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The Simultineous Enzymatic Hydrolysis of Tapioca Starch for Instant Formation of Glucose

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Abstract: This study investigated the possibility of simultaneous reactions of the gelatinization, liquefaction and saccharification (SGLS) carried out at two reaction temperatures of saccharification 55 and 60°C for instant glucose production as well as controlling low viscosity of solute over the hydrolysis period. At 55°C, 10% (w/w) of the tapioca starch and 0.9 mL L^{-1} of a blending mixture of α-amylase and amyloglocosidase, the viscosity was kept low below 2.2×10^{-3} pa-s throughout the hydrolysis process. The conversion of the tapioca starch to glucose was as high as 65% (w/w) over 28 h of the hydrolysis time. Increasing the temperature to 60°C did not increase the conversion but, (1) increased the maximum rate of reaction from 8.89g L^{-1} h^{-1} to 13.3 g $L^{-1}h^{-1}$ (2) reduced the time to reach a half of the final glucose concentration from 6.1 to 5 h and also (3) slightly increased the earlier stage of solute viscosity without affecting the entire process.

Key words: Starch hydrolysis, amylase enzyme, instant glucose production, low temperature process affecting the entire process

INTRODUCTION

The conventional production of glucose from starch by the batch process has begun with mixing the starch powder with water to produce the starch slurry. The starch slurry is pumped through an extruder at high temperature above 105°C for five minutes retention time to completely gelatinising the starch. After the gelatinisation process, the gelatinised starch is mixed with a thermo stable α-amylase in a stir tank reactor and leave to react at 95°C around two hours the retention time (Linko and Javanainen, 1996). This reaction process is called liquefaction process that cuts specific bonding of polymers to produce short chains. The following process is saccharifacation process. This reaction needs low temperature about 60°C and 72 h retention time to complete the reaction Paolucci-Jeanjean (2000b).

It is clear that the limitation is of the conventional process are (1) formation of high viscous solute after the gelatinisation reaction requires high energy for mixing, (2) high temperature increases enzyme decay rate, (3) a heat exchanger may be required to reduce the temperature from 105°C during liquefaction reaction to 60°C for saccharification reaction to take place and (4) when the membrane reactor is to be used, a high viscous solute increases the gel layer fouling.

In this study, the batch simultaneous gelatinization, liquefaction and saccharification (SGLS) carried out at the optimum temperature of saccharification was proposed to replace the sequential enzymatic starch hydrolysis for glucose production. When the liquefying and saccharifying enzymes are added simultaneously in a stir tank reactor containing the starch milk and then heated to an optimum temperature of saccharification reaction, the simultaneous reaction between gelatinisation and liquefaction reactions should give a low solute viscosity provided the liquefaction reaction is slightly faster or the same rate as the gelatinisation rate. Furthermore, this simultaneous reaction scheme should also consume less reaction time as the continuous conversion of the intermediate substrate (gelatinised starch and liquefied starch) over time will keep high ratio of enzymes to substrates and with instantly produce the glucose as the final product.

EXPERIMENTAL SETUP

Commercial tapioca starch isolated from Manihot plant supplied by the Thai World Import and Export Co., Ltd was used throughout the experiment. Commercial enzymes of α-amylase (EC3.2.1.1) and amyloglocosidase (AMG) was prepared and supplied by Nova Nordisk, derived from *Basillus licheniformis* and *Aspergillus niger* (EC 3.2.1.3), respectively.

Simultaneous batch hydrolysis: The batch enzymatic hydrolysis of tapioca starch with a concentration of 10 % (w/v) was carried out in 250 mL flasks. Starch milk was prepared by adding 10 g tapioca starch powder in 100 mL RO water containing 50 ppm calcium chloride dehydrate. In the first experiment, different enzymes concentration were used; (1) 0.3 mL L^{-1} α-amylase enzyme, (2) an equal blending of α-amylase and amyloglucosidase enzymes with 0.15, 0.30, 0.45 and 0.60 mL L^{-1} and (3) the control experiment with no enzymes added. All flasks were covered with aluminium foil, incubated for 28 h at 55°C and agitated at 350 RPM. During hydrolysis process, 1 mL aliquots were withdrawn periodically using pipettes.

