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Role of Starch Pre-treatment Temperature on types of Cyclodextrins produced from Raw Tapioca Starch

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ABSTRACT The effect of starch pre-treatment temperature and exposure duration of raw tapioca starch on cyclodextrins production using thermostable cyclodextrin glycosyltransferase was investigated in this study. Maximum concentration of cyclodextrins was produced after an hour of exposure duration on raw tapioca starch at 80°C of the starch pretreatment temperature. Employment of this critical condition as the starch pretreatment procedure promotes high cyclodextrins production as well as better preservation on the starch granule structure. As a matter of fact, the starch critical pretreatment temperature also increased 93% and 94% of swelling power and leached amylose, respectively. All types of cyclodextrin produced revealed significant linear relationship with starch pretreatment temperature.

(Cyclodextrins, Starch pretreatment, Tapioca starch, Thermostable cyclodextrin glycosyltransferase, Statistical analysis)

INTRODUCTION

Tapioca contains higher amylopectin and amylose molecules compared to other starch sources, thus implying the solution becomes more viscous especially at high temperatures and with the presence of substantial starch concentrations. However, the amylopectin component which is responsible in causing viscosity is actually an advantage that is beneficial in producing high yield of cyclodextrins. Cyclodextrins are produced from intramolecular transglycosylation reaction through degradation of substrate by cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) enzyme [1, 2]. Traditionally, starch was liquefied by using amylase prior to additional of CGTase. However, additional liquefying enzyme would reduce cyclodextrins production due to the acceleration of other CGTase reactions through coupling reaction and degradation of cyclodextrins [3]. Thus, alternative methods were introduced by several researchers to overcome these problems, which included the enzymatic and physical methods [4, 5]. For example, the enzymatic method involved liquefaction controlled process, which was introduced and patented by Yano *et al.* [6] and the usage of CGTase as the liquefying enzyme to minimize the starch degradation as suggested by Masanobu *et al.* [7]. While the physical methods were recommended by Hitoshi *et al.* [8] using sonification method, by Yoshinao *et al.* [9] using extrusion method, by Lee *et al.* [10] using milling method, Kim *et al.* [11] introduced the moderate heat treatment method. In this study, moderate heat treated starch is employed for pretreatment process. This type of starch pretreatment method is simple and efficient for large scale of cyclodextrins production, besides the unreacted starch can be further recycled for the next reaction process.

Previous studies done by many researchers generate cyclodextrins using corn starch [10 - 13], potato starch [14, 15], wheat starch [16] and sago starch [17, 18] as a substrate. However, not many studies have been carried out the employment of tapioca starch as a substrate. Furthermore, there is still little agreement on the contribution of pre-treatment temperature in the type of cyclodextrins produced. Thus, this study focused on determining the effect of tapioca starch pre-treatment temperature using a large scale of enzymatic reactor on the amount of cyclodextrins production as well as the types of cyclodextrins produced.

MATERIALS AND METHODS

Heating Stage Microscopy

The thermal response (swelling and gelatinization) was directly observed using an optical microscope (Nikon Eclipe, Nikon Inc.) attached with a 40x lens. A 8% w/v starch suspension in water was heated from 30°C to 80°C at a heating rate of 10°C/min. The images were analyzed with Image-Pro Plus 3.0 (Media Cybernatic).

Differential Scanning Calorimetry (DSC)

Thermal characterization of starch samples was performed by using a Differential Scanning Calorimetry or DSC. Starch suspension (18 to 20 mg, 50% moisture) was sealed in a stainless steel pan (Mettler Toledo). An empty pan was used as a reference and the DSC was calibrated using indium. Each sample was heated from 30°C to 100°C at 10°C/min. Samples were measured in duplicates.

Determination of the absorbance of amylose-I_2 complex

Starch samples with 8% w/v were heated at desired temperature (30 - 90°C) for 30 min in a water bath, cooled to room temperature and centrifuged (78.25 *x g* for 15 min). 50 µl of the supernatant was mixed with 50 µl of Iodine reagent (0.2g I_2 dissolved in 100 ml 2% w/v KI). The volume was made up to 5 ml with deionized water. The absorbance of amylose-I_2 complex at 630 nm and the amylopectin- I_2 complex at 545 nm was measured 15 min after the iodine reagent was added using a UV/VIS spectrophotometer (Hitachi Model U-100) [19 - 21].

