CHAPTER III

RESULTS

3.1 The optimum culturing condition for high glucansucrase production

Culturing condition was modified from Ammar *et al.*, 2002. The parameters which were not changed were 0.4 % supplemented ovalbumin and pH 6.5 of the medium since our preliminary result showed that they were suitable. However, the carbon source, temperature and time of cultivation should be optimized in this work for best production of the enzyme.

3.1.1 Optimum carbon source

The effect of various carbon source on growth and glucansucrase (GS) production of *Bacillus licheniformis* TH 4-2 was performed as described in section 2.5.2. As shown in Figure 3.1, the microorganisms grew well on all type of C sources tested, soluble starch from cassava, soluble starch from potato, and sucrose did not result in much difference in bacterial growth. In contrast, GS production was significantly affected by the type of C source. Highest activity was observed when, soluble starch from cassava was used and the GS activity was two and eight times higher when used soluble starch from potato and sucrose, respectively.

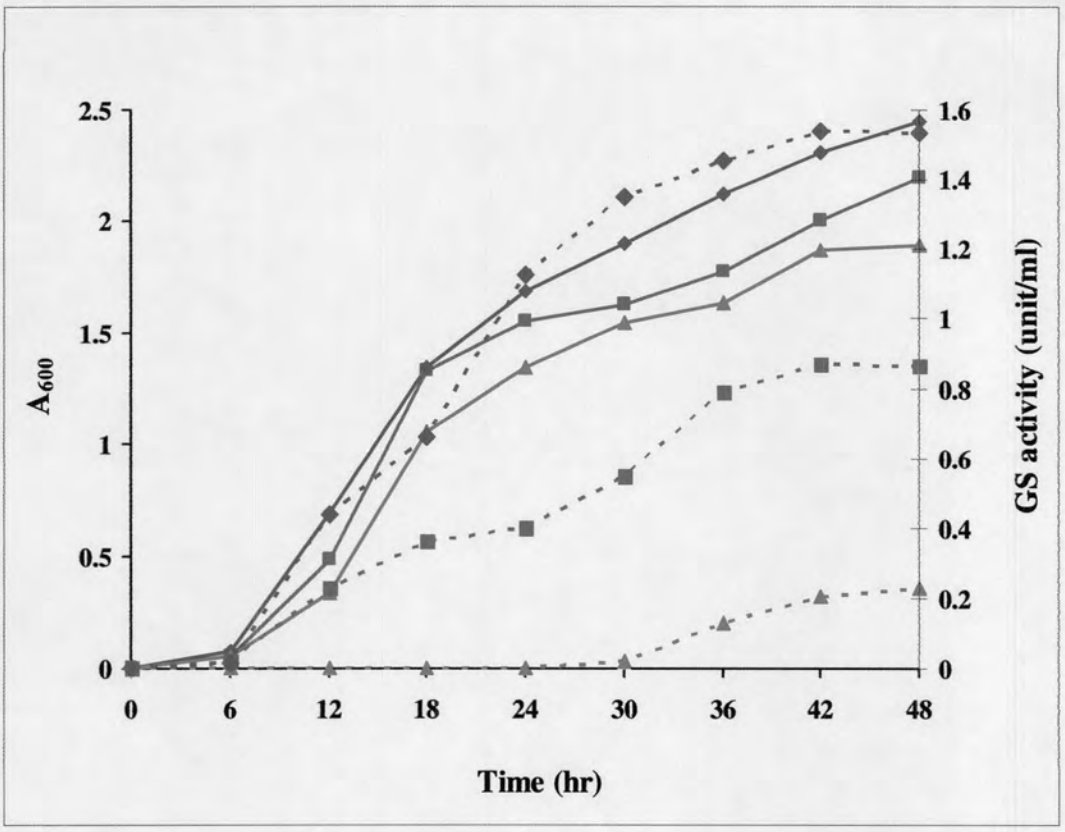


Figure 3.1 Effect of various carbon source on growth and glucansucrase (GS) production

——— Growth ..... GS Activity

◆ soluble starch from cassava

■ soluble starch from potato

▲ sucrose

3.1.2 Optimum concentration of carbon source

The effect of the concentration of the selected carbon source on bacterial growth and glucansucrase production was performend as described in section 2.5.3. Concentrations of soluble starch from cassava were varied at 2, 5 and 10 % (w/v). As shown in Figure 3.2, 2-10 % soluble starch gave approximately similar growth. At 5 % concentration, highest GS production was obtained, the activity was 2 times higher than when 2 % or 10 % cassava soluble starch was used. Thus, the concentration of soluble starch from cassava at 5 % was used for the next experiments.

3.1.3 Optimum cultivation temperature

The optimum cultivation temperature for bacterial growth and enzyme production was determined as described in section 2.5.4. Cultivation temperatures were varied at 45 °C and 50 °C. As shown in Figure 3.3., not much difference in growth was observed, though it seemed to be higher at 45 °C. But GS activity was only observed at 45 °C, no activity at all was detected at 50 °C. Thus, the cultivation at 45 °C was used for the next experiments.

3.1.4 Optimum cultivation time

The optimum cultivation time for bacterial growth and enzyme production was determined as described in section 2.5.5. The cell culture was cultivated at 45 °C for 0-72 hours. As shown in Figure 3.4, growth and GS activity rapidly increased with

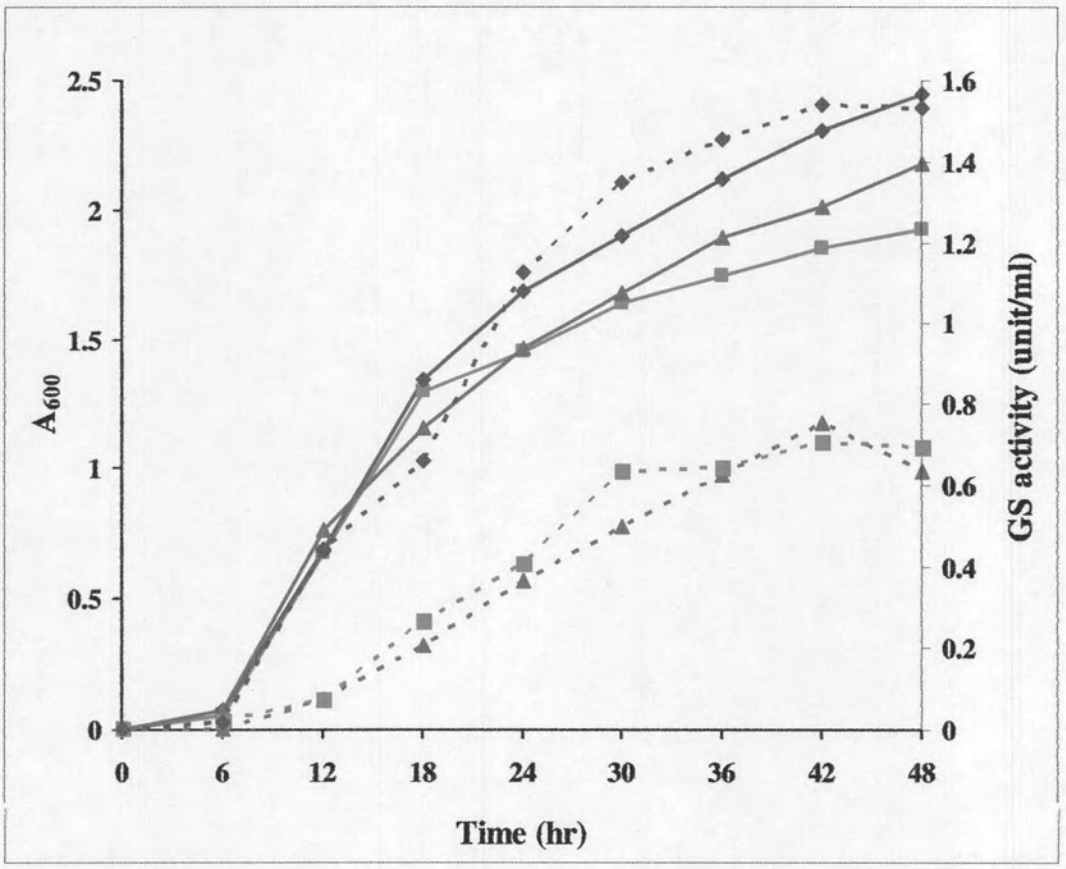


Figure 3.2 Effect of the concentration of soluble starch from cassava on bacterial growth and glucansucrase production

——— Growth ..... GS Activity

■ 2 %

◆ 5 %

▲ 10 %

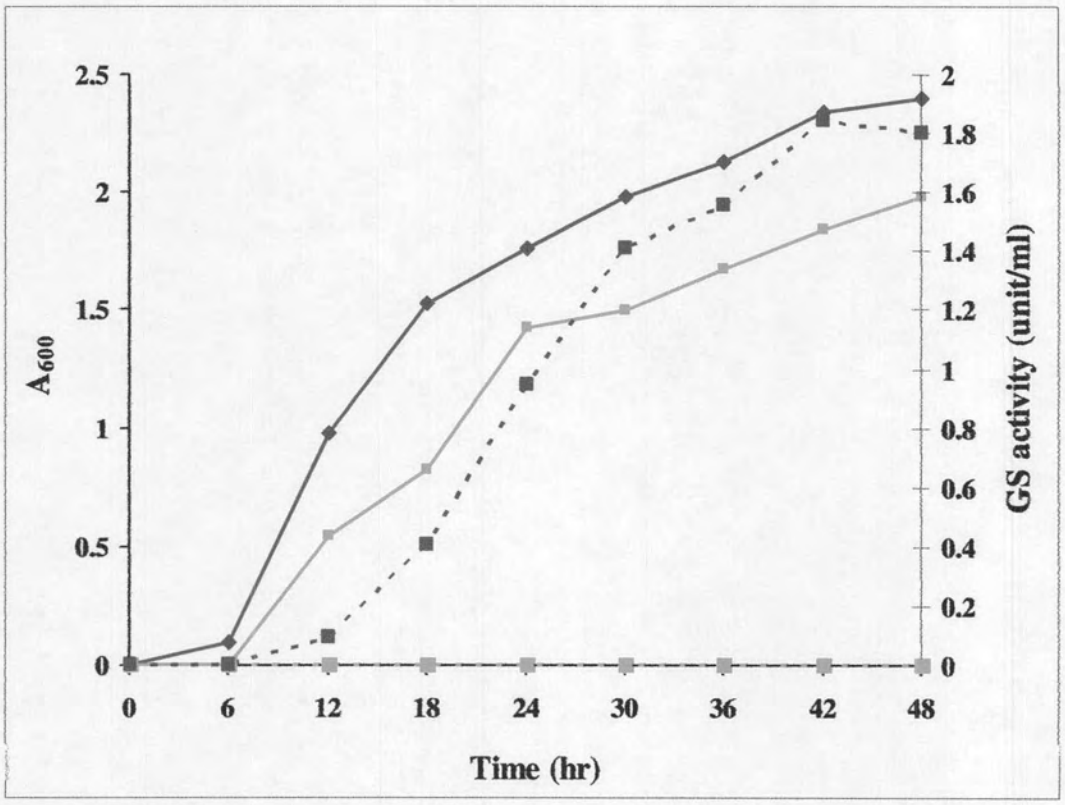


Figure 3.3 Effect of cultivation temperature on bacterial growth and glucansucrase production

——— Growth ..... GS Activity

◆ 45 °C

■ 50 °C

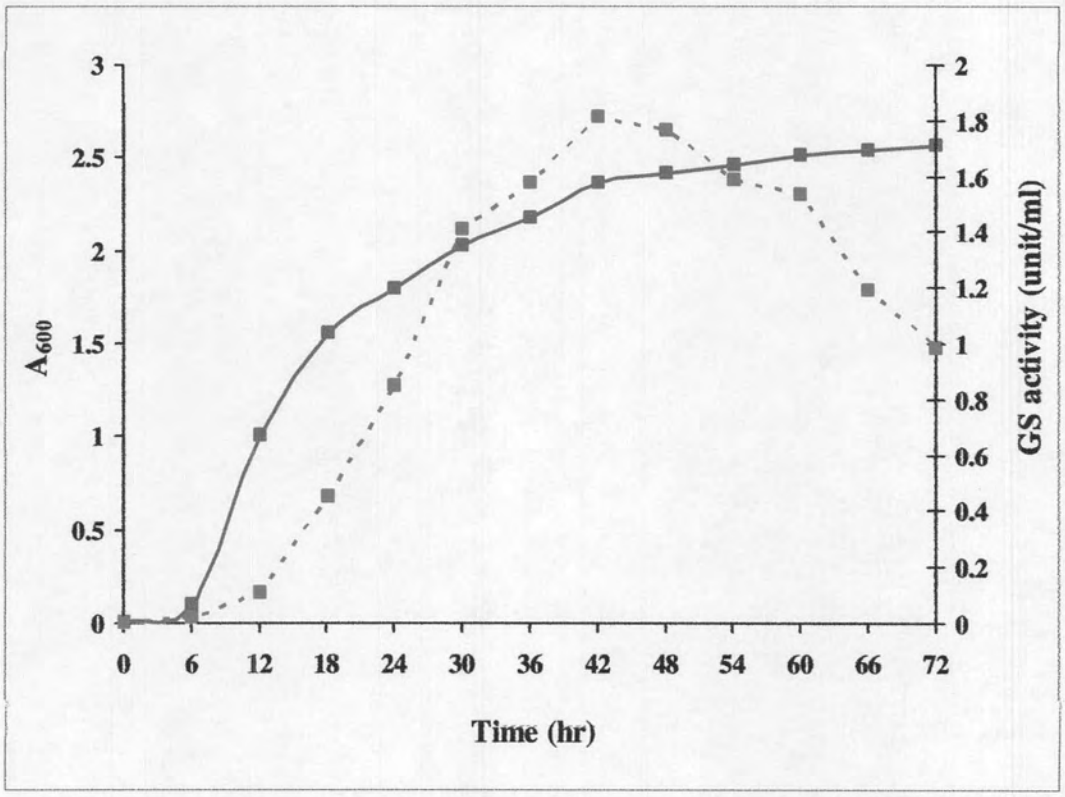


Figure 3.4 Effect of cultivation time on bacterial growth and glucansucrase production

——— Growth ..... GS Activity

increasing of cultivation time from 6 to 42 hours. After that, the activity was significantly decreased while the growth was leveled off. The highest GS activity of 1.81 unit/ml was detected at 42 hours. Therefore, the cultivation time of 42 hours was selected.

From the above results, the optimum condition for glucansucrase production of *Bacillus licheniformis* TH 4-2 was cultivation of *Bacillus licheniformis* TH 4-2 in 5 % soluble starch from cassava at 45 °C for 42 hours.

3.2 Purification of glucansucrase

3.2.1 Preparation of crude enzyme

After cultivation of *Bacillus licheniformis* TH 4-2 in enzyme production medium containing 5 % soluble starch from cassava supplemented with 0.4 % ovalbumin , pH 6.5 at 45 °C for 42 hours , the cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. The crude enzyme in the supernatant fraction was collected. Crude enzyme solution contained 3.99×10^3 mg protein and 4.89×10^3 units of glucansucrase activity. Thus, the specific activity was 1.23 unit/mg protein.

3.2.2 Enzyme purification steps

3.2.2.1 Ammonium sulfate precipitation

In the first step of purification, crude extract was purified by ammonium sulfate precipitation as described in section 2.7.1. To determine the proper

ammonium sulfate concentration for enzyme precipitation, the concentration ranges using 0-30 % and 30-60 % saturation were tried. The result showed that most of enzyme activity was found in the 30-60 % fraction. Therefore, proteins from 30-60 % saturated ammonium sulfate fraction was collected and dialysed against 20 mM sodium acetate buffer, pH 6.0. The recovered protein and enzyme activity were 1.31x 10^3 mg protein and 4.39 x 10^3 units, respectively. Thus, the specific activity of the enzyme from this step was 3.35 unit/mg protein. The enzyme was purified 2.7 fold with 90 % yield.

