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BIOCONVERSION OF STARCH TO MALTOOLIGOSACCHARIDES (MOS) BY THE REACTION OF MALTOGENIC AMYLASE

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Abstract

Maltogenic amylase is one of the significant enzymes in oligosaccharides synthesis. Its ability to utilise multiple substrates and catalyse hydrolysis and transglycosylation reactions simultaneously makes it a unique biocatalyst. The catalysis could be exploited in many ways to obtain oligosaccharides of different lengths and various modified sugars. Nonetheless, one of the major drawbacks of substrate hydrolysis to produce oligosaccharides is the low production of MOS with higher degree of polymerisation. To address this issue, reaction parameter optimisation was performed via one-factor-at-a-time (OFAT) approach on the production of MOS from soluble starch hydrolysis using maltogenic amylase from *Bacillus lehensis* G1 (MAG1). Optimisation of MAG1 loading, soluble starch loading, temperature, time and pH resulted in the production of 84.87 mg/g MOS with polymerisation degree of 3 to 7 compared to that of 51.60 mg/g obtained before the optimisation process, which recorded 1.64-fold increment. Among all parameters, soluble starch loading gave the most significant impact on the MOS production as the reaction equilibrium is highly affected by substrate concentration. The occurrence of MOS with polymerisation degree of 4 and above, which resulted from starch hydrolysis further confirms the endo-type of MAG1. Because starch is an abundant and inexpensive source of carbohydrate in the world, this study provides a cost-effective MOS production process which is highly relevant for industry.

Keywords: Maltogenic amylase, maltooligosaccharides, starch, degree of polymerisation

Abstrak

Maltogenik amilase merupakan antara enzim terpenting dalam penghasilan oligosakarida. Enzim ini mempunyai keunikan tersendiri kerana kebolehannya menggunakan beberapa jenis substrat dan memangkin tindak balas hidrolisis dan transglikosilasi secara serentak. Ciri ini boleh dieksploitasi dengan pelbagai cara bagi mendapatkan oligosakarida berbeza saiz serta pelbagai gula termodifikasi. Namun, antara kekangan utama di dalam hidrolisis substrat bagi penghasilan oligosakarida adalah penghasilan MOS berdarjah pempolimeran tinggi yang rendah. Bagi mengatasi isu ini, pengoptimuman parameter tindak balas menggunakan kaedah satu-faktor-pada-setiap-masa (OFAT) bagi penghasilan MOS daripada tindakbalas hidrolisis kanji dijalankan menggunakan maltogenik amilase daripada *Bacillus lehensis* G1 (MAG1). Penghasilan sebanyak 84.87 mg/g MOS dengan panjang rantaian polimer daripada 3 ke 7 unit telah direkodkan menerusi pengoptimuman beban MAG1, beban kanji larut, suhu, masa dan pH, dibandingkan dengan hanya 51.60 mg/g MOS yang terhasil sebelum proses pengoptimuman. Ini menunjukkan peningkatan sebanyak 1.64 kali ganda. Beban kanji larut merupakan parameter yang paling memberi kesan kepada penghasilan MOS. Ini kerana keseimbangan tindak balas amat dipengaruhi oleh faktor tersebut. Penghasilan MOS dengan panjang rantaian polimer 4 unit ke atas membenarkan bahawa MAG1 adalah enzim yang mempunyai ciri endo. Hasil kajian ini mencadangkan satu proses penghasilan MOS yang lebih murah dan relevan dengan industri kerana melibatkan penggunaan bahan mentah iaitu kanji yang murah dan mudah didapati.

Kata kunci: Maltogenik amilase, maltooligosakarida, kanji, darjah pempolimeran

1.0 INTRODUCTION

Oligosaccharides are carbohydrate polymer made up of several numbers of monosaccharide generally from three to ten monomer units that are linked by glycosidic bonds. Its attributes are determined by polymerization degree, composition and glycosidic linkages [1]. Oligosaccharides have broad applications as food ingredients, prebiotic supplements, drug delivery, cosmetics, animal feed and agrochemicals. Of late, this carbohydrate has garnered tremendous attention due to consumer preference for food beneficial for health [2, 3].

