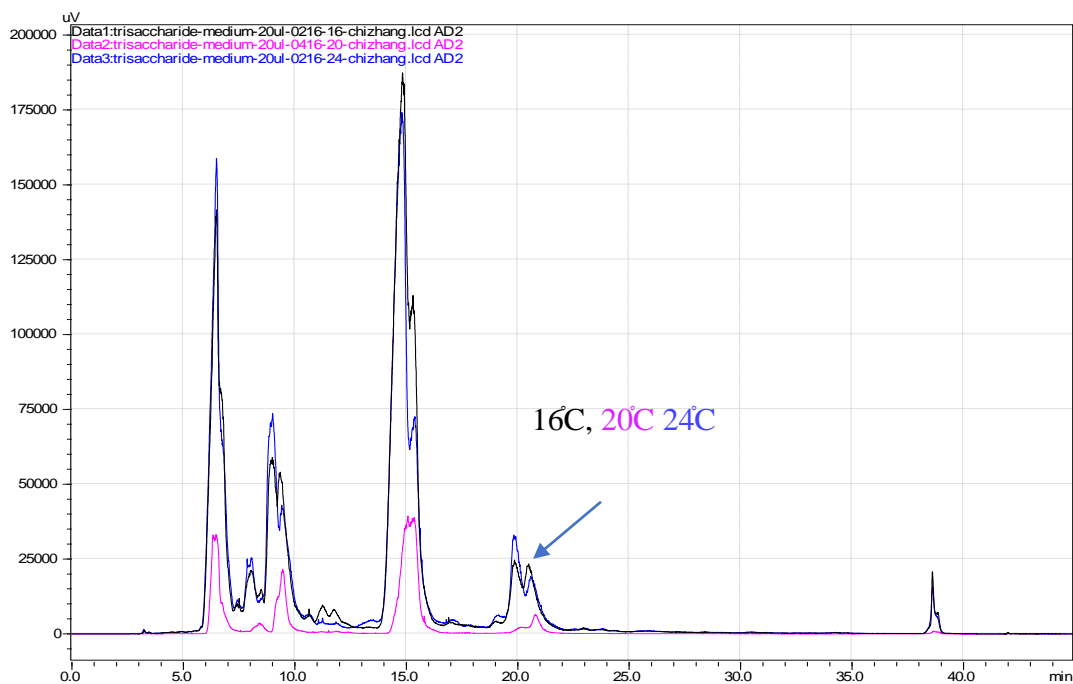


Figure 7 HPLC result of trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) (in medium)

After the simple two steps purification, the product from all three induction temperatures in 4L flasks and the 2L fermenter was also identified at 20mins (Figure 8).

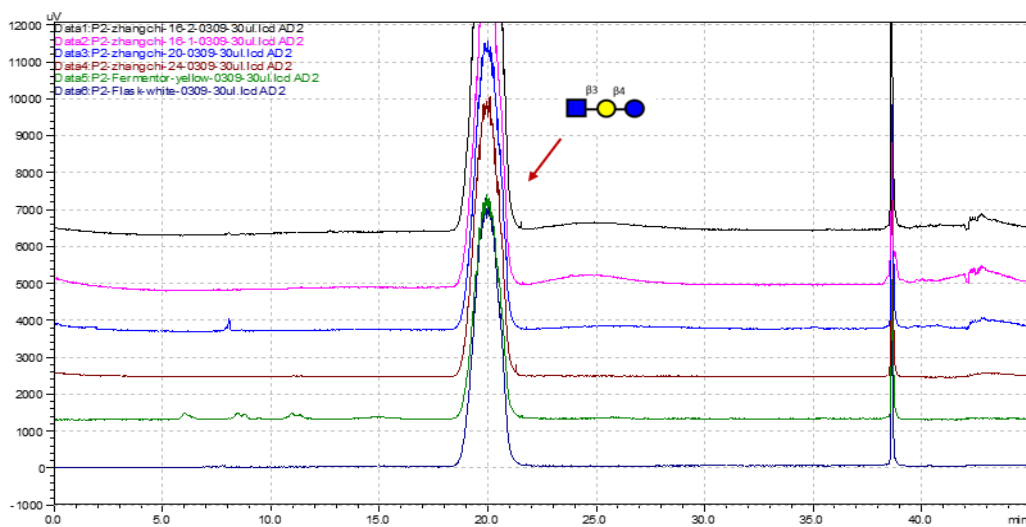


Figure 8 HPLC result of purified trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc)

The trisaccharide synthesized from flasks and the fermenter is confirmed by MALDI-  
TOP (Figure 9)

