ENZYMATIC CONVERSION STRATEGIES OF STARCH TO MALTOOLIGOSACCHARIDES BY MALTOGENIC AMYLASE AND CYCLODEXTRIN GLUCANOTRANSFERASE

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ABSTRACT

In nature, *Bacillus lehensis* G1 utilizes extracellular cyclodextrin glucanotransferase (CGTase) to degrade starch into cyclodextrins (CDs). This is followed by hydrolysis of CDs by intracellular maltogenic amylase (MAG1) into glucose, maltose and maltooligosaccharides (MOS). The MOS are potential prebiotic for human consumption. In industries, amylases are used to produce MOS from starch. However, the conversion of starch directly to MOS using MAG1 has several limitations such as low specificity towards starch compared to β-cyclodextrin (β-CD) and low productivity of MOS. In order to overcome these drawbacks, two strategies involving optimization of reaction parameters using statistical method and synergism of enzyme mixture approach, were applied. In this study, the optimization of enzymatic reaction parameters for enhanced MOS production by MAG1 using soluble starch as a substrate was performed. In the first strategy, the effects of reaction parameters (enzyme loading, substrate loading, temperature, reaction time and pH) on MOS yield was investigated using one-factor-at-a-time (OFAT) method and 2^{5-1} fractional factorial design. Based on the 2^{5-1} fractional factorial design results, three parameters namely substrate loading, reaction time and pH were found to have a significant effect and was used in central composite design under response surface methodology (RSM). The MOS production was successfully optimized by the RSM. Under the optimized conditions (0.25 % (w / v) of substrate loading, 0.5 h of reaction time and pH 7.45) by RSM, the MOS yield was 107.29 mg / g of substrate which was 1.3-fold higher compared to the value after OFAT analysis which was only 84.87 mg / g of substrate. In the second strategy, the synergistic effect of MAG1 and CGTase for improving the MOS production process had also been studied using two different approaches which were asynchronous and synchronous methods. For the asynchronous method, the cyclization and hydrolysis reaction of CGTase and MAG1, were carried out in two separated steps respectively. Whereas, for the synchronous method, the two enzymes were added simultaneously and became a one-pot enzymatic reaction. The results from the studies conducted show the capability of the synchronous method was capable to convert the soluble starch (1.5 % (w / v)) into MOS with higher yield than the asynchronous method. The optimum conditions were obtained when MAG1 loading to CGTase loading in 3 U: 7 U ratio, with the reaction temperature of 40 ºC and pH 7.0. Based on these optimum conditions, the total yield of MOS attained was 307.86 mg / g of substrate after 2 h, which was 2.1-fold higher than the asynchronous method (146.78 mg / g) and 2.9-fold higher compared to the reaction of MAG1 alone. The used of CGTase and MAG1 synchronously enable the direct conversion of soluble starch to higher yield of MOS.

ABSTRAK

Secara semulajadi, *Bacillus lehensis* G1 menggunakan enzim siklodekstrin glukanotransferase (CGTase) di luar sel untuk menukarkan kanji kepada siklodekstrin (CD). Diikuti dengan hidrolisis CD oleh amilase maltogenik (MAG1) di dalam sel menjadi glukosa, maltosa dan maltooligosakarida (MOS). MOS adalah prebiotik yang berpotensi untuk kegunaan manusia. Didalam industri, amilase digunakan untuk menghasilkan MOS daripada kanji. Walau bagaimanapun, penukaran kanji secara langsung kepada MOS menggunakan MAG1 mempunyai beberapa kekangan seperti pengkhususan yang rendah terhadap kanji berbanding β-siklodekstrin (β-CD) dan produktiviti MOS yang rendah. Untuk mengatasi kelemahan ini, dua strategi yang melibatkan pengoptimuman pembolehubah tindakbalas dengan menggunakan kaedah statistik dan pendekatan sinergi campuran enzim, telah digunakan. Dalam kajian ini, pengoptimuman terhadap pembolehubah tindakbalas enzimatik bagi meningkatkan penghasilan MOS oleh MAG1 dengan menggunakan kanji boleh larut sebagai substrat telah dilakukan. Dalam strategi yang pertama, kesan pembolehubah tindakbalas (muatan enzim, muatan substrat, suhu, masa tindakbalas dan pH) terhadap penghasilan MOS telah dikaji menggunakan kaedah satu-faktor-pada-satu-masa (OFAT) dan reka bentuk pemfaktoran pecahan 2^{5-1}. Berdasarkan kepada keputusan reka bentuk pemfaktoran pecahan 2^{5-1}, tiga pembolehubah iaitu muatan substrat, masa tindakbalas dan pH didapati mempunyai kesan yang ketara dan digunakan dalam reka bentuk komposit berpusat di bawah kaedah sambutan permukaan (RSM). Penghasilan MOS berjaya dioptimumkan dengan RSM. Di bawah keadaan yang dioptimakan (0.25 % (w / v) muatan substrat, 0.5 jam masa tindakbalas dan pH 7.45) oleh RSM, hasil MOS adalah 107.29 mg / g substrat yang mana 1.3 kali lebih tinggi berbanding dengan nilai selepas analisis OFAT iaitu hanya 84.87 mg / g substrat. Dalam strategi yang kedua, kesan sinergistik MAG1 dan CGTase dalam memperbaiki proses penghasilan MOS juga telah dikaji dengan menggunakan dua pendekatan yang berbeza iaitu kaedah tidak serentak dan serentak. Bagi kaedah tidak serentak, tindakbalas pensiklikan dan hidrolisis CGTase dan MAG1, masing-masing telah dijalankan dalam dua langkah berasingan. Manakala, untuk kaedah serentak, kedua-dua enzim telah ditambah secara serentak dan menjadi tindakbalas enzimatik satu bekas. Hasil daripada kajian yang telah dijalankan menunjukkan kemampuan kaedah serentak mampu menukarkan kanji boleh larut (1.5 % (w / v)) kepada MOS dengan hasil yang lebih tinggi daripada kaedah tidak serentak. Keadaan optima diperolehi apabila muatan MAG1 kepada muatan CGTase dalam nisbah 3 U: 7 U, dengan suhu tindakbalas 40 ºC dan pH 7.0. Berdasarkan keadaan optima ini, hasil keseluruhan MOS yang dicapai adalah 307.86 mg / g substrat selepas 2 jam, iaitu 2.1 kali ganda lebih tinggi daripada kaedah tidak serentak (146.78 mg / g) dan 2.9 kali ganda lebih tinggi berbanding tindakbalas oleh MAG1 sahaja. Penggunaan CGTase dan MAG1 secara serentak membolehkan penukaran kanji boleh larut terus kepada hasil MOS yang lebih tinggi.

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LIST OF ABBREVIATIONS AND SYMBOLS

A_{600}	-	absorbance at optical density of 600 nm
Asp or D	-	Aspartic acid
B. lehensis	-	*Bacillus lehensis*
B. licheniformis	-	*Bacillus licheniformis*
B. stearothermophilus	-	*Bacillus stearothermophilus*
BSA	-	bovine serum albumin
C	-	carbon
CGTase	-	Cyclodextrin glucanotransferase
CD	-	cyclodextrin
E. coli	-	*Escherichia coli*
EC	-	Enzyme Commission
g	-	gram
G. thermoleovorans	-	*Geobacillus thermoleovorans*
GH	-	Glycosyl Hydrolase
Glu or E	-	glutamic acid
h	-	hour
H	-	hydrogen
HPLC	-	high-performance liquid chromatography
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
kDa	-	kilodalton
l	-	liter
L. gasseri	-	*Lactobacillus gasseri*
LB	-	Luria-Bertani
m	-	mili
M	-	molar
M1	-	glucose
M2	-	maltose

M3	-	maltotriose
M4	-	maltotetraose
M5	-	maltopentaose
M6	-	maltohexaose
M7	-	maltoheptaose
MAG1	-	maltogenic amylase from Bacillus lehensis G1
mg	-	milligram
min	-	minute
ml	-	milliliter
MW	-	molecular weight
NEB	-	New England Biolabs
nm	-	nanomete
O	-	oxygen
OH	-	hydroxyl
PMSF	-	phenylmethylsulfonyl fluoride
rpm	-	revolutions per minute
s	-	second
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel
sp	-	species
U	-	unit
UV	-	ultraviolet
V	-	volt
w/v	-	weight per volume
w/w	-	weight per weight
α	-	alpha
α -CD	-	α- cyclodextrin
β	-	beta
β-CD	-	β-cyclodextrin
°C	-	degree celcius
γ	-	gamma
γ- CD	-	γ-cyclodextrin
µ	-	micro

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CHAPTER 1

INTRODUCTION

1.1 Background

Starch is a polysaccharide that resulted from the photosynthesis process of green plants. Starch is the most abundant form of storage in cereals, legumes and mainly tubers. In the plant, the starch is stored within the cells as partially crystalline granules (Englyst *et al.*, 2006). According to Van Der Maarel *et al.* (2002), starch also can be used to produce glucose, maltose, dextrin, maltooligomer mixture and oligosaccharides, by chemically or enzymatically. Nowadays, the use of enzymes for catalyzing various biochemical reactions is more preferable than chemical such as acid because it is safer and environmental-friendly. Since the starch is naturally existed in a form of an insoluble starch granules, therefore, most of the research on starch degrading enzyme, has been conducted on gelatinized starch, which is starch granules was heating in water to produce starch solution (Taniguchi and Honnda, 2009).