3.2.2.2 DEAE-cellulose column chromatography

The enzyme from 30-60 % saturated ammonium sulfate precipitation was loded into DEAE-cellulose column as described in section 2.7.2. The chromatographic profile was shown in Figure 3.5. The unbound proteins were eluted from the column by 20 mM sodium acetate buffer, pH 6.0, whereas the bound proteins were eluted with linear salt gradient of 0 to 0.3 M sodium chloride in the same buffer as indicated in the profile. Glucansucrase fraction was pooled, dialysed against the buffer, concentrated by aquasorb to reduce enzyme volume. This step resulted in 0.07 x 10^3 mg protein and the activity of 3.22 x 10^3 units. Thus, the specific activity of the enzyme from this step was 46 unit/mg protein. The enzyme was purified 37 fold with 66 % yield.

3.2.2.3 Sephadex G-100 chromatography

The pool activity fraction from DEAE-cellulose column was applied onto Sephadex G-100 chromatography as described in section 2.7.3. Figure 3.6

showed Sephadex G-100 chromatography column profile of glucansucrase which was well separated from other contaminated proteins of larger and smaller size.

The overall purification steps of glucansucrase from *Bacillus licheniformis* TH 4-2 are summarized in Table 3.1. The glucansucrase was purified to 112 fold with the yield of 28 %. The specific activity was increased from 1.23 of the crude enzyme to 137 unit/mg protein of the purified enzyme.

3.2.3 Determination of enzyme purity

The enzyme purity and native protein pattern from each purification step were investigated by non-denaturing polyacrylamide gel electrophoresis as described in section 2.10.1. The result of protein staining was shown in Figure 3.7A, the single band from Sephadex G-100 column in lane 4 indicated that the enzyme was purified to homogeneity. The activity staining was shown in Figure 3.7B, the single red band in lane 1 and 2 confirmed that the protein band which was purified was glucansucrase.

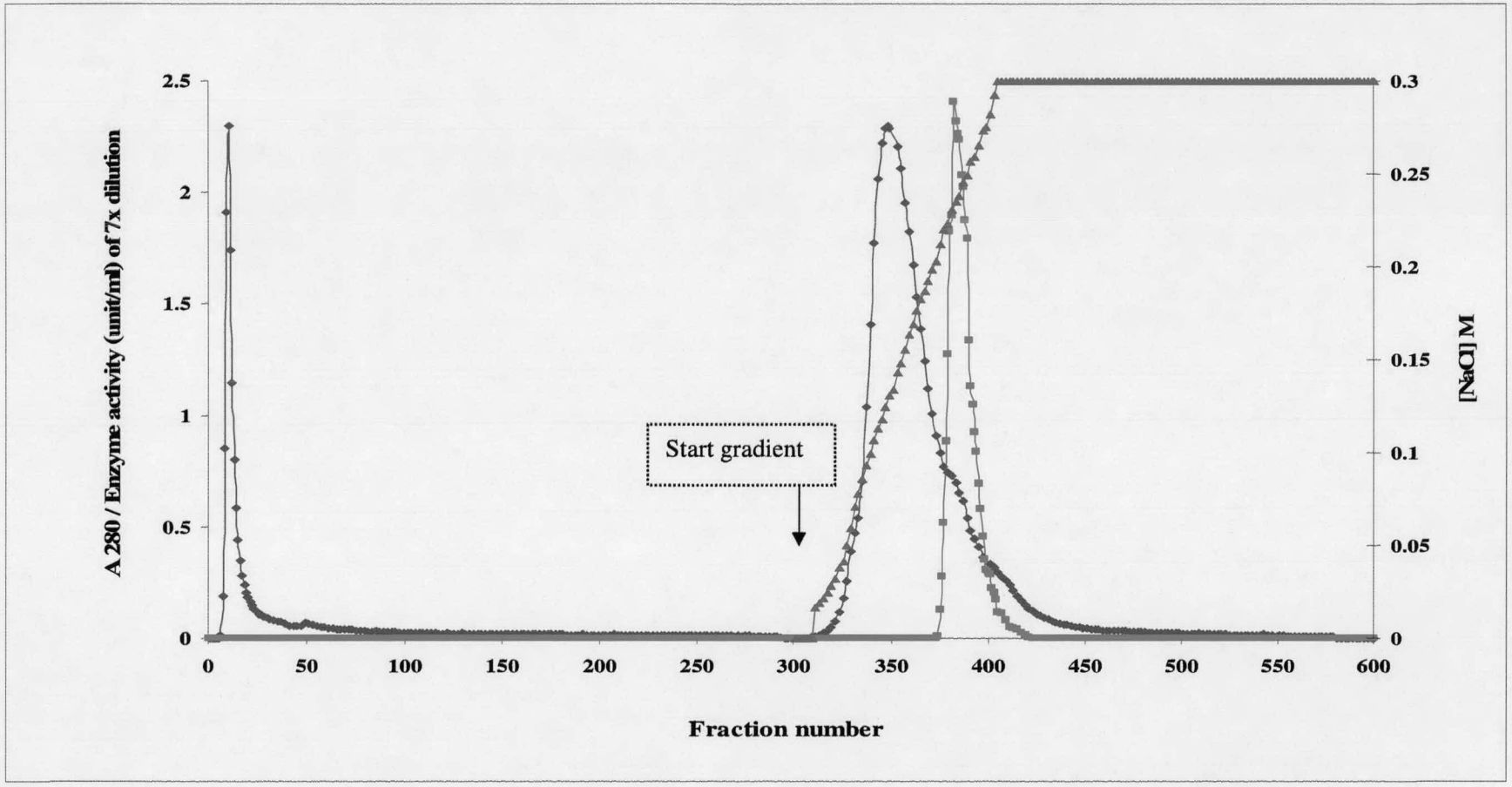


Figure 3.5 DEAE- cellulose chromatography of glucansucrase. Column size 1.5 x 27 cm, flow rate 45 ml/hours, elution was by 0-0.3 M NaCl in sodium acetate buffer pH 6, fractions of 5ml were collected. ◆ A 280, ■ enzyme activity, ▲ conductivity

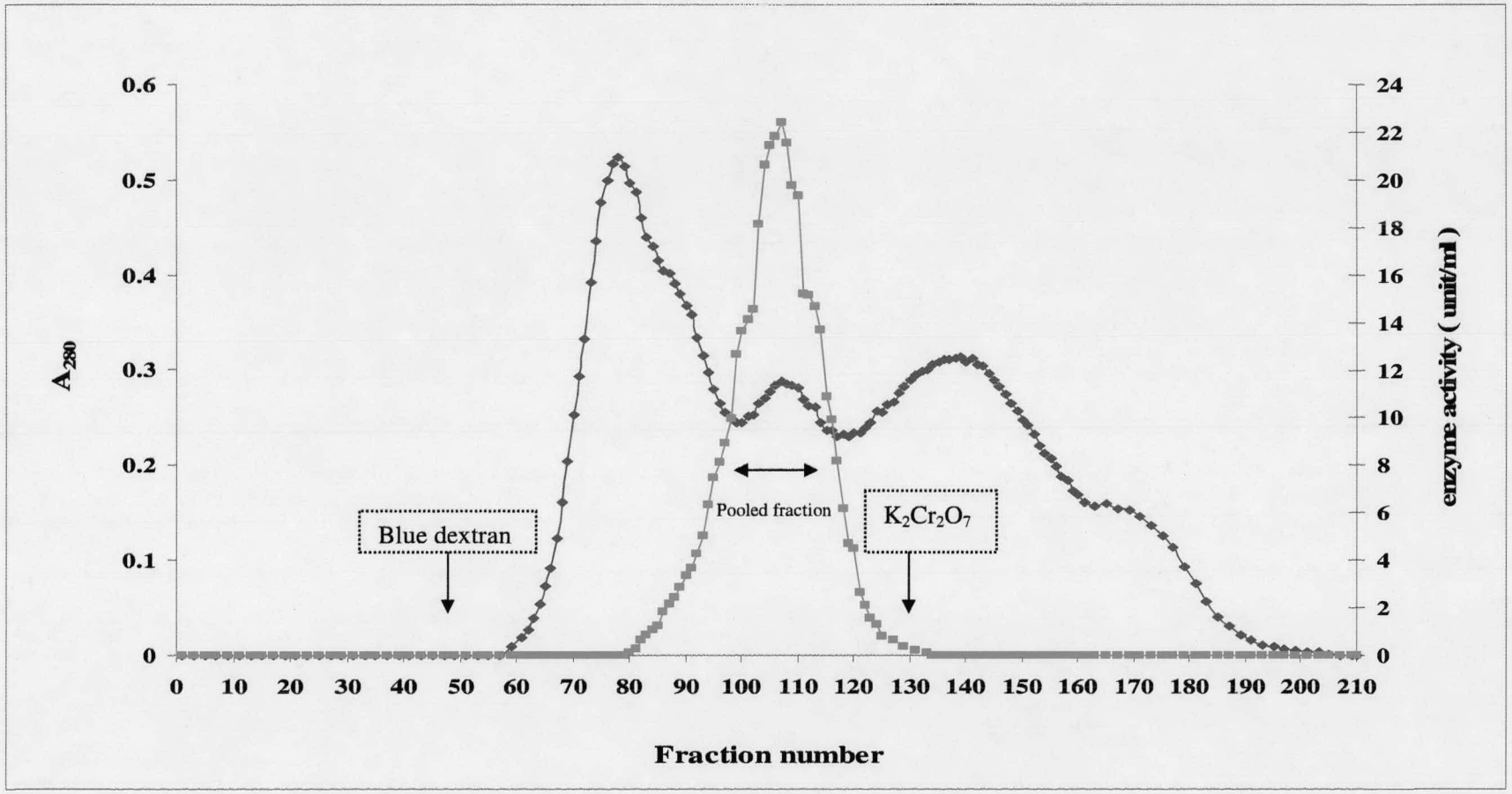


Figure 3.6 Sephadex G-100 chromatography of glucansucrase. Column size 1.9 x 90 cm, flow rate 20 ml/hours and sodium acetate buffer pH 6.0 was used. Fractions of 2ml were collected. ◆A 280, ■ enzyme activity,

Table 3.1 Purification of glucansucrase from *Bacillus licheniformis* TH 4-2

Purification step	Total Volume (ml)	Protein		Glucansucrase Activity				
		Concentration (mg/ml)	Total Protein (mg) $x10^3$	Activity (unit/ml)	Total Activity (unit) $x10^3$	Specific Activity (unit/mg protein)	Fold	Yield (%)
Crude enzyme	1940	2.06	3.99	2.52	4.89	1.23	1	100
Ammonium sulfate 30-60%	350	3.74	1.31	12.53	4.39	3.35	2.7	90
DEAE – cellulose	300	0.24	0.07	10.75	3.22	46.0	37	66
Sephadex G-100	75	0.13	0.01	18.22	1.37	137	112	28

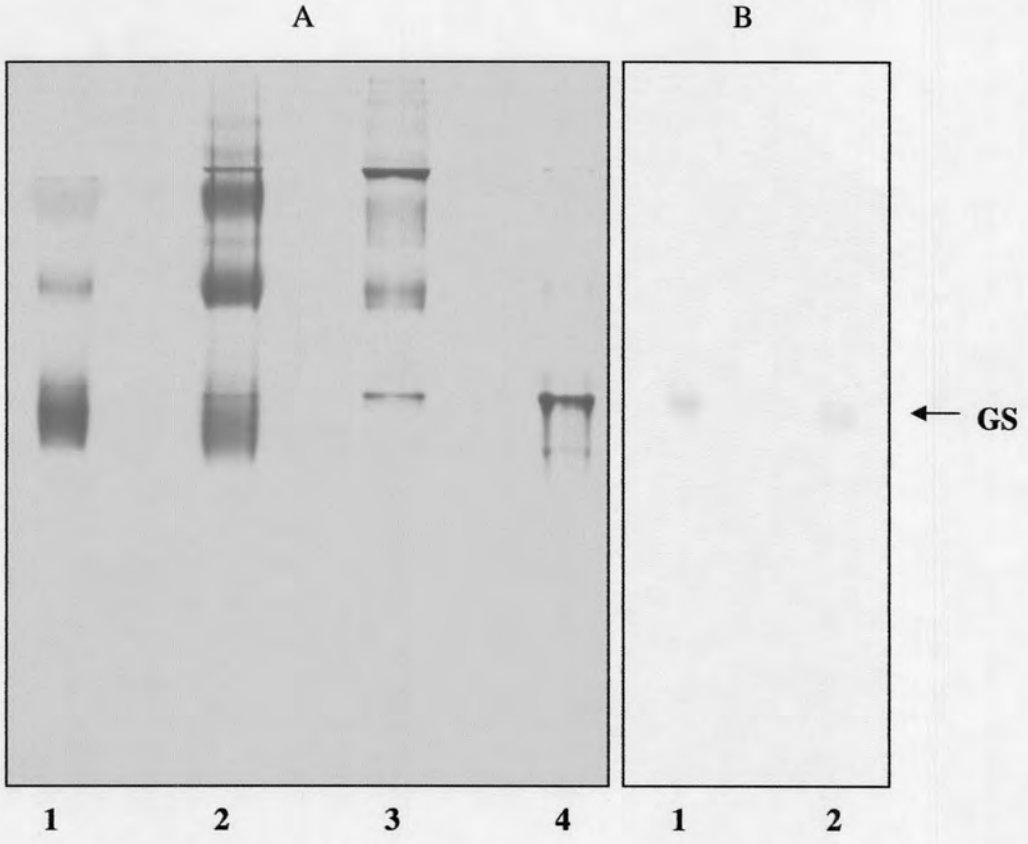


Figure 3.7 Non-denaturing PAGE of glucansucrase from different purification step

A. Protein staining

Lane 1 : Crude enzyme (25 μg)

Lane 2 : Ammonium sulfate precipitate (30-60 %) (15 μg)

Lane 3 : GS pool from DEAE- cellulose column (15 μg)

Lane 4 : Purified GS from Sephadex G-100 column (15 μg)

B. Activity staining

Lane 1 : GS pool from DEAE- cellulose column (3 unit)

Lane 2 : Purified GS from Sephadex G-100column (3 unit)

3.3 Characterization of purified glucansucrase

3.3.1 Molecular weight determination of glucansucrase

The molecular weight of the enzyme was determined from molecular weight calibration curve obtained by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Figure 3.8. The result indicated that, the molecular weight of the enzyme was estimated to be 64 kDa by its mobility in SDS-PAGE compared with those of standard proteins as shown in Figure 3.9.

3.3.2 Optimum pH

The effect of pH on the enzyme activity was performed as described in section 2.11.2. The pH was varied from 5.0 to 9.0 at 45 °C. The result was shown in Figure 3.10, the activity was presented as % relative activity with the highest activity referred as 100 % . Optimum activity was allowed at pH6.0 with both sodium acetate and phosphate buffer, and broad optimum activity profile from pH 6.0 to 7.0 was observed.

3.3.3 Optimum temperature

The effect of temperature on the enzyme activity was performed as described in section 2.11.3. The temperature was varied from 30 °C to 60 °C at optimum pH. The result was shown in Figure 3.11, the activity was presented as % relative activity with the highest activity referred as 100 %. The enzyme performed the highest activity at 45 °C and the activity was lost significantly when temperature was higher than 50 °C .