Determination of the starch swelling power (SP)

The swelling power of tapioca starch was determined using methods of Tsai *et al.* [22] and Li and Yeh [23]. Starch solution containing 0.8 mg of tapioca starch in 10 ml of distilled water was incubated at 30 to 90°C for an hour. The reaction mixture was cooled to room temperature in an iced water bath and centrifuged (125.2 *x g*) for 20 min. The supernatant was poured out from the tube. The precipitate material was weighed (W_p), while the supernatant was dried to constant weight (W_s) in an air oven at 100°C. The water soluble index (WSI) and swelling power (SP) were calculated by using the equation below:

$$WSI(\%) = (W_s / 0.1) \times 100\% \quad (1)$$

$$SP(g/g) = W_p / [0.1(100\% - WSI)] \quad (2)$$

Scanning Electron Microscopy

A digital scanning electron microscopy (SEM) (Philips SEMEDAX; XL 40; PW6822/10) was used to study the qualitative surface of raw and treated tapioca starch granule. The sample of the tapioca starch was put on the stark and coated with a gold-palladium before the photographs were taken.

Production of cyclodextrins

A 10 L enzymatic reactor was filled with 8 L of starch solution (480 g of raw tapioca starch in 10mM phosphate buffer pH 6.0) with continuous stirring at 3.13 *x g*. Prior to enzyme addition, the starch was pre-heated at certain temperature and exposure duration as to get the optimum condition for reactive structure of starch. The pretreatment temperature was conducted under starch gelatinization temperature in order to avoid starch from becoming more viscous and difficult to handle. Then, the 0.5% of heat stable CGTase (Toruzyme 3.0 l produced by Novozyme Sdn Bhd.) was added in a free form to the tapioca starch solution and the reaction was carried out for 4 hours.

Analysis of CD

The concentrations of CD were determined by using HPLC system (Water Assoc.) with separation carried out using an Econosphere NH2 (5 µm, 250mm×4.6 mm) column. The peaks were eluted with acetonitrile: water (70:30) at 1.0

mL/min and detected by refractive index detector (Waters 410). The column temperature was controlled at 30°C. All samples were filtered with Whatman ® nylon membrane filter (0.2 µm pore size, 13 mm diameter) before injection.

Statistical analysis

Analysis of variance (ANOVA), Fisher's test ($p < 0.05$) and regression analysis were performed employing Minitab statistical software (Minitab Release 13.20) to determine if there exist a relationship between reaction parameters and type of cyclodextrins produced.

RESULTS AND DISCUSSION

The composition and gelatinization of tapioca starch used in cyclodextrins production was characterized and shown in Table 1 and 2. Based on Table 3 the employed tapioca starch started to gelatinize at temperature of 80.4°C. Note that gelatinization of tapioca starch is referred to the loss of structural order as tapioca starch granule is swelling in heated aqueous solution as shown in Figure 1. Gelatinization depends on temperature, time and grain size. This could be reasoned dûe to the hydrogen bonds among the constituent molecules that were weakened until it reached the point whereby the granules can absorb more water [24]. At this temperature, which is called as the initial gelatinization temperature, T_o, the granules swelled and started to loss their birefringence, following by the loss of crystallinity. As the temperature increased, the starch granules swell up to several times from the original volume without disintegration. Subsequently, the linear macromolecule (amylose) was leached out from the granules and initiated a collapse of the tapioca starch granules, increased numbers of water molecules attaching to exposed OH-groups on the molecules and consequently intensified in viscosity of the slurry [25 - 26].

Table 1. The characteristics of the tapioca starch used in this study

COMPOSITION	AVERAGE*
Water (%)	13.43 ± 0.263
Ash (%)	0.23 ± 0.021
Protein (%)	0.065 ± 0.005
Lipid (%)	0
Carbohydrate (%)	86.35 ± 0.397

