CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Instruments/Model	Company/Country
Autoclave, Model HA 30	Hirayama Manufacturing Corperation, Japan
Autopipette, Pipetman	Gilson, France
Bench-top centrifuge	Kokusan Enshiki Co., Ltd., Japan
Brookfield viscometer DV-II+	Brookfield Engineering Laboratories, USA.
Diaflo ultrafiltration:Stirrer Ultrafiltration Cell 8050	Amicon W.R. Grace Cooperation, USA.
Filter Hoider	Gelman Sciences, Inc., USA.
Freeze-dryer	Stone Ridge, New York, USA.
Fraction collector, model 2211	Pharmacia LKB, Sweden
High Performance Liquid chromatography, Model LC-3A	Shimadzu Coorperation, Japan
Incubator	Haraeus, Germany
Incubator shaker, controlled environment, Psyco-Therm	New Brunswick Scientific Co., USA
Peristaltic pump	Pharmacia LKB, Sweden
pH meter, PHM 83 Autocal pH meter	Radiometer, Denmark
Refrigerated centrifuge, Model J-21C	Beckman Instrument Inc, USA
Scanning electron microscope JSM-35CF	JEOL,Japan
Spectrophotometer, Spectronic 20D	Bauch & Lomb, USA
Vortex: Model K-550-GE	Scientific Industries, USA
Water bath	Charles Hearson Co., Ltd., England
Water bath,shaking	Heto Lab Equpment, Denmark

2.2 Materials

Materials	Company/country
Filter paper No.1	Whatman International Ltd., England
Regenerated cellulose MW. Cut off 30,000	Amicon W.R. Grace Corporation, USA
Filter membrane, cellulose acetate 0.45 μm	Satorious AG, Germany
Scintillation vial	Hewlett Packard, USA

2.3 Chemicals

Chemicals	Company /country
Beef extract	Difco Laboratories,USA
Bacto-peptone	Difco Laboratories,USA
Amylopectin from corn (practical grade)	Sigma Chemical Company, USA
Amylose from corn (practical grade)	Sigma Chemical Company, USA
β-amylase, type I-B from sweet potato	Sigma Chemical Company, USA
Bovine serum albumin fraction V	Sigma Chemical Company, USA
Glucose	Sigma Chemical Company, USA
Maltotriose	Sigma Chemical Company, USA
Maltotetraose	Sigma Chemical Company, USA
Maltopenaose	Sigma Chemical Company, USA
Maltohexaose	Sigma Chemical Company, USA
Maltoheptaose	Sigma Chemical Company, USA
Soluble starch (potato)	Sigma Chemical Company, USA
Standard amylose	Sigma Chemical Company, USA
Standard amylopectin	Sigma Chemical Company, USA
Standard α-, β-, and γ-cyclodextrins	Sigma Chemical Company, USA
Acetonitrile (HPLC grade)	BDH Laboratory Chemical– Division, England
Coomassie Blue G-250	BDH Laboratory Chemical– Division, England

Soluble starch, potato	BDH Laboratory Chemical– Division, England
Trichloethylene	BDH Laboratory Chemical– Division, England
D (+)-maltose monohydrate	Fluka AG, Switzerland
α-amylase (BAN 240 L)	Novo Nordisk, Denmark
Pullulanase (Promozyme® 400 L)	Novo Nordisk, Denmark
Fungal α-amylase (Fungamyl®)	Novo Nordisk, Denmark
Maltogenic α-amylase (Malogenase™)	Novo Nordisk, Denmark

The other chemicals were obtained from Carlo, Fluka, Sigma or BDH as reagent grade. Cassava starch was gift from National Starch and Chemical (Thailand) Co. Raw rice starch (three-head elephant brand), corn starch (Maizena) were locally purchased.

2.4 Bacteria

Bacillus circulans A11, isolated from South-East Asian soil, was obtained from the Division of Microbe Exporation, Fermentation Research Institute, Japan (Pongsawasdi and Yagisawa, 1987).

2.5 Cultivation of bacterial

2.5.1 Media preparation

2.5.1.1 Medium I (Pongsawasdi and Yagisawa, 1987)

The composition of the medium is as follows:

beef extract	5	g/l
Peptone	10	g/l
NaCl	2	g/l
Yeast extract	2	g/l
Soluble starch	2	g/l

All ingredients were dissolved in distilled water, and the pH was

adjusted to pH 7.2 with 1 M NaOH. For solid medium, Bacto agar was added to make 1.5% agar. Sterilization was performed by autoclaving at 121 °C for 15 minutes.

2.5.1.2 Cultivation medium (Horikoshi's medium) (Techaiyakul, 1991)

The composition of Hirokoshi's medium is as follows:

rice starch	10	g/l
Peptone	5	g/l
yeast extract	5	g/l
K_2HPO_4	1	g/l
$MgSO_4.7H_2O$	0.2	g/l
Na_2CO_3	7.5	g/l

All ingredients were dissolved in distilled water, then the pH was adjusted to pH 10.1 with 1 M NaOH. The medium was then autoclaved at 121 °C for 15 minutes.