The experiment was repeated by fixing the ratio of enzymes to starch concentrations at 6 mL -enzymes per kilogram-starch to produce the concentration of the enzymes in the system consistent. Starch concentrations used were 5, 10, 15 and 20% (w/v) and an equal blending of enzymes at 0.15, 0.3, 0.45 and 0.6 mL L^{-1} were added, respectively. The batch hydrolysis experiment was also repeated for 60°C reaction temperature with 10% (w/v) tapioca starch and 0.9 mL L^{-1} of equal blending of enzymes.

Determination of viscosity: Aliquots collected from the batch SGLS and control experiments were submerged into a cooled glycol solution ($T<4$°C) to stop further enzymatic reaction and starch swelling. Viscosity of the samples was determined using a Bohlin CS Rheometer machine, type BR CS-50 at a constant temperature (30°C) and a cone spindle of 4° cone angle and a standard diameter of 20 mm.

Glucose determination by HPLC: The SGLS samples collected from the flask were diluted to 1/10 ratio with hydrochloric acid (*HCl*) solution fixed at pH 3-3.5 and $T<4$°C. Low pH and temperature should effectively stop the gelatinisation and enzymatic reactions as well as destroy the enzymes over a sufficient contact time. The treated sample was kept in a cold room at $T<4$°C for more then 3 h. Samples were then neutralised with sodium hydroxide solution to pH 6-8, filtered through a 0.45 μm nylon filter using a syringe into vials and analysed using HPLC. A Shimadzu HPLC with a refractometer index detector (RID-10A) and a sodium column with a guard were used for determining sugar concentrations. The column's operating temperature was maintained at 80°C. The eluent was 0.05% sodium azide in RO water and the flow rate was 0.3 mL min^{-1}.

RESULTS AND DISCUSSION

Viscosity of the SGLS: In the SGLS, viscosity of the solute can be controlled low when the concentration of the gelatinised starch is controlled. The factors that determine the concentration of the gelatinised starch are (1) the initial starch concentration, (2) the operating temperature and (3) the liquefied enzyme concentration. This study was to show that the solute viscosity of the SGLS can be kept low throughout the study period. As can be seen in Table 1, the sample's viscosity of the SGLS hydrolysed at reaction temperature of 55°C and then tested at a constant shear stress of 0.181 pa show an effectively constant over the period studied. Average shear rate was 83/s while the average viscosity was 2.2×10^{-3} pa-s.

Table 2 shows the viscosity of the control experiment. The result shows that the solute's viscosity has increased and the shear rate was reduced when a constant heating of 55°C was applied to the sample. These results clearly indicate that with the SGLS the sample's viscosity can be kept low over the entire hydrolysis process.

Furthermore, when a high temperature is used, high viscous of solute will be formed in the beginning of the hydrolysis process as reported by Paolucci-Jeanjean *et al*. (2000b).

Evaluation of pH during SGLS: Figure 1 shows change of pH with time recorded during the SGLS. As can be seen, the pH was initially unchanged for about 3 h before it exponentially decreased to a plateau giving a pH 4.3 after 25 h of the hydrolysis time. In the sequential starch hydrolysis, the pH of the reactant should be fixed in respect to the optimum pH required by the specific enzyme using buffer solutions. However, in the SGLS reaction, fixing the pH may not necessary since the change of pH was recorded over the entire hydrolysis process has naturally reduced.

In Fig. 1, it shows that the first three hours the pH was optimum for fast liquefaction reaction therefore buffers the increased viscosity and then when the substrate was depleted the pH decreased to the optimum pH for fast saccharification reaction therefore rapidly converted the liquefied starch into glucose.