* samples are measured in triplicate

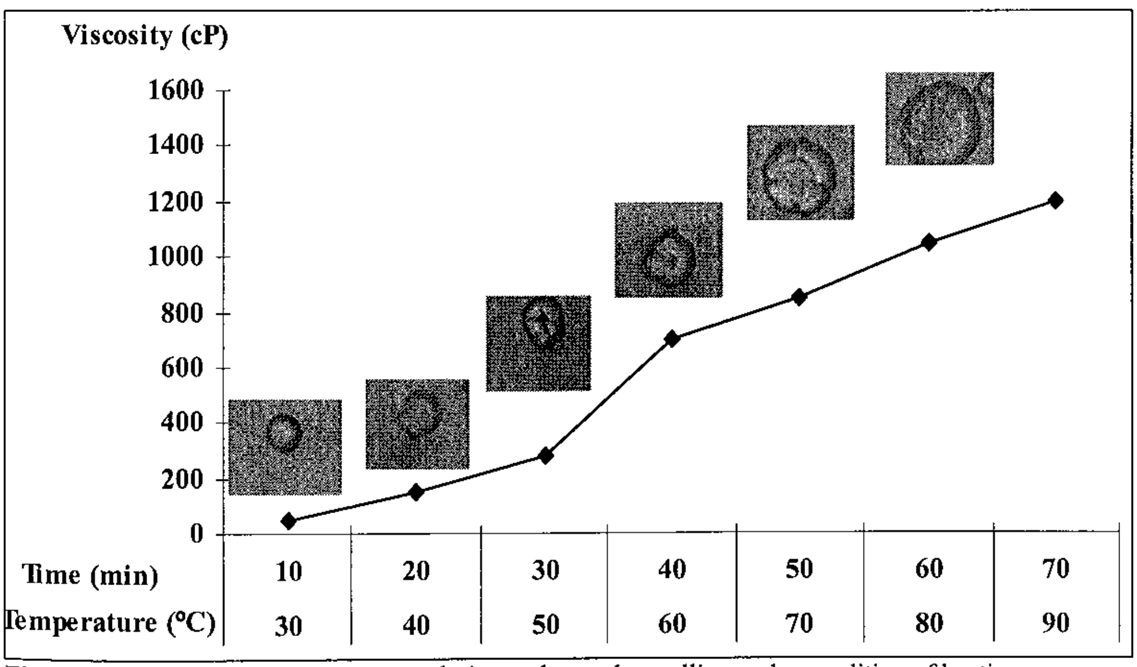

Figure 1. Viscosity of the aqueous solution and granule swelling under condition of heating temperature and time

Table 2. The gelatinization property of tapioca starch

	AVERAGE
Onset temperature, T_o (°C)	81.43 ± 0.702
Gelatinization range, R (°C)	80.4 to 118.3
Gelatinization enthalpy, ΔH ($J.g^{-1}$)	13.3 ± 0.351

* samples are measured in triplicate

Effect of starch pre-treatment temperature on the cyclodextrins production

Starch pre-treatment temperature is one of the parameters that influences the production of cyclodextrins. In this study, temperature ranging from 40 to 80°C (below the tapioca starch gelatinization temperature, T_{gel}, 90°C) was used for tapioca starch pretreatment. The effect of pretreatment temperature on cyclodextrins production is shown in Figure 2 where low pretreatment temperature (below 60°C), attributes to small amount of cyclodextrins produced. This phenomenon was due to the starch granules that were not fully converted into reactive structures at temperature below 60°C. It can also be seen that the amounts of cyclodextrins produced at low pretreatment temperature were slightly equivalent to the amount of cyclodextrins produced without pretreatment. Therefore, pretreatment temperature below 60°C was found to be insignificant as it resulted in poor cyclodextrins yield (Table 3). This result was found to be similar with Kim *et al.* [27] who reported that pretreatment of corn starch below 60°C exhibited low amount of cyclodextrins produced. However, it was observed that, cyclodextrins concentrations appeared to increase with the incremental of pretreatment temperature (60°C, 70°C and 80°C). The tapioca starch which was initially heat treated at 80°C induced the greatest rate of cyclodextrins production as well as demonstrated a maximum amount of cyclodextrins of 34 g.

Therefore, application of critical pretreatment temperature (below 80°C) was considerably practical in order to generate cyclodextrins in the continuous system. Concisely, pretreatment temperature was directly proportional to cyclodextrin production since higher pretreatment temperature resulted in greater expansion of the tapioca starch granule. As a result amylopectin melts, expands and releases the residual amylose that was trapped between the amylopectin layers and within the crystalline amylopectin [28]. In other words, higher heat treatment, the structures of tapioca starch granules opened up and became more susceptible to CGTase action. This explanation was well supported by Figures 3, 4 and 5.