2.5.2 Maintenance of bacterial cultures

2.5.2.1 Short term maintenance

Bacillus circulans A11 was maintained by subculture on medium I slant agar every month and kept in refrigerator at 4 °C.

2.5.2.2 Long term maintenance

Bacillus circulans A11 was maintained by storing in sterilized glycerol 50 % v/v in freezer at -20 °C.

2.6 Enzyme preparation

2.6.1 Starter inoculum

A single colony from medium I plate was inoculated into slant agar

medium I, incubated in incubator at 37 °C for 18 hours. One full loop culture from the slant medium was further inoculated into a 10 ml medium I broth, incubated in a rotary shaking water bath with 250 rpm at 37 °C until A_{420} reached 0.3-0.5 and used as starter.

2.6.2 Enzyme production

Starter bacterial were transferred to 500 ml Horikoshi's medium in 1 l Erlenmeyer flask to make a final concentration of 1% starter. The bacteria were cultivated in a rotary shaking water bath with 250 rpm at 37°C for 72 hours. Cells were removed by centrifugation at 5,000 rpm for 15 minutes at 4°C. The crude supernatants was collected and checked for enzyme activity and protein content, before further purified by starch adsorption.

2.6.3 Partial purification of CGTase

CGTase was purified from the culture broth of *Bacillus circulans*A11 by starch adsorption method of Kato and Horokoshi (1984) with slight modification (Rutchtom, 1993) (Figure 9). Corn starch was oven dried at 100°C for 30 minutes and cooled to room temperature. All subsequent steps were conducted at 4°C. Fifty gram of corn starch was added into stirring crude CGTase broth (1liter) and stirred for 3 hours. The corn starch cake which adsorbed the enzyme was collected by centrifugation at 5,000 rpm for 30 minutes and washed twice with 200 ml of 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake by stirring for 30 minutes with 125 ml of TB1 buffer containing 0.2 M maltose twice. CGTase was recovered by centrifugation at 5,000 rpm for 30 minutes. The enzyme solution was concentrated by passing through an ultrafiltration membrane filter with molecular weight cut-off 10,000 daltons. The partially purified enzyme was again checked for enzyme activity and protein content.The enzyme was kept at 4°C for further study of cyclodextrin production.