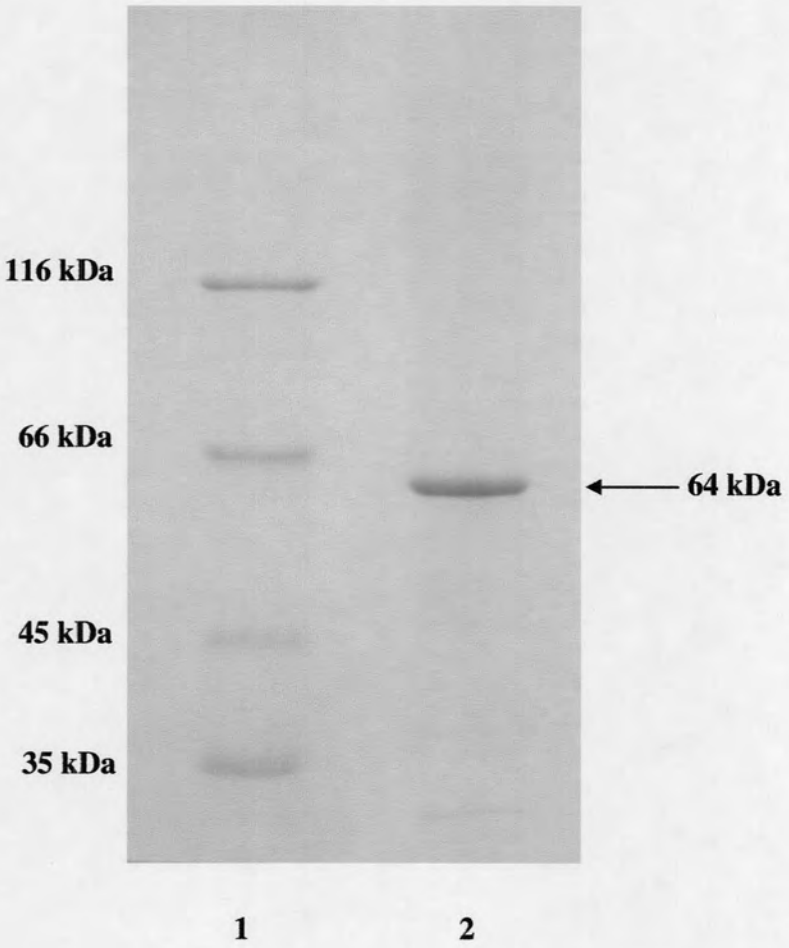


Figure 3.8 SDS- PAGE of glucansucrase from different purification step

Lane 1 : Protein molecular weight markers

β- galactosidase	(MW 116,000)
bovine serum albumin	(MW 66,200)
ovalbumin	(MW 45,000)
lactate dehydrogenase	(MW 35,000)

Lane 2 : Purified GS from Sephadex G-100 column (3 μg)

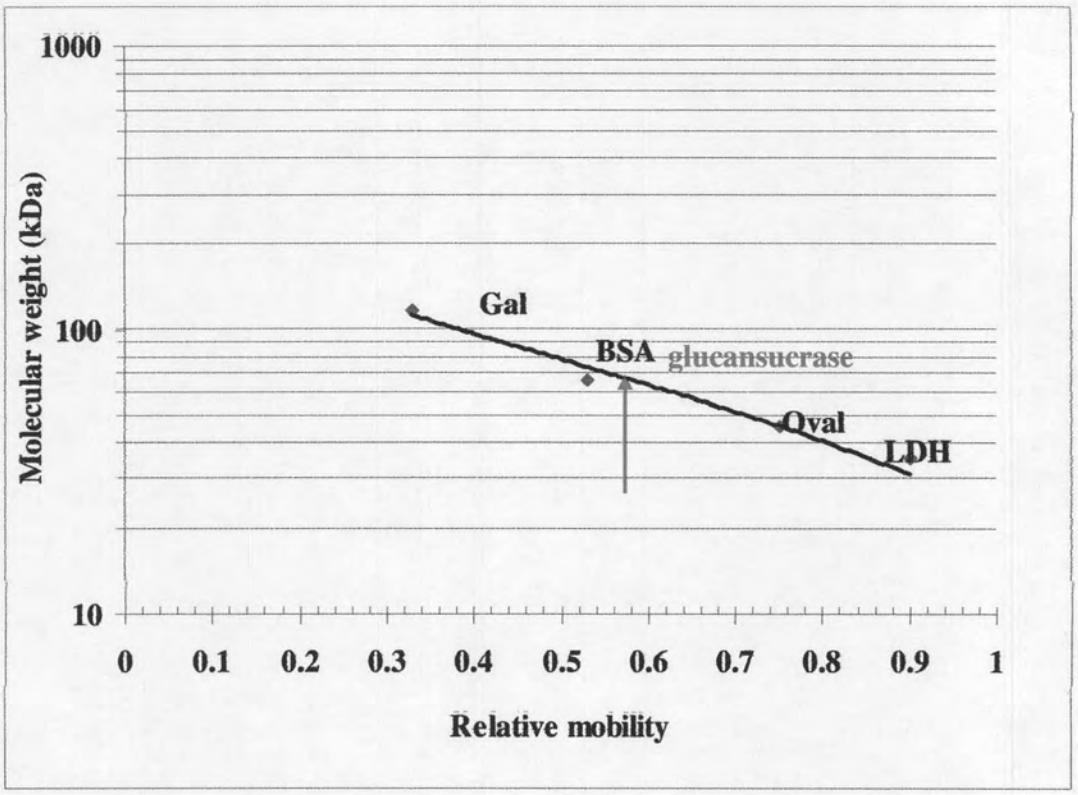


Figure 3.9 Calibration curve for molecular weight of glucansucrase on SDS-polyacrylamide gel electrophoresis

Gal	:	β- galactosidase	(MW 116,000)
BSA	:	bovine serum albumin	(MW 66,200)
Oval	:	ovalbumin	(MW 45,000)
LDH	:	lactate dehydrogenase	(MW 35,000)

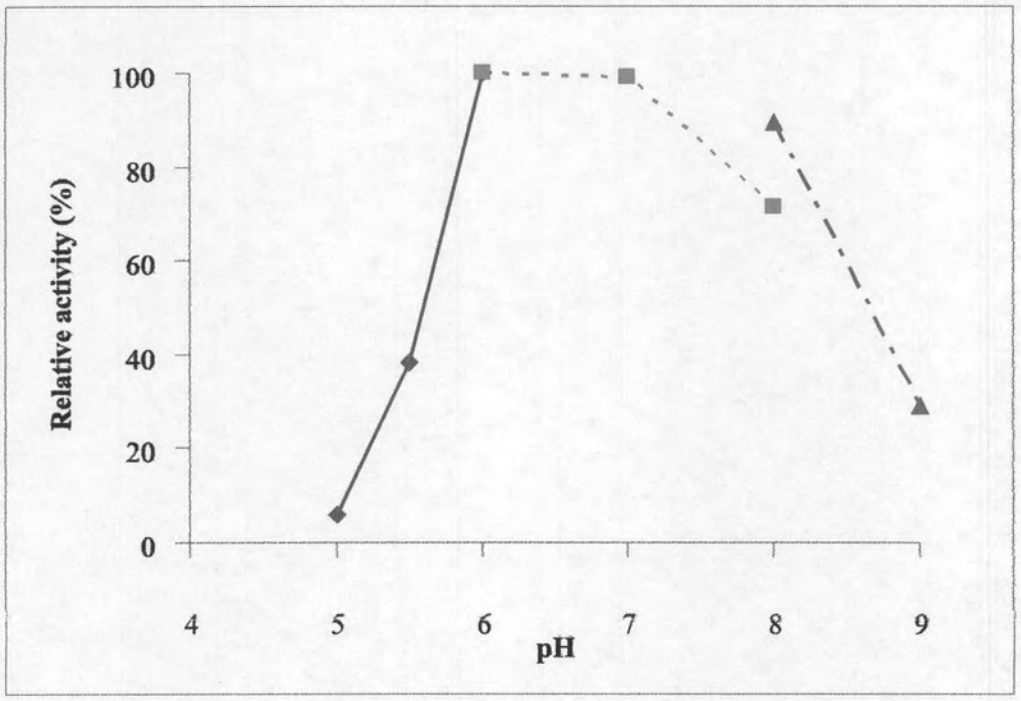


Figure 3.10 Effect of pH on enzyme activity ◆ Sodium acetate buffer (pH 5-6) ■ Phosphate buffer (pH 6-8) ▲ Borate buffer (pH 8-9)

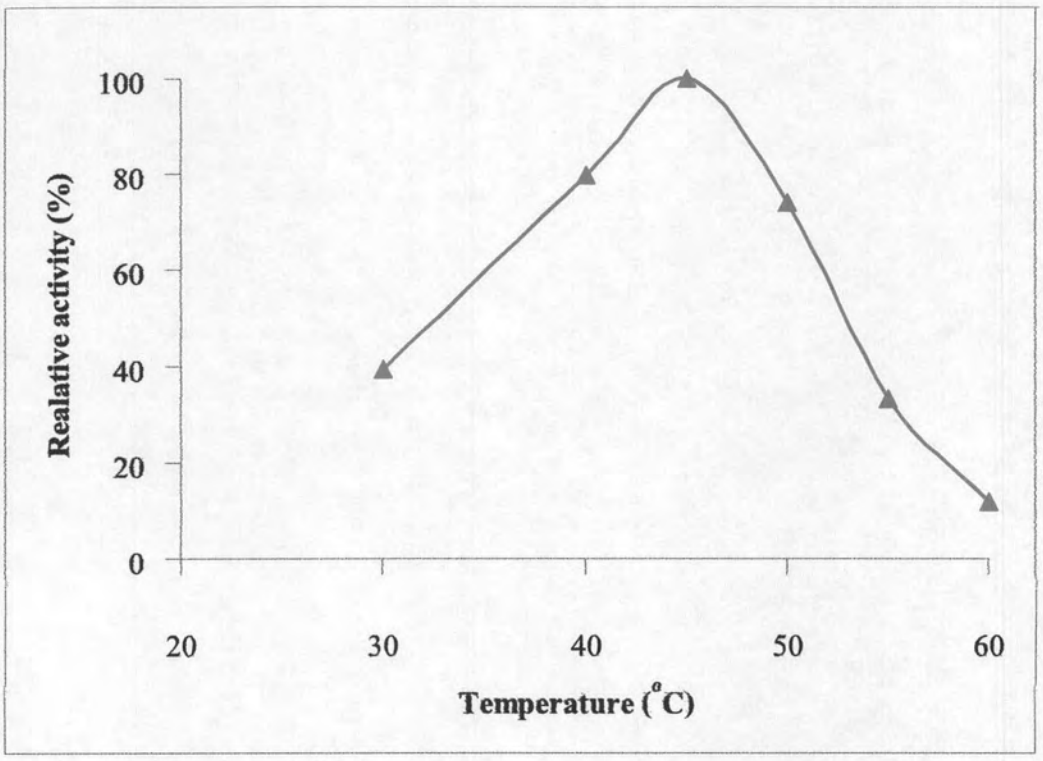


Figure 3.11 Effect of temperature on enzyme activity

3.3.4 Kinetics of glucansucrase

3.3.4.1 Determination of K_m and V_{max} for sucrose substrate

A steady-state kinetic analysis was carried out to investigate the kinetic parameters. Initial velocity (v_0) studies of glucansucrase was determined by sucrose hydrolysis activity assayed with various concentrations of sucrose substrate at 45 °C for 10 min and measured the activity as described in section 2.8. The Lineweaver-Burk plot of glucansucrase was shown in Figure 3.12, the Michaelis constant (K_m) and the maximum velocity (V_{max}) for sucrose substrate were calculated to be 38.14 mM and 0.042 μmole/min, respectively.

3.3.4.2 Determination of K_m and V_{max} for melibiose acceptor

A steady-state kinetic analysis was carried out to investigate the kinetic parameters. Initial velocity (V_0) of transglucosylation activity of glucansucrase was determined using sucrose as donor with various concentrations of melibiose acceptor at 45°C, pH 6.0 for 180 min and the peak area of oligosaccharide product was determined as described in section 2.12.2.2. The Lineweaver-Burk plot of glucansucrase was shown in Figure 3.12-B, the Michaelis constant (K_m) and the maximum velocity (V_{max}) for melibiose acceptor were calculated to be 148 mM and 0.072 μmole/min, respectively.

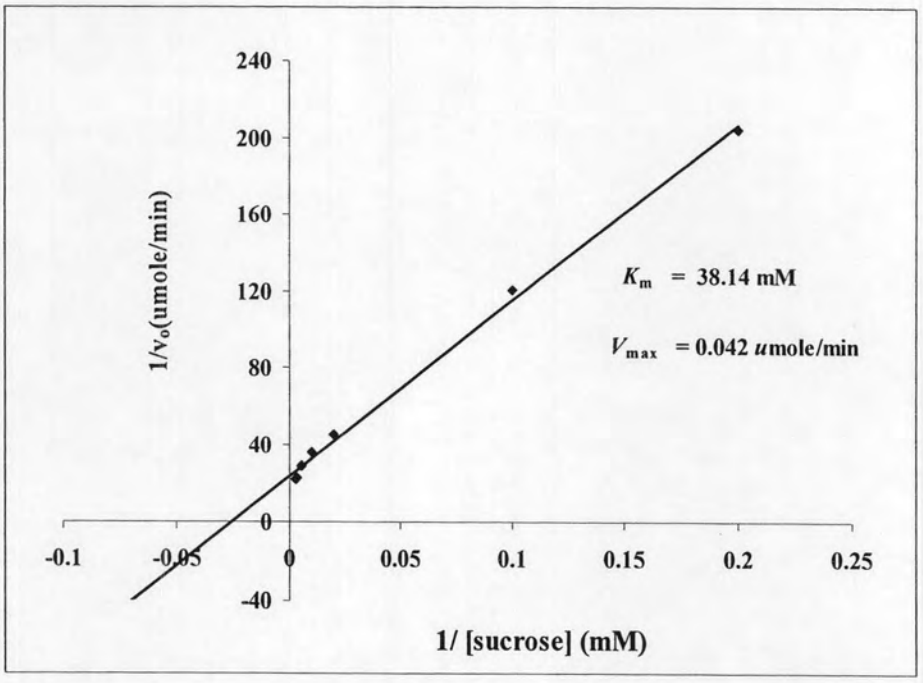


Figure 3.12-A Lineweaver- Burk plot of glucansucrase activity with sucrose as donor substrate

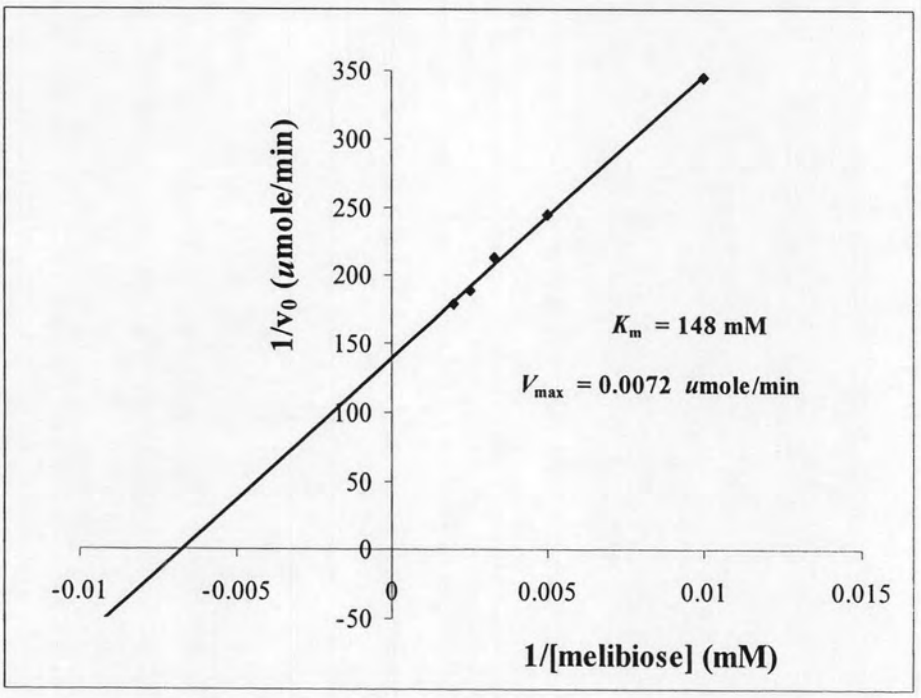


Figure 3.12-B Lineweaver -Burk plot of glucansucrase activity with melibiose as glucosyl acceptor