Oligosaccharides are procured by isolation from natural sources such as fruits, vegetables and milk by approaches that take advantage of size, stability or combination of these and other properties of the molecule of interest [4, 5]. Furthermore, it can also be synthesized by physical, chemical extraction method and/or enzymatic process [6, 7].

Nonetheless, various approaches have been explored in order to meet the increasing market demand for oligosaccharides [8]. Nowadays, enzymatic synthesis has been recognized as alternative tools for oligosaccharides synthesis as it offers stereochemical specificity and involves simpler steps as oppose to the tedious procedures required in chemical synthesis [9]. Besides, it is more environmentally friendly, without the use of noxious chemicals and being aligned with the perspective of a biodegradable process [10]. Glycosyl transferases (EC 2.4) and glycosyl hydrolases (EC 3.2) are the classes of enzymes that able to synthesise oligosaccharides [11].

Gylcosyl hydrolases utilise inexpensive, simple and easy-to-obtain saccharides such as starch into smaller saccharide units for instance glucose, maltodextrin and maltooligosaccharides (MOS) [12]. Sugar producing-enzymes such as α-amylase [EC 2.3.1.1], maltogenic amylase (EC 3.2.1.133), cyclodextrinase (EC 3.2.1.54), cyclodextrin glucanotransferase (EC 2.4.1.19) and pullulanase (EC 3.2.1.4) are from the same family yet demonstrated to have different substrate specificity [13]. The former enzyme exhibited affinity towards starch [14] whilst the others were revealed to have inclination towards cyclodextrin, pullulan and starch as substrates [11].

Maltogenic amylase from glycosyl hydrolase family 13 (GH13) is a potential catalyst for maltooligosaccharide production [11]. This enzyme catalysed the formation of glycosidic linkages to yield oligosaccharides and various modified sugars. In addition, it also exhibited transglycosylation activity that forms various length sugar molecules [15, 16]. Despite that, the major drawback of using this enzyme for oligosaccharide production is the ineluctable hydrolysis activity that hydrolysed the newly synthesised oligosaccharides.

Formation of oligosaccharides via enzymatic method is influenced by various reaction conditions such as substrates, substrate concentration, enzyme concentration, temperature, pH and time. Previously, maltogenic amylase from *Bacillus lehensis* G1 (MAG1) has successfully been used to produce maltooligosaccharides from hydrolysis of beta-cyclodextrin (17). This present article reports the optimisation of reaction parameters enhancing MOS production specifically MOS with degree of polymerization (DP) 3 to DP7 by maltogenic amylase from MAG1 using soluble starch as the substrate. The effects of MAG1 loading, soluble starch loading, temperature, time and pH on the production of MOS were investigated.

2.0 METHODOLOGY

2.1 Bacterial Strains and Media

Recombinant maltogenic amylase (MAG1) encoding gene from *B. lehensis* G1 (GenBank accession number: KJ416416) that was prepared in previous study by Abdul Manas *et al*. (2014) was used. pET21a(+) from Novagen (Merck KGaA, Darmstadt, Germany) and *E. coli* BL21 (DE3) (Promega, Madison, WI, USA) were used respectively as the expression plasmid and expression host. Cells harboring the recombinant plasmid were grown in Luria-Bertani (LB) media supplemented with 100 µg/ml ampicillin.

2.2 Enzyme Production and Purification

MAG1 protein was expressed in *E. coli* BL21 (DE3) and was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after the OD_{600} reached ~0.5-0.7. The culture was grown in LB medium supplemented with 100 µg/ml ampicillin at 30 °C for 12 hours in shaking condition. Then, the cells were harvested by centrifugation at 8,000 ×g for 20 min. Affinity chromatography purification was performed using nickel-nitrilotriacetic acid (Ni-NTA) column HisTrap™ HP (GE Healthcare, Little Chalfont, Buckinghamshire, UK) via ÄKTAPrime Plus chromatography system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Phosphate-buffered saline containing 20 mM Na_2HPO_4 and 500 mM NaCl, pH 7.4 was used to remove the unbound proteins whilst the same buffer with the additional of 500 mM imidazole was used to elute MAG1 by gradient elution. The efficiency of the purification was determined by SDS–PAGE [18].