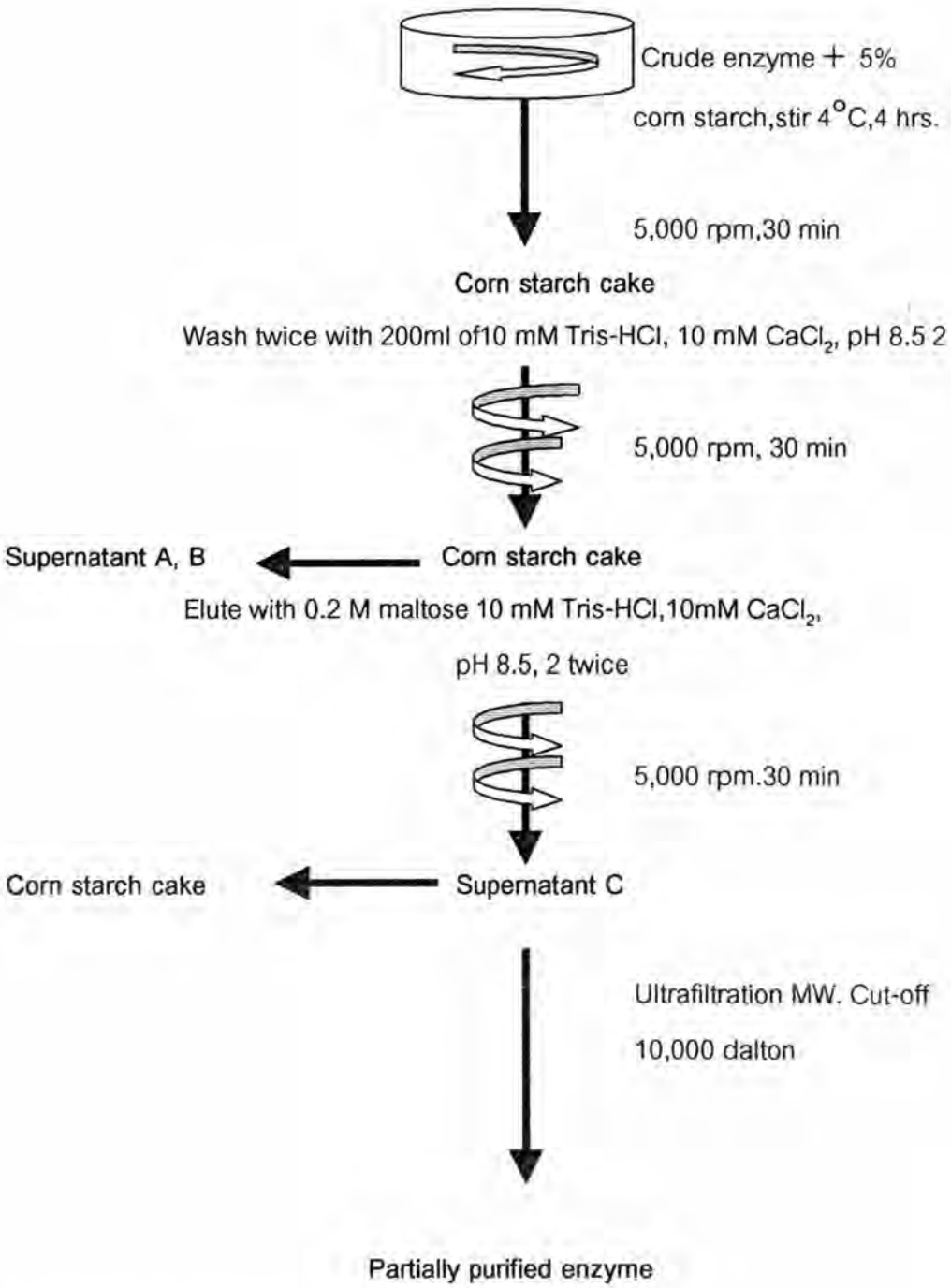

Figure 9 Flowsheet for partial purification of CGTase

2.7 Enzyme assay

CGTase activity was screened either by assay of amylolytic (dextrinizing) activity, or precipitation of cyclodextrin with tricloroethylene (TCE). For cyclodextrin production, the products were measured by high performance liquid chromatography (HPLC) technique.

2.7.1 Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the method of Fuwa (1944) with slight modification (Techaiyakul, 1991). The method measured the dextrinizing power of the enzyme that degraded α-1,4 glycosidic bond of starch by detecting the decrease in color intensity of the iodine-starch complex. The dextrinizing activity was non-specific because other enzymes which also hydrolyzed α-1,4 glycosidic bond such as α-amylase could also yield the same activity.

The assay mixture, contained 10 µl of enzyme and 0.3 ml of 0.2 % soluble potato starch in 0.2 M phosphate buffer pH 6.0, was incubated at 40°C for 10 minutes. The reaction was stopped with 4 ml of 0.2 M HCl. Half ml of iodine reagent (0.02 g% I_2 in 0.2 g% KI) was added. The mixture was diluted to final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, the enzyme sample was added after treatment with HCl.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex in one minute under the described conditions.

2.7.2 Cyclodextrin-trichloroethylene (CD-TCE) assay

Cyclodextrin-trichloroethylene (CD-TCE) activity was determined by the method of Nomoto, et al., (1986). This method measured β-cyclodextrin, which formed from CGTase, complexes with TCE and precipitated at the interphase of water and TCE.

The enzyme solution was diluted by serial double dilution with 0.2 M phosphate buffer, pH 6.0 (1:2, 1:4, 1:8.....1: 2^n). Half ml of each diluted enzyme was mixed with 2.5 ml of a 2% soluble potato starch, then the mixture was incubated at 40°C for 24 hours. After incubation, 0.5 ml of trichloroethylene (TCE) was added with

vigorously shaking and the suspension was left overnight at room temperature in the dark.

The CD-forming activity was expressed by the dilution limit ($1:2^n$) where "n" is the highest dilution that can produce observable which CD-TCE precipitate between upper starch solution layer and lower TCE layer.

2.8 Protein determination

Protein concentration was determined by the Coomassie Blue micromethod according to Bradford (1976).

The reaction mixture, containing 100 µl of enzyme solution and 1 ml of Bradford's reagent (Appendix A) was mixed and left at room temperature for 5 minutes before measuring the absorbance at 595 nm. The quantity of protein in the enzyme was determined using using bovine serum albumin fraction V as standard (10 µg/µl).

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

2.9 Cyclodextrin production

All cassava starch for process were gelatinized starch.

2.9.1 Process for cyclodextrin production

The standard process for CD production (slightly modified from the method of Malai,1995) was as shown in Figure 10. In brief, 2 g% of starch in 10 mM phosphate buffer pH 6 was gelatinized in boiling water for 10 minutes. The dissolved starch was cooled to 40 ^{o}C and incubated with partial purified CGTase (500 U/ g starch) at 40 ^{o}C pH 6.0 for 17 hours with continuous shaking at 150 rpm, then kept in boiling water for 10 minutes to inactivate the enzyme. The mixture was incubated with β-amylase (200 U/ g starch) at 25^{o}C for 3 hours in order to completely digest the small oligosaccharides in the mixture (Delbourg et al., 1993). Finally, the mixture was boiled