Evaluations of the kinetic of instant production of glucose: The reaction rate kinetic of the SGLS is mainly determined by gelatinised and enzymatic reactions. The initial starch concentration (water content) and the

Table 1: The shear stress, shear rate and solute's viscosity of the SLGS

Time (min)	Shear stress (pa)	Shear rate (1/s)	Viscosity pa-s
0	0.181	80.7	2.24×10^{-3}
30	0.181	82.8	2.18×10^{-3}
105	0.181	93.3	1.94×10^{-3}
230	0.181	88.7	2.04×10^{-3}
390	0.181	69.1	2.62×10^{-3}
Avg.		82.9	2.20×10^{-3}

Condition: Starch concentration 10% (w/v), Enzymes concentration 0.9 mL L^{-1} and reaction temperature 55°C

Table 2: The shear stress, shear rate and solute's viscosity of the control experiment

Time (min)	Shear stress (pa)	Shear rate (1/s)	Viscosity pas
0	0.181	99.60	1.81×10^{-3}
15	5.050	4.00	1.26
30	5.050	2.13	2.38
110	5.050	1.98	1.97
250	5.050	0.50	10.10

Condition: Starch concentration 10% (w/v) and heating temperature 55°C

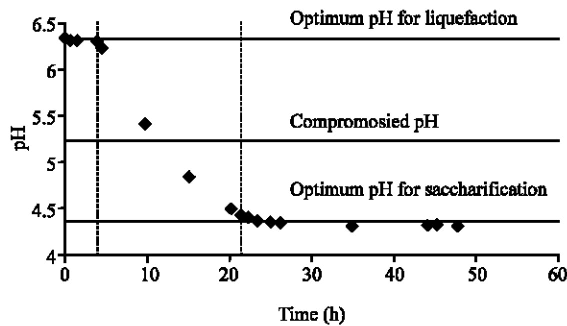

Fig.1: Change of pH with time recorded during the SGLS. (Condition: Starch concentration 10% (w/v), Enzymes concentration 0.9 mL L^{-1} and reaction temperature 55°C)

reaction temperature control the gelatinised starch concentration, while the enzymatic reaction relies on the intermediate substrate concentration and the number of active molecules of enzymes.

The enzyme activity or specific enzymatic reaction rate rely on the time for active enzyme molecules to move from one substrate to another (momentum and mass diffusivity) and the effective interaction time between the molecule of enzyme and the substrate to complete the reaction (relaxation time) which can be lumped sum as the effective enzymatic reaction time (EERT).

Either mass transport or relaxation time would limit the reaction rate; further investigation for a specific condition is required. Nonetheless, controlling the reaction temperature should give a constant of the relaxation time average. Enzyme activities also differ due to the momentum and diffusivity of the molecule of

Table 3: Summary of the kinetic parameters of instant production of glucose at different blending amylases concentration (Condition: starch concentration 100 g $L^{-1}$and T = 55°C)

	$\sigma_{E01} = 0.3$	$\sigma_{E0}^{mix} = 0.3$	$\sigma_{E0}^{mix} = 0.6$	$\sigma_{E0}^{mix} = 0.9$	$\sigma_{E0}^{mix} = 1.2$
X_A	0.018	0.27	0.61	0.64	0.65
τ(h)	7.200	8.00	7.00	6.10	5.00
Γ_{max}	0.270	2.88	6.67	8.89	10.80

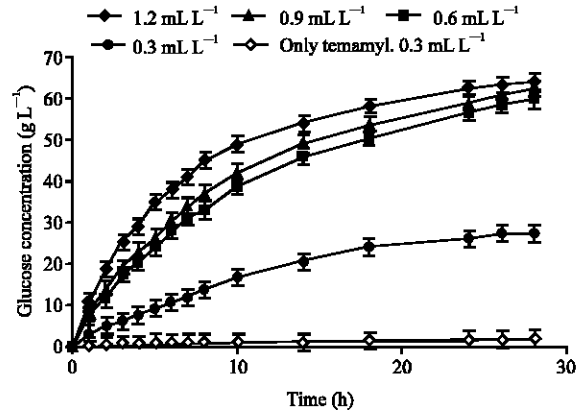

Fig. 2: The kinetic of instant production of glucose at different blending amylases concentration (Condition: starch concentration 100 g L^{-1} and T = 55°C)

enzyme and substrate moving from one to another, which also proportionate to e.g. Power number, Sherwood number, Reynolds number and Schmidt number.