2.3 Protein Concentration Measurement and Enzyme Assay

MAG1 concentration was determined by using Bradford method [19] with bovine serine albumin as the standard. MAG1 activity was measured according to Miller, (1959) dinitrosalicylic acid (DNS) method [20]. β-cyclodextrin (β-CD) in 50 mM potassium phosphate buffer, pH 7.0 was used as the

substrate. A mixture of 100 µl enzyme, 250 µl 1% β-CD (w/v) and 150 µl buffer was incubated at 40 °C for 10 min. Subsequently, 500 µl DNS reagent was added and the reaction mixture was immediately boiled for 5 min to stop the reaction. The mixture was then cooled to room temperature. The amount of reducing sugars released was analysed at 575 nm by UV–Vis spectrophotometer using maltose as a standard. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of maltose per min under optimal conditions.

2.4 MOS Quantification

The samples resulted from enzymatic hydrolysis of soluble starch were filtered through a 0.2 mm syringe filter prior to injection into the high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA). A Rezex RSO-Oligosaccharide Ag^+ 4% guard column (60 mm x 10.00 mm, Phenomenex, Torrance, CA, USA) in line with a Rezex RSO-Oligosaccharide Ag^+ 4% analytical column (200 mm x 10.00 mm, Phenomenex) was used to analyse the hydrolysis product with ultra-pure water as the mobile phase. The elution was monitored using a refractive index (RI) detector at 75 °C with 0.2 mL/min flowrate.

2.5 Optimisation of MOS Production Using One-Factor-At-a-Time (OFAT) Method

To obtain the optimal reaction condition for enhanced MOS production, five factors including enzyme loading, substrate loading concentrations as well as temperature, time and pH were investigated by the OFAT method.

3.0 RESULTS AND DISCUSSION

3.1 Purification of MAG1

MAG1 was expressed in *E. coli* BL21 (DE3) and purified to homogeneity via affinity chromatography (Figure 1). High level of MAG1 expression was shown by the high activity of crude enzyme which was 756.84 U/mL (Table 1). The crude enzyme was purified to homogeneity as exhibited by SDS-PAGE at the expected size of 68 kDa (Figure 1). The purification of recombinant MAG1 resulted in the recovery of the enzyme as high as 69.89 % from the total activity of MAG1 with a 34.17-fold increase in the specific activity.

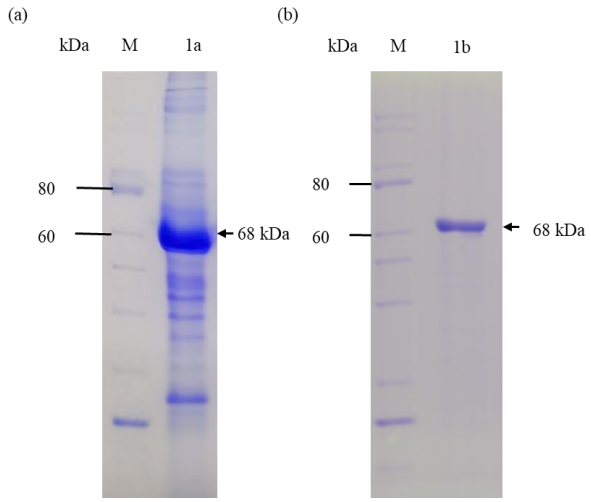

Figure 1 SDS-PAGE profile of (a) crude MAG1 and (b) purified MAG1. Lane M: Protein marker; Lane 1a: Crude MAG1; Lane 1b: Purified MAG1. The size of MAG1 is approximately 68 kDa

3.2 Effect of MAG1 Loadings on Soluble Starch Hydrolysis

Different MAG1 loadings (0.5 U to 8.0 U) were studied to determine its effect on soluble starch hydrolysis. From this experiment, it was revealed that the specific product of MAG1 could be achieved by manipulating the enzyme loading. 1.0 U of enzyme loading resulted in the highest MOS (M3-M7) production which was 71.38 mg/g soluble starch (Figure 2a) and it was used for further substrate loading study. However, the MOS yield decreased gradually as the enzyme concentration increased. Conversely, the appearances of higher DP of MOS (M5 and M6) were more apparent in higher unit of MAG1.