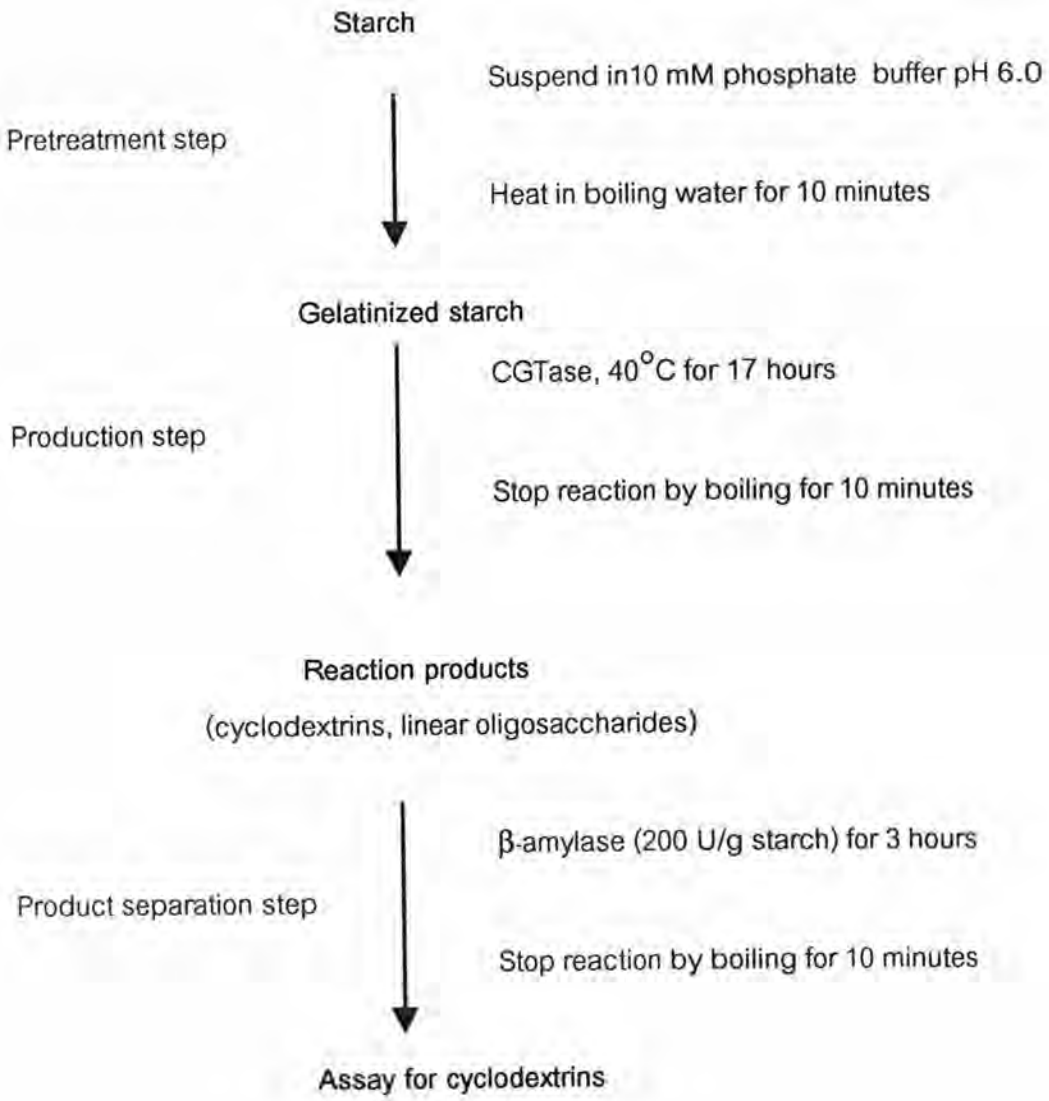

Figure 10 Flowsheet for the production of cyclodextrins

in boiling water for 10 minute to stop reaction of β-amylase. The amount of CDs formed was quantitated by HPLC technique.

2.9.2 Determination of cyclodextrins by High Performance Liquid Chromatography

The analysis of cyclodextrins by HPLC was performed as described by (Rutchtorn, 1993). The HPLC system was a Shimadzu LC-3A equipped with Spherisorb-NH_2 column (0.46×25 cm.) and using Shimadzu RID-3A refractometer as detector. Before injection, the mixture was filtered through a 0.45 μm membrane filter and then lyophilized to dryness. The dry mixture was adjusted to 500 μl with ultrapure water. The mixture was injected and eluted with acetonitrile-water (75:25, v/v) using a flow rate of 2 ml/min. The CD peak was identified by comparing the retention time with that of standard CDs, which composed of α-,β- and γ-CD mixture (20 mg/ml). For quantitative analysis, peak area corresponded with each cyclodextrin was determined from standard curve (Appendix F, G,H). Cyclodextrins formed will be expressed as % conversion from starch.

$$\% \text{ Conversion} = \frac{\text{Concentration of cyclodextrins detected (g/l)} \times 100}{\text{Concentration of starch substrate (g/l)}}$$

2.10 Determination of amylose and amylopectin contents

2.10.1 Standard amylose and amylopectin

2.10.1.1 Preparation of standard solution

Solutions of 8 μg/ml amylose, 32 μg/ml amylopectin and 8 μg/ml amylose plus 32 μg/ml amylopectin mixture were prepared as follows:

Standard amylose (20 mg) or amylopectin (20 mg) was dissolved in 9 ml 1 N NaOH and 1 ml 95% ethanol. The mixture was boiled in boiling water for 10 minutes. The volume was made to 100 ml with distilled water. Next, 4 ml of amylose solution, 16 ml of amylopectin and the mixture containing 4 ml of amylose solution and 16 ml of amylopectin solution were neutralized with 0.8, 3.2 and 4.0 ml of 1 N acetic acid,

respectively. Then the mixtures were mixed with 2 ml of iodine reagent (0.2g I_2 in 100 ml 2%KI) and the volume was made up to 100 ml with distilled water. These solutions were used for scanning the absorption spectra to obtain the six characteristic wavelengths. To obtain stable absorbance values, the absorbance was measured 15 minutes after the iodine reagent was added.