Glucan-1,4-α-maltohydrolase (EC 3.2.1.133) is usually known as maltogenic amylase. It is an amylolytic enzyme from glycosyl hydrolase family 13 (GH 13). It is one of the maltooligosaccharide- forming amylase (MFAse) enzyme used in the commercial production of maltose and novel oligosaccharides for food and pharmaceutical applications. In addition to hydrolysis, maltogenic amylase also known to have transglycosylation ativity to produce sugar molecules with various lengths. Maltogenic amylase exhibits broad substrates specificity toward cyclodextrins (CDs), starch and pullulan and favours CDs as a substrate (Kim *et al*., 1999).

Oligosaccharides have potential benefits for human health and diverse applications in improving physicochemical properties of foods. Conventionally, the oligosaccharides were synthesized using chemical such as acid. However, later the use of enzymes for oligosaccharides production is more preferable than chemical because it is safer, environmental-friendly, low cost and give high yield. Glycoside hydrolases and glycosyltransferases have been used, but glycoside hydrolase enzymes are favoured due to cheap and easy-to-get saccharides, such as starch as their substrates.

However, excessive hydrolysis could be one of the drawback resulting in low yield of oligosaccharides. The control of the reaction condition will affect the reaction and product yield. Furthermore, less substrate specificity of starch would also results in the low yield of oligosaccharides. Over a decade, interest in oligosaccharides is growing and research are being carried out to improve the production of oligosaccharides. The development for synergism of enzyme mixture is an alternative for enhancing the production of oligosaccharides. Previous findings such as Kuriki *et al*. (1993) and Lee *et al*. (2002a) have provided a guidance for improving the direct conversion of starch to achieve the objective of the current study.

1.2 Problem Statement

To date, numerous enzymes are being discovered and some are available commercially to degrade starch for producing oligosaccharides. Thus, the use of enzymes is almost completely replace chemical techniques for synthesizing oligosaccharides in the industry. Glycoside hydrolase enzyme is preferred to be employed over the glycosyltransferase enzyme. However, the low production of maltooligosaccharides (MOS) by maltogenic amylase (MAG1) from starch is the major obstacle. Various strategies have been employed to overcome the problem, including the control of thermodynamic equilibrium of the enzyme reaction by manipulating the range of parameters used in reaction mixture (Abdul Manas *et al*., 2014). However, no statistical study on the influence of reaction conditions for improving MOS production by maltogenic amylase has been reported to date. Enzyme

mixture approach has also become a recently practice to improve the production of oligosaccharides (Li *et al*., 2017). The substrate specificity of MAG1 towards CD compared to starch also can be a significant drawback for direct conversion of starch into MOS. Therefore, the synergistic enzyme of MAG1 and cyclodextrin glucanotransferase (CGTase) is expected to enhanced the direct conversion or used of starch for MOS production.

1.3 Research Objectives

The objective of this research is to improve the production of MOS by MAG1 from soluble starch via optimization of reaction parameters using statistical method and synergism of enzyme mixture approach.

1.4 Scopes of the Study

This study focusses on the improvement of the production of MOS by appropriate strategies. Therefore, the following scopes were outlined to achieve the objective:

(a) Expression of MAG1 and CGTase in *Escherichia coli* expression system and purification using AKTAprime plus purification system.

(b) Determination of the significant ranges of reaction parameters (enzyme loading, substrate loading, reaction time, temperature and pH) on MOS production using a one-factor-at-a-time (OFAT) method and screening of the reaction conditions (enzyme loading, substrate loading, reaction time, temperature and pH) affecting MOS production by Fractional Factorial Design (FFD). Optimization of the reaction conditions (substrate loading, reaction time and pH) by central composite design (CCD) towards the achievement of highest MOS yield.

(c) Screening of the reaction parameters (pH, substrate loading, CGTase loading and reaction time) on β-CD production through OFAT in the first step of the asynchronous method. Screening of the reaction parameters (MAG1 loading and reaction time) on MOS production via OFAT in the second step of the asynchronous method. Screening of the reaction parameters (temperature, MAG1: CGTase ratio, substrate loading and reaction time) on MOS production by OFAT in the synchronous method.

1.5 Novelties of the Study

The novelties of the study are as follows:

1. The optimization of reaction conditions for the enhanced MOS yield by maltogenic amylase from *Bacillus lehensis* G1 using statistical tool has not been reported.

2. To the best of our knowledge, this is the first report on direct conversion of starch using the synergistic action of maltogenic amylase and CGTase from *B. lehensis* G1 to improve the production of MOS.

REFERENCES

Abdul Manas, N. H. (2016). *Reaction Control and Protein Engineering of Bacillus lehensis G1 Maltogenic Amylase for higher Malto-Oligosaccharide Synthesis*. Universiti Teknologi Malaysia.

Abdul Manas, N. H., Abu Bakar, F. D., and Md. Illias, R. (2016). Computational docking , molecular dynamics simulation and subsite structure analysis of a maltogenic amylase from Bacillus lehensis G1 provide insights into substrate and product specificity. *Journal of Molecular Graphics and Modelling*, *67*, 1–13.

Abdul Manas, N. H., Md. Illias, R., and Mahadi, N. M. (2018). Strategy in manipulating transglycosylation activity of glycosyl hydrolase for oligosaccharide production. *Critical Reviews in Biotechnology*, *38*(2), 272–293.

Abdul Manas, N. H., Pachelles, S., Mahadi, N. M., and Md Illias, R. (2014). The Characterisation of an Alkali-Stable Maltogenic Amylase from Bacillus lehensis G1 and Improved Malto-Oligosaccharide Production by Hydrolysis Suppression. *PLoS One*, *9*(9), 1–13.

Biwer, A., Antranikian, G., and Heinzle, E. (2002). Enzymatic production of cyclodextrins. *Applied Microbiology and Biotechnology*, *59*(6), 609–617.

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, *72*(1–2), 248-254.

Cha, H. J., Yoon, H. G., Kim, Y. W., Lee, H. S., Kim, J. W., Kweon, K. S., Oh, B. H., and Park, K. H. (1998). Molecular and enzymatic characterization of a maltogenic amylase that hydrolyzes and transglycosylates acarbose. *European Journal of Biochemistry*, *253*(1), 251–262.

Cho, M.-H., Park, S.-E., Lee, M.-H., Ha, S.-J., Kim, H.-Y., Kim, M.-J., Lee, S., Madsen, S. M., and Park, C.-S. (2007). Extracellular secretion of a maltogenic amylase from Lactobacillus gasseri ATCC33323 in Lactococcus lactis MG1363 and its application on the production of branched maltooligosaccharides. *Journal of Microbiology and Biotechnology*, *17*(9), 1521–1526.

Christophersen, C., Otzen, D. E., Norman, B. E., Christensen, S., Schäfer, T., and Denmark, B. (1998). Enzymatic Characterisation of Novamyl, a Thermostable alpha -Amylase. *Starch/Sta¨rke*, *50*(1), 39–45.

Chung, C. T., Niemela, S. L. and Miller, R. H. (1989). One-Step Preparation of Competent Escherichia Coli: Transformation and Storage of Bacterial Cells in the Same Solution. *Proceedings of the National Academy of Sciences*, *86*(7), 2172-2175.