From this finding, it can be explained that when high enzyme concentration is supplied, the hydrolysis occurs in two steps; hydrolysis of starch to MOS and followed by hydrolysis of MOS to produce maltose and glucose. This is verified by the higher amount of glucose and maltose produced in 8.0 U of MAG1 compared to lower units of MAG1 (0.5 U and 1.0 U) (Figure 2b). When higher unit of MAG1 was used, in the excess of active site condition, M3 and M4 were proceed to subsequent hydrolysis steps to produce glucose and maltose. Simultaneously, it could be observed that the production of M5 and M6 were increased and accumulated. Thus, it was suggested, that transglycosylation also occurred at the same time by MAG1.

Table 1 Purification table of MAG1 using nickel affinity chromatography

Fraction	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	10	756.84	7568.40	106.19	71.27	1.00	100.00
Purified	2	2643.42	5289.85	2.17	2435.54	34.17	69.89

Maltooligosaccharide-forming amylase (MAF) from *Bacillus circulans* was reported to exhibit similar characteristics as it produced G5 as major product at the early stage of hydrolysis. After the reaction time was prolonged, the high concentration of enzyme further hydrolysed the product into glucose, maltose and G3 as the final products [21]. Other research also reported similar action pattern of MAF where G2 and G4 were accumulated as the final products whilst G5 and G6 were hydrolysed [22]. Excessive hydrolysis was undesirable as it leads to low yields of oligosaccharides. It is important to prevent oligosaccharides from being further hydrolysed to smaller sugars.

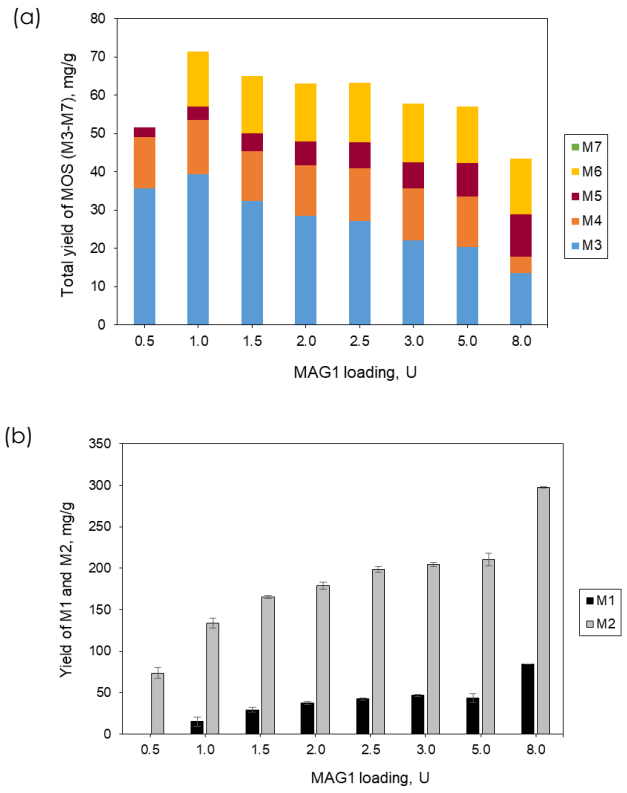

Figure 2 (a) Effect of different MAG1 loadings on the soluble starch hydrolysis (b) Yield of glucose (M1) and maltose (M2)

3.3 Effect of Substrate Loadings on the Soluble Starch Hydrolysis

Different starch concentrations in the range of 0.25 to 2 % (w/v) were screened. Figure 3a indicates that 0.5 % (w/v) soluble starch used resulted in the highest MOS production. A total of 72 mg/g MOS produced consisting of 38.67 mg/g M3, 20.93 mg/g M4, 4.2 m/g M5 and 8.2 mg/g M6. It was observed that further increase in the starch concentration resulted in a decrease of total MOS production. However, the appearances of higher DP (M6) of MOS was more apparent in higher concentrations of starch (1.0 % and 2.0 % (w/v)) used.