2.10.1.2 Determination of absorptivity of standard amylose and amylopectin

The characteristic absorption spectra of standard amylose solution, standard amylopectin solution and standard amylose plus amylopectin mixture in iodine (2.9.1) were determined by Beckman Du 600 diode-array spectrophotometer against water blank. From the spectra, six wavelengths were chosen as follows:

$\lambda 1$: wavelength of greatest difference between the amylose and amylopectin spectra and the absorbance due to amylopectin was greater than that due to amylose.

$\lambda 2$: amylopectin peak.

$\lambda 3$: peak for 20% amylose and 80% amylopectin.

$\lambda 4$: amylose peak.

$\lambda 5$: wavelength of greatest difference between amylose and amylopectin spectra and the absorbance due to amylose was greater than that due to amylopectin.

$\lambda 6$: wavelength of greatest absorbance due to amylose and the absorbance due to amylopectin approaches zero.

After the six wavelengths were chosen, standard amylose (concentration varied from 4 to 10 µg/ml) and standard amylopectin (concentration varied from 32 to 128 µg/ml) were measured for their absobances at each wavelength. The absorptivity was calculated as shown in equation (1) and (2).

$$Abs1 = E_0am1 \times C_{am} + E_0ap1 \times C_{ap} \qquad (1)$$

$$Abs2 = E_0am2 \times C_{am} + E_0ap2 \times C_{ap} \qquad (2)$$

Where C_{am} = amylose concentration (μg/ml)

C_{ap} = amylopectin concentration (μg/ml)

Abs1 = sample absorbance at the first wavelength

Abs2 = sample absorbance at the second wavelength

E_0am1 = absorptivity of amylose at the first wavelength

E_0am2 = absorptivity of amylose at the second wavelength

E_0ap1 = absorptivity of amylopectin at the first wavelength

E_0ap2 = absorptivity of amylopectin at the second wavelength

2.10.2 Starch sample

2.10.2.1 Removal of lipid from starches

Lipid extraction of starch was carried out according to a slightly modified procedure of Sievet (1993). Two gram starch sample were refluxed with 1-propanol/water (3:1v/v, 250 ml) in a Soxhlet extractor for 26 hours and then dried under vacuum overnight at 30°C. Starches used in this thesis are amylose, amylopectin, potato, cassava, sago, rice (long grain), rice (short grain), wheat starch, corn starch, arrow root, rye and Thai glutinous rice.

2.10.2.2 Determination of amylose content

Amount of varied concentration of defatted starch samples (20-30 mg from 2.10.2.1 were dissolved and mixed with iodine reagents as described in 2.10.1.1 and measured for absorbancy at six wavelengths as standard. The amylose content was calculated according to equation by Jarvis and Walker (1993).

$$C_{am} = \frac{\{(Abs2 \times E_0ap2 / E_0ap2) - Abs1\}}{\{(E_0am1 \times E_0ap1 / E_0ap2) - E_0am1\}} \qquad (3)$$

A PC-computer spreadsheet program was used to solve these simultaneous equations for C_{am} for each of the 15 combinations of the six wavelengths and to calculate the mean and standard deviation of the estimates.

2.11 Property of cassava starch

2.11.1 Cassava starch morphology

The morphology of cassava starch was determined by using scanning electron microscopy (Fitt and Snyder, 1984). Dried native starch and starch hydrolysate samples were sprinkled on to double-sided adhesive tape attached to a circular specimen stub and coated with approximately 300 A° of gold-palladium using a Baltzer SCD 004 sputter coater. The samples were viewed and photographed using a JEOL JSM-35CF scanning electron microscope at 10 kV.

2.11.2 Swelling power and solubility determination

Swelling power and solubility determination were done at the temperature range 40°C-90 °C by the procedure of Leach et al., (1956). Three gram percent cassava starch was suspended in water in 250ml centrifuge bottle and heated in varied thermostate water bath from 40°C-90 °C for 30 minutes, slowly stirred being continued during this period. The determination of swelling power and solubility were run at temperature interval of 5°C. The swelling cassava starch was centrifuged at 3,000 rpm in room temperature for 15 minutes. The clear supernatant solution carefully drawn off by transfer pipettes about 20 ml into petridish. An aliquot of this supernate was evaporated to dryness in the oven at 80°C and weighed. The remaining aqueous layer above the sedimented paste was then removed by pipettes and starch were weighed as wet weight. The bottle and sedimented paste were weighed. The swelling power and % solubility calculated according to equation (5) and (6) respectively.

