CHAPTER III

RESULTS

3.1 Preparation of partially purified CGTase from *Bacillus circulans* A 11

Bacillus circulans A11 was cultivated in Horikoshi' s medium by incubation at 37°C and 250 rpm for 72 hours. The culture was centrifuged to remove cells and crude CGTase in the supernatant was collected. Then, the crude CGTase was partially purified by starch adsorption as described in Section 2.8 and concentrated by ultrafiltration. Through these steps, the % recovery of CGTase and purification fold were 57.13% and 94.9 respectively, as shown in Table 5 the final specific activity was 2996.38 U/mg. The partial purified CGTase was then used for the study on cyclodextrin production.

3.2 Cyclodextrins production from different kinds of starch

To compare the efficacy of starch for cyclodextrin production, 2 g% of each kind of starch were gelatinized in boiling water for 10 minutes and incubated with partial purified CGTase (500 U/g starch) at 40 °C for 17 hours and the amount of cyclodextrins were quatitated by HPLC as described in Section 2.9.1 and 2.9.2. The results in Figure 11 showed that amylopectin (corn) is a better substrate for CGTase than amylose with the yield of 37% and 25%, respectively. Soluble starch and starches from potato and sago gave the highest yield of cyclodextrin (40% or more). Rye produced the lowest amount of cyclodextrin, yielding only 27%, which is comparable to amylose (corn). The result also showed that cassava starch, as well as potato, long grain rice, and Thai glutinous rice were also good for cyclodextrin production (37% yield).

Using CGTase from *Bacillus circulans* A 11, all starches gave β-CD as the major products (11-22%), except for Thai glutinous rice and rye which gave comparable amounts of α- and β-CD. Noticably, Thai glutinous rice gave the highest yield of α-CD (16%) and soluble starch (from potato) gave the highest yield of γ-CD (7%).

The ratio of α:β:γ cyclodextrins from cassava starch was 2.8: 3.7:1.

Table 5 Partial purification of CGTase from *Bacillus circulans* A11

Step	Volume (ml)	Enzyme activity		Total protein (mg)	Specific activity* (U/mg)	Recovery (%)	Purification fold**
		Dextrinizing (total unit)	CD-TCE (dilution limit)				
Crude enzyme	1000	17840	2^6	565	31.57	100	1
Partial purified enzyme	207	15673.4	2^8	4.79	2280.01	87.9	72.2
Ultrafiltrated enzyme	100	10187.7	2^{10}	3.4	2996.38	57.13	94.9

* Specific activity $= \frac{\text{Dextrinizing activity (U)}}{\text{Protein (mg)}}$

** Purification fold $= \frac{\text{Specific activity of each preparation}}{\text{Specific activity of crude enzyme}}$

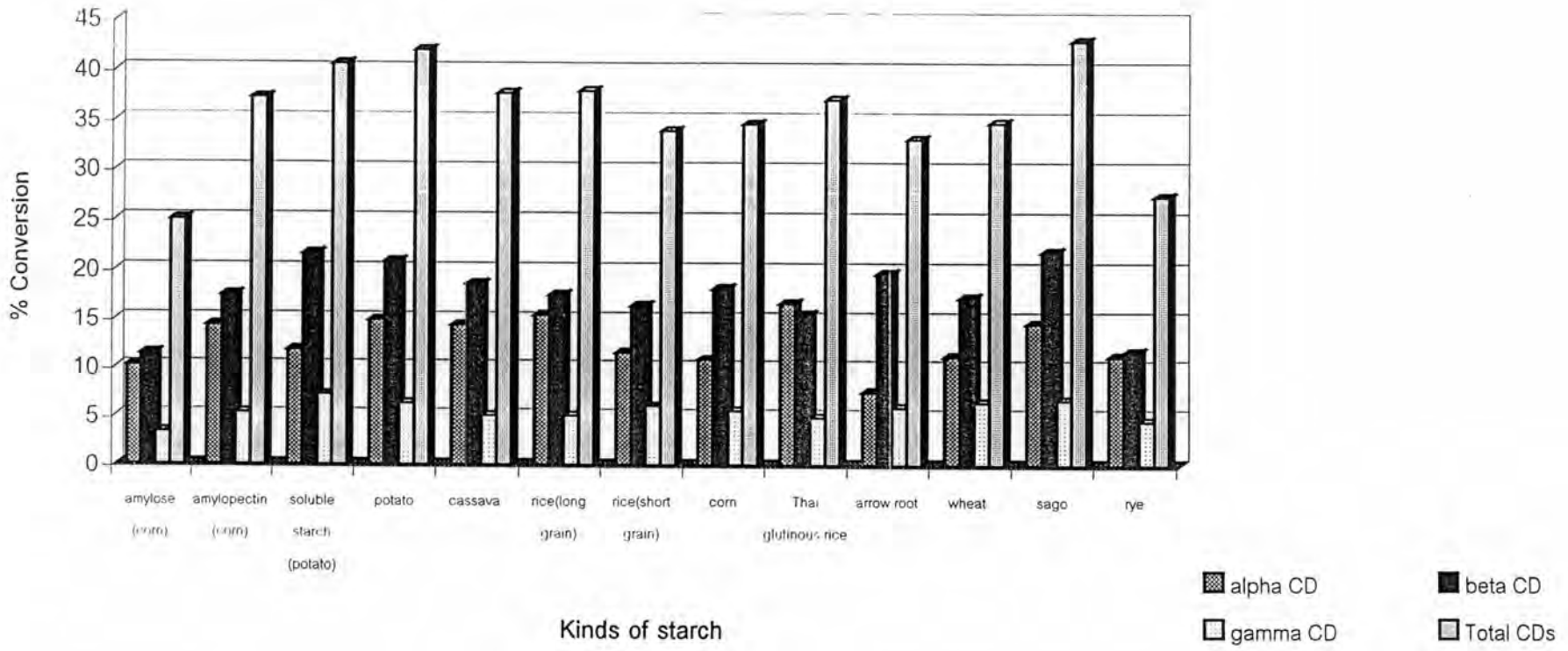


Figure 11 Production of cyclodextrins from various kinds of starch.

Two gram percent of starch was incubated with CGTase 500 U/g starch for 17 hr and cyclodextrins were determined by HPLC as described in Section 2.9.1 and 2.11.2.

3.3 Determination of amylose and amylopectin content

From our results on the production of cyclodextrins from different kinds of starch gave different yields because of the different amylose or amylopectin content, so amylose and amylopectin in starch were determined by colorimetric assay.

3.3.1 Absorption spectra

The characteristic absorption spectra of 20 μg/ml iodine solution, 8 μg/ml standard amylose solution, 32 μg/ml standard amylopectin solution and mixture of 8 μg/ml standand amylose solution plus 32 μg/ml standard amylopectin solution reacted with iodine were determined by diode - array spectrophotometer against a water blank (2.10.1.1). The absorption was shown in Figure 12. Iodine absorbs quite strongly up to 500 nm, and the amylose (λmax 602 nm) and amylopectin (λmax 535 nm) both exhibit broad overlapping spectral peaks. The six wavelengths (2.10.1.2) were chosen as follows: λ1= 526 nm, λ2 = 535 nm, λ3 = 564 nm, λ4 = 602 nm, λ5 = 700 nm, λ6 = 800 nm. Table 6 showed the absorptivity of amylose and amylopectin at the six chosen wavelengths using the formula in equations (1) and (2) as indicated in Section 2.10.1.2.

3.3.2 Amylose and amylopectin contents in starches

Starch samples were extracted with 1 - Propanol/ water (3:1, v/v, 250 ml) according to Section 2.10.2.1. Each sample was reacted with iodine and the absorbancy was measured at six wavelengths. The amylose concentration in starch samples was calculated from the equation as explained in Method (2.10.2.2) and the result was shown in Table 7. The amylopectin content was also reported. Amylose content in amylose starch from corn was highest of all starches whereas amylose content in amylopectin starch from corn and Thai glutanious rice were lowest. Amylose content in cassava starch was 16.6±0.31% lower than amylose content in potato and corn which usually use for cyclodextrin production. Preparation of commercial amylose and amylopectin (corn, Sigma) were used as standards. Of the various starches tested,

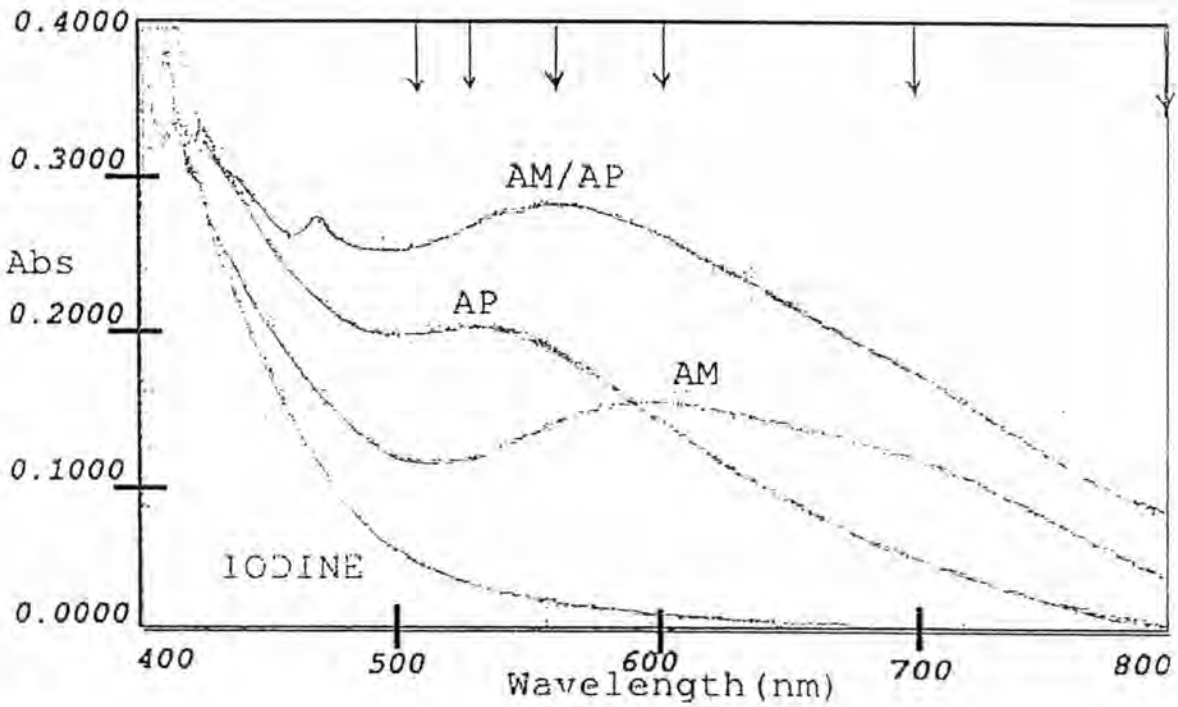


Figure 12 Visible light absorption spectra of 20 μl/ml iodine solution, 8μg/ml standard amylose solution (AM), 32 μg/ml standard amylopectin solution (AP) and the mixture of 8 μg/ml standard amylose solution plus 32 μg/ml standard amylopectin solution (AM+AP). Arrows indicate wavelengths used for multi-component analysis.

Table 6 Absorptivity of amylose and amylopectin

Wavelength	Absorptivity (ml/μg.cm)	
	Amylose	Amylopectin
λ1 526	0.011	0.006
λ2 535	0.0123	0.006
λ3 564	0.0161	0.0059
λ4 602	0.0188	0.0049
λ5 700	0.0153	0.0019
λ6 800	0.0048	0.0004

Table 7 Amylose and amylopectin contents in starch/flour

Kind of starch/flour	Amylose (%)	Amylopectin (%)*	%Conversion
Amylose (corn, practical grade , Sigma)**	48.88±0.77	51.12	25.01
Amylopectin (corn, practical grade, Sigma)**	8.47±0.41	91.53	37.24
Potato**	24.19±0.29	75.81	41.95
Sago**	20.31±0.37	79.69	42.87
Cassava**	16.6±0.31	83.4	37.67
Wheat***	28.34±0.33	71.66	34.71
Corn***	23.13±0.42	76.87	34.57
Arrow root***	21.00±0.32	79	33.01
Rice (long grain)***	18.0±0.6	82	37.91
Rye***	14.78±0.32	85.22	27.26
Rice (short grain)***	13.58±0.42	86.42	33.86
Thai glutinous rice***	8.35±0.78	91.65	37.03

* Approximate amylopectin content (%) was estimated from 100 - amylose (%)

** Starch

*** Flour

± = range , n = 4

starches from potato, corn, arrow root, wheat and sago showed highest amylose content (20-28%); rice (long and short grain), cassava starch, and rye had moderate amylose content (13-18%); whereas Thai glutinous rice had remarkably low amylose (8%) comparable to the standard amylose preparation.

3.4 Property of cassava starch

This thesis aims to study on the use of cassava starch for cyclodextrin production, therefore, some properties of cassava starch were also studied.

3.4.1 Starch morphology

Dried starch was sprinkled onto a double back Scotch tape attached to circular specimen stub and coated with gold. The samples were viewed and photographed using scanning electron microscopy. The protocol was described in 2.11.1. As shown in Figure 13a, the cassava starch granules were considerably irregular shapes consisting of spherical, cap–shaped and truncated egg shaped. The granular size of cassava starch was within the range of 6-17 μm. Figure 13 b-f showed starch gel treated with amylolytic enzyme and also showed difference in the surface granule.

3.4.2 Swelling power and solubility

As shown in Figure 14, The trend of swelling power and solubility were increased in parallel. At 90°C, the swelling power and %solubility were 63% and 55% respectively.

3.4.3 Pasting profile

The pasting characteristics of cassava starch can be determined by using Brabender Viscoamylograph. Pasting temperature of cassava starch was 68.6± 0.125 °C. Peak viscosity occurred at 79.2 ±0.2 °C and viscosity of cassava paste was 642.5±32.5 BU as shown in Table 8. The Brabender Viscoamylograph rheological profile was given in Figure 15.

Figure 13 Scanning electron micrographs of native cassava starch

(a) raw native cassava starch

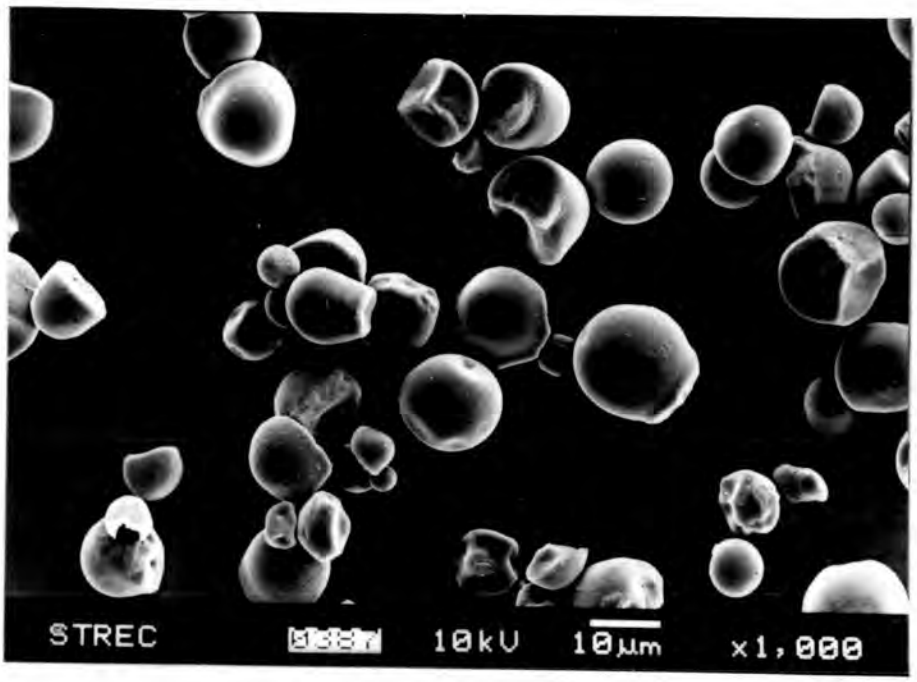
(b) cassava gel

(c) cassava gel treated with pullulanase (96 U/g starch) for 24 hours

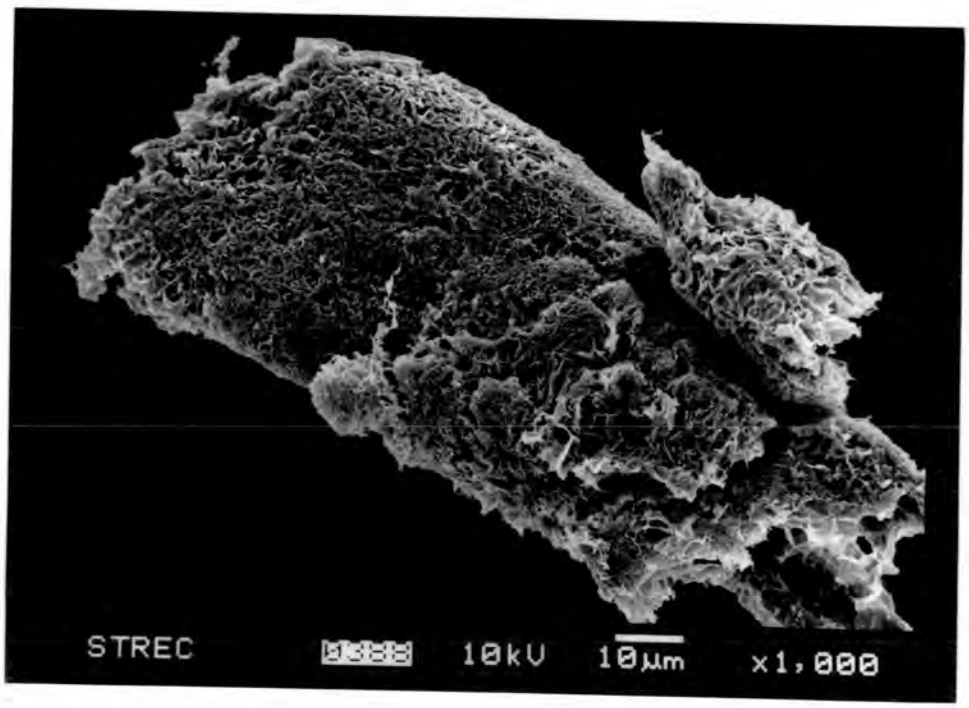
(d) cassava gel treated with α-amylase (0.0024 U/g starch) for 24 minutes