Contesini, F. J., de Lima, E. A., Mandelli, F., Borin, G. P., Alves, R. F., and Terrasan, C. R. F. (2018). Carbohydrate Active Enzymes Applied in the Production of Functional Oligosaccharides. In *Encyclopedia of Food Chemistry*, *2*, 30–34.

Crittenden, R. ., and Playne, M. . (1996). Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science & Technology*, *7*, 353–361.

Cruz-Guerrero, A. E., Gómez-Ruiz, L., Viniegra-González, G., Bárzana, E., and García-Garibay, M. (2006). Influence of water activity in the synthesis of galactooligosaccharides produced by a hyperthermophilic β-glycosidase in an organic medium. *Biotechnology and Bioengineering*, *93*, 1123–1129.

Davies, G., and Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure*, *3*(9), 853–859.

Deutschmann, R., and Dekker, R. F. H. (2012). From plant biomass to bio-based chemicals: Latest developments in xylan research. *Biotechnology Advances*, *30*, 1627–1640.

Doukyu, N., Ogino, H., Kuwahara, H., Furuki, N., and Yamagishi, W. (2007). Purification and characterization of a maltooligosaccharide-forming amylase that improves product selectivity in water-miscible organic solvents, from dimethylsulfoxide-tolerant *Brachybacterium* sp. strain LB25. *Extremophiles*, *11*(6), 781–788.

Englyst, K. N., Hudson, G. J., and Englyst, H. N. (2006). Starch Analysis in Food. *Encyclopedia of Analytical Chemistry*, 4246–4262.

Fathiyah Sy Mohamad, S., Mohd Said, F., Sakinah Abdul Munaim, M., Mohamad, S., and Mohd. Azizi Wan Sulaiman, W. (2019). Fractional Factorial Analysis for Identifying Significant Factors in Extraction of Jacalin From Jackfruit Seeds Using Anionic Reverse Micellar System. *Materials Today: Proceedings*, *19*, 1638–1646.

Fernández-Arrojo, L., Marín, D., Gómez De Segura, A., Linde, D., Alcalde, M., Gutiérrez-Alonso, P., Ghazi, I., Plou, F. J., Fernández-Lobato, M., and Ballesteros, A. (2007). Transformation of maltose into prebiotic isomaltooligosaccharides by a novel α-glucosidase from Xantophyllomyces dendrorhous. *Process Biochemistry*, *42*(11), 1530–1536.

Gan, C. Y., Alkarkhi, A. F. M., and Easa, A. M. (2009). Using response surface methodology to optimize process parameters and cross-linking agents for production of combined-cross-linked bovine serum albumin gels. *Journal of Bioscience and Bioengineering*, *107*(4), 366–372.

González-Delgado, I., López-Muñoz, M. J., Morales, G., and Segura, Y. (2016). Optimisation of the synthesis of high galacto-oligosaccharides (GOS) from lactose with β-galactosidase from Kluyveromyces lactis. *International Dairy Journal*, *61*, 211–219.

Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., and Gras, S. L. (2010). Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry*, *121*(2), 307–318.

Hansson, T., Kaper, T., Van Oost, J. Der, De Vos, W. M., and Adlercreutz, P. (2001). Improved oligosaccharide synthesis by protein engineering of β-glucosidase CelB from hyperthermophilic Pyrococcus furiosus. *Biotechnology and Bioengineering*, *73*(3), 203–210.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, *280*(2), 309–316.

Hesta, M., Debraekeleer, J., Janssens, G. P. J., and De Wilde, R. (2001). The effect of a commercial high-fibre diet and an iso-malto-oligosaccharide-supplemented diet on post-prandial glucose concentrations in dogs. *Journal of Animal Physiology and Animal Nutrition*, *85*(7–8), 217–221.

Hondoh, H., Kuriki, T., and Matsuura, Y. (2003). Three-dimensional structure and substrate binding of Bacillus stearothermophilus neopullulanase. *Journal of Molecular Biology*, *326*(1), 177–188.

Illias, R. M., Fen, T. S., Abdulrashid, N. A., Yusoff, W. M. W., Hamid, A. A., Hassan, O., and Kamaruddin, K. (2002). Cyclodextrin Glucanotransferase Producing Alkalophilic Bacillus sp. G1: its Cultural Condition and Partial Characterization of the Enzyme. *Pakistan Journal of Biological Sciences*, *5*(6), 688–692.

Kamon, M., Sumitani, J. ichi, Tani, S., Kawaguchi, T., Kamon, M., Sumitani, J., Tani, S., and Kawaguchi, T. (2015). Characterization and gene cloning of a maltotriose-forming exo-amylase from Kitasatospora sp. MK-1785. *Applied Microbiology and Biotechnology*, *99*(11), 4743–4753.

Kang, G.-J., Kim, M.-J., Kim, J.-W., and Park, K. H. (1997). Immobilization of Thermostable Maltogenic Amylase from Bacillus stearothermophilus for Continuous Production of Branched Oligosaccharides. *Journal of Agricultural and Food Chemistry*, *45*(10), 4168–4172.

Karmakar, M., and Ray, R. R. (2011). A Maltotriose producing thermostable amylase from Bacillus sp KR11. *Journal of Microbiology and Biotechnology Research*, *1*(3), 91–99.

Kaya, F., Heitmann, J. A., and Joyce, T. W. (2000). Influence of lignin and its degradation products on enzymatic hydrolysis of xylan. *Journal of Biotechnology*, *80*(3), 241–247.

Ketabi, A., Dieleman, L. A., and Ga, M. G. (2011). Influence of isomalto-oligosaccharides on intestinal microbiota in rats. *Journal of Applied Microbiology*, *110*, 1297–1306.

Khanahmadi, S., Yusof, F., Amid, A., Mahmod, S. S., and Mahat, M. K. (2015). Optimized preparation and characterization of CLEA-lipase from cocoa pod husk. *Journal of Biotechnology*, *202*, 153–161.

Kim, J.-W., Kim, Y. H., Lee, H. S., Yang, S. J., Kim, Y. W., Lee, M. H., Kim, J. W., Seo, N. S., Seok, P. C., and Park, K. H. (2007). Molecular Cloning and Biochemical Characterization of the First Archaeal Maltogenic Amylase from the Hyperthermophilic Archaeon Thermoplasma Volcanium Gss1. *Biochimica et Biophysica Acta*, *1774*, 661–669.

Kim, T., Kim, M., Kim, B., Kim, J., Cheong, T., Kim, J., and Park, K. (1999). Modes of Action of Acarbose Hydrolysis and Transglycosylation Catalyzed by a Thermostable Maltogenic Amylase , the Gene for Which Was Cloned from a Thermus Strain. *Applied and Environmental Microbiology*, *65*(4), 1644–1651.

Kumar, S., and Khare, S. K. (2012). Purification and characterization of maltooligosaccharide-forming α-amylase from moderately halophilic Marinobacter sp. EMB8. *Bioresource Technology*, *116*, 247–251.

Kuriki, T., Yanase, M., Takata, H., Takesada, Y., Imanaka, T., and Okada, S. (1993). A new way of producing isomalto-oligosaccharide syrup by using the transglycosylation reaction of neopullulanase. *Applied and Environmental Microbiology*, *59*(4), 953–959.

Lee, H.-S., Kang, M.-H., Kim, T.-J., Kim, M.-J., Kim, J.-W., Yoon, H.-G., Auh, J.-H., Hong, S.-S., Park, K.-H., Park, J.-H., Moon, T.-W., and Lee, H.-S. (2002a). Cooperative Action of αlpha-Glucanotransferase and Maltogenic Amylase for an Improved Process of Isomaltooligosaccharide (IMO) Production. *Journal of Agricultural and Food Chemistry*, *50*(10), 2812–2817.

Lee, H.-S., Kim, M., Cho, H., Kim, J., Kim, T., Choi, J., Park, C., Lee, H., Oh, B., and Park, K. (2002b). Cyclomaltodextrinase , Neopullulanase , and Maltogenic Amylase Are Nearly Indistinguishable from Each Other. *The Journal of Biological Chemistry*, *277*(24), 21891–21897.

Lee, S., Park, G. G., Jang, J. K., and Park, Y. S. (2018). Optimization of oligosaccharide production from leuconostoc lactis using a response surface methodology and the immunostimulating effects of these oligosaccharides on macrophage cells. *Molecules*, *23*(1–12).