$$\text{Swelling power (g/g)} = \frac{\text{Wet weight of sedimented paste}}{\text{Dry weight of dry starch}} \qquad (5)$$

$$\text{Solubility (\%)} = \frac{\text{Solid in supernate}}{\text{Dry weight}} \times 100 \qquad (6)$$

2.11.3 Pasting profile

Pasting profile of 6g% cassava starch were recorded on a Brabender Viscoamylograph. The Brabender Viscoamylograph monitored continuously the viscosity

cooking and cooling the starch paste. Thirty grams of cassava starch (dry weight) was suspended in 470 ml of distilled water in an amylograph cup and heated from 50°C-95°C at an increment rate of 1.5 °C/minute with stirring at 75 rpm. At 95°C, the sample was held at this temperature for 15 minutes, after which it was reduced to 50°C at the rate of -1.5°C/min and the sample was held at 50°C for a further 15 minute.

2.11.4 Analysis of linamarin

Linamarin content was determined by the activity of linamarase using the method of Eksittikul (1986). The linamarase catalyzed hydrolysis of linamarin, yielding glucose and acetone cyahydrin, which further breaks down into acetone and hydrogen cyanide. The HCN reacted with picrate to isopurpurin, a deep orange colour complex, which could be determined at 515 nm.

The reaction mixture consisted of 0.25 g of cassava starch into total volume 2 ml of 20 mM potassium phosphate buffer, pH 6.8. The reaction was performed in scintillation vial holding a center tube, which contained 0.4 ml of 0.5% picric acid in 2.5% sodium carbonate. The linamarase enzyme was added last to initiate the reaction and the vial was immediately sealed with a serum cap. The reaction vial was incubated at 37°C in shaking water bath for 1 hour, then the reaction was stopped by injecting 0.5 ml of ice-cold 0.5 N HCl into the reaction mixture and the vial was further incubated at room temperature for 30 min with moderate shaking. The released cyanide was trapped by picrate solution and was determined by measuring the absorbance at 515 nm. Cyanide standard curve (Appendix C) was constructed by incubating a set of closed vial, which contained potassium cyanide as standard.

2.12 Production of cyclodextrin from cassava starch

2.12.1 Optimization of condition for cyclodextrin production

2.12.1.1 Starch concentration

Cassava starch was varied from 1-30g% in 10 mM phosphate buffer pH

6.0 and incubated with partial purified CGTase (500 U/g starch) at 40 °C for 17 hours. Cyclodextrins were rid from non–cyclic products and quantitated by HPLC as described in Section 2.9.2.

2.12.1.2 Incubation temperature

Appropriate concentration of cassava starch from the result in 2.12.1.1 was incubated with CGTase (500 U/g starch) under the standard conditions (Section 2.9.1) but the incubation temperature was varied from 25-50°C. The amount of CDs formed was determined by HPLC as described in Section 2.9.2.

2.12.1.3 CGTase concentration

Using optimum cassava starch concentration and temperature from Section 2.12.1.1 and 2.12.1.2, cassava starch was incubated with varied amount of CGTase (25-2,000 U/g starch). The other procedure were as described in Section 2.9.1. The amount of CD formed was quantitated by HPLC as described in Section 2.9.2.

2.12.1.4 Incubation time

The starch concentration, temperature and the CGTase concentration were fixed at those optimum conditions. Optimization of the time of incubation was determined by varying incubation time from 1-48 hours. Cyclodextrins were separated from non-cyclic products in the Section 2.9.1 and further the amount of CDs formed was quantitated by HPLC as described in Section 2.9.2.

2.12.2 Pretreatment of cassava starch with hydrolytic enzymes

In order to obtain the proper substrate for CGTase, pretreatment of cassava starch in the presence of hydrolytic enzymes such as α-amylase (BAN 240L), pullulanase (Promozyme®), maltogenic α-amylase (Maltogenase™) and fungal α-amylase (Fungamyl®) were studied. The degree of hydrolysis was represented by the increased DE value.

2.12.2.1 Dextrose Equivalent determination

Dextrose Equivalent (DE) is an indication of total reducing sugars calculated as D-glucose on a dry-weight basis. The DE value is inversely related to the degree of polymerization (DP). Unhydrolyzed starch has a DE of virtually zero, whereas the DE of anhydrous D-glucose id defined as 100. (Lloyd and Nelson, 1984).

$$DE = \frac{\text{Reducing sugar (g/l)} \times 100}{\text{Dry weight (g/l)}} \qquad (7)$$

2.12.2.2 Determination of reducing sugar

Reducing sugar was determined by the method of Somogyi-Nelson (Nelson, 1944), using arsenomolybdate-copper reagent. Copper (II) was initially reduced to the cuprous form (Cu^{+}) by heating with the hydrolysate starch in alkaline condition and the resulting Cu^{+} further reduced by the arsenomolybdate to molybdenum blue, which could be determined at 520 nm.