3.4 Synthesis and detection of prebiotic oligosaccharide products

3.4.1 Acceptor specificity

Acceptor specificity was determined by using various saccharides as glucosyl acceptor. After incubation of glucansucrase (0.5 unit/ml) with 5 % sucrose donor and 5 % of various acceptors (G1; Glucose to G7; maltoheptaose, lactose, melibiose, cellobiose, raffinose, palatinose and lactulose) at 45 °C for 24 hours, reaction products were analyzed by thin-layer chromatography (TLC). The result was shown in Figure 3.13 and 3.14. From TLC chromatogram (Figure 3.13), the migration of standard sugars in this TLC system was in the order of fructose ≃ G1 (lane 2, 3), sucrose (lane 1), G2 to G4 (lane 3) and G5 to G7 (lane 4). Fructose and G1 showed the highest Rf value. When analyzing the products from G2 to G7 as acceptors, after 24 hours of incubation, the spots expected to be reaction products were observed (lane 8, 10, 12, 14, 16 and 18). G1 acceptor seemed to give no product (lane 6) while G2 to G7 gave 1 to 3 observable products. When other sugars were used as acceptor (Figure 3.14), the migration of standard sugars in this TLC system was in the order of fructose ≃ glucose (lane 2, 3), sucrose (lane 1), palatinose (lane 16), cellobiose (lane 10), lactulose (lane 19), lactose (lane 4) and melibiose ≃ raffinose (lane 7, 13). After 24 hours of incubation with various sugar acceptors, the spots expected to be reaction products were observed (lane 6, 9, 12, 15, 18, and 21). The spots expected to be products from glucosyl acceptor were observed at a slightly lower position than their acceptor counterparts. The Rf values of all standards and products were shown in Table 3.2 and the total number of products from various acceptors were summarized in Table 3.3. The result showed that, glucansucrase from *Bacillus licheniformis* TH 4-2 was able to synthesize a variety of prebiotic OS products from sucrose donor

using various saccharides as glucosyl acceptors. G1 could not act as acceptor. Melibiose was one of the best glucosyl acceptors judging from clearly observable and resolvable product spot. Melibiose has been reported to be an acceptor for GS .The results indicated that dextransucrase from *Leuconostoc mesenteroides* B-512F could synthesize α-D- glucopyranosyl -(1- 4) -α-D- galactopyranosyl -(1- 6) -D-glucose. In addition when used alternansucrase, the structure of product was identified as α-D-glucopyranosyl-(1-3)-α-D-galactopyranosyl-(1-6)-D-glucose (Fu *et al.*, 1990 ; Cote *et al.*, 2000). From HPLC analysis of reaction products in Fig 3.15, two products at Rt 7.1 and 8.3 min were clearly observed. The main product at Rt 8.3 min (later proved to be glucosylated transfer product of melibiose, see section 3.5.1) was named product A. While the product at Rt 7.1 min was named product X but later proved to be transfer product from sucrose, not from melibiose acceptor (see section 3.5.1). However, in the preliminary identification of our product from melibiose acceptor (Product A at Rt 8.3 min), when treated with glucoamylase (20 unit/ml) at 37 °C for 6 hours, HPLC result showed that our main product was resistant to glucoamylase (data not shown). The result suggested that our product might be a novel structure . Thus, we decided to use melibiose as acceptor for glucosyl transfer by GS *Bacillus licheniformis* TH 4-2 in this work.

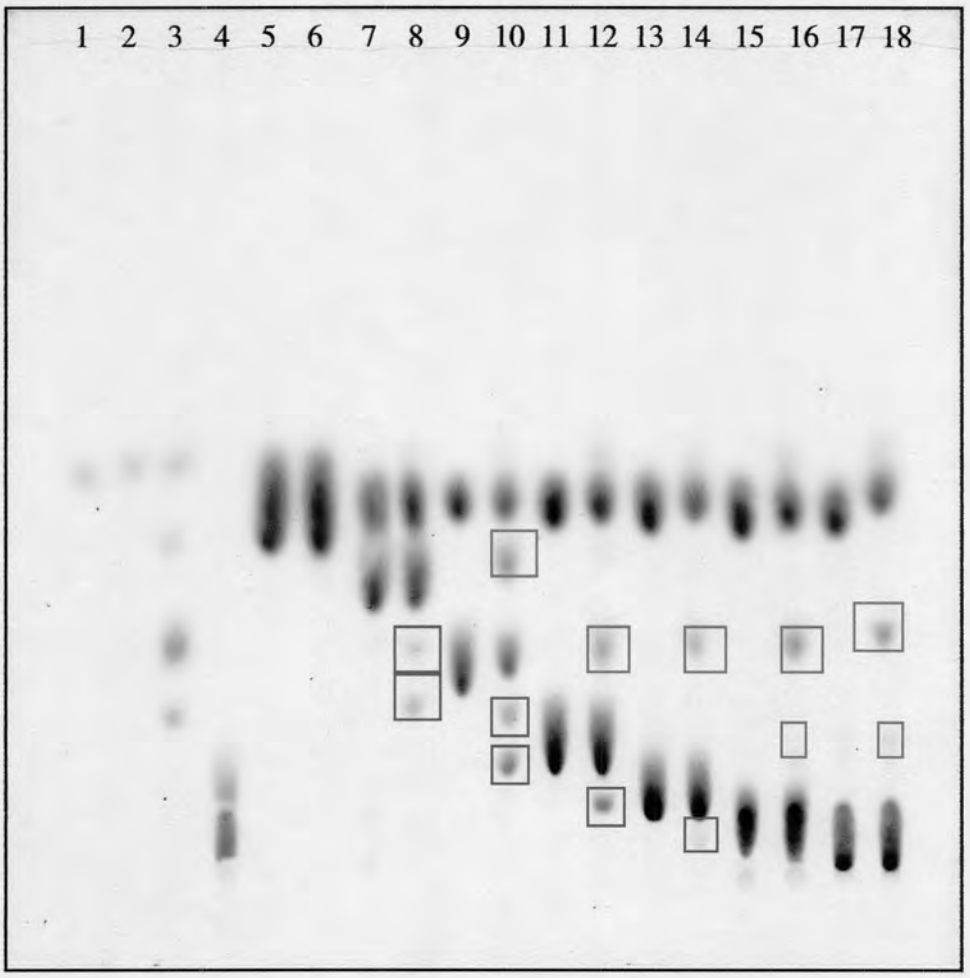


Figure 3.13 TLC chromatogram of reaction products of glucansucrase incubated with sucrose donor and various acceptors (G1 to G7) for 24 hours

Lane 1 :	Standard sucrose (20 μg)
Lane 2 :	Standard fructose (20 μg)
Lane 3 :	Standard G1-G4 (20 μg each)
Lane 4 :	Standard G5-G7 (20 μg each)
Lane 5-6 :	G1 as acceptor, 0 and 24 hr
Lane 7-8 :	G2 as acceptor, 0 and 24 hr
Lane 9-10 :	G3 as acceptor, 0 and 24 hr
Lane 11-12 :	G4 as acceptor, 0 and 24 hr
Lane 13-14 :	G5 as acceptor, 0 and 24 hr
Lane 15-16 :	G6 as acceptor, 0 and 24 hr
Lane 17-18 :	G7 as acceptor, 0 and 24 hr

Square -red box (□) show reaction products from sucrose donor
-blue box (□) show reaction products from acceptor after transglucosylation

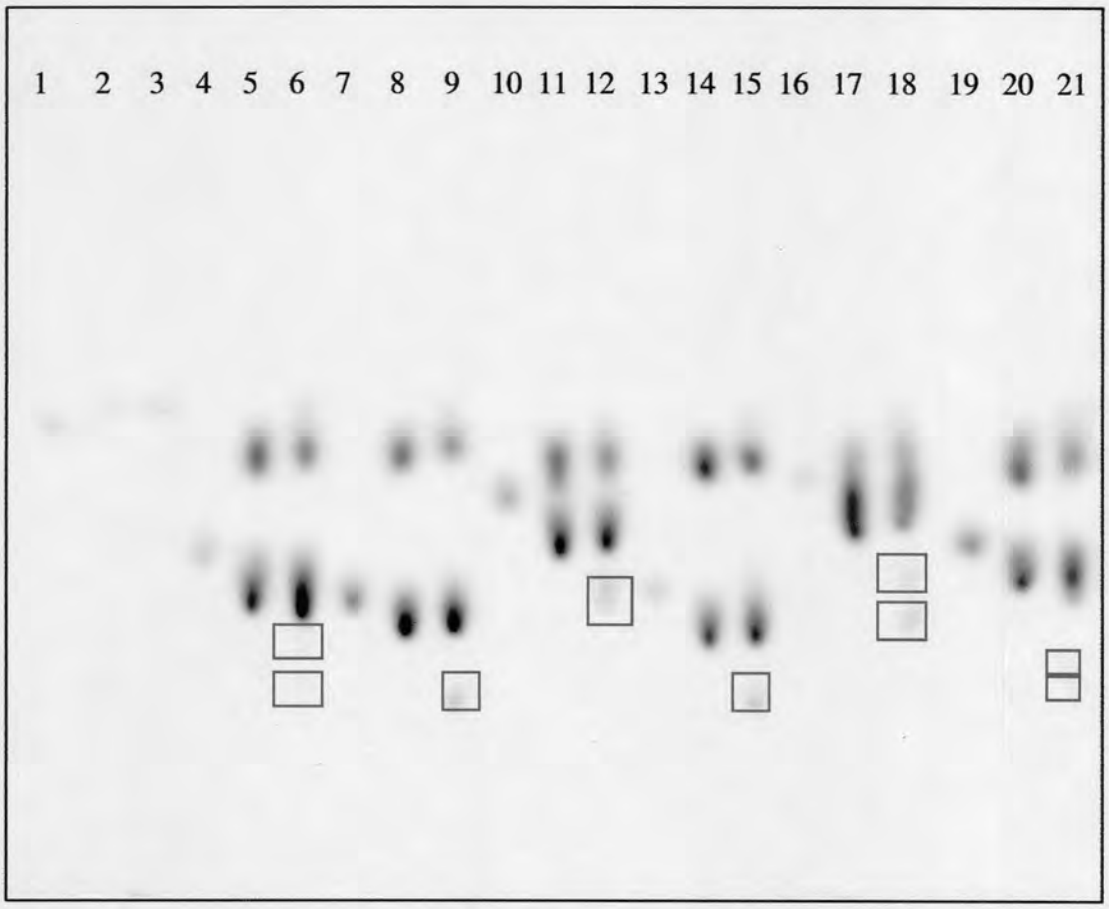


Figure 3.14 TLC chromatogram of reaction products of glucansucrase incubated with sucrose donor and non-maltooligosaccharide acceptors (lactose, melibiose, cellobiose, raffinose, palatinose and lactulose) for 24 hours

Lane 1-4 :	Standard sucrose, fructose, glucose, lactose (20 μg each)
Lane 5-6 :	Lactose as acceptor, 0 and 24 hr
Lane 7 :	Standard melibiose (20 μg)
Lane 8-9 :	Melibiose as acceptor, 0 and 24 hr
Lane 10 :	Standard cellobiose (20 μg)
Lane 11-12 :	Cellobiose as acceptor, 0 and 24 hr
Lane 13 :	Standard raffinose (20 μg)
Lane 14-15 :	Raffinose as acceptor , 0 and 24 hr
Lane 16 :	Standard palatinose (20 μg)
Lane 17-18 :	Palatinose as acceptor, 0 and 24 hr
Lane 19 :	Standard lactulose (20 μg)
Lane 20-21 :	Lactulose as acceptor, 0 and 24 hr

Square -blue box (□) show reaction products from acceptor after transglucosylation

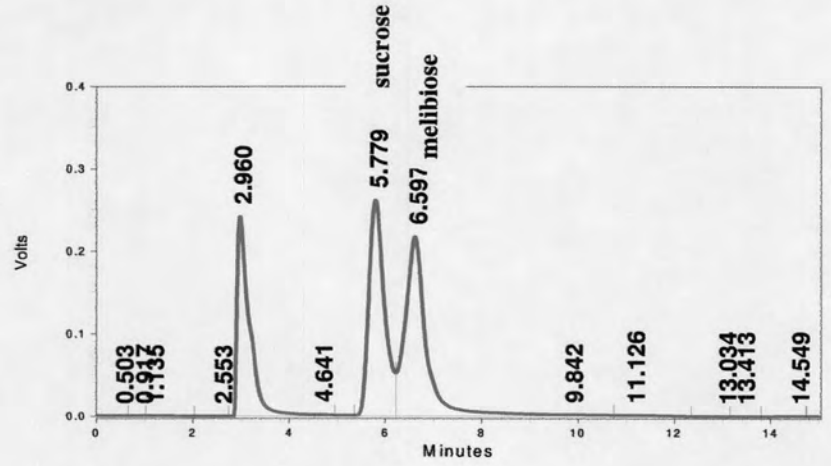
Table 3.2 Rf values from TLC analysis of standard saccharides , various acceptors and the reaction products

Saccharide/Acceptor		Rf		
		Standard	Product	
			From sucrose	From acceptor
Saccharides	Sucrose	0.53	-	-
	Fructose	0.55	-	-
	Glucose	0.55	-	-
Acceptors	G1	0.55	-	-
	G2	0.46	-	0.35, 0.28
	G3	0.35	0.44	0.27, 0.22
	G4	0.27	0.35	0.17
	G5	0.19	0.35	0.14
	G6	0.14	0.35, 0.24	Origin
	G7	0	0.35, 0.24	Origin
	Lactose	0.39	-	0.29, 0.25
	Melibiose	0.35	-	0.23
	Cellobiose	0.46	-	0.34
	Raffinose	0.35	-	0.23
	Palatinose	0.49	-	0.37, 0.32
	Lactulose	0.41	-	0.28, 0.24

Table 3.3 The total number of products from TLC analysis of the reaction products obtained from various acceptors

Acceptor	number of product from sucrose	number of product from acceptor
G1	-	-
G2	-	2
G3	1	2
G4	1	1
G5	1	1
G6	2	-
G7	2	-
Lactose	-	2
Melibiose	-	1
Cellobiose	-	1
Raffinose	-	1
Palatinose	-	2
Lactulose	-	2

A.



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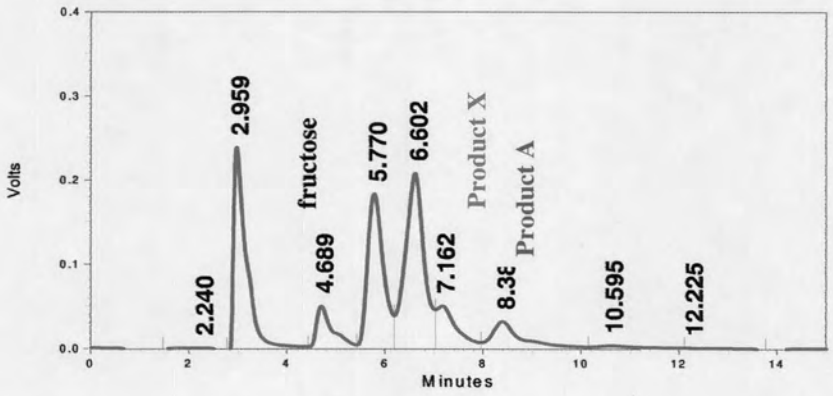


Figure 3.15 HPLC chromatogram of reaction products of glucansucrase with sucrose donor and melibiose acceptor (before optimization)

A. at 0 hour of incubation

B. at 24 hours of incubation

3.5 Optimization of transglucosylation reaction

The suitable condition for the production of glucosyl melibiose was determined using sucrose as glucosyl donor. The reaction was performed as described in section 2.13 and reaction products were analyzed by HPLC. During optimization, the yield of products was determined from peak areas of products at different conditions. After that, the optimum condition was judged from the maximum percent yield of transglusylated product, calculated from ratio of peak area of product to that of initial concentration of melibiose (see section 2.12.3).