Lee, S., Yoo, S., Kim, M., Kim, J., Seok, H., and Park, K. (1995). Production and Characterization of Branched Oligosaccharides from Liquefied Starch by the Action of B. Licheniformis Amylase. *Starch - Stärke*, *47*(4), 127–134.

Leemhuis, H., Dijkstra, B. W., and Dijkhuizen, L. (2003). Thermoanaerobacterium thermosulfurigenes cyclodextrin glycosyltransferase: Mechanism and kinetics of inhibition by acarbose and cyclodextrins. *European Journal of Biochemistry*, *270*(1), 155–162.

Li, Z., Ji, K., Zhou, J., Ye, X., Wang, T., Luo, X., Huang, Y., Cao, H., Cui, Z., and Kong, Y. (2017). A debranching enzyme IsoM of Corallococcus sp . strain EGB with potential in starch processing. *International Journal of Biological Macromolecules*, *105*, 1300–1309.

Li, Z., Wu, J., Zhang, B., Wang, F., Ye, X., Huang, Y., Huang, Q., and Cui, Z. (2015). AmyM, a Novel Maltohexaose-Forming α-Amylase from Corallococcus sp. Strain EGB. *Applied and Environmental Microbiology*, *81*, 1977–1987.

Ling, H. L., How Lie, L., Rahmat, Z., Abdul Murad, A. M., Mahadi, N. M., and Md. Illias, R. (2017). Proteome-based identification of signal peptides for improved secretion of recombinant cyclomaltodextrin glucanotransferase in Escherichia coli. *Process Biochemistry*, *61*, 21.

Liu, B., Wang, Y., and Zhang, X. (2006). Characterization of a Recombinant Maltogenic Amylase from Deep Sea Thermophilic Bacillus Sp. Wpd616. *Enzyme and Microbial Technology*, *39*, 805–810.

Maalej, H., Ben Ayed, H., Ghorbel-Bellaaj, O., Nasri, M., and Hmidet, N. (2014). Production and biochemical characterization of a high maltotetraose (g4) producing amylase from Pseudomonas stutzeri AS22. *BioMed Research International*, *2014*.

Mabrouk, S. Ben, Messaoud, E. Ben, Ayadi, D., Jemli, S., Roy, A., Mezghani, M., and Bejar, S. (2008). Cloning and sequencing of an original gene encoding a maltogenic amylase from Bacillus sp. US149 strain and characterization of the recombinant activity. *Molecular Biotechnology*, *38*(3), 211–219.

Mackenzie, L., Wang, Q., Warren, R., and Withers, S. G. (1998). Glycosynthases: Mutant glycosidases for glycoside synthesis. *Journal of American Chemical Society*, *120*, 5583–5584.

Mehta, D., and Satyanarayana, T. (2013). Dimerization Mediates Thermo-Adaptation, Substrate Affinity and Transglycosylation in a Highly Thermostable Maltogenic Amylase of Geobacillus thermoleovorans. *PLoS One*, *8*(9).

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426-428.

Montgomery, D. C. (2013). *Design and Analysis of Experiments* (Eighth). John Wiley & Sons, Inc.

Moon, I.-S., and Cho, G. (1997). Production of Maltooligosaccharides from Starch and Separation of Maltopentaose by Adsorption of Them on Activated Carbon (I). *Biotechnology and Bioprocess Engineering*, *2*(1), 19–22.

Muniandy, K., Ummirul, K., Chun, C., Yen, C., Poh, G., and Kian, G. (2013). Application of statistical experimental design for optimization of novel α-amylase production by Anoxybacillus species. *Journal of Biological Sciences*, 13, 605–613.

Murphy, O. (2001). Non-polyol low-digestible carbohydrates : food applications and functional benefits. *British Journal of Nutrition*, *85*, 47–53.

Musa, H., Chien, P., Ha, F., Gopinath, S. C. B., and Azmier, M. (2017). Turning oil palm empty fruit bunch waste into substrate for optimal lipase secretion on solid state fermentation by Trichoderma strains. *Process Biochemistry*, *63*(2017), 35–41.

Mussatto, S. I., Dragone, G., Fernandes, M., Milagres, A. M. F., and Roberto, I. C. (2008). The effect of agitation speed, enzyme loading and substrate concentration on enzymatic hydrolysis of cellulose from brewer's spent grain. *Cellulose*, *15*(5), 711–721.

Mussatto, S. I., and Mancilha, I. M. (2007). Non-digestible oligosaccharides: A review. *Carbohydrate Polymers*, *68*(3), 587–597.

Nakamura, A., Haga, K., and Yamane, K. (1994). Four Aromatic residue in the active center of cyclodextrin glucanotransferase from alkalophilic Bacillus sp. 1011: Effects of replacements on substrate binding and cyclization characteristics. *Biochemistry*, *33*, 9929–9936.

Nobre, C., Alves Filho, E. G., Fernandes, F. A. N., Brito, E. S., Rodrigues, S., Teixeira, J. A., and Rodrigues, L. R. (2018). Production of fructo-oligosaccharides by Aspergillus ibericus and their chemical characterization. *LWT - Food Science and Technology*, *89*, 58–64.

Noman, A., Xu, Y., AL-Bukhaiti, W. Q., Abed, S. M., Ali, A. H., Ramadhan, A. H., and Xia, W. (2018). Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and functional properties of protein hydrolysate obtained from Chinese sturgeon (Acipenser sinensis) by using papain enzyme. *Process Biochemistry*, *67*, 19–28.

Nor, N. M., Mohamed, M. S., Loh, T. C., Foo, H. L., Rahim, R. A., Tan, J. S., and Mohamad, R. (2017). Comparative analyses on medium optimization using one-factor-at-a-time , response surface methodology , and artificial neural network for lysine – methionine biosynthesis by Pediococcus pentosaceus RF-1. *Biotechnology and Biotechnological Equipment*, *31*(5), 935–947.

Oh, K. W., Kim, M. J., Kim, H. Y., Kim, B. Y., Baik, M. Y., Auh, J. H., and Park, C. S. (2005). Enzymatic characterization of a maltogenic amylase from Lactobacillus gasseri ATCC 33323 expressed in Escherichia coli. *FEMS Microbiology Letters*, *252*(1), 175–181.

Olano-martin, E., Mountzouris, K. C., Gibson, G. R., and Rastall, R. A. (2000). In vitro fermentability of dextran , oligodextran and maltodextrin by human gut bacteria. *British Journal Of Nutrition*, *83*, 247–255.

Onishi, N., and Tanaka, T. (1995). Purification and properties of a novel thermostable galacto- oligosaccharide-producing β-galactosidase from Sterigmatomyces elviae CBS8119. *Applied and Environmental Microbiology*, *61*(11), 4026–4030.

Otieno, D. O., and Ahring, B. K. (2012). The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: Xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannooligosaccharides (MOS). *Carbohydrate Research*, *360*, 84–92.

Ovissipour, M., Thunnus, T., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R., and Shahiri, H. (2009). The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (Acipenser persicus) viscera. *Food Chemistry*, *115*(1), 238–242.

Pachelles, S. (2013). *Expression and Biochemical Characterization of Maltogenic amylase from Bacillus lehensis G1*. Universiti Teknologi Malaysia.

Park, K.-H., Kim, T.-J., Cheong, T.-K., Kim, J.-W., Oh, B.-H., and Svensson, B. (2000). Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the αlpha-amylase family. *Biochimica et Biophysica Acta*, *1478*, 165–185.

Park, S. H., Na, Y., Kim, J., Kang, S. D., and Park, K.-H. (2018). Properties and applications of starch modifying enzymes for use in the baking industry. *Food Science and Biotechnology*, *27*(2), 299–312.

Patel, S., and Goyal, A. (2011). Functional oligosaccharides: Production, properties and applications. *World Journal of Microbiology and Biotechnology*, *27*(5), 1119–1128.

Prapulla, S. G., Subhaprada, V., and Karanth, N. G. (2000). *Microbial production of oligosaccharides: A review*. *47*, 299–343.

Qi, Q., and Zimmermann, W. (2005). Cyclodextrin glucanotransferase: From gene to applications. *Applied Microbiology and Biotechnology*, *66*(5), 475–485.

Rastall, R. A. (2010). Functional Oligosaccharides: Application and Manufacture. *Annual Review of Food Science and Technology*, *1*(1), 305–339.

Reddy, N. ., Annapoorna, N., and K., R. S. S. R. (2003). An overview of the microbial alpha-amylase family. *African Journal of Biotechnology*, *2*(12), 645–648.