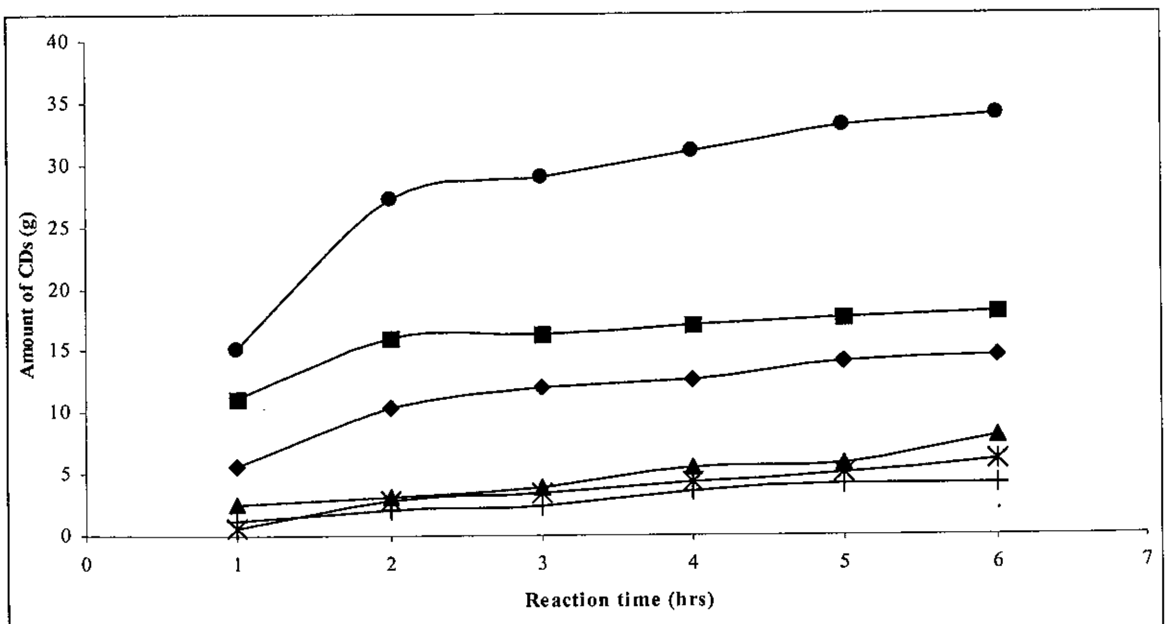

Figure 2. Effect of starch pretreatment temperature on cyclodextrins production. The starch pretreatment temperature was: ●, 80°C; ■, 70°C; ♦, 60°C; ▲, 50°C; *, without starch pretreatment; +, 40°C

Table 3. Effect of starch pretreatment temperature on conversion yield

REACTION TIME (hrs)	PRETREATMENT TEMPERATURE					
	WITHOUT PRETREATMENT	40°C	50°C	60°C	70°C	80°C
1	0.9	2.0	4.2	9.3	18.3	25.0
2	4.7	3.3	5.2	17.3	26.7	45.3
3	5.7	3.8	6.3	20.0	27.2	49.3
4	7.2	5.8	9.2	20.8	28.3	54.2
5	8.3	6.8	9.7	23.3	29.3	57.3
6	10.0	7.0	13.3	24.2	30.0	58.4