3.5.1 Effect of concentration of melibiose acceptor

When incubating 0.5 unit/ml of glucansucrase with 5 % (w/v) sucrose donor and various concentrations of melibiose 0-22.5 % (w/v) in 20 mM acetate buffer pH 6.0 at 45 °C for 24 hours (as described in section 2.13.1), it was found that number of products and product yields varied with melibiose concentrations (Table 3.4). At 0 % (w/v) melibiose concentration, two products from sucrose at Rt 7.1 min (product X) and Rt 8.9 min (product Y) were found, and yields of these two products were decreased when increases melibiose concentration until at 12.5 % (w/v) where they were disappeared. When melibiose was present, the yield of product A at Rt 8.3 min was increased when increased melibiose concentration from 1 to 22.5 % (w/v). Thus product A should be glucosyl melibiose. The yield of product A was rapidly increased when melibiose concentration was increased from 0-5 %. However, the increase was less with melibiose from 5-15 % while no increase was observed when melibiose concentration was in the range of 15 to 22.5 % (w/v) (Fig 3.16). Thus 15 % melibiose was chosen to be the optimum concentration.

Table 3.4 Yields of transglucosylated products determined from peak areas at different melibiose concentrations

Melibiose concentration (%,w/v)	Product yield (peak area x10^6)		
	from sucrose		from melibiose
	Product X	Product Y	Product A (main product)
	Rt 7.1	Rt 8.9	Rt 8.3
0	1.65	0.34	-
1	1.48	0.33	0.32
2.5	1.43	0.30	0.63
5	1.34	-	1.37
7.5	1.20	-	1.60
10	1.00	-	1.87
12.5	-	-	1.92
15	-	-	2.32
17.5	-	-	2.32
20	-	-	2.45
22.5	-	-	2.45

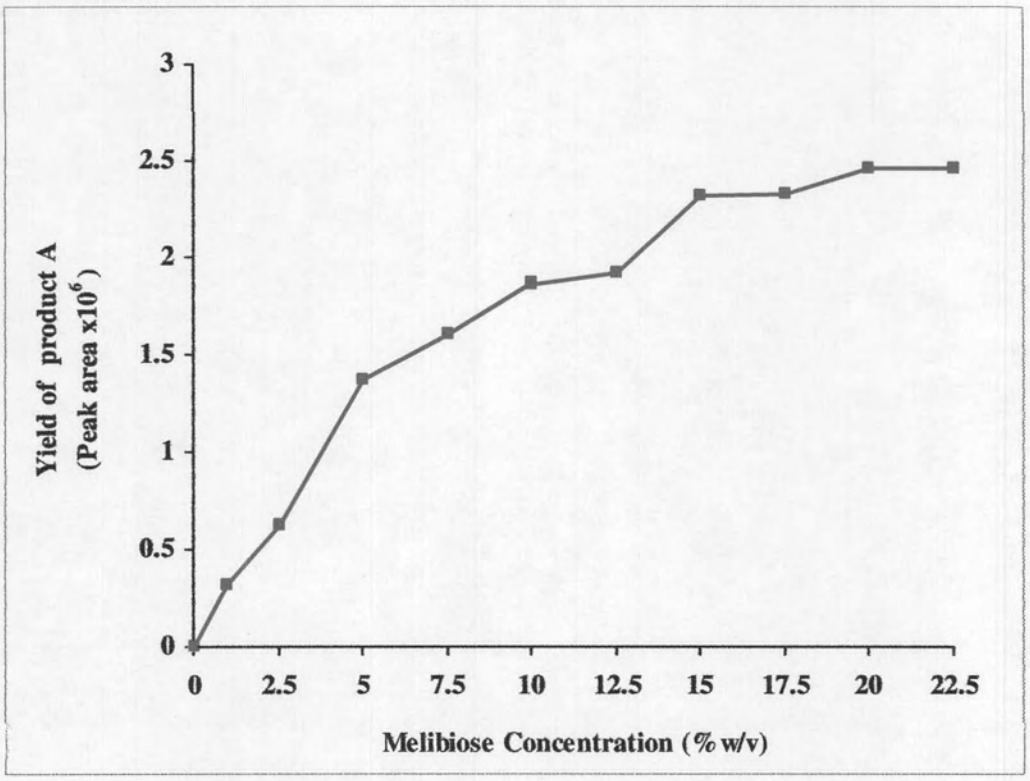


Figure 3.16 Effect of melibiose concentration on transglucosylation yield

3.5.2 Effect of concentration of sucrose donor

Using the condition as mentioned in section 3.5.1 with various sucrose concentrations, it was found that product yields varied with sucrose concentration. Products X at Rt 7.1 min and Y at Rt 8.9 min were not observed since all reactions contained 15 % melibiose. The yields of Product A at Rt 8.3 min were shown in Table 3.5 and Figure 3.17. The yield increased rapidly when sucrose was increased from 0 to 2.5 % and the maximum yield was obtained at 5 % (w/v) of sucrose concentration. Thus, 5 % sucrose was chosen to be the optimum concentration.

3.5.3 Effect of enzyme concentration

Using the condition as mentioned in section 3.5.2 with various concentrations of glucansucrase, it was found that the yields of product A was significantly increased when GS was increased from 0 to 0.5 unit/ml. The maximum yield was obtained at 5 unit/ml of glucansucrase (Table 3.6 and Figure 3.18). Further increase in enzyme concentration did not result in increase in yield of product A. In addition, when the reaction mixtures were incubated with 1.25 to 10 unit/ml of glucansucrase, a new product was observed at Rt 10.3 min (product B). However, product B was detected with the very low yield when compared to product A, the main product. The maximum yield of both product A and product B was observed at 5 unit/ml GS. Thus, this enzyme concentration was chosen to be the optimum concentration.

Table 3.5 Yields of transglucosylated products expressed as peak areas and product yield (%) at different sucrose concentrations

Sucrose concentration (%,w/v)	Product yield			
	(peak area x10^6)			(%)
	from sucrose		from melibiose	from melibiose
	Product X	Product Y	Product A (main product)	Product A (main product)
	Rt 7.1	Rt 8.9	Rt 8.3	Rt 8.3
0	-	-	-	-
1	-	-	0.49	2.47
2.5	-	-	2.28	11.4
5	-	-	2.48	12.4
7.5	-	-	2.44	12.2
10	-	-	2.26	11.3
15	-	-	2.10	10.3

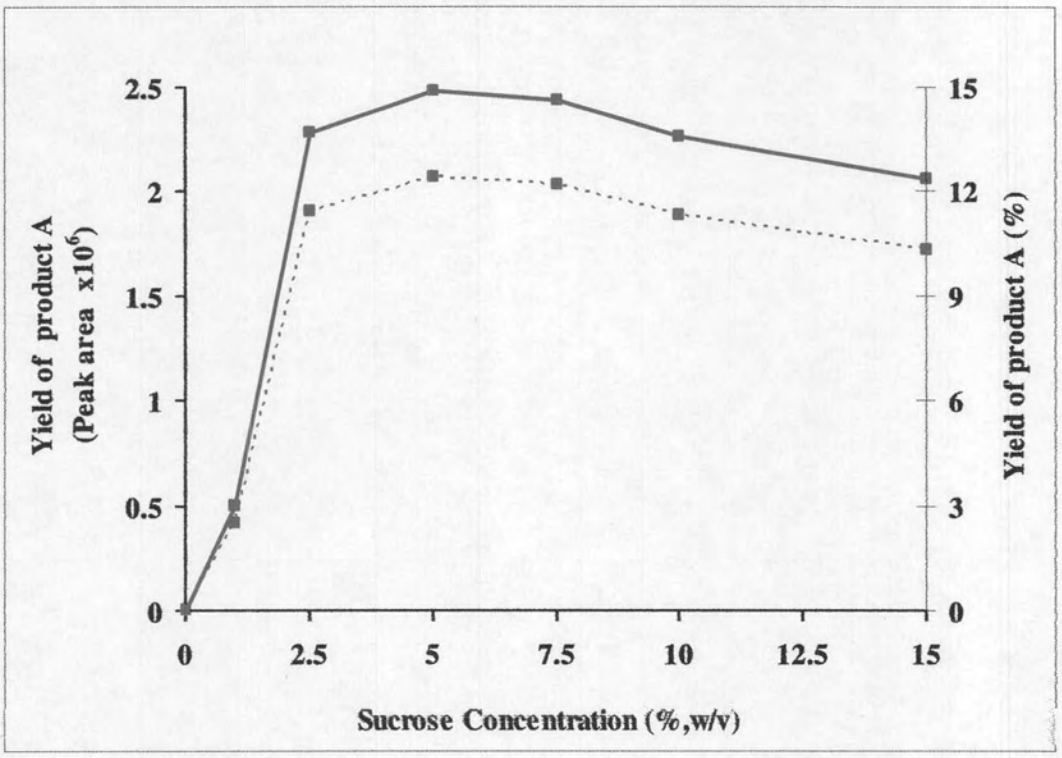


Figure 3.17 Effect of sucrose concentration on transglucosylation yield

——— **Peak area** ..... **(%)**

Table 3.6 Yields of transglucosylated products expressed as peak areas and product yield (%) at different GS concentrations

Glucansucrase Concentration (unit/ml)	Product yield					
	(peak area x10^6)				(%)	
	from sucrose		from melibiose		from melibiose	
	Product X	Product Y	Product A (main product)	Product B (minor product)	Product A (main product)	Product B (minor product)
	Rt 7.1	Rt 8.9	Rt 8.3	Rt 10.3	Rt 8.3	Rt 10.3
0	-	-	-	-	-	-
0.10	-	-	0.33	-	1.75	-
0.25	-	-	1.81	-	10.0	-
0.50	-	-	2.36	-	12.7	-
1.25	-	-	2.69	0.45	14.7	2.49
2.5	-	-	2.98	0.54	17.0	3.08
5.0	-	-	3.29	0.69	18.2	4.23
7.5	-	-	3.18	0.66	17.6	3.81
10	-	-	3.15	0.64	17.5	3.68

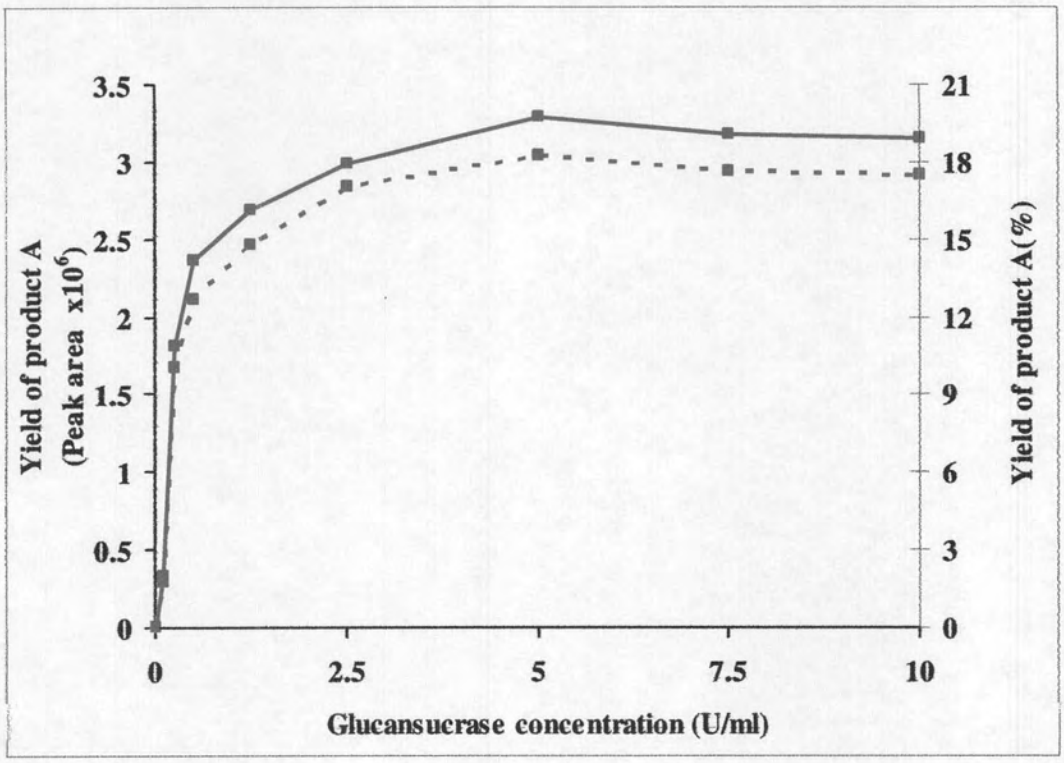


Figure 3.18 Effect of glucansucrase concentration on transglucosylation yield

——— **Peak area** ..... **(%)**

3.5.4 Effect of pH

Using the condition as mentioned in section 3.5.3 at various pHs, it was found that product yields varied with pH. The yields of transglucosylated products were shown in Table 3.7 and Figure 3.19, the maximum yield of main product (product A) at Rt 8.3 min and of minor product (product B) at Rt 10.3 min was obtained when the reaction mixture was performed in acetate buffer pH 6.0. However, the yields of products in the buffer at pH 6.0 (acetate buffer), pH 6.0 to pH 7.0 (phosphate buffer) and pH 8.0 (borate buffer) were not significantly different. It was observed that the yields of product A and B were also maximum at these pHs. Acetate buffer at pH 6.0 was chosen to be the optimum concentration.

3.5.5 Effect of temperature

Using the condition as mentioned in section 3.5.4 with various temperatures, it was found that product yields significantly varied with temperature. The yields of transglucosylated products were shown in Table 3.8 and Figure 3.20, the maximum yield of main product (product A) at Rt 8.3 min and minor product (product B) at Rt 10.3 min was obtained at 45 °C. However, the yields of products when incubated at 40 °C to 50 °C were not significantly different. The increase of temperature to 60 °C was obviously decreased the total yield. Thus, 45 °C was chosen to be the optimum temperature.

Table 3.7 Yields of transglucosylated products expressed as peak areas and product yield (%) at different pH

Buffer	pH	Product yield					
		(peak area x10^6)				(%)	
		from sucrose		from melibiose		from melibiose	
		Product X	Product Y	Product A (main product)	Product B (minor product)	Product A (main product)	Product B (minor product)
		Rt 7.1	Rt 8.9	Rt 8.3	Rt 10.3	Rt 8.3	Rt 10.3
Acetate	5.0	-	-	0.15	-	0.79	-
	5.5	-	-	2.46	0.39	12.9	2.02
	6.0	-	-	3.26	0.69	16.5	3.63
Phosphate	6.0	-	-	3.23	0.57	16.3	2.96
	7.0	-	-	3.22	0.54	16.4	2.82
	8.0	-	-	3.22	0.59	16.4	3.08
Borate	8.0	-	-	3.16	0.45	16.5	2.37
	9.0	-	-	1.82	0.37	9.52	1.91

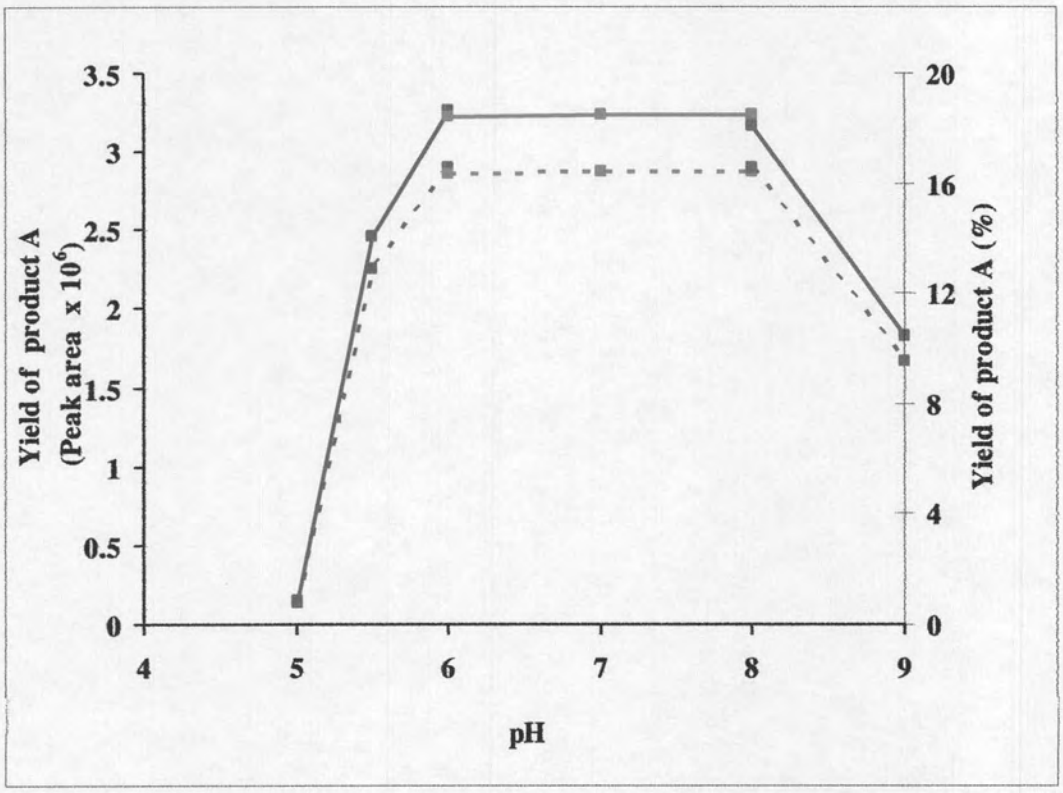


Figure 3.19 Effect of pH on transglucosylation yield

■ **Sodium acetate buffer (pH 5.0 – 6.0)**

■ **Phosphate buffer (pH 6.0-8.0)**

■ **Borate buffer (pH 8.0-9.0)**

——— **Peak area** ..... **(%)**

Table 3.8 Yields of transgluctosylated products expressed as peak areas and product yield (%) at different incubation temperatures