One ml of sample was mixed with 1 ml of alkaline copper reagent (Appendix D). After the solution was heated for 15 minutes, the solution was immediately cooled in a pan of ice cold. One ml of Nelson reagent was then added, and the solution was left for 30 minutes at room temperature. Finally 5 ml of distilled water was added and the mixture was left for another 20 minutes before measuring absorbance at 520 nm. The quantity of reducing sugar in sample was determined using glucose (0-250 µg/ml) as standard.

2.12.2.3 Dry weight determination

One gram of dry diatomaceous earth was placed in a petridish of know weight and then 10 ml of hydrolyzed starch was transferred to the same petridish. The hydrolyzed starch with diatomaceous earth (mixture sample) was heated in the oven at 100-105°C for 4 hours and left cool in a desiccator. This mixture sample was dry in oven for three times for stable weight. The weight of total solid was calculated in g/l units.

2.12.3 Enzymatic treatment of cassava starch

2.12.3.1 Treatment with α-amylase

Five gram percent of cassava starch suspended in 10 mM phosphate buffer pH 6.0 was gelatinized in boiling water for 10 minutes. The gelatinized starch was cooled to 60 °C and incubated with α-amylase (BAN 240 L) by varying the amount of the enzyme from 0.0024 –0.096 U/g starch at 60°C for 20 minutes. After incubation, the hydrolyzed starch was placed in boiling water for 10 minutes. The dextrose equivalent was determined as described in the Section 2.12.2.1.

2.12.3.2 Treatment with pullulanase

Five gram percent of cassava starch suspended in 10 mM phosphate buffer pH 6.0 was gelatinized in boiling water for 10 minutes. The gelatinized starch was cooled to 60 °C and incubated with by varying the amount of pullulanase (Promozyme® 400 L) (24- 96 U/g starch) at 60°C for 24 hours. After incubation, the hydrolyzed starch was placed in boiling water for 10 minutes. The dextrose equivalent was determined as described in the Section 2.12.2.1.

2.12.3.3 Treatment with pullulanase and α-amylase

The procedure for preparation of gelatinized starch was same as 2.12.3.2 and the gelatinized starch was then incubated with pullulanase (Promozyme® 400 L) (96 U/g starch) at 60°C for 24 hours. After inactivation of pullulanase by heating in boiling for 10 minutes, varying amount of α-amylase (BAN 240 L) (0.0024 - 0.096 U/g starch) was added and incubated at 60°C for 20 minutes. After incubation, the hydrolyzed starch was placed in boiling water for 10 minutes in order to inactivate the enzyme. Value of dextrose equivalent was determined as described in the Section 2.12.2.1.

2.12.3.4 Treatment with pullulanase and maltogenic α-amylase or fungal α-amylase

The procedure for preparation of gelatinized starch was same as

2.12.3.1 and the gelatinized starch was then incubated with pullulanase (Promozyme®) (96 U/g starch) at 60°C for 24 hours. After inactivation of the enzyme by heating in boiling water for 10 minutes, varying amount of maltogenic α-amylase (Maltogenase™) (3.2-32 U/g starch) or fungal α-amylase (Fungamyl®) (3-30 U/g starch) were incubated respectively at 60°C for 12 hours. After incubation, the hydrolyzed starch were placed on boiling water for 10 minutes in order to inactivate enzyme. The dextrose equivalent was determined as described in the Section In the experiment, 2.12.2.1.

A detailed description of determination of enzyme activity from Novo Nordisk was shown in Appendix K.

2.12.3.5 Production of cyclodextrin from starch hydrolysate

Preparation of hydrolysate starch from 2.12.3.1-2.12.3.4 were incubated with CGTase (500 U/g starch) at 40 °C, pH 6.0, for 10 hours and assay for cyclodextrin products as described in Section 2.9.1 and 2.9.2.

2.13 Preparation of short chain cassava starch

2.13.1 Starch hydrolysis by α-amylase

Starch hydrolysate were were prepared by heating 5g of cassava starch which was dispersed in 95 ml of 10 mM phosphate buffer pH 6.0 for 10 min. The solution was cooled to 60°C and added 62.5 µl of α-amylase (BAN 240 L) for 0.024 U/g starch. The mixture was incubated at 60°C for 20 minutes. After stopping the reaction by boiling in the boiled water, the hydrolyzed cassava starch was filtered by ultrafiltration (molecular weight cut off 30,000). The filtrate was lyophilized and kept in desiccator until used.

2.13.2 Starch hydrolysate by pullulanase

Approximately 1 g of cassava starch 99 ml of 10 mM phosphate buffer pH 6.0 was heated in water bath at 96°C for 10 min. The solution was cooled to 60°C and added 24 µl of pullulanase (Promozyme® 400L) for 96 U/g starch. The mixture was incubated in a shaker bath at 60°C for 24 hours. The digest was heated in boiling water

for 10 minutes to inactivate the enzyme. The starch hydrolysate was used for loading to gel filtration chromatography.