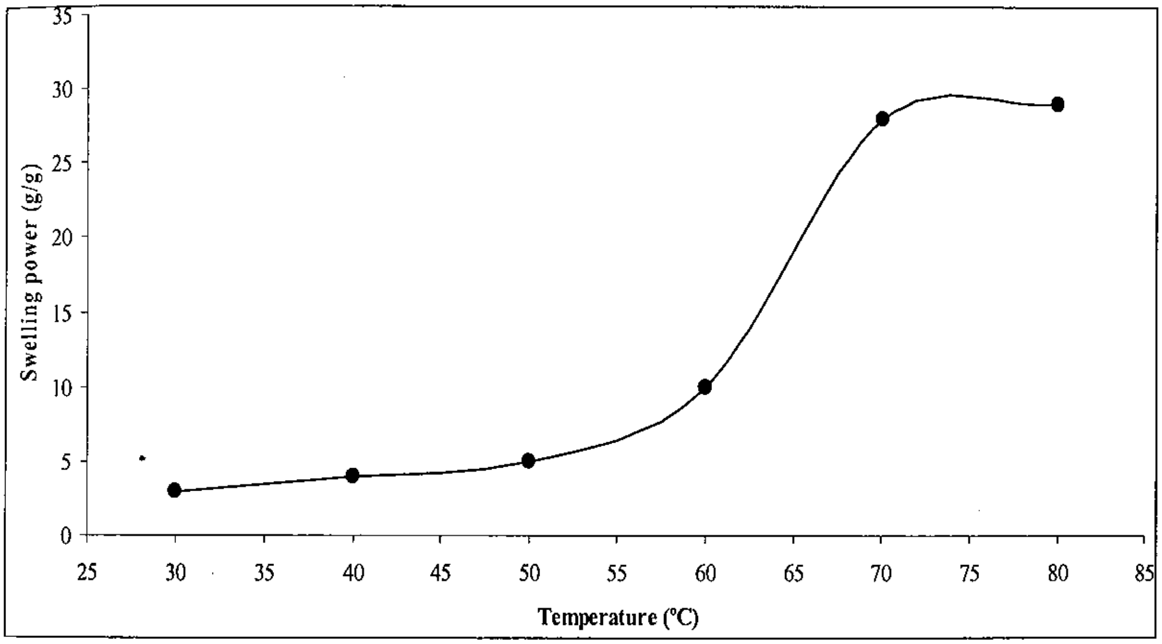

Figure 3. Temperature effects on swelling powers of tapioca starch

As shown in Figure 3, the swelling power of tapioca starch used increased with temperature. The swelling power of tapioca starch indicated the sharp incline after 60°C of exposure temperature. However, the swelling power of tapioca starch was observed to be slightly static after 70°C of exposure temperature. The results indicated that the swelling power of tapioca starch can be divided into three phases. At the first phase, which was below 60°C, the starch granule started to swell and free amylose begins to dissolve into water as this amylose attributed as hydrophilic molecule. Then, the starch granule rapidly swelled as the temperature increased for the second phase. Finally, the starch granule started to melt [23]. The transformation of starch granule is shown in Figure 4.

Figure 4 exhibited raw tapioca starch granule and tapioca starch granule treated at 80°C being observed under SEM. The starch granule under 1000 magnification clearly showed the starch granule was totally broke down and this phenomenon was an irreversible process. As the starch breaks down, the CGTase could be easily reacted with, and produced greater cyclodextrins. This is because increasing temperature would induce greater expansion of starch granule which makes the amylopectin melt, expand and release the residual amount of amylose that was trapped between the amylopectin layers and within the crystalline amylopectin [29]. As the swollen amylopectin contained more surface area for the enzyme to react with, the leached amylose was freely and easily reacts with enzyme in aqueous solution to produce more cyclodextrins.