Temperature (°C)	Product yield					
	(peak area x10^6)				(%)	
	from sucrose		from melibiose		from melibiose	
	Product X	Product Y	Product A (main product)	Product B (minor product)	Product A (main product)	ProductB (minor product)
	Rt 7.1	Rt 8.9	Rt 8.3	Rt 10.3	Rt 8.3	Rt 10.3
30	-	-	2.72	0.28	15.1	1.56
40	-	-	3.19	0.54	17.1	3.00
45	-	-	3.25	0.66	17.4	3.65
50	-	-	3.22	0.61	17.3	3.35
55	-	-	2.87	0.42	15.9	2.35
60	-	-	0.11	-	0.61	-

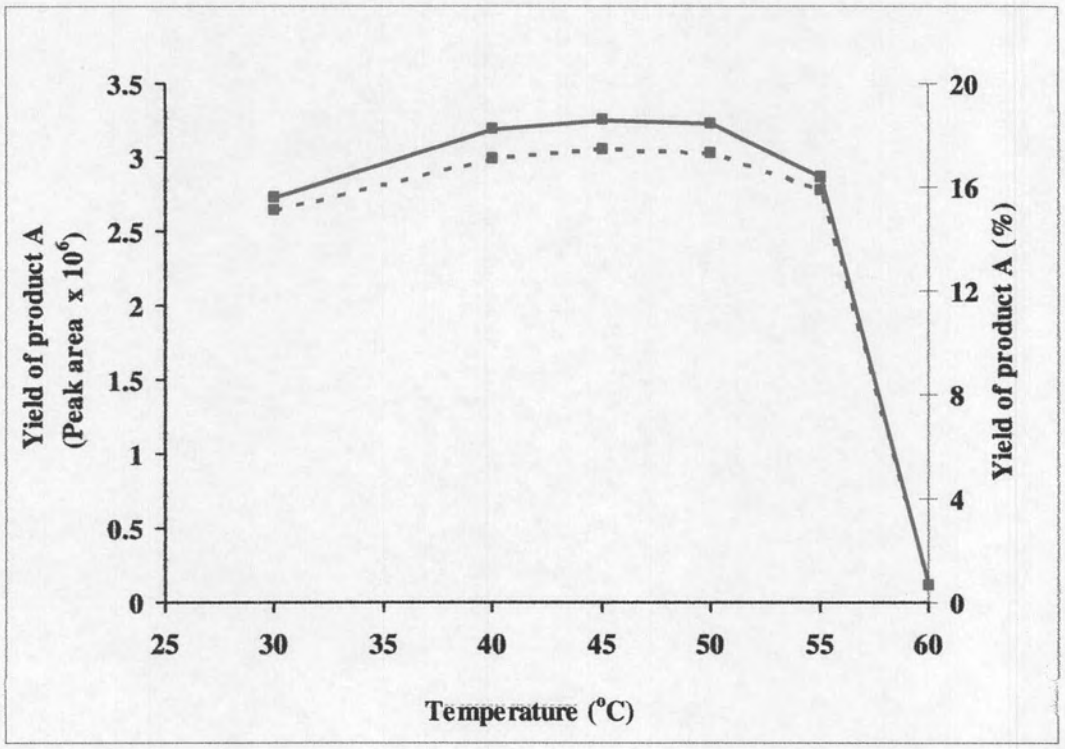


Figure 3.20 Effect of incubation temperature on transglucosylation yield

——— **Peak area** ..... **(%)**

3.5.6 Effect of incubation time

Using the condition as mentioned in section 3.5.5 with various incubation times, it was found that product yields were clearly affected. Product yields of transglucosylated products were shown in Table 3.9 and Figure 3.21, The yield of main product (product A) at Rt 8.3 min was rapidly increased when incubation time was in the range of 0-12 hours. The maximum yield of main product A and minor product (product B) at Rt 10.3 min was obtained when incubation time was in the range of 24-48 hours. To save on time, the 24 hours was chosen as optimum incubation time.

Therefore, the optimum condition for transglucosylation by glucansucrase from *Bacillus licheniformis* TH 4-2 which gave glucosyl melibiose products yield at the maximum was: incubation of 5 unit/ml of glucansucase with 5 % (w/v) sucrose (donor) and 15 % (w/v) melibiose (acceptor) in 20 mM acetate buffer pH 6.0 at 45 °C for 24 hours. Two products were obtained from glucosyl transfer to melibiose acceptor, product A and product B with the yields of 17.2 % and 3.3 %, respectively. The profiles of reaction products before and after optimization were compared in Figure 3.22. Only the main product at Rt 8.3 min was further characterized and determined for its biological property.

Table 3.9 Yields of transglucosylated products expressed as peak areas and product yield (%) at different incubation times

Incubation time (hours)	Product yield					
	(peak area x10^6)				(%)	
	from sucrose		from melibiose		from melibiose	
	Product X	Product Y	Product A (main product)	Product B (minor product)	Product A (main product)	Product B (minor product)
	Rt 7.1	Rt 8.9	Rt 8.3	Rt 10.3	Rt 8.3	Rt 10.3
0	-	-	-	-	-	-
3	-	-	2.54	-	12.6	-
6	-	-	2.74	0.46	13.6	2.27
12	-	-	3.27	0.57	16.2	3.31
24	-	-	3.46	0.67	17.2	3.32
36	-	-	3.54	0.69	17.5	3.92
48	-	-	3.55	0.68	17.6	3.90

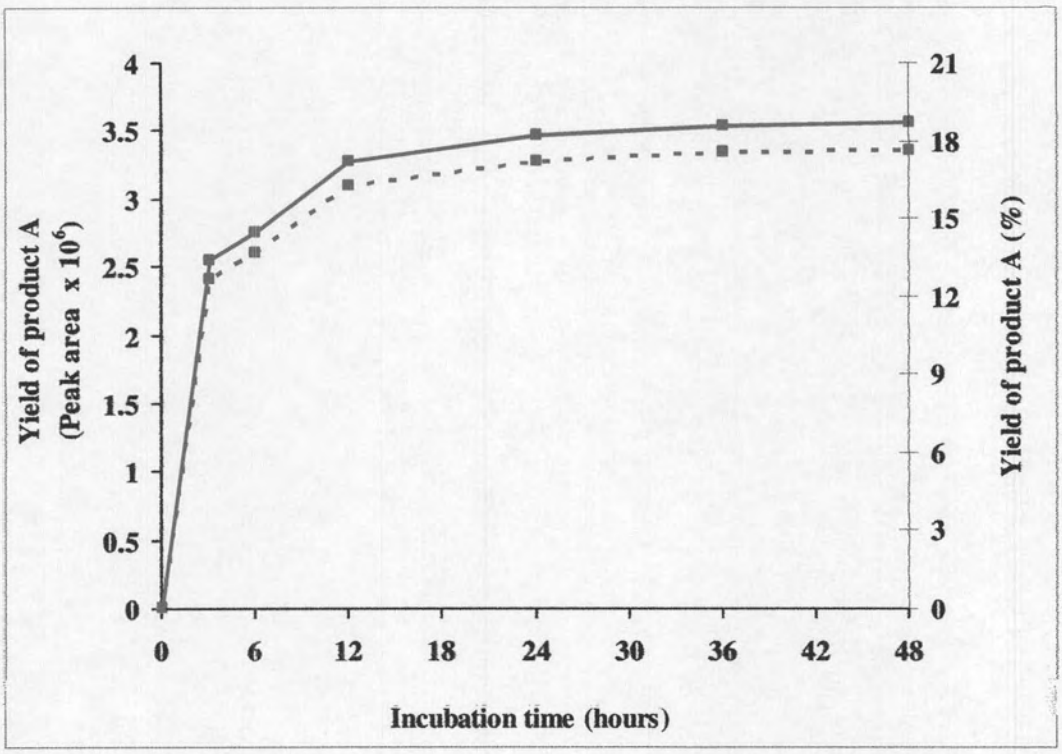
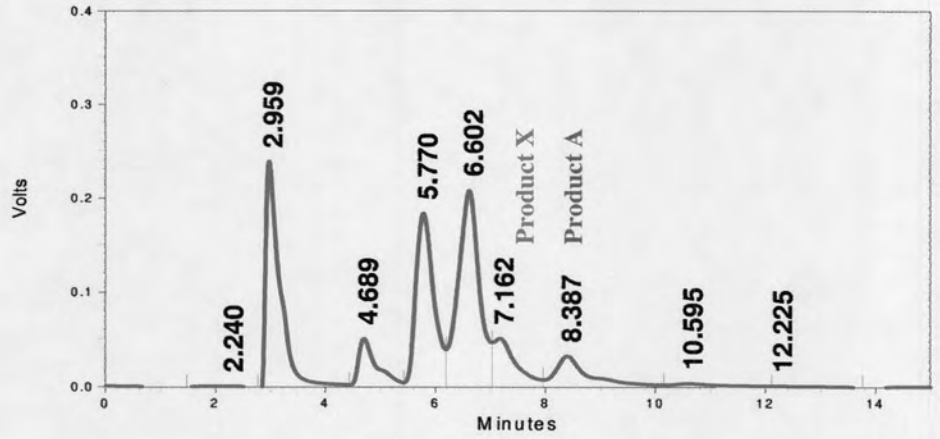


Figure 3.21 Effect of incubation time on transglucosylation yield

A.



B.

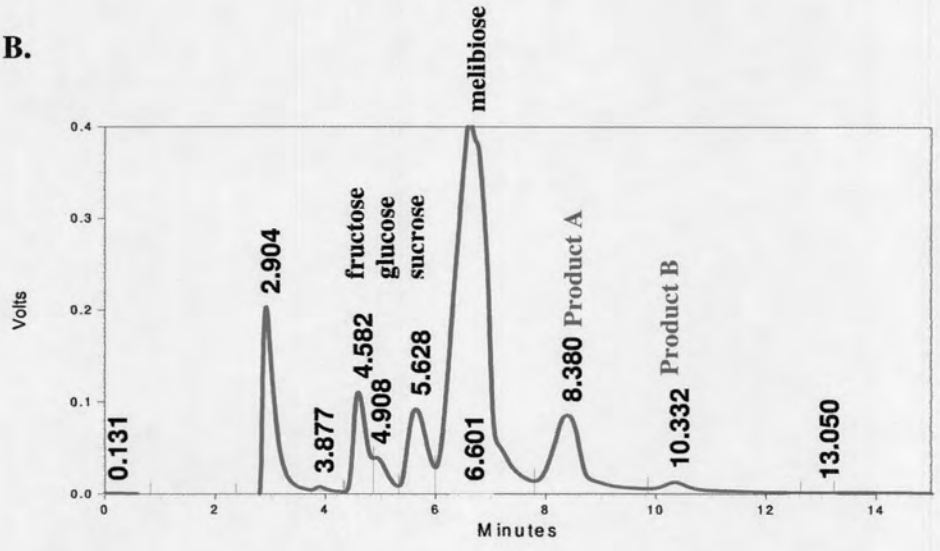


Figure 3.22 HPLC chromatogram of reaction products of glucansucrase with melibiose acceptor and sucrose donor

A. before optimization

B. after optimization

3.6 Larger scale preparation and isolation of glucosyl melibiose products

For production of higher amounts of glucosyl melibiose products, the larger scale of reaction mixture (10 ml) was prepared as described in section 2.14 using optimum condition for transglucosylation obtained in section 3.5. After transglucosylation reaction, the enzyme invertase (20 unit/ml) was added to the reaction mixture and incubation was performed at 37 °C for 3 hours. After treated with invertase, the reaction mixture of 0.5 ml was applied on to Sephadex LH-20 column. The column was eluted with 70%(v/v) n- propanol at a flow rate of 10 ml/hour, fraction size 1 ml. The fractions containing sugar were followed by assay of reducing sugars using Somagyi-Nelson's method (1990) as described in section 2.8. Sugar identification was by HPLC as described in section 2.12.2.2. The Sephadex LH-20 column profile of reaction products was shown in Figure 3.23. Three main peaks (I, II, III) were observed: they were identified by HPLC as product A, melibiose acceptor, glucose and fructose, respectively. Glucose and fructose could not be separated by the column in the condition used. In addition, no peak of sucrose donor was observed because sucrose was hydrolyzed by invertase and became glucose and fructose before applied to the column. The result of the analysis of each peak by HPLC was shown in Figure 3.24

Peak I (product A at Rt 8.4 min), Peak II (melibiose acceptor at Rt 6.1 min), and peak III (glucose at Rt 4.6 min and fructose at Rt 4.3 min) were identified. respectively. The Rt values of all sugars in this Figure 3.24 were some what shifted from those values in Fig.3.15 where product A, melibiose, glucose, and fructose were at Rt 8.3, 6.5, 5.0, and 4.6 min, respectively. These may be due to differences in sugar

concentration. Then, product A at Rt 8.4 min was pooled and concentrated with a rotary evaporator. The concentrated product A was further characterized for size, structure and biological activity. In addition melibiose acceptor was pooled and concentrated and kept for reuse.

3.7 Characterization of Product

3.7.1 Mass spectrometry

The molecular weight of the synthesized product was elucidated by mass spectrometry as described in section 2.15.1. The molecular weight of the product A at Rt 8.3 min was estimated to be 504 daltons by ESI-TOF mass spectrometry with [M+Na]+ at m/z of 527 (Figure 3.25), This corresponded to the size of a trisaccharide of melibiose (gal-glu) with one molecule of glucose.

3.7.2 Nuclear Magnetic Resonance

For the structural elucidation of product A at Rt 8.3 min. Information from ^{1}H- NMR , ^{13}C-NMR, HSQC and HMBC were used to determine the information of linkage. ^{1}H-NMR displayed characteristic signal for the α-anomeric proton at δ = 4.778 ppm (J = 3.6 Hz) and δ= 4.753 ppm (J = 3.6 Hz) (Figure3.26). The coupling constant (J = 3.6 Hz) suggested that glucosyl residue was joined to melibiose (gal α1-6 glu) by α-linkage. HMBC displayed characteristic correlation between anomeric proton at C1 position (δ = 4.778 ppm and δ= 4.753 ppm) and methylene carbon (CH_2) at C6 position (δ = 66.32 ppm and δ= 65.50 ppm), respectively (Figure 3.29), suggesting the glucosyl residue was attached to melibiose acceptor by α-1, 6 linkage.