(e),(f) cassava gel treated with CGTase (500 U/g starch) for 10 hours

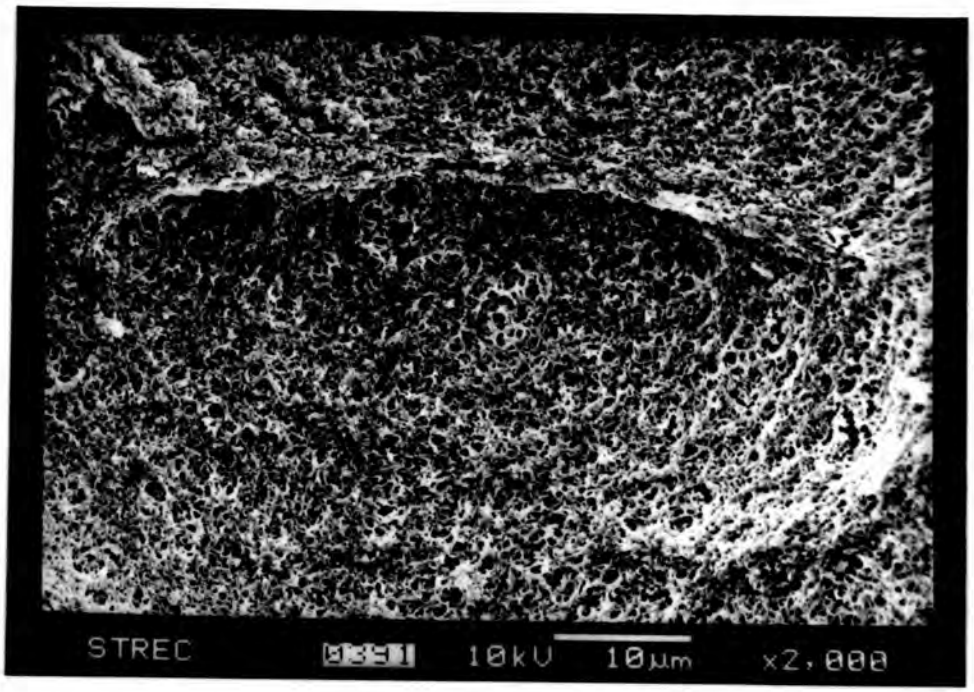
(a)



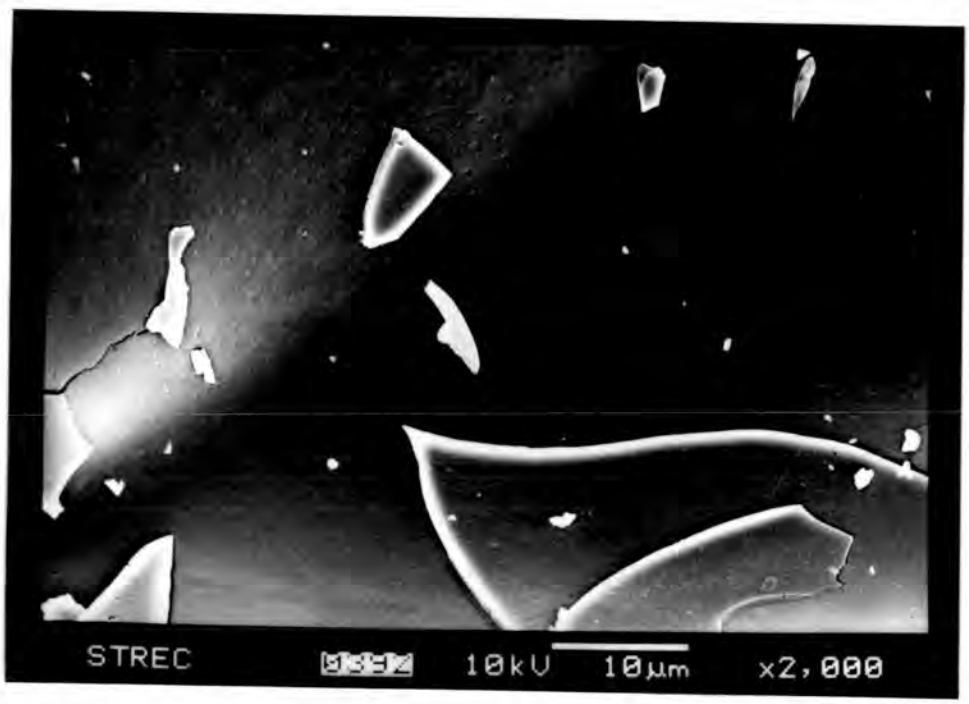
(b)



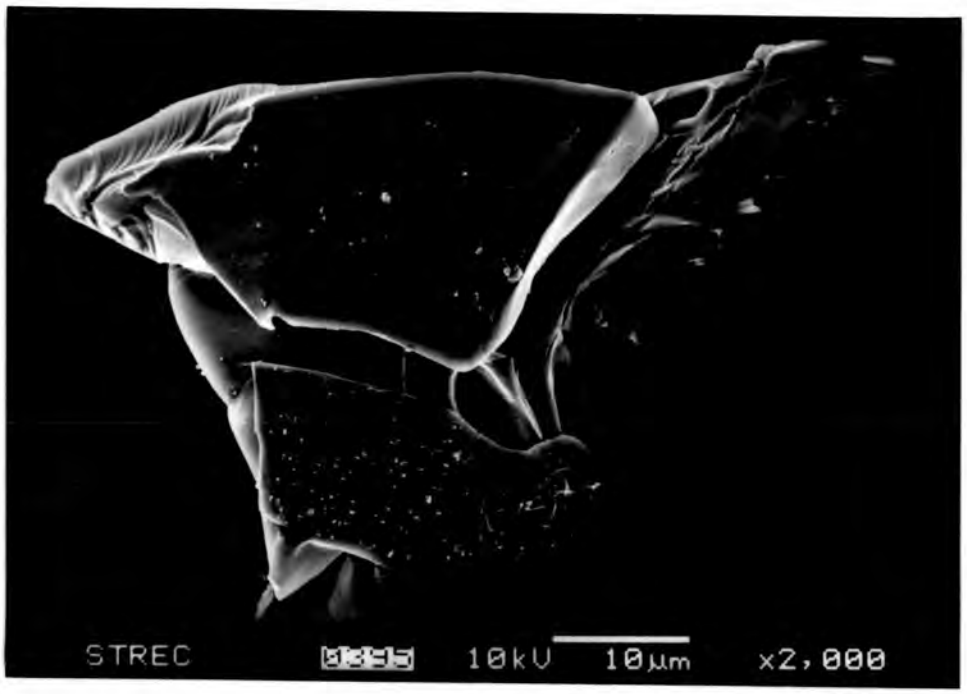
(c)



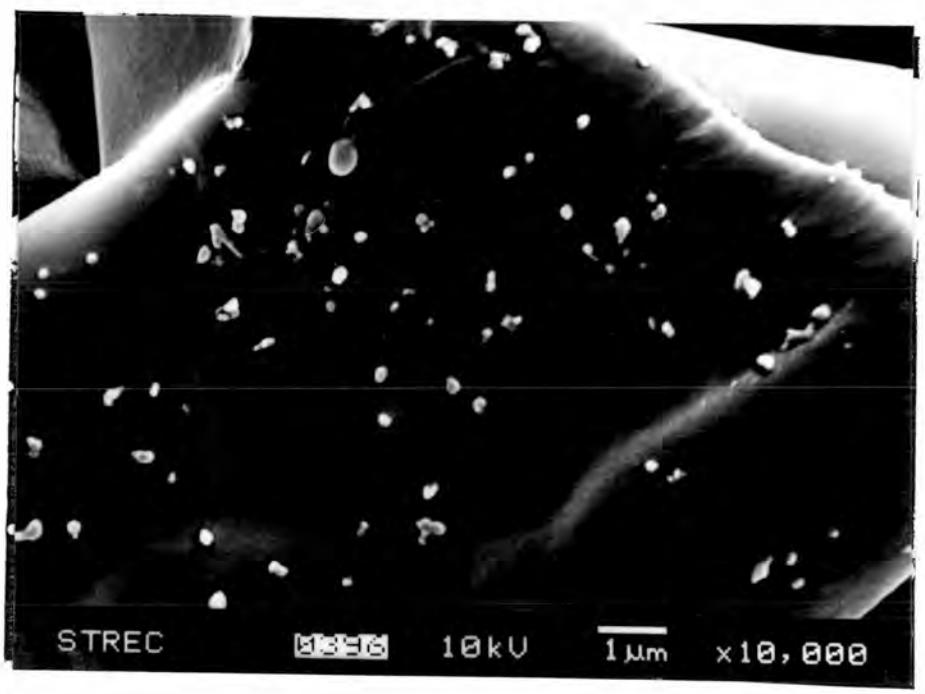
(d)



(e)



(f)



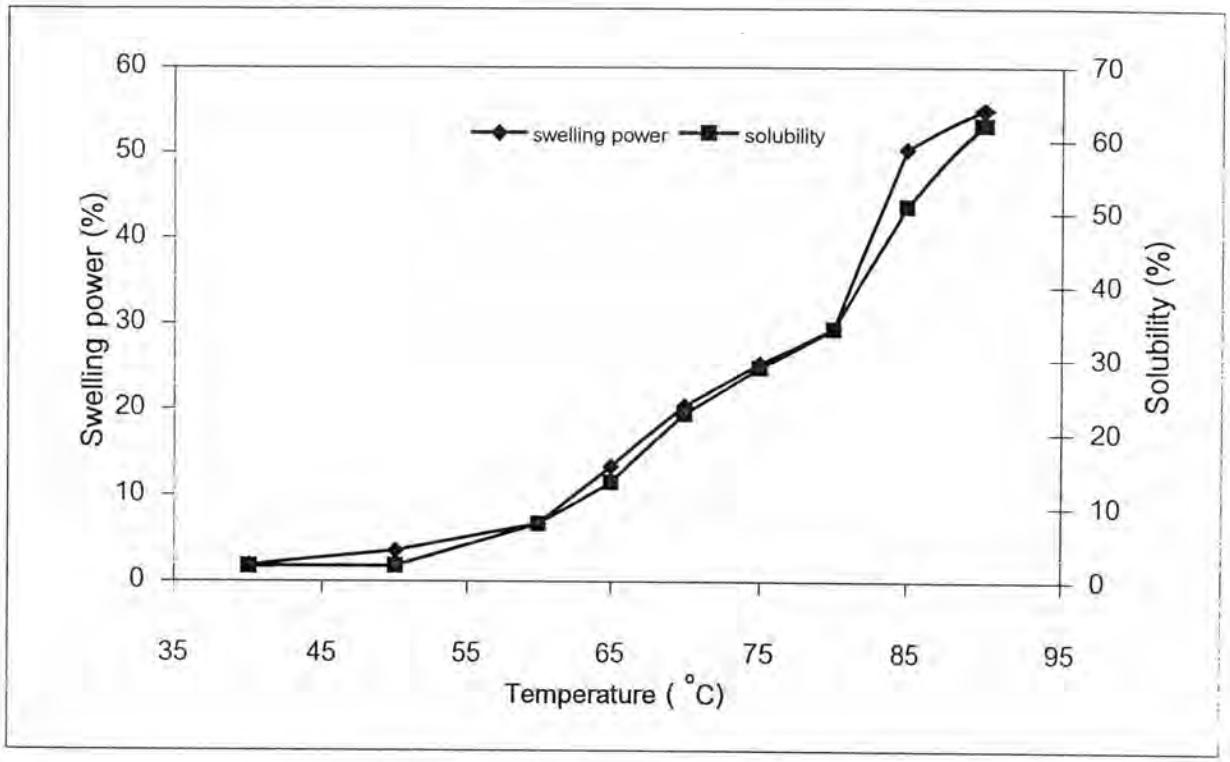


Figure 14 Swelling power and solubility profile of cassava starch.

The procedure was described in Section 2.11.3.

Table 8 Pasting properties of 6 g%(w/v) cassava starch in distilled water

	Torque (BU)	Temperature (°C)
Pasting temp	17.5±7.5	68.62±0.125
Peak viscosity	642.5±32.5	79.2±0.2
Trough	235±25	90
Final viscosity	415±25	50
Breakdown	405±7.07	93.5
Setback	185±5	50

BU ; Brabender Unit

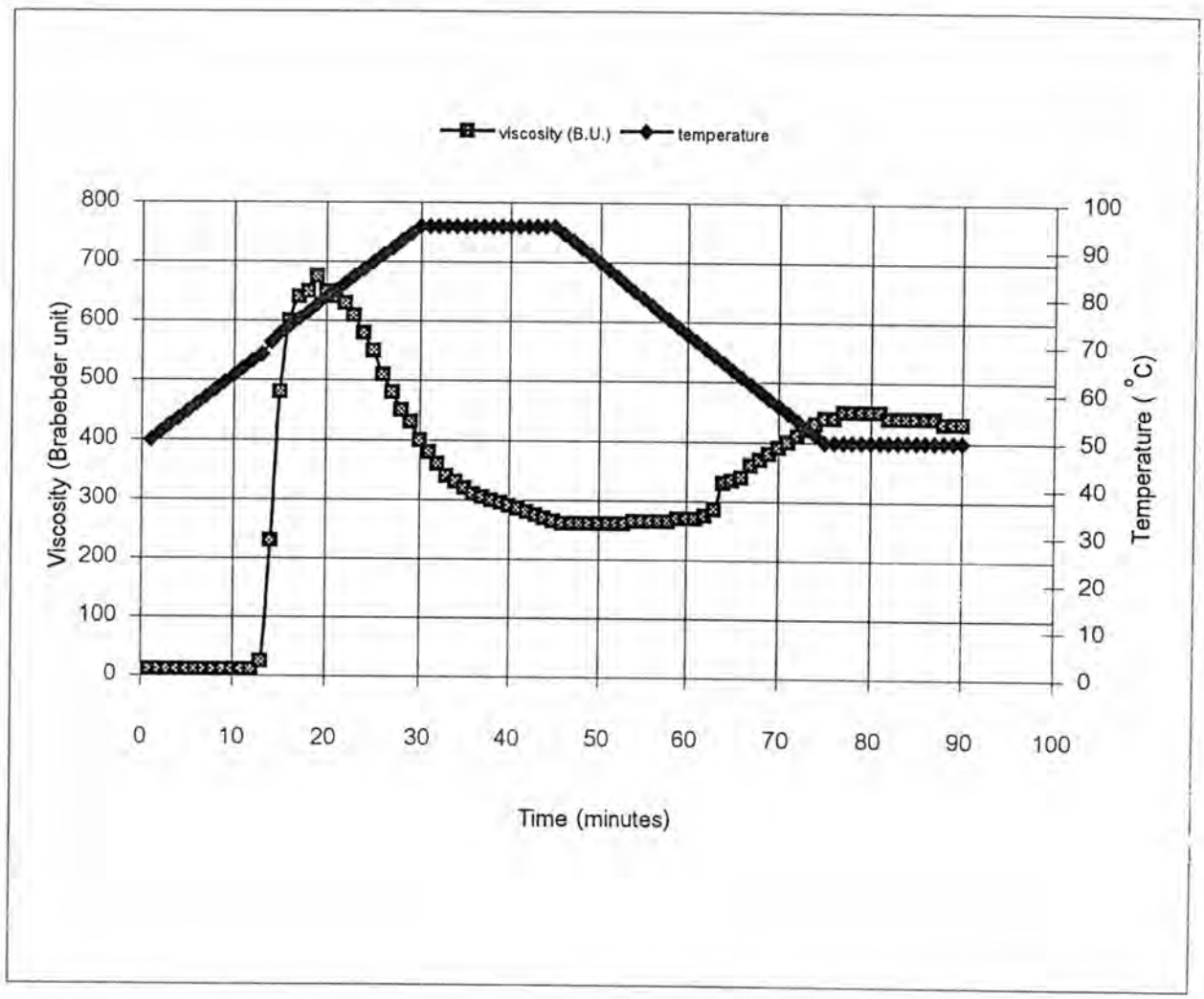


Figure 15 Pasting profile of 6 %(w/v) cassava in distilled water by Brabender Viscoamylograph.

The procedure was described in Section 2.11.3.

3.4.4 Linamarin contents

The linarnarin contents in starch were assayed by the method as described in Section 2.11.4 and expressed as mg/kg dry weight. Using a cyanide standard curve (Appendix C), linamarin contents was 0.05 mg / kg dry weight and is within the acceptable level (not exceed 10 mg/kg; CODEX standard 176-1995).