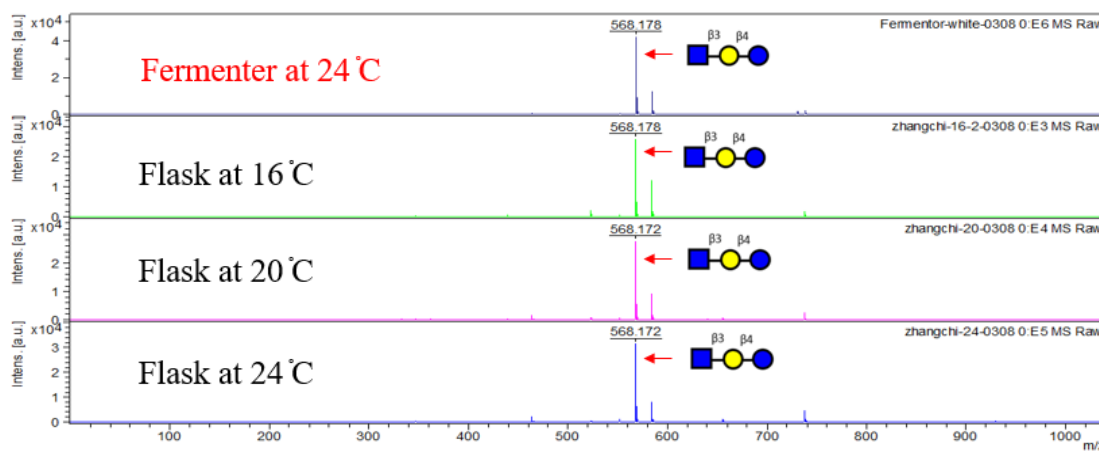


Figure 9 MALDI-TOP result of purified trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc)

After the two step purification, trisaccharide from flasks and fermenter reached purity  
above 90% (Table 4).

Table 4 Final product purity

	Induction Temperature	Area	Total area	Purity (%)
4L Flask	16	895129	979645	91.37
4L Flask	24	592995	628997	94.28
2L Fermenter	24	460403	495686	92.88

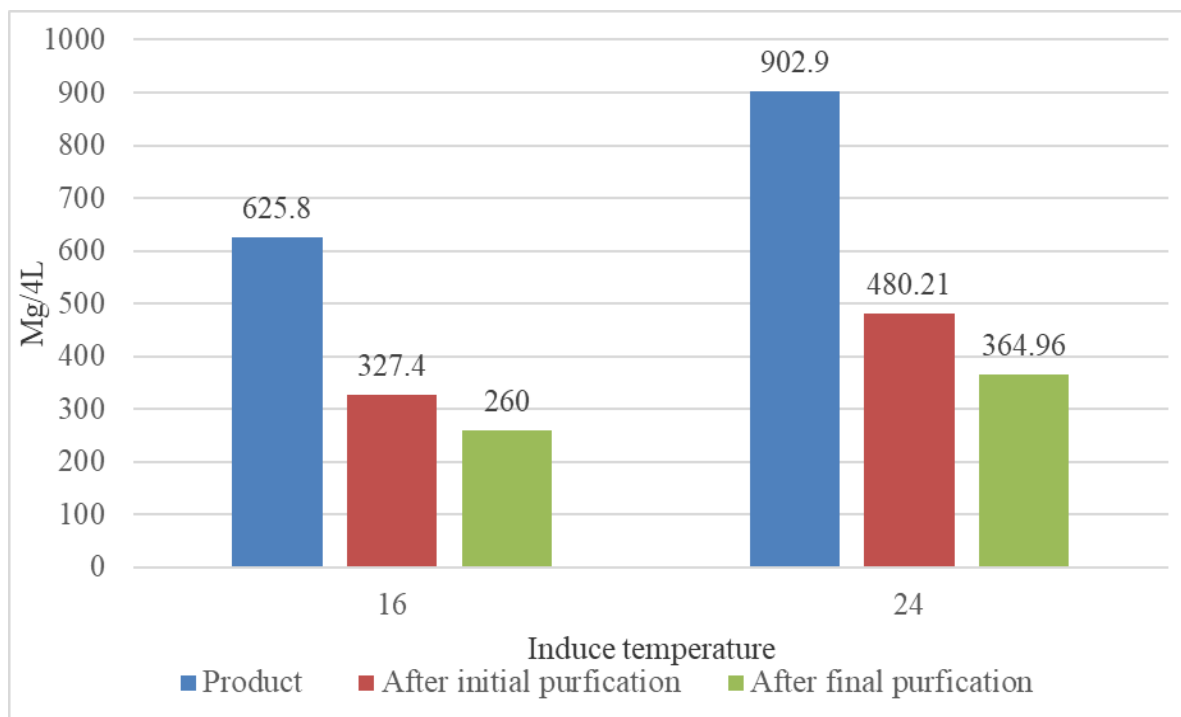


Figure 10 Final production yield from 4L flasks

Based on the yield of crude product, there are about 50% lost after initial purification, about less than 30% lost after final purification (Figure 10). However, because the purification method and its efficiency were not suitable for multi-gram scale synthesis of oligosaccharides, therefore, the final production yield from 2L fermenter was estimated by comparing to the 4L flask production/purification yield (Figure 11).