Rezaei, P. S., Darzi, G. N., and Shafaghat, H. (2010). Optimization of the fermentation conditions and partial characterization for acido-thermophilic α-amylase from Aspergillus niger NCIM 548. *Korean Journal of Chemical Engineering*, *27*(3), 919–924.

Romsaiyud, A., Songkasiri, W., Nopharatana, A., and Chaiprasert, P. (2009). Combination effect of pH and acetate on enzymatic cellulose hydrolysis. *Journal of Environmental Sciences*, *21*(7), 965–970.

Roy, D., Daoudi, L., and Azaola, A. (2002). Optimization of galacto-oligosaccharide production by Bifidobacterium infantis RW-8120 using response surface methodology. *Journal of Industrial Microbiology and Biotechnology*, *29*(5), 281–285.

Sako, T., Matsumoto, K., and Tanaka, R. (1999). Recent progress on research and applications of non-digestible. *International Dairy Journal*, 69–80.

Sambrook, J. and Russell, D. W. (2001). Molecular Cloning : A Laboratory Manual. Cold Spring Harbor, N.Y; Cold Spring Harbor Laboratory.

Seidle, H. F., and Huber, R. E. (2005). Transglucosidic reactions of the Aspergillus niger Family 3 β-glucosidase: Qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. *Archives of Biochemistry and Biophysics*, *436*(2), 254–264.

Sian, H. K., Said, M., Hassan, O., Kamaruddin, K., Ismail, A. F., Rahman, R. A., Mahmood, N. A. N., and Illias, R. M. (2005). Purification and characterization of cyclodextrin glucanotransferase from alkalophilic Bacillus sp. G1. *Process Biochemistry*, *40*(3–4), 1101–1111.

Takaku, H. (1988). Handbook of Amylases and Related Enzymes: Their Sources, Isolation Methods, Properties and Applications. In *The amylase research society of Japan, Osaka, Japan, Pergamon Press*.

Takasaki, Y. (1983). An amylase producing maltotetraose and maltopentaose from Bacillus circulans. *Agricultural and Biological Chemistry*, *47*(10), 2193–2199.

Talluri, V. P., Lanka, S. S., and Saladi, V. R. (2019). Statistical Optimization of Process Parameters by Central Composite Design (CCD) for an Enhanced Production of L-asparaginase by Myroides gitamensis BSH-3 , a Novel Species. *Avicenna Journal of Medical Biotechnology*, *11*(1), 59–66.

Taniguchi, H., and Honnda, Y. (2009). Amylases. *Applied Microbiology: Industrial | Amylases*, 159–173.

Tester, R. F., Karkalas, J., and Qi, X. (2004). Starch -composition, fine structure and architecture. *Journal of Cereal Science*, *39*, 151–165.

Torres, D. P. M., Gonçalves, M. do P. F., Teixeira, J. A., and Rodrigues, L. R. (2010). Galacto-Oligosaccharides: Production, properties, applications, and significance as prebiotics. *Comprehensive Reviews in Food Science and Food Safety*, *9*(5), 438–454.

Uitdehaag, J. C. M., Van Der Veen, B., Dijkhuizen, L., and Dijkstra, B. W. (2002). Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the αlpha-amylase family. *Enzyme and Microbial Technology*, *30*(3), 295–304.

Van Der Maarel, M. J. E. C., Van Der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., and Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. *Journal of Biotechnology*, *94*(2), 137–155.

Van der Veen, B. A., Uitdehaag, J. C. M., Dijkstra, B. W., and Dijkhuizen, L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, *1543*(2), 336–360.

Vénica, C. I., Bergamini, C. V., and Perotti, M. C. (2017). Response surface methodology as a tool for modelling galacto-oligosaccharide production. *Journal of Dairy Research*, *84*(4), 464–470.

Voragen, A. G. J. (1998). Technological aspects of functional food-related carbohydrates. *Trends in Food Science & Technology*, *9*, 328–335.

Wahid, Z., and Nadir, N. (2013). Improvement of one factor at a time through design of experiments. *World Applied Sciences Journal*, *21*, 56–61.

Wan Azelee, N. I. (2015). *Optimisation of Kenaf Pretreatment and Enzymatic Reaction Conditions of Kenaf Hemicellulose for xylooligosaccharides Production*. Universiti Teknologi Malaysia.

Wan Azelee, N. I., Jahim, J. M., Ismail, A. F., Fuzi, S. F. Z. M., Rahman, R. A., and Md Illias, R. (2016). High xylooligosaccharides (XOS) production from pretreated kenaf stem by enzyme mixture hydrolysis. *Industrial Crops and Products*, *81*, 11–19.

Wang, L.-X., and Huang, W. (2009). Enzymatic transglycosylation for glycoconjugate synthesis. *Current Opinion in Chemical Biology*, *13*(5–6), 592–600.

Weijers, C. A., Franssen, M. C., and Visser, G. M. (2008). Glycosyltransferase-catalyzed synthesis of bioactive oligosaccharides. *Biotechnology Advances*, *26*, 436–456.

Yoo, S., Kweon, M.-R., Kim, M., Auh, J., Jung, D., KIM, J., Yook, C., Kim, J., and Park, K. (1995). Branched Oligosaccharides Concentrated by Yeast Fermentation and Effectiveness as a Low Sweetness Humectant. *Journal of Food Science*, *60*(3), 516–521.

Zhu, X., Tian, Y., Xu, W., Guang, C., Zhang, W., Zhang, T., and Mu, W. (2018). Bioconversion of sucrose to maltooligosaccharides by the synergistic action of amylosucrase and α-amylase. *Process Biochemistry*, *74*, 71–76.

LIST OF PUBLICATIONS

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APPENDIX A

Medium and Buffers Preparation

Medium Preparation for *E. coli* Culturing

Luria-Bertani (LB) Medium/Agar

Composition: 1 % (w/v) tryptone, 0.5 % yeast extract, 0.5 % NaCl and 1.5 % agar.

For 1 liter LB medium/agar:

1) The following ingredients were dissolved in 950 ml distilled water: 10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar. For LB medium, agar was excluded.
2) The pH of the solution was adjusted to 7.5 using 5 M NaOH solution. Distilled water was added to bring the volume to 1 liter.
3) The solution was autoclaved for 15 min at 121 °C.
4) When the solution has cooled to ~ 55 °C, antibiotic was added appropriately. For LB agar, the solution was poured into the petri dish and left to solidify. Plates were stored at 4 °C.

Ampicillin Stock Solution

Composition: 100 mg/ml ampicillin.

For 10 ml ampicillin stock solution:

1) The ampicillin powder was weighed to 1 g and dissolved in 10 ml distilled water.
2) The solution was filtered using a sterile 0.2 μm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in – 20 °C and thawed prior to use.

IPTG Stock Solution

Composition: 0.1 M IPTG.

For 10 ml IPTG stock solution:

1) The IPTG powder was weighed to 0.24 g and dissolved in 10 ml distilled water.
2) The solution was filtered using a sterile 0.2 μm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in – 20 ℃ and thawed prior to use

Buffers for Molecular Works

50X TAE Electrophoresis Buffer

Composition: 2M Tris base, 50 mM EDTA

For 1 liter 50X TAE electrophoresis buffer:

1) The following ingredients were dissolved in distilled water: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0).
2) Distilled water was added to bring the volume to 1 liter.
3) The solution was diluted to 1X prior to use by mixing 20 ml of 50X TAE buffer with 980 ml distilled water.

EDTA Solution

Composition: 0.5 M EDTA (pH 8.0).

For 500 ml EDTA solution:

1) EDTA was weighed to 93.05 g and put into 350 ml distilled water.
2) The solution was mixed vigorously using a magnetic stirrer and the pH was adjusted to 8.0 by titrating 5 M NaOH solution. The EDTA powder will only soluble in solution when the pH reaches 8.0.
3) Distilled water was added to bring the volume to 500 ml.

RNAase A Stock Solution

Composition: 10 mg/ml RNAase A.

For 10 ml RNAase A stock solution:

1) The RNAase A powder was weighed to 0.1 g and dissolved in 10 ml distilled water.
2) The solution was filtered using a sterile 0.2 μm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in – 20 °C and thawed prior to use.

TE Buffer

Composition: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0.

The solution was autoclaved and stored at room temperature

Lysozyme Stock Solution

Composition: 50 mg/ml lysozyme.