3.5 Production of cyclodextrins from cassava starch

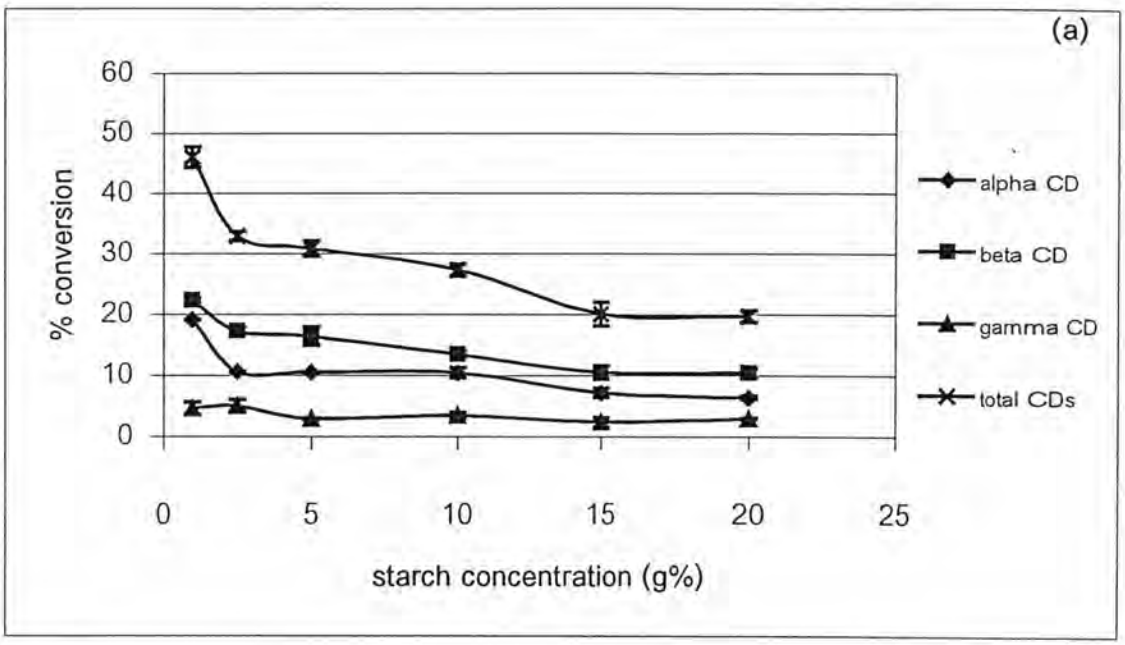
In order to obtain good conversion of cyclodextrin from cassava starch, its production conditions were optimized. All experiments were carried out pH 6.0.

3.5.1 Starch concentration

In this work, different amount of cassava starch, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 %(w/v), were used for cyclodextrin production. Cassava starch in varied concentration were gelatinized, and then gelatinized starch was incubated with partial purified CGTase (500 U/ g starch) for 17 hours and products were determined according to Section 2.9.1 and 2.9.2. The % conversion decreased from 45 to 20 percents with increasing concentrations of cassava starch from 1.0 to 20g% as shown in Figure 16. It should also be noticed that CGTase from *Bacillus circulans* was a β-CGTase but it also formed a relatively significant amount of α-CD (6-19%) in every starch concentration. The γ-CD was almost constant except for a little increased at 1-2.5 g%starch in every cassava starch concentration. The α-CD yield leveled off when the cassavas starch concentration increased from 5% to 20%. From this experiment, 2.5 g% of starch was selected for further production. With increasing cassava starch concentration, stirring of the gelatinized suspension will be more difficult and energy consuming.

3.5.2 Incubation temperature

After the concentration of cassava starch was chosen at 2.5g%, the incubating temperature was varied from 25-50 ^{o}C. Gelatinized starch (2.5g%) was incubated with CGTase at various temperature and the product were quantitated as



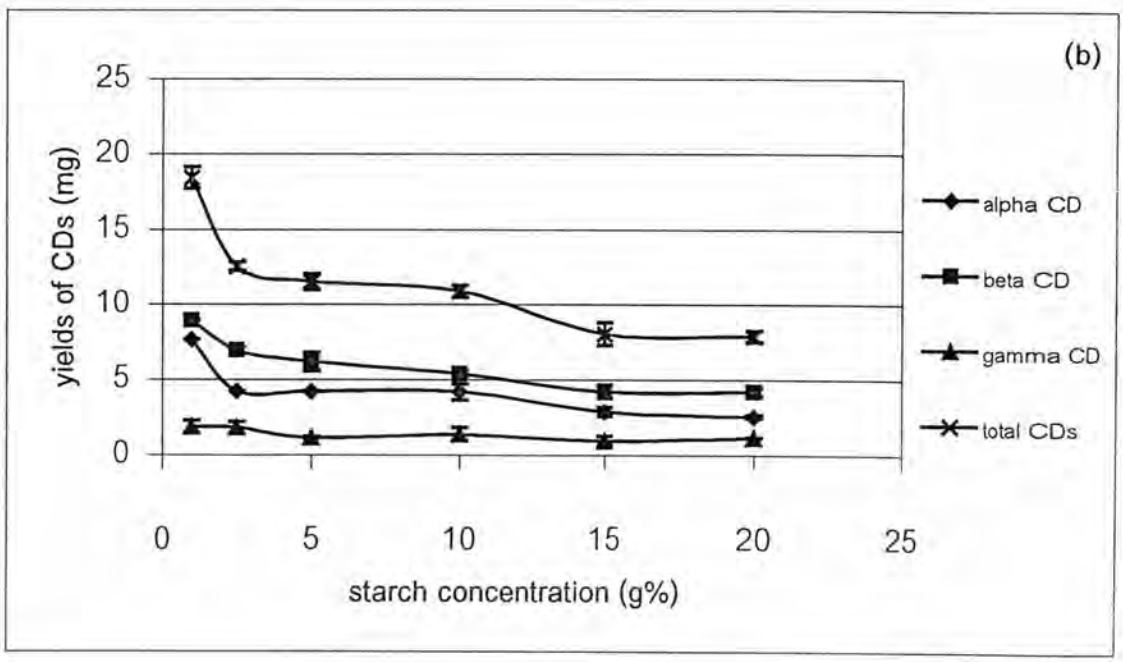


Figure 16 Effect of starch concentration on cyclodextrin production.

Cassava starch was incubated with CGTase 500 U/g starch for 17 hr. The procedures were described in section 2.9.1 and 2.9.2.

(a) % conversion

(b) yield in mg

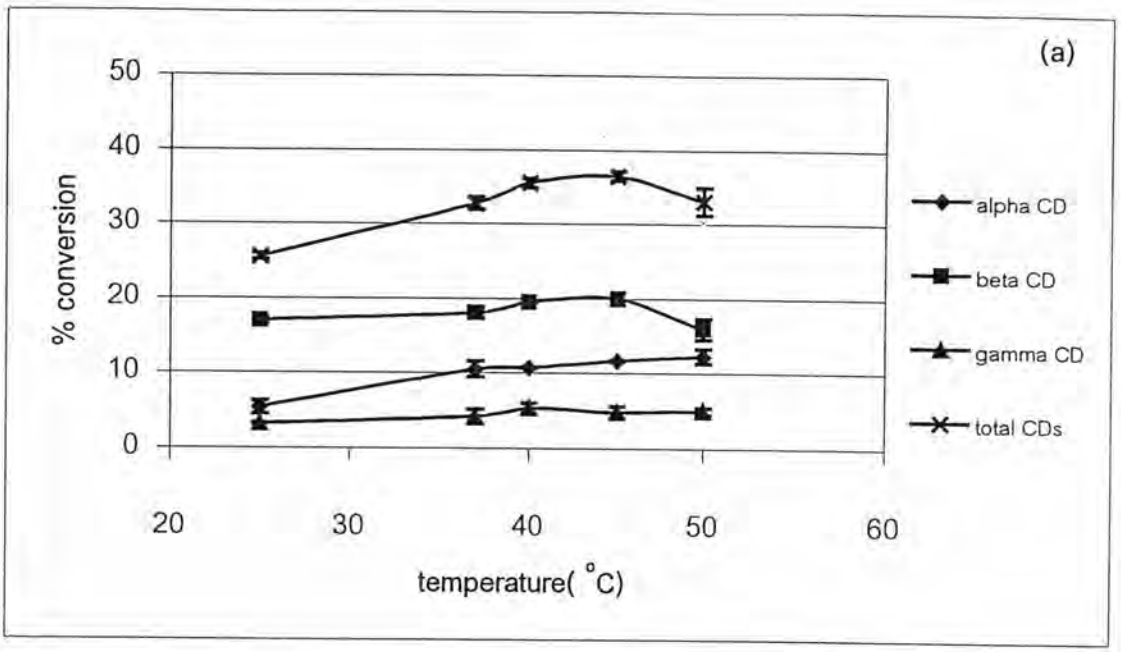
described in Section 2.9.1 and 2.9.2. As shown in Figure 17, total and individual cyclodextrins yields were maximum at temperature 40 -45 ^{o}C. Raising the temperature to 50^{o}C decreased the total and β-CD yields significantly. Hence the appropriate incubation temperature was 40^{o}C.

3.5.3 CGTase concentration

In order to determine the optimum ratio of enzyme to starch, the concentration of cassava starch and the incubation temperature were fixed at 2.5 g% and 40^{o}C respectively. The gelatinized cassava starch was incubated with vary amount of partial purified CGTase (25-2,000 U/g starch) for 17 hours. Cyclodextrin products were then assayed by HPLC technique (Section 2.9.1 and 2.9.2). Figure 18 demonstrated that 500 U/g starch CGTase gave maximum cyclodextrins production providing α: β: γ CD approximately 3:9:2. When increased the enzyme concentration, the ratio of α-, β-, and γ-CD was changed. At all concentration of CGTase, β-CD was the major products, peaking at 500 U/g starch. At low amount of CGTase (< 250 U/g starch) γ-CD was more than α-CD, whereas higher α: γ CD was observed at high amount of CGTase (1,000 - 2,000 U/g starch). The data also that at high CGTase (> 1,000 U/g starch), the amount of α- and β- produced are almost the same. Gamma cyclodextrin was low constant at all concentration tested. CGTase at 500 U/g starch was chosen for cyclodextrin production in consideration of total cyclodextrins and β-CD obtained.

3.5.4 Incubation time

In this work, the incubation time was varied from 1 to 48 hours using the above calibrated conditions. Figure 19 showed that total yield rapidly increased in the first 8 hours, then leveled off, while α, β and γ-CD yield were maximum at around 12,8 and 8 hours respectively. With prolonged incubation time, the ratio of α-, β- and γ-CD was changed from 1: 30: 16 at 1 hour to 1.3: 2.7: 1 at 24 hours.



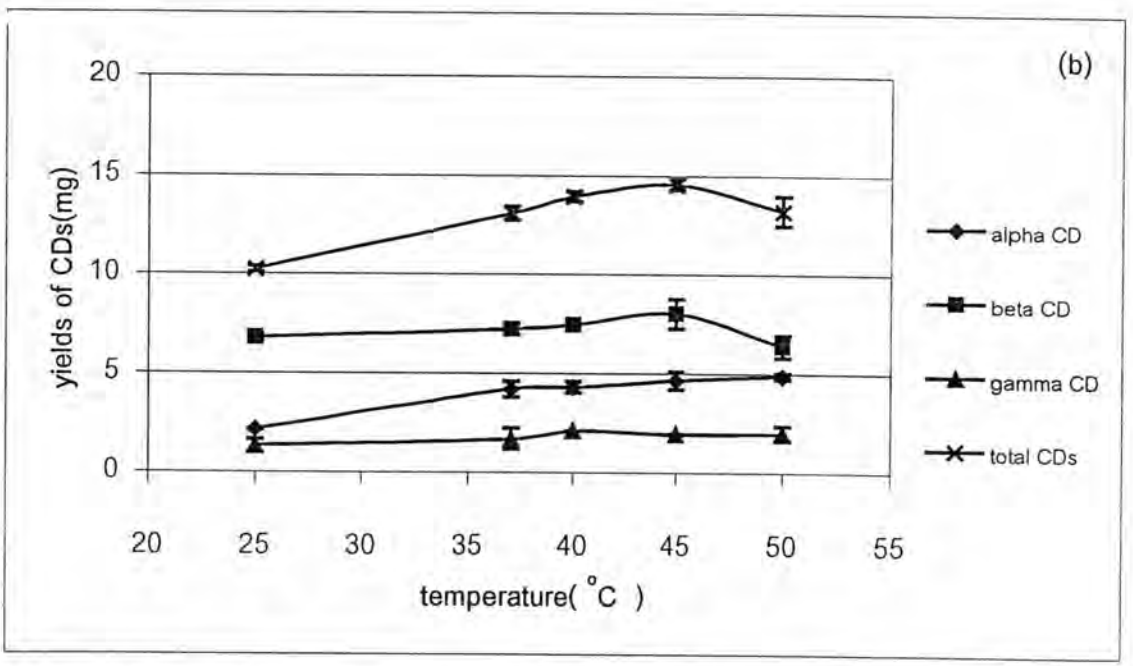
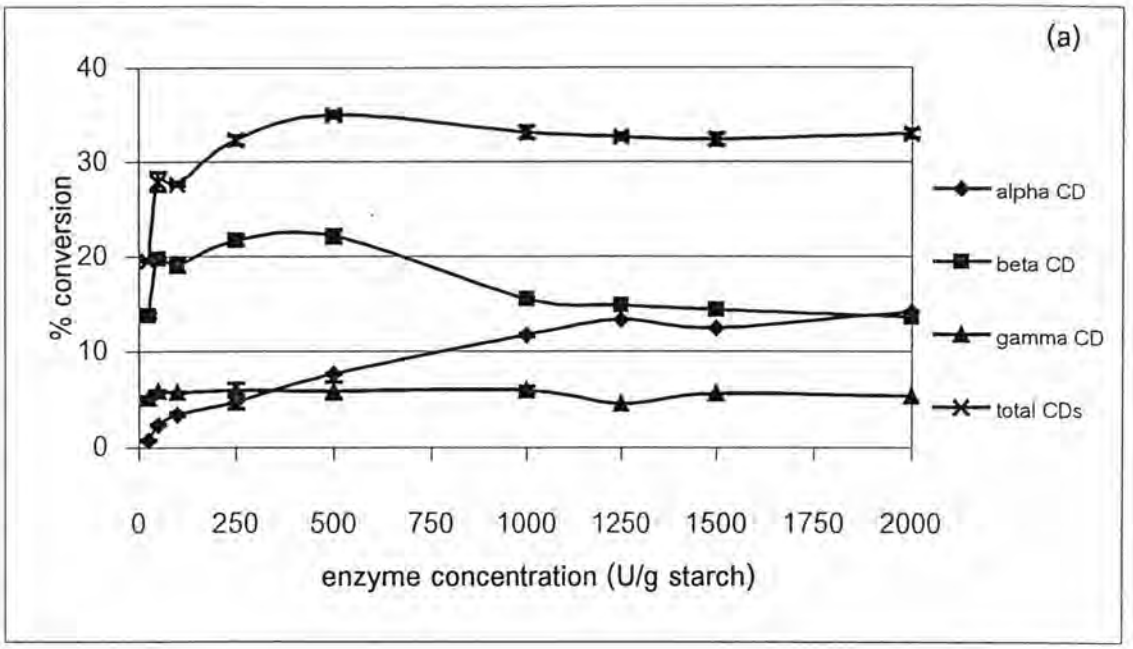


Figure 17 Effect of temperature on cyclodextrin production

Cassava starch (2.5 g%) was incubated with CGTase (500 U/ g starch) for 17 hr. The procedures were described in Section 2.9.1 and 2.9.2.