Effect of enzymes concentration: To study the effect of enzyme concentration on the rate of reaction, experiment was designed under a fixed starch concentration, a constant reaction temperature and a constant agitation rate. Figure 2 shows the effect of the blending enzymes concentration on the kinetic of instant production of glucose. As can be seen, when sufficient enzymes concentration (>0.6 m L L^{-1}) was used, the maximum substrate conversion to glucose over the period studied has slightly changed ranging from the value of X_A from 0.58 to 0.64, while using 0.3 mL L^{-1} produced lower final glucose concentration with X_A = 0.28.

However, using the Termamyl enzyme (α-amylase) individually without adding a saccharifying enzyme gave the lowest maximum glucose concentration with less than 2 g L^{-1} of glucose (X_A = 0.02). Table 3 shows the kinetic parameters of final glucose concentration, time to obtain half of glucose concentration and the maximum reaction rate obtained from Fig. 2. This evaluation shows that when more than 0.6 mL $L^{-1}$of a blending amylases enzyme was used, the half time to obtain the maximum glucose concentration decreased and the maximum rate of reaction increased.

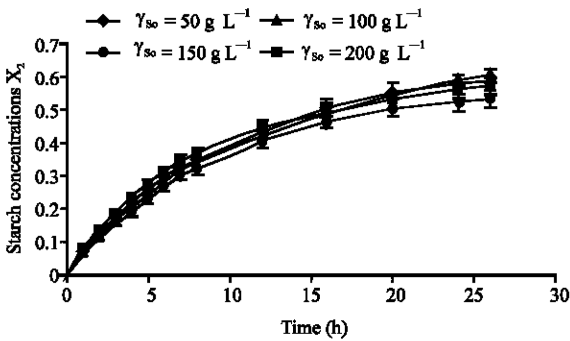

Fig. 3: The kinetic of glucose conversion at different starch concentrations and at 6 mL kg^{-1} enzymes to starch ratio and T = 55°C

This result indicates that the minimal blending amylases enzyme concentration should not be less than 0.6 mL L^{-1} for producing more or less the same glucose concentration; otherwise the mass transfer would limit the conversion rate.

Increasing the enzymes concentration increased the number of active enzymes thus increasing the maximum reaction rate however the final conversion remained the same that due to the amount of the gelatinised starch stayed constant which was limited by the initial starch concentration which speeds up and the reaction temperature.

Despite a very high enzymes concentration fasters the reaction rate, it will increase some sort of enzyme-enzyme inhibition (Ozbek *et al*., 2001) and enzyme competition to the substrate thus decreasing the enzyme activity and increasing the cost of production.

Effect of the starch concentration: To study the effect of starch concentration, the experiment was designed under a fixed ratio of the blending of amylases enzyme to starch at 6 mL kg^{-1} starch, a reaction temperature and an agitation rate. Figure 3 shows the effect of different starch concentrations on the kinetic of instant glucose production. As can be seen, 50 g L^{-1} substrate gave slightly low final conversion at 0.53 than the others, which reached effectively, the same final conversion at 0.6 over the period studied.

Table 3 shows the kinetic's parameters of the final glucose concentration, time to obtain half of glucose concentration and the maximum reaction rate obtained from Fig. 3. As can be seen, when the starch concentration was changed from 50-200 g L^{-1} and the enzymes concentration was fixed to 6 mL kg^{-1} of starch, the maximum reaction rate increased from 2.81 to 12.8 g L^{-1}h. The time to obtain half of maximum production showed low when 50 g L^{-1} of starch was used. However, it increased to a maximum value when 100 g L^{-1} of starch use and then decreased again when starch of less than 100 g L^{-1} used.