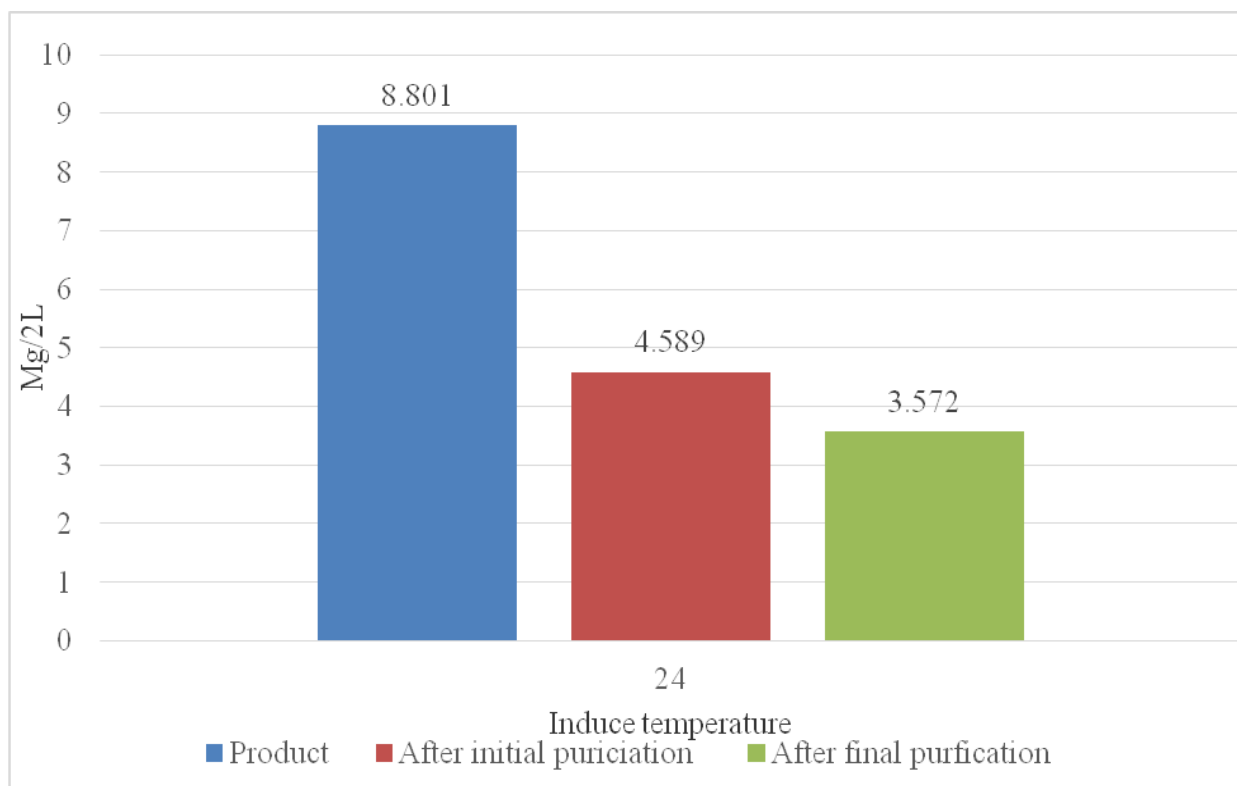


Figure 11 Estimated yield from 2L fermenter

### 3 CONCLUSIONS

From this study, superbug technology was used to successfully synthesize trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) at three different induction temperatures, 16°C, 20°C, and 24°C. The purity of the purified products from *E. coli* grown at the three temperatures is all above 90%. However, the percentage yield of trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) at 24°C showed a limited success than the other two induction temperatures. After repeating this method at 24°C induction temperature in a 2-L fermenter, the production yield and the experimental efficiency has improved. As a result, 24°C induction temperature using a fermenter is an improved method for the synthesis of HMO starting structure (trisaccharide (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc)). By using this improved method using a fermenter, the cost of HMOs' production may be decreased and the demand of HMOs' study and applications in relating areas may increase.



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