(a) % conversion

(b) yield in mg



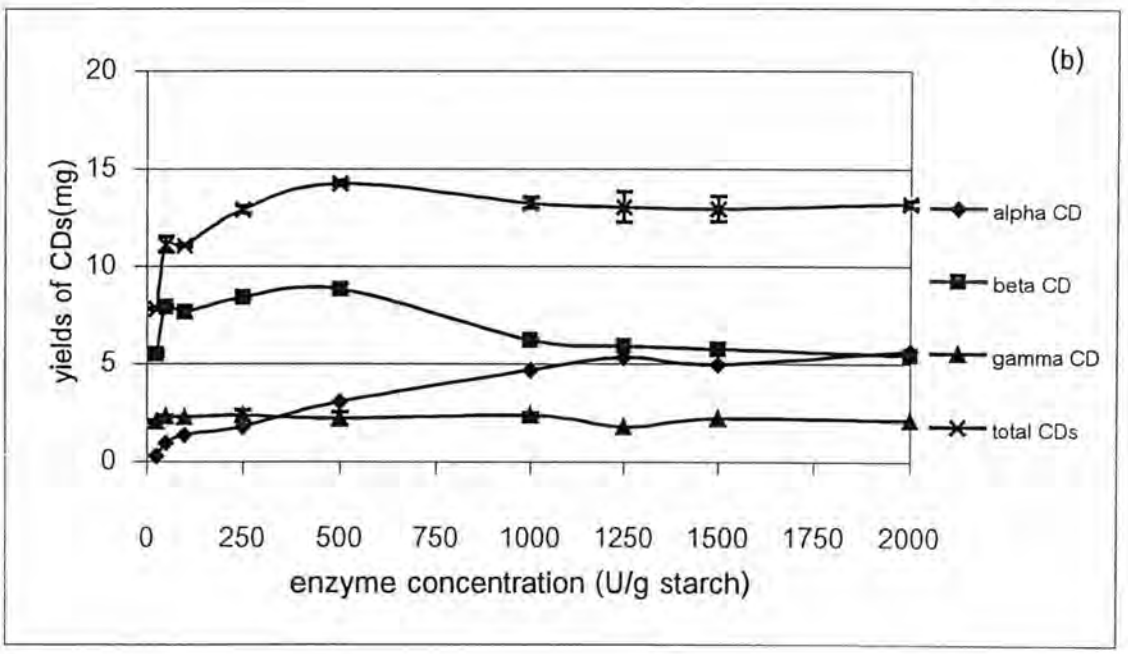
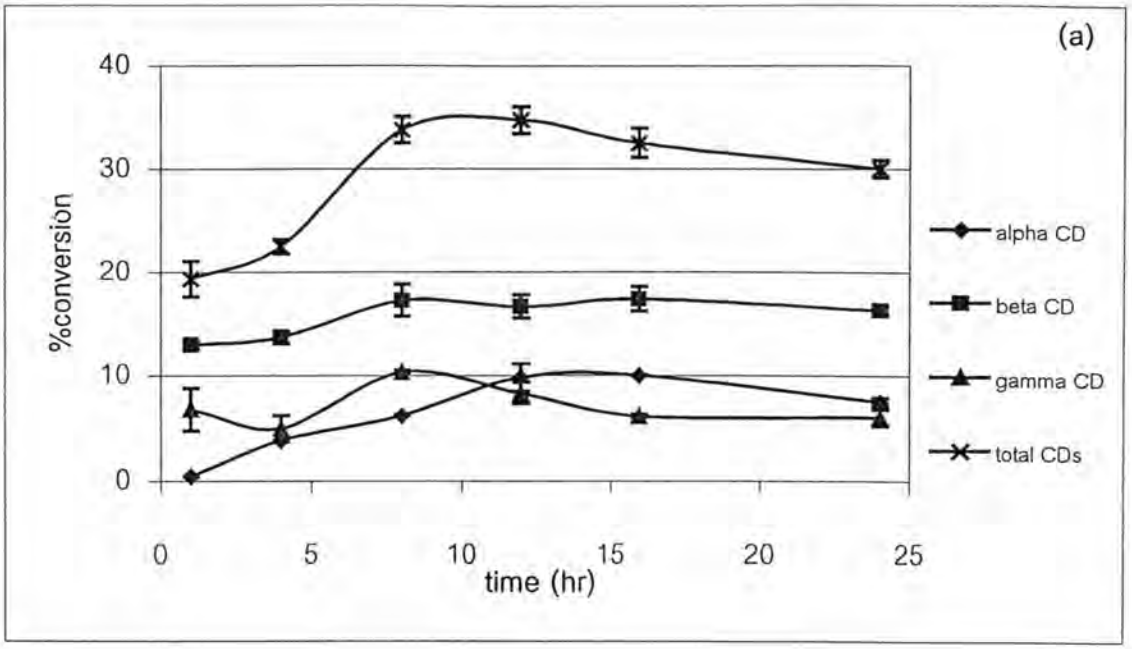


Figure 18 Effect of enzyme concentration on cyclodextrin production.

Cassava starch (2.5g%) was incubated with vary amounts of CGTase for 17 hr. The procedures were described in Section 2.9.1 and 2.9.2.

(a) % conversion

(b) yield in mg



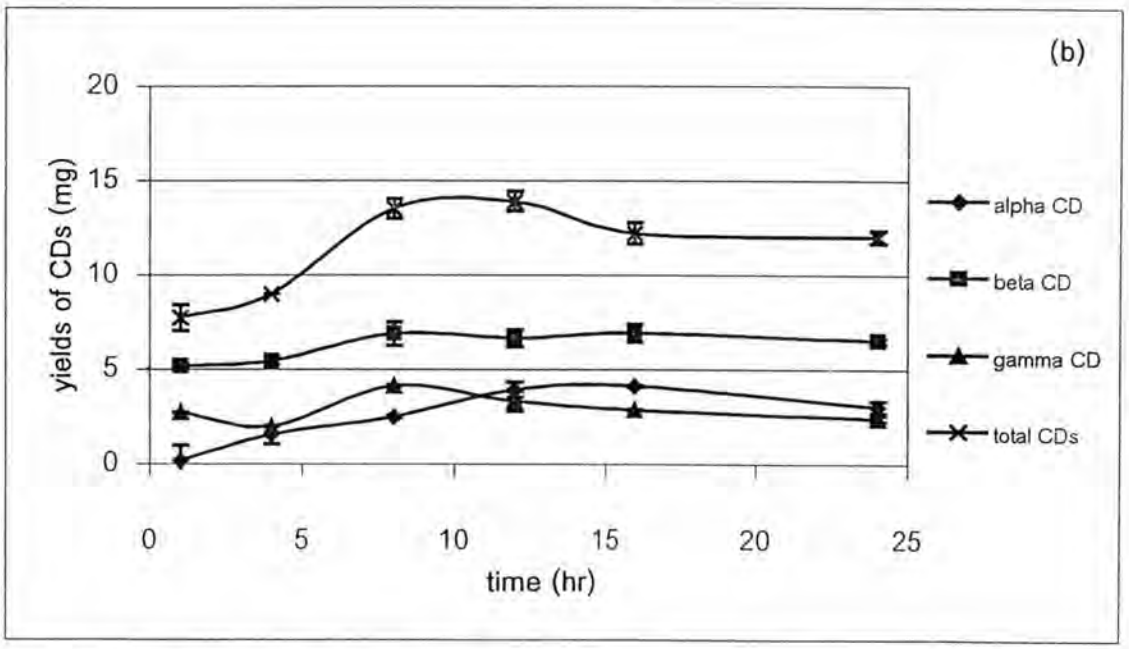


Figure 19 Effect of incubation time on cyclodextrin production.

Cassava starch (2.5g%) was incubated with CGTase (500 U/g starch) at 40 °C by varying incubation time 1-24 hr. The procedures were described in section 2.9.1 and 2.9.2.

(a) % conversion

(b) yield in mg

When CGTase 1,250 U/g starch was used (Figure 20), it was shown that the product yield rapidly increased in the first 16 hours, then decreased to a steady level after 24 hours. The yield of α-CD and β-CD was maximum at around 16 hours while γ-CD was maximum at around 8 hours. This result also showed that after 8 hours of incubation, the amount of α-CD was either higher or comparable to β-CD. With the long incubation time, the ratio of α-, β- and γ-CD was changed from 2: 3: 1 at 1 hour to 14: 12: 1 at 48 hours. The maximum ratio was 10.82: 12.75: 1 at 16 hours.

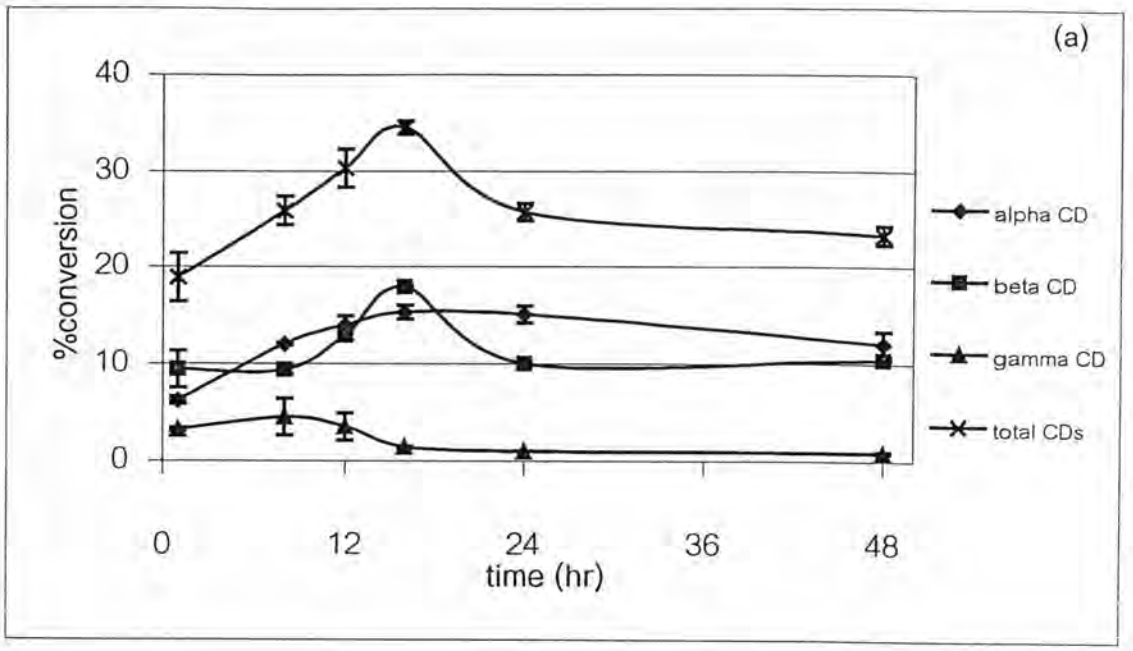
In both experiments, the maximum total % conversion was about 35%.

3.6 Pretreatment of cassava starch with enzymes

Cassava starch composed of amylose (16.6%) and amylopectin (83.4%) when using high concentration starch gave the high viscosity, CGTase was difficult to digest gelatinized starch. Therefore, the amylolytic enzyme such as α-amylase (BAN 240 L), pullulanase (Promozyme® 400 L), maltogenic α-amylase (Maltogenase™) and fungal α-amylase (Fungamyl®) were studied in order to digest cassava starch to the proper substrate for CGTase. Moreover, partial hydrolysis improved the solubility and lower the viscosity of solution. To study the treatment with enzyme, the DE values were determined. After that, starch hydrolysate was incubated with CGTase and determined the cyclodextrin yields from these conditions.

3.6.1 Treatment with α-amylase

In the experiment, 5g% cassava starch was treated with α-amylase (0.0024 - 0.096 U/g starch) and the dextrose equivalent was determined as described in Section 2.12.2.1. Thereafter, starch hydrolysate was incubated with CGTase 500 U/g starch at 40°C for 10 hours and the cyclodextrins were determined according to protocol in Section 2.9.1 and 2.9.2. As shown in Figure 21, the total production yield from cassava treated with α-amylase at 0.0024 and 0.0012 U/g starch (31.65 – 32.64%) was more than the total production yield from the untreated one (28.10%). When



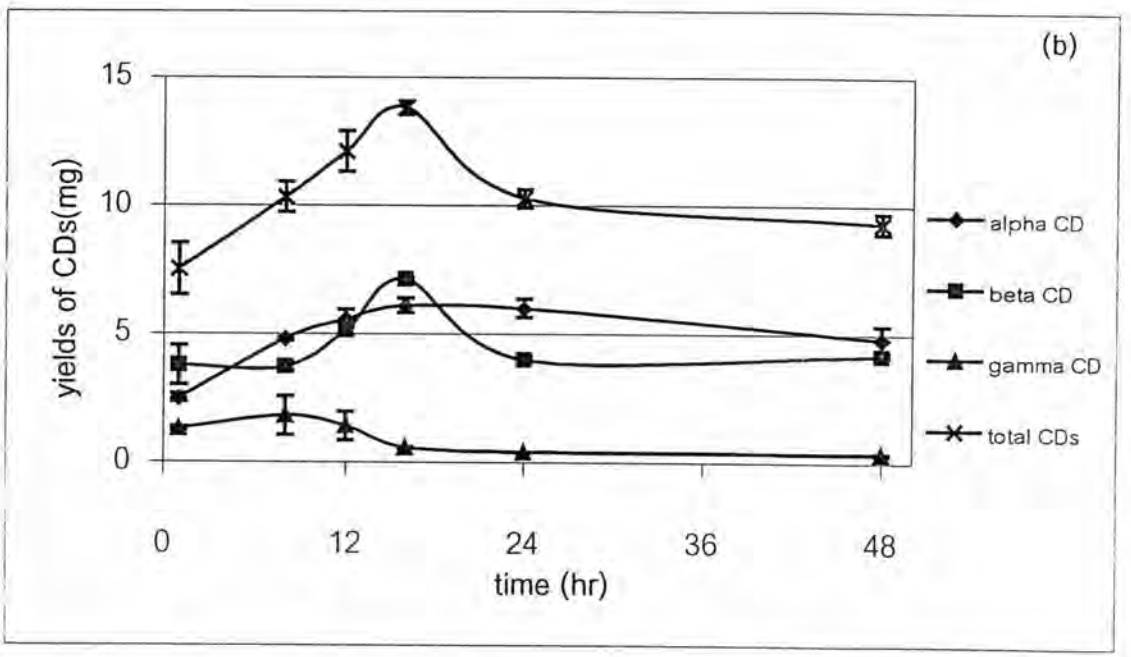


Figure 20 Effect of incubation time on cyclodextrin production.

Cassava starch (2.5g%) was incubated with CGTase (1,250 U/g starch) by varying incubation time 1-48 hr. The procedures were described in Section 2.9.1 and 2.9.2.

(a) % conversion

(b) yield in mg

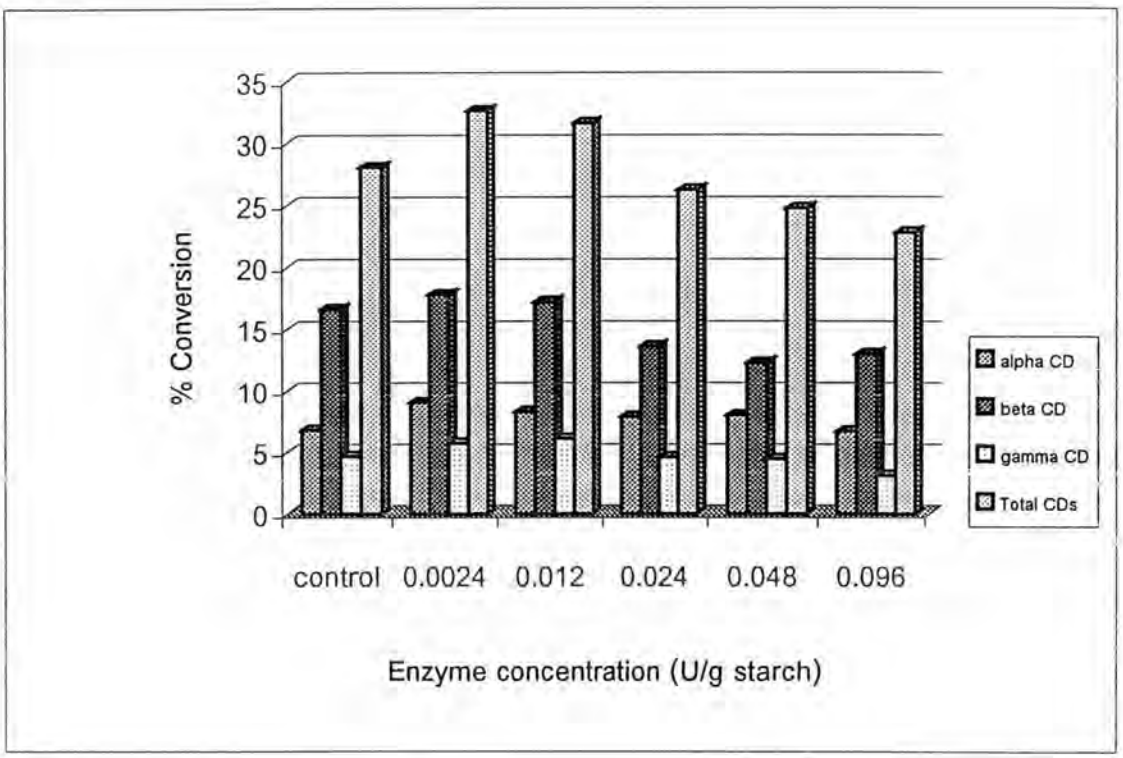


Figure 21 Effect of α-amylase concentration on cyclodextrin production.

5 g% cassava starch liquefied with α-amylase at various concentration. Thereafter, liquefied starch was incubated with CGTase 500 U/g starch for 10 hours. The procedures were described in Section 2.12.3.1 and 2.12.3.5.

the α-amylase concentration was increased beyond 0.024 U/g starch the total yield decreased from 31.64% to 22.8%. The appropriate α-amylase concentration was 0.0024 U/g starch. It was found that total cyclodextrins decreased when DE of dissolved starch increased, especially at DE > 6.21 as shown in Table 9. Thus, considering the kind of CD, the β-CD was produced mainly.

3.6.2 Treatment with pullulanase

In the experiment, 5 g% cassava starch was treated with pullulanase (24 - 96 U/g starch) and the dextrose equivalent were determined as described in Section 2.12.2.1. Thereafter, starch hydrolysate was incubated with CGTase 500 U/g starch at 40°C for 10 hours and the cyclodextrins were determined according to protocol in Section 2.9.1 and 2.9.2. As show in Figure 22 the total yield from cassava treatment with pullulanase at 48 and 96 U/g starch (32.09–37.00%) was more than total yield from non treated with pullulanase (28.10%). The yields increased 14.2-24.8% compared with non-treated with pullulanase. With the increased pullulanase concentration, total yield was increased from 28.16 – 37.00%.