From this finding, it can be explained that when low starch concentration is supplied, MOS were concurrently hydrolysed to produce maltose and glucose. This is proven by the higher amount of glucose and maltose produced in 0.25 % and 0.5 % (w/v) starch compared to 1.0 % and 2.0 % (w/v) starch (Figure 3b). When higher concentration of starch was supplied in the limited active site condition (at the same enzyme concentration), higher DP of MOS was produced and accumulated but did not proceed to subsequent hydrolysis steps to produce glucose and maltose. This is proved by lower glucose and maltose produced at higher starch concentration.

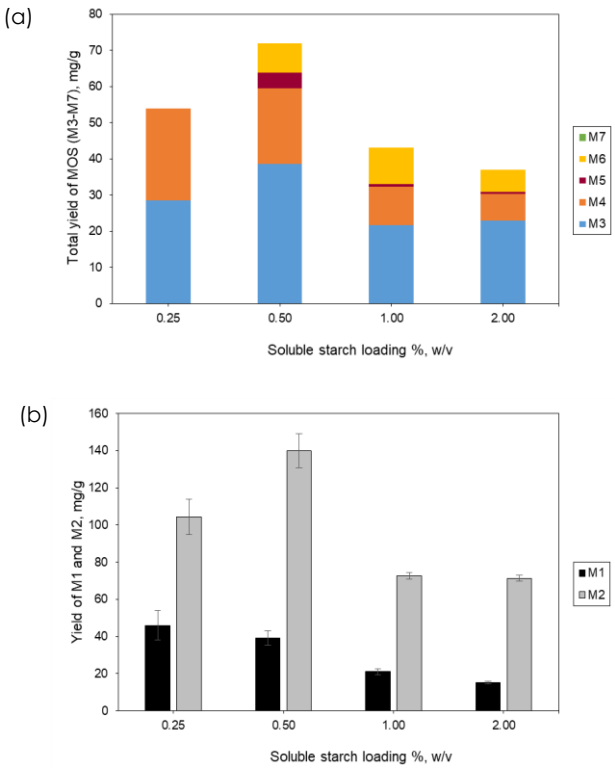

Figure 3 (a) Effect of substrate loadings on the soluble starch hydrolysis (b) Yield of glucose (M1) and maltose (M2)

MAG1 is an endo-acting enzyme that does not require reducing sugar end to perform hydrolysis. It was proven by its ability to hydrolyse β-cyclodextrin, a cyclic carbohydrate polymer consists of seven glucose molecules [17]. The finding in this study further confirms the endo-type characteristic of MAG1 when it was able to produce MOS with DP of more than two by its hydrolysis activity, although the active site only extends until +2 subsite [23]. Other study also reported the similar endo-type behaviour of maltogenic α-amylase [24].

3.4 Effect of Incubation Time on the Soluble Starch Hydrolysis

The effects of different incubation time ranging from 0.17 hours (10 minutes) to 24 hours were analysed (Figure 4a). Enzyme loading and substrate loading were fixed to 1.0 U and 0.5 % (w/v), respectively. The yield of MOS (M3-M7) increased and become constant after 1 hour of incubation. Interestingly, the amount of M6 increased significantly at 1 hour and remained almost constant with further incubation. Prolonged incubation provided sufficient time for complete hydrolysis reaction to occur. From this result, it was suggested that the substrate was completely hydrolysed by MAG1 after one hour. It was proven by the constant yield of glucose, maltose and MOS produced after 1 hour of incubation (Figure 4b). Therefore, it can be concluded that 1 hour was the optimum incubation time for maximum MOS production.