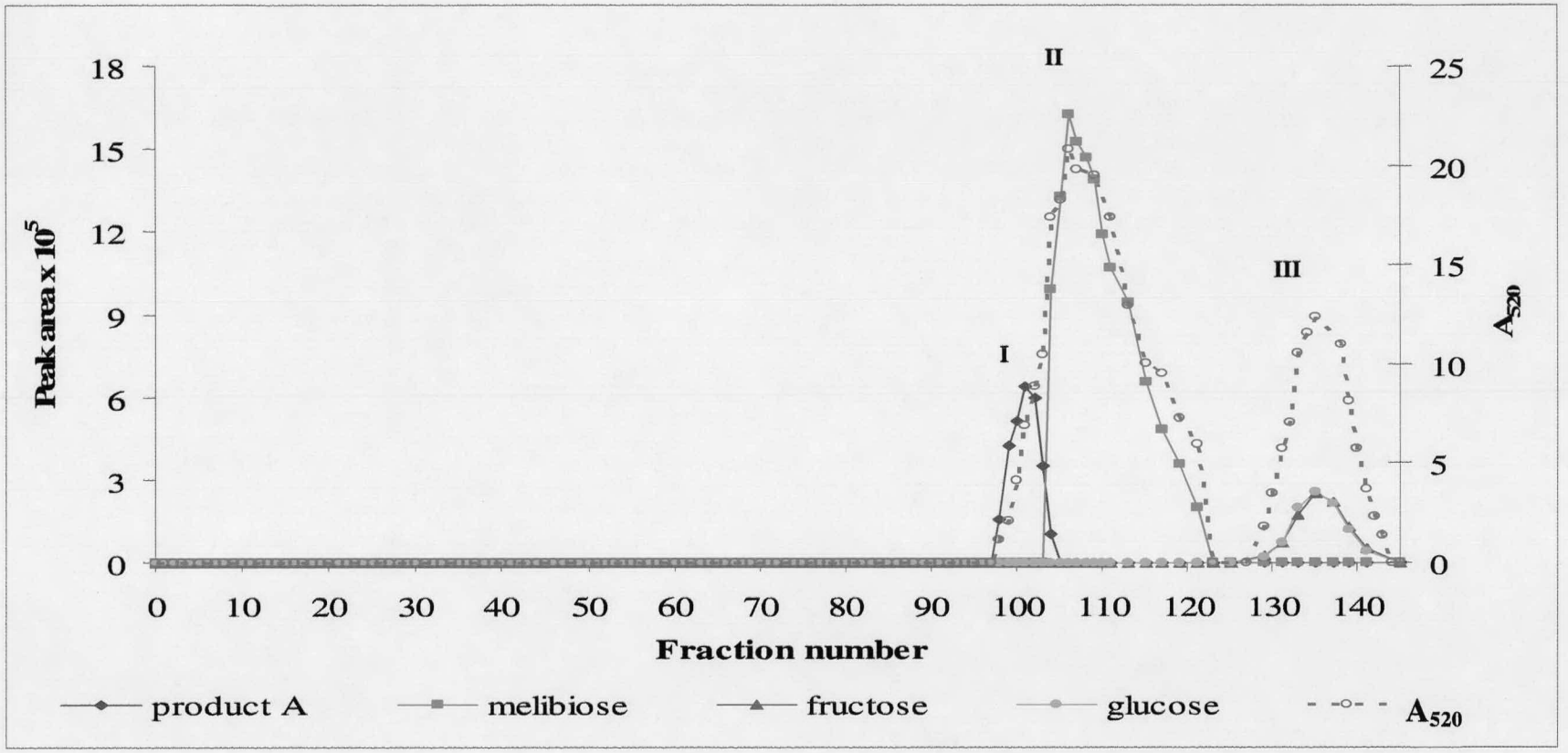
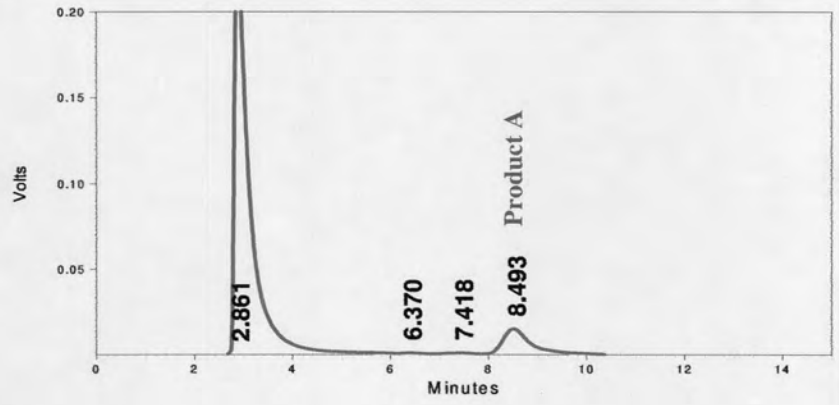
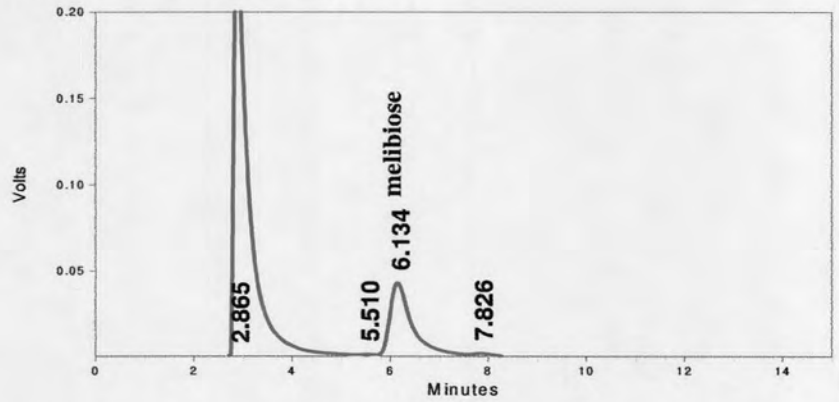


Figure 3.23 Sephadex LH-20 column profile of reaction products, column size 1.2x120 cm, flow rate 10 ml/hour, fraction size 1 ml, 70% n-propanol as eluent