3.6.3 Treatment with pullulanase and α-amylase

Five gram percent of cassava starch was incubated with pullulanase Promozyme® 400 L) with 96 U/g starch at 60°C for 24 hours. After inactivate enzyme by heating in boiling water for 10 minutes, α-amylase (BAN 240 L) was added at varied concentrations from 0.0024–0.096 U/g starch. After incubation with α-amylase, the dextrose equivalent was determined as described in the Section 2.12.2.1. Further, hydrolyzed starch was incubated with CGTase (500 U/g starch) at 40°C for 10 hours and cyclodextrins were determined according to protocol in Section 2.9.1 and 2.9.2. Figure 23 showed that total yield decreased from 38.7% at 0.0024 U/g starch to 30.1% at 0.096 U/g starch. The total yield from starch hydrolysate treated with only pullulanase (96 U/g strach) was comparable to the total yield from starch hydrolysate treated with

Table 9 Effect of enzyme treatment on the starch and total CD production

Types of enzyme	Enzyme (U/g starch)	DE average of treated starch	DP average of treated starch	Total CDs (mg)	% Conversion
Untreated (control)	0	-	-	11.24±0.006	28.10±0.02
α-Amylase (BAN 240 L)	0.0024	1.52±0.05	65.79	13.056±0.28	32.64±0.7
	0.012	6.12±0.2	16.34	12.66±0.08	31.65±0.2
	0.024	8.86±0.2	11.25	10.51±0.08	26.27±0.2
	0.048	15.25±0.14	6.56	9.9±0.55	24.76±1.38
	0.096	17.5±0.8	5.71	9.12±0.175	22.8±0.43
Pullulanase (Promozyme® 400 L)	24	-	-	11.26±0.36	28.16±0.04
	48	-	-	12.83±0.368	32.09±.92
	96	1.0±0.25	100	14.82±0.034	37.0±0.034
Pullulanase (96 U/g starch) + α-Amylase (BAN 240 L)	0.0024	5.27±0.35	18.97	15.48±0.012	38.7±0.03
	0.012	9.69±0.5	10.32	14.92±0.064	37.31±0.16
	0.024	11.85±0.7	8.44	15.23±0.28	39.57±0.7
	0.048	14.78±0.6	6.76	14.35±0.068	35.87±0.17
	0.096	19.65±0.4	5.09	12.04±0.51	30.1±1.27
Pullulanase (96 U/g starch) + Maltogenase™	3.2	44.57±0.68	2.24	4.49±0.15	11.22±0.37
	32	50.39±0.87	1.98	4.154±0.386	10.38±0.96
Pullulanase + Fungamyl®	3.0	47.12±0.75	2.12	4.38±0.23	10.96±0.58
	30	65.0±0.64	1.54	4.78±0.162	11.95±0.4

α-amylase (BAN 240 L) activity unit defined as U unit

Pullulanase (Promozyme® 400 L) activity unit defined as U unit

Maltogenase™ activity unit defined as U unit

Fungamyl® activity unit defined as U unit

- = not available

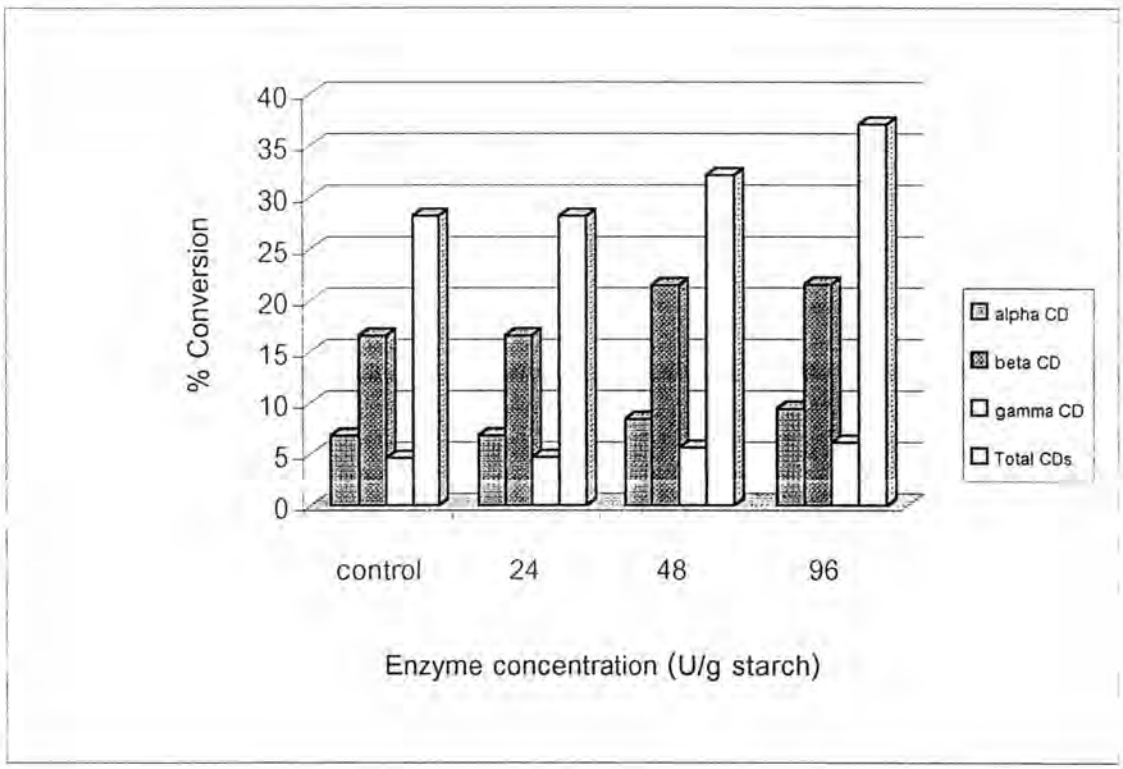


Figure 22 Effect of pullulanase treatment on cyclodextrin production.

5 g% cassava starch liquefied with pullulanase at various concentration. Thereafter, liquefied starch was incubated with CGTase 500 U/g starch for 10 hours. The procedures were described in Section 2.12.3.1 and 2.12.3.5.

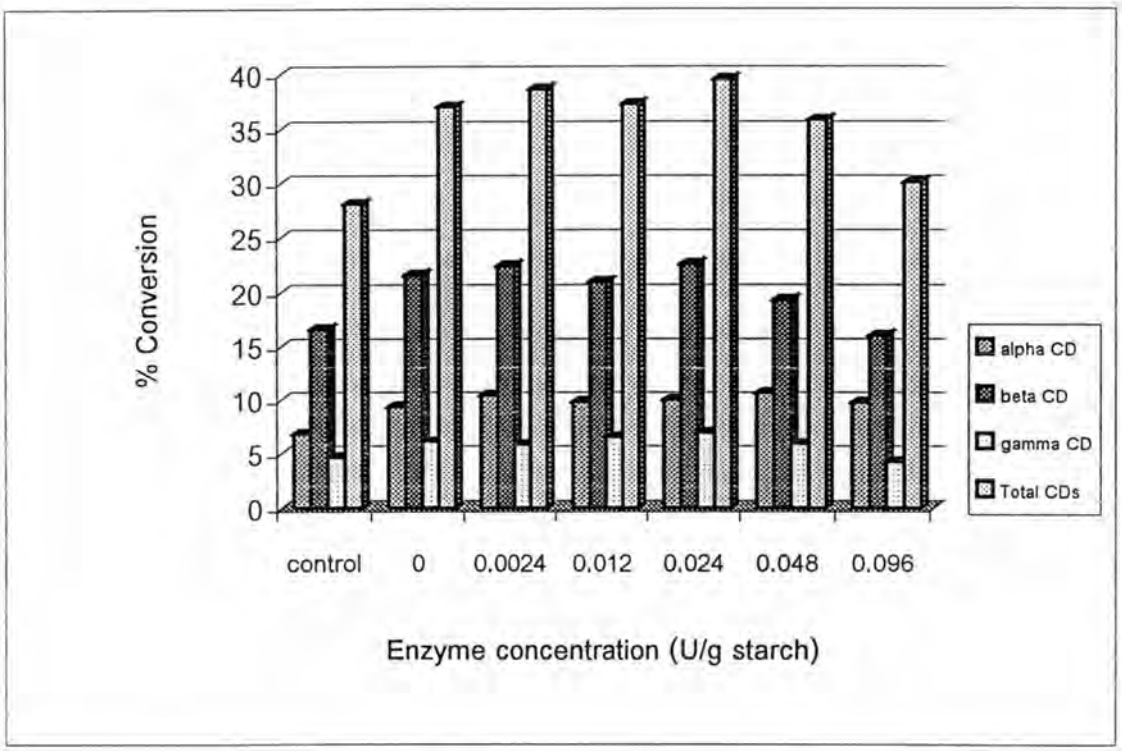


Figure 23 Effect of α-amylase and pullulanase treatment on cyclodextrin production

5 g% cassava starch was firstly digested by pullulanase (96 U/g starch) before subjecting to vary amount of α-amylase. Thereafter, liquefied starch was incubated with CGTase 500 U/g starch for 10 hours. The procedures were described in Section 2.12.3.1 and 2.12.3.5.

pullulanase and α-amylase (0.0024-0.0024 U/g starch or DE value of 5.27-11.85) whereas starch hydrolysate treated with pullulanase and α-amylase (0.048-0.096 U/g starch or DE values of 14.78-19.65) gave lower total yield than treated with only pullulanase (96 U/g starch). The DE value of hydrolyzed starch increased after increasing α-amylase as shown in Table 9. The β-CD dramatically decreased but α- and γ-CD leveled of when the α-amylase increased.

3.6.4 Treatment with pullulanase and maltogenase α-amylase or fungal α-amylase

Five gram percent of cassava starch was incubated with pullulanase in the procedure as described in Section 2.12.3.2. After inactivation of pullulanase, varying amount of maltogenase α-amylase (3.2–32 U/g starch) or fungal α-amylase (3-30 U/g starch) were used. The dextrose equivalent was determined as described in the Section 2.12.2.1. Thereafter, starch hydrolysate was incubated with CGTase (500 U/g starch) at 40°C for 10 hours. Cyclodextrins were determined by HPLC as described in Section 2.9.1 and 2.9.2. Figure 24,25 showed that the total yields of all treatment with pullulanase and maltogenase α-amylase or fungal α-amylase were 11%, therefore these total yields were lower than the total yields from all treatment with α-amylase or pullulanase and α-amylase. From these results, the total yields decreased 60.8% compared with control. The DE values of starch hydrolysate treated with pullulanase and maltogenase α-amylase or fungal α-amylase were 44.57-65 as shown in Table 9. The α-CD was the main products all of treatment.

From cassava starch treated with pullulanase and α-amylase, the total yields of all treatments were higher than the total yields of the treatment with α-amylase and with pullulanase and maltogeic α-amylase or fungal α-amylase. The average DE values of average DP values starch hydrolysate from treatment with α-amylase ware 1.5-6.16 and 16-66 respectively, gave more total yield than control. In addition, the average DE values and DP values of starch hydrolysate from treatments with pullulanase were 5.27-14.78 and 6-19 respectively, gave more total yield than control. However, starch hydrolysates treated with amylolytic enzyme ware mixed in various chain lengths of polymers.

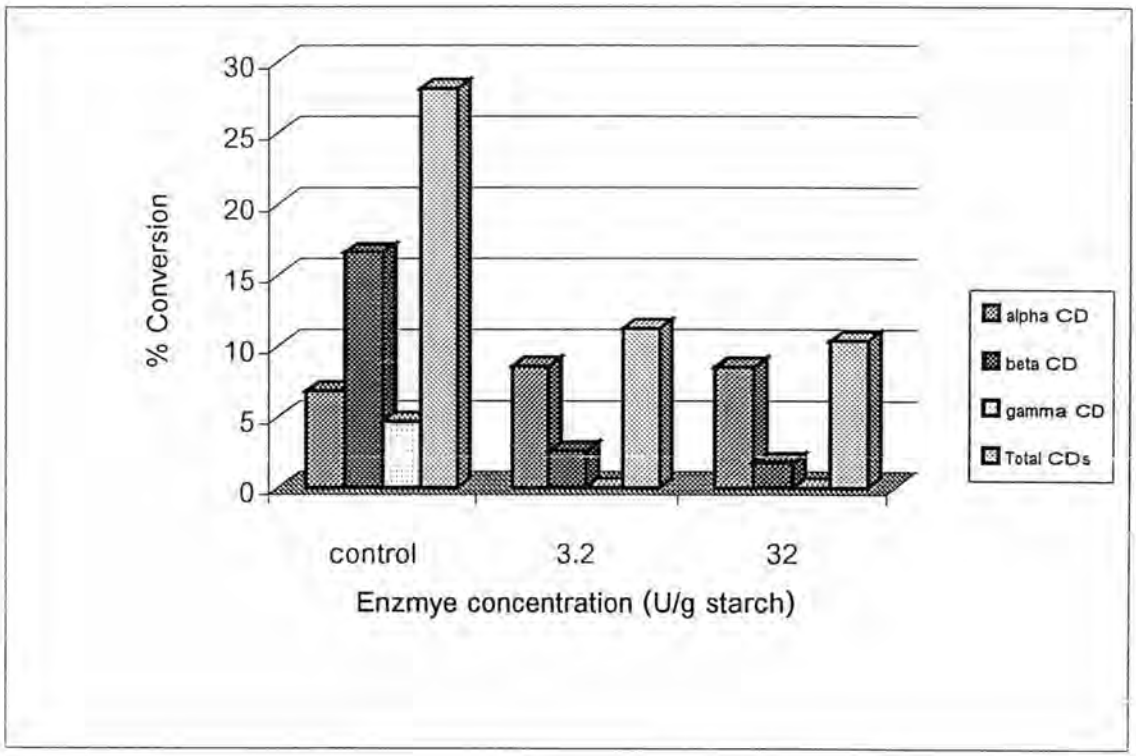


Figure 24 Effect of maltogenic α-amylase and pullulanase treatment on cyclodextrin production

5 g% cassava starch was firstly digested by pullulanase (96 U/g starch) before subjecting to vary amount of maltogenic α-amylase. Thereafter, liquefied starch was incubated with CGTase 500 U/g starch for 10 hours. The procedures were described in Section 2.12.3.1 and 2.12.3.5.

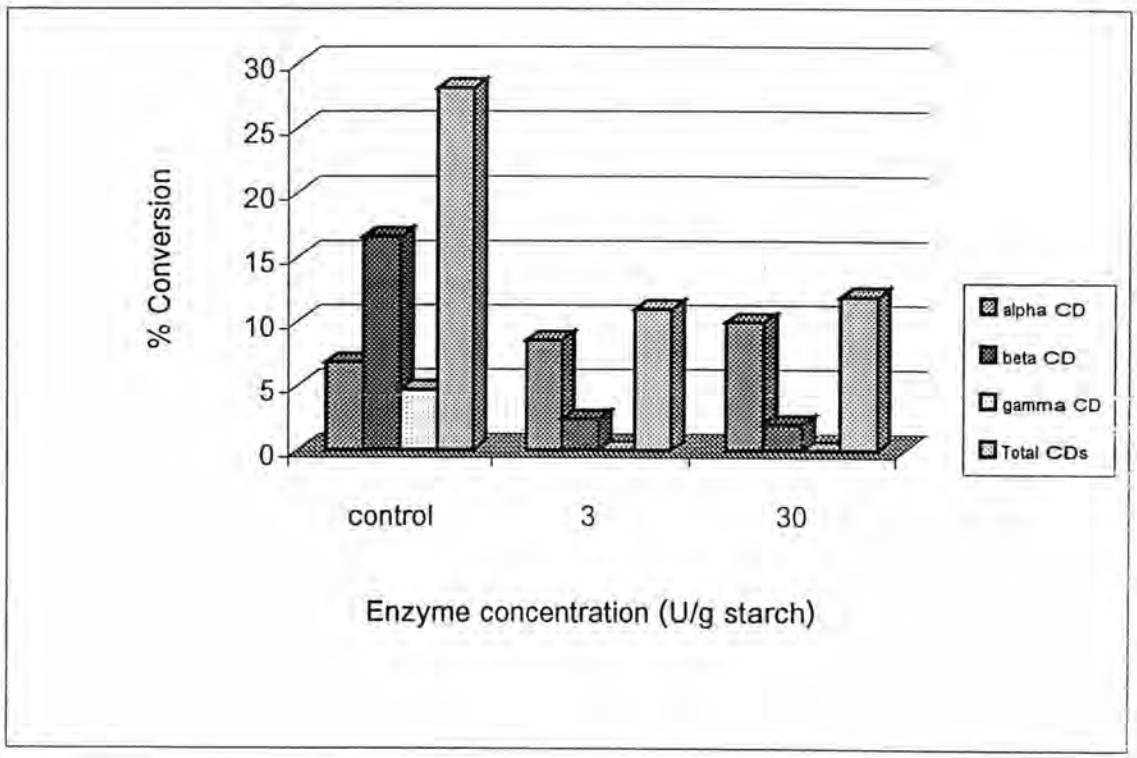


Figure 25 Effect of fungal α-amylase and pullulanase treatment on cyclodextrin production.

5 g% cassava starch was firstly digested by pullulanase (96 U/ g starch) before subjecting to vary amounts of fungal α-amylase. Thereafter, liquefied starch was incubated with CGTase 500 U/g starch for 10 hours. The procedures were described in Section 2.12.3.1 and 2.12.3.5.

3.7 Production of cyclodextrins from starch hydrolysate

From our studies, the yields were enhanced by treatment with α-amylase and pullulanase. By treatment with enzyme, the chain lengths of substrate were mixed and were also difficult to identify the chain length of substrate. There are reports that long chain substrate containing 16-80 glucoses were suitable for cyclization. On the other hand, the long chain more than 100 glucoses were not suitable for cyclization. Thus in this work, starch hydrolysate were fractionated and production of cyclodextrin from fractions were studied.

3.7.1 Production of cyclodextrins from cassava starch treated with α-amylase

Cassava starch was treated with α- amylase (BAN 240 L) as described in Section 2.13.2. The hydrolyzed starch was fractionated by Biogel P-10 chromatography according to Section 2.13.3.2. In the experiment, carbohydrate profile was followed and every other 5 fractions were pooled for determination of DP and cyclodextrin production. Figure 26 showed that with α- amylase treated cassava starch could be fractionated in 1 large peak with average DP values of 4-9. Higher polymers were also observed (average DP 194, 109, 82, 56, 34 and 21). Pooled fractions of each DP value were incubated with CGTae (condition in Section 2.12.1). The results in Figure 27 demonstrated that oligosaccharides of DP 9 gave highest yield and β-CD was the major product. It was surprising that oligosaccharides of DP 4 favored mainly γ-CD product and β-CD was very scare in the contrast to other DP starch. The longer chain oligosaccharides (DP>20) was converted only to α-CD and β-CD. It should be noted here that production from DP of 34-82 favored more α-CD than β-CD, while DP of 109 and 194 which were comparable amount of α-and β-CD.