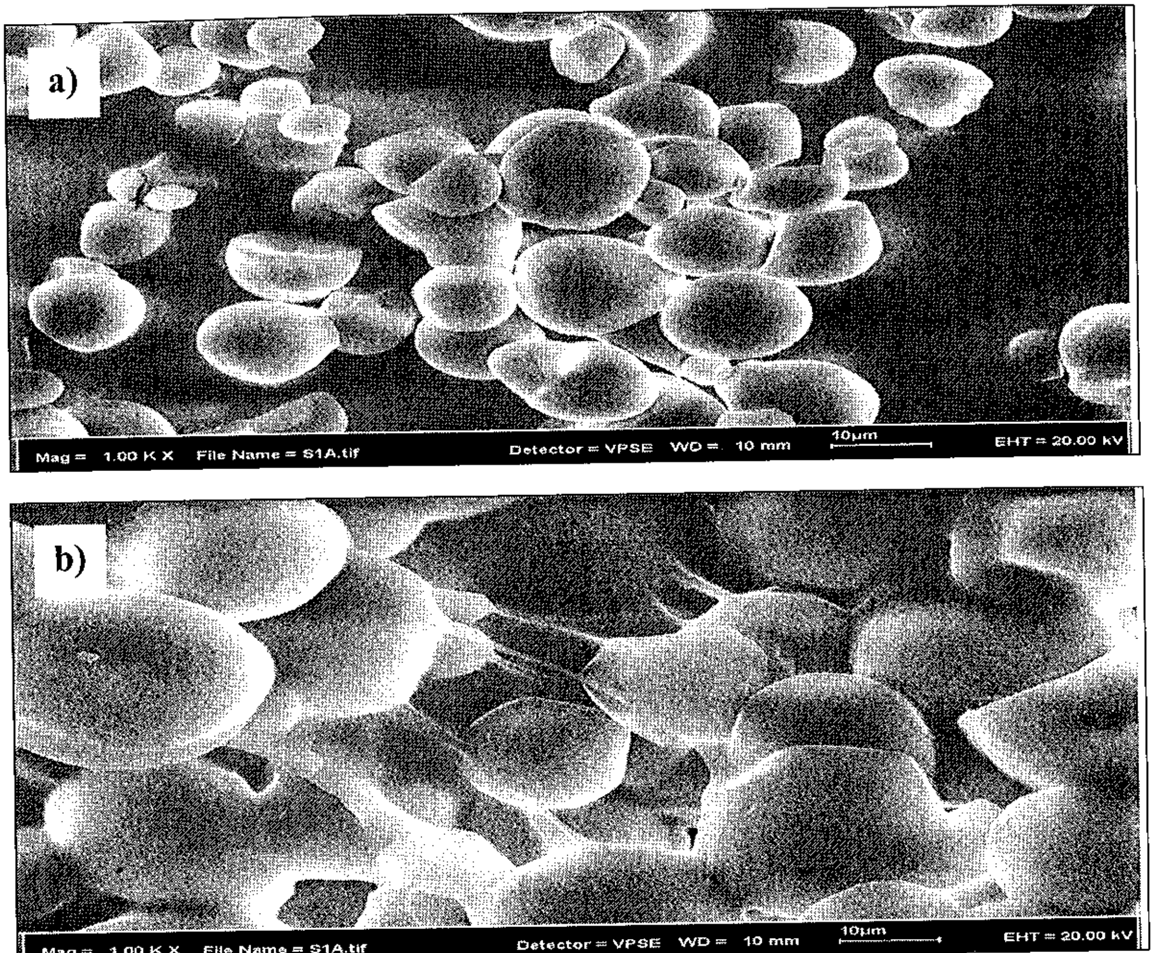

Figure 4. SEM micrograph of (a) raw tapioca starch granule and (b) tapioca starch granule treated at 80°C (magnification 1000x)

The leaching of amylose from starch granules could be proved by Figure 5 which depicted the quantitative data of amylose-iodine and amylopectin-iodine as a function of temperature. In Figure 5, the 630 nm and 545 nm absorbance of the soluble fraction (supernatant) of heated starch in excess water started with sharp inclination after 60°C of the starch pretreatment temperature. At the critical starch pretreatment temperature (80°C), the absorbance of the amylose-iodine complex achieved about 94% more than the absorbance at the initial temperature, while the amylopectin-iodine complex achieved about 92% more than the absorbance at the initial temperature. This was due to the increased number of leached amylose attached with a small amount of iodine and produced a blue color solution. The similar result was also revealed from the amount of cyclodextrins as a function of starch pretreatment temperature. Increments in starch pre-treatment temperature (80°C), resulted in increase in the amount of cyclodextrins up to 94% compared to the cyclodextrins production amount at the initial starch pretreatment temperature (30°C).

Apparently, the results indicate that cyclodextrins production depends on the starch pretreatment temperature as well as the structure transformation of starch granule. Employment of critical starch pretreatment temperature (80°C) promotes high cyclodextrins production (85% higher compared to without starch pretreatment) as well as better preservation of the starch granule structure (Figure 2). In addition, this condition provided additional advantage since the CGTase that was employed in this study (Toruzyme^{TM} which was obtained from Novozyme Sdn.Bhd.) possesses optimum temperature at 80°C. Subsequently, higher amount of cyclodextrins has been produced by employing the starch pretreatment of 80°C. In contrast, application of starch pre-treatment temperature above 80°C resulted in very viscous aqueous solution with most of the starch particle structure having collapsed, thus unsuitable to be used for recycled reaction purposes [3, 18].