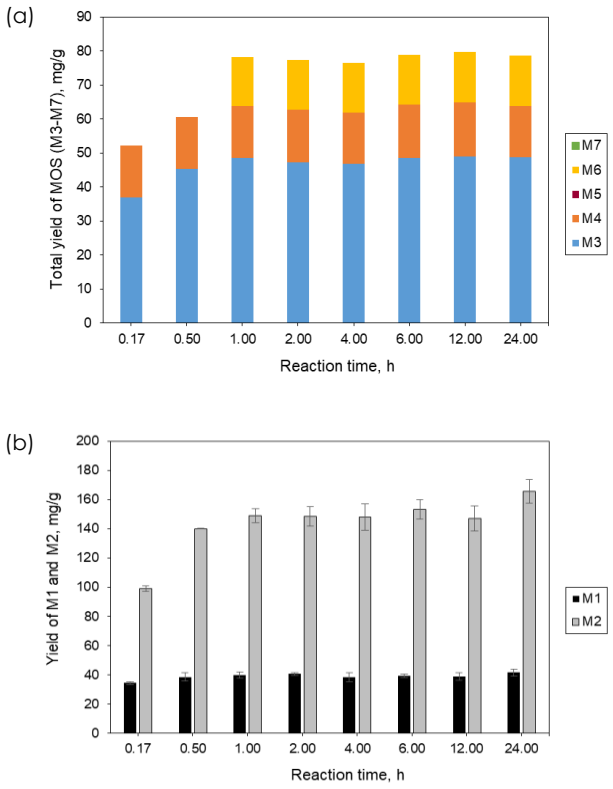

Figure 4 (a) Effect of incubation time on the soluble starch hydrolysis (b) Yield of glucose (M1) and maltose (M2)

3.5 Effect of Temperature on Soluble Starch Hydrolysis

The influence of different temperatures (20, 30, 40, 45, 50 and 60 °C) was tested for the hydrolysis of soluble starch with MAG1. It was observed, higher DP of MOS were only apparent in moderate temperatures in the range of 20-40 °C, providing a maximum MOS yield in the experiment carried out at 40 °C (Figure 5). A total of 83.40 mg/g MOS produced that consists of 40.47 mg/g M3, 21.73 mg/g M4 and 21.20 mg/g M6. Though, at an increase reaction temperature of 45-60 °C resulted in a decrease of MOS production.

This result can be related to the lack of thermal stability of maltogenic amylase from *B. lehensis* G1, for which such stability at this temperature has been described previously [11]. MAG1 was reported to retain about 50 % of its activity at 40 °C within 10 minutes. MAG1 stability decreased after 40 °C causing a decline in its activity, thus, the MOS production was also reduced. It was verified by the yield of glucose, maltose and MOS produced at 45-60 °C. Therefore, 40 °C was selected as the temperature for the hydrolytic condition of the MOS production because it provides maximum MOS yield with moderate glucose and maltose production.

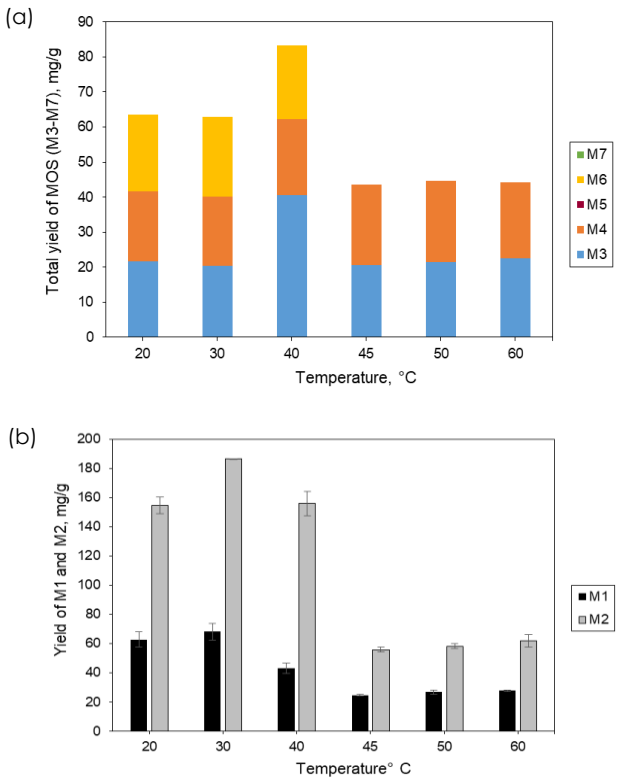

Figure 5 (a) Effect of temperature on the soluble starch hydrolysis (b) Yield of glucose (M1) and maltose (M2)