3.7.2 Production of cyclodextrins from cassava starch treated with pullulanase

Cassava starch was treated with pullulanase (Promozyme®), as described in Section 2.13.2. The hydrolyzed starch was fractionated by Biogel P-10 chromatography according to Section 3.13.3.2. Carbohydrate profile was monitored and desired fractions were pooled for DP analysis and cyclodextrin producttion.

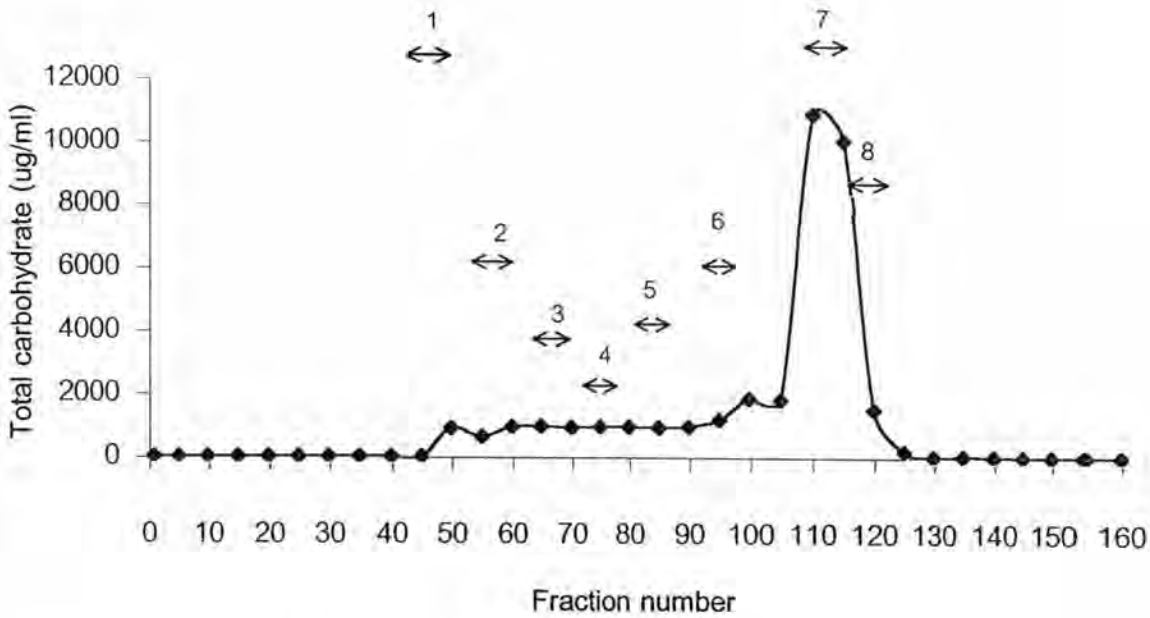


Figure 26 Fractionation of cassava hydrolysate by Biogel P-10 chromatography

Alpha-amylase treated starch was loaded onto Biogel P-10 column (1.9x85 cm) and eluted with 10 mM phosphate buffer at the flow rate of 10 ml/hr. Carbohydrate profile was monitored by phenol-sulfuric method. The DP values were determined as described in Section 2.12.4.

(1) F 48-53 = DP 194, (2) F 58-64 = DP 109,
(3) F 68-72 = DP 82, (4) F 78-82 = DP 55,
(5) F 88-92 = DP 34, (6) F 100-105 = DP 20,
(7) F 110-115 = DP 9, (8) F 120-125 = DP 4

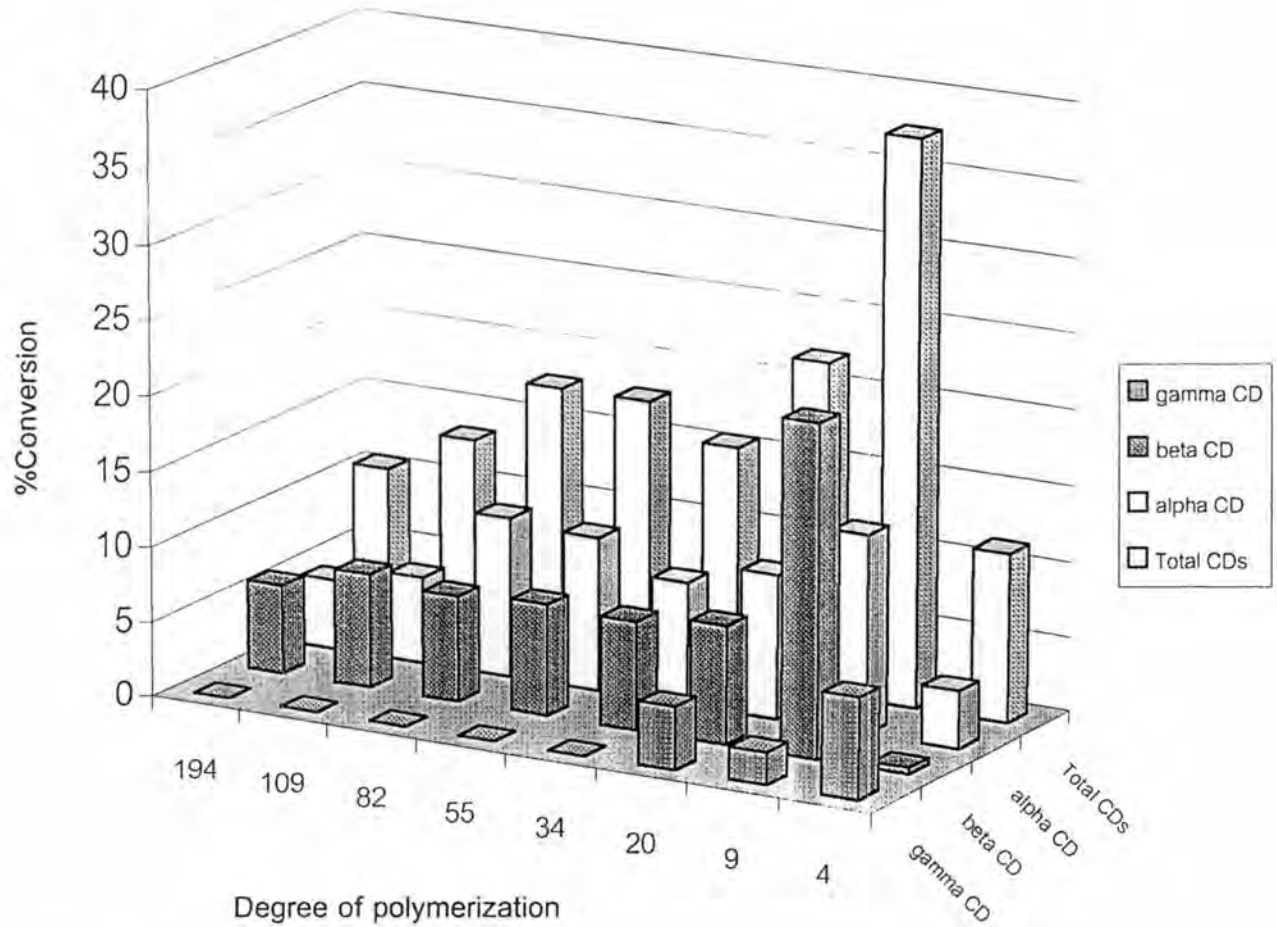


Figure 27 Production of cyclodextrins from alpha-amylase treated starch hydrolysate.

0.2 g% of cassava treated with alpha-amylase was incubated with CGTase (500 U/ starch) for 10 hr. at 40 °C, pH 6.0

Figure 28 showed that cassava starch treated with pullulanase could be fractionated into major fractions with average degree of polymerization of id (unable to determined), 75, and 26. When each fractions were incubated with CGTase (condition in Section 2.12.1), the results in Figure 29 demonstrated that hydrolysate of DP 26 gave highest yield and β-CD was the major product. Hydrolysate of DP 75 and id were produced only α-CD and β-CD. Very large polymers (id) were not suitable for cyclodextrin productrion.

From the above studies, the production of cyclodextrins was further performed by using polymer of DP 75, 26 from cassava starch treated with pullulanase and oligosaccharides of DP 9 from cassava starch treated with α-amylase as substrate.

3.7.3 Optimization of cyclodextrin production from starch hydrolysate

To investigate cyclodextrin production from short, medium and long chain polymers fraction with average DP 9, 26 and 75 were used.

3.7.3.1 Production of cyclodextrin from starch hydrolysate (DP 9)

Fractions from Biogel P-10 desired DP 9 were incubated with varying amount of CGTase (50, 500 and 1,500 U/g starch) at 40°C for 10 hours and varying incubation time (10 and 24 hours). The results in Figure 30a demonstrated that total and β-CD were highest when the amount of enzyme were 500 U/g starch. At 1,500 U/g starch, the amounts of α-CD were equal to β-CD whereas γ-CD was not produced. The lowest yield was incubated with CGTase 50 U/g starch. Prolong incubation time did significantly decreased the yield (Figure 30b).

3.7.3.2 Production of cyclodextrin from starch hydrolysate (DP 26)

Fractions from Biogel P-10 desired DP 26 were incubated with varying amount of CGTase (50, 500 and 1,500 U/g starch) at 40°C for 10 hours and varying incubation time (10 and 24 hours). The result in Figure 31a showed that total and β-CD were highest when the amount of enzyme was 500 U/g starch. To produce more α- and γ-CD, 1500 units per gram starch should be used at the sacrifice of β-CD. Decreasing

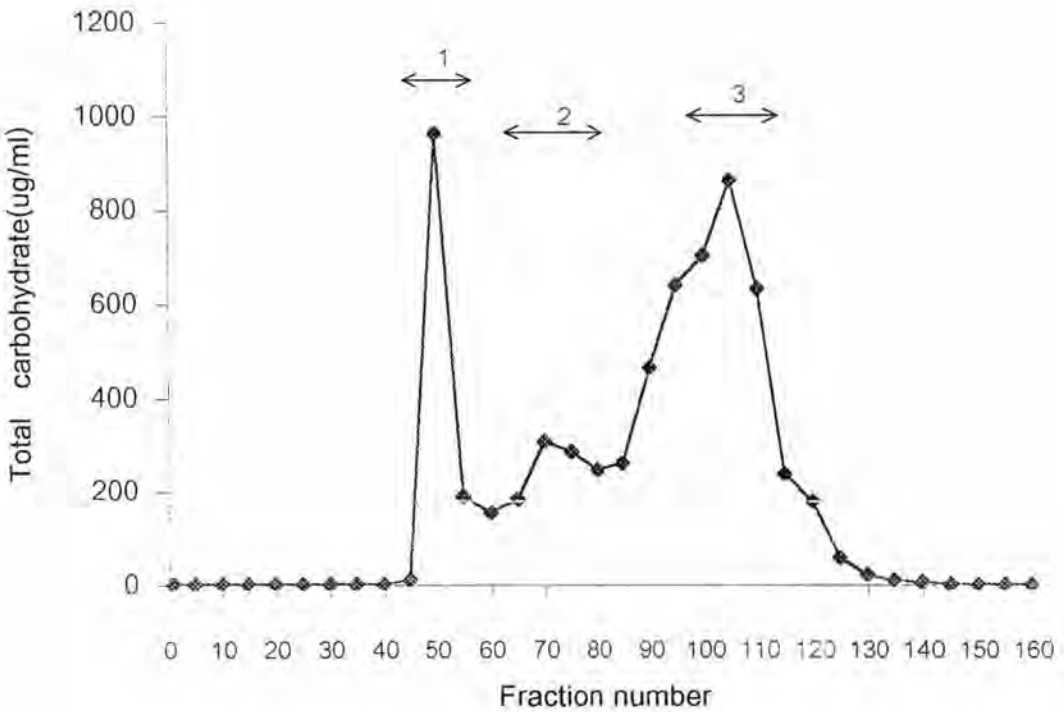


Figure 28 Fractionation of cassava hydrolysate with Biogel P-10 chromatography

Pullulanase treated starch was loaded onto Biogel P-10 column (1.9x85 cm) and eluted with 10 mM phosphate buffer, pH 6.0 at flow rate of 15 ml/hr. Carbohydrate profile was monitored by phonol-sulfuric method. The DP values were determined as described in Section 2.12.4.

(1) F 45-60 = ID (unable to determined),

(2) F 65-802 = DP 75,

(3) F 95-110 = DP 26

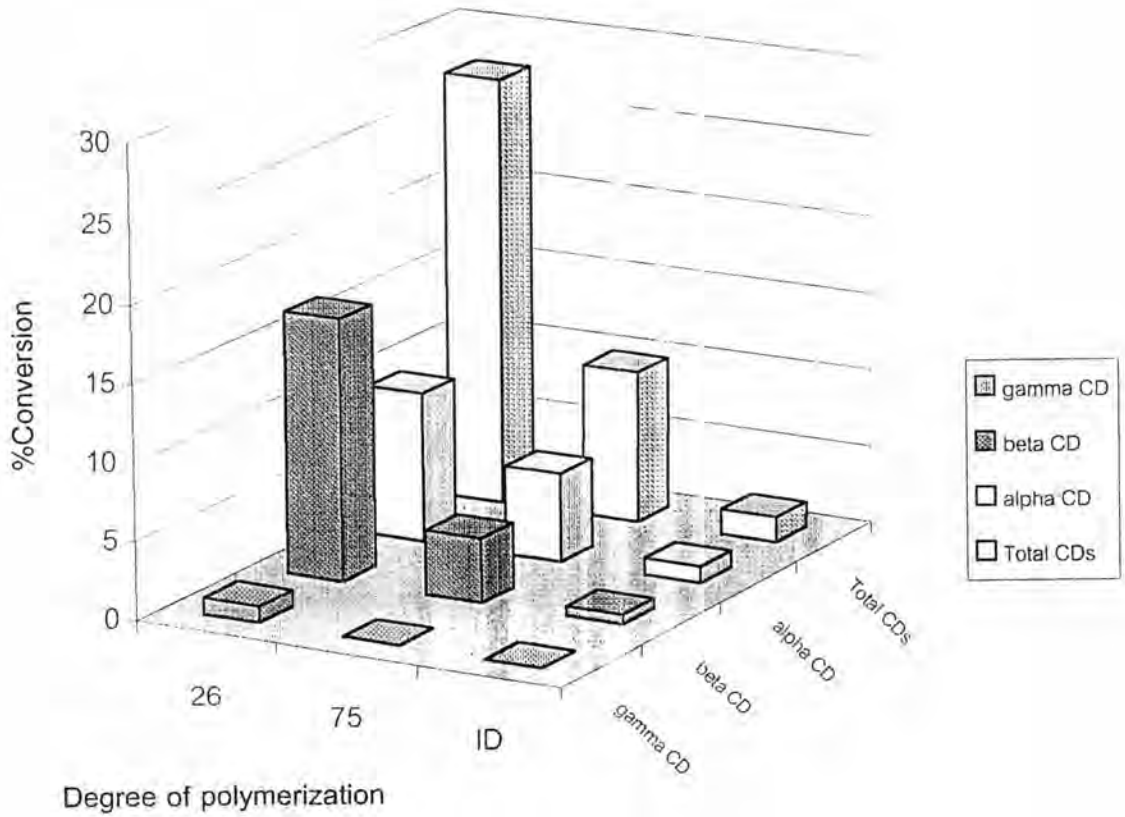


Figure 29 Production of cyclodextrins from pullulanase treated starch hydrolysate

0.2 g% of cassava treated with pullulanase was incubated with CGTase (500 U/g starch) for 10 hr at 40 °C, pH 6.0. The procedure was performed as described in Section 2.14.1.