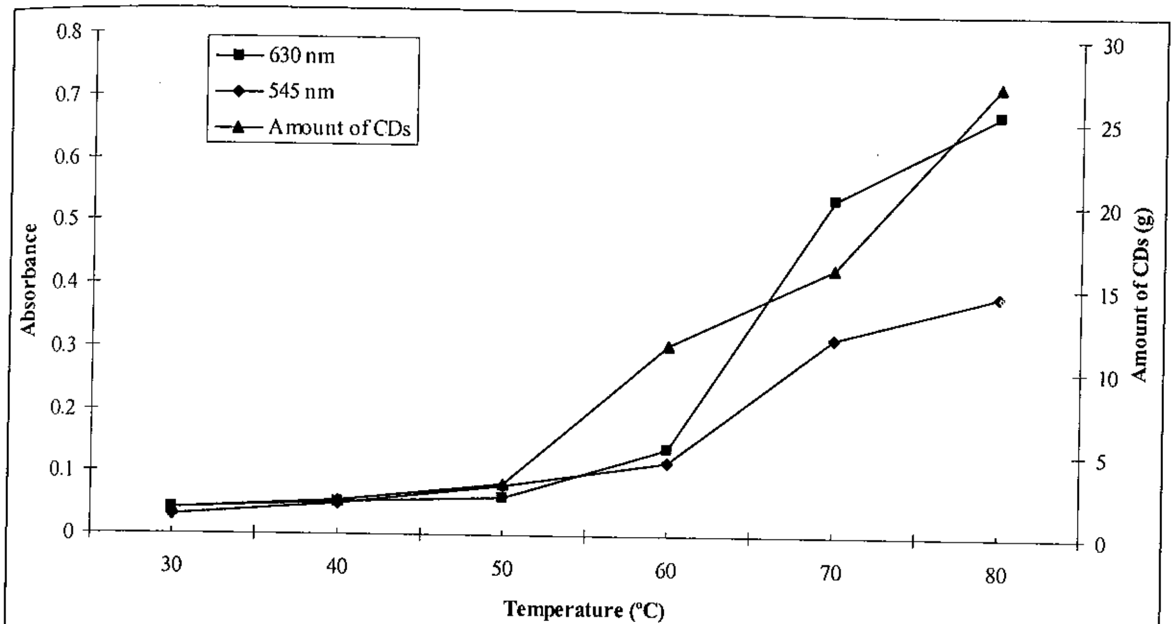

Figure 5. Absorbance of amylose-iodine complex (630 nm) and amylopectin-iodine complex (545 nm) obtained from the supernatant and the amount of cyclodextrins as a function of temperature

Effect of the starch pretreatment temperature on the types of cyclodextrins produced

In order to further identify the relationship between starch pretreatment temperature and types of cyclodextrins produced, a statistical analysis method (ANOVA) was employed. The relationship between the amounts of total cyclodextrins, α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin produced at various starch pretreatment temperature are shown in Figure 6. As seen in Figure 6, all types of produced cyclodextrins exhibited linear relationship with the starch pretreatment temperature. It is worth to note here that increase in starch pre-treatment temperature has been found to significantly influence the ratio of cyclodextrins mode produced. Apparently, the amount of α-cyclodextrin produced at the pretreatment temperature below 60°C was found to be higher than β-cyclodextrin, and γ-cyclodextrin produced. In contrast, increasing starch pretreatment temperature higher than 60°C resulted in greater conversion amount of β-cyclodextrin. In addition, the ANOVA analysis for the total amount of produced cyclodextrin, α-, β- and γ-cyclodextrin after three hours reaction time signified that the starch pretreatment temperature imparted significant effect to the total amount of cyclodextrin, α-cyclodextrin β-cyclodextrin and γ-cyclodextrin (the value of Prob>F less than 0.05). In fact, increasing the starch pretreatment temperature consequently increased the cyclodextrin amount. Besides that, all types of cyclodextrin also revealed strong relationship with all variables with Pearson correlation for the total amount of cyclodextrin, α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin of 0.970, 0.988, 0.951 and 0.895, respectively.

This could be reasoned due to the low temperature of starch pretreatment (below 60°C), a small quantity of α-1,4 bond (amylose and amylopectin) contained in the aqueous solution which reacts with CGTase to produce more low molecular weight of cyclodextrin (α-cyclodextrin). Nevertheless, increased starch pretreatment temperature (above 60°C), produced more quantity of α-1,4 bond (came from the expansion of amylopectin molecule and the amount of leached amylose that was trapped between amylopectin layers and within crystalline amylopectin) promoted better condition for CGTase to perform cyclization reaction with α-1,4 bond and subsequently generated high molecular weight of cyclodextrin (β-cyclodextrin).

Moreover, high degree of transformation will provide the starch structure to be reactive with CGTase. A mechanism of expanded amylopectin and leached-out amylose from starch granule were suggested in this study to further elaborate the above result as shown in Figure 7. The amylopectin was initially expanding with the escalation of exposure duration of starch pretreatment. Then, the residual amount of amylose that was trapped between the amylopectin layers and within the crystalline amylopectin started to leach out from the starch granule. Finally, the free amylose and the expanded amylopectin reacted with CGTase in aqueous solution to produce simple structure of cyclodextrins such as α-cyclodextrin.