For 10 ml 50 mg/ml lysozyme:

1) The lysozyme powder was weighed to 0.5 g and dissolved in 10 ml distilled water.
2) The solution was filtered using a sterile 0.2 μm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in – 20 °C and thawed prior to use.

Transformation and Storage Solution (TSS)

Composition: 10% (w/v) polyethyleneglycol (PEG), 5% (v/v) dimethyl sulfoxide (DMSO), 50 mM MgCl2

Preparation:

1) The components were weighed and pipetted accordingly and dissolved in LB medium at final pH of 6.5.
2) The solution was autoclaved and stored at 4 °C

Buffers for Protein Analysis

50 mM Potassium Phosphate Buffer

Table A1 Preparation of 100 ml of 50 mM potassium phosphate buffer at 25 °C

pH	Volume of 1 M K_2HPO_4 (ml)	Volume of 1 M KH_2PO_4 (ml)	Volume of distilled water (ml)
6.0	8.5	91.5	100.0
7.0	61.5	38.5	100.0
8.0	94.0	6.0	100.0

50 mM Glycine - NaOH Buffer

For 100 ml preparation of 50 mM glycine-NaOH buffer.

1) 0.38 g of glycine was weighed and dissolved in distilled water.
2) The pH was titrated to desired pH (9.0 and 10.0) using 5 M NaOH.
3) Distilled water was added to 100 ml.

Binding Buffer/ Buffer A (Ni^{2+} Affinity Chromatography)

Composition: 20 mM phosphate, 0.5 M NaCl, pH 7.4

For 1 liter preparation of binding buffer:

1) 3.12 g of NaH2PO4.2H2O and 29.22 g of NaCl was weighed.
2) The powder was dissolved in distilled water by stirring.
3) After the powder was completely dissolved, the pH was adjusted to 7.5 using 5 M NaOH or 5 M HCl.
4) Distilled water was added to 1 liter.
5) The solution was degassed using vacuum filter prior to use for the protein purification.

Elution Buffer/ Buffer B (Ni^{2+} Affinity Chromatography)

Composition: 20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4

For 500 ml preparation of elution buffer:

1) 1.56 g of NaH2PO4.2H2O, 14.62 g of NaCl and 17.02 g of imidazole was weighed.
2) The powder was dissolved in distilled water by stirring.
3) After the powder was completely dissolved, the pH was adjusted to 7.5 using 5 M NaOH or 5 M HCl.
4) Distilled water was added to 500 ml.
5) The solution was degassed using vacuum filter prior to use for the protein purification.

Polyacrylamide Stacking Gel (SDS-PAGE)

Composition: 5% polyacrylamide in Tris-HCl pH 6.8.

For 5 ml preparation of stacking gel:

1) 0.83 ml of 30% acrylamide mix solution, 0.63 ml of 1.0 M Tris-HCl (pH 6.8) stock solution and 0.05 ml of 10% SDS stock solution was dissolved in 3.4 ml distilled water.
2) 0.05 ml of 10% ammonium persulfate and 0.005 ml TEMED were added prior to polymerization.

Polyacrylamide Resolving Gel (SDS-PAGE)

Composition: 12% polyacrylamide in Tris-HCl pH 8.8.

For 15 ml preparation of resolving gel:

1) 6.0 ml of 30% acrylamide mix solution, 3.8 ml of 1.5 M Tris-HCl (pH 8.8) stock solution and 0.15 ml of 10% SDS stock solution was dissolved in 4.9 ml distilled water.
2) 0.15 ml of 10% ammonium persulfate and 0.006 ml TEMED were added prior to polymerization.

Polycrylamide Mix Stock Solution

Composition: 30% (w/v) Acrylamide, 0.8 (w/v) Bis-acrylamide

Ammonium Persulfate Stock Solution

Composition: 10% ammonium persulfate.

For 2 ml preparation of ammonium persulfate stock solution:

1) 0.2 g ammonium persulfate was weighed and dissolved in 2 ml distilled water.
2) The solution was kept in 4 °C.

Note: Stable for months in a capped tube in refrigerator.

5X Tris-glycine Electrophoresis Buffer (SDS-PAGE)

For 1 liter preparation of 5X Tris-glycine electrophoresis buffer:

1) 15.1 g of Tris-base, 72.0 g of glycine and 5.0 g of SDS were weighed.
2) The powder mixture was dissolved in 1 liter distilled water.
3) The solution was diluted to 1X before use.

2X SDS – Gel Loading Buffer (SDS-PAGE)

Composition: 100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT

For 50 ml preparation of 2X SDS – gel loading buffer:

1) 0.6055 g of tris base, 2 g of SDS and 0.1 g bromophenol blue were weighed.
2) 10 ml glycerol was pipetted and all components were dissolved in distilled water.
3) The pH was adjusted to 6.8 and distilled water was added to 50 ml.
4) DTT was freshly added to final concentration prior to loading the protein sample.

Staining Solution A

Composition: 50% ethanol, 10% acetic acid.

For 500 ml preparation of staining solution A:

1) 263 ml of 95% ethanol and 50 ml of acetic acid glacial were measured and dissolved in distilled water.
2) Distilled water was added to 500 ml.

Staining Solution B

Composition: 5% ethanol, 7.5% acetic acid.

For 500 ml preparation of staining solution B:

1) 26.3 ml of 95% ethanol and 37.5 ml of acetic acid glacial were measured and dissolved in distilled water.
2) Distilled water was added to 500 ml.

Coomassie Blue Solution

Composition: 0.25% coomassie blue

For 50 ml preparation of coomassie blue solution:

1) 0.125 g coomassie blue was weighed and dissolved in 50 ml ethanol.
2) The solution was kept in a dark bottle at room temperature.

Gel Filtration Buffer

Composition: 50mM NaH_2PO_4, 0.15 M NaCl, pH 7.4

For 1 liter preparation of gel filtration buffer.

1) 6.90 g of $NaH_2PO_4.H_2O$ and 8.77 g of NaCl were weighed and dissolved in distilled water.
2) The pH was adjusted to 7.5 using 5 M NaOH or 5 M HCl.
3) Distilled water was added to 1 liter.
4) The solution was degassed using vacuum filter prior to use for the protein purification.

DNS Reagent

For 500 ml preparation of DNS reagent:

1) 5 g dinitrosalicylic acid was weighed and dissolved in 300 ml distilled water in a tin foil-covered beaker.
2) 8 g NaOH was weighed and gradually added into the DNS solution and stirred to dissolve.
3) 150 g Rochelle salt (sodium potassium tartarate) was weighed and slowly added into the mixture solution and stirred until dissolved at temperature between 40-100 °C.
4) Distilled water was added to 500 ml and the solution was kept in a dark bottle at room temperature.

APPENDIX B

Example of Experimental Data Calculation

Maltogenic Amylase Activity Calculation (DNS Assay)

One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 µmol maltose per min under the optimum conditions.

Unit activity per volume enzyme (U/ml) was calculated using the equation below:

$$\text{Activity}, U/ml = \frac{A_{575,\,enzyme} - A_{575,\,control}}{A575/\mu mol\ maltose \times 10\,\text{min} \times 0.1 ml} \times DF_{enzyme} \times DF_{assay}$$

Where,

$A_{575,\,enzyme}$: the absorbance of assay with the enzyme read at 575 nm

$A_{575,\,control}$: the absorbance of assay without the enzyme read at 575 nm

$A_{575}/\mu mol\ maltose$: standard curve equation of maltose

10 min : incubation time

0.1 ml : volume of the enzyme used in the assay

DF_{enzyme} : dilution factor of the enzyme used for the assay

DF_{assay} : dilution factor of the enzyme in the assay

$$\text{Standard deviation} = \sqrt{\sum \frac{(x-\bar{x})^2}{n}}$$

Where, x = sample

$\bar{x}$ = mean sample

n = number of sample

An example of activity calculation is shown below:

Given,

$A_{575,\ enzyme}$ = 0.136, 0.117, 0.115 (triplicate readings)

$A_{575,\ control}$ = 0.000, 0.000, 0.000 (triplicate readings)

$A_{575}/\mu mol\ maltose$ = 0.6028 (from the standard curve)

DF_{enzyme} = 100

DF_{assay} = 5 (0.1 ml enzyme in 0.5 ml assay volume)