2.13.3 Gel filtration column chromatography

2.13.3.1 Preparation Biogel P-10 column

Fifty grams of Biogel P-10 were swelled in 500 ml water a in 1 litre beaker by boiling water for 3 hours with gentle stirring. After hydration, the swollen gel was left to cool in room temperature. Fine particles were removed by decanting. The swollen gel was washed twice with 500 ml of 10 mM phosphate buffer pH 6.0 and degassed with suction pump for 30 minutes. The gel slurry was poured carefully into a glass column (1.9×85 cm), and equilibrated with two column volume of degassed 10 mM phosphate buffer pH 6.0. The flow rate was adjusted to 15 ml/hr. Before loading the sample, the column was checked for homogeneity by 2 mg/ml Blue Dextran 2000 and 1 mg/ml $K_2Cr_2O_7$. The zone of the marker dyes must move through the bed smoothly and showed straight horizontal bands.

2.13.3.2 Chromatography of starch hydrolysate on Biogel P-10

Hydrolysate from α-amylase or pullulanase (150 mg in 2.0 ml distilled water or 10 mg in 5.0 ml, respectively) was loaded into Biogel P-10 column, eluted with 10 mM phosphate buffer pH 6.0 at a flow rate of 15 ml/hr. Fractions of 2.0 ml were collected. Carbohydrate profile was monitored by measurement of total carbohydrate content. Desired fractions were pooled and estimated for DP value. These pooled fractions were lyophilized and kept for further study.

2.13.4 Determination of DP

The average DP of hydrolysate in effluent fractions was determined by dividing the total carbohydrate by its reducing value (equation 8). The total carbohydrate was analyzed by the phenol sulfuric acid method (Dubois et al., 1956). The reducing value was measured by Somogyi-Nelson (Nelson, 1954) as shown in Section 2.12.2.2.

$$DP = \frac{\text{Total carbohydrate (mg/ml)}}{\text{Reducing sugar (mg/ml)}} \quad (8)$$

2.13.4.1 Carbohydrate determination

Carbohydrate was determined by the phenol - sulfuric acid Method (Dubois et al., 1956). The action of strong acid on carbohydrates leads to the formation of colored decomposition such as furfural and hydroxymethylfurfural. These substances condensed with phenol to produce a yellow color, its intensity being proportional to carbohydrate concentration.

The phenol - sulfuric acid method, determined the total carbohydrate content. In brief, 0.5 ml of 5% phenol, 2.5 ml of concentrated H_2SO_4 and 0.5 ml of hydrolyzed starch were mixed. The absorbance was measured at 490 nm after 20 minutes incubation at temperature. The carbohydrate content in the sample was determined using glucose as a standard (0- 50 µg/ml) (Appendix E).

2.13.4.2 Reducing sugar determination

The reducing sugar was determined by Somogyi Nelson (Nelson 1944). One ml of the starch hydrolysate from each pooled of fraction Biogel P-10 chromatography was used and performed as described in Section 2.12.2.2.

2.14 Production of cyclodextrins from starch hydrolysate

2.14.1 Production of cyclodextrins from fractionated starch hydrolysate

Lyophilized starch hydrolysate from Section 1.13.3.2 was dissolved in distilled water to make a concentration of 0.2g%, then incubated with CGTase (500 U/g starch) at 40 ^{o}C, pH 6.0, for 10 hours and assay for cyclodextrin products as described in Section 2.9.1 and 2.9.2.

2.14.2 Optimazation of cyclodextrin production from cassava starch hydrolysate

Fractions of DP 9 from α-amylase treated starch (Section 1.13.3.2) or DP 75 and DP 26 from pullulanase treated starch (Section 1.13.3.2) were used in this study.

2.14.2.1 CGTase concentration

Starch samples were incubated with varying amount of CGTase (50, 500, 1500 U/g starch). The incubation condition was performed as in Section 2.9.1 and the amount of cyclodextrins formed was quantitated by HPLC as described in Section 2.9.2.

2.14.2.2 Incubation time

Starch samples were incubated with CGTase as described in Section 2.14.2.1 except the incubation time was varied to 10 and 25 hours. The amount of cyclodextrins formed was quantitated by HPLC as described in Section 2.9.2.

2.15 Production of cyclodextrins in the presence of complexant

Cyclodextrin production in the presence of complexant was performed. In brief, complexant was added to the reaction mixture. After incubation, the mixture was heated in boiling water for 30 minutes to evaporated the complexant. The amount of cyclodextrins formed was determined by HPLC as described in Section 2.9.2.

2.15.1 Effect of ethanol concentration on cyclodextrin production

The cassava starch concentration, temperature, enzyme concentration and incubation time on cyclodextrin production were fixed at 2.5g%, 40°C, 500 U/g starch and 10 hours. To determine the suitable ethanol concentration, 1-30 %(v/v) of ethanol were added to the reaction mixture.

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

Optimal ethanol concentration from 2.15.1 was used. To determine the incubation time on cyclodextrin production, incubation time was varied from 1-72 hours.

2.15.3 Effect of aliphatic alcohol and CGTase concentration on cyclodextrin production

The conditions were performed using the concentration of complexant and incubation times derived from experiments in Section 2.15.1 and 2.15.2.

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).