The time to obtain a half of the maximum glucose production was low when 50 g L^{-1} starch was used. This is due to the low final substrate to glucose conversion at 0.53. Low conversion of 50 g L^{-1} starch within the period studied was corresponding to a low gelatinised starch and enzymes concentrations in the system which increased the required time for effective momentum and diffusivity. Extending the hydrolysis time of 50 g L^{-1} starch should lead to an increase in starch to glucose conversions to an effectively the same level with others.

These results suggest that within a certain range of the enzymes and starch concentrations, the final conversion will be effectively the same. Furthermore, lowering the starch and enzymes concentrations lead to a low in the final conversion.

Very high enzymes and starch concentrations may lead in producing a lower final conversion due to enzymes-enzymes inhibition (Ozbek *et al*., 2001), product inhibition (Lim *et al*., 2003) and insufficient water content for starch swelling and gelatinisation (Tester and Sommerville, 2000).

Enzymes activity: Enzymes activity analysis will look closely at the productivity of the enzymes at molecule level. The highest specific molecule productivity, the so called the highest enzymes activity, is relative to the highest glucose and is produced over a short period of time by every milliliter of the enzymes as shown in Equation 1 below.

$$E_{activity} = \frac{\Gamma_{max}}{\sigma_{E_0}^{mix}} \quad (1)$$

Where:

$E_{activity}$ = The enzyme activity.
Γ_{max} = The maximum reaction rate.
$\sigma_{E_0}^{mix}$ = The blending concentration of amylases.

As can be seen in Fig. 4, when the starch concentration was fixed at 100 g L^{-1}, the ratio of 3 mL enzymes per kilogram substrate gave low enzyme activity of 9.6 g mL^{-1} h. The enzymes activity was increased to the highest value of 11.12 g mL^{-1} h when the ratio was 6 mL kg^{-1} and then decreased again to the lowest value of 9 g mL^{-1} h when the ratio of 12 mL kg^{-1} was used.

When the ratio of enzymes to starch was fixed at 6 mL kg^{-1}, enzymes activity was low when 0.3 mL L^{-1} of the enzymes and 50 g L^{-1} of the substrate were used. The enzyme activity increased to the highest value of

Table 4: Summary of the kinetic parameters of instant production of glucose at different starch concentration (Condition: $\sigma_{E0}^{mix}/\gamma_{so}$ = 6 mL kg^{-1} and T = 55°C)

	γ_{So} = 50	γ_{So} = 100	γ_{So} = 150	γ_{So} = 200
X_A	0.53	0.61	0.60	0.58
τ(h)	6.00	7.00	6.50	5.50
Γ	2.81	6.67	10.00	12.8

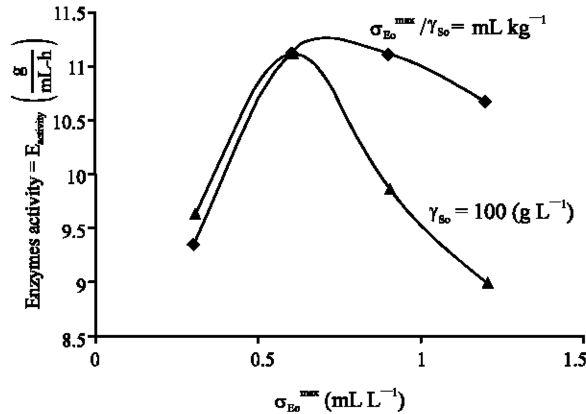

Fig. 4: Plots of enzymes activity at T = 55°C

11.4 g mL^{-1} h when the enzymes concentration kept within the range of 0.6 to 0.9 mL L^{-1} and the substrate within 100 to 150 g L^{-1}. Enzymes activity is then decreased to 10.67 g mL^{-1} h when 1.2 mL L^{-1} of enzymes and 200g L^{-1} of substrate were used.

The low activity of the enzymes when less than 0.6 mL L^{-1} l of enzymes is due to mass transfer limiting the contact time between the enzymes and substrate. When the starch concentration was fixed and the enzymes concentration was increased, the enzymes activity is reduced due to low supply of the gelatinised starch in the system. However, when the ratio of the enzymes to substrate was fixed, the enzymes activity was slightly lower at high concentration, which may be due to insufficient water content in the system and enzymes-enzymes inhibition.