Therefore,

$$\text{Activity}_{1} = \frac{0.136 - 0.000}{0.6028 \times 10\,\text{min} \times 0.1 ml} \times 100 \times 5 = 112.81\,U/ml$$

$$\text{Activity}_{2} = \frac{0.117 - 0.000}{0.6028 \times 10\,\text{min} \times 0.1 ml} \times 100 \times 5 = 97.05\,U/ml$$

$$\text{Activity}_{3} = \frac{0.115 - 0.000}{0.6028 \times 10\,\text{min} \times 0.1 ml} \times 100 \times 5 = 95.39\,U/ml$$

$$\text{Activity}_{\text{mean}} = \frac{112.81 + 97.05 + 95.39}{3} = 101.75\,U/ml$$

$$\text{Standard deviation} = \sqrt{\frac{(112.81 - 101.75)^2}{3} + \frac{(97.05 - 101.75)^2}{3} + \frac{(95.39 - 101.75)^2}{3}} = 7.85$$

Enzyme activity = 101.75 ± 7.85 U/ml

Cyclodextrin Glucanotransferase Activity Calculation

$A = (\%OD\ decrease \times Y_p \times D_f \times 10^3) / (MW \times t_i)$

Where,

A = Enzyme activity (U/ml or μmol/ml) of protein sample

%OD decrease = [(ODcontrol−ODsample)/ ODcontrol] × 100%

Y_p = mg of β-CD equivalent to 100% OD decrease of the standard curve

D_f = Dilution factor

MW = Molecular weight of β-CD, 1135

t_i = Time of incubation

Example of calculation:

% OD decrease:

OD_{550} for control (in triplicate) = (1.438+1.424+1.438)/3 = 1.433

OD_{550} for sample (in triplicate) = (1.225+1.226+1.205)/3 = 1.219

Hence, % OD decrease = [(1.433−1.219)/1.433] × 100% = 14.9%

Y_p:

When y = 100, y = 66.712x + 6.8336 (refer Figure E1)

100 = 66.712x + 6.8336

x = 1.4

Hence, Y_p = 1.4

D_f:

Sample taken for assay = 100 μl

Hence, Df = 10

MW of β-CD = 1135 g/mol

t_i = 10 min

Therefore, CGTase activity of the sample is:

A = [(14.9)(1.4)(10)(103)]/[(1135)(10)]

= 18.4 U/ml

Protein Concentration Calculation (Bradford Assay)

Protein concentration was calculated using the equation below:

$$\text{Protein concentration}, mg/ml = \frac{A_{595}}{A_{595}/mg\ ml^{-1} BSA} \times DF$$

Where, A_{595} = the absorbance of Bradford assay read at 575 nm

$A_{595}/mg\ ml^{-1}\ BSA$ = standard curve equation of BSA

DF = dilution factor of the protein

An example of activity calculation is shown below:

Given,

A_{595} = 0.107, 0.132, 0.112

$A_{595}/ mg\ ml^{-1}\ BSA$ = 0.2815

DF = 10

Therefore,

$$\text{Protein concentration}_{1} = \frac{0.107}{0.2815} \times 10 = 3.80\ mg/ml$$

$$\text{Protein concentration}_{2} = \frac{0.132}{0.2815} \times 10 = 4.69\ mg/ml$$

$$\text{Protein concentration}_{3} = \frac{0.112}{0.2815} \times 10 = 3.98\ mg/ml$$

$$\text{Protein concentration}_{\text{mean}} = \frac{3.80 + 4.69 + 3.98}{3} = 4.16\ mg/ml$$

$$\text{Standard deviation} = \sqrt{\frac{(3.80 - 4.16)^2}{3} + \frac{(4.69 - 4.16)^2}{3} + \frac{(3.98 - 4.16)^2}{3}} = 0.38$$

Protein concentration = 4.16 ± 0.38 *mg/ml*

APPENDIX C

Standard Curves

DNS Assay

Table C1 Absorbance at OD of 575 nm for maltose standards

[Maltose], mM	Maltose mass, µmol	Absorbance, A_{575}			Average
0	0	0	0	0	0
1	0.5	0.228	0.223	0.227	0.226
2	1.0	0.536	0.530	0.542	0.536
3	1.5	0.849	0.868	0.884	0.867
4	2.0	1.169	1.181	1.190	1.180
5	2.5	1.538	1.496	1.539	1.524
6	3.0	1.815	1.832	1.847	1.831
7	3.5	2.109	2.117	2.137	2.121
8	4.0	2.418	2.431	2.430	2.426

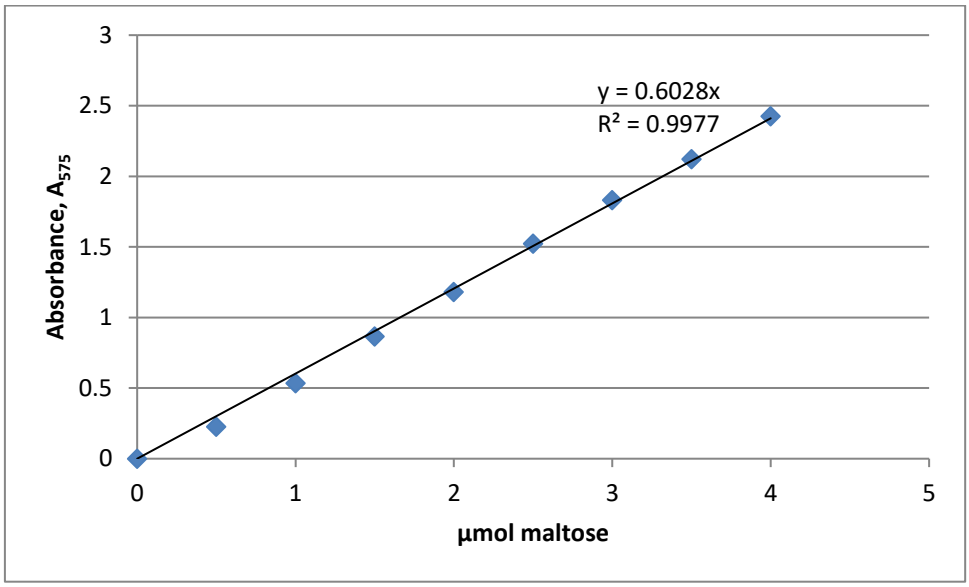

Figure C1 The standard curve of absorbance, A_{575} versus maltose mass, µmol

CGTase Assay

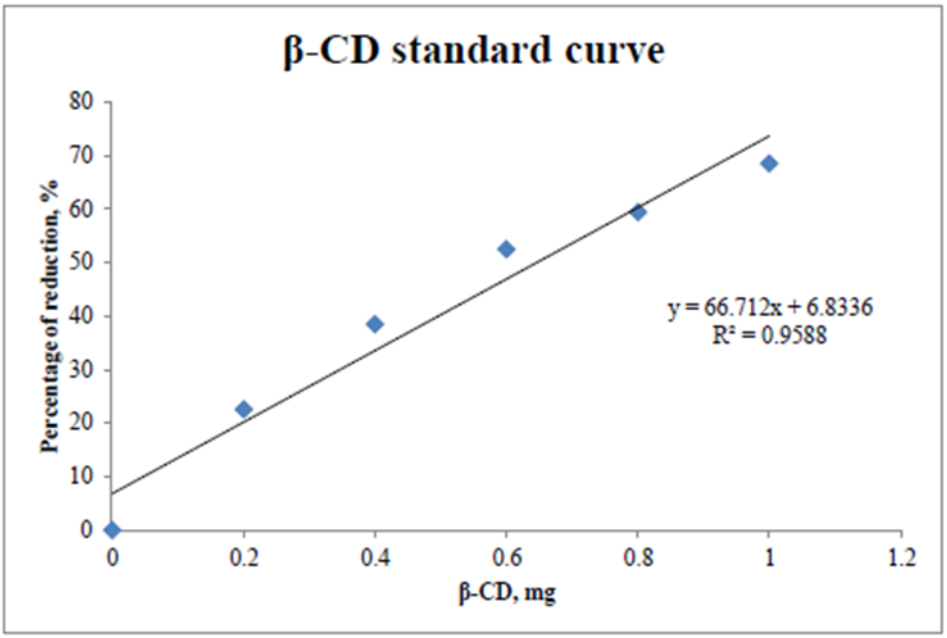

Figure C2 β-CD standard curve for the calculation of CGTase assay

Bradford Assay

Table C3 Absorbance at OD of 595 nm for BSA standards

[BSA], mg/ml	Absorbance, A_{595}			Average
2.0	0.516	0.586	0.594	0.565
1.4	0.403	0.407	0.426	0.412
1.2	0.333	0.341	0.345	0.340
1.0	0.171	0.283	0.294	0.249
0.8	0.214	0.243	0.213	0.223
0.6	0.172	0.168	0.183	0.174
0.4	0.101	0.115	0.113	0.110
0.2	0.057	0.057	0.051	0.055