A.peakI



B. peak II



C. peak III

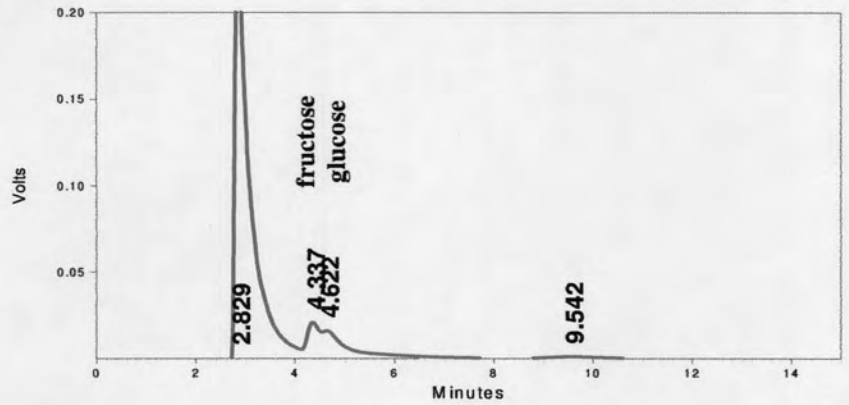


Figure 3.24 HPLC chromatogram of peak I to III from Sephadex LH-20 column

A. peak I (product A, Rt 8.4 min)

B. peak II (melibiose, Rt 6.1 min)

C. peak III (glucose and fructose, Rt 4.6 and 4.3 min)

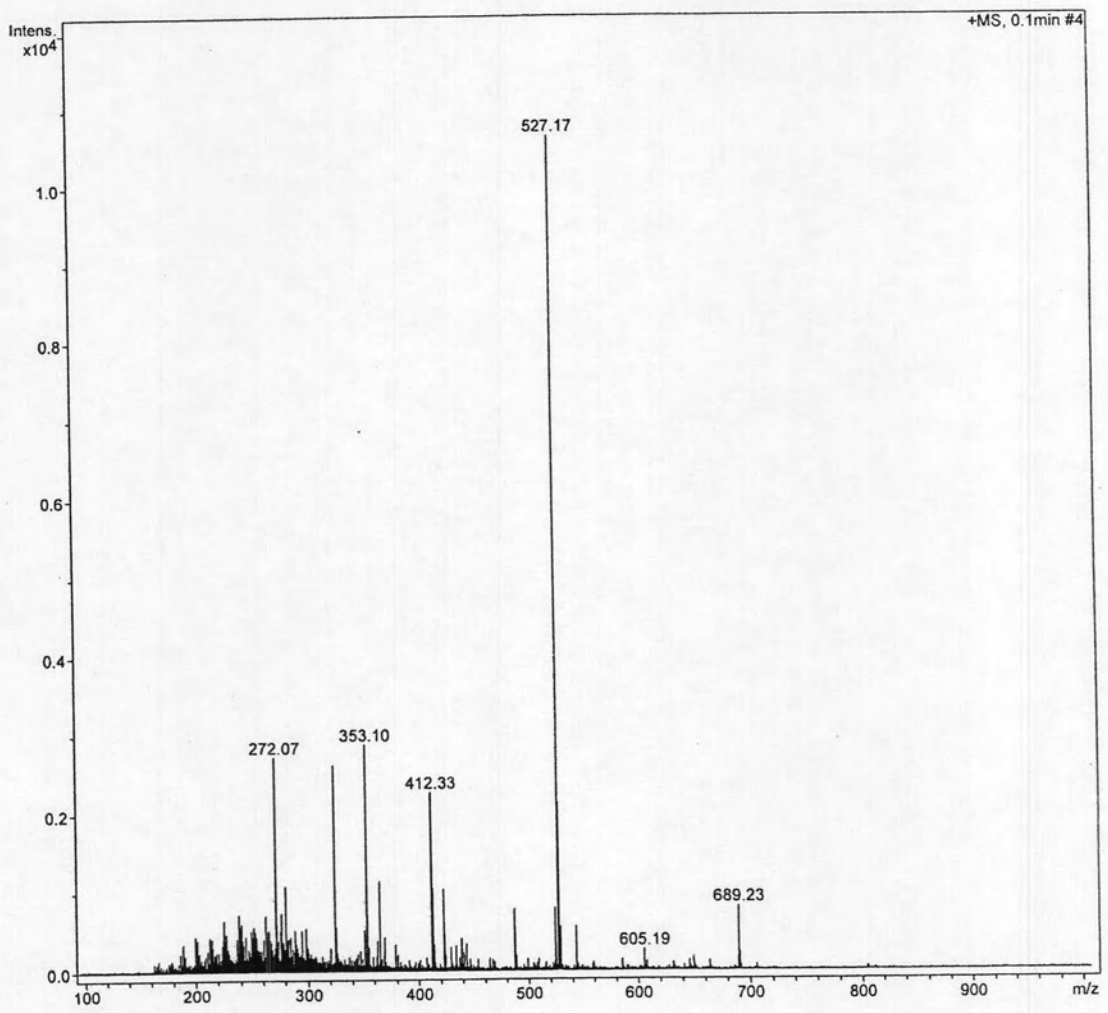


Figure 3.25 ESI-TOF mass spectrum of the product A at Rt 8.3 min

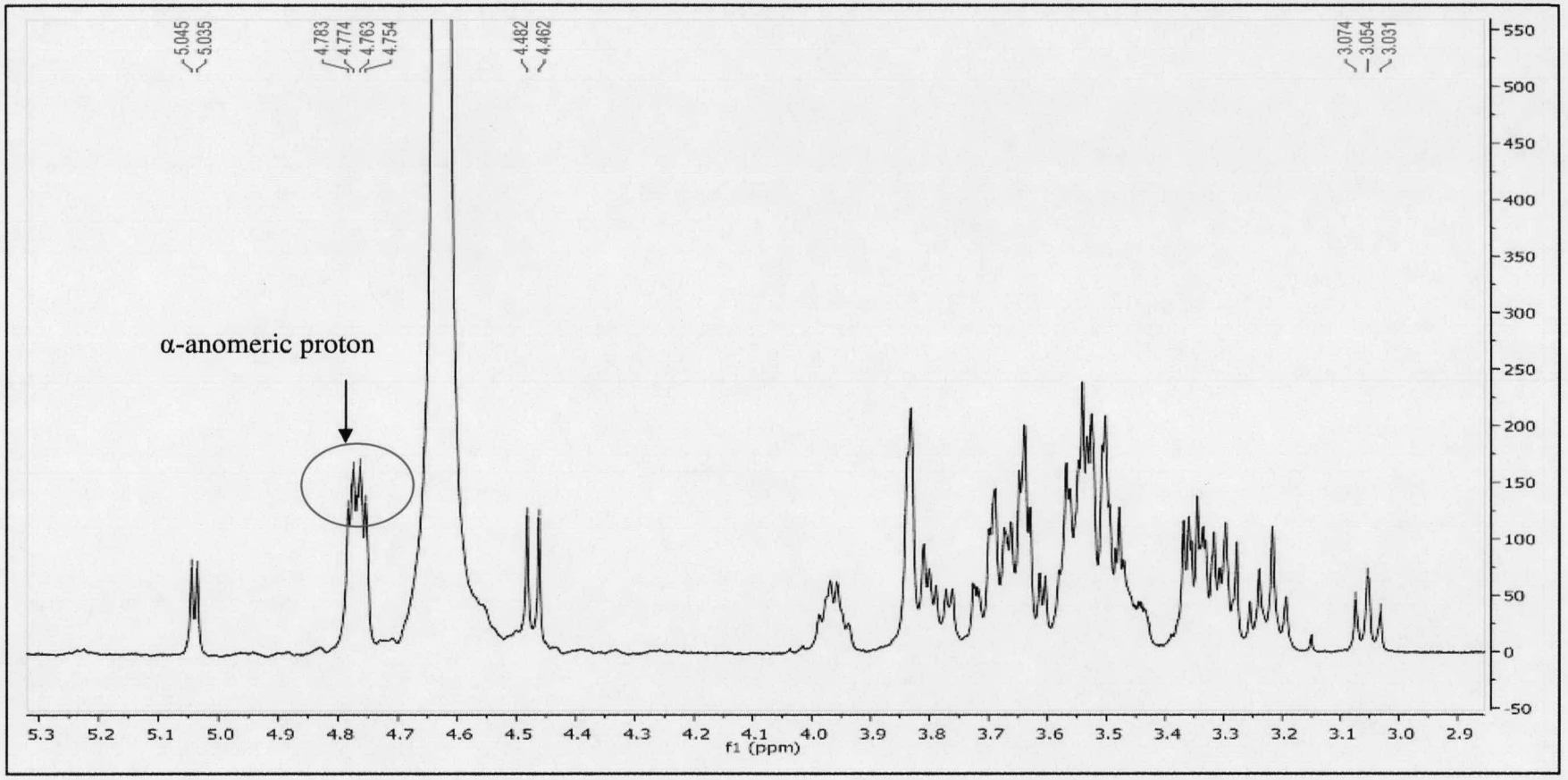


Figure 3.26 The 400 MHz ^{1}H-NMR spectrum of the product A at Rt 8.3 min

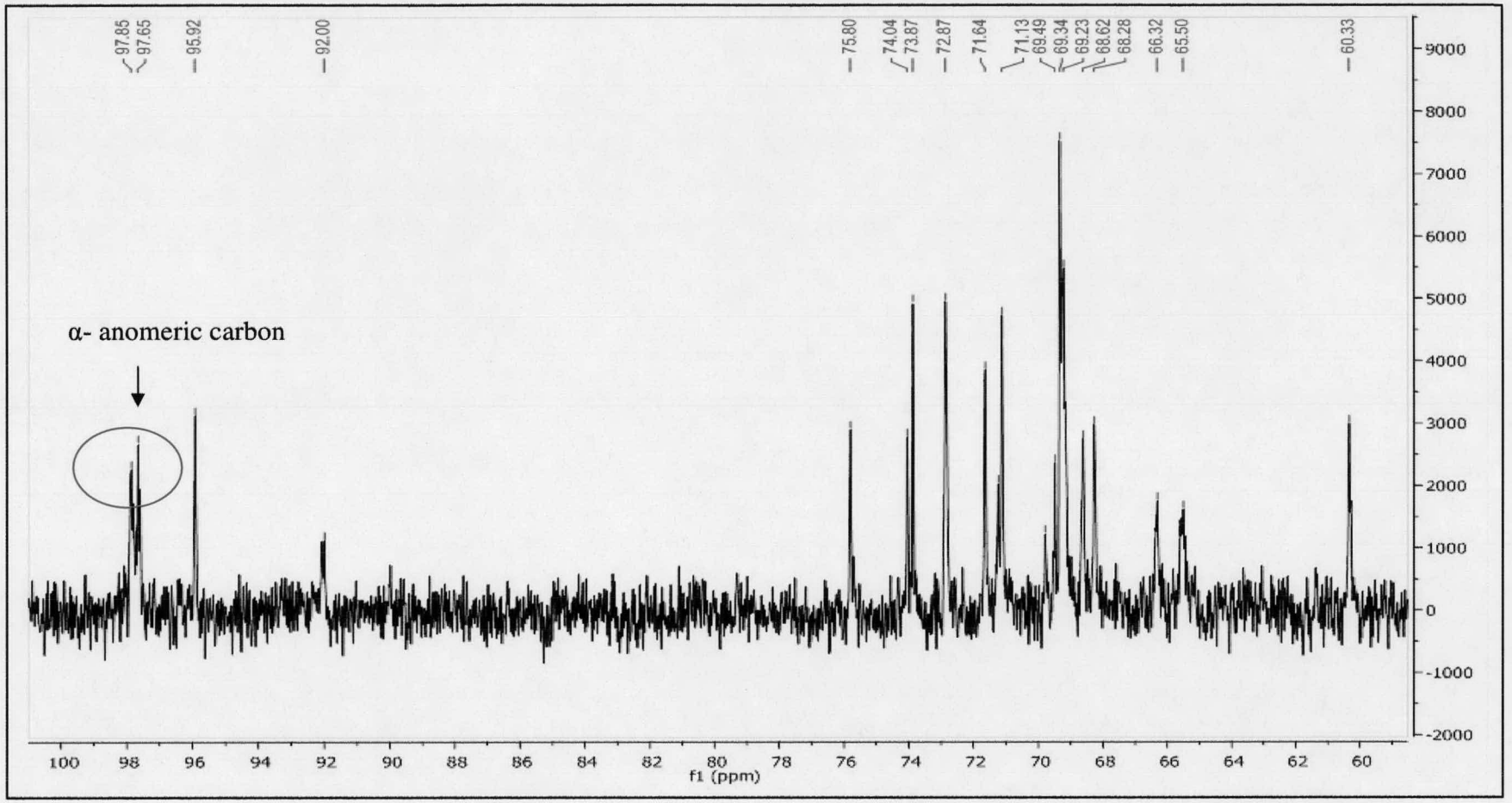


Figure 3.27 The 100 MHz ^{13}C-NMR spectrum of the product A at Rt 8.3 min

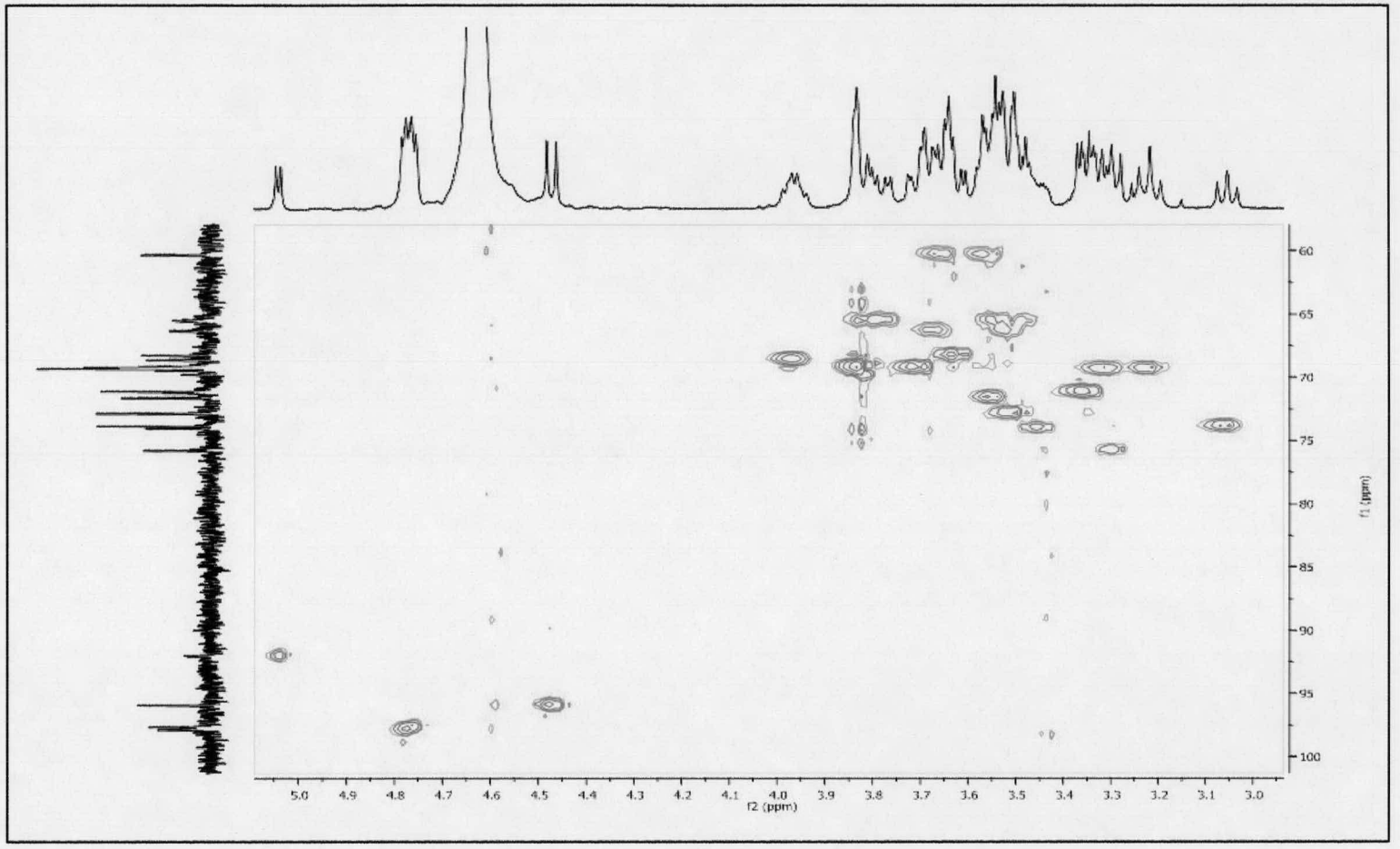


Figure 3.28 HSQC spectrum of the product A at Rt 8.3 min

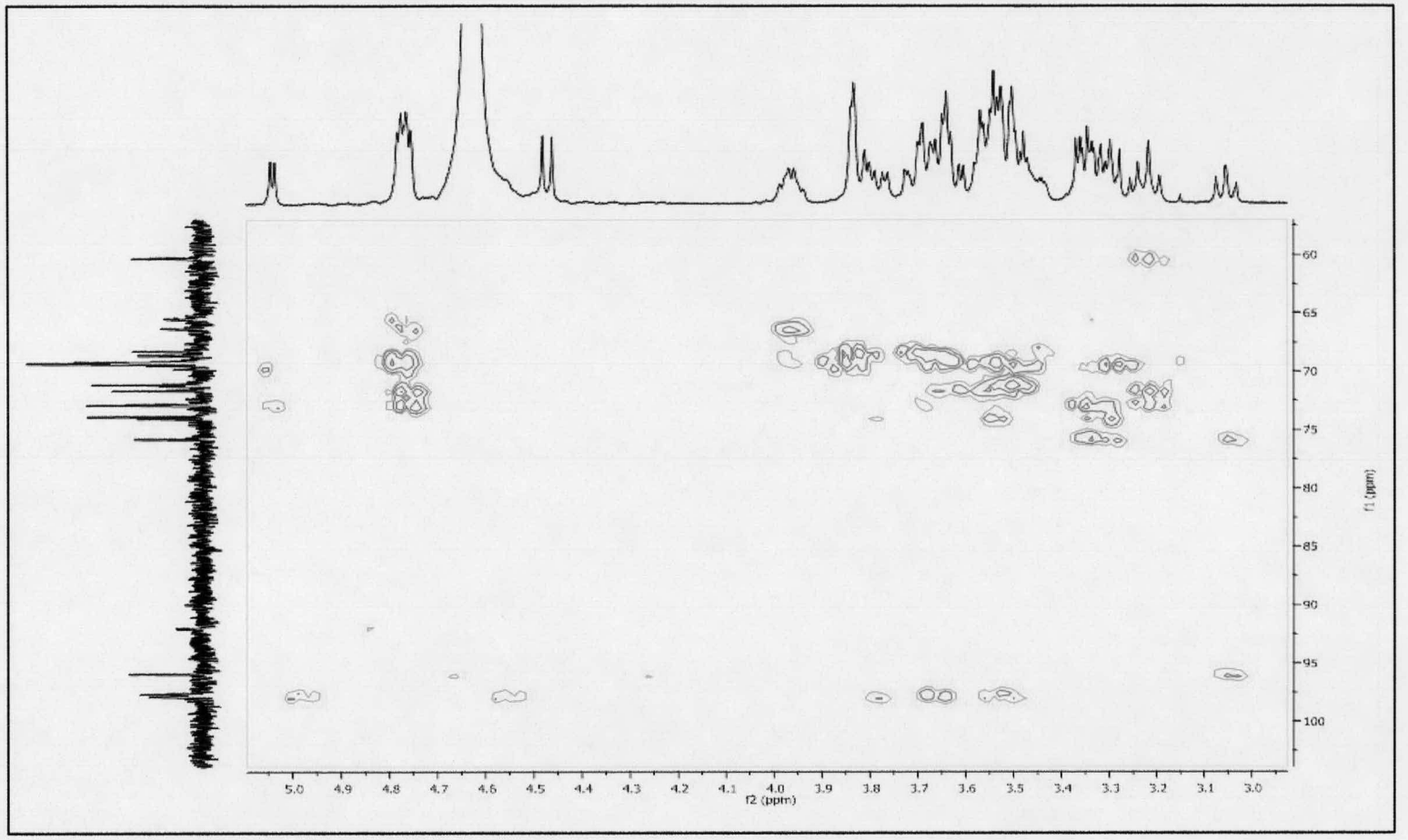


Figure 3.29 HMBC spectrum of the product A at Rt 8.3 min

3.8 Determination of biological activity of product

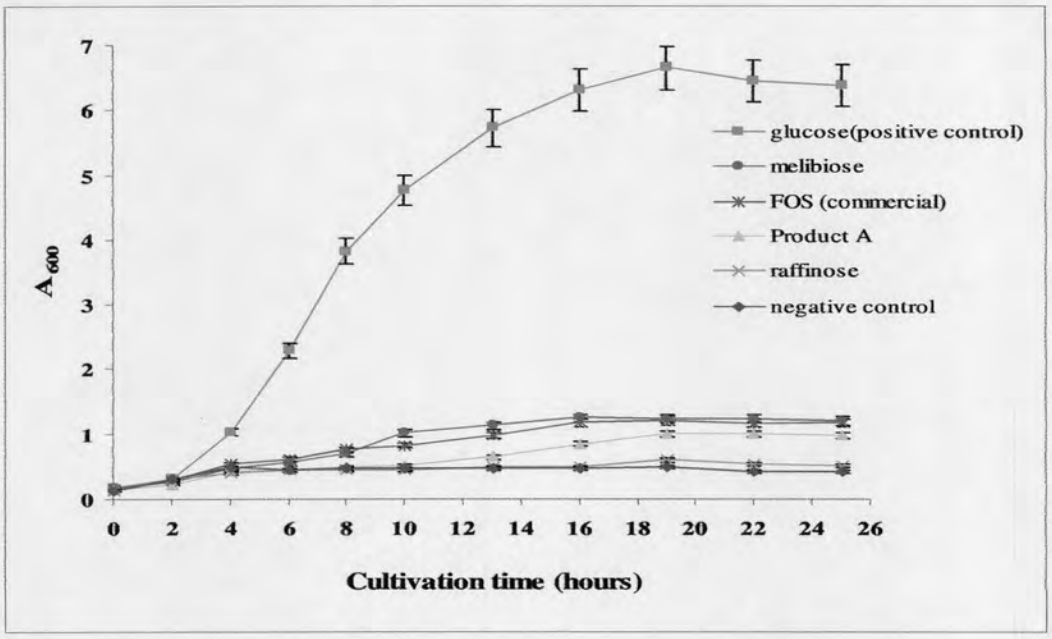
3.8.1 Monitoring of biological activity of OS product in supporting growth of *Lactobacillus acidophilus*

3.8.1.1 Various supplements

Growth of *Lactobacillus acidophilus*, a probiotic organism, was monitored in MRS medium with various types of supplement at a concentration of 2 % as described in section 2.16.1. The result showed that *Lactobacillus acidophilus* grew maximally on glucose (positive control),with overnight culture reaching A_{600} level of more than 6.0 (Fig 3.30A). In MRS medium supplemented with melibiose, FOS (commercial) and product A, this probiotic organism could grow better than the negative control with A_{600} reaching 1.0-1.2 at 24 hours. The lowest growth (overnight cultures reaching A_{600} level less than 0.6) was observed in the medium with no supplements (negative control) and when raffinose was added.

For a clearer comparison, Figure 3.30 B shows the expanded growth curves from A when the positive control was left out. The result indicates that product A can support significant growth of *Lactobacillus acidophilus* but at a lower rate than melibiose and FOS (commercial). Raffinose, on the contrary, did not support growth of this bacteria.

A.



B.

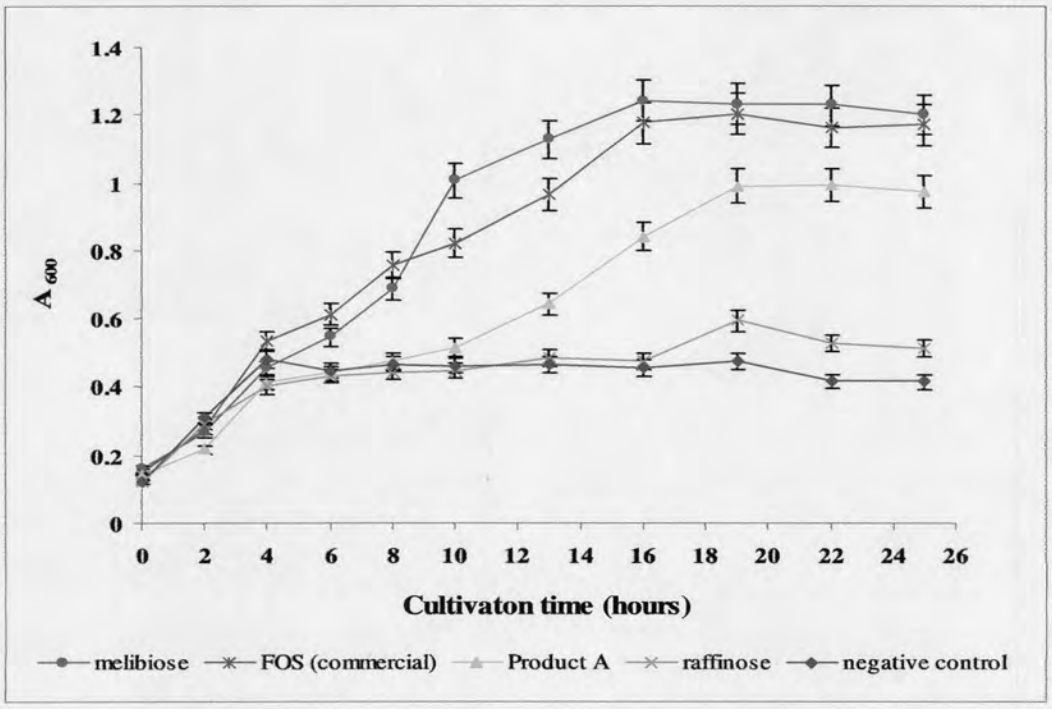


Figure 3.30 A. The growth curves of *Lactobacillus acidophilus* grown in MRS medium supplemented with various supplements at 2% w/v.

B. The expanded growth curves from A when the positive control was left out.