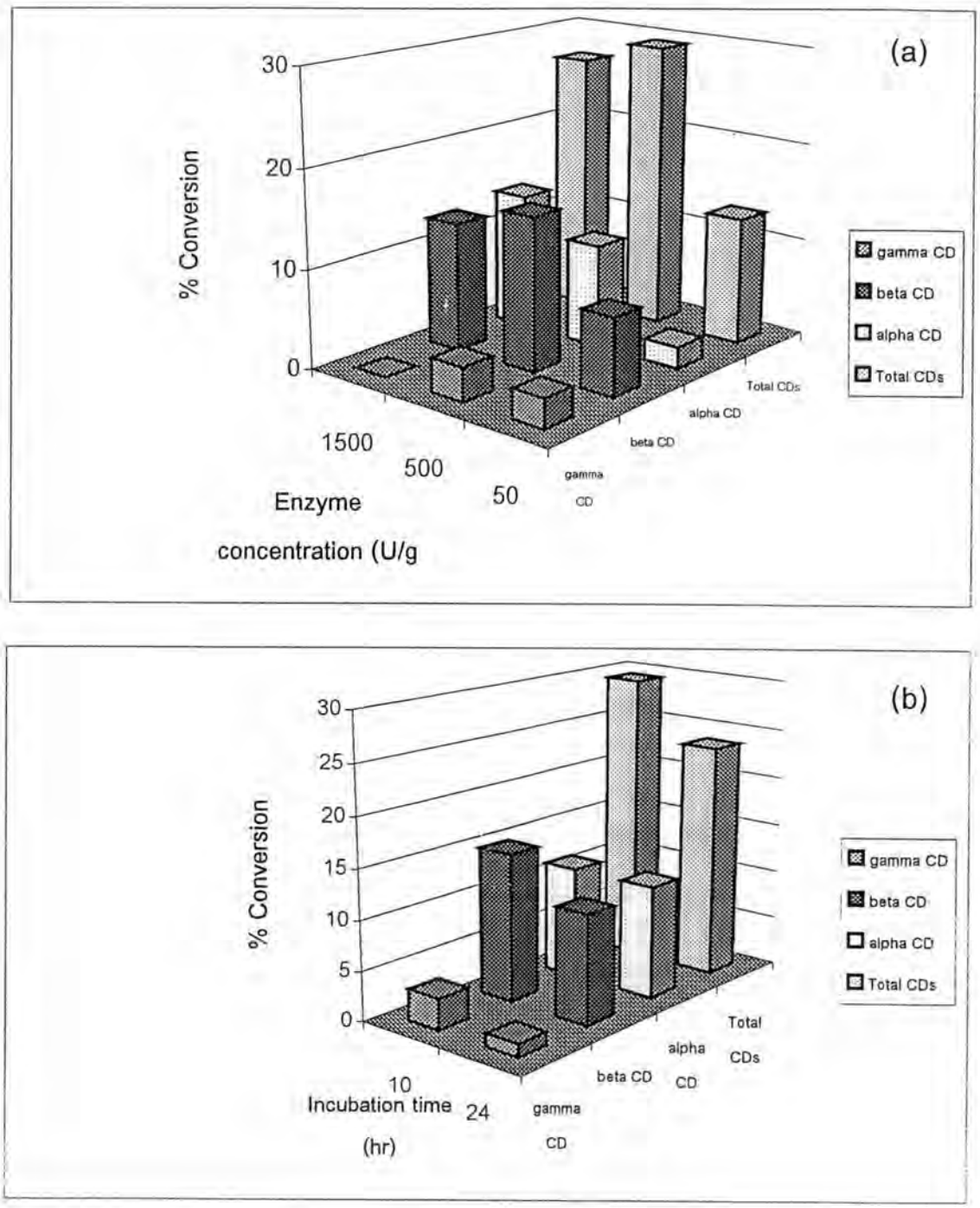
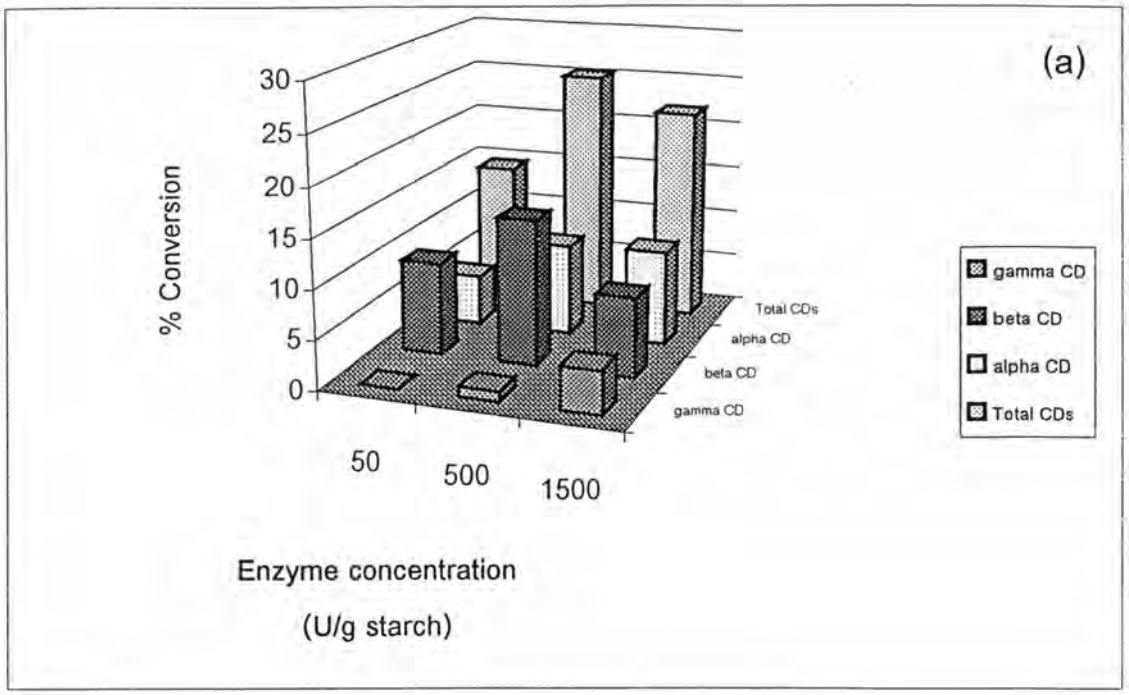


Figure 30 Cyclodextrin production from DP 9 fractions

(a) effect of CGTase concentration

(b) incubation time course

The procedures were described in Section 2.14.2.



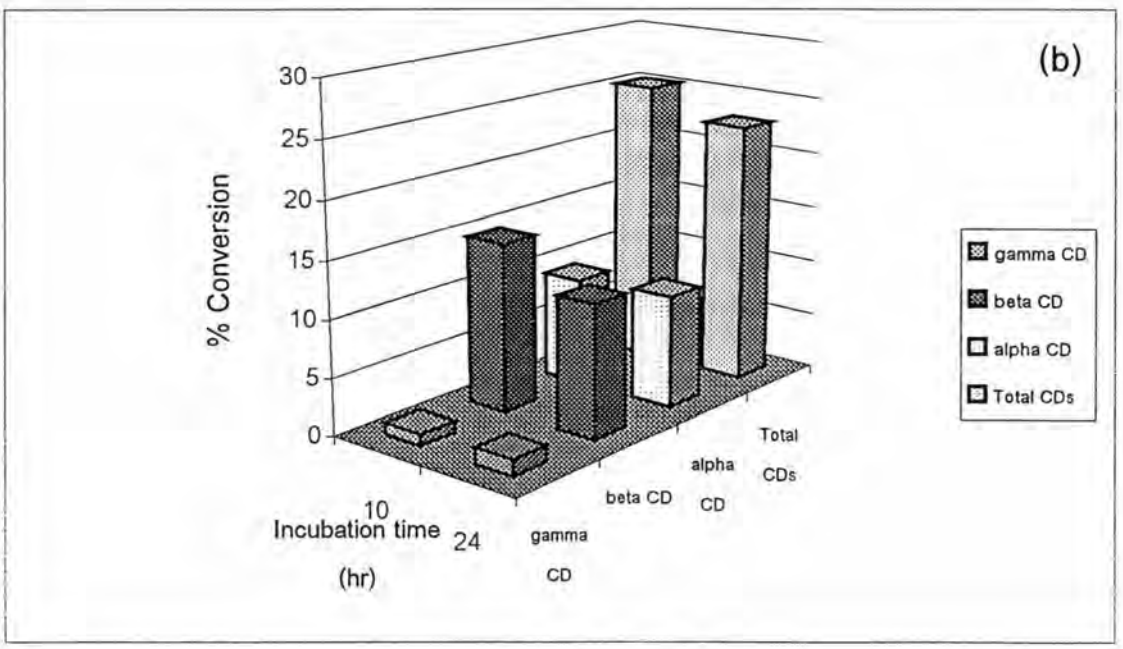


Figure 31 Cyclodextrin production from DP 26 fractions

(a) effect of CGTase concentration

(b) incubation time course

The procedures were described in Section 2.14.2.

the enzyme to 50 U/g starch would diminish γ-CD entirely. Prolong incubation time did not significantly increase the yield (Figure 31b).

3.7.3.3 Production of cylodextrin from starch hydrolysate (DP 75)

Fractions from Biogel P-10 desired DP 75 were incubated with varying amount of CGTase (50, 500 and 1,500 U/g starch) at 40°C for 10 hours and varying incubation time (10 and 24 hours). The result in Figure 32a showed that total cyclodextrin were highest at concentration 500 and1500 U/ g starch whereas α-CD were highest at concentration 1500 U/g starch. In every concentration of CGTase did not produce γ-CD. Increasing incubation time, the yield was decreased with a half of the total yield at 10 hours (Figure 32b).

3.8 Production of cyclodextrins in the presence of complexant

Cyclodextrin production in the presence of complexant was performed. The cassava starch concentration, temperature and enzyme concentration on cyclodextrin production were fixed at 2.5 g%, 40°C and 500 U/g starch, respectively. After incubation, The amount of CDs formed was determined as described in Section 2.11.1. and 2.11.2.

3.8.1 Effect of ethanol concentration on cyclodxtrin production

Different concentrations of ethanol (1-30%, v/v) were added to the reaction mixture. The yield of cyclodextrins was shown in Figure 33. For control the ratio of α: β: was 10:17:5 and a total %conversion of 33. The presence of 10-30% ethanol in the incubation mixture markedly increased the β-CD product although the total yield was only slightly different. However, at these concentrations, α-CD production was inhibited (35.73 to 74.29% inhibitions) and γ-CD was abolished.

3.8.2 Effect of incubation time on cyclodextrin production in the presence of ethanol

Incubation time for cyclodextrin production in the presence of ethanol was also studied. Ethanol concentrations were used at 20%. Figure 34 was

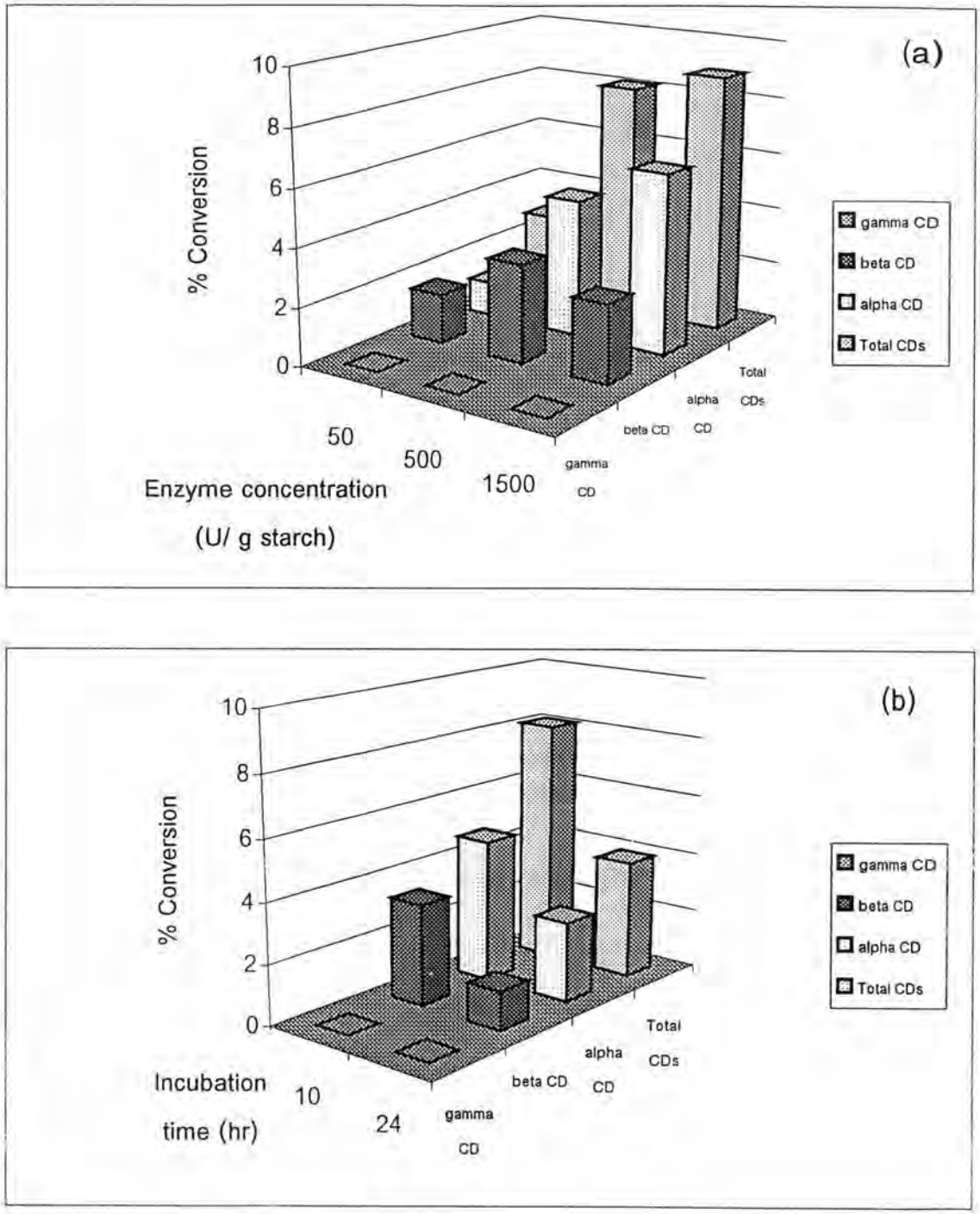


Figure 32 Cyclodextrin production from DP 75 fractions

(a) effect of CGTase concentration

(b) incubation time course

The procedures were described in Section 2.14.2.

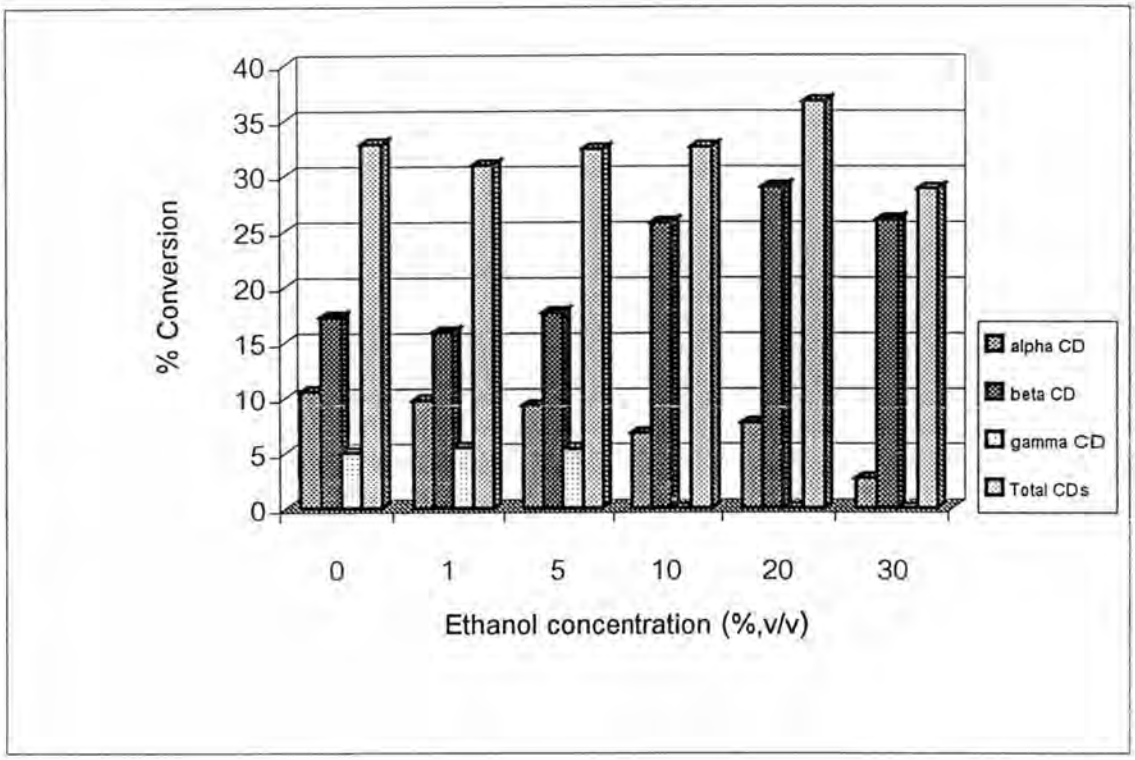


Figure 33 Effect of ethanol concentration on cyclodextrin production.

2.5 g% cassava starch was incubated with CGTase 500 U/g starch for 10 hours and ethanol was added to the reaction mixture in various concentration.

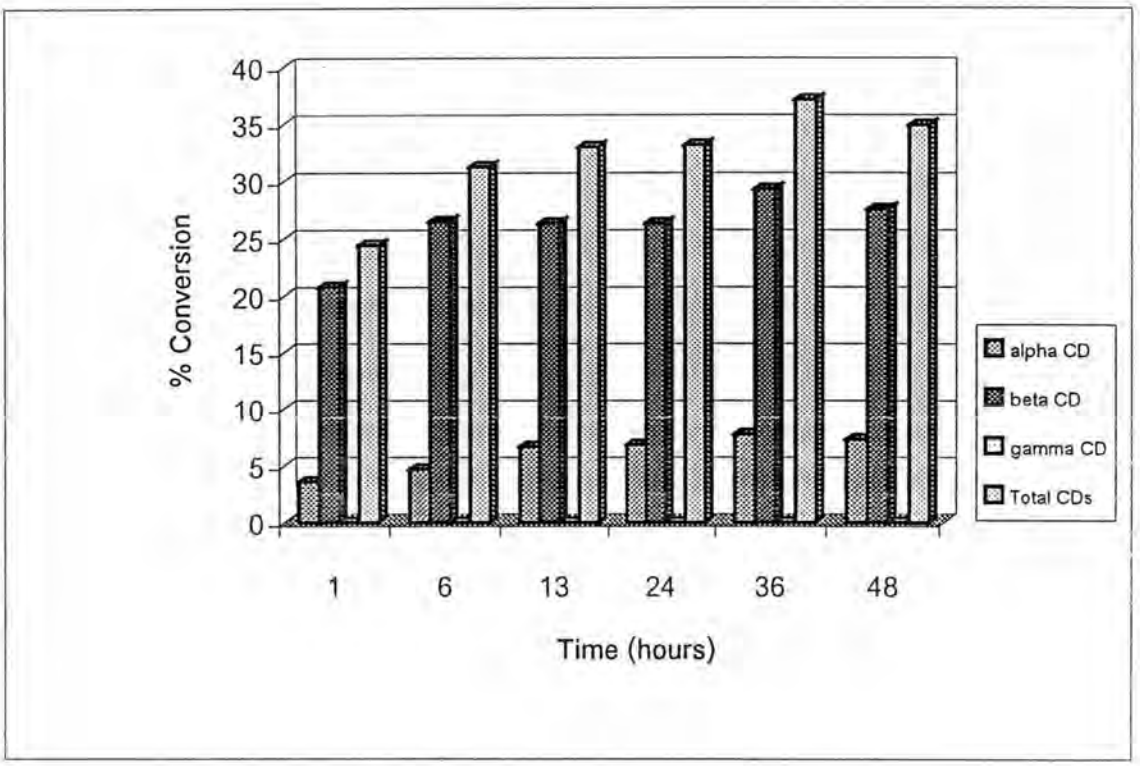


Figure 34 Effect of incubation time on cyclodextrin production in the presence of ethanol.