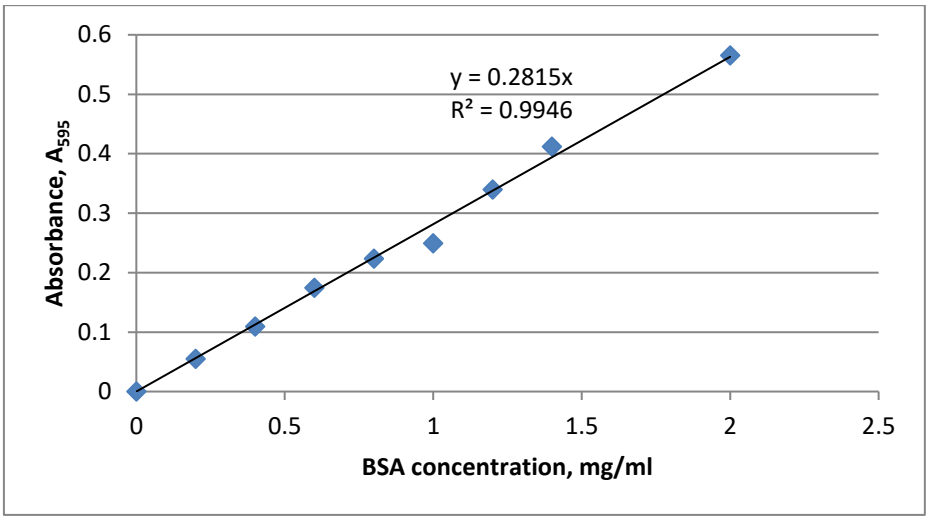

Figure C3 The standard curve of absorbance, A_{595} versus BSA concentration, mg/ml

APPENDIX D

Example of Peaks from HPLC Analysis (Standard)

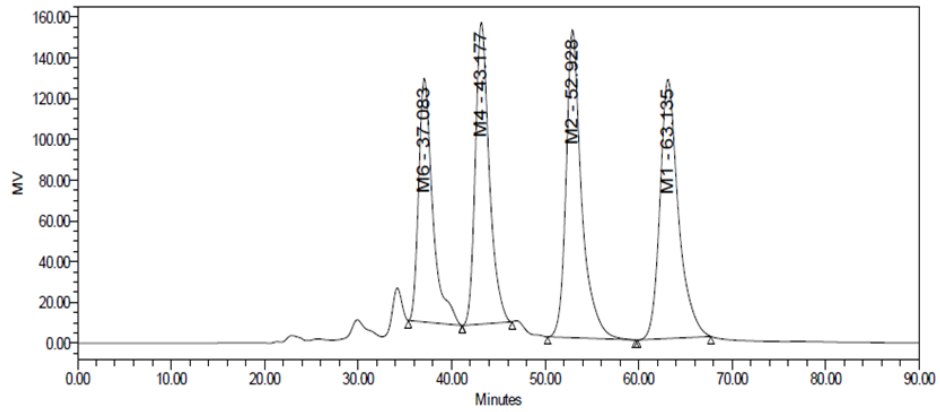

Figure D1 Peaks from standard curve of glucose (M1), maltose (M2), maltotetraose (M4) and maltohexaose (M6) with concentration of 5 mg/ml

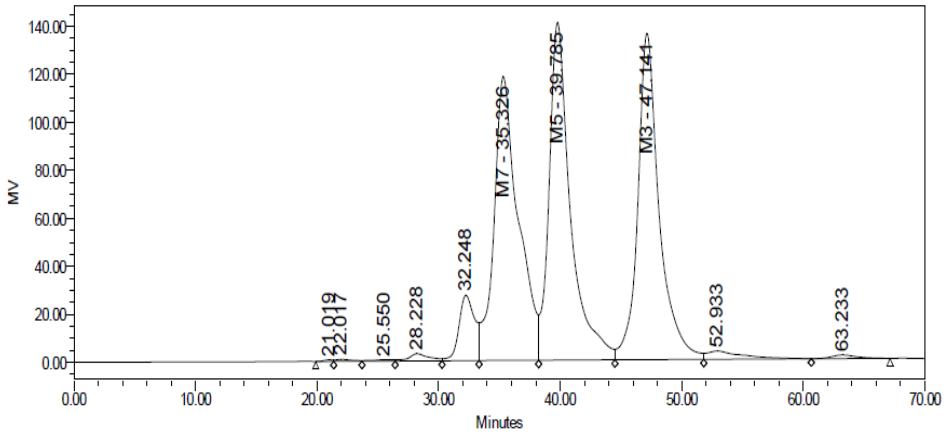

Figure D2 Peaks from standard curve of maltotriose (M3), maltopentaose (M5) and maltoheptaose (M7) with concentration of 5 mg/ml

APPENDIX E

Standard Operational Procedures for Instruments

AKTA Prime

1. Distilled water, binding buffer, elution buffer and 20% ethanol were prepared and degassed by vacuum filter using 0.2 μm nylon filter.
2. The AKTAPrime and computer were switched on and the PrimeView program was opened.
3. Both lines A and B were put in a bottle containing distilled water.
4. The system was washed using distilled water by running the 'System Wash' program (Template >Application template>System wash method).
5. After the system wash has finished, the flow rate was increased by using manual run (Manual run> Set flow rate 0.5 ml/min> Pressure limit 0.3 MPa> Start run).
6. The column was connected to the system via wet connection (drop to drop). Connections were checked for any leakage.
7. The flow rate was increased to 5 ml/min. The column was washed by allowing five column volume of distilled water to flow. The flow was end by pressing the 'End' button.
8. Line A was put into binding buffer and line B was put into elution buffer.
9. The system was washed using buffer by running the 'System Wash' program.
10. The column was equilibrated by allowing two column volume of binding buffer to flow using 'Manual Run'. The flow was end by pressing the 'End' button.
11. The program for running the desired protein purification was selected and all parameters were set.
12. The sample loop was connected to the system and the loop was washed using binding buffer.

13. The sample was filtered through a 0.2 μm nylon syringe filter and loaded into the sample loop. Any bubbles in the syringe containing the samples were removed prior to sample loading.
14. The fraction collectors were filled in with collection tubes.
15. The appropriate method was run.
16. After finish, both lines A and B were put in distilled water.
17. The system was washed using distilled water by running the 'System Wash' method.
18. The column was washed by allowing five column volume of distilled water to flow. The flow was end by pressing the 'End' button.
19. Steps 16 to 18 were repeated with 20% ethanol.
20. The flow rate was set to 0.5 ml/min by 'Manual Run'.
21. The bottom part of the column was detached from the system followed by the top.
22. The line was re-connected to the UV detector. Hit End.
23. The AKTAPrime and computer were switched off.

Always set pressure limit to 0.3MPa to protect your column from damage.

High Performance Liquid Chromatography (Waters)

1. Column and guard column were attached to the appropriate lines.
2. Deionized water was degassed using vacuum filter and the probe was inserted into the water.
3. The degasser, pump and detector were switched on.
4. The computer was switched on and the Breeze software was opened.
5. The line for the flow of the mobile phase was set to 100%.
6. Pump prime was carried out using a syringe.
7. The pump valve was pushed to the right and pump purge was started.
8. Method was set by opening a new method in the Breeze software.
9. The system was equilibrated for a few seconds to recognize the new set method.
10. The flow rate was increased to 0.05 ml/min and pressure was observed. After the pressure was stable, the flow rate was increased gradually. Connecting lines were checked for any leakage.
11. When the flow rate reached half of the desired flow rate, the oven was switched on.
12. The flow rate was increased gradually until the desired flow rate was reached.
13. After flow rate and temperature reached the set up conditions, detector was purged until the reading was stable (until +0000 reading was shown on the detector's screen).
14. The detector was unpurged.
15. The system was equilibrated until a stable baseline was achieved.
16. After finished equilibration, standards and samples can be injected into the column.