Effects of temperature: Previously, Marchal *et al.* (1999) studied the simultaneous gelatinisation and liquefaction hydrolysis for amylopectin potato starch at different temperatures of 50, 70 and 90°C using bacillus *licheniformis* α-amylase. They also found that the glucose concentration has not significantly increased by increasing the temperature but has effectively increased the concentration of high degree polysaccharides. Linko and Javanainen (1996) carried out the simultaneous liquefaction, saccharification and lactic acid fermentation on barley starch.

The simultaneous hydrolysis at temperatures of 37 and 60°C has produced an effectively the same glucose concentration over 25 h hydrolysis time. However, during 5 h hydrolysis time, the starch hydrolysed to glucose at temperature of 60°C was about 50% while at temperature of 37°C it was only about 25%. This result suggests that low hydrolysis temperature would give a low glucose production rate but the final conversion is remained the same.

Table 5: Summary of the glucose production rate by two temperatures. (Condition initial starch concentration, γ_{so} = 100 g L^{-1} and enzymes blending concentration, σ_{Eo}^{mix} = 0.9 mL L^{-1})

	55°C	60°C
$\gamma_{pglucose}^{max}/\gamma_{So}$	0.63	0.640
τ(h)	6.10	5.000
Γ_{max}	8.89	13.30
$E_{acticity}^{max}$(g mL^{-1} h)	9.88	14.78

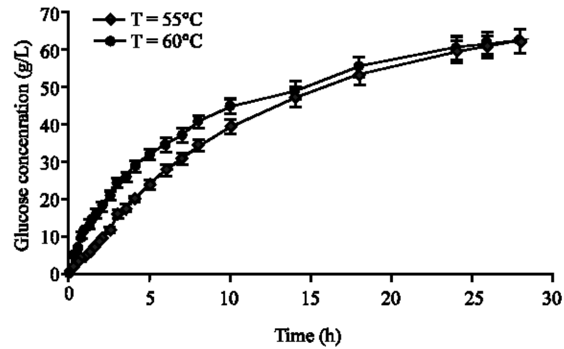

Fig. 5: Temperature effects on the kinetic of instant glucose production. (Condition initial starch concentration, γ_{so} = 100 g L^{-1} and enzymes blending concentration, σ_{Eo}^{mix} = 0.9 mL L^{-1})

Figure 5 shows the glucose production by SGLS at T = 55 and 60°C. The initial starch concentration was fixed at 100 g L^{-1} and the enzymes to starch ratio was fixed at 0.9 mL L^{-1} As can be seen, initially the glucose concentration at T = 60°C was high and reached faster the maximum glucose concentration at 65 g L^{-1} than when T = 55°C was used. However, the maximum glucose concentration effectively reached the same level. Similar results were reported by other authors but different operating conditions were used (Linko and Javanainen, 1996; Paolucci-Jeanjean *et al.*, 2001).

These results suggest that a higher temperature increases the enzymes activity and the initial starch conversion rate to gelatinised starch, but the maximum gelatinised starch conversion would not change with small changes of temperature, thus the maximum glucose concentration reaches an effectively the same level.

The temperature effect on the maximum reaction rate, the substrate conversion and τ value is shown in Table 5. As can be seen, when the γ_{So} at 100 g L^{-1} and the σ_{Eo}^{mix} at 0.9 mL L^{-1} were fixed, increasing the temperature

from 55 to 60°C has reduced the τ value from 6.1 to 5 h. The highest enzymes activity was also increased from 8.89 to 14.78 g mL^{-1} h.

CONCLUSION

In conclusion, the simultaneous reaction of SGLS carried out at optimum temperature for saccharification can produce instantly the glucose as high as 65% conversion and the solute's viscosity can be kept low below 2.2×10^{-3} pa-s over the entire of hydrolysis process. As the SGLS can produce glucose instantly as well as low solute's viscosity throughout; these advantages suggest to use the membrane process for continuous production of glucose.

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