2.5 g% cassava starch was incubated with CGTase 500 U/g starch with ethanol 20 % (v/v) and incubated in various incubation time.

demonstrated that the amounts of total CD, α-CD and β-CD would increased with incubation period, reaching maximum at 36 hours. However, only little change was observed after 6 hours of incubation except for α-CD product. Gamma CD was not observed due to the 20% ethanol used in this experiment.

3.8.3 Effect of aliphatic alcohol and CGTase concentration on cyclodextrin production

Besides ethanol many other alcohols with number of carbon atoms ranging from 1-4 were also tested for their effect on cyclodextrin products. The conditions were performed according to Section 2.11.1 and 2.11.2 and the complexant concentration and incubation times were fixed at 20% and 36 hours. Complexants (methanol, ethanol, 1-propanol, 2-propanol,1- butanol and 2-butanol) were mixed in the reaction mixtures and the CGTase concentration was varied (50, 500 and 1500 U/ g starch). Table 10 summarized the results on the effect of alcoholic complexant.

Figure 35 showed that the condition in the presence of aliphatic alcohol gave higher yield than in the absence of aliphatic alcohol. The mixture composed of 2-propanol and 1-butanol. Using aliphatic alcohol β-CD was the major product. It is remarkable than the condition of 50 U/g starch with the presence of aliphatic alcohol produced more γ-CD than α-CD. Results showed that when alcohol complexants were added, the β-yields increased 1.3-1.7 times.

Figure 36 showed that the condition in the presence of aliphatic alcohol yielded more total yield than in the absence of aliphatic alcohol except in the presence of methanol, the total yield were highest when the absence of aliphatic alcohol. Total and β-CD yield were highest when the condition in the presence of 1-propanol, 1-butanol and 2-butanol. The condition in the presence of aliphatic alcohol produced mainly α-and β-CD. In contrast to, the condition in the presence of methanol produced three types of CDs. Results showed that when alcohol complexants were added, the β-CD yields increased 1.8-3 times.

Table 10 Summerized results on the effect of alcoholic complexant

Alcoholic complexant	Yields of cyclodextrins (%)											
	CGTase (Units/ g starch)											
	50				500				1500			
	α	β	γ	T	α	β	γ	T	α	β	γ	T
Control	0.56±0.1	15.97±0.5	4.77±0.77	21.3±0.22	10.61±0.2	17.1±0.3	4.88±0.23	32.6±0.4	15.31±0.2	13.15±0.7	0	28.46±0.5
Methanol	0.36±0.2	20.71±0.2	8.52±0.03	29.59±0.2	4.28±0.35	12.89±0.4	6.78±0.19	32.56±0.3	10.96±0.1	15.3±0.17	6.75±0.15	33.0±0.2
Ethanol	1.72±0.2	22.46±0.7	8.86±0.9	33.02±1.4	7.81±0.81	31.22±1.7	0	39.0±0.9	13.18±0.1	22.98±1.2	0	36.16±1.1
1-Propanol	0.74±0.1	17.5±0.5	7.51±0.7	25.76±1.1	2.64±0.6	42.12±0.8	0	44.76±0.8	10.24±0.3	33.21±0.1	0	43.45±0.2
2-Propanol	2.31±0.0	25.2±0.2	8.97±1.1	36.48±0.8	5.2±0.42	34.42±0.7	0	39.62±1.1	10.24±0.4	32.46±0.2	0	42.71±0.6
1-Butanol	1.11±0.5	26.72±0.4	8.7±0.3	36.53±0.1	4.41±0.18	44.37±0.9	0	48.78±1.1	10.17±0.6	36.52±0.3	0	46.7±0.25
2-Butanol	0.75±0.2	23.44±0.6	6.95±0	31.15±0.4	2.59±0.23	49.31±0.1	0	51.9±0.12	11.11±1.0	39.55±1.1	0	50.67±0.1

α = α-CD, β = β-CD, γ = γ-CD, T = Total CDs

control = The result of cyclodextrin production with no alcoholic complexant

Condition: 2.5 g% cassava starch was incubated with CGTase 50, 500 and 1500 U/g starch in the presence of alcoholic complexants 20% (v/v) for 36 hours

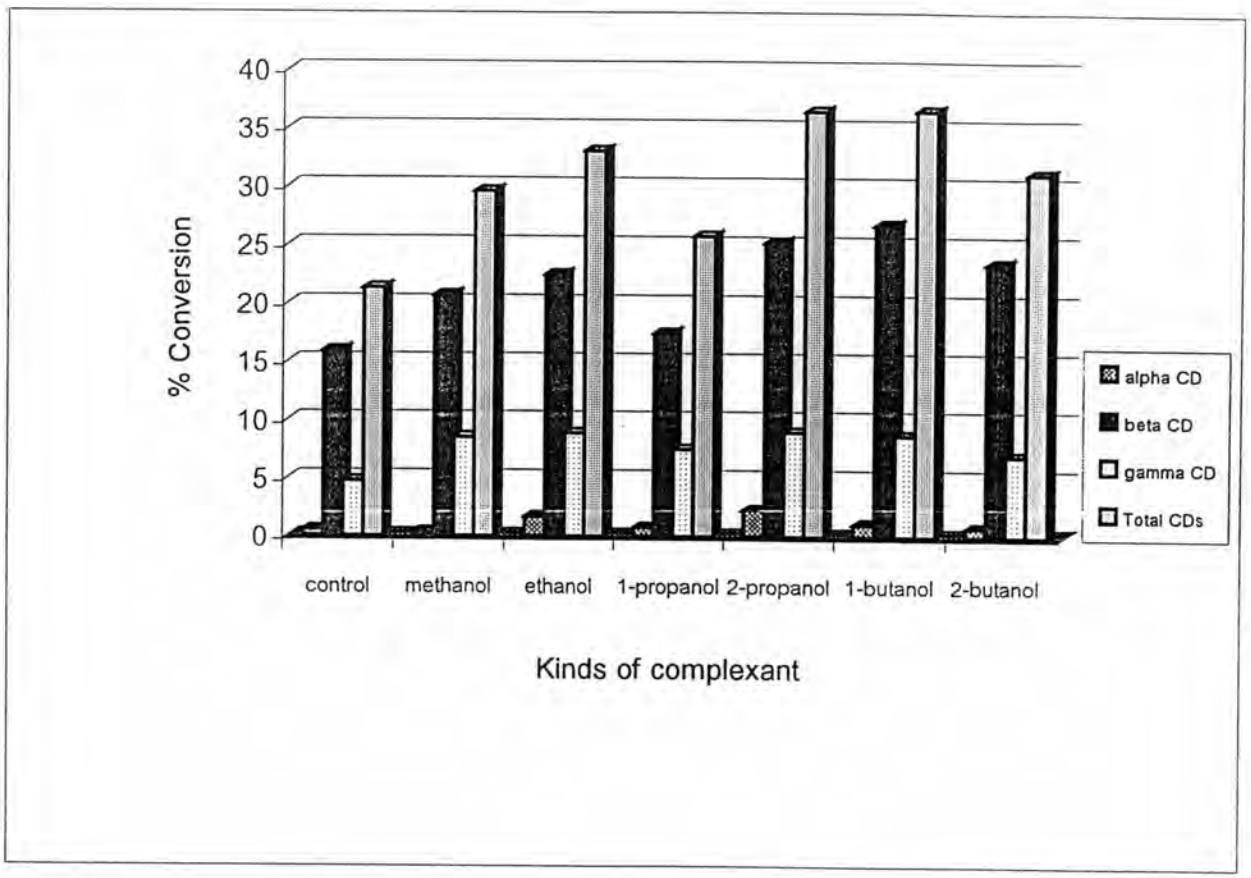


Figure 35 Effect of alcoholic complexants on cyclodextrin production

2.5 g% cassava starch was incubated with CGTase 50 U/g starch. Alcoholic complexants were added 20%(v/v) to reaction mixture and incubated for 36 hr. The procedure was described in Section 2..15.3.

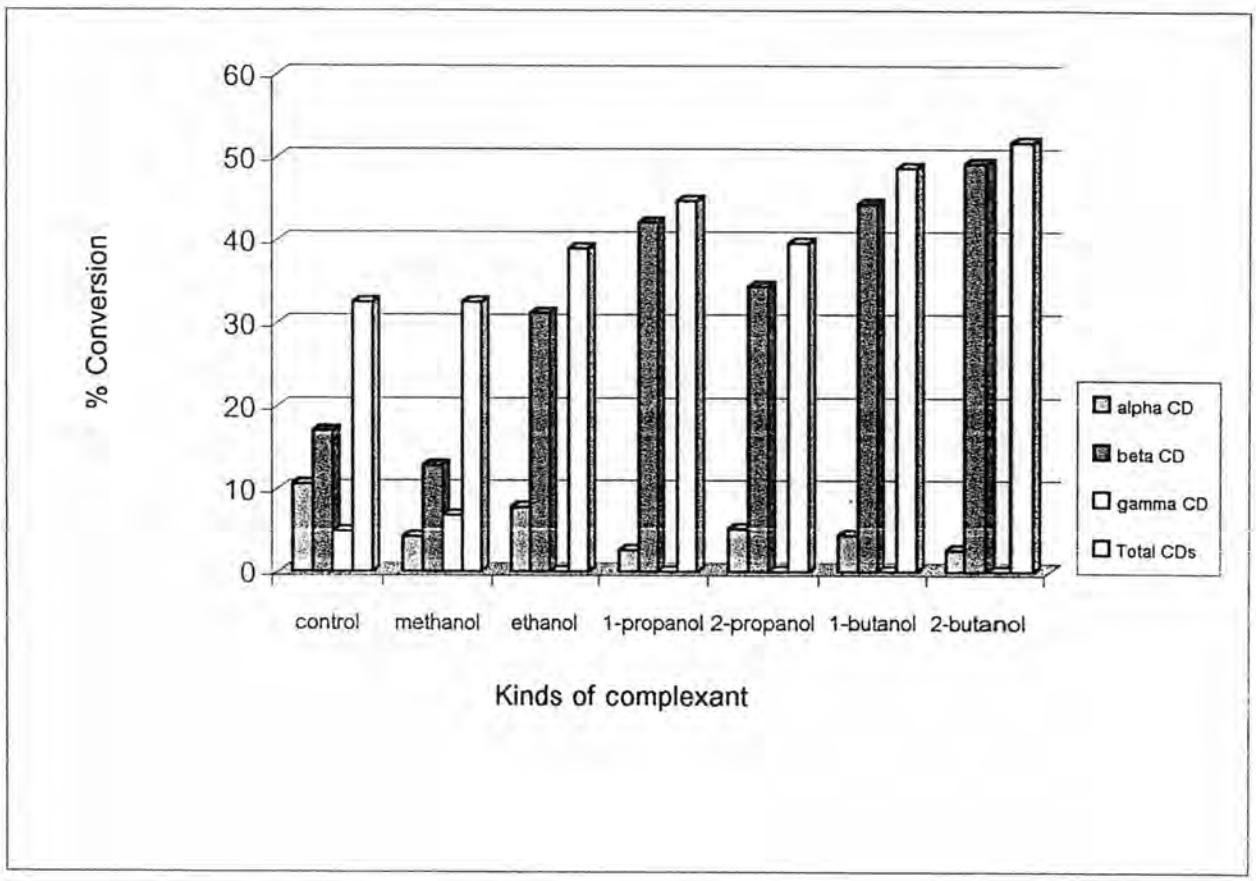


Figure 36 Effect of alcoholic complexants on cyclodextrin production

2.5 g% cassava starch was incubated with CGTase 500 U/g starch. Alcoholic complexants were added 20%(v/v) to reaction mixture and incubated for 36 hr. The procedure was described in Section 2.15.3.

Figure 37 showed that the condition in presence of aliphatic alcohol yielded more total yields than in the absence of aliphatic alcohol. Total and β-CD yields were highest when the 2-butanol was added. The condition the presence of aliphatic alcohol gave mainly α-and β-CD. On the other hand, the condition in the presence of method gave three type of CDs. From these results, the β-CD yields increased 1.2–3 times.

When consider in the position of –OH group, the presence of 1-propanol in the incubation mixture with 50 U/g starch gave higher total yield than in the presence of 2-propanol in the incubation mixture. The incubation mixture with 500 U/g starch in the presence of 1-propanol gave lower total yield than in the presence of 2-propanol. In addition if either 1-propanol or 2-propanol was added into the incubation mixture (1,500 U/g starch), the total yields were equal. In the presence of 1-butanol in the incubation mixture (50 U/g starch) gave higher the total yield than in the presence of 2-butanol whereas in presence of 1-butanol in the incubation mixture (500 and 1,500 U/g starch) gave lower the yield than in the presence of 2-butanol.

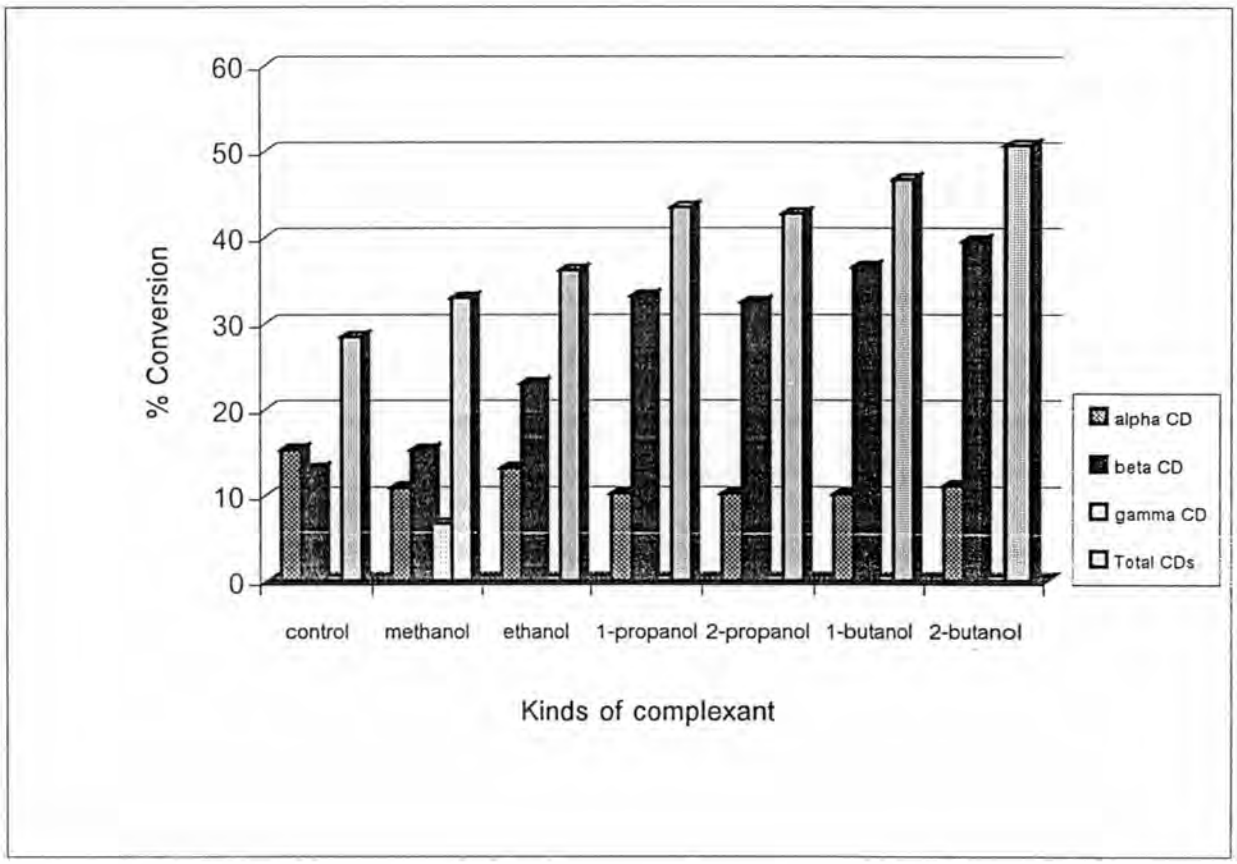


Figure 37 Effect of alcoholic complexants on cyclodextrin production

2.5 g% cassava starch was incubated with CGTase 1500 U/g starch. Alcoholic complexants were added 20%(v/v) to reaction mixture and incubated for 36 hr. The procedure was described in Section 2.15.3