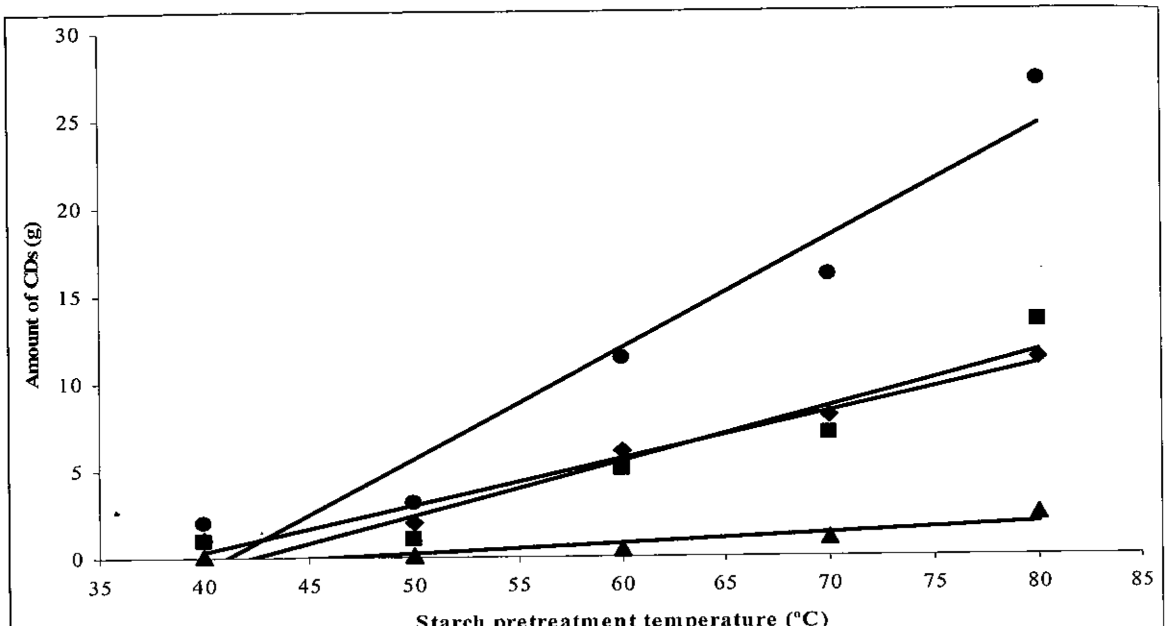

Figure 6. The effect of starch pretreatment temperature on type of produced cyclodextrins for an hour of exposure duration of starch pretreatment and 3 hours of reaction time. The amount of CD was: ●, Total CDs; ♦, α-CD; ■, β-CD; ▲, γ-CD

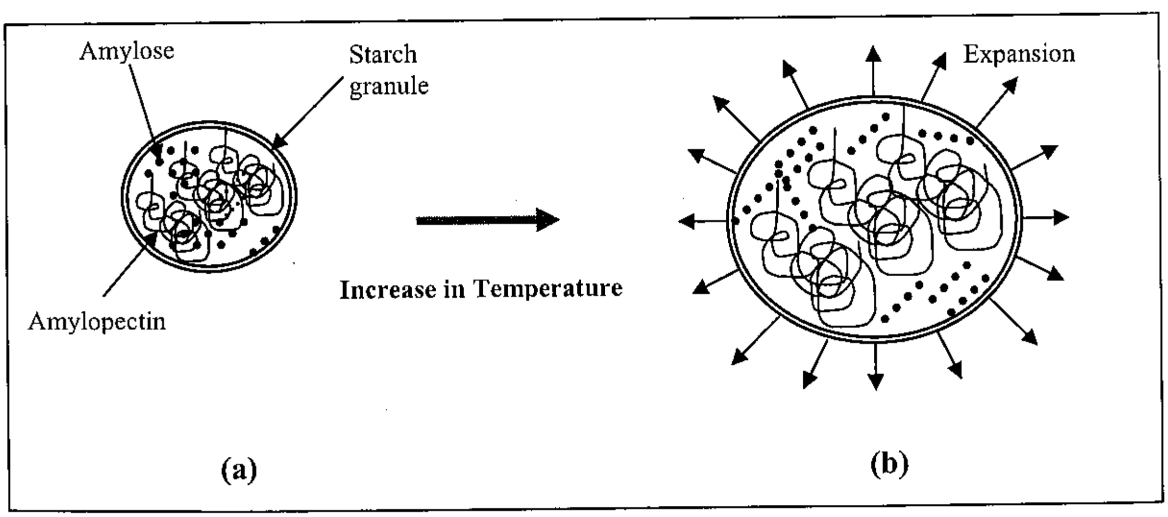

Figure 7. Schematic diagram of the expansion of amylopectin and amylose leaching mechanisms during starch pretreatment

CONCLUSION

This study showed that it is feasible to employ tapioca starch as a substrate to produce cyclodextrins using thermostable CGTase.

Low starch pretreatment temperature (below 60°C) produces small amount of cyclodextrins.

Increase in starch pretreatment temperature (higher than 60°C) resulted in greater conversion amount of β-cyclodextrin.

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