3.6 Effect of pH on Soluble Starch Hydrolysis

At a fixed value of enzyme loading, substrate loading, reaction time and temperature obtained

from the previous screening, the effects of different pH values ranging from 6 to 10 were analysed for soluble starch hydrolysis (Figure 6a). From pH 6 to pH 7, there were no significant differences (<10 %) observed on MOS production. A gradual decrease in maltooligosaccharides production was observed from pH 8 to pH 10. Lowest MOS was detected at pH 10 with the yield of 47.53 mg/g. Such decline was possibly resulted from the enzyme structure denaturation at higher pH [25]. As observed, MOS was highly produced at pH 7 (84.87 mg/g) (Figure 6a), while glucose and maltose were highly produced at pH 8 (51.47 and 190.90 mg/g, respectively) (Figure 6b).

These variations were caused by the changes in ionization states of important residues in the active site when pH was manipulated. In fact, the changes would alter the active site conformation [26]. This would consequently affect binding mode of the substrates in the enzyme active site, hence the product variation. This finding suggests that MAG1 hydrolysis was best performed at neutral condition for which the highest MOS was produced. MAG1 was also found to be the most active and stable in this condition [17]. Judging from the high level of MOS produced (Figure 6a) and moderate glucose and maltose level (Figure 6b), pH 7 was chosen as the optimum pH to produce high MOS content from starch.

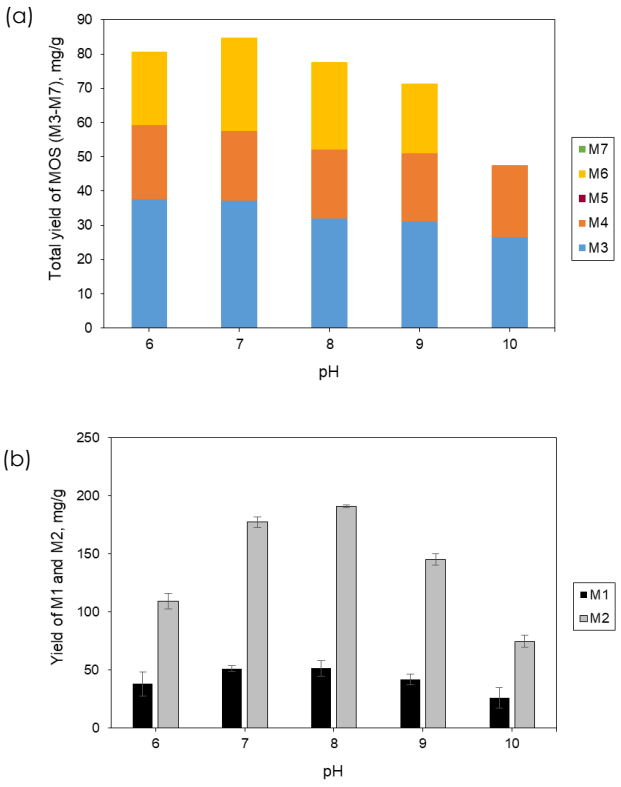

Figure 6 (a) Effect of pH on the soluble starch hydrolysis (b) Yield of glucose (M1) and maltose (M2)

4.0 CONCLUSION

MAG1 exhibits dual catalytic reaction (hydrolysis and transglycosylation) owing to its unique structural properties. It is a proficient biocatalyst that yields maltooligosaccharides of various lengths. MAG1 reactions in the optimised conditions were able to enhance maltooligosaccharides production in particular MOS with degree of polymerisation 3 to 7 